Moonlighting Proteins

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections

Edited by Brian Henderson

Division of Infection and Immunity, University College London, London, UK

WILEY Blackwell

Published by John Wiley & Sons, Inc., Hoboken, New Jersey

Published simultaneously in Canada

Library of Congress Cataloging-in-Publication Data

Names: Henderson, Brian (Professor) editor.
Title: Moonlighting proteins : novel virulence factors in bacterial infections / Edited by Brian Henderson.
Description: Hoboken, New Jersey : John Wiley & Sons, Inc., [2017] | Includes bibliographical references and index.
Identifiers: LCCN 2016049475 (print) | LCCN 2016051302 (ebook) | ISBN 9781118951118 (cloth) | ISBN 9781118951125 (pdf) | ISBN 9781118951132 (epub)
Subjects: | MESH: Bacterial Proteins–physiology | Virulence Factors | Bacterial Infections
Classification: LCC RA644.B32 (print) | LCC RA644.B32 (ebook) | NLM QW 52 | DDC 614.5/7–dc23
LC record available at https://lccn.loc.gov/2016049475

Cover Image: © SEBASTIAN KAULITZKI/SCIENCE PHOTO LIBRARY/Getty Images
Cover Design: Wiley

Set in 10/12pt Warnock by SPi Global, Pondicherry, India

10 9 8 7 6 5 4 3 2 1

Contents

List of Contributors

Angela Barbosa
Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil.

Simone Bergmann
Department of Infection Biology, Institute of Microbiology, Technische Universität Braunschweig, Braunschweig, Germany.

Vishant M. Boradia
Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Punjab, India; Present address: Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland, USA.

Janine Z. Bowring
Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK.

Catherine A. Brissette
Department of Microbiology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota, USA.

Yung-Fu Chang
Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA.

Jonathan D. Cook
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Canada.

Sayoni Das
Institute of Structural and Molecular Biology, University College London, Gower Street, London, UK.

Roger Dumke
Technical University Dresden, Dresden, Germany.

Paula Ferreira
ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal; Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

Kathleen Friedrich
Technical University Dresden, Dresden, Germany.

Marcus Fulde
Department of Infectious Diseases, Institute for Microbiology, University

of Veterinary Medicine Hannover, Hannover, Germany; Present address: Centre for Infection Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany.

Rafael A. Garduño
Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada; Present address: Canadian Food Inspection Agency, Dartmouth Laboratory, Dartmouth, Nova Scotia, Canada.

Anne Gründel
Technical University Dresden, Dresden, Germany.

Brian Henderson
Department of Microbial Diseases, UCL-Eastman Dental Institute, University College London, UK.

Tomoe Ichikawa
Department of Microbial Science and Host Defense, Meiji Pharmaceutical University, Tokyo, Japan.

Riikka Ihalin
Department of Biochemistry, University of Turku, Turku, Finland.

Reiko Ikeda
Department of Microbial Science and Host Defense, Meiji Pharmaceutical University, Tokyo, Japan.

Chaaya Iyengar Raje
Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Punjab, India.

Enno Jacobs
Technical University Dresden, Dresden, Germany.

Constance J. Jeffery
Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois, USA.

Shigetada Kawabata
Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan.

Ishita Khan
Department of Computer Science, Purdue University, North University Street, West Lafayette, Indiana, USA.

Daisuke Kihara
Department of Biological Sciences, Purdue University, Martin Jischke Drive, West Lafayette, Indiana, USA.

Hideki Kinoshita
Department of Food Management, School of Food, Agricultural and Environmental Sciences, Miyagi University, Miyagi, Japan; Present address: Laboratory of Food Biochemistry, Department of Bioscience, School of Agriculture, Tokai University, Kumamoto, Japan.

Chih-Jung Kuo
College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan.

Jeffrey E. Lee
Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Canada.

Alberto Marina
Instituto de Biomedicina de Valencia, IBV-CSIC, Valencia, Spain; CIBER de Enfermedades Raras (CIBERER-ISCIII).

Lois E. Murray
Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada.

Neil J. Oldfield
Molecular Bacteriology and Immunology Group, School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK.

Christine Orengo
Institute of Structural and Molecular Biology, University College London, Gower Street, London, UK.

Vijay Pancholi
Department of Pathology, The Ohio State University College of Medicine and Wexner Medical Center, Columbus, Ohio, USA.

José R. Penadés
Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK.

Melanie Pfeiffer
Technical University Dresden, Dresden, Germany.

Christopher P. Ptak
Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA.

Nuria Quiles-Puchalt
Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK.

Manoj Raje
Council of Scientific and Industrial Research (CSIR) Institute of Microbial Technology (CSIR-IMTECH), Chandigarh, India.

Angela L. Riveroll
Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada; Present address: Solarvest BioEnergy Inc. Summerville, PE, Canada.

Peter Robertson
Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada; Present address: Canadian Food Inspection Agency, Dartmouth Laboratory, Dartmouth, Nova Scotia, Canada.

Natalia Salazar
Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil.

Michael A. Sirover
Department of Pharmacology, Temple University School of Medicine, Philadelphia, USA.

Fariza Shams
Molecular Bacteriology and Immunology Group, School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK.

Patrick Trieu-Cuot
Unité de Biologie des Bactéries Pathogènes à Gram-positif, Institut Pasteur, CNRS ERL 3526, 28 rue du Dr ROUX, Paris, France.

David P.J. Turner
Molecular Bacteriology and Immunology Group, School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK.

Karla N. Valenzuela-Valderas
Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada.

Willem van Eden
Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands.

Karl G. Wooldridge
Molecular Bacteriology and Immunology Group, School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK.

Masaya Yamaguchi
Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan.

Preface

Bacterial infection with exogenous pathogens can be thought of as a result of the evolution of specific bacterial behaviors that outwit the vast panoply of host immune defenses. Such interactions occur against the background of the vast colonization of *Homo sapiens* with a phylogenetically complex bacterial microbiota, whose interactions with the human host can only be guessed at. At its simplest, bacterial infection can be seen as a dynamic and evolutionarily constrained competition between the host and the genetically dynamic bacterial population of the environment. The major defining factor is bacterial virulence which could be defined simply as the population number required to infect a host organism. The fewer organisms required, the more virulent the organism. However, this has to be seen as a simplistic view of virulence, which is a systems-based phenomenon with emergent properties. Virulence is a systems-based concept which is dependent on the generation, by the bacterium, of molecules which can allow the bacterium to: (1) colonize; (2) survive the initial colonization process; (3) grow and, potentially, form biofilms; (4) defeat the approaches of the innate immune system; (5) deal with the adaptive immune cells; and finally (6) survive without killing the host.

The concept of virulence has given rise to the "virulence factor." These will be best known in terms of the terrors of bacterial infection with gas gangrene (caused by *Clostridium perfringen*), the flesh-eating bacterium (mainly describing *Streptococcus pyogenes*) with both pathologies being caused by enzymes, and flaccid and tetanic muscle spasms caused by *Clostridium botulinum* and *Clostridium tetani* toxins. Toxins are the main factor that comes to mind when thinking of bacterial virulence. However, they are only one of a range of molecules that aid the bacterium in its colonization and growth in the human organism. A range of other bacterial virulence factors include molecules which aid bacterial adhesion to matrices and cells, promote bacterial invasion of tissues and cells, control bacterial growth by binding essential metals, enable bacterial evasion of host immunity, and allow bacteria to enter low-growth states (e.g., dormancy) which decrease their molecular signatures in the host. The formation of the bacterial biofilm involves a range of other virulence factors including those involved in quorum sensing, biofilm dispersion, and so on.

The renaissance of Bacteriology over the last 30 years (largely due to the upsurge in antibiotic resistance) has seen the identification of a wide range of

bacterial virulence mechanisms and the discovery of a large number of molecularly distinct virulence factors, many of which are proteins. Since the early 1990s, it has become clear that among these distinct virulence proteins there exists a substantial number of proteins whose main function has nothing to do with bacterial virulence. Cytoplasmic proteins such as the glycolytic enzymes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase have been identified on the surface of a wide range of Gram-positive and Gram-negative bacteria, and have been reported to have a surprising number of diverse biological actions which are assumed to contribute to bacterial virulence. Indeed, where assessed using gene inactivation/upregulation, it has been established that these proteins have a direct role to play in bacterial virulence. These proteins are known as *moonlighting proteins*, which are defined as proteins with more than one unique biological action. Some of the bacterial moonlighting protein families from different bacterial species, although sharing >90% sequence identity, can produce quite distinct biological actions, thus increasing the virulence "range" of these proteins.

At the time of writing, around 90 bacterial proteins have been reported to exhibit more than one biological activity, with the moonlighting function being related to some virulence phenomenon. Many of these proteins are actually found in all three domains of life, and can therefore be thought of as shared signals. The discovery of the role of moonlighting proteins in bacterial interactions with their hosts reveals the plasticity of protein evolution as it relates to protein function and bacterial communication. Indeed, there are a small number of examples of human moonlighting proteins playing a role in enhancing bacterial colonization and virulence.

This book brings together the leading experts in the study of pathogenicity of bacterial moonlighting proteins. The book is divided into a number of related parts. In Part 1, the reader is introduced to the concept of protein moonlighting and is provided with current concepts in the evolution of protein moonlighting, its structural biological underpinnings, and its potential role in terms of cellular complexity and systems biology. Part 2 focuses on moonlighting in prokaryotes in a general sense, and includes chapters on general moonlighting proteins in bacterial infection and the role of moonlighting bacterial proteins in autoimmunity. Part 3 of this book discusses, in some detail, the various moonlighting proteins that are known to function as virulence factors, including: molecular chaperones and protein-folding catalysts (Parts 3.1 and 3.2); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Part 3.3); enolase (Part 3.4); other glycolytic enzymes (Part 3.5); other metabolic enzymes (Part 3.6); miscellaneous proteins (Part 3.7); and bacterial moonlighting proteins that function to bind cytokines (Part 3.8). Finally, in Part 3.9 the subject switches to novel findings in bacteriophage and virus biology in which moonlighting proteins are able either to promote bacterial virulence or aid in viral infection.

This book will be of interest to a range of scientists. Clearly the major reader will be the bacteriologist/cell biologist interested in the mechanisms of bacterial virulence and in the possible role of bacterial moonlighting proteins as therapeutic targets. Given the widespread use of some of these moonlighting proteins as

virulence determinants by many of the bacterial pathogens of *Homo sapiens*, this is a possibility. Other readers will include immunologists, biochemists, molecular biologists, and pathologists focusing on the biology of the cell stress response and those interested in the diversity of protein structure and function. The finding that bacteria encode high-affinity binding proteins for key proinflammatory cytokines such as IL-1β and TNFα also suggests a therapeutic potential for such molecules.

About the Editor

Brian Henderson is Professor of Biochemistry in the Department of Microbial Diseases at the UCL-Eastman Dental Institute, University College London. He has worked in academia, both in the UK and North America, and also in the pharmaceutical and biopharmaceutical industry. He has been a cell biologist, immunologist, and pharmacologist and over the past 20 years has focused on bacteria–host interactions in relation to human infection and the maintenance of the human microbiota. This is known as the discipline of Cellular Microbiology, and Henderson published the first book on this subject in 1999. At the inception of his career as a cellular microbiologist, he discovered a potent bone-destroying protein secreted by a pathogenic oral bacterium. This protein turned out to be the cell stress protein, heat-shock protein (Hsp)60. This was one of the earliest bacterial moonlighting proteins discovered, and is the reason that the editor has spent the last 20 years exploring the role of protein moonlighting in the life of the bacterium and its interactions with its human host. Henderson has written or edited 17 books and monographs and was the senior editor of the Cambridge University Press monograph series *Advances in Molecular and Cellular Microbiology*. His last book, published in 2013, was entitled *Moonlighting Cell Stress Proteins in Microbial Infections*.

Part I

Overview of Protein Moonlighting

1

What is Protein Moonlighting and Why is it Important?

Constance J. Jeffery

Department of Biological Sciences, University of Illinois at Chicago, Chicago, Illinois, USA

1.1 What is Protein Moonlighting?

Moonlighting proteins exhibit more than one physiologically relevant biochemical or biophysical function *within one polypeptide chain* (Jeffery 1999). In this class of multifunctional proteins, the multiple functions are not due to gene fusions, multiple RNA splice variants or multiple proteolytic fragments. The moonlighting proteins do not include pleiotropic proteins, where a protein has multiple downstream cellular roles in different pathways or physiological processes that result from a single biochemical or biophysical function of a protein. Moonlighting proteins also do not include families of homologous proteins if the different functions are performed by different members of the protein family.

Some of the first moonlighting proteins to be identified were taxon-specific crystallins in the lens of the eye. These proteins, including the delta 2 crystallin/arginosuccinate lyase in the duck (Wistow and Piatigorsky 1987), upsilon crystallin/lactate dehydrogenase A in the duckbill platypus (van Rheede *et al*. 2003), eta-crystallin/cytosolic aldehyde dehydrogenase (ALDH class 1) in the elephant shrew (Bateman *et al*. 2003), and several others, are ubiquitous soluble enzymes that were adopted as structural proteins in the lens. Other well-known moonlighting proteins include soluble enzymes in biochemical pathways that also bind to DNA or RNA to regulate transcription or translation. Human thymidylate synthase (TS), a cytosolic enzyme in the *de novo* synthesis of the DNA precursor thymidylate, also binds to mRNA encoding TS to inhibit translation (Chu *et al*. 1991). The *Salmonella typhimurium* PutA protein is an enzyme with proline dehydrogenase and proline oxidase pyrroline-5-carboxylic acid dehydrogenase activity when it is bound to the inner side of the plasma membrane (Menzel and Roth 1981*a*, *b*), but it also binds to DNA and moonlights as a transcriptional repressor of the *put* operon (Ostrovsky de Spicer *et al*. 1991; Ostrovsky de Spicer and Maloy 1993). The *E. coli* BirA biotin synthase is an enzyme in the biotin biosynthetic pathway that is also a *bio* operon suppressor

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

(Barker and Campbell 1981). *Saccharomyces cerevisiae* N-acetylglutamate kinase/N-acetylglutamyl-phosphate reductase (Arg5,6p) is an enzyme in the arginine biosynthetic pathway (Boonchird *et al*. 1991; Abadjieva *et al*. 2001) and also binds to mitochondrial and nuclear DNA to regulate expression of several genes (Hall *et al*. 2004). *Kluyveromyces lactis* galactokinase (GAL1) phosphorylates galactose and is also a transcriptional activator of genes in the *GAL* operon (Meyer *et al*. 1991).

Perhaps even more surprising than the fact that some proteins can perform such different functions is that such a large variety of proteins moonlight. Over the past few decades, hundreds of proteins have been shown to moonlight (Mani *et al*. 2015; moonlightingproteins.org). They include many types of proteins: enzymes, scaffolds, receptors, adhesins, channels, transcription and translation regulators, extracellular matrix proteins, growth factors, and many others. They are active in a variety of physiological processes and biochemical pathways, are found in the cytoplasm, nucleus, mitochondria, on cell surface, and other cellular compartments, and some are secreted. They are also expressed in many different cell types within a species. They are found in a variety of species from throughout the evolutionary tree. They are common in eukaryotes in humans and other placental and monotreme (i.e., platypus) mammals, reptiles, birds, amphibians, fish, worms, insects, plants, fungi, and protozoans. A few are found in archea and many more have been identified in eubacteria, including pathogenic species (*Clostridium difficile*, *Helicobacter pylori*, *Pseudomonas aeruginosa*, *Staphylococcus*, etc.) as well as nonpathogenic, commensal bacteria, including health-promoting or "pro-biotic" species (*Bifidobacterium*). A few moonlighting proteins have even been found in viruses.

The variety also extends to the combinations of functions that are observed. Many of the known moonlighting proteins are cytosolic enzymes, chaperones, or other proteins that exhibit a second function in other cellular locations, for example as a receptor on the cell surface. Several proteins described in more detail in other chapters are cytosolic enzymes or chaperones that are secreted to serve as growth hormones or cytokines. For example, an enzymatic function and an extracellular cytokine function are found in phosphoglucose isomerase/autocrine motility factor (Gurney *et al*. 1986*a*, *b*; Chaput *et al*. 1988; Faik *et al*. 1988; Watanabe *et al*. 1996; Xu *et al*. 1996). Many of the moonlighting proteins have surprisingly unrelated functions, such as the PHGPx (glutathione peroxidase), a soluble enzyme that is also a sperm structural protein (Scheerer *et al*. 2007), adopted during evolution for its structural characteristics in the same way as taxon-specific crystallins above. Other proteins can exhibit two functions even within the same cellular compartment and may change function as cellular conditions change, for example changes in pH, or the concentration of a ligand, substrate, cofactor, or product. Still other moonlighting proteins have one function as a monomer or homo-multimer but interact with other proteins in a multiprotein complex, such as the proteasome or ribosome. Some proteins even have more than two functions, for example glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase.

1.2 Why is Moonlighting Important?

1.2.1 Many More Proteins Might Moonlight

The diverse examples of moonlighting proteins already identified suggest that many more moonlighting proteins are likely to found. The ability of one protein to perform multiple functions greatly expands the possible number of functions that can be performed by the proteome. In addition, the study of the molecular mechanisms and regulation of moonlighting functions helps broaden our understanding of protein biochemistry and suggests additional activities that might be encoded by genomes.

1.2.2 Protein Structure/Evolution

X-ray crystal structures and other biochemical and biophysical studies of some of the moonlighting proteins have added to our understanding of how one protein can perform two different functions and, in some cases, provided information about the triggers and molecular mechanisms involved in switching between two activities (several examples reviewed in Jeffery 2004, 2009).

In cases where the function of the protein changes in response to changes in the environment, moonlighting proteins provide examples of how a protein can sense and respond to these changes, and thereby provide interesting examples of regulation of protein function. The hemagglutinin-neuraminidase of paramyxovirus, which causes mumps, has different conformations at high- and low-pH conditions. The protein first enables binding of the virus to the surface of host cells. A change in pH promotes the movement of several amino acid side-chains and a loop in the active site to switch between the protein's sialic acid binding and hydrolysis functions so that it can cleave the glycosidic linkages of neuraminic acids (Crennell *et al.* 2000). The *E. coli* periplasmic serine endoprotease/heat-shock protein DegP (Protease Do) switches from a peptidase at high temperatures to a protein-folding chaperone at lower temperatures (Krojer *et al.* 2002).

In some moonlighting proteins the two functional sites are located distant from each other on the protein surface and the protein can perform both functions simultaneously, but in other proteins the functional sites are close to each other or even overlapping. *Streptomyces coelicolor* albaflavenone monooxygenase/synthase has a heme-dependent monooxygenase activity to catalyze the reaction (+)-epi-isozizaene + 2 NADPH + 2 O(2) < = > albaflavenone + 2 NADP(+) + 3 H(2) O and has a typical cytochrome P450 fold. However, the protein was also found to exhibit terpene synthase activity. After solution of its X-ray crystal structure a second active site pocket, for terpene synthase activity, was identified in an alpha-helical barrel near the monooxygenase active site (Zhao *et al.* 2008, 2009). It was recently found that the fructose-1,6-bisphosphate aldolase/phosphatase enzymes from the hyperthermophiles *Thermoproteus neutrophilususes* and *Sulfolobus tokodaii* utilize a single active site pocket to catalyze two reactions in the same biochemical pathway (Du *et al.* 2011; Fushinobu *et al.* 2011). After completion of the first catalytic function, several loops undergo conformational changes in order to bind the second substrate and perform the second catalysis.

A much larger conformational change occurs in cytosolic aconitase that renders the protein unable to perform one function but able to perform a second function. Aconitase is an enzyme in the citric acid cycle that uses an active site-bound 4Fe-4S cluster to catalyze the interconversion of citrate to isocitrate when cellular iron levels are high. When cellular iron concentrations decrease, the enzyme loses its 4Fe-4S cluster and becomes the iron-responsive element-binding protein (IRE-BP), which binds to iron-responsive elements (IREs) in 5'- or 3'-untranslated regions of mRNAs that encode proteins that are involved in iron uptake and use (Kennedy *et al*. 1992). This change in function involves a huge change in protein conformation. Domain 4 rotates 32° and translates 14 Å relative to the rest of the protein, and domain 3 rotates 52° and translates 13 Å relative to the rest of the protein. All four protein domains then interact with the IREs. In fact, the RNA binding and active sites overlap extensively, and several conserved active site amino acids are also important in mRNA binding (Philpott *et al*. 1994; Walden *et al*. 2006).

Other moonlighting proteins perform one function as a monomer or homomultimer but are incorporated into a larger multiprotein complex (i.e., ribosome, proteasome) to perform a second, sometimes structural role, and in those cases the moonlighting polypeptide might or might not undergo a large conformational change. The ferredoxin-dependent glutamate synthase in spinach chloroplasts (FdGOGAT) catalyzes the reaction 2 L-glutamate + 2 oxidized ferredoxin = > L-glutamine + 2-oxoglutarate + 2 reduced ferredoxin in L-glutamate biosynthesis and is also a subunit of the UDP-sulfoquinovose synthase (SQD1) (Shimojima *et al*. 2005), a multiprotein complex that catalyzes the transfer of sulfite to UDP-glucose in the synthesis of UDP-sulfoquinovose, which is the head group donor in the biosynthesis of sulfoquinovosyldiacylglyerol, a plant sulfolipid. Human delta-aminolevulinic acid dehydratase (porphobilinogen synthase, ALADH) converts 5-aminolevulinate to porphobilinogen in the biosynthesis of protoporphyrin-IX (Gibbs and Jordan 1986; Wetmur *et al*. 1986). It also interacts with the proteasome as a proteasome inhibitory subunit that blocks proteolysis of specific protein substrates (Li *et al*. 1991; Guo *et al*. 1994). *Tetrahymena thermophile* citrate synthase, a soluble enzyme from the citric acid cycle, is a protein in the 14 nm cytoskeletal filament (Numata 1996).

Questions remain as to how these proteins obtained a second function. Many of the known moonlighting proteins are ubiquitous enzymes in central metabolism or ubiquitous chaperone proteins (Jeffery 1999, 2009). These proteins first arose billions of years ago and are expressed in many species and cell types. They are likely to have been adopted for a second function because organisms evolve by utilizing and building upon components they already possess, and these proteins are available in many organisms.

Binding to another protein is the key characteristic of the second, or more recently acquired, function of many of the known moonlighting proteins, and a new binding function can result if a protein's structure is modified to create a new binding site on the protein surface. Modification of a short amino acid sequence on a surface exposed loop could be all that is needed for the formation of a new protein–protein interaction site. Enolase is an ubiquitous cytoplasmic enzyme in glycolysis that moonlights in many species. In several bacterial

species it is found on the cell surface where it can bind to plasminogen. The plasminogen-binding site of *Streptococcus pneumoniae* enolase has been identified as a nonapeptide (248-FYDKERKVY-256). In X-ray crystal structures of *S. pneumoniae* enolase (PDB-ID = 1W6T), this sequence motif is found to be on the solvent exposed surface of the octamer, in surface loop near the active site pocket. Three loops, L1 (residues 38–45), L2 (152–159), and L3 (244–265), fold over the active site pocket in the substrate-bound state. The plasminogen binding site is located in L3 (Bergmann *et al*. 2003; Ehinger *et al*. 2004).

In general, enzymes appear to contain many more amino acids than are required to form an active site pocket, leaving a lot of surface amino acids that are not involved in the original function and are therefore not under as much selective pressure. This has been illustrated by considering the X-ray crystal structure of phosphoglucose isomerase, an enzyme that is nearly ubiquitous in evolution (Jeffery *et al*. 2000). In an alignment of 136 PGI sequences, 47 residues were found to be conserved. The conserved amino acids include those that interact with substrate as well as others that help form the shape of the active site pocket and position the catalytic amino acids, and these are the amino acids that have been conserved for three billion years of evolution to maintain the isomerase activity. At the same time, during three billion years of evolution most of the other residues changed. As is the case with most proteins, many of the amino acids that have undergone changes are located on the protein's surface. In this large dimeric protein of more than 1000 amino acids, numerous entire helices and other surface features are made up of nonconserved residues. It is quite possible that one of these surface features could have gained an additional binding function during evolution. As long as that new function did not interfere with the isomerase activity of the protein, it had the potential to benefit the organism and its offspring and was perhaps kept during evolution. This is one possible way to explain how a protein can evolve a moonlighting function.

Additional insight into how a protein can gain a novel function is provided by the identification of several "neomorphic moonlighting proteins," proteins that gain a second function through a single amino acid substitution (reviewed in Jeffery 2011). These proteins are not true moonlighting proteins because a second function is performed only by a mutant form of the protein and is not a normal physiological function of the protein. Several of these gain-of-function mutations have been identified because they result in disease.

Dihydrolipoamide dehydrogenase (DLD) is a flavin-dependent oxidoreductase that is found in several multienzyme complexes, including the pyruvate, alpha-ketoglutarate, and branched chain amino acid dehydrogenase complexes. Wild-type DLD is a dimer that catalyzes the conversion of dihydrolipoic acid to lipoic acid along with the reduction of NAD+ to NADH. Because of its critical role in energy and redox balance in the cell, genetic mutations that cause a deficiency of enzyme activity result in severe disorders in infancy; the symptoms are however variable and due to the specific mutation found in each case. Some single amino acid substitutions in the homodimer interface result in hypertrophic cardiomyopathy, which is not observed in patients with other mutations in the protein. Surprisingly, these mutations cause a decrease in dimer formation and reveal a protease active site that enables the enzyme to cleave protein substrates, which

might contribute to the observed symptoms (Babady *et al*. 2007; Brautigamet *et al*. 2005). The neomorphic moonlighting proteolytic activity was shown to be independent of the DLD activity because a S456A amino acid substitution, which is in the catalytic dyad of the protease active site, abolished protease activity but did not affect DLD activity.

Mutations in isocitrate dehydrogenases result in a novel product of catalysis that promotes cancer (Yan *et al*. 2009; Figueroa *et al*. 2010). The NADP+-dependent isocitrate dehydrogenases (IDH1 and IDH2) catalyze the oxidative decarboxylation of isocitrate to alpha-ketoglutarate (alpha-KG) in the Krebs cycle. The cause of some gliomas and some cases of acute myeloid leukemia (AML) was found to be an amino acid substitution at R132 in the catalytic pocket of IDH1. In other gliomas, substitutions were found at the equivalent R172 position in IDH2. Instead of knocking out enzyme activity, the single amino acid substitution mutation causes a neomorphic moonlighting enzymatic activity. In place of producing the usual alpha-ketoglutarate product, the mutant enzyme reduces alpha-KG to the R-enantiomer of 2-hydroxyglutartate ((R)-2HG R(2)-2-hydroxyglutarate; 2HG; Dang *et al*. 2010; Xu *et al*. 2011; Lu *et al*. 2012). The 2HG product is an oncometabolite that works by inhibiting alpha-KG-dependent dioxygenases, including proteins involved in histone and DNA demethylation, thereby affecting the epigenetic state of the cells and blocking cellular differentiation.

The fact that single amino acid substitutions in DLD and IDH can cause a gain of function (although a pathological "neomorphic" moonlighting function and not a "true" moonlighting function) that results in changes in the cell suggests that, at least in some cases, very small changes in a protein sequence and structure might be all that is needed for a protein to gain a true moonlighting function.

It is also interesting to note that an ancestral protein can gain different moonlighting proteins in different evolutionary lineages. Enolase and GAPDH are enzymes and plasminogen or extracellular matrix-binding proteins in several species, as described above. In the sea lamprey (*Petromyzon marinus*), enolase is an enzyme and has been adopted to be tau-crystallin in the lens of the eye (Stapel and de Jong 1983; Williams *et al*. 1985; Jaffe and Horwitz 1992). In the diurnal gecko (*Phelsuma*), GAPDH is an enzyme and also pi-crystallin (Jimenez-Asensio *et al*. 1995). The protein-folding chaperonin 60 has also been adopted for many different moonlighting functions in different species (Henderson *et al*. 2013).

Further discussion of the current knowledge of evolution of protein moonlighting and its structural biological underpinnings are the topics of Chapters 2 and 3.

1.2.3 Roles in Health and Disease

1.2.3.1 Humans

In humans, many of the known moonlighting proteins function in cellular processes that can go wrong in cancer, diabetes, and other common diseases or are important in disease treatment, for example: DNA synthesis or repair; chromatin and cytoskeleton structure; angiogenesis; amino acid, protein, sugar, and lipid metabolic pathways; and as growth factors or cytokines (reviewed in Jeffery 2003*a*, *b*). Phosphoglucose isomerase/autocrine motility factor/neuroleukin in

glycolysis and thymidine phosphorylase/platelet-derived endothelial cell growth factor (PDGF) in dTMP biosynthesis via the salvage pathway are two cytosolic enzymes that function as growth factors outside the cell (Gurney *et al*. 1986*a*, *b*; Chaput *et al*. 1988; Faik *et al*. 1988; Watanabe *et al*. 1991, 1996; Furukawa 1992; Xu *et al*. 1996). Phosphoglycerate kinase is another glycolytic enzyme that has a second role when secreted. Outside the cell it is a disulfide bond reductase that reduces plasmin, which enables the cleavage of plasmin to produce an angiogenesis inhibitor, angiostatin (Lay *et al*. 2000). Human thymosin beta-4 sulfoxide inhibits actin polymerization in the cytoplasm by sequestering G-actin (monomeric actin) (Safer *et al*. 1997). It is secreted in response to glucocorticoids and serves as an immunomodulatory signal to limit inflammation due to cell injury (Young *et al*. 1999).

Histone H1, which plays a structural role in chromatin fibers and is also involved in regulation of gene expression, has another function as a cell-surface receptor for thyroglobulin, which helps in the production of the thyroid hormones thyroxine (T4) and triiodothyronine (T3) (Brix *et al*. 1998). SMC-3 (stuctural maintenance of chromosome 3) is part of a complex that maintains proper sister chromatid cohesion throughout the cell cycle and during mitosis to ensure accurate chromosome segregation (Darwiche *et al*. 1999; Wu and Yu 2012). It is also a component of the Engelbreth–Holm–Swarm tumor matrix, the renal mesangial matrix, and the basement membrane of other tissues and is involved in the control of cell growth and transformation (Couchman *et al*. 1996; Ghiselli *et al*. 1999). Human Hsp60 is a mitochondrial heat-shock protein that aids in protein import into the mitochondria, correct protein folding, and the prevention of protein misfolding. It can also be displayed on the cell surface where it serves as a receptor for HDL by binding to the apolipoprotein apoA-II (Bocharov *et al*. 2000). The folate receptor alpha (FRalpha) is a GPI-anchored protein on the cell surface that is important for binding folate and its derivatives and bringing them into the cell through endocytosis, where it can play a role in preventing neural tube defects during embryogenesis and help prevent other diseases in adults. It was also recently found to be a transcription factor in the nucleus where it binds to the cis-regulatory elements of the promoter regions of the Fgfr4 and Hes1 genes to regulate their expression (Boshnjaku *et al*. 2012).

As the number of known moonlighting proteins increases, it is becoming clear that many also have key roles related to cellular complexity and coordinating cellular activities; these are important in systems biology and also discussed further in Chapter 4. Because of their dual functions and ability to switch between functions, a moonlighting protein can help the cell to respond to changing conditions in its environment or to changes in concentrations of metabolites within a cell as a feedback mechanism, or to help coordinate the actions of proteins with similar or complementary functions. The cystic fibrosis transmembrane conductance regulator (CFTR) is a chloride channel that also regulates other channels (Stutts *et al*. 1995), including the outwardly rectifying chloride channel (ORCC) and a sodium channel (ENaC). In this way, moonlighting proteins can contribute to cellular homeostasis. They can also promote homeostasis throughout the organism because some moonlighting proteins are also involved in communication between different cell types within an organism.

Some of these moonlighting proteins are the focus of a great deal of study because one or more of their functions plays a key role in disease. As described above, a functioning CFTR protein that is properly targeted to the plasma membrane helps promote epithelial cell ion homeostasis through its actions as a chloride channel and regulator of other channels. Mutations in the CFTR result in the genetic disease cystic fibrosis, and alleviating all the symptoms of the disease will require re-acquisition of the multiple functions of the protein. In cancer, the moonlighting protein PGI/AMF has been found to be involved in breast cancer metastasis because it can affect tumor cell motility, but it can also cause differentiation of some leukemia cell lines. The P-glycoprotein is a transmembrane flippase that exports hydrophobic substances from the cell and also helps regulate volume-activated ion channels in response to cell swelling (Hardy *et al.* 1995). It can become a serious problem in cancer treatment, especially when it becomes overexpressed in cancer cells, because it can also export anti-cancer drugs, resulting in multidrug resistance and a decrease in effectiveness of chemotherapy.

For these human moonlighting proteins, especially those that might be potential therapeutic targets, understanding all their functions is important in alleviating disease as well as avoiding toxicity and side effects.

1.2.3.2 Bacteria

Another type of disease in which many moonlighting proteins are involved is infections. Pathogenic bacteria, fungi, worms, and other species use moonlighting proteins for colonization, invasion, defeating the host's immune system, forming biofilms, quorum sensing, acquiring nutrients, or producing toxins. A large subset of moonlighting proteins found in bacteria have a canonical biochemical function inside the cell and perform a second biochemical function on the cell surface or when secreted. In some pathogenic bacteria, the extracellular function plays a key role in infection or virulence or, in the case of some non-pathogenic or "pro-biotic" species, in commensal interactions with a host species. Colonization of the host requires attachment of the bacterium to the host, and many of the intracellular/cell-surface moonlighting proteins have been shown to bind to proteins in the extracellular matrix or directly to host cells, while some play other roles in invasion of host tissues.

Streptococcus oralis 6-phosphofructokinase from glycolysis (Kinnby *et al.* 2008), *Candida albicans* alcohol dehydrogenase (Crowe *et al.* 2003), *Haemophilus influenza* aspartate ammonia lyase (aspartase) (Sjstrm *et al.* 1997), *Staphylococcus aureus* enolase (as well as enolase from many other species) (Antikainen *et al.* 2007), *Mycobacterium tuberculosis* DnaK (a protein chaperone) (Xolalpa *et al.* 2007), and *Pseudomonas aeruginosa* Ef-Tu (an elongation factor during protein synthesis) (Kunert *et al.* 2007) are examples of several dozen cytosolic proteins that have a second role in some pathogenic bacterial species as a receptor for plasminogen on the cell surface. Once bound to the receptor the plasminogen is converted to the active protease plasmin, which is used to degrade host tissues and aid in tissue invasion. Other cytoplasmic proteins are also found moonlighting on the cell surface where they aid the bacteria in attaching to host cells or extracellular matrix. A few will be described here, but many more are included in Chapter 5 and described in other articles (Henderson and Martin 2011, 2013; Henderson 2014; Amblee and Jeffery 2015). Alcohol acetaldehyde dehydrogenase/Listeria adhesion

protein (LAP) enables *Listeria monocytogenes* to bind to intestinal epithelial cells (Jagadeesan *et al*. 2010). *Streptococcus pneumonia* 6-phosphogluconate dehydrogenase (Daniely *et al*. 2006) is also an adhesin, and anti-6PGD-antibodies inhibited 90% of *S. pneumoniae* adhesion to A459 type-II lung carcinoma cultured cells in a concentration-dependent manner. *Listeria monocytogenes* alcohol acetaldehyde dehydrogenase (Jagadeesan *et al*. 2010) also promotes bacterial adhesion by binding to Hsp60 on enterocyte-like Caco-2 cells (Wampler *et al*. 2004). *Enteamoeba histolytica* alcohol dehydrogenase (EhADH2) binds fibronectin, laminin, and type-II collagen (Yang *et al*. 1994). *Lactobacillus plantarum* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) binds salivary mucin and also directly to cells (Kinoshita *et al*. 2008). *Streptococcus pyogenes* GAPDH also binds to cells, using the uPAR/CD87 receptor on human cells (Jin *et al*. 2005) as well as binding to fibronectin and plasminogen (Lottenberg 1992). Some of these proteins are found to moonlight on the surface of multiple species, sometimes with different extracellular functions in different species. It is possible that many of the other cytoplasmic/cell-surface moonlighting proteins mentioned above also bind to multiple proteins – plasminogen, components of the extracellular matrix, cell-surface receptors – but only some of the binding partners have been identified to date.

Further examples of moonlighting proteins with roles in autoimmunity, virulence, etc. are discussed in Chapters 5–23. The potential of some of these proteins as therapeutic targets is especially important because bacterial drug resistance is growing and new therapeutics are greatly needed.

1.3 Current questions

Even though a great deal has been learned about moonlighting proteins in the past few decades, many questions still remain and are the topics of current study. Some of the key questions include the following.

1.3.1 How Many More Proteins Moonlight?

The MoonProt Database contains 270 entries (Mani *et al*. 2015; moonlightingproteins.org), but more and more moonlighting proteins are found every year. Their diverse characteristics and widespread presence in the evolutionary tree suggest that moonlighting could be found in many more proteins. This raises the question "do most proteins moonlight?"

1.3.2 How Can We Identify Additional Proteins That Moonlight and all the Moonlighting Functions of Proteins?

In many cases, the multiple functions of a moonlighting protein were discovered by serendipity. There is currently no good method of amino acid sequence analysis or structural analysis that can predict which of the millions of amino acid sequences that are available from genome projects, or the over 100,000 protein structures in the Protein Data Bank, correspond to moonlighting proteins. Sequence or structural homology is often not enough to identify moonlighting proteins, in part because sequence homologs of moonlighting proteins can share one, both, or neither function.

1.3.3 In Developing Novel Therapeutics, How Can We Target the Appropriate Function of a Moonlighting Protein and Not Affect Other Functions of the Protein?

The ability to identify all of the functions of moonlighting proteins becomes especially important if the protein plays a key role in disease. A therapeutic that targets one function of a moonlighting protein could adversely affect other functions of the protein as well, including those not involved in the disease, and thereby result in toxicity or side effects. It would be helpful to be able to identify all the functions of a moonlighting protein and make sure that only the function involved in disease is targeted.

In addition, many of the moonlighting proteins that have been identified as being important for infection and virulence in pathogens have orthologs in humans. How can we target the pathogen's function involved in infection and virulence without affecting the human ortholog? Can we find sufficient differences between the structure and function of the bacterial proteins and the human proteins that can be exploited to develop effective and specific drugs?

1.3.4 How do Moonlighting Proteins get Targeted to More Than One Location in the Cell?

For example, cytosolic "housekeeping" enzymes in central metabolism that moonlight on the cell surface as a receptor not only need a binding site with which to interact with another protein, but they also they need a mechanism to be transported across the cell membrane and a mechanism to become attached to the cell surface. None of the intracellular/cell-surface moonlighting proteins have been found to possess a signal peptide for targeting to the cell membrane or other sequence motifs associated with other mechanisms of secretion. It is also important to note that only a small portion of each protein is partitioned to the cell surface while most of the protein remains in the cell cytoplasm. Once outside the cell, the intracellular/cell-surface proteins need a mechanism for attachment to the cell surface. There are several known sequence motifs for this purpose, for example the LPXTG motif that is involved in attaching proteins to the cell surface in Gram-positive bacteria (Schneewind *et al.* 1993); however, the intracellular/cell-surface moonlighting proteins do not possess any of these known cell-surface attachment motifs. How these intracellular proteins end up located outside of the cell and attached to the cell surface is an active area of inquiry in this field. In the case of bacterial pathogens, some of these processes might involve previously unknown mechanisms of secretion or attachment that could be targeted in the development of novel therapeutics.

1.3.5 What Changes in Expression Patterns Have Occurred to Enable the Protein to be Available in a New Time and Place to Perform a New Function?

In addition to changes in the protein sequence or structure itself, or association with new binding partners, changes in the expression pattern of the protein are often needed for a protein to perform a new function, for example, expression in

multiple cell types and/or expression at additional times during development. These may be the result of changes in the promoter region or other regulatory sequences of the gene.

1.4 Conclusions

With such a large variety of moonlighting proteins with key roles in many normal physiological processes and in disease, including cancer and genetic diseases in humans, as well as roles in infection and virulence by many pathogens, it is important to learn more about these proteins. We must continue to identify additional proteins that moonlight, and learn about the mechanisms of their functions as well as their methods to regulate those functions. There is still a great deal to be learned from these intriguing proteins. Building on what has been learned in the past few decades about the structures and functions of moonlighting proteins, a growing number of laboratories are addressing the topics and questions discussed above. Many of these topics are discussed in more detail in the remainder of the book.

References

Abadjieva A, Pauwels K, Hilven P, Crabeel M (2001) A new yeast metabolon involving at least the two first enzymes of arginine biosynthesis: acetylglutamate synthase activity requires complex formation with acetylglutamate kinase. *Journal of Biological Chemistry* 276: 42869–80.

Amblee V, Jeffery CJ. (2015) Physical features of intracellular proteins that moonlight on the cell surface. *PloS One* 10(6), doi: 10.1371/journal.pone.0130575.

Antikainen J, Kuparinen V, Lahteenmaki K, Korhonen TK (2007) Enolases from Gram–positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunology and Medical Microbiology* 51: 526–34.

Babady NE, Pang YP, Elpeleg O, Isaya G (2007) Cryptic proteolytic activity of dihydrolipoamidedehydrogenase. *Proceedings of the National Academy of Science, USA* 104: 6158–6163.

Barker DF, Campbell AM (1981) Genetic and biochemical characterization of the birA gene and its product: evidence for a direct role of biotin holoenzyme synthetase in repression of the biotin operon in *Escherichia coli*. *Journal of Molecular Biology* 146: 469–92.

Bateman OA, Purkiss AG, van Montfort R, Slingsby C, Graham C, Wistow G (2003) Crystal structure of eta-crystallin: adaptation of a class 1 aldehyde dehydrogenase for a new role in the eye lens. *Biochemistry* 42: 4349–56.

Bergmann S, Wild D, Diekmann O, Frank R, Bracht D, Chhatwal GS, Hammerschmidt S (2003) Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. *Molecular Microbiology* 49: 411–423.

Bocharov AV, Vishnyakova TG, Baranova IN, Remaley AT, Patterson AP, Eggerman TL (2000) Heat shock protein 60 is a high-affinity high-density lipoprotein binding protein. *Biochemical and Biophysical Research Communications* 277: 228–35.

Boonchird C, Messenguy F, Dubois E (1991) Determination of amino acid sequences involved in the processing of the ARG5/ARG6 precursor in *Saccharomyces cerevisiae*. *European Journal of Biochemistry* 199: 325–335.

Boshnjaku V, Shim KW, Tsurubuchi T, Ichi S, Szany EV, Xi G, Mania-Farnell B, McLone DG, Tomita T, Mayanil CS (2012) Nuclear localization of folate receptor alpha: a new role as a transcription factor. *Scientific Reports* 2: 980.

Brautigam CA, Chuang JL, Tomchick DR, Machius M, Chuang DT (2005) Crystal structure of human dihydrolipoamide dehydrogenase: NAD1/NADH binding and the structural basis of disease causing mutations. *Journal of Molecular Biology* 350: 543–552.

Brix K, Summa W, Lottspeich F, Herzog V (1998) Extracellularly occurring histone H1 mediates the binding of thyroglobulin to the cell surface of mouse macrophages. *Journal of Clinical Investigation* 102: 283–93.

Chaput M, Claes V, Portetelle D, Cludts I, Cravador A, Burny A, Gras H, Tartar A (1988) The neurotrophic factor neuroleukin is 90% homologous with phosphohexose isomerase. *Nature* 332: 454–5.

Chu E, Koeller DM, Casey JL, Drake JC, Chabner BA, Elwood PC, Zinn S, Allegra CJ (1991) Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. *Proceedings of the National Academy of Science, USA* 88: 8977–81.

Couchman JR, Kapoor R, Sthanam M, Wu RR (1996) Perlecan and basement membrane-chondroitin sulfate proteoglycan (bamacan) are two basement membrane chondroitin/dermatan sulfate proteoglycans in the Engelbreth–Holm–Swarm tumor matrix. *Journal of Biological Chemistry* 271: 9595–602.

Crennell S, Takimoto T, Portner A, Taylor G (2000) Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. *Nature Structural and Molecular Biology* 7: 1068–74.

Crowe JD, Sievwright IK, Auld GC, Moore NR, Gow NA, Booth NA (2003) *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. *Molecular Microbiology* 47: 1637–51.

Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD, Cantley LC, Thompson CB, Vander Heiden MG, Su SM (2010) Cancer-associated IDH1 mutations produce 2–hydroxyglutarate. *Nature* 465: 966.

Daniely D, Portnoi M, Shagan M, Porgador A, Givon-Lavi N, Ling E, Dagan R, Mizrachi Nebenzahl Y (2006) Pneumococcal 6-phosphogluconate-dehydrogenase, a putative adhesin, induces protective immune response in mice. *Clinical and Experimental Immunology* 144: 254–63.

Darwiche N, Freeman LA, Strunnikov A (1999) Characterization of the components of the putative mammalian sister chromatid cohesion complex. *Gene* 233: 39–47.

Du J, Say RF, Lu W, Fuchs G, Einsle O (2011) Active-site remodelling in the bifunctional fructose-1,6-bisphosphate aldolase/phosphatase. *Nature* 478: 534–7.

Ehinger S, Schubert WD, Bergmann S, Hammerschmidt S, Heinz DW (2004) Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. *Journal of Molecular Biology* 343: 997–1005.

Faik P, Walker JIH, Redmill AAM, Morgan MJ (1988) Mouse glucose-6-phosphate isomerase and neuroleukin have identical 3' sequences. *Nature* 332: 455–57.

Figueroa ME, Abdel-Wahab O, Lu C, Ward PS, Patel J, Shih A, Li Y, Bhagwat N, Vasanthakumar A, Fernandez HF, Tallman MS, Sun Z, Wolniak K, Peeters JK, Liu W, Choe SE, Fantin VR, Paietta E, Löwenberg B, Licht JD, Godley LA, Delwel R, Valk PJ, Thompson CB, Levine RL, Melnick A (2010) Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* 18: 553–67.

Furukawa T, Yoshimura A, Sumizawa T, Haraguchi M, Akiyama S, Fukui K, Ishizawa M, Yamada Y (1992) Angiogenic factor. *Nature* 356: 668.

Fushinobu S, Nishimasu H, Hattori D, Song HJ, Wakagi T (2011) Structural basis for the bifunctionality of fructose-1,6-bisphosphate aldolase/phosphatase. *Nature* 478: 538–41.

Ghiselli G, Siracusa LD, Iozzo RV (1999) Complete cDNA cloning, genomic organization, chromosomal assignment, functional characterization of the promoter, and expression of the murine Bamacan gene. *Journal of Biological Chemistry* 274: 17384–93.

Gibbs PNB, Jordan PM (1986) Identification of lysine at the active site of human 5-aminolaevulinate dehydratase. *Biochemical Journal* 236: 447–51.

Guo GG, Gu M, Etlinger JD (1994) 204-kDa proteasome Inhibitor (CF–2) is identical to aminolevulinic acid dehydratase. *Journal of Biological Chemistry* 269: 12399–402.

Gurney ME, Apatoff BR, Spear GT, Baumel MJ, Antel JP, Bania MB, Reder AT (1986*a*) Purification of a survival factor for spinal neurons from mouse salivary gland. *Science* 234: 574–81.

Gurney ME, Heinrich SP, Lee MR, Yin HS (1986*b*) Molecular cloning and expression of neuroleukin, a neurotrophic factor for spinal and sensory neurons. *Science* 234: 566–74.

Hall DA, Zhu H, Zhu X, Royce T, Gerstein M, Snyder M (2004) Regulation of gene expression by a metabolic enzyme. *Science* 306: 482–4.

Hardy SP, Goodfellow HR, Valverde MA, Gill DR, Sepulveda V, Higgins CF (1995) Protein kinase C-mediated phosphorylation of the human multidrug resistance P-glycoprotein regulates cell volume–activated chloride channels. *EMBO Journal* 14: 68–75.

Henderson B (2014) An overview of protein moonlighting in bacterial infection. *Biochemical Society Transactions* 42: 1720–27.

Henderson B, Martin A (2011) Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity* 79: 3476–91.

Henderson B, Martin A (2013) Bacterial moonlighting proteins and bacterial virulence. *Current Topics in Microbiology and Immunology* 358: 155–213.

Henderson B, Fares MA, Lund PA (2013) Chaperonin 60: a paradoxical, evolutionarily conserved protein family with multiple moonlighting functions. *Biological Reviews of the Cambridge Philosophical Society* 88: 955–987.

Jaffe NS, Horwitz J (1992) Lens and cataract. In *Textbook of Ophthalmology* (eds SM Podos and M Yanoff). Gower Medical Publishing, London.

Jagadeesan B, Koo OK, Kim KP, Burkholder KM, Mishra KK, Aroonnual A, Bhunia AK (2010) LAP, an alcohol acetaldehyde dehydrogenase enzyme in Listeria, promotes bacterial adhesion to enterocyte-like Caco-2 cells only in pathogenic species. *Microbiology* 156(9): 2782–95.

Jeffery CJ (1999) Moonlighting proteins. *Trends in Biochemical Science* 24: 8–11.

Jeffery CJ (2003*a*) Moonlighting proteins: old proteins learning new tricks. *Trends in Genetics* 19: 415–7.

Jeffery CJ (2003*b*) Multifunctional proteins: examples of gene sharing. *Annals of Medicine* 35: 28–35.

Jeffery CJ (2004) Molecular mechanisims of multitasking: recent crystal structures of moonlighting proteins. *Current Opinion in Structural Biology* 14: 663–8.

Jeffery CJ (2009) Moonlighting proteins – an update. *Molecular BioSystems* 5: 345–50.

Jeffery CJ (2011) Neomorphic moonlighting functions in disease. *IUBMB Life* 63(7): 489–94.

Jeffery CJ, Bahnson B, Chien W, Ringe D, Petsko GA (2000) Crystal structure of rabbit phosphoglucose isomerase, a glycolytic enzyme that moonlights as neuroleukin, autocrine motility factor, and differentiation mediator. *Biochemistry* 39: 955–64.

Jimenez-Asensio J, Gonzalez P, Zigler JS Jr, Garland DL (1995). Glyceraldehyde 3-phosphate dehydrogenase is an enzyme-crystallin in diurnal geckos of the genus Phelsuma. *Biochemical and Biophysical Research Communications* 209: 796–802.

Jin H, Song YP, Boel G, Kochar J, Pancholi V (2005) Group A streptococcal surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *Journal of Molecular Biology* 350: 27–41.

Kennedy MC, Mende-Mueller L, Blondin GA, Beinert H (1992) Purification and characterization of cytosolic aconitase from beef liver and its relationship to the iron-responsive element binding protein. *Proceedings of the National Academy of Science, USA* 89: 11730–4.

Kinnby B, Booth NA, Svensater G (2008) Plasminogen binding by oral streptococci from dental plaque and inflammatory lesions. *Microbiology* 154: 924–931.

Kinoshita H, Uchida H, Kawai Y, Kawasaki T, Wakahara N, Matsuo H, Watanabe M, Kitazawa H, Ohnuma S, Miura K, Horii A, Saito T (2008) Cell surface *Lactobacillus plantarum* LA 318 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) adheres to human colonic mucin. *Journal of Applied Microbiology* 104: 1667–1674.

Krojer T, Garrido-Franco M, Huber R, Ehrmann M, Clausen T (2002) Crystal structure of DegP (HtrA) reveals a new protease-chaperone machine. *Nature* 416: 455–9.

Kunert A, Losse J, Gruszin C, Huhn M, Kaendler K, Mikkat S, Volke D, Hoffmann R, Jokiranta TS, Seeberger H, Moellmann U, Hellwage J, Zipfel PF (2007) Immune evasion of the human pathogen *Pseudomonas aeruginosa*: elongation factor Tuf is a factor H and plasminogen binding protein. *Journal of Immunology* 2179: 2979–88.

Lay AJ, Jiang XM, Kisker O, Flynn E, Underwood A, Condron R, Hogg PJ (2000) Phosphoglycerate kinase acts in tumour angiogenesis as a disulphide reductase. *Nature* 408: 869–73.

Li X, Gu M, Etlinger JD (1991) Isolation and characterization of a novel endogenous inhibitor of the proteasome. *Biochemistry* 30: 9709–9715.

Lottenberg R, Broder CC, Boyle MD, Kain SJ, Schroeder BL, Curtiss R (1992) Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. *Journal of Bacteriology* 174: 5204–10.

Lu C, Ward PS, Kapoor GS, Rohle D, Turcan S, Abdel-Wahab O, Edwards CR, Khanin R, Figueroa ME, Melnick A, Wellen KE, O'Rourke DM, Berger SL, Chan TA, Levine RL, Mellinghoff IK, Thompson CB (2012) IDH mutation impairs histone demethylation and results in a block to cell differentiation. *Nature* 483: 474–8.

Mani M, Chen C, Amblee V, Liu H, Mathur T, Zwicke G, Zabad S, Patel B, Thakkar J, Jeffery CJ (2015) MoonProt: a database for proteins that are known to moonlight. *Nucleic Acids Research* 43: D277–82.

Menzel R, Roth R (1981*a*) Purification of the putA gene product: a bifunctional membrane-bounded protein from *Salmonella typhimurium* responsible for the two-step oxidation of proline to glutamate. *Journal of Biological Chemistry* 256: 9755–9761.

Menzel R, Roth R (1981*b*) Enzymatic properties of the purified putA protein from *Salmonella typhimurium*. *Journal of Biological Chemistry* 256: 9762–6.

Meyer J, Walker-Jonah A, Hollenberg CP (1991) Galactokinase encoded by GAL1 is a bifunctional protein required for induction of the GAL genes in *Kluyveromyces lactis* and is able to suppress the gal3 phenotype in *Saccharomyces cerevisiae*. *Molecular Cell Biology* 11: 5454–61.

Numata O (1996) Multifunctional proteins in Tetrahymena: 14-nm filament protein/citrate synthase and translation elongation factor-1 alpha. *Internal Review of Cytology* 164: 1–35.

Ostrovsky de Spicer P, Maloy S (1993) PutA protein, a membrane–associated flavin dehydrogenase, acts as a redox-dependent transcriptional regulator. *Proceedings of the National Academy of Science, USA* 90: 4295–8.

Ostrovsky de Spicer P, O'Brien K, Maloy S (1991) Regulation of proline utilization in *Salmonella typhimurium*: a membrane-associated dehydrogenase binds DNA in vitro. *Journal of Bacteriology* 173: 211–9.

Philpott CC, Klausner RD, Rouault TA (1994) The bifunctional iron-responsive element binding protein/cytosolic aconitase: the role of active-site residues in ligand binding and regulation. *Proceedings of the National Academy of Science, USA* 91: 7321–5.

Safer D, Sosnick TR, Elzinga M (1997) Thymosin beta 4 binds actin in an extended conformation and contacts both the barbed and pointed ends. *Biochemistry* 36: 5806–16.

Scheerer P, Borchert A, Krauss N, Wessner H, Gerth C, Hohne W, Kuhn H (2007) Structural basis for catalytic activity and enzyme polymerization of phospholipid hydroperoxide glutathione peroxidase-4 (GPx4). *Biochemistry* 46(31): 9041–49.

Schneewind O, Mihaylova-Petkov D, Model P (1993) Cell wall sorting signals in surface proteins of gram-positive bacteria. *EMBO Journal* 12: 4803–11.

Shimojima M, Hoffmann Benning S, Garavito RM, Benning C (2005) Ferredoxin-dependent glutamate synthase moonlights in plant sulfolipid biosynthesis by forming a complex with SQD1. *Archives of Biochemistry and Biophysics* 436: 206–14.

Sjstrm I, Grndahl H, Falk G, Kronvall G, Ullberg M (1997) Purification and characterisation of a plasminogen-binding protein from *Haemophilus influenzae*. Sequence determination reveals identity with aspartase. *Biochimica et Biophysica Acta* 1324: 182–90.

Stapel SO, de Jong WW (1983) Lamprey 48-kDa lens protein represents a novel class of crystallins. *FEBS Letters* 162: 305–9.

Stutts MJ, Canessa CM, Olsen JC, Hamrick M, Cohn JA, Rossier BC, Boucher RC (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science* 269(5225): 847–50.

van Rheede T, Amons R, Stewart N, de Jong WW (2003) Lactate dehydrogenase A as a highly abundant eye lens protein in the platypus (*Ornithorhynchus anatinus*): upsilon crystallin. *Molecular Biology and Evolution* 20: 994–8.

Walden WE, Selezneva AI, Dupuy J, Volbeda A, Fontecilla-Camps JC, Theil EC, Volz K (2006) Structure of dual function iron regulatory protein 1 complexed with ferritin IRE-RNA. *Science* 314: 1903–8.

Wampler JL, Kim KP, Jaradat Z, Bhunia AK (2004) Heat shock protein 60 acts as a receptor for the Listeria adhesion protein in Caco-2 cells. *Infection and Immunity* 72: 931–6.

Watanabe H, Carmi P, Hogan V, Raz T, Silletti S, Nabi IR, Raz A (1991) Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. *Journal of Biological Chemistry* 266: 13442–8.

Watanabe H, Takehana K, Date M, Shinozaki T, Raz A (1996) Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. *Cancer Research* 56: 2960–3.

Wetmur JG, Bishop DF, Cantelmo C, Desnick RJ (1986) Human sigma-aminolevulinate dehydratase: Nucleotide sequence of a full-length cDNA clone. *Proceedings of the National Academy of Science, USA* 83: 7703–7.

Williams LA, Ding L, Horwitz J, Piatigorsky J (1985) tau-Crystallin from the turtle lens: purification and partial characterization. *Experimental Eye Research* 40: 741–9.

Wistow G, Piatigorsky J (1987) Recruitment of enzymes as lens structural proteins. *Science* 236: 1554–6.

Wu N, Yu H (2012) The Smc complexes in DNA damage response. *Cell and Bioscience* 2: 5.

Xolalpa W, Vallecillo AJ, Lara M, MendozaHernandez G, Comini M, Spallek R, Singh M, Espitia C (2007) Identification of novel bacterial plasminogen-binding proteins in the human pathogen Mycobacterium tuberculosis. *Proteomics* 7: 3332–41.

Xu W, Seiter K, Feldman E, Ahmed T, Chiao JW (1996) The differentiation and maturation mediator for human myeloid leukemia cells shares homology with neuroleukin or phosphoglucose isomerase. *Blood* 87: 4502–6.

Xu W, Yang H, Liu Y, Yang Y, Wang P, Kim SH, Ito S, Yang C, Wang P, Xiao MT, Liu LX, Jiang WQ, Liu J, Zhang JY, Wang B, Frye S, Zhang Y, Xu YH, Lei QY, Guan

KL, Zhao SM, Xiong Y (2011) Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of α-ketoglutarate-dependent dioxygenases. *Cancer Cell* 19: 17–30.

Yan H, Parsons DW, Jin G, McLendon R, Rasheed BA, Yuan W, Kos I, Batinic-Haberle I, Jones S, Riggins GJ, Friedman H, Friedman A, Reardon D, Herndon J, Kinzler KW, Velculescu VE, Vogelstein B, Bigner DD (2009) IDH1 and IDH2 mutations in gliomas. *New England Journal of Medicine* 360: 765–73.

Yang W, Li E, Kairong T, Stanley SL Jr (1994) *Entamoeba histolytica* has an alcohol dehydrogenase homologous to the multifuctional adhE gene product of *Escherichia coli*. *Molecular and Biochemical Parasitology* 64: 253–60.

Young JD, Lawrence AJ, MacLean AG, Leung BP, McInnes IB, Canas B, Pappin DJ, Stevenson RD (1999) Thymosin beta 4 sulfoxide is an anti-inflammatory agent generated by monocytes in the presence of glucocorticoids. *Nature Medicine* 5: 1424–7.

Zhao B, Lin X, Lei L, Lamb DC, Kelly SL, Waterman MR, Cane DE (2008) Biosynthesis of the sesquiterpene antibiotic albaflavenone in *Streptomyces coelicolor* A3(2). *Journal of Biological Chemistry* 283: 8183–9.

Zhao B, Lei L, Vassylyev DG, Lin X, Cane DE, Kelly SL, Yuan H, Lamb DC, Waterman MR (2009) Crystal structure of albaflavenone monooxygenase containing a moonlighting terpene synthase active site. *Journal of Biological Chemistry* 284: 36711–9.

2

Exploring Structure–Function Relationships in Moonlighting Proteins

Sayoni Das[1], Ishita Khan[2], Daisuke Kihara[3], and Christine Orengo[1]

[1] *Institute of Structural and Molecular Biology, University College London, Gower Street, London, UK*
[2] *Department of Computer Science, Purdue University, North University Street, West Lafayette, Indiana, USA*
[3] *Department of Biological Sciences, Purdue University, Martin Jischke Drive, West Lafayette, Indiana, USA*

2.1 Introduction

As the availability of large-scale genomic data and the technical advancements in high-throughput biological experiments reach an astonishingly high level, the functional characterization of proteins becomes more sophisticated than ever. Consequently, an increasing number of proteins have been found to moonlight, that is, perform multiple independent cellular functions within a single polypeptide chain (see Chapter 1 for more details of the definition of protein moonlighting).

The functional diversity of moonlighting proteins is not a consequence of gene fusions, splice variance, proteins performing different functions in different cellular contexts, varying post-transcriptional modifications, homologous but non-identical proteins, or multiple photolytic fragments. The multiple roles of moonlighting proteins are not restricted to certain organisms or protein families, nor do they have a common mechanism through which they switch between different functions. Experimentally identified moonlighting proteins have been shown to switch functions as a consequence of changes in cellular locations within and outside the cell, expression in different cell types, oligomerization states, ligand binding locations, binding partners, and complex formation [1–3].

A large number of moonlighting proteins have been found to be involved in bacterial virulence, DNA synthesis or repair, cancer cell motility, and angiogenesis, among others. As an example, neuropilin is a moonlighting protein that is known to show diverse functions due to changes in cellular contexts. In endothelial cells, it is a vascular endothelial cell growth factor (VEGF) receptor and a major regulator of angiogenesis, vasculogenesis, and vascular permeability. However, in nerve axons, it is a receptor for a different ligand (Semaphorin III) and mediates neuronal cell guidance.

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

More than 300 moonlighting proteins are known in the literature today (see Chapter 1); however, the rapid increase in the number of identified moonlighting proteins suggests that the phenomenon may be common in all kingdoms of life. So far, the moonlighting function(s) of the known proteins have mostly been discovered by serendipity and little is known about the molecular mechanisms of such moonlighting actions [2]. Consequently, any efforts to characterize the molecular mechanisms of such proteins and understand their structure–function relationship would aid in identifying new moonlighting functions and help to better understand of the complex functional interplay of moonlighting proteins in the cell.

In this chapter, we first briefly discuss the different contexts in which protein function can be described, the complex structure–function relationship in proteins, followed by the current approaches used in identifying and characterizing moonlighting proteins. We then propose a classification of moonlighting proteins based on the structure–function analysis of selected moonlighting proteins. A few examples of moonlighting proteins in each classification are described in detail, many of which are implicated in bacterial virulence. Finally, we describe some general trends observed in the analysis which will, we hope, be valuable in understanding how a moonlighting protein can perform more than one unrelated function.

2.2 Multiple Facets of Protein Function

The phrase "protein function" is very ambiguous, as the functional role of a protein can be described in many different contexts. It can be described in terms of: (1) the molecular function of the protein; (2) its role in biological pathway(s); or (3) its cellular location. Natural language annotations in databases and the literature are too vague and unspecific to accurately describe the function(s) of a protein. This has led to the development of a common organized protein annotation vocabulary such as the Enzyme Commission (EC) number and Gene Ontology (GO) [4], which are the most commonly used protein function annotation resources.

The Enzyme Commission (EC) number [5] system is a numerical classification system for enzymes that uses a hierarchical set of four numbers separated by periods to represent the catalytic reaction that it carries out. For example, the EC number 5.3.1.9 describes an isomerase (EC 5.-.-.-) that acts as an intramolecular oxidoreductase (EC 5.3.-.-) and interconverts aldoses and ketoses (EC 5.3.1.-) using glucose-6-phosphate as the substrate (EC 5.3.1.9).

The Gene Ontology (GO) [4] is the most comprehensive and widely used resource of protein annotations. GO annotation can be used to assign functional terms to both enzymes and non-enzymes from three structured, non-overlapping ontologies in a species-independent manner: (1) molecular function ontology (MFO) describes the biochemical activity of the protein at the molecular level; (2) biological process ontology (BPO) describes the cellular processes and pathways in which the protein is involved; and (3) cellular component ontology (CCO) describes the compartment(s) of the cell in which the protein performs its action.

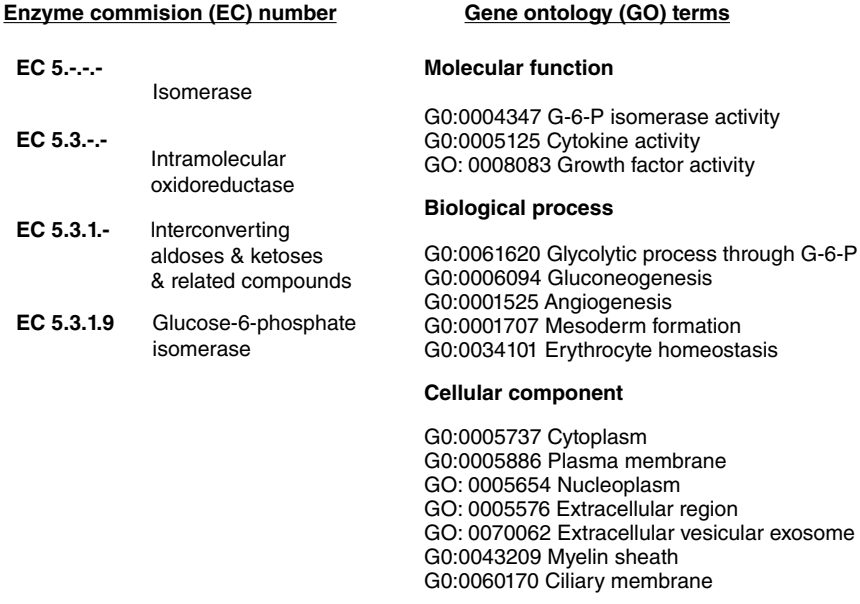

Figure 2.1 Function annotations for the mouse protein, glucose-6-phosphate isomerase (Uniprot Accession no. P06745) from the Enzyme Commision (EC) number system and Gene Ontology.

The sources of these annotations can be literature references, experimental results, author statements, database references, or computational outputs.

Figure 2.1 shows the functional annotations for the enzyme Phosphoglucose isomerase (PGI) in the mouse, which can moonlight as a tumor-secreted cytokine and angiogenesis factor and also as a neurotrophic factor. The EC number can only describe the catalytic function of PGI; however, the GO annotations from the three ontologies are sufficient to completely describe the functions of the moonlighting protein (see Figure 2.1).

2.3 The Protein Structure–Function Paradigm

Knowledge of the three-dimensional structure of a protein plays an important role in understanding the molecular mechanisms underlying its function. The three-dimensional structures of proteins can often provide more functional insight than simply knowing the protein's sequence (Fig. 2.2). For example, the structure reveals the overall conformation of the protein along with the biological multimeric state of the protein. It reveals the binding sites, interaction surfaces and the spatial relationships of catalytic residues. Protein–ligand complexes provide details of the nature of the ligand and its precise binding site, which helps in postulating the catalytic mechanism. The PDBsum resource [6] provides pictorial analyses for every structure in the PDB along with detailed information extracted from various resources such as SwissProt, Catalytic Site Atlas (CSA), Pfam, and CATH, which are beneficial for structure–function studies.

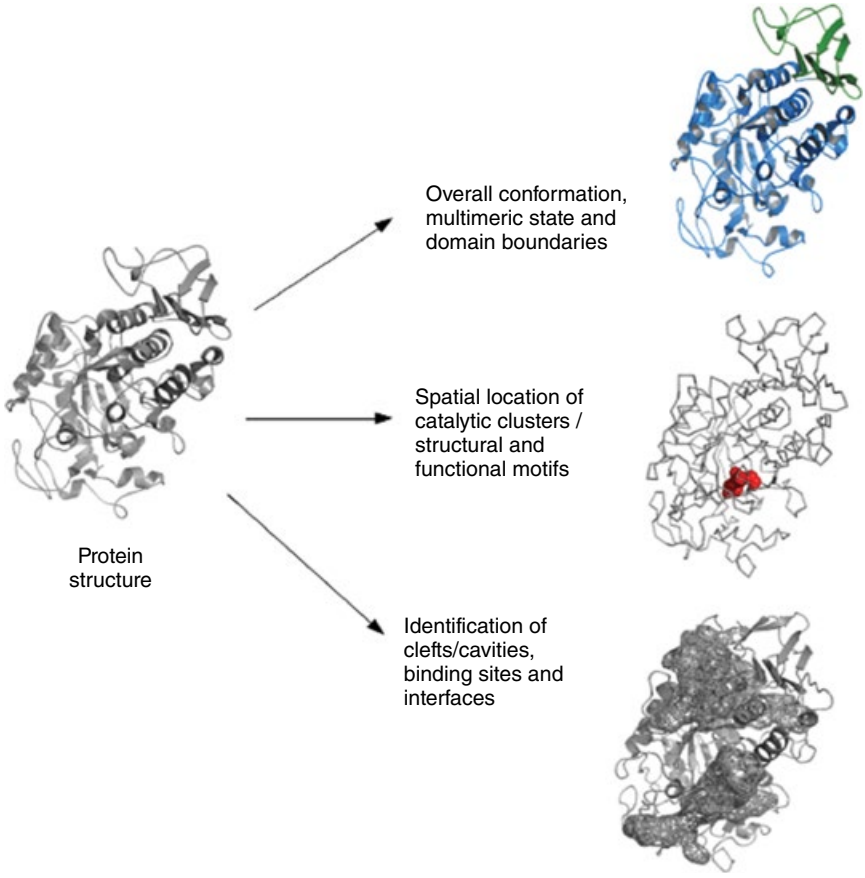

Figure 2.2 From protein structure to function.

Furthermore, *ab initio* prediction of binding pockets and clefts on the protein structure, using methods such as pvSOAR, CASTp, SURFACE, SiteEngine, and THEMATICScan, also provides useful information about protein function.

Proteins are composed of one or more building blocks called domains which are distinct, compact units of protein structure. These domains often combine in a mosaic manner in multidomain proteins (domain shuffling), generating new or modified functions [7]. Structural similarity between homologous proteins is more highly conserved during evolution than sequence and is therefore helpful in recognizing even distantly related proteins. Domains are considered to have the same fold if they share the same orientation and connectivity of the secondary structures. Specific domains within a protein are often found to have distinct functional roles, but sometimes more than one domain may be involved in a particular function, for example where an enzyme's active site is formed at the interface between two domains.

Evolutionarily related proteins having high fold similarity often share functional similarity. As a result, protein functions can sometimes be inferred by comparing the structure of the query protein with that of an experimentally

characterized protein. Structural relationships can be captured by using various well-established algorithms, for example DALI [8], SSAP [9], STRUCTAL [10], CE [11], MAMMOTH [12], FATCAT [13] and CATHEDRAL [14].

Protein structure classification databases such as CATH and SCOP have enabled detailed analysis of structure–function relationships between evolutionarily related proteins. CATH and SCOP extract structural information from the PDB and classify domains into different classes, folds, and homologous superfamilies in a hierarchical manner based on their structural relationships and evolutionary origin. Studies based on these resources have shown that the structure–function relationship of proteins is very complex and fold similarity may not always be sufficient to conclude functional similarity [15]. For example, some folds such as the Rossmann fold and TIM barrels can carry out a large number of different functions, and many different folds can be associated with the same function.

At the time of writing, the CATH database comprises around 1400 protein folds and approximately 3000 homologous domain superfamilies. These have been found to comprise at least 70% of protein domain sequences [16], which suggests that a relatively limited number of folds carry out the huge diversity of functions observed in protein function space [17–19]. For the majority of the domain superfamilies in CATH (>90%), domains have highly similar structures and functions. However, these conserved superfamilies tend to be small and highly specific to certain species or subkingdoms of life. Most of the remaining superfamilies can incorporate large amounts of structural and functional diversity and are highly populated, accounting for >50% of all known domains. Structural and functional diversity between domains in enzyme superfamilies can be attributed to the use of different sets of residues in their active site, the addition of secondary structure embellishments to the core domain structure, or domain recruitment [20].

A recent study on the diversity of functional sites in CATH superfamilies by [21] showed that, for most superfamilies, the spatial locations of functional sites are limited. By contrast, members of the most diverse superfamilies show a considerable amount of functional plasticity, as their relatives can exploit different sites for interacting with their protein partners or for binding small-ligands.

By subclassifying these diverse superfamilies into functional families [16], it is possible to group relatives sharing a common functional site and similar functional properties. Structural similarity with a protein in a CATH functional family can therefore be used to infer functional properties more accurately. Functional family classification also provides a means to understand the mechanisms of its functional divergence during evolution.

2.4 Computational Approaches for Identifying Moonlighting Proteins

Existing computational approaches to the analysis of moonlighting proteins explore different aspects of proteins characteristics, varying from sequence data, protein–protein interaction (PPI) data, to structural properties. Two recent studies have investigated how well the current sequence-based methods characterize the

functional diversity of moonlighting proteins [22, 23]. Gomez *et al*. [24] have also conducted an analysis on the PPI networks of moonlighting proteins and statistically quantified if the interacting partners of the moonlighting proteins can identify the moonlighting functions. Hernandez *et al*. [25] explored structural aspects of moonlighting proteins and analyzed whether the diverse functions are caused by conformational fluctuations in the disordered regions of their structures. Becker *et al*. [26] analyzed the human PPI network and developed a novel clustering method that can decompose a network into multiple overlapping clusters. They reported that proteins that belong to the overlapping clusters are more central in the network compared to mono-clustered proteins and contain multiple domains; they are therefore candidates for multitasking proteins. There exist two publicly available databases that serve as a repository of moonlighting proteins: MoonProt [27] and MultitaskProtDB [28]. These databases store the primary and secondary functions of known moonlighting proteins, UniProt accessions, species information, and PDB codes (if available), among other information. Computational works of moonlighting proteins were recently summarized in a review article [29].

Khan *et al*. [30] explored the functional diversity of moonlighting proteins in a computational framework and identified multiple proteomics characteristics of these proteins. Initially the existing gene ontology (GO) annotations of experimentally known moonlighting proteins were explored and the diversity of GO terms of a protein in the hierarchical GO structure quantified. Based on this GO similarity/distance, GO terms of moonlighting proteins were then clustered into groups characterized by their multiple functions. This allowed the identification of novel moonlighting proteins in the *Escherichia coli* genome and a subset of these were confirmed by literature analysis. Subsequently, context-driven analysis of moonlighting proteins was performed to explore how moonlighting proteins interacted with other proteins in a physical interaction network, through similar gene expression profiles, phylogenetic profiles, and by means of genetic interactions. This led to two notable observations: moonlighting proteins tend to interact with a more functionally diverse group of proteins than non-moonlighting proteins, and most interacting partners of moonlighting proteins share their primary function. Moreover, a significant number of interacting partners of the moonlighting proteins had indications of being moonlighting proteins themselves. In other -omics-scale analyses (i.e., based on gene expression), phylogenetically related proteins, and genetic interactions, the weak trend that on average moonlighting proteins interact with more functionally diverse proteins was observed. Structural characteristics of moonlighting proteins (i.e., ligand-binding sites and intrinsic disordered regions) were also investigated. In several cases, the ligand-binding sites for distinct functions are located in separate regions of the protein's tertiary structure.

2.5 Classification of Moonlighting Proteins

In this section we examine the structural data that are available for moonlighting proteins and propose a classification of moonlighting proteins based on the spatial locations of the experimentally verified functional sites exploited by a protein to

Table 2.1 Proteins having distinct sites for different functions in the same domain.

Protein (organism)	Function 1	Function 2	Structure	Refs
Enolase (*S. pneumoniae*)	Enolase (EC 4.2.1.11)	Binds plasminogen	1W6T	[31]
Albaflavenone monooxygenase (*S. coelicolor A3(2)*)	Albaflavenone monooxygenase (EC 1.14.13.106)	Terpene synthase (EC 4.2.3.47)	3EL3	[32, 33]
MAPK1/ERK2 (*H. sapiens*)	Mitogen-activated protein kinase 1 (EC 2.7.11.24)	Transcriptional repressor (binds DNA)	4G6N	[34]
1-Cys Peroxiredoxin (*H. sapiens*)	Phospholipase A2 (EC 3.1.1.4)	Glutathione peroxidase (EC 1.11.1.15)	1PRX	[35]
Cytochrome C (*S. cerevisiae*)	Electron carrier protein in electron transport chain	Promotes apoptosis (binds Apaf-1)	1YCC	[36]
GCN4 (*S. cerevisiae*)	Transcription factor (binds DNA)	Ribonuclease (EC 3.1.27.5)	1YSA	[37]
I-Anii (*A. nidulans*)	Homing endonuclease (EC 3.1.-.-)	Transcriptional repressor (binds DNA)	3EH8	[3, 38]

perform its primary and moonlighting function(s). The primary and moonlighting function(s) of the proteins are referred to as 'Function 1' and 'Function 2' in the following. The moonlighting proteins were taken from the database of moonlighting proteins, MoonProt [27], and recently published papers which had known structural information along with experimentally verified functional sites responsible for the primary and moonlighting function(s) of the protein. Information on catalytic site residues for the proteins was extracted from the Catalytic Site Atlas (CSA), and additional functional annotation was extracted from PDBsum [6] and SwissProt. The various categories are proteins: (1) having distinct sites for different functions in the same domain (Table 2.1 [31–38]); (2) having distinct sites for different functions in different domains (Table 2.2; [39–44]); (3) using the same residues for different functions (Table 2.3; [45–47]); (4) using different residues in the same/overlapping site for different functions (Table 2.4; [48–53]); and (5) using different structural conformations or folds for different functions (Table 2.5; [54–56]).

2.5.1 Proteins with Distinct Sites for Different Functions in the Same Domain

These are single domain or multidomain proteins (listed in Table 2.1) that use distinct spatial functional sites of a single domain for carrying out their primary and moonlighting function(s).

2.5.1.1 α-Enolase, *Streptococcus pneumonia*

α-Enolase (EC 4.2.1.11) from *Streptococcus pneumoniae* is a key glycolytic enzyme (Function 1) that is also expressed on the bacterial cell surface, where it binds to human plasminogen to facilitate the host invasion process during infection

Table 2.2 Proteins having distinct sites for different functions in different domains.

Protein (organism)	Function 1	Function 2	Structure	Refs
Malate synthase (*M. tuberculosis*)	Malate synthase (EC 2.3.3.9)	Binds laminin	2GQ3	[39]
BirA (*E. coli*)	Biotin holoenzyme synthetase (EC 6.3.4.15)	Bio repressor	1BIB	[40]
MRDI (*H. sapiens*)	MTR-1-P isomerase (EC 5.3.1.23)	Mediator of cell invasion	4LDQ	[41]
Hexokinase 2 (*S. cerevisiae*)	Hexokinase 2 (EC 2.7.1.1)	Glucose sensor (interacts with transcriptional repressor Mig1)	1IG8	[42]
Neuropilin-I (*H. sapiens*)	Semaphorin binding	VEGF binding	2QQN	[43]
ATF2 (*H. sapiens*)	Transcription factor	DNA damage response	1T2K	[44]

Table 2.3 Proteins using the same residues for different functions.

Protein (organism)	Function 1	Function 2	Structure	Refs
GAPDH (*E. coli*)	GAPDH (EC 1.2.1.12)	NAD ribosylating activity	1DC5	[45]
Leukotriene A-4 hydrolase (*H. sapiens*)	Leukotriene A-4 hydrolase (EC 3.3.2.6)	Aminopeptidase (EC 3.4.11.24)	2R59	[46]
Hemagglutinin (*Paramyxovirus*)	Hemagglutinin binding	Neuraminidase (EC 3.2.1.18)	1E8T	[47]

Table 2.4 Proteins using different residues in the same/overlapping site for different functions.

Protein (organism)	Function 1	Function 2	Structure	Refs
Phosphoglucose isomerase (*O. cuniculus*, *H. sapiens*)	Phosphoglucose isomerase (EC 5.3.1.9)	Autocrine motility factor, neuroleukin, differentiation, and maturation mediator	1DQR, 1IAT	[48, 49]
Fructose-bisphosphate aldolase (*P. falciparum*)	Fructose-bisphosphate aldolase (EC 4.1.2.13)	Attaches actin to trap proteins	2PC4	[50]
Gpx4 (*H. sapiens*)	Phospholipid hydroperoxide glutathione peroxidase (EC 1.11.1.12)	Polymerized form has structural role in spermatozoa	2OBI	[51]
S1O ribosomal/ protein (*E. coli*)	Component of ribosomal 30S subunit	Part of transcription antitermination complex	1O9J	[52]
Lens crystallin/retinal DH (*E. edwardii*)	Lens crystallin	Retinal DH (EC 1.2.1.3)	1O9J	[53]

Table 2.5 Proteins using different folds for different functions.

Protein (organism)	Function 1	Function 2	Structure	Refs
RfaH (*E. coli*)	Transcription factor	Translational regulator	2OUG, 2LCL	[54, 55]
Lymphotactin (Ltn) (*H. sapiens*)	Chemokine (activates XCR1)	Binds cell-surface glycosaminoglycans	2JP1	[56]

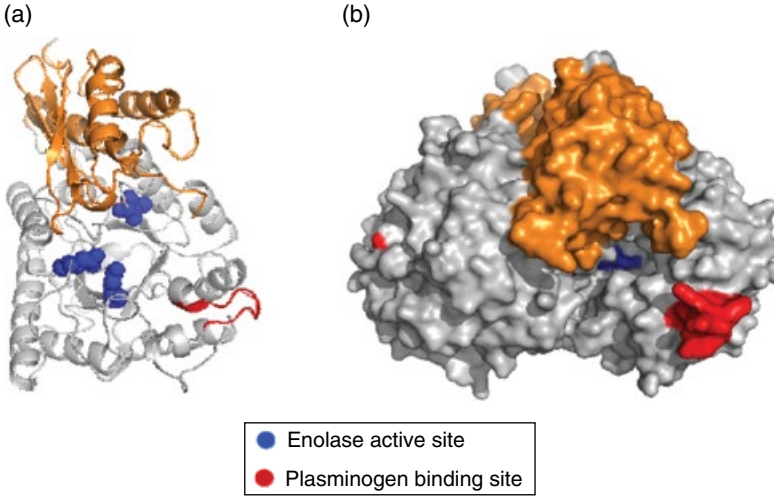

Figure 2.3 α-Enolase. (a) Single chain of Enolase showing the enzyme active site in blue and the plasminogen-binding site in red. (b) Enolase monomer displayed as surface. Different domains are colored in gray and orange (PDB:1W6T). (*See color plate section for the color representation of this figure.*)

(Function 2) [31]. The protein is known to exist in an octameric state both in the cytoplasm and on the cell surface. Each monomer of α-enolase consists of a 2-layer αβ sandwich domain and a TIM barrel domain (Fig. 2.3). The structurally conserved α-enolase active site is located in the TIM barrel which comprises the catalytic residues Glu164, Glu205, and Lys342 in *S. pneumoniae*, which are located in a pocket. Two plasminogen-binding sites have also been found in the TIM barrel domain at sites distinct from the active site that includes a nine-residue internal motif (248 FYDKERKYV256) and terminal lysine residues (433KK434). The former site has been shown to have a more important role in interacting with plasminogen than the latter. The last lysine residue is not included in the structure as it is disordered.

2.5.1.2 Albaflavenone monooxygenase, *Streptomyces coelicolor A3(2)*

Albaflavenone monooxygenase (CYP170A1) (EC 1.14.13.106) from *Streptomyces coelicolor A3(2)* catalyzes the last two steps in the biosynthesis of the antibiotic albaflavenone (Function 1) [32]. Study of the crystal structure of albaflavenone monooxygenase showed that it exists as a dimer having two chains, each consisting of a single, α orthogonal bundle (Fig. 2.4). These studies also revealed a second,

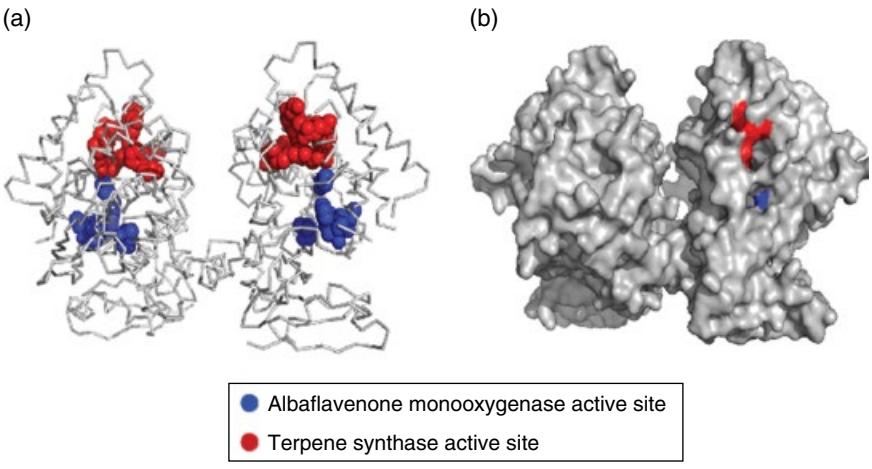

Figure 2.4 Albaflavenone monooxygenase. The monooxygenase and terpene synthase active sites are shown in blue and red respectively in the (a) cartoon and (b) surface representation of Albaflavenone monooxygenase (PDB: 3EL3). (*See color plate section for the color representation of this figure.*)

completely distinct, catalytic activity of a terpene synthase (EC 4.2.3.47) [33] which is involved in the synthesis of farnesene isomers from farnesyl diphosphate (Function 2), by identification of signature sequences and motifs associated with terpene synthases. The residues Trp92, Pro274, Val338, Ile447, and Thr448 are involved in the monooxygenase activity, whereas the residues Arg116, Leu244, Leu248, Glu263, Val268, Leu271, Ile 272, and Phe-415 are associated with the terpene synthase activity which are located in different pockets in the protein. The monooxygenase activity was found to be optimal over the pH range 7.2–8 and was found to decline at lower pH values which favor terpene synthase activity (pH 5.5–6.5). This suggests that the two different enzymatic states of the protein possess optimal conformations at distinct pHs.

2.5.1.3 MAPK1/ERK2, *Homo sapiens*

Studies to characterize the human protein-DNA interactome revealed the human mitogen-activated protein kinase 1 (MAPK1) or extracellular signal-r kinase 2 (ERK2) (Function 1) as a DNA-binding transcriptional repressor (Function 2) that regulates interferon gamma signaling [34]. The crystal structure of the human MAPK1 exists as a monomer which contains two domains: a discontinuous αβ 2-layer sandwich domain and a mainly α-orthogonal bundle domain (Fig. 2.5). The kinase active site residues involve Asp147, Lys149, Ser151, and Asn152. The motif that has been found to help in binding DNA are 259 K ARN Y LLSLP H K N K V P W N R277. It can be seen from Figure 2.5 that the kinase active site is located far from the DNA-binding motif.

2.5.2 Proteins with Distinct Sites for Different Functions in More Than One Domain

The second category of moonlighting proteins are multidomain proteins (listed in Table 2.2) which utilize functional sites in separate domains for carrying out their primary and moonlighting function(s).

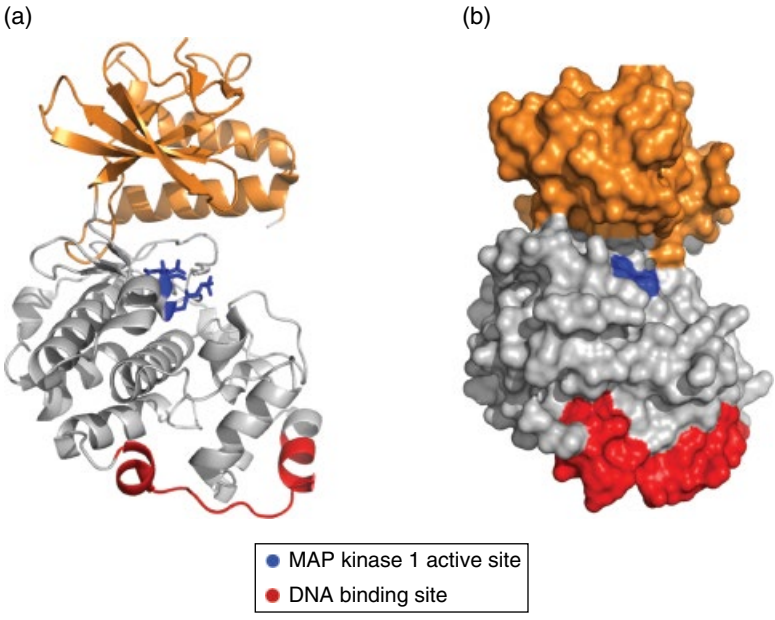

Figure 2.5 Human MAPK1/ERK2. The MAPK1 active site is shown in blue and the DNA-binding motif is highlighted in red. Different domains are shown in gray and orange (PDB:4G6N). (*See color plate section for the color representation of this figure.*)

2.5.2.1 Malate synthase, *Mycobacterium tuberculosis*

Malate synthase (EC 2.3.3.9) is a cytoplasmic enzyme (Function 1) involved in the glyoxalate pathway [57]. In *M. tuberculosis* it has also been found on the cell wall, adapted to function as an adhesin that binds laminin and fibrinogen which may contribute to *M. tuberculosis* virulence by promoting infection and dissemination (Function 2) [58]. The structure of the *M. tuberculosis* malate synthase consists of two identical chains each of which consists of four domains: an α-orthogonal bundle, a TIM barrel, a mainly β complex domain, and an α up-down bundle [58]. The malate synthase active site residues are Glu273, Asp274, Arg339, Glu434, Leu461, Asp462, and Glu633 (highlighted as blue sticks) and the residues that are associated with binding laminin or fibrinogen are Gln696–Glu727 (highlighted in red) (Fig. 2.6). Both the sites are present in different domain regions of the protein.

2.5.2.2 BirA, *Escherichia coli*

The *E. coli* BirA protein performs different functions depending on its dimeric state [40]. As a heterodimer with biotin carboxyl carrier protein (BCCP), a subunit of acetyl-CoA carboxylase, it functions as a biotin protein ligase (Function 1); as a homodimer, it functions as a biotin operon repressor (Function 2) that binds to DNA [59]. The BirA structure consists of three domains: an α-orthogonal bundle, an α/β 2-layer sandwich domain and a mainly β SH3-type fold (Fig. 2.7). The residues responsible for the two functions of BirA are located in distinct sites in the protein. The catalytic residues for the ligase activity of the protein are Arg118, Lys183, and Arg317, and are found in a pocket formed between the αβ sandwich, the SH3 domain, and a helix-turn-helix (H-T-H) motif (residues 22–46) which is responsible for the binding DNA found in the α-orthogonal bundle (Fig. 2.7) [40].

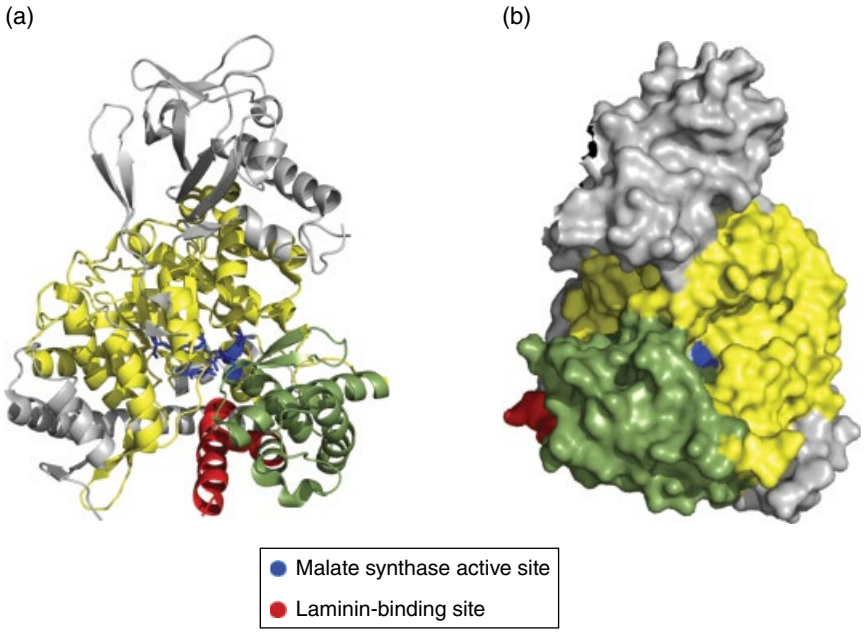

Figure 2.6 Malate synthase. The enzyme active site is shown in blue and the laminin- binding site is shown in red. Different domains are shown in different colors (PDB:2GQ3). (*See color plate section for the color representation of this figure.*)

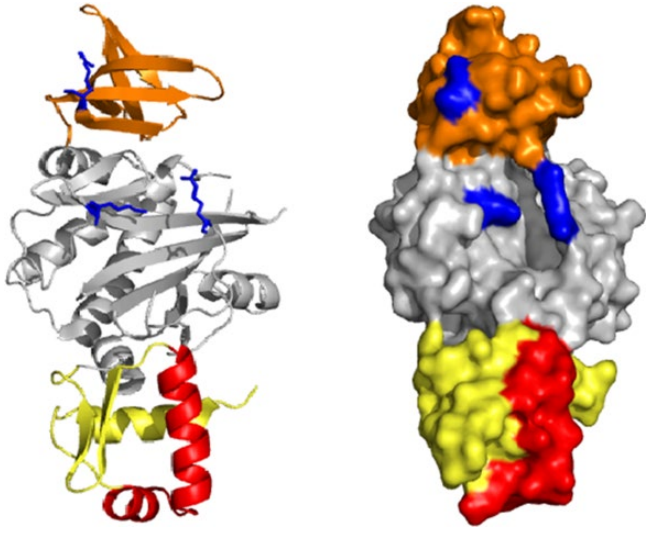

Figure 2.7 BirA. The catalytic site residues are shown in blue while the H-T-H motif involved in binding DNA (moonlighting function) is shown in red. Different domains are shown in different colors (PDB:1BIB). (*See color plate section for the color representation of this figure.*)

2.5.2.3 MRDI, *Homo sapiens*

The protein mediator of RhoA-dependent invasion (MRDI) is a moonlighting protein found in humans [41]. It acts both as a methylthioribose-1-phosphate (MTR-1-P) isomerase (EC 5.3.1.23) (Function 1) and a mediator of melanoma cell invasion (Function 2). The MRDI structure consists of two chains, each comprising a 4-helix bundle and a Rossmann fold (Fig. 2.8). The catalytic residues of MRDI are Cys168 and Asp248 (shown in blue), which are located in the base of a pocket. A potential binding site has been identified in MRDI by mutational analysis: Ser283 and Arg109 (shown in red). This is located distinct from the catalytic site in another pocket of the protein, formed between the two domains of each chain.

2.5.3 Proteins Using the Same Residues for Different Functions

These are multidomain proteins (listed in Table 2.3) which utilize the same functional site for carrying out their primary and moonlighting function(s).

2.5.3.1 GAPDH *E. coli*

The *E. coli* glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12) (Function 1) is a multifunctional housekeeping protein. It also catalyzes its own NAD+ -dependent ADP-ribosylation which has been implicated in host–pathogen interactions (Function 2) [45]. GAPDH consists of two chains, each comprising a Rossmann fold and a α3β5 sandwich domain [60]. The three catalytic residues of GAPDH are Cys149, His179, and Ser238, which are located in the sandwich domain (Fig. 2.9). However, mutational analyses have shown that the catalytic Cys149 (shown in red) is also the target residue of the ADP-ribosylation.

2.5.3.2 Leukotriene A4 hydrolase, *Homo sapiens*

Leukotriene A4 hydrolase (EC 3.3.2.6) is a bifunctional zinc metalloenzyme that converts the fatty acid epoxide leukotriene A4 (LTA4) into a potent chemoattractant, leukotriene B4 (LTB4) (Function 1) and also exhibits an anion-dependent aminopeptidase activity (EC 3.4.11.24) (Function 2) [46]. Both the enzymatic activities require the presence of the catalytic zinc which is coordinated by the

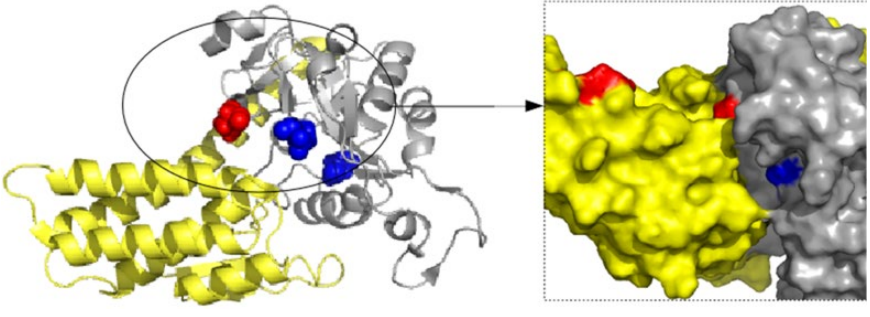

Figure 2.8 Human MRDI. The active site residues are shown in blue while the residues implicated in controlling invasion (moonlighting function) is shown in red. Different domains are shown in different colors (PDB:4LDQ). (*See color plate section for the color representation of this figure.*)

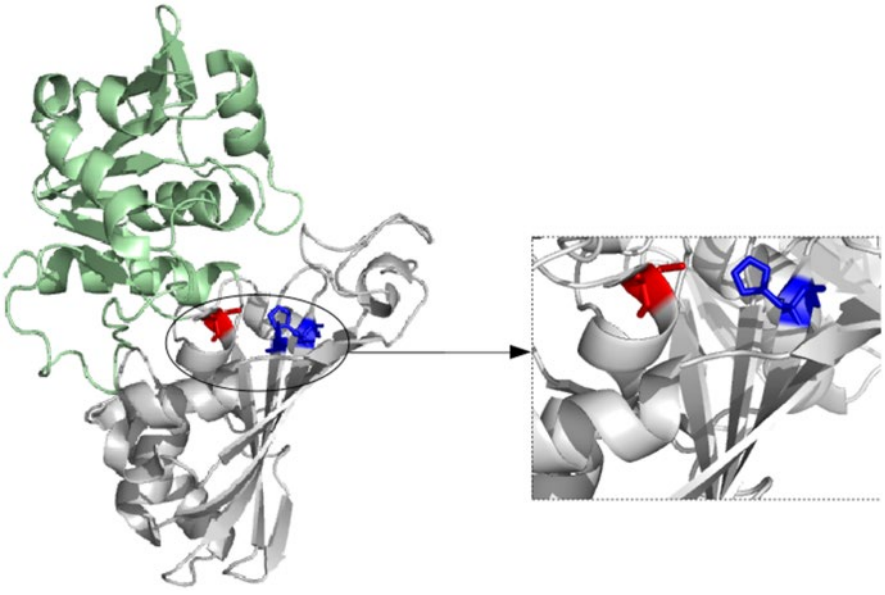

Figure 2.9 GAPDH. The catalytic site residue Cys149 (shown in red) is the residue known to be involved for both the canonical and moonlighting functions of *E. coli* GAPDH. The other catalytic residue His179 is shown in blue (PDB:1DC5). (*See color plate section for the color representation of this figure.*)

three zinc-binding residues His295, His299, and Glu318. The crystal structure of the LTA4 hydrolase consists of three domains: a β-sandwich, an α-orthogonal bundle, and a α-α superhelix (Fig. 2.10) [61]. It also contains the 269 GX M EN272 motif in the M1 family of zinc peptidases. Mutation of the catalytic residues Glu296 or Tyr383 resulted in loss of the aminopeptidase activity, and mutation of the catalytic residue Glu271 abolished both the epoxide hydrolase activity and the aminopeptidase activity. Glu271 is a unique example of a catalytic residue that has distinct roles in two separate catalytic reactions for two chemically different substrates. Based on the LTA4 hydrolase structure and structure activity studies, two mechanistic models for the role of Glu271 in the epoxide hydrolase activity and in the aminopeptidase reaction were proposed [62].

2.5.4 Proteins Using Different Residues in the Same/Overlapping Site for Different Functions

Moonlighting proteins in this category are multidomain proteins (listed in Table 2.4) which utilize overlapping functional sites for carrying out their primary and moonlighting function(s).

2.5.4.1 Phosphoglucose isomerase, *Oryctolagus cuniculus, Mus musculus, Homo sapiens*

Phosphoglucose isomerase (PGI, EC 5.3.1.9) is a glycolytic enzyme which catalyses the interconversion of glucose-6-phosphate and fructose-6-phosphate (Function 1). It is known to moonlight as an autocrine motility factor (tumor-secreted cytokine that promotes cellular growth and motility), neuroleukin (a neurotrophic factor

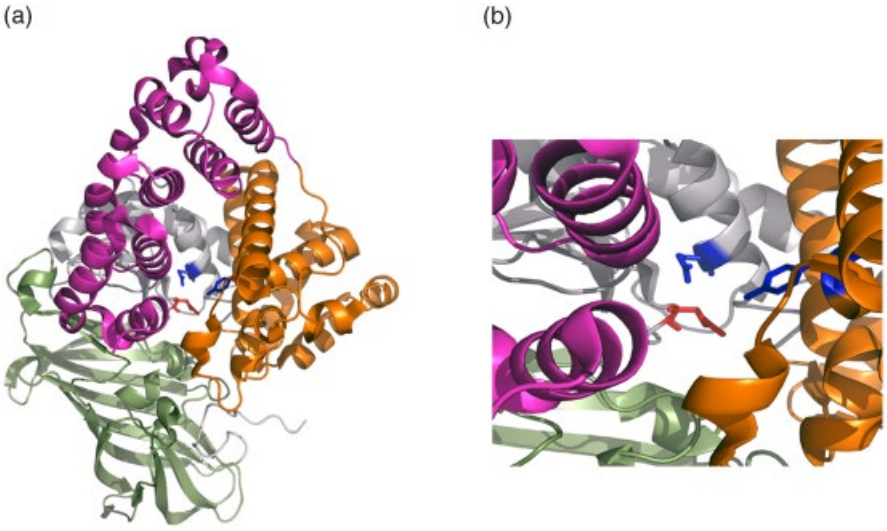

Figure 2.10 Leukotriene A4 hydrolase. The LTA4 catalytic site residues Glu296 and Tyr383 are shown in blue. The catalytic site residue Glu271, involved in two separate functions in two different catalytic reactions is shown in red (PDB:2R59). (*See color plate section for the color representation of this figure.*)

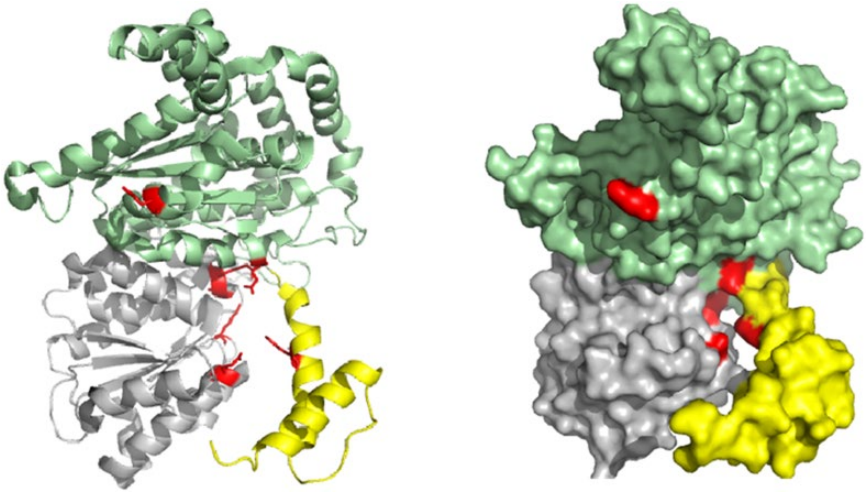

Figure 2.11 Phosphoglucose isomerase (PGI). Catalytic residues are shown as red sticks. Inhibition of enzymatic and AMF functions of PGI by the PGI inhibitor and mutational analysis of the catalytic residues have indicated overlapping regions of both functions in the human PGI (PDB:1IAT). (*See color plate section for the color representation of this figure.*)

for neurons), and differentiation mediator in mammals (Function 2) [48]. The human PGI exists as a dimer comprising three domains: two (one large and one small) Rossman fold domains and an α-orthogonal bundle [49] (Fig. 2.11). The known catalytic site residues are Lys210, Glu216, Gly271, Arg272, Glu357, His388, and Lys518 (1IAT). The PGI inhibitor erythrose-4-phosphate (E4P) is known to inhibit both the enzymatic and cell motility activities of PGI. Moreover,

mutation of the catalytic residues resulted in significant reduction in the AMF or cell-motility-stimulating activity.

2.5.4.2 Aldolase, *Plasmodium falciparum*

The fructose-bisphosphate aldolase (EC 4.1.2.13; Function 1) from apicomplexan parasites such as *P. falciparum* and *P. vivax* also provides a bridge between the actin filaments and TRAP (thrombospondin-related anonymous protein), which is critical for the host invasion machinery of the malaria parasite (Function 2) [50]. The *P. falciparum* aldolase structure consists of four chains, each consisting of a TIM barrel domain (Fig. 2.12). The aldolase active site residues and the residues involved in binding actin or TRAP overlap are located in the centre of the TIM barrel. The aldolase active site comprises the residues Asp39, Lys112, Glu194, and Lys236. The actin-binding residues of aldolase are Arg48, Lys112, Arg153, and Lys236 and the TRAP binding residues are Glu40, Lys47, Arg48, Lys151, Arg153, Arg309, and Gln312.

2.5.5 Proteins with Different Structural Conformations for Different Functions

These are "transformer proteins" [63] (listed in Table 2.5) which utilize different fold states for carrying out their primary and moonlighting function(s).

2.5.5.1 RfaH, *E. coli*

RfaH is a bacterial antitermination protein which binds to the RNA polymerase (RNAP) and suppresses pausing, Rho-dependent inhibition, and intrinsic termination at a subset of sites (Function 1) [54]. As a result, termination signals

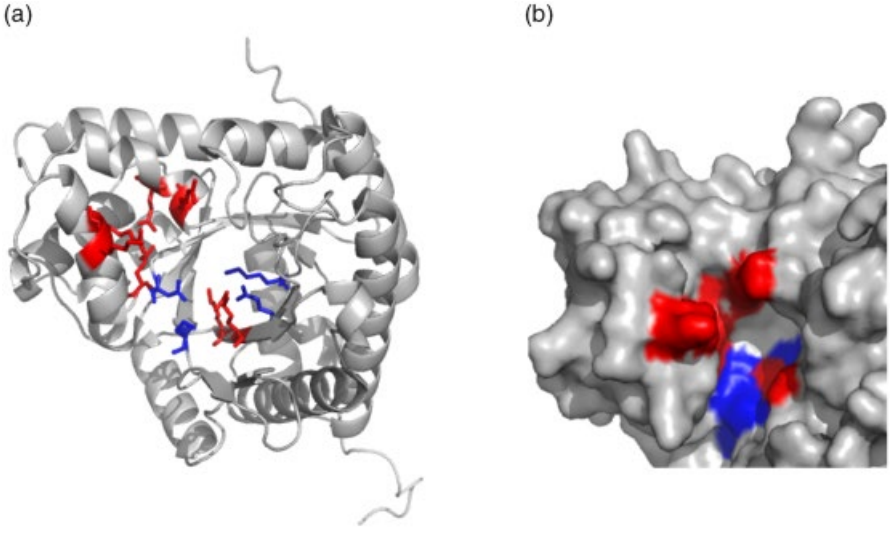

Figure 2.12 Aldolase. The enzyme active site is shown in blue and the actin-binding site is shown in red (PDB:2PC4). (*See color plate section for the color representation of this figure.*)

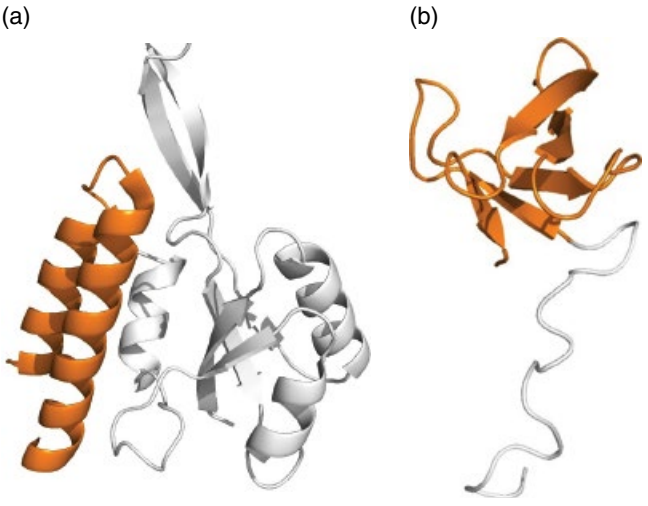

Figure 2.13 RfaH The Rfah CTD is colored in orange. In the closed form of RfaH (a), the CTD (α-helix form) and NTD tightly interacts and works as a transcription factor (PDB:2OUG). The subsequent (or simultaneous) refolding of the CTD into a (b) β-barrel transforms RfaH into a translation factor (PDB:2LCL). (*See color plate section for the color representation of this figure.*)

are bypassed, which allows complete synthesis of long RNA chains. RfaH is a two-domain protein, and the two domains are observed to interact closely in the crystal structure. The RfaH N-terminal domain (NTD) has a central antiparallel sheet surrounded by helices and the C-terminal domain (CTD) in the crystal structure is an all-helical domain. However, the solution structure of the protein, solved by NMR, showed that the RfaH CTD folds into a helical structure when it interacts with the RfaH NTD and transforms into an all-sheet fold in the absence of NTD (Fig. 2.13). These two different fold states allow the protein to perform alternative functions. When the CTD exists in the all-sheet state, it can stimulate translation by recruiting a ribosome to an mRNA lacking a ribosome-binding site (Function 2) [55].

2.6 Conclusions

From the detailed analysis of the moonlighting proteins examined in this chapter, we can see considerable structural diversity in the types of domains that have evolved a moonlighting function, together with significant diversity in the different types of moonlighting functions that have evolved in these proteins. Some of the moonlighting proteins utilize different sites for their primary and moonlighting functions; however, there are others which use overlapping regions with their primary functional site or even the same site for both functions. Our investigation of moonlighting proteins revealed two general trends of functional site utilization in moonlighting proteins.

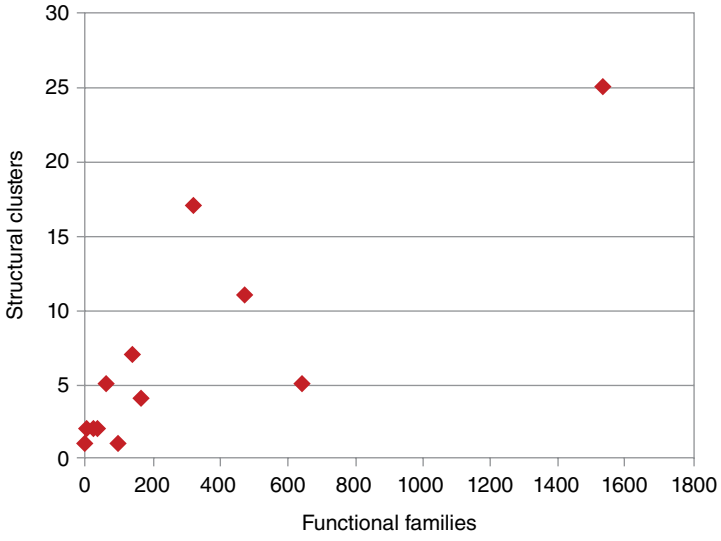

Figure 2.14 Structural diversity v. functional diversity of CATH domain superfamilies represented in the moonlighting proteins studied in this chapter. Structural diversity is represented by the number of structural clusters (domains clustered at 5Å RMSD) in the superfamilies and the functional diversity is represented by the number of functional families identified in the superfamily.

1) Type 1: The primary functional site resides in the largest pocket of the protein while the moonlighting functional site is present on a distinct exposed surface of the protein. This is true for proteins for which binding to other proteins facilitates their moonlighting function. Examples include enolase, perodoxin, MAPK1, PutA, and I-Anil.
2) Type 2: The primary and moonlighting functional sites are present on two pockets or clefts in the protein structure. These sites can be utilized for two different enzymatic reactions or one enzymatic reaction along with a binding function. Examples include cytochrome c and albaflavenone monooxygenase.

Figure 2.14 shows the structural and functional diversity of the CATH domain superfamilies that are represented in the moonlighting proteins discussed in this chapter. We observe that these proteins belong to superfamilies ranging from very low to high structural and functional diversity. Knowledge of the structural and functional diversity sheds some light on the possible routes by which they may have acquired their moonlighting function. For example, for domain superfamilies with high structural diversity, it is more likely that the new functions can emerge through structural embellishments. By contrast, domain superfamilies having low structural diversity are more likely to evolve a new function by domain recruitment or use of different amino acids.

Given the diversity of moonlighting proteins in terms of functionality, physical locations in cell, mechanisms to moonlight, and the type of genomes in which they are found to exist, moonlighting proteins seem to be abundant in nature. Moonlighting could be a more common way of reutilizing or repurposing

proteins than the currently understood method. However, only around 300 moonlighting proteins are currently known, and <40% of these have known structures. As the number of moonlighting proteins with solved structures increases, we hope to pursue these structural analyses further.

References

[1] Constance J Jeffery. Moonlighting proteins. *Trends in Biochemical Sciences*, 24(1): 8–11, 1999.

[2] Constance J Jeffery. Molecular mechanisms for multitasking: recent crystal structures of moonlighting proteins. *Current Opinion in Structural Biology*, 14(6): 663–668, 2004.

[3] Constance J Jeffery. Moonlighting proteins: complications and implications for proteomics research. *Drug Discovery Today: Targets*, 3(2): 71–78, 2004.

[4] Michael Ashburner, Catherine A Ball, Judith A Blake, David Botstein, Heather Butler, J Michael Cherry, Allan P Davis, Kara Dolinski, Selina S Dwight, Janan T Eppig, *et al*. Gene ontology: tool for the unification of biology. *Nature Genetics*, 25(1): 25–29, 2000.

[5] Amos Bairoch. The enzyme data bank. *Nucleic Acids Research*, 22(17): 3626–3627, 1994.

[6] Tjaart AP de Beer, Karel Berka, Janet M Thornton, and Roman A Laskowski. Pdbsum additions. *Nucleic Acids Research*, 42(D1): D292–D296, 2014.

[7] Matthew Bashton and Cyrus Chothia. The generation of new protein functions by the combination of domains. *Structure*, 15(1): 85–99, 2007.

[8] Liisa Holm and Chris Sander. Dali: a network tool for protein structure comparison. *Trends in Biochemical Sciences*, 20(11): 478–480, 1995.

[9] Christine A Orengo and William R Taylor. SSAP: sequential structure alignment program for protein structure comparison. *Methods in Enzymology*, 266: 617–635, 1996.

[10] Michael Levitt and Mark Gerstein. A unified statistical framework for sequence comparison and structure comparison. Proceedings of the National Academy of Sciences, 95(11): 5913–5920, 1998.

[11] Ilya N Shindyalov and Philip E Bourne. Protein structure alignment by incremental combinatorial extension (ce) of the optimal path. *Protein Engineering*, 11(9): 739–747, 1998.

[12] Angel R Ortiz, Charlie EM Strauss, and Osvaldo Olmea. Mammoth (matching molecular models obtained from theory): an automated method for model comparison. *Protein Science*, 11(11): 2606–2621, 2002.

[13] Yuzhen Ye and Adam Godzik. Fatcat: a web server for flexible structure comparison and structure similarity searching. *Nucleic Acids Research*, 32(suppl 2): W582–W585, 2004.

[14] Oliver C Redfern, Andrew Harrison, Tim Dallman, Frances MG Pearl, and Christine A Orengo. Cathedral: a fast and effective algorithm to predict folds and domain boundaries from multidomain protein structures. *PLoS Computational Biology*, 3(11): e232, 2007.

[15] Andrew CR Martin, Christine A Orengo, E Gail Hutchinson, Susan Jones, Maria Karmirantzou, Roman A Laskowski, John BO Mitchell, Chiara Taroni, and Janet M Thornton. Protein folds and functions. *Structure*, 6(7): 875–884, 1998.

[16] Ian Sillitoe, Tony E Lewis, Alison Cuff, Sayoni Das, Paul Ashford, Natalie L Dawson, Nicholas Furnham, Roman A Laskowski, David Lee, Jonathan G Lees, *et al*. Cath: comprehensive structural and functional annotations for genome sequences. *Nucleic Acids Research*, 43(D1): D376–D381, 2015.

[17] Christine A Orengo, David T Jones, and Janet M Thornton. Protein superfamilies and domain superfolds. *Nature*, 372(6507): 631–634, 1994.

[18] Chao Zhang and Charles DeLisi. Estimating the number of protein folds. *Journal of Molecular Biology*, 284(5): 1301–1305, 1998.

[19] Sridhar Govindarajan, Ruben Recabarren, and Richard A Goldstein. Estimating the total number of protein folds. *Proteins: Structure, Function, and Bioinformatics*, 35(4): 408–414, 1999.

[20] Annabel E Todd, Christine A Orengo, and Janet M Thornton. Evolution of function in protein superfamilies, from a structural perspective. *Journal of Molecular Biology*, 307(4): 1113–1143, 2001.

[21] Benoit H Dessailly, Natalie L Dawson, Kenji Mizuguchi, and Christine A Orengo. Functional site plasticity in domain superfamilies. *Biochimica et Biophysica Acta (BBA), Proteins and Proteomics*, 1834(5): 874–889, 2013.

[22] Antonio Gomez, Nuria Domedel, Juan Cedano, Jaume Piñol, and Enrique Querol. Do current sequence analysis algorithms disclose multifunctional (moonlighting) proteins? *Bioinformatics*, 19(7): 895–896, 2003.

[23] Ishita K Khan, Meghana Chitale, Catherine Rayon, and Daisuke Kihara. Evaluation of function predictions by pfp, esg, and psi-blast for moonlighting proteins. In BMC Proceedings, volume 6, page S5. BioMed Central Ltd, 2012.

[24] Antonio Gomez, Sergio Hernandez, Isaac Amela, Jaume Piñol, Juan Cedano, and Enrique Querol. Do protein–protein interaction databases identify moonlighting proteins? *Molecular BioSystems*, 7(8): 2379–2382, 2011.

[25] Sergio Hernandez, Isaac Amela, Juan Cedano, Jaume Piñol, JosepAntoni Perez-Pons, Angel Mozo-Villarias, and Enrique Querol. Do moonlighting proteins belong to the intrinsically disordered protein class. *Journal of Proteomics and Bioinformatics*, 5(11), 2012.

[26] Emmanuelle Becker, Benôit Robisson, Charles E Chapple, Alain Gúenoche, and Christine Brun. Multifunctional proteins revealed by overlapping clustering in protein interaction network. *Bioinformatics*, 28(1): 84–90, 2012.

[27] Mathew Mani, Chang Chen, Vaishak Amblee, Haipeng Liu, Tanu Mathur, Grant Zwicke, Shadi Zabad, Bansi Patel, Jagravi Thakkar, and Constance J Jeffery. Moonprot: a database for proteins that are known to moonlight. *Nucleic Acids Research*, 43: D277–282, 2014.

[28] Sergio Hernandez, Gabriela Ferragut, Isaac Amela, JosepAntoni Perez-Pons, Jaume Piñol, Angel Mozo-Villarias, Juan Cedano, and Enrique Querol. MultitaskprotDB: a database of multitasking proteins. *Nucleic Acids Research*, 42(D1): D517–D520, 2014.

[29] Ishita K Khan and Daisuke Kihara. Computational characterization of moonlighting proteins. *Biochemical Society Transactions*, 42(6): 1780–1785, 2014.

[30] Ishita Khan, Yuqian Chen, Tiange Dong, Xiaowei Hong, Rikiya Takeuchi, Hirotada Mori, and Daisuke Kihara. Genome-scale identification and characterization of moonlighting proteins. *Biology Direct*, 9(1): 30, 2014.

[31] Stefanie Ehinger, Wolf-Dieter Schubert, Simone Bergmann, Sven Hammerschmidt, and Dirk W Heinz. Plasmin (ogen)-binding α-enolase from Streptococcus pneumoniae: Crystal structure and evaluation of plasmin (ogen)-binding sites. *Journal of Molecular Biology*, 343(4): 997–1005, 2004.

[32] Bin Zhao, Xin Lin, Li Lei, David C Lamb, Steven L Kelly, Michael R Waterman, and David E Cane. Biosynthesis of the sesquiterpene antibiotic albaflavenone in streptomyces coelicolor a3 (2). *Journal of Biological Chemistry*, 283(13): 8183–8189, 2008.

[33] Bin Zhao, Li Lei, Dmitry G Vassylyev, Xin Lin, David E Cane, Steven L Kelly, Hang Yuan, David C Lamb, and Michael R Waterman. Crystal structure of albaflavenone monooxygenase containing a moonlighting terpene synthase active site. *Journal of Biological Chemistry*, 284(52): 36711–36719, 2009.

[34] Shaohui Hu, Zhi Xie, Akishi Onishi, Xueping Yu, Lizhi Jiang, Jimmy Lin, Hee-sool Rho, Crystal Woodard, Hong Wang, Jun-Seop Jeong, *et al*. Profiling the human protein-dna interactome reveals erk2 as a transcriptional repressor of interferon signaling. *Cell*, 139(3): 610–622, 2009.

[35] Aron B Fisher. Peroxiredoxin 6: a bifunctional enzyme with glutathione peroxidase and phospho- lipase a2 activities. *Antioxidants & Redox Signaling*, 15(3): 831–844, 2011.

[36] Maria LR Lim, May-Ghee Lum, Tania M Hansen, Xavier Roucou, and Phillip Nagley. On the release of cytochromec from mitochondria during cell death signaling. *Journal of Biomedical Science*, 9(6): 488–506, 2002.

[37] Yaroslav Nikolaev, Christine Deillon, Stefan RK Hoffmann, Laurent Bigler, Sebastian Friess, Renato Zenobi, Konstantin Pervushin, Peter Hunziker, and Bernd Gutte. The leucine zipper domains of the transcription factors gcn4 and
c-jun have ribonuclease activity. *PloS One*, 5(5): e10765, 2010.

[38] Ryo Takeuchi, Michael Certo, Mark G Caprara, Andrew M Scharenberg, and Barry L Stoddard. Optimization of in vivo activity of a bifunctional homing endonuclease and maturase reverses evolutionary degradation. *Nucleic Acids Research*, 37(3): 877–890, 2009.

[39] Arvind G Kinhikar, Diana Vargas, Hualin Li, Spencer B Mahaffey, Laura Hinds, John T Belisle, and Suman Laal. Mycobacterium tuberculosis malate synthase is a laminin-binding adhesin. *Molecular Microbiology*, 60(4): 999–1013, 2006.

[40] Keith P Wilson, Lisa M Shewchuk, Richard G Brennan, Anthony J Otsuka, and Brian W Matthews. Escherichia coli biotin holoenzyme synthetase/bio repressor crystal structure delineates the biotin- and dna-binding domains. Proceedings of the National Academy of Sciences, 89(19): 9257–9261, 1992.

[41] Paul D Templeton, Elizabeth S Litman, Sandra I Metzner, Natalie G Ahn, and Marcelo C Sousa. Structure of mediator of rhoa-dependent invasion (mrdi) explains its dual function as a metabolic enzyme and a mediator of cell invasion. *Biochemistry*, 52(33): 5675–5684, 2013.

[42] Carlos Gancedo and Carmen-Lisset Flores. Moonlighting proteins in yeasts. *Microbiology and Molecular Biology Reviews*, 72(1): 197–210, 2008.

[43] Brent A Appleton, Ping Wu, Janice Maloney, JianPing Yin, Wei-Ching Liang, Scott Stawicki, Kyle Mortara, Krista K Bowman, J Michael Elliott, William Desmarais, *et al*. Structural studies of neuropilin/antibody complexes provide insights into semaphorin and vegf binding. *The EMBO Journal*, 26(23): 4902–4912, 2007.

[44] Anindita Bhoumik, Shoichi Takahashi, Wolfgang Breitweiser, Yosef Shiloh, Nic Jones, and Zeev Ronai. Atm-dependent phosphorylation of atf2 is required for the dna damage response. *Molecular Cell*, 18(5): 577–587, 2005.

[45] Laura Aguilera, Rosa Giménez, Josefa Badia, Juan Aguilar, and Laura Baldoma. Nad + -dependent post-translational modification of escherichia coli glyceraldehyde-3-phosphate dehydrogenase. *International Microbiology*, 12(3): 187–192, 2010.

[46] Jesper Z Haeggstrom. Leukotriene a4 hydrolase/aminopeptidase, the gatekeeper of chemotactic leukotriene b4 biosynthesis. *Journal of Biological Chemistry*, 279(49): 50639–50642, 2004.

[47] Susan Crennell, Toru Takimoto, Allen Portner, and Garry Taylor. Crystal structure of the multifunctional paramyxovirus hemagglutinin-neuraminidase. *Nature Structural & Molecular Biology*, 7(11): 1068–1074, 2000.

[48] Constance J Jeffery, Brian J Bahnson, Wade Chien, Dagmar Ringe, and Gregory A Petsko. Crystal structure of rabbit phosphoglucose isomerase, a glycolytic enzyme that moonlights as neuroleukin, autocrine motility factor, and differentiation mediator. *Biochemistry*, 39(5): 955–964, 2000.

[49] Jon Read, Jake Pearce, Xiaochun Li, Hilary Muirhead, John Chirgwin, and Christopher Davies. The crystal structure of human phosphoglucose isomerase at 1.6 °a resolution: implications for catalytic mechanism, cytokine activity and haemolytic anaemia. *Journal of Molecular Biology*, 309(2): 447–463, 2001.

[50] Jürgen Bosch, Carlos A Buscaglia, Brian Krumm, Bjarni P Ingason, Robert Lucas, Claudia Roach, Timothy Cardozo, Victor Nussenzweig, and Wim GJ Hol. Aldolase provides an unusual binding site for thrombospondin-related anonymous protein in the invasion machinery of the malaria parasite. Proceedings of the National Academy of Sciences, 104(17): 7015–7020, 2007.

[51] Patrick Scheerer, Astrid Borchert, Norbert Krauss, Helga Wessner, Christa Gerth, Wolfgang Höhne, and Hartmut Kuhn. Structural basis for catalytic activity and enzyme polymerization of phospholipid hydroperoxide glutathione peroxidase-4 (gpx4). *Biochemistry*, 46(31): 9041–9049, 2007.

[52] Xiao Luo, He-Hsuan Hsiao, Mikhail Bubunenko, Gert Weber, Donald L Court, Max E Gottesman, Henning Urlaub, and Markus C Wahl. Structural and functional analysis of the e. coli nusb-s10 transcription antitermination complex. *Molecular Cell*, 32(6): 791–802, 2008.

[53] OA Bateman, AG Purkiss, R Van Montfort, C Slingsby, C Graham, and G Wistow. Crystal structure of η-crystallin: adaptation of a class 1 aldehyde dehydrogenase for a new role in the eye lens. *Biochemistry*, 42(15): 4349–4356, 2003.

[54] Georgiy A Belogurov, Marina N Vassylyeva, Vladimir Svetlov, Sergiy Klyuyev, Nick V Grishin, Dmitry G Vassylyev, and Irina Artsimovitch. Structural basis

for converting a general transcription factor into an operon-specific virulence regulator. *Molecular Cell*, 26(1): 117–129, 2007.

[55] Bjorn M Burmann, Stefan H Knauer, Anastasia Sevostyanova, Kristian Schweimer, Rachel A Mooney, Robert Landick, Irina Artsimovitch, and Paul Rösch. An
α helix to β barrel domain switch transforms the transcription factor rfah into a translation factor. *Cell*, 150(2): 291–303, 2012.

[56] Robbyn L Tuinstra, Francis C Peterson, Snjezana Kutlesa, E Sonay Elgin, Michael A Kron, and Brian F Volkman. Interconversion between two unrelated protein folds in the lymphotactin native state. Proceedings of the National Academy of Sciences, 105(13): 5057–5062, 2008.

[57] NE Tolbert. Metabolic pathways in peroxisomes and glyoxysomes. *Annual Review of Biochemistry*, 50(1): 133–157, 1981.

[58] David M Anstrom and S James Remington. The product complex of m. tuberculosis malate synthase revisited. *Protein Science*, 15(8): 2002–2007, 2006.

[59] Constance J Jeffery. Proteins with neomorphic moonlighting functions in disease. *IUBMB Life*, 63(7): 489–494, 2011.

[60] Mikyung Yun, Cheon-Gil Park, Ji-Yeon Kim, and Hee-Won Park. Structural analysis of glycer-aldehyde 3-phosphate dehydrogenase from escherichia coli: direct evidence of substrate binding and cofactor-induced conformational changes. *Biochemistry*, 39(35): 10702–10710, 2000.

[61] Fredrik Tholander, Ayumo Muroya, Bernard-Pierre Roques, Marie-Claude Fournié-Zaluski, Marjolein MGM Thunnissen, and Jesper Z Haeggstrom. Structure-based dissection of the active site chemistry of leukotriene a4 hydrolase: implications for m1 aminopeptidases and inhibitor design. *Chemistry & Biology*, 15(9): 920–929, 2008.

[62] Peter C Rudberg, Fredrik Tholander, Marjolein MGM Thunnissen, and Jesper Z Haeggström. Leukotriene a4hydrolase/aminopeptidase glutamate 271 is a catalytic residue with specific roles in two distinct enzyme mechanisms. *Journal of Biological Chemistry*, 277(2): 1398–1404, 2002.

[63] Stefan H Knauer, Irina Artsimovitch, and Paul Rösch. Transformer proteins. *Cell Cycle*, 11(23): 4289–4290, 2012.

Part II

Proteins Moonlighting in Prokarya

3

Overview of Protein Moonlighting in Bacterial Virulence

Brian Henderson

Department of Microbial Diseases, UCL-Eastman Dental Institute, University College London, UK

3.1 Introduction

It is intriguing that, in spite of the estimates of our planet harboring between 10 million and one billion species of bacteria (Schloss and Handelsman 2004), only a tiny handful of these organisms are capable of causing human disease (Wilson *et al*. 2005). Both exogenous and endogenous bacterial pathogens cause disease through the possession, and expression, of various classes of proteins known as virulence factors. A large number of molecularly distinct bacterial virulence factors are now recognized (Sansonetti 2010). It is therefore surprising that, over the past 20 years, a growing number of moonlighting proteins have been shown to be involved in bacterial virulence (Henderson and Martin 2011, 2013; Henderson 2014). This chapter performs the task of explaining the background to the rest of the book, and will introduce the reader to the concept of bacterial virulence and bacterial virulence factors, and will provide a brief overview of the role of bacterial moonlighting proteins as virulence determinants.

3.2 The Meaning of Bacterial Virulence and Virulence Factors

Bacterial virulence is a systems biology-based term which can be quantitatively assessed in terms of the numbers of any particular bacterium able to cause disease or kill an infected host. Bacterial infection, at least for an exogenous pathogen, requires a number of hurdles to be jumped before the infecting organism can be said to have "infected." The first step in infection is for the bacterium to contact the host and bind to it. This may be the end of the infectious process for organisms that only infect the outer surfaces of their hosts. However, most bacteria must invade their hosts and this requires that the organisms can enter into and travel through the epithelial or other cell barriers that protect the host. Once the

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

bacterium has exited from an epithelial cell of a mucosal barrier, it must then invade through the basement membranes on which epithelial cells are supported. There may be further invasion of bacteria through blood vessel walls, allowing the infecting bacterium to enter into the bloodstream, to either infect the blood, or journey to specific sites where the bacterium can bind and grow. Growth of the infecting bacterium has to contend with the fact that essential nutrients, such as iron and vitamins, are normally sequestered by the host; in addition, bacteria also have to survive the immune response initiated in response to infections.

Bacterial infection therefore requires that an exogenous bacterium: (1) bind to the host; (2) invade the host; (3) attain essential nutrients; and (4) evade the immune response of the host. These key barriers to infection have been dealt with by bacteria evolving a range of virulence factors (generally proteins) which allow bacteria to: (1) bind to host cells or tissues (called **adhesins**); (2) invade cell and tissue barriers (called **invasins**); (3) obtain essential nutrients (no term is available and these will be called **nutritins** in this chapter); and (4) evade immune responses (termed **evasins**). Invasion and evasion are often helped by the best known of the bacterial virulence factors: the **toxins** (Henkel *et al.* 2010). The term suggests that bacterial toxins are proteins able to destroy host cells and tissues. Some toxins have such properties. However, others have the role of subverting some essential host cell function for the purpose of aiding bacterial invasion or evasion of immunity (Lemonnier *et al.* 2007).

Adhesins, invasins, nutritins, evasins, and toxins therefore represent the major bacterial virulence factors and these various cell surface and secreted products of the invading bacterium act together dynamically, in time and space, to aid the infectious process and maintain the infection (Fig. 3.1). The overall interaction of

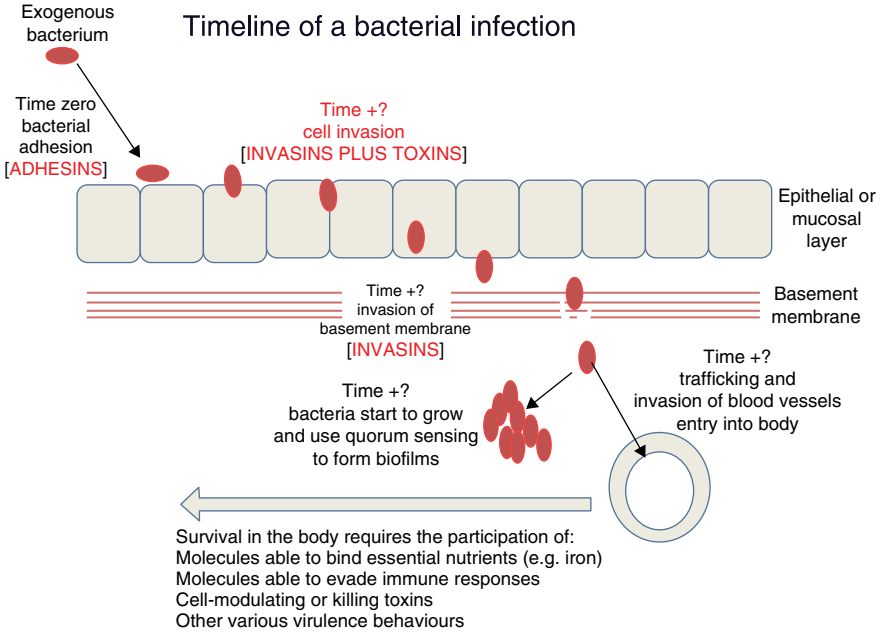

Figure 3.1 A schematic diagram showing the dynamic "timeline" of a typical bacterial infection and the requirements for the major virulence factors in the process.

these various virulence factors creates a system, with emergent properties, which is currently little understood in any detail. In addition to these key virulence factors, other systems are employed to aid bacterial infection. This includes the quorum sensing systems used by most bacteria as an aid in biofilm formation (Garg *et al*. 2014) and systems which enable bacteria to enter some form of dormant state in response to nutrient depletion or other stresses (Ayrapetyan *et al*. 2015). It is likely that other virulence systems still await detection.

Moonlighting proteins have been found to replicate most of these distinct forms of virulence factors and, where able to be analyzed, have been shown to play important roles in bacterial infection and survival. In addition to bacterial moonlighting proteins, it is also emerging that certain host moonlighting proteins also play key roles in aiding bacterial infection.

3.3 Affinity as a Measure of the Biological Importance of Proteins

While the literature does not reveal it, one of the criticisms aimed at those working on protein moonlighting is that these proteins are only interacting *nonspecifically* with their target ligands (generally other proteins). If a moonlighting protein is identified as an adhesin, binding to a specified host component, the K_D of the interaction would be low and therefore unable to fulfill the biological function ascribed to it. A careful perusal of the literature has identified a number of reports in which the affinity of interaction of moonlighting proteins with their *moonlighting* protein ligands has been measured, normally by surface plasmon resonance, using the Biacore apparatus. The equilibrium dissociation constant K_D describes the strength of binding of the test protein to its receptor/ligand. In a classic equilibrium reaction, with a protein (P) and its ligand (L) (L is generally also a protein), we have $P + L \leftarrow \rightarrow PL$. The equilibrium dissociation constant K_D is defined:

$$K_D = [P][L]/[PL].$$

Very strong binding is described by small K_D values, as the bound complex [PL] (denominator) will be vastly in excess of the individual components [P] and [L] in the numerator. Picomolar K_D values are 1000 times stronger than nanomolar K_Ds, and so on. Our understanding of the affinities of protein–ligand interactions is still in its infancy, but the available data would suggest that K_D values of 10^{-9}–10^{-12} M are high/very high affinity interactions and 10^{-7}–10^{-9} M are moderate to high affinity associations. Many biologically relevant protein–ligand interactions are in the range 10^{-7}–10^{-5} M (Kumar *et al*. 2009). Nonspecific interactions of proteins with "ligands" have K_Ds in the order of 10^{-2}–10^{-4} M (Jenuwine and Shaner 1996). The available kinetic analysis of moonlighting proteins is shown in Tables 3.1 and 3.2, where it is clearly seen that the majority of moonlighting proteins have K_D values in the nanomolar range; this reveals that these proteins are involved in high-affinity interactions with their target ligands. As many of these protein interactions are involved in bacterial virulence then it can be stated, with confidence, that these interactions are biologically relevant. What

Table 3.1 Binding affinities of moonlighting proteins to various protein ligands.

Bacterium	Moonlighting protein	Ligand bound	K_D (M)	Reference
M. tuberculosis	GAPDH	Epidermal growth factor	2×10^{-10}	Bermudez *et al.* 1996
L. monocytogenes	Alcohol acetaldehyde dehydrogenase	Human Hsp60	5.4×10^{-8}	Kim *et al.* 2006
Pasteurellaceae	ComE1 proteins	Fibronectin	150×10^{-9}	Mullen *et al.* 2008*a*, *b*
Yersinia pestis	Caf1	IL-1 receptor	5.4×10^{-10}	Abramov *et al.* 2001
Y. pestis	Caf1A usher protein	IL-1β	1.4×10^{-10}	Zav'yalov *et al.* 1995
Y. pestis	Lcrv	IFNγ	32×10^{-9}	Abramov *et al.* 2007; Gendrin *et al.* 2010
Strep. pyogenes	Protein H	Fibronectin	1.6×10^{-8}	Frick *et al.* 1995
Strep. pyogenes	Serum opacity factor 2	Fibulin-1	1.6×10^{-9}	Courtney *et al.* 2009
Strep. pneumonia	GAPDH	C1q	3×10^{-10}	Terrasse *et al.* 2012

is surprising is that evolution has resulted in the moonlighting sites in these proteins having such high affinity. This suggests that the evolution of moonlighting sites in proteins is under the same constraints as the evolution of the so-called "primary" function of moonlighting proteins.

3.4 Moonlighting Bacterial Virulence Proteins

The various chapters in this book will describe individual moonlighting virulence proteins or the use of moonlighting proteins in the virulence of specific bacteria. This chapter seeks to provide a general overview of the field of moonlighting virulence proteins in 2016 as a foundation for the rest of this volume. A word of caution must be introduced at this point. Evidence that a particular protein is involved in bacterial virulence is normally obtained by the inactivation of the gene encoding the protein of interest, and the demonstration that this isogenic mutant (with appropriate controls for polar effects) is less virulent than the wild-type organism within a relevant host species. This is often not possible with moonlighting proteins, as many are essential proteins required for bacterial survival. With many moonlighting proteins, the evidence that they are involved in bacterial virulence depends purely on *in vitro* experimentation. This is the normal starting point for the work on any virulence factor, and the evidence supporting a role in virulence can often be substantiated by means other than *in vivo* studies, such as antibody neutralization, use of protein mutagenesis, and so on. Those studies that verify the role of moonlighting proteins in *in vivo* virulence will be briefly described.

Table 3.2 Binding affinities of moonlighting proteins to plasmino(gen).

Bacterium	Moonlighting proteins binding plasmin(ogen)	K_D plasminogen (M)	K_D plasmin (M)	Reference
B. burgdorferi	Enolase	12.5×10^{-8}		Floden *et al.* 2011
Strep. pyogenes	Enolase	$K_{D1} = 1.3 \times 10^{-9}$	$K_{D1} = 2.2 \times 10^{-9}$	Derbis *et al.* 2004
		$K_{D2} = 7.4 \times 10^{-9}$	$K_{D2} = 2.7 \times 10^{-8}$	Cork *et al.* 2009
Strep. pneumonia	Enolase	$K_{D1} = 8.6 \times 10^{-8}$		Bergmann *et al.* 2003, 2005
		$K_{D2} = 5.5 \times 10^{-10}$		Ehinger *et al.* 2004
Strep. suis	Enolase	1.4×10^{-8}		Esgleas *et al.* 2008
Strep. pneumonia	GAPDH	$K_{D1} = 4.3 \times 10^{-7}$	$K_{D1} = 2.8 \times 10^{-8}$	
		$K_{D2} = 1.6 \times 10^{-10}$	$K_{D2} = 5.2 \times 10^{-8}$	
Strep. equi	GAPDH	22×10^{-8}	2.5×10^{-8}	
Bacillus stearothermophilus	GAPDH	56×10^{-8}	9.8×10^{-8}	
Mycobacterium tuberculosis	Aldolase	6.7×10^{-9}		de la Paz Santangelo *et al.* 2011
Bifidobacter animalis	DnaK	12×10^{-9}		
Leptospira spp.	Ompl	37×10^{-8}		
Leptospira interrogans	Lsa44	54×10^{-9}		
L. interrogans	Lsa45	38×10^{-9}		

Currently almost 100 bacterial species have been identified as utilizing specific moonlighting proteins in behavior that is likely to be involved in the process of bacterial virulence (Henderson *et al.* in press). These include: *Acinetobacter baumannii*, a Gram-negative opportunistic nosocomial pathogen; *Bacillus anthracis*; *Francisella tularensis*, the causative agent of tularaemia; *Listeria monocytogenes*; a range of streptococci; and the insect pathogen *Xenorhabdus nematophila*. Gram-negative and Gram-positive organisms, organisms such as mycobacteria which are Gram-indeterminate, and the mycoplasmas all utilize moonlighting proteins in virulence and survival. The use of moonlighting proteins for virulence and survival is therefore a widely dispersed phenomenon in bacteria. Certain bacterial genera seem to particularly favor the

use of moonlighting proteins, as both multiple species are found to utilize these proteins and multiple proteins are employed. Examples include mycobacteria, mycoplasma, and streptococci, with the former and latter employing between 15 and 20 moonlighting proteins.

A list of the bacterial proteins identified as having moonlighting activity is provided in Table 3.3. Here we see that almost 100 bacterial proteins have evolved functions which fit within the actions of the major classes of virulence factors. Note that at least half of these proteins are actually shared between bacteria and eukaryotes, and many of them are known to be eukaryotic moonlighting proteins. Examples include GAPDH, enolase, chaperonin 10, chaperonin 60, Hsp70, cyclophilin A, and so on (Henderson *et al*. in press). The remainder of the proteins are a diverse collection of bacterial proteins with no apparent common theme, at least that this author can see. This probably hints at the commonality of protein moonlighting in the bacteria.

An interesting divide in the bacterial moonlighting proteins is between those which exhibit multiple biological actions and those which appear to have only one moonlighting function. Table 3.4 shows some of the multiple biological actions of GAPDH, enolase, chaperonin 60, Hsp70 (DnaK), and peptidylprolyl isomerase (PPI). Other bacterial moonlighting proteins with multiple actions include chaperonin 10, dihydrolipoamide dehydrogenase, and Ef-Tu (elongation factor thermal unstable). Now, it cannot be ruled out that all moonlighting proteins do not have multiple functions. This depends on how much study has been devoted to each protein, and to a degree of experimental serendipity. However, it is interesting that most of these multi-moonlighting proteins are evolutionarily ancient, suggesting the hypothesis that in the last common universal ancestor (LUCA), and "organisms" preceding LUCA, protein heterogeneity was limited and evolution favored polypeptide chains with multiple biological activities. The assumption is that these have been retained and amplified through deep time.

3.4.1 Bacterial Proteins Moonlighting as Adhesins

Of the five major classes of virulence proteins dealt with in this book – adhesins, invasins, nutritins, evasions, and toxins – the process of bacterial adhesion utilizes more moonlighting proteins than any other virulence activity (Table 3.5). In this table, only one example of the individual moonlighting proteins acting as adhesins is provided. At the time of writing, 13 bacteria are known to employ GAPDH as either a protein able to interact with host components or as a true adhesin. Similarly, at the time of writing, eight bacteria employ enolase as an adhesin. Where possible, the host receptors for these proteins are provided. As can be seen in Table 3.5, a surprising number of bacterial moonlighting proteins function as adhesins, binding to a range of host cell and host extracellular matrix proteins. One of the key questions that needs to be raised in relation to protein moonlighting is whether this process generates unique biology. Do moonlighting proteins have actions not replicated by "non-moonlighting" proteins (i.e., proteins evolved as virulence factors), and do these proteins expand the functionality of the bacterial proteome? A number

Table 3.3 The distribution of bacterial moonlighting proteins into functional groups. Proteins in bold are those produced both by bacteria and eukaryotes.

Function	Functional grouping	Example
Metabolic enzymes	Glycolytic pathway	**GAPDH**
		Enolase
		Phosphoglucoisomerase (PGI)
		Phosphofructokinase (PFK)
		Aldolase
		Triosephosphate isomerase (TPI)
		Phosphoglycerate kinase (PGK)
		Phosphoglycerate mutase (PGM)
		Pyruvate kinase
	Tricarboxylic acid (TCA) cycle	**Pyruvate dehydrogenase**
		Dihydrolipoamide dehydrogenase
		Aconitase
		Isocitrate dehydrogenase
		Succinyl coA synthetase
		Pyruvate oxidase
	Hexose monophosphate shunt	**6-Phosphogluconate dehydrogenase**
	Glyoxylate cycle	**Malate synthase**
Miscellaneous enzymes		**Alcohol acetaldehyde dehydrogenase**
		Adenylyltransferase GlnE
		Arginine deiminase
		Aspartase
		Glucosyltransferase
		Glutamine synthetase
		Glutamate racemase
		NAD-synthetase
		Poly gamma glutamate synthetase
		Pyruvate formate lyase
		Superoxide dismutase
Proteases		**Glutamyl aminopeptidase**
		Endopeptidase O
Intracellular signaling proteins		**cAMP phosphodiesterase**
		Serine/threonine phosphatase

(*Continued*)

Table 3.3 (Continued)

Function	Functional grouping	Example
Nucleic acid-interacting proteins		**Adenosine kinase**
		Adenylate kinase
		DNA-binding (histone-like) protein HU
		Ef-Tu
		DNA polymerase III θ
		Nuclease
		Nucleoside diphosphate kinase
		Polynucleotide phosphorylase
Transporter proteins		**Oligonucleotide permease**
Molecular chaperones		**Chaperonin (Hsp) 10**
		Hsp18
		Hsp20
		Chaperonin (Hsp) 60
		DnaK (Hsp70)
		HtpH (Hsp90)
		HtrA
Protein-folding catalysts		**Peptidylprolyl isomerase (PPI)**
		Thioredoxin (Trx)
Bacterial proteins		ABC transporter
		BCAM0223 (trimeric autotransporter)
		BCAM0224 (trimeric autotransporter)
		Albaflavenone synthase
		Anthrax lethal toxin
		Autolysins
		Caf1 usher protein
		Caf1A
		Cell and mucus binding protein (CmbA)
		Cholesterol-dependent cytolysin
		Choline binding protein
		ComEA
		Endopeptidase O

Table 3.3 (Continued)

Function	Functional grouping	Example
		Fibronectin binding protein A (FnbpA)
		Fimbrial subunit protein
		67 kDa Fimbrillin
		Hemin-binding protein
		Hemoglobin-receptor protein
		Hemoprotein receptor
		Heme transport protein (HbpA)
		Heparin-binding hemagglutinin (HBHA)
		Harpin
		Histone-like protein A (HlpA)
		Lcrv
		Lsa44
		Lsa45
		Lysozyme LytC
		Lysozyme-like protein from actinobacteria (rpfs)
		Lysozyme-like proteins from *L. monocytogenes* (rpfs)
		Manganese transport protein
		Mhp182
		MPT51 (FbpC1)
		Multivalent adhesion molecule (MAM)7
		Mycobacterial DNA-binding protein-1
		Mycolyl transferases
		Neisseria meningitis NhhA
		OprF
		Outer membrane protein P5
		Ompl
		Porins
		Protein A
		Protein H
		Serum opacity factor 2
		S-layer protein (Slp)
		TonB-dependent OMP
		Type IV pilus

Table 3.4 Some bacterial moonlighting proteins with (some of their) multiple moonlighting functions. Biological actions are not in any particular order, and not all the biological functions of these proteins are shown.

GAPDH	Enolase	Chaperonin	60DnaK	PPI
Plasminogen-binding protein	Plasminogen-binding protein	Osteolytic protein	Plasminogen-binding protein	Collagen-binding protein
Inhibitor of C5a activity	Cytokeratin-binding protein	Promotes epithelial cell migration	Epithelial cell adhesin	Invasin
C1q-binding protein	Fibronectin-binding protein	Virus-binding protein	Cytokine-inducer	Macrophage infectivity protein
Induces macrophage apoptosis	Induces neutrophil trap formation	Inhibits epithelial cell apoptosis	Inhibits alkaline phosphatase activity	Induces TH17 inflammation
Heme-binding protein	Interacts with CvfA modulates virulence	Stimulates oxidation of LDL	Adhesin for sulfatide	Induces epithelial apoptosis
DNA repair protein	Laminin-binding protein	Induces endothelial cell apoptosis	Binds DC-SIGN on dendritic cells	IL-6 inducer via NF-kB
Mucin-binding protein	Complement-evasion protein	Vascular endothelial cell growth factor	Inhibits HIV infection of cells	Involved in competence
Binds blood group antigens	Interacts with degradosome	Promotes endothelial cell VCAM synthesis	Binds yeast mannan	Cell adhesin
Phosphorylates host proteins	Mucin-binding protein	Histidine kinase activity	Binds CD40/ induces dendritic cell maturation	Induces intracellular bacterial persistence
Erythrocyte adhesion	Erythrocyte adhesion	Modulates actin cytoskeleton	Induces chemokine synthesis	

of moonlighting adhesins bind to the same host proteins/nonproteins as do the "natural" bacterial adhesins. Host receptors include fibronectin, vitronectin, heparan, mucin, and so on. However, a number of moonlighting adhesins interact with host receptors not normally recognized as being part of the bacterial adhesive process. These include the binding of *Strep. pyogenes* GAPDH to uPAR/CD87 (Jin *et al*. 2005), *Mycobacterium tuberculosis* DnaK binding to the chemokine receptor CCR5 (Floto *et al*. 2006), and *M. tuberculosis* chaperonin 60.2 binding to CD43 (Hickey *et al*. 2010). It is likely that other unusual pairings are also in the literature (and await discovery). These data are revealing that the use of moonlighting proteins by bacteria extends the virulence landscape of the bacterium.

Table 3.5 Bacterial moonlighting proteins acting as bacterial adhesins.

Protein	Organism	Host receptor	Reference
GAPDH	*Strep. pyogenes*	uPAR/CD87	Jin *et al.* 2005
Enolase	*Strep. gallolyticus*	cytokeratin 8	Boleij *et al.* 2011
Phosphoglucoisomerase	*L. crispatus*	type I collagen	Kainulainen *et al.* 2012
Aldolase	*Strep. pneumoniae*	flamingo cadherin	Blau *et al.* 2007
Triose phosphate isomerase	*Staph. aureus*	oligosaccharides	Furuya and Ikeda 2009
Phosphoglycerate kinase	*Spiroplasma citri*	actin	Labroussaa *et al.* 2010
2011)			
Pyruvate kinase	Lactic acid bacteria	yeast mannans	Katakura *et al.* 2010
Pyruvate dehydrogenase	*L. plantarum*	fibronectin	Vastano *et al.* 2014
Succinyl CoA synthetase	*Bartonella henselae*	receptor unknown	Chang *et al.* 2011
Pyruvate oxidase	*Strep. pneumoniae*	receptor unknown	Spellerberg *et al.* 1996
6-phosphogluconate dehydrogenase	*Haemophilus parasuis*	receptor unknown	Fu *et al.* 2012
Malate synthase	*M. tuberculosis*	laminin	Kinhikar *et al.* 2006
Hsp20	*H. pylori*	receptor unknown	Du and Ho 2003
Cpn60	*E. coli*	Lox-1	Zhu *et al.* 2013
Cpn60.2	*M. tuberculosis*	CD43	Hickey *et al.* 2010
DnaK	*H. influenza*	sulfatide	Hartmann *et al.* 2001
Htra	*C. jejuni*	receptor unknown	Bæk *et al.* 2011
PPI	*L. pneumophila*	type IV collagen	Wagner *et al.* 2007
Alcohol acetaldehyde dehydrogenase	*L. monocytogenes*	chaperonin 60	Kim *et al.* 2006
Glutamine synthetase	*L. crispatus*	receptor unknown	Kainulainen *et al.* 2012
Glutamyl aminopeptidase	*Myc. hypopneumoniae*	heparin	Robinson *et al.* 2013
Endopeptidase O	*Strep. pneumoniae*	fibronectin	Agarwal *et al.* 2013
Ef-Tu	*Acinetobacter baumannii*	fibronectin	Dallo *et al.* 2012
Nuclease	*Myc. pneumoniae*	receptor unknown	Somarajan *et al.* 2010
Polynucleotide phosphorylase	*C. jejuni*	receptor unknown	Haddad *et al.* 2012

(*Continued*)

Table 3.5 (Continued)

Protein	Organism	Host receptor	Reference
Oligopeptide permease	*Myc. hominis*	receptor unknown	Hopfe *et al*. 2011
ABC transporter	*Strep. suis*	receptor unknown	Zhang *et al*. 2011
Aae	*Staph. epidermidis*	fibronectin/ vitronectin	Heilmann *et al*. 2003
Cell- and mucus-binding protein	*L. reuteri*	mucin	Jensen *et al*. 2014
Choline-binding protein	*Strep. pneumonia*	receptor unknown	Rosenow *et al*. 1997
ComEA	Pasteurella spp.	DNA/fibronectin	Mullen *et al*. 2008*a*
b)			
Hemin-binding protein	*P. gingivalis*	receptor unknown	Hiratsuka *et al*. 2010
Hemoprotein receptor	*Strep. pyogenes*	laminin	Dahesh *et al*. 2012
Histone-like protein A (HlpA)	*Strep. gallolyticus*	heparin	Boleij *et al*. 2009
Leukotoxin ED	*Staph. aureus*	CCR5	Alonzo *et al*. 2013
Leukotoxin ED	*Staph. aureus*	CXCR1/CXCR2	Reyes-Robles *et al*. 2013
Lsa44 (Leptospiral surface adhesin)	*L. interrogans*	laminin/ plasmin(ogen)	Fernandes *et al*. 2014
Lsa45	*L. interrogans*	laminin/ plasmin(ogen)	Fernandes *et al*. 2014
Mhp182	*Myc. hypopneumoniae*	fibronectin	Seymour *et al*. 2012
MPT51 (FbpC1)	*M. tuberculosis*	fibronectin	Wilson *et al*. 2004
Multivalent adhesion molecule (MAM)7	Many bacterial species	fibronectin/ phosphatidic acid	Krachler and Orth 2011
Mycobacterial DNA-binding protein 1	*M. tuberculosis*	hyaluronan	Aoki *et al*. 2004
Mycolyl transferases	*M. tuberculosis*	fibronectin	Abou-Zeid *et al*. 1991; Kuo *et al*. 2012
Ompl	*Leptospira* spp.	ECM components	Fernandes *et al*. 2012
Omps (various)	*A. baumanni*	fibronectin	Smani *et al*. 2012
Omps	*H. pylori*	heparin sulfate	López-Bolaños *et al*. 2009
Protein H	*Strep. pyogenes*	fibronectin	Frick *et al*. 1995
Serum opacity factor	*Strep. pyogenes*	fibulin-1	Courtney *et al*. 2009
S-layer protein (Slp)	*L. acidophilus*	receptor unknown	Buck *et al*. 2005
TonB-dependent OMP	*Bacteroides fragilis*	fibronectin	Pauer *et al*. 2009

3.4.2 Bacterial Moonlighting Proteins That Act as Invasins

Bacterial invasion of nonphagocytic cells is dependent on the interaction of specific bacterial proteins (invasins) with host cell-surface receptors, or may be through the injection of cell modulators to alter host cell function (Pizarro-Cerdá and Cossart 2006). Only a small number of bacterial moonlighting proteins have been demonstrated to function as invasins. These include *Strep. pyogenes* GAPDH, which binds to uPAR/CD87 to enable cell entry (Jin *et al*. 2005), the chaperonin 60 protein of *Legionella pneumophila* (Chong *et al*. 2009; see also Chapter 6), and a range of peptidyl prolyl isomerases (Norville *et al*. 2011; Rasch *et al*. 2014; see also Chapter 7). Many of the receptors for these moonlighting invasins are themselves moonlighting proteins (e.g., uPAR/CD87) and this will be discussed in more detail in Section 3.5.

3.4.3 Bacterial Moonlighting Proteins Involved in Nutrient Acquisition

Bacterial invasion into the human body would be thought to be like a starving man arriving at a feast. All the nutrients that the bacterium needed would be available from the host. Nutrient acquisition is essential for bacterial growth and is therefore an evolutionary target for antibacterial defense. The best-known example of this is the essential metal, iron. While the average human has 4–5 g of iron in their bodies, the level of the free metal in human biological fluids is kept extremely low through its binding to two high-affinity iron-binding proteins: transferrin and lactoferrin (Skaar 2010). This nutritional defense mechanism has been countered by the evolution, by bacteria, of proteins that bind host iron-binding proteins (transferrin, lactoferrin, hemoglobin, haptoglobin, etc.), heme, and also small organic molecules termed siderophores which bind directly to free iron (Morgenthau *et al*. 2013). For example, the Neisseria, which do not manufacture siderophores, utilize two transferrin-binding proteins (A and B) to take up iron (Noinaj *et al*. 2012).

When we turn to moonlighting proteins and iron binding, the first such report was of the GAPDH of *Staphylococcus aureus* acting as a transferrin receptor (Modun and Williams 1999). This finding was not confirmed by Taylor and Heinrichs (2002) and an explanation for this may be that *Staph. aureus* has two genes encoding GAPDH. In one study, only the glyceraldehyde 3-phosphate dehydrogenase protein known as GapC was found to contribute to bacterial pathogenicity (Kerro-Dego *et al*. 2012); this difference in the literature could be due to the two groups employing different GAPDHs. Since these early studies, the GADPH of *Streptococcus suis* (a pig pathogen) has been shown to function as a heme-binding protein (Hannibal *et al*. 2012) and, as explained in Chapter 11, the *Mycobacterium tuberculosis* GAPDH functions as a transferrin-binding protein (Boradia *et al*. 2014). In addition, the peroxiredoxin of *Strep. agalactiae* functions as a heme-binding protein (Lechardeur *et al*. 2011) and certain of the porins of enteropathogens function as transferrin-binding protein (Sandrini *et al*. 2013). Moonlighting proteins therefore appear to contribute to the ability of bacteria to capture iron. To show the unexpected nature of protein moonlighting, it has recently been reported that the *Staph. aureus* manganese transport protein, MntC, binds plasminogen and extracellular matrix proteins (Salazar *et al*. 2014).

3.4.4 Bacterial Moonlighting Proteins Functioning as Evasins

We are all taught that immunity is our defense against the bacterium. What we are not taught is how effective bacteria have been at evolving evasive strategies against both innate and adaptive immunity. Indeed, the most effective bacterial evasion mechanism would seem to be the bacterial biofilm (Hanke and Kielian 2012). Our immune system has evolved to kill individual bacteria, largely by phagocytosis of antibody/complement-opsonized bacteria. However, bacteria within biofilms are relatively resistant to all the overtures of immunity, and one of the major paradoxes in bacteriology is how bacterial biofilms are prevented from forming or the bacteria killed if they form. The major problem is the fact that in biofilms, bacteria are coated in an extracellular matrix which interlinks the bacteria and protects them from outside agents, such as complement and phagocytes. The biofilm matrix can contain polysaccharides, proteins, DNA, and so on. It is only recently that it has been found that the major human pathogen *Staphylococcus aureus* employs a number of moonlighting proteins, and in particular GAPDH and enolase, in the formation of biofilms (Foulston *et al.* 2014). It is possible that these proteins in the biofilm also express other moonlighting activities, such as immune evasion.

Bacterial immune evasion strategies generally influence: (1) the B lymphocyte (B cell superantigens); (2) the T lymphocyte (T cell superantigens); (3) the antibody molecule (Ig-binding proteins and Ig proteases); (4) complement activation and action (by a variety of mechanisms); (5) phagocyte activity and viability (by a variety of mechanisms); and (6) the actions of neutrophil nets (neutrophil extracellular traps). As can be seen in Table 3.6, bacterial moonlighting proteins can generate most of these actions except, at the time of writing, the mimicking of bacterial superantigens. A range of proteins are involved including metabolic enzymes and molecular chaperones, found both in prokaryotes and eukaryotes, as well as a range of proteins found only in bacteria. One of the most unexpected findings from the literature is the striking range of actions of the GAPDH family, which can inhibit complement by targeting C1q or C5a, induce macrophage apoptosis, or even function as a polyclonal B cell activator (see references in Tables 3.4, 3.6). Now this range of actions is even more unusual, given that the only GAPDH proteins to be examined seem to be those from the streptococci. These streptococcal proteins have >90% sequence identity. Either these varied moonlighting proteins are discriminated on the basis of very small changes in sequence, or all these proteins share this range of immune-modulatory activity. A number of these proteins seem to have unusual, potentially unique, actions. The chaperonin 60.1 protein of *Mycobacterium tuberculosis* seems able to induce the formation of the multinucleate giant cell that is a hallmark of the tuberculoid granuloma (Hu *et al.* 2008), while also being a potent inhibitor of the formation of the osteoclast (Winrow *et al.* 2008). These activities are not classically immune evasive, but reveal the range of influence of bacterial moonlighting proteins on immune cells. Many of these proteins, including GAPDH, enolase, phosphoglycerate kinase, dihydrolipoamide dehydrogenase, endopeptidase O, Ef-Tu and a number of specific bacterial proteins, function to inhibit the complement system at various points in this complex cascade.

Table 3.6 Bacterial moonlighting proteins functioning as evasins.

Moonlighting protein	Species	Biological activity	Reference
GAPDH	*Strep. pyogenes*	Inhibits complement-mediated killing	Jin *et al.* 2005
GAPDH	*Strep. pyogenes*	Inhibits effect of C5a on neutrophils	Terao *et al.* 2006
GAPDH	*Strep. agalactiae*	Polyclonal B cell stimulator and inducer of IL-10 synthesis whose overexpression enhances virulence	Madueira *et al.* 2007
GAPDH	*Strep. agalactiae*	Immunization against GAPDH protects mice from *Strep. agalactiae* infection	Madueira *et al.* 2011
GAPDH	*Strep. agalactiae*	Induces macrophage apoptosis	Oliveira *et al.* 2012
GAPDH	*Strep. pneumoniae*	C1q binding/inhibiting protein inhibits complement activation	Terrasse *et al.* 2012
Enolase	*Strep. pneumonia*	Induces cell death of neutrophils	Mori *et al.* 2012
Enolase	*Strep. pneumoniae*	Binds to complement component C4B and inhibits complement activity	Agarwal *et al.* 2012
Phosphoglycerate kinase	*Strep. pneumoniae*	Inhibits complement membrane-attack complex	Blom *et al.* 2014
Dihydrolipoamide dehydrogenase	*Anaplasma phagocytophilum*	Immunomodulatory effects on neutrophils and monocytes resulting in increased infection	Chen *et al.* 2012
Dihydrolipoamide dehydrogenase	*Ps. aeruginosa*	Binds complement factor H, FHL-1, and CFHR1 to produce immune evasion	Hallström *et al.* 2012
Chaperonin 10	*M. tuberculosis*	Administration suppresses models of arthritis in mice and rats	Jorgensen *et al.* 1988; Ragno *et al.* 1996; Agnello *et al.* 2002
Chaperonin 10	*M. tuberculosis*	Administration inhibits allergic asthma in mouse	Riffo-Vasquez *et al.* 2004
Chaperonin 60.1	*M. tuberculosis*	Administration inhibits allergic asthma in mouse; Cpn60.2 paralog inactive	Riffo-Vasquez *et al.* 2004
Chaperonin 60.1	*M. tuberculosis*	Inhibitor of leukocyte diapedesis in allergic asthma model	Riffo-Vasquez *et al.* 2012
Chaperonin 60.2	*M. leprae*	Administration inhibits allergic asthma in mouse	Rha *et al.* 2002
Chaperonin 60.2	*M. leprae*	Inhibits allergic asthma in mouse by modulating dendritic cell function	Shin *et al.* 2012
Cpn60.1	*M. tuberculosis*	Inactivation of cpn60.1 gene results in isogenic mutant unable to induce granulomatous inflammation in mice and guinea pigs	Hu *et al.* 2008
Cpn60.1	*M. tuberculosis*	Inhibits the formation of osteoclasts	Winrow *et al.* 2008

(*Continued*)

Table 3.6 (Continued)

Moonlighting protein	Species	Biological activity	Reference
NAD-synthetase	*Strep. sobrinus*	Polyclonal B cell activator	Veiga-Malta *et al.* 2004
Endopeptidase 0	*Strep. pneumoniae*	Binds C1q and blocks complement and also uses this interaction to promote bacterial adhesion	Agarwal *et al.* 2014
Adenylate kinase	*Ps. aeruginosa*	Secreted macrophage cytotoxic factor	Markaryan *et al.* 2001
DNA-binding protein HU	*A. actinomycetemcomitans*	Binds to IL-1β	Paino *et al.* 2012
Ef-Tu	*Leptospira spp.*	Binds complement factor	Wolff *et al.* 2013
Ef-Tu	*Ps. aeruginosa*	Binds complement factor	Kunert *et al.* 2007
Nucleoside diphosphate kinase	*M. tuberculosis*	Secreted macrophage cytotoxic factor	Chopra *et al.* 2003
BCAM0224 (trimeric autotransporter)	*Burkholderia cenocepacia*	Inhibits complement activation	Mil-Homens *et al.* 2014
Caf1 usher protein	*Y. pestis*	High-affinity IL-1β-binding protein	Zav'yalov *et al.* 1995
Caf1A	*Y. pestis*	High-affinity IL-1R binding protein	Abramov *et al.* 2001
Lcrv	*Y. pestis*	High-affinity IFNγ-binding protein	Gendrin *et al.* 2010; Abramov *et al.* 2007
Oprf	*Ps. aeruginosa*	Receptor for IFNγ	Wu *et al.* 2005
Protein A	*S. aureus*	Stimulates release of TNFR1 receptor and acts as anti-inflammatory signal	Giai *et al.* 2013
Slp	*L. acidophilus*	Modulator of dendritic cells and T-cells	Konstantinov *et al.* 2008
Type IV pilus	*N. meningitides*	Binds TNFα and IL-8 and induces changes in bacterial virulence	Mahdavi *et al.* 2013
LytA	*Strep. pneumoniae*	Complement inhibitor	Ramos-Sevillano *et al.* 2015
Streptococcal collagen-like protein 1	*Strep. pyogenes*	Evades actions of neutrophil NETs	Döhrmann *et al.* 2014
Leptospira immunoglobulin -like proteins (Lig)	*Leptospira spp.*	Adhesins which also inhibit complement	Castiblanco-Valencia *et al.* 2012
YadA	*Y. enterocolitica*	Adhesin and complement inhibitor	Schindler *et al.* 2012

An emerging literature suggests that bacteria have evolved proteins which bind to specific host cytokines. As cytokines are among the most important controlling factors in immunity, it is possible that these cytokine-binding proteins have roles to play in immune evasion. However, this has not yet been established. What is surprising is that the majority of these cytokine-binding proteins are moonlighting proteins. These include the proteins shown in Table 3.6 plus the GAPDH of *M. tuberculosis*, which is a very-high-affinity binding receptor for epidermal growth factor (EGF) (Bermudez *et al*. 1996). Only one of these proteins has been shown to interfere with cytokine networks. This is the *Yersinia pestis* capsular protein, Caf1a, which is a high-affinity binding protein to the IL-1 receptor and can antagonize the binding of IL-1 to its receptor (Abramov *et al*. 2001). Of interest, the binding of cytokines to these bacterial cytokine binding proteins can alter bacterial global transcription and influence bacterial virulence (Mahdavi *et al*. 2013). This suggests an additional level of communication between bacteria and the immune system which may play key roles in immune evasion. See Chapter 21 by Riikka Ihalin for more details on bacterial cytokine binding.

Bacterial T cell superantigens activate and kill (or make anergic) large percentages of circulating T lymphocytes, knocking holes in the hosts T cell repertoire. Williams *et al*. (2000) identified a family of superantigen-like proteins in *Staph. aureus*, now numbering 14 proteins (Langley *et al*. 2010). Over the past 15 years these proteins have been shown to exhibit a very wide variety of biological actions, a number of which are important in immune evasion (Langley *et al*. 2010). This is clearly a moonlighting family of bacterial proteins, with actions covering much of the virulence protein spectrum.

3.4.5 Bacterial Moonlighting Proteins with Toxin-like Actions

Bacterial toxins have been feared since the early days of bacteriology. The toxin concept rose from the study of diphtheria, a disease localized in the throat, but with systemic manifestation. This was explained by the bacterium releasing a "poison" (toxin) which could cause widespread damage to the host (Lax 2005). Since these early days, a very large number of bacterial toxins have been discovered and we now appreciate that many of these proteins are not simple cell or tissue killers, but are precision controllers of cell functionality by specifically targeting host cell surface or intracellular proteins. The infamous clostridial neurotoxins are metalloproteases which target SNARE proteins to influence neuronal function (Popoff and Poulain 2010). Other toxins cause pore formation, inhibit protein synthesis, activate specific intracellular signaling pathways, activate T lymphocytes, and so on (Lax 2005).

A growing number of bacterial moonlighting proteins have functions which would seem to overlap with the actions we would expect from a 'modern' toxin. The clearest examples of this come from bacteria that target insects. These have evolved to use the molecular chaperone, chaperonin 60, as a toxin. The endosymbiotic bacterium, *Enterobacter aerogenes*, which exists in the saliva of the doodlebug (a larval form of insects known as the *Myrmeleonidae*) secretes its chaperonin 60 to be used as an insect neurotoxin by its host (Yoshida *et al*. 2001). This

chaperonin 60 protein is active at nanomolar concentrations. The highly homologous *E. coli* chaperonin 60 protein, GroEL, has no neurotoxic action, but a single residue mutation can turn this protein into a potent neurotoxin (Yoshida *et al*. 2001). Various *Xenorhabdus* species also use their chaperonin 60 proteins as toxins, in this case the target tissue is the gut (e.g., Joshi *et al*. 2008). Another insecticidal toxin is employed by *Paenibacillus larvae* which infects larvae of the honeybee (*Apis mellifera*), causing the condition American Foulbrood. In this condition, the enolase of the bacterium functions as a toxin, being directly toxic to the larvae (Antúnez *et al*. 2011). In addition, the fimbrial subunit of *Xenorhabdus nematophila* also functions as a pore-forming toxin (Banerjee *et al*. 2006).

Other proteins with toxin-like actions include the dihydrolipoamide dehydrogenase of *Mycobacterium bovis*, which binds to the actin-binding protein coronin causes the arrest of phagosome maturation, thus preventing killing of the bacterium within the macrophage (Deghmane *et al*. 2007) and the *Pseudomonas aeruginosa* adenylate kinase, which is a secreted cytotoxic factor for macrophages (Markaryan *et al*. 2001).

3.5 Bacterial Moonlighting Proteins Conclusively Shown to be Virulence Factors

As stated in Section 3.4, while *in vitro* experiments can suggest that a particular moonlighting protein is involved in virulence because it mimics the action of known virulence factors, the true test is to inactivate the gene and show that the organism has decreased in virulence. With most of the early discovered bacterial moonlighting proteins being essential metabolic enzymes or molecular chaperones, it proved difficult to generate isogenic mutants. However, a trickle of publications is now starting to appear where either novel approaches have been taken to generate gene knockouts, where the moonlighting protein is a nonessential homolog, or where the bacterium does not utilize a key metabolic pathway containing a moonlighting protein.

The first "gene knockout" of a moonlighting protein gene was of the glycolytic protein, GAPDH, in *Streptococcus pyogenes*. In what is still a fascinating technical achievement, Vijay Pancholi's group knocked out the chromosomal copy of the *gapdh* gene. This would render the bacterium nonviable and so the trick was to replace the chromosomal copy of the gene with a plasmid-based version which was modified. Pancholi predicted that generating the GAPDH protein with a small C-terminal hydrophobic peptide would: (1) generate an active enzyme; and (2) prevent the enzyme being secreted by the bacterium. This turned out to be the case, and the "cell-surface mutant" was viable but contained only a fraction of the cell-surface GAPDH. Moreover, this mutant was altered in its cellular adhesion and susceptibility to complement (Boël *et al*. 2005). It was later shown that this cell-surface GAPDH mutant was avirulent in mice. This may be related to the finding that many of the virulence genes failed to be switched on in this mutant, perhaps suggesting that secreted GAPDH is a stimulatory autocrine factor (Jin *et al*. 2011). The reader should refer to Chapter 9 for more detailed discussion.

The chaperonin 60 protein is a multifunctional moonlighting protein (Henderson *et al*. 2013). *Mycobacterium tuberculosis* is one of the large fraction of bacteria to contain more than one gene encoding chaperonin 60 proteins (Lund 2009), in this case termed chaperonin 60.1 and 60.2 (Hsp65). The gene encoding chaperonin 60.1 is in an operon with the gene encoding the co-chaperone, chaperonin 10, and so was thought to be the major cellular molecular chaperone (Lund 2009). Both of these proteins have a range of moonlighting actions (Henderson *et al*. 2010). In an attempt to determine the role the three chaperonins played in *M. tuberculosis* pathology, these three genes were individually inactivated. Loss of the genes encoding chaperonin 60.2 or chaperonin 10 resulted in nonviable organisms unless the genes were replaced with plasmid-based copies (Hu *et al*. 2008). However, unexpectedly, the gene encoding the chaperonin 60.1 protein could be inactivated, with no change in bacterial growth *in vitro* and with no change in bacterial responses to a variety of stressors. However, when used to infect mice or guinea pigs, the chaperonin 60.1 isogenic mutant failed to induce the classic tubercular granulomatous response, which was not due to lack of *in vivo* bacterial growth (Hu *et al*. 2008). Using a human blood granuloma assay it was shown that the chaperonin 60.1 mutant was virtually unable to induce the formation of the multinucleate giant cells that are a hallmark of the tuberculoid granuloma, suggesting that this protein is responsible for the induction of these cells (whose function is unknown) in tuberculosis (Cehovin *et al*. 2010). Related to these findings, but in a way that is currently not explicable, is the finding that the *M. tuberculosis* chaperonin 60.1 is a potent inhibitor of the formation of the naturally multinucleate myeloid cell of bone: the osteoclast. This is via inhibition of the synthesis of a key osteoclast transcription factor, NFATc1 (Winrow *et al*. 2008). This suggests chaperonin 60.1 from this bacterium is a controlling factor in myeloid cell multinucleation.

The glycolytic enzyme fructose-1,6-bisphosphate aldolase (FBA) is now recognized as a virulence factor of a number of bacterial pathogens (Shams *et al*. 2014). For example, in *M. tuberculosis*, it is a cell-surface plasminogen receptor. Knockout of the gene encoding FBA in *M. tuberculosis* did not generate viable mutants, showing this enzyme to be essential (de la Paz Santangelo *et al*. 2011). In *Neisseria meningitidis*, the glycolytic enzyme phosphofructokinase is missing, thus rendering the glycolytic pathway redundant (Baart *et al*. 2007). It would be expected that in bacteria lacking the use of the glycolytic pathway, the genes for the individual glycolytic enzymes would be lost. However, this has not happened in the neisseriae, and it has been found that FBA functions as an adhesin for binding to host cells (Tunio *et al*. 2010). Inactivation of the *fba* gene in *N. meningitidis* resulted in a viable organism which grew normally but had a decreased ability to bind to the target cells (Tunio *et al*. 2010). It still remains for this mutant organism to be compared to the wild-type bacterium in *in vivo* studies.

The endopeptidase O of *Strep. pneumoniae* is a moonlighting plasminogen- and fibronectin-binding protein involved in both adhesion and cell invasion. Inactivation of the gene for this protein resulted in an isogenic mutant that had decreased *in vitro* adhesive and cell invasive activities (Agarwal *et al*. 2013).

Knockout of the gene encoding the moonlighting autotransporter of *Burkolderia cenocepacia*, termed BCAM0223, blocks many of the virulence

attributes of this protein, including adhesion, serum resistance, and hemagglutination (Mil-Homens and Fialho 2012). Of importance, the isogenic mutant lacking this autotransporter had attenuated virulence when the organism was used to infect the moth, *Galleria mellonella* (Mil-Homens and Fialho 2012). The *N. meningitidis* autotransporter NhhA is an adhesin for human epithelial cells, and also laminin and heparan sulfate. Inactivation of the gene resulted in decreased bacterial adhesion to host cells *in vitro* (Scarselli *et al*. 2006)

The *Helicobacter pylori* peptidyl prolyl cis,trans isomerase, HP0175, was shown to be a secreted virulence factor able to induce apoptosis of gastric epithelial cells (Basak *et al*. 2006). Further research on the mechanism of action of HP0175 revealed that it does this partly by inducing autophagy in the gastric cells. A mutant *H. pylori* strain lacking the gene encoding HP0175 is compromised in its ability to induce autophagy and apoptosis (Halder *et al*. 2015).

3.6 Eukaryotic Moonlighting Proteins That Aid in Bacterial Virulence

Surprising as it is that bacterial moonlighting proteins are involved in the virulence process, it is even more surprising that host moonlighting proteins have also evolved to aid the infection of certain bacteria. Perhaps the first example of this was the report that transferrin enhanced the binding of *Neisseria gonorrhoea* to human endometrial cells (Heine *et al*. 1996). More recently, it has been shown that transferrin also aids the uptake of viruses, such as hepatitis C virus (Martin and Uprichard 2013), into cells. Other human moonlighting proteins already discussed which aid bacterial binding and possible invasion are uPAR/CD87, which binds the GAPDH of *Strep. pyogenes* (Jin *et al*. 2005), and CD43, which binds to the cell-surface-expressed chaperonin 60.2 protein of *M. tuberculosis* (Hickey *et al*. 2010). Here we have a bacterial chaperonin 60 protein acting as a ligand for a host cell receptor. *Listeria monocytogenes* uses a cell-surface acetaldehyde-alcohol dehydrogenase (a molecule known as Listeria adhesion protein or LAP) to bind to host epithelial cells. Here the host receptor is the human chaperonin 60 protein (better known in this context as Hsp60). Binding between these two proteins has a low nanomolar K_D, suggesting this is a biologically relevant protein–protein pairing (Kim *et al*. 2006).

Other host moonlighting proteins which appear to aid in bacterial colonization are the molecular chaperones Hsp70, Hsp90, and Gp96. The former two function as part of a multiprotein complex for the proinflammatory major Gram-negative cell-wall component, lipopolysaccharide (LPS) (Triantafilou *et al*. 2001). It is not clear if this binding forms part of an adhesive process. However, the EEVD motif of another Hsp70 protein, Hsc70 (HSPA8), on trophoblast giant cells is involved in the uptake of abortion-inducing pathogenic bacteria (Watanabe *et al*. 2009).

The endoplasmic reticulum molecular chaperone, Gp96, also forms part of an adhesive and invasive process for strains of *E. coli* (K1) that can invade the brain through the microvasculature and cause meningitis. This organism binds to human brain microvascular endothelial cells through the pairing of the bacterial

protein, outer membrane protein (Omp)A, and the host endoplasmic reticulum chaperone, Gp96, which is present on the outer cell membrane of these endothelial cells (Maruvada *et al.* 2008). Binding of these two proteins activates signaling through the transcription factor STAT3, leading to invasion of the bacteria into the endothelial cells.

A number of bacterial toxins require to interact with host moonlighting proteins such as Hsp90 and peptidyl prolyl isomerases to translocate to the correct cellular compartment where they exert their toxic actions. These include the binary actin-ADP-ribosylating toxins from *Clostridium difficile* and *Clostridium perfringens* (Kaiser *et al.* 2011) and *Photorhabdus luminescens* ADP-ribosyltransferases (Lang *et al.* 2014). Another molecular chaperone, the Hsp70 protein family member BiP, is the only target of the *E. coli* subtilase cytotoxin and is responsible for cellular changes leading to apoptosis (Paton *et al.* 2013).

To conclude this section the recent review article by Dunphy *et al.* (2013) on *Ehrlichia chaffeensis*, an obligately intracellular Gram-negative bacterium that targets and invades human mononuclear phagocytes and is the causative agent of monocytotropic erlichiosis (Anderson *et al.* 1991), is likely to be describing the paradigm for the complexity of the interaction of moonlighting proteins between prokaryotes and their eukaryotic hosts.

3.7 Conclusions

Bacterial virulence is a consequence of complex networks of interactions between the bacterial cell surface and secreted molecules (largely proteins) and host components, principally cell surface and extracellular proteins (unless the pathogen is intracellular). This generates networks of overlapping protein–ligand interactions which have evolved to enable bacteria to colonize their hosts and maintain survival and growth within this, potentially hostile, environment. Among the networks of bacteria–host interactions involved in bacterial virulence, we find a surprising number of bacterial moonlighting proteins and also a growing number of moonlighting host proteins. What is interesting is that many of the bacterial moonlighting proteins are homologs of human proteins, examples being GAPDH, enolase, aldolase, chaperonin 60, Hsp70, peptidyl prolyl isomerases, and so on. The human versions of these proteins also exhibit another network of moonlighting actions; it is not clear how these networks of protein moonlighting interact, and if such interactions aids the pathogen or the host.

A number of questions about bacterial moonlighting proteins are starting to find answers. The obvious question is how important are moonlighting proteins to bacterial virulence? To some extent this is answered by the facts that many human pathogens utilize one or other moonlighting proteins in their interactions with the host. A second point suggesting their importance is the finding that many different bacterial species employ the same moonlighting protein (e.g., GAPDH, enolase, chaperonin 60, Hsp27, etc.) as virulence factors. A third and (the author believes) crucial fact is that, where measured, the affinity of bacterial moonlighting proteins for their ligands is of high affinity. This strongly supports the hypothesis that the biological actions of moonlighting proteins are important

in host–pathogen interactions. That the interactions of bacterial moonlighting proteins with the host is important in virulence is also supported by the growing number of examples in which the genes encoding moonlighting proteins have been inactivated and the bacterium is seen to lose its virulence attributes in *in vitro* studies. Currently, only three gene inactivation studies have been tested *in vivo*, but all reveal that the specific moonlighting protein plays a key role in virulence. A final point is the realization that bacterial moonlighting proteins can exhibit novel biological properties not attributable to the population of specific bacterial virulence factors. Moonlighting proteins are therefore able to expand the functional proteome of the bacterium. As some bacteria are currently known to use up to 20 different moonlighting proteins, this suggests that in the future we are likely to see the bacterial "moonlight-ome" being much larger than it is at present.

We live in a world of increasing antibiotic resistance in which novel targets are required to deal with bacterial infections. Moonlighting proteins, either bacterial- or host-derived, are potential future therapeutic targets. With so many bacteria utilizing GAPDH or enolase, targeting these proteins could generate "wide-spectrum" agents able to deal with multiple Gram-negative and Gram-positive pathogens. Host moonlighting proteins could also be targeted. For example, it has been shown that angiotensin II receptor type 1 (AT1R) associates with plasma membrane Gp96 (extracellular (e)Gp96). The small molecular inhibitor of AT1R, telmisartan, inhibits *E. coli* K1 invasion into human brain microvascular endothelial cells; administration of this compound to newborn mice before infection with *E. coli* prevented the onset of meningitis (Krishnan *et al*. 2014). This shows the potential therapeutic value of understanding the infection biology of moonlighting proteins.

References

Abou-Zeid C, Garbe T, Lathigra R, Wiker HG, Harboe M, Rook GA, Young DB (1991) Genetic and immunological analysis of *Mycobacterium tuberculosis* fibronectin–binding proteins. *Infection and Immunity* 59: 2712–18.

Abramov VM, Vasiliev AM, Vasilenko RN, Kulikova NL, Kosarev IV, Khlebnikov VS, Ishchenko AT, MacIntyre S, Gillespie JR, Khurana R, Korpela T, Fink AL, Uversky VN (2001) Structural and functional similarity between *Yersinia pestis* capsular protein Caf1 and human interleukin-1 beta. *Biochemistry* 40: 6076–84.

Abramov VM, Khlebnikov VS, Vasiliev AM, Kosarev IV, Vasilenko RN, Kulikova NL, Khodyakova AV, Evstigneev VI, Uversky VN, Motin VL, Smirnov GB, Brubaker RR (2007) Attachment of LcrV from *Yersinia pestis* at dual binding sites to human TLR-2 and human IFN-gamma receptor. *Journal of Proteome Research* 6: 2222–31.

Agarwal V, Hammerschmidt S, Malm S, Bergmann S, Riesbeck K, Blom AM (2012) *Enolase of Streptococcus pneumoniae* binds human complement inhibitor C4b–binding protein and contributes to complement evasion. *Journal of Immunology* 189: 3575–84.

Agarwal V, Kuchipudi A, Fulde M, Riesbeck K, Bergmann S, Blom AM (2013) *Streptococcus pneumoniae* endopeptidase O (PepO) is a multifunctional plasminogen- and fibronectin-binding protein, facilitating evasion of innate immunity and invasion of host cells. *Journal of Biological Chemistry* 288: 6849–63.

Agarwal V, Sroka M, Fulde M, Bergmann S, Riesbeck K, Blom AM (2014) Binding of *Streptococcus pneumoniae* endopeptidase O (PepO) to complement component C1q modulates the complement attack and promotes host cell adherence. *Journal of Biological Chemistry*, published online 16 April 2014, doi: 10.1074/jbc.M113.530212.

Agnello D, Scanziani E, Di GM, Leoni F, Modena D, Mascagni P, Introna M, Ghezzi P, Villa P (2002) Preventive administration of Mycobacterium tuberculosis 10-kDa heat shock protein (hsp10) suppresses adjuvant arthritis in Lewis rats. *International Immunopharmacology* 2: 463–474.

Alonzo F 3rd, Kozhaya L, Rawlings SA, Reyes-Robles T, DuMont AL, Myszka DG, Landau NR, Unutmaz D, Torres VJ (2013) CCR5 is a receptor for *Staphylococcus aureus* leukotoxin ED. *Nature* 493: 51–5.

Anderson BE, Dawson JE, Jones DC, Wilson KH (1991) *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. *Journal of Clinical Microbiology* 29: 2838–42.

Antúnez K, Anido M, Arredondo D, Evans JD, Zunino P (2011) *Paenibacillus larvae* enolase as a virulence factor in honeybee larvae infection. *Vetinary Microbiology* 147: 83–9.

Aoki K, Matsumoto S, Hirayama Y, Wada T, Ozeki Y, Niki M, Domenech P, Umemori K, Yamamoto S, Mineda A, Matsumoto M, Kobayashi K (2004) Extracellular mycobacterial DNA-binding protein 1 participates in mycobacterium-lung epithelial cell interaction through hyaluronic acid. *Journal of Biological Chemistry* 279: 39798–806.

Ayrapetyan M, Williams TC, Oliver JD (2015) Bridging the gap between viable but non-culturable and antibiotic persistent bacteria. *Trends in Microbiology* 23(1): 7–13.

Baart GJ, Zomer B, de Haan A, van der Pol LA, Beuvery EC, Tramper J, Martens DE (2007) Modelling Neisseria meningitidis metabolism: from genome to metabolic fluxes. *Genome Biology* 8: R136.

Bæk KT, Vegge CS, Brøndsted L (2011) HtrA chaperone activity contributes to host cell binding in *Campylobacter jejuni*. *Gut Pathogens* 3: 13.

Banerjee J, Singh J, Joshi MC, Ghosh S, Banerjee N (2006) The cytotoxic fimbrial structural subunit of *Xenorhabdus nematophila* is a pore-forming toxin. *Journal of Bacteriology* 188: 7957–62.

Basak C, Pathak SK, Bhattacharyya A, Pathak S, Basu J, Kundu M (2005) The secreted peptidyl prolyl cis,trans-isomerase HP0175 of *Helicobacter pylori* induces apoptosis of gastric epithelial cells in a TLR4- and apoptosis signal-regulating kinase 1-dependent manner. *Journal of Immunology* 174: 5672–80.

Bergmann S, Wild D, Diekmann O, Frank R, Bracht D, Chhatwal GS, Hammerschmidt S (2003) Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. *Molecular Microbiology* 49: 411–23.

Bergmann S, Rohde M, Preissner KT, Hammerschmidt S (2005) The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration. *Thrombosis and Haemostasis* 94: 304–11.

Bermudez LE, Petrofsky M, Shelton K (1996) Epidermal growth factor-binding protein in *Mycobacterium avium* and *Mycobacterium tuberculosis*: a possible role in the mechanism of infection. *Infection and Immunity* 64: 2917–22.

Blau K, Portnoi M, Shagan M, Kaganovich A, Rom S, Kafka D, Chalifa Caspi V, Porgador A, Givon-Lavi N, Gershoni JM, Dagan R, Mizrachi Nebenzahl Y (2007) Flamingo cadherin: a putative host receptor for *Streptococcus pneumoniae*. *Journal of Infectious Diseases* 195: 1828–37.

Blom AM, Bergmann S, Fulde M, Riesbeck K, Agarwal V (2014) *Streptococcus pneumoniae* phosphoglycerate kinase is a novel complement inhibitor affecting the membrane attack complex formation. *Journal of Biological Chemistry* 289: 32499–511.

Boël G, Jin H, Pancholi V (2005) Inhibition of cell surface export of group A streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties. *Infection and Immunity* 73: 6237–48.

Boleij A, Schaeps RM, de Kleijn S, Hermans PW, Glaser P, Pancholi V, Swinkels DW, Tjalsma H (2009) Surface-exposed histone-like protein a modulates adherence of *Streptococcus gallolyticus* to colon adenocarcinoma cells. *Infection and Immunity* 77: 5519–27.

Boleij A, Laarakkers CM, Gloerich J, Swinkels DW, Tjalsma H (2011) Surface-affinity profiling to identify host-pathogen interactions. *Infection and Immunity* 79: 4777–83.

Boradia VM, Malhotra H, Thakkar JS, Tillu VA, Vuppala B, Patil P, Sheokand N, Sharma P, Chauhan AS, Raje M, Raje CI (2014). *Mycobacterium tuberculosis* acquires iron by cell-surface sequestration and internalization of human holo-transferrin. *Nature Communications* 5: 4730.

Buck BL, Altermann E, Svingerud T, Klaenhammer TR (2005) Functional analysis of putative adhesion factors in *Lactobacillus acidophilus* NCFM. *Applied and Environmental Microbiology* 71, 8344–51.

Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, Strobel S, Józsi M, Isaac L, Barbosa AS (2012) Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *Journal of Infectious Diseases* 205: 995–1004.

Cehovin A, Coates AR, Hu Y, Riffo-Vasquez Y, Tormay P, Botanch C, Altare F, Henderson B (2010) Comparison of the moonlighting actions of the two highly homologous chaperonin 60 proteins of *Mycobacterium tuberculosis*. *Infection and Immunity* 78: 3196–206.

Chang CC, Chen YJ, Tseng CS, Lai WL, Hsu KY, Chang CL, Lu CC, Hsu YM (2011). A comparative study of the interaction of *Bartonella henselae* strains with human endothelial cells. *Vetinary Microbiology* 149: 147–56.

Chen G, Severo MS, Sakhon OS, Choy A, Herron MJ, Felsheim RF, Wiryawan H, Liao J, Johns JL, Munderloh UG, Sutterwala FS, Kotsyfakis M, Pedra JH (2012) *Anaplasma phagocytophilum* dihydrolipoamide dehydrogenase 1 affects host-derived immunopathology during microbial colonization. *Infection and Immunity* 80: 3194–205.

Chong A, Lima CA, Allan DS, Nasrallah GK, Garduno RA (2009) The purified and recombinant *Legionella pneumophila* chaperonin alters mitochondrial trafficking and microfilament organization. *Infection and Immunity* 77: 4724–39.

Chopra P, Singh A, Koul A, Ramachandran S, Drlica K, Tyagi AK, Singh Y (2003) Cytotoxic activity of nucleoside diphosphate kinase secreted from Mycobacterium tuberculosis. *European Journal of Biochemistry* 270: 625–634.

Cork AJ, Jergic S, Hammerschmidt S, Kobe B, Pancholi V, Benesch JL, Robinson CV, Dixon NE, Aquilina JA, Walker MJ (2009) Defining the structural basis of human plasminogen binding by streptococcal surface enolase. *Journal of Biological Chemistry* 284: 17129–37.

Courtney HS, Li Y, Twal WO, Argraves WS (2009) Serum opacity factor is a streptococcal receptor for the extracellular matrix protein fibulin-1. *Journal of Biological Chemistry* 284: 12966–71.

Dahesh S, Nizet V, Cole JN (2012) Study of streptococcal hemoprotein receptor (Shr) in iron acquisition and virulence of M1T1 group A streptococcus. *Virulence* 3: 566–75.

Dallo SF, Zhang B, Denno J, Hong S, Tsai A, Haskins W, Ye JY, Weitao T (2012) Association of *Acinetobacter baumannii* EF-Tu with cell surface, outer membrane vesicles, and fibronectin. *Scientific World Journal* 2012: 128705.

de la Paz Santangelo M, Gest PM, Guerin ME, Coinçon M, Pham H, Ryan G, Puckett SE, Spencer JS, Gonzalez-Juarrero M, Daher R, Lenaerts AJ, Schnappinger D, Therisod M, Ehrt S, Sygusch J, Jackson M (2011) Glycolytic and non-glycolytic functions of *Mycobacterium tuberculosis* fructose-1,6-bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. *Journal of Biological Chemistry* 286: 40219–31.

Deghmane AE, Soualhine H, Bach H, Sendide K, Itoh S, Tam A, Noubir S, Talal A, Lo R, Toyoshima S, Av-Gay Y, Hmama Z (2007) Lipoamide dehydrogenase mediates retention of coronin-1 on BCG vacuoles, leading to arrest in phagosome maturation. *Journal of Cell Science* 120: 2796–806.

Derbise A, Song YP, Parikh S, Fischetti VA, Pancholi V (2004) Role of the C-terminal lysine residues of streptococcal surface enolase in Glu- and Lys-plasminogen-binding activities of group A streptococci. *Infection and Immunity* 72: 94–105.

Döhrmann S, Anik S, Olson J, Anderson EL, Etesami N, No H, Snipper J, Nizet V, Okumura CY (2011) Role for streptococcal collagen-like protein 1 in M1T1 group A Streptococcus resistance to neutrophil extracellular traps. *Infection and Immunity* 82: 4011–20.

Döhrmann S, Anik S, Olson J, Anderson EL, Etesami N, No H, Snipper J, Nizet V, Okumuru CY (2014) Role of streptococcal collagen-like protein 1 in M1T1 group A streptococcus resistance to neutrophil extracellular traps. *Infection and Immunity* 82: 4011–4020.

Du RJ, Ho B (2003) Surface localized heat shock protein 20 (HslV) of *Helicobacter pylori*. *Helicobacter* 8: 257–67.

Dunphy PS, Luo T, McBride JW (2013) *Ehrlichia* moonlighting effectors and interkingdom interactions with the mononuclear phagocyte. *Microbes and Infection* 15: 10.

Ehinger S, Schubert WD, Bergmann S, Hammerschmidt S, Heinz DW (2004) Plasmin(ogen)-binding α-Enolase from *Streptococcus pneumoniae*: crystal

structure and evaluation of plasmin(ogen)-binding sites. *Journal of Molecular Biology* 343: 997–1005.

Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD, Gottschalk M (2008) Isolation and characterization of α-enolase, a novel fibronectin-binding protein from *Streptococcus suis*. *Microbiology* 154: 2668–79.

Fernandes LG, Vieira ML, Kirchgatter K, Alves IJ, de Morais ZM, Vasconcellos SA, Romero EC, Nascimento AL (2012) OmpL1 is an extracellular matrix- and plasminogen-interacting protein of Leptospira spp. *Infection and Immunity* 80: 3679–92.

Fernandes LG, Vieira ML, Alves IJ, de Morais ZM, Vasconcellos SA, Romero EC, Nascimento AL (2014) Functional and immunological evaluation of two novel proteins of Leptospira spp. *Microbiology* 160: 149–64.

Floden AM, Watt JA, Brissette CA (2011) *Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein. *PLoS One* 6: e27502.

Floto RA, MacAry PA, Boname JM, Mien TS, Kampmann B, Hair JR, Huey OS, Houben EN, Pieters J, Day C, Oehlmann W, Singh M, Smith KG, Lehner PJ (2006) Dendritic cell stimulation by mycobacterial Hsp70 is mediated through CCR5. *Science* 314: 454–8.

Foulston L, Elsholz AK, DeFrancesco AS, Losick R (2014) The extracellular matrix of *Staphylococcus aureus* biofilms comprises cytoplasmic proteins that associate with the cell surface in response to decreasing pH. *MBio* 5: e01667–14.

Frick IM, Crossin KL, Edelman GM, Björck L (1995) Protein H: a bacterial surface protein with affinity for both immunoglobulin and fibronectin type III domains. *EMBO Journal* 14: 1674–79.

Fu S, Yuan F, Zhang M, Tan C, Chen H, Bei W (2012) Cloning, expression and characterization of a cell wall surface protein, 6-phosphogluconate dehydrogenase, of *Haemophilus parasuis*. *Research in Vetinary Science* 93: 57–62.

Furuya H, Ikeda R (2009) Interaction of triosephosphate isomerase from the cell surface of *Staphylococcus aureus* and alpha-(1–>3)-mannooligosaccharides derived from glucuronoxylomannan of *Cryptococcus neoformans*. *Microbiology* 155: 2707–13.

Garg N, Manchanda G, Kumar A (2014) Bacterial quorum sensing: circuits and applications. *Antonie Van Leeuwenhoek* 105: 289–305.

Gendrin C, Sarrazin S, Bonnaffé D, Jault JM, Lortat-Jacob H, Dessen A (2010) Hijacking of the pleiotropic cytokine interferon-γ by the type III secretion system of *Yersinia pestis*. *PLoS One* 5: e15242.

Giai C, Gonzalez C, Ledo C, Garofalo A, Di Genaro MS, Sordelli DO, Gomez MI (2013) Shedding of tumor necrosis factor receptor 1 induced by protein A decreases tumor necrosis factor alpha availability and inflammation during systemic Staphylococcus aureus infection. *Infection and Immunity* 81: 4200–4207.

Haddad N, Tresse O, Rivoal K, Chevret D, Nonglaton Q, Burns CM, Prévost H, Cappelier JM (2012) Polynucleotide phosphorylase has an impact on cell biology of *Campylobacter jejuni*. *Frontiers in Cellular and Infection Microbiology* 2: 30.

Halder P, Datta C, Kumar R, Sharma AK, Basu J, Kundu M (2015) The secreted antigen, HP0175 of Helicobacter pylori links the unfolded protein response (UPR) to autophagy in gastric epithelial cells. *Cell Microbiology* 17(5): 714–29.

Hallström T, Mörgelin M, Barthel D, Raguse M, Kunert A, Hoffmann R, Skerka C, Zipfel PF (2012) Dihydrolipoamide dehydrogenase of *Pseudomonas aeruginosa* is a surface-exposed immune evasion protein that binds three members of the factor H family and plasminogen. *Journal of Immunology* 189: 4939–50.

Hanke ML, Kielian T (2012) Deciphering mechanisms of staphylococcal biofilm evasion of host immunity. *Frontiers in Cellular and Infection Microbiology* 2: 62.

Hannibal L, Collins D, Brassard J, Chakravarti R, Vempati R, Dorlet P, Santolini J, Dawson JH, Stuehr DJ (2012) Heme binding properties of glyceraldehydes-3-phosphate dehydrogenase. *Biochemistry* 51: 8514–29.

Hartmann E, Lingwood CA, Reidl J (2001) Heat-inducible surface stress protein (Hsp70) mediates sulfatide recognition of the respiratory pathogen *Haemophilus influenzae*. *Infection and Immunity* 69: 3438–41.

Heilmann C, Thumm G, Chhatwal GS, Hartleib J, Uekötter A, Peters G (2003) Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. *Microbiology* 149: 2769–78.

Heine RP, Elkins C, Wyrick PB, Sparling PF (1996) Transferrin increases adherence of iron–deprived *Neisseria gonorrhoeae* to human endometrial cells. *American Journal of Obstetrics and Gynecology* 174: 659–66.

Henderson B (2014) An overview of protein moonlighting in bacterial infection. *Biochemical Society Transactions* 42: 1720–27.

Henderson B, Martin A (2011) Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity* 79: 3476–91.

Henderson B, Martin A (2013) Bacterial moonlighting proteins and bacterial virulence. *Current Topics in Microbiology and Immunology* 358: 155–213.

Henderson B, Lund PA, Coates AR (2010) Multiple moonlighting functions of mycobacterial molecular chaperones. *Tuberculosis (Edinb)* 90: 119–24.

Henderson B, Fares MA, Lund PA (2013) Chaperonin 60: a paradoxical, evolutionarily conserved protein family with multiple moonlighting functions. *Biological Reviews of the Cambridge Philosophical Society* 88: 955–87.

Henderson B, Fares M, Martin A (in press) *Protein Moonlighting in Biology and Medicine: Biological and Biomedical Consequences of Protein Moonlighting*. Wiley-Blackwell.

Henkel JS, Baldwin MR, Barbieri JT (2010) Toxins from bacteria. *EXS* 100: 1–29.

Hickey TB, Ziltener HJ, Speert DP, Stokes RW (2010) *Mycobacterium tuberculosis* employs Cpn60.2 as an adhesin that binds CD43 on the macrophage surface. *Cell Microbiology* 12: 1634–47.

Hiratsuka K, Kiyama-Kishikawa M, Abiko Y (2010) Hemin-binding protein 35 (HBP35) plays an important role in bacteria-mammalian cells interactions in *Porphyromonas gingivalis*. *Microbial Pathogenesis* 48: 116–23.

Hopfe M, Dahlmanns T, Henrich B (2011) In *Mycoplasma hominis* the OppA-mediated cytoadhesion depends on its ATPase activity. *BMC Microbiology* 11: 185.

Hu Y, Henderson B, Lund PA, Tormay P, Ahmed MT, Gurcha SS, Besra GS, Coates AR (2008) A *Mycobacterium tuberculosis* mutant lacking the groel homologue Cpn60.1 is viable but fails to induce an inflammatory response in animal models of infection. *Infection and Immunity* 76: 1535–1546.

Jensen H, Roos S, Jonsson H, Rud I, Grimmer S, van Pijkeren JP, Britton RA, Axelsson L (2014) Role of *Lactobacillus reuteri* cell and mucus-binding protein A (CmbA) in adhesion to intestinal epithelial cells and mucus *in vitro*. *Microbiology* 160: 671–81.

Jenuwine ES, Shaner SL (1996) Equilibrium constants for nonspecific binding of proteins to DNA may be obtained by quantitative zonal DNA affinity chromatography. *Analytical Biochemistry* 242: 228–33.

Jin H, Song YP, Boel G, Kochar J, Pancholi V (2005) Group A streptococcal surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *Journal of Molecular Biology* 350: 27–41.

Jin H, Agarwal S, Agarwal S, Pancholi V (2011) Surface export of GAPDH/SDH, a glycolytic enzyme, is essential for *Streptococcus pyogenes* virulence. *MBio* 2: e00068–11.

Jorgensen C, Gedon E, Jaquet C, Sany J (1998) Gastric administration of recombinant 65 kDa heat shock protein delays the severity of type II collagen induced arthritis in mice. *Journal of Rheumatology* 25: 763–767.

Joshi MC, Sharma A, Kant S, Birah A, Gupta GP, Khan SR, Bhatnagar R, Banerjee N (2008). An insecticidal GroEL protein with chitin binding activity from *Xenorhabdus nematophila*. *Journal of Biological Chemistry* 283: 28287–96.

Kainulainen V, Loimaranta V, Pekkala A, Edelman S, Antikainen J, Kylväjä R, Laaksonen M, Laakkonen L, Finne J, Korhonen TK (2012) Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37. *Journal of Bacteriology* 194: 2509–19.

Kaiser E, Kroll C, Ernst K, Schwan C, Popoff M, Fischer G, Buchner J, Aktories K, Barth H (2011) Membrane translocation of binary actin-ADP-ribosylating toxins from *Clostridium difficile* and *Clostridium perfringens* is facilitated by cyclophilin A and Hsp90. *Infection and Immunity* 79: 3913–21.

Katakura Y, Sano R, Hashimoto T, Ninomiya K, Shioya S (2010) Lactic acid bacteria display on the cell surface cytosolic proteins that recognize yeast mannan. *Applied Microbiology and Biotechnology* 86: 319–26.

Kerro-Dego O, Prysliak T, Perez-Casal J, Potter AA (2012) Role of GapC in the pathogenesis of *Staphylococcus aureus*. *Vetinary Microbiology* 156: 443–7.

Kim KP, Jagadeesan B, Burkholder KM, Jaradat ZW, Wampler JL, Lathrop AA, Morgan MT, Bhunia AK (2006) Adhesion characteristics of Listeria adhesion protein (LAP)-expressing *Escherichia coli* to Caco-2 cells and of recombinant LAP to eukaryotic receptor Hsp60 as examined in a surface plasmon resonance sensor. *FEMS Microbiology Letters* 256: 324–32.

Kinhikar AG, Vargas D, Li H, Mahaffey SB, Hinds L, Belisle JT, Laal S (2006) *Mycobacterium tuberculosis* malate synthase is a laminin-binding adhesin. *Molecular Microbiology* 60: 999–1013.

Konstantinov SR, Smidt H, de Vos WM, Bruijns SC, Singh SK *et al.* (2008) S layer protein A of Lactobacillus acidophilus NCFM regulates immature dendritic cell and T cell functions. Proceedings of the National Academy of Science, USA 105: 19474–19479.

Krachler AM, Orth K (2011) Functional characterization of the interaction between bacterial adhesin multivalent adhesion molecule 7 (MAM7) protein and its host cell ligands. *Journal of Biological Chemistry* 286: 38939–47.

Krishnan S, Shanmuganathan MV, Behenna D, Stoltz BM, Prasadarao NV (2014) Angiotensin II receptor type 1: a novel target for preventing neonatal meningitis in mice by *Escherichia coli* K1. *Journal of Infectious Diseases* 209: 409–19.

Kumar P, Han BC, Shi Z, Jia J, Wang YP, Zhang YT, Liang L, Liu QF, Ji ZL, Chen YZ (2009) Update of KDBI: Kinetic Data of Bio-molecular Interaction database. *Nucleic Acids Research* 37(Database issue): D636–641.

Kunert A, Losse J, Gruszin C, Hühn M, Kaendler K, Mikkat S, Volke D, Hoffmann R, Jokiranta TS, Seeberger H, Moellmann U, Hellwage J, Zipfel PF (2007) Immune evasion of the human pathogen Pseudomonas aeruginosa: elongation factor Tuf is a factor H and plasminogen binding protein. *Journal of Immunology* 179: 2979–2988.

Kuo CJ, Bell H, Hsieh CL, Ptak CP, Chang YF (2012) Novel mycobacteria antigen 85 complex binding motif on fibronectin. *Journal of Biological Chemistry* 287: 1892–902.

Labroussaa F, Arricau-Bouvery N, Dubrana MP, Saillard C (2010) Entry of *Spiroplasma citri* into *Circulifer haematoceps* cells involves interaction between spiroplasma phosphoglycerate kinase and leafhopper actin. *Applied and Environmental Microbiology* 76: 1879–86.

Lang AE, Ernst K, Lee H, Papatheodorou P, Schwan C, Barth H, Aktories K (2014) The chaperone Hsp90 and PPIases of the cyclophilin and FKBP families facilitate membrane translocation of *Photorhabdus luminescens* ADP-ribosyltransferases. *Cell Microbiology* 16: 490–503.

Langley R, Patel D, Jackson N, Clow F, Fraser JD (2010) Staphylococcal superantigen super-domains in immune evasion. *Critical Reviews in Immunology* 30: 149–65.

Lax AJ (2005) Toxins and the interaction between bacterium and host. In Bacterial Protein Toxins (ed. Lax AJ), pp. 1–6. Cambridge University Press, Cambridge.

Lechardeur D, Fernandez A, Robert B, Gaudu P, Trieu-Cuot P, Lamberet G, Gruss A (2011) The 2-Cys peroxiredoxin alkyl hydroperoxide reductase c binds heme and participates in its intracellular availability in *Streptococcus agalactiae*. *Journal of Biological Chemistry* 285: 16032–41.

Lemonnier M, Landraud L, Lemichez E (2007) Rho GTPase-activating bacterial toxins: from bacterial virulence regulation to eukaryotic cell biology. *FEMS Microbiology Reviews* 31: 515–34.

López-Bolaños CC, Guzmán-Murillo MA, Ruiz-Bustos E, Ascencio F (2009) The role of heparan sulfate on adhesion of 47 and 51 kDa outer membrane proteins of *Helicobacter pylori* to gastric cancer cells. *Canadian Journal of Microbiology* 55: 450–6.

Lund PA (2009) Multiple chaperonins in bacteria––why so many? *FEMS Microbiology Reviews* 33: 785–800.

Madureira P, Baptista M, Vieira M, Magalhães V, Camelo A, Oliveira L, Ribeiro A, Tavares D, Trieu-Cuot P, Vilanova M, Ferreira P (2007) *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. *Journal of Immunology* 178: 1379–87.

Madureira P, Andrade EB, Gama B, Oliveira L, Moreira S, Ribeiro A, Correia-Neves M, Trieu-Cuot P, Vilanova M, Ferreira P (2011) Inhibition of IL-10 production by maternal antibodies against Group B Streptococcus GAPDH confers immunity to offspring by favoring neutrophil recruitment. *PLoS Pathogens* 7: e1002363.

Mahdavi J, Royer PJ, Sjölinder HS, Azimi S, Self T, Stoof J, Wheldon LM, Brännström K, Wilson R, Moreton J, Moir JW, Sihlbom C, Borén T, Jonsson AB, Soultanas P, Ala'Aldeen DA (2013) Pro-inflammatory cytokines can act as intracellular modulators of commensal bacterial virulence. *Open Biology* 3: 130048.

Markaryan A, Zaborina O, Punj V, Chakrabarty AM (2001) Adenylate kinase as a virulence factor of *Pseudomonas aeruginosa*. *Journal of Bacteriology* 183: 3345–52.

Martin DN, Uprichard SL (2013) Identification of transferrin receptor 1 as a hepatitis C virus entry factor. *Proceedings of the National Academy of Science, USA* 110: 10777–82.

Maruvada R, Argon Y, Prasadarao NV (2008) *Escherichia coli* interaction with human brain microvascular endothelial cells induces signal transducer and activator of transcription 3 association with the C-terminal domain of Ec-gp96, the outer membrane protein A receptor for invasion. *Cell Microbiology* 10: 2326–38.

Mil-Homens D, Fialho AM (2012) A BCAM0223 mutant of *Burkholderia cenocepacia* is deficient in hemagglutination, serum resistance, adhesion to epithelial cells and virulence. *PLoS One* 7: e41747.

Mil-Homens D, Leça MI, Fernandes F, Pinto SN, Fialho AM (2014) Characterization of BCAM0224, a multifunctional trimeric autotransporter from the human pathogen Burkholderia cenocepacia. *Journal of Bacteriology* 196: 1968–1979.

Modun B, Williams P (1999) The staphylococcal transferrin-binding protein is a cell wall glyceraldehyde-3-phosphate dehydrogenase. *Infection and Immunity* 67: 1086–92.

Morgenthau A, Pogoutse A, Adamiak P, Moraes TF, Schryvers AB (2013) Bacterial receptors for host transferrin and lactoferrin: molecular mechanisms and role in host–microbe interactions. *Future Microbiology* 8: 1575–85.

Mori Y, Yamaguchi M, Terao Y, Hamada S, Ooshima T, Kawabata S (2012) α-Enolase of *Streptococcus pneumoniae* induces formation of neutrophil extracellular traps. *Journal of Biological Chemistry* 287: 10472–81.

Mullen LM, Nair SP, Ward JM, Rycroft AN, Williams RJ, Robertson G, Mordan NJ, Henderson B (2008a) Novel adhesin from *Pasteurella multocida* that binds to the integrin-binding fibronectin FnIII9-10 repeats. *Infection and Immunity* 76: 1093–1104.

Mullen LM, Bossé JT, Nair SP, Ward JM, Rycroft AN, Robertson G, Langford PR, Henderson B (2008b) Pasteurellaceae ComE1 proteins combine the properties of fibronectin adhesins and DNA binding competence proteins. *PLoS One* 3: e3991.

Noinaj N, Buchanan SK, Cornelissen CN (2012) The transferrin-iron import system from pathogenic Neisseria species. *Molecular Microbiology* 86: 246–57.

Norville IH, Harmer NJ, Harding SV, Fischer G, Keith KE, Brown KA, Sarkar-Tyson M, Titball RW (2011) A *Burkholderia pseudomallei* macrophage infectivity potentiator-like protein has rapamycin-inhibitable peptidylprolyl isomerase activity and pleiotropic effects on virulence. *Infection and Immunity* 79: 4299–307.

Oliveira L, Madureira P, Andrade EB, Bouaboud A, Morello E, Ferreira P, Poyart C, Trieu-Cuot P, Dramsi S (2012). Group B streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. *PLoS One* 7: e29963.

Paino A, Lohermaa E, Sormunen R, Tuominen H, Korhonen J, Pöllänen MT, Ihalin R (2012) Interleukin-1β is internalised by viable Aggregatibacter actinomycetemcomitans biofilm and locates to the outer edges of nucleoids. *Cytokine* 60: 565–574.

Paton AW, Wang H, Paton JC (2013) BiP (Grp78): A target for *Escherichia coli* subtilase cytotoxin. In Moonlighting Cell Stress Proteins in Microbial Infections (ed. Henderson B), pp. 309–322. Springer Science: Dordrecht, Heidelberg, New York, London.

Pauer H, Ferreira Ede O, dos Santos-Filho J, Portela MB, Zingali RB, Soares RM, Domingues RM (2009) A TonB-dependent outer membrane protein as a *Bacteroides fragilis* fibronectin-binding molecule. *FEMS Immunology and Medical Microbiology* 55: 388–95.

Pizarro-Cerdá J, Cossart P (2006) Bacterial adhesion and entry into host cells. *Cell* 124: 715–27.

Popoff MR, Poulain B (2010) Bacterial toxins and the nervous system: neurotoxins and multipotential toxins interacting with neuronal cells. *Toxins (Basel)* 2: 683–737.

Ragno S, Winrow VR, Mascagni P, Lucietto P, Di Pierro F, Morris CJ, Blake DR (1996) A synthetic 10-kD heat shock protein (hsp10) from Mycobacterium tuberculosis modulates adjuvant arthritis. *Clinical and Experimental Immunology* 103: 384–390.

Ramos-Sevillano E, Urzainqui A, Campuzano S, Moscoso S, *et al*. (2015) Pleiotropic effects of cell wall amidase Lyta on Streptococcus pneumoniae sensitivity to the host immune response. *Infection and Immunity* 83: 591–603.

Rasch J, Unal CM, Steinert M (2014) Peptidylprolyl cis-trans isomerases of Legionella pneumophila: virulence, moonlighting and novel therapeutic targets. *Biochemical Society Transactions* 42: 1728–33.

Reyes-Robles T, Alonzo F 3rd, Kozhaya L, Lacy DB, Unutmaz D, Torres VJ (2013) *Staphylococcus aureus* leukotoxin ED targets the chemokine receptors CXCR1 and CXCR2 to kill leukocytes and promote infection. *Cell Host and Microbe* 14: 453–9.

Rha YH, Taube C, Haczku A, Joetham A, Takeda K, Duez C, Siegel M, Aydintug MK, Born WK, Dakhama A, Gelfand EW (2002) Effect of microbial heat shock proteins on airway inflammation and hyperresponsiveness. *Journal of Immunology* 169: 5300–5307.

Riffo-Vasquez Y, Spina D, Page C, Tormay P, Singh M, Henderson B, Coates A (2004) Effect of Mycobacterium tuberculosis chaperonins on bronchial eosinophilia and hyper-responsiveness in a murine model of allergic inflammation. *Clinical and Experimental Allergy* 34: 712–719.

Riffo-Vasquez Y, Coates AR, Page CP, Spina D (2012) Mycobacterium tuberculosis chaperonin 60.1 inhibits leukocyte diapedesis in a murine model of allergic lung inflammation. *American Journal of Respiratory Cell and Molecular Biology* 47: 245–252.

Robinson MW, Buchtmann KA, Jenkins C, Tacchi JL, Raymond BB, To J, Roy Chowdhury P, Woolley LK, Labbate M, Turnbull L, Whitchurch CB, Padula MP,Djordjevic SP (2013) MHJ_0125 is an M42 glutamyl aminopeptidase that moonlights as a multifunctional adhesin on the surface of *Mycoplasma hyopneumoniae*. *Open Biology* 3: 130017.

Rosenow C, Ryan P, Weiser JN, Johnson S, Fontan P, Ortqvist A, Masure HR (1997) Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Molecular Microbiology* 25: 819–29.

Salazar N, Castiblanco-Valencia MM, Silva LB, Castro IA, Monaris D, Masuda HP, Barbosa AS, Arêas AP (2014) *Staphylococcus aureus* manganese transport Protein C (MntC) is an extracellular matrix- and plasminogen-binding protein. *PLoS One* 9: e112730.

Sandrini S, Masania R, Zia F, Haigh R, Freestone P (2013) Role of porin proteins in acquisition of transferrin iron by enteropathogens. *Microbiology* 159: 2639–50.

Sansonetti P (2010) *Bacterial Virulence: Basic Principles, Models and Global Approaches*. Wiley-VCH Verlag GmbH.

Scarselli M, Serruto D, Montanari P, Capecchi B, Adu-Bobie J, Veggi D, Rappuoli R, Pizza M, Aricò B (2006) *Neisseria meningitidis* NhhA is a multifunctional trimeric autotransporter adhesin. *Molecular Microbiology* 61: 631–44.

Schindler MK, Schütz MS, Mühlenkamp MC, Rooijakkers SH, Hallström T, Zipfel PF, Autenrieth IB (2012) *Yersinia enterocolitica* YadA mediates complement evasion by recruitment and inactivation of C3 products. *Journal of Immunology* 189: 4900–8.

Schloss PD, Handelsman J (2004) Status of the microbial census. *Microbiology and Molecular Biology Research* 68: 686–91.

Seymour LM, Jenkins C, Deutscher AT, Raymond BB, Padula MP, Tacchi JL, Bogema DR, Eamens GJ, Woolley LK, Dixon NE, Walker MJ, Djordjevic SP (2012) Mhp182 (P102) binds fibronectin and contributes to the recruitment of plasmin(ogen) to the *Mycoplasma hyopneumoniae* cell surface. *Cell Microbiology* 14: 81–94.

Shams F, Oldfield NJ, Wooldridge KG, Turner DP (2014) Fructose-1,6-bisphosphate aldolase (FBA): a conserved glycolytic enzyme with virulence functions in bacteria: 'ill met by moonlight'. *Biochemical Society Transactions* 42: 1792–5.

Shin YS, Takeda K, Shiraishi Y, Jeong YY, Domenico J, Jia Y, Han J, Spallek R, Singh M, Lucas JJ, Gelfand EW (2012) Microbial heat shock protein 65 attenuates airway hyperresponsiveness and inflammation by modulating the function of dendritic cells. *Journal of Immunology* 189: 3404–3410.

Skaar EP (2010) The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathogens* 6: e1000949.

Smani Y, McConnell MJ, Pachón J (2012) Role of fibronectin in the adhesion of *Acinetobacter baumannii* to host cells. *PLoS One* 7: e33073.

Somarajan SR, Kannan TR, Baseman JB (2010) *Mycoplasma pneumoniae* Mpn133 is a cytotoxic nuclease with a glutamic acid-, lysine- and serine-rich region essential for binding and internalization but not enzymatic activity. *Cell Microbiology* 12: 1821–31.

Spellerberg B, Cundell DR, Sandros J, Pearce BJ, Idanpaan-Heikkila I, Rosenow C, Masure HR (1996) Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*. *Molecular Microbiology* 19: 803–13.

Taylor JM, Heinrichs DE (2002) Transferrin binding in *Staphylococcus aureus*: involvement of a cell wall-anchored protein. *Molecular Microbiology* 43: 1603–14.

Terao Y, Yamaguchi M, Hamada S, Kawabata S (2006) Multifunctional glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pyogenes* is essential for evasion from neutrophils. *Journal of Biological Chemistry* 281: 14215–23.

Terrasse R, Tacnet-Delorme P, Moriscot C, Pérard J, Schoehn G, Vernet T, Thielens NM, Di Guilmi AM, Frachet P (2012) Human and pneumococcal cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins are both ligands of human C1q protein. *Journal of Biological Chemistry* 287: 42620–33.

Triantafilou K, Triantafilou M, Dedrick RL (2001) A CD14-independent LPS receptor cluster. *Nature Immunology* 2: 338–45.

Tunio SA, Oldfield NJ, Berry A, Ala'Aldeen DA, Wooldridge KG, Turner DP (2010) The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. *Molecular Microbiology* 76: 605–15.

Vastano V, Salzillo M, Siciliano RA, Muscariello L, Sacco M, Marasco R (2014) The E1 beta-subunit of pyruvate dehydrogenase is surface-expressed in *Lactobacillus plantarum* and binds fibronectin. *Microbiology Research* 169: 121–7.

Veiga-Malta I, Duarte M, Dinis M, Madureira P, Ferreira P, Videira A (2004) Identification of NAD+ synthetase from Streptococcus sobrinus as a B-cell-stimulatory protein. *Journal of Bacteriology* 186: 419–426.

Wagner C, Khan AS, Kamphausen T, Schmausser B, Unal C, Lorenz U, Fischer G, Hacker J, Steinert M (2007) Collagen binding protein Mip enables *Legionella pneumophila* to transmigrate through a barrier of NCI-H292 lung epithelial cells and extracellular matrix. *Cell Microbiology* 9: 450–62.

Watanabe K, Tachibana M, Kim S, Watarai M (2009) EEVD motif of heat shock cognate protein 70 contributes to bacterial uptake by trophoblast giant cells. *Journal of Biomedical Science* 16: 113.

Williams RJ, Ward JM, Henderson B, Poole S, O'Hara BP, Wilson M, Nair SP (2000) Identification of a novel gene cluster encoding staphylococcal exotoxin-like proteins: characterization of the prototypic gene and its protein product, SET1. *Infection and Immunity* 68: 4407–15.

Wilson M, McNab R, Henderson B (2005) Bacterial *Disease Mechanisms*. Cambridge University Press, Cambridge.

Wilson RA, Maughan WN, Kremer L, Besra GS, Fütterer K (2004) The structure of *Mycobacterium tuberculosis* MPT51 (FbpC1) defines a new family of non-catalytic alpha/beta hydrolases. *Journal of Molecular Biology* 335: 519–30.

Winrow VR, Mesher J, Meghji S, Morris CJ, Maguire M, Fox S, Coates AR, Tormay P, Blake DR, Henderson B (2008) The two homologous chaperonin 60 proteins of *Mycobacterium tuberculosis* have distinct effects on monocyte differentiation into osteoclasts. *Cell Microbiology* 10: 2091–104.

Wolff DG, Castiblanco-Valencia MM, Abe CM, Monaris D, Morais ZM, Souza GO, Vasconcellos SA, Isaac L, Abreu PA, Barbosa AS (2013) Interaction of leptospira

elongation factor tu with plasminogen and complement factor h: a metabolic leptospiral protein with moonlighting activities. *PLoS One* 8: e81818.

Wu L, Estrada O, Zaborina O, Bains M, Shen L, Kohler JE, Patel N, Musch MW, Chang EB, Fu YX, Jacobs MA, Nishimura MI, Hancock RE, Turner JR, Alverdy JC (2005) Recognition of host immune activation by Pseudomonas aeruginosa. *Science* 309: 774–777.

Yoshida N, Oeda K, Watanabe E, Mikami T, Fukita Y, Nishimura K, Komai K, Matsuda K (2001) Protein function. Chaperonin turned insect toxin. *Nature* 411: 44.

Zav'yalov VP, Chernovskaya TV, Navolotskaya EV, Karlyshev AV, MacIntyre S, Vasiliev AM,Abramov VM (1995) Specific high affinity binding of human interleukin 1 beta by Caf1A usher protein of *Yersinia pestis*. *FEBS Letters* 371: 65–8.

Zhang W, Liu G, Tang F, Shao J, Lu Y, Bao Y, Yao H, Lu C (2011) Pre-absorbed immunoproteomics: a novel method for the detection of *Streptococcus suis* surface proteins. *PLoS One* 6: e21234.

Zhu H, Lee C, Zhang D, Wu W, Wang L, Fang X, Xu X, Song D, Xie J, Ren S, Gu J (2013) Surface-associated GroEL facilitates the adhesion of *Escherichia coli* to macrophages through lectin-like oxidized low-density lipoprotein receptor-1. *Microbes and Infection* 15: 172–80.

4

Moonlighting Proteins as Cross-Reactive Auto-Antigens

Willem van Eden

Department of Infectious Diseases and Immunology, Utrecht University, Utrecht, The Netherlands

4.1 Autoimmunity and Conservation

Vaccine developers avoid the inclusion of conserved antigens in vaccines for safety reasons. However, molecules such as heat-shock protein (HSP) 60, a particularly conserved protein, have been present in various vaccines such as whole-cell pertussis vaccines. Immune responses to HSP60 have been documented in children immunized with whole-cell pertussis vaccines (Del Giudice *et al*. 1993). Although more recently the latter vaccines were replaced with (unfortunately, less effective) subunit vaccines without HSP60, there was no evidence of any unwanted side-effects caused by HSP60 in these earlier *Bordetella pertussis* vaccines. Pertussis may just have demonstrated that conserved microbial antigens, with sometimes extensive molecular mimicries with self, are not inducing unwanted or detrimental auto-reactivities in most cases.

However, HSP60 can also be used to illustrate an interesting extra complexity to the issues of molecular mimicry and the associated risks it harbors. HSP60 is one of the immuno-dominant antigens of *Mycobacterium tuberculosis*, and this molecule has been associated with auto-immune arthritis in both rodents and humans. Adjuvant arthritis in rats is induced easily with heat-killed *M. tuberculosis* (Mtb) in mineral oil (Whitehouse 2007) and the repeated instillation of live BCG in the bladder of patients with bladder cancer is known to lead to a transient, but severe, arthritis in a significant number of the cases (Bernini *et al*. 2013). Based on the cross-reactivity of an arthritogenic T-cell clone that was found to be specific for HSP60 of Mtb, a mimicry of HSP60 with proteoglycans of cartilage has been suggested to have an etiologic role in the induction of arthritis (van Eden *et al*. 1985). However, HSP60 by itself was never seen to induce arthritis, also not in the presence of adjuvants such as DDA (dimethyl dioctadecyl ammoniumbromide) or incomplete Freund's adjuvant. If real, the arthritogenic potential of HSP60 apparently depended on the further molecular context of this protein in the complexity of the mycobacterial cell walls.

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

Interestingly, immunizations with HSP60 were observed to induce T-cell responses that protected against subsequent induction of arthritis in various experimental models (van Eden *et al*. 2005).

4.2 Immunogenicity of Conserved Proteins

A common antigen of Gram-negative bacteria was already known to exist before HSP60 was identified and cloned. Later on this common and dominant immunogen was characterized as being HSP60 (Thole *et al*. 1988). The immuno-dominant behavior of microbial HSP60 can be taken as an illustration of the fact that a conserved molecular composition is not automatically associated with low immunogenicity. In other words, a high degree of self-cross-reactivity is not imposing immunological tolerance on elements of the immune system with specific receptors for the conserved molecule. Once we raised and mapped T-cell clones with specificity for Mtb HSP60 in the rat, it was identified that there were clones recognizing very conserved sites in the molecule and at the same time clones recognizing nonconserved sites (Anderton *et al*. 1995). Apparently, the immune system does not behave like the vaccine developer; it does not shy away from handling conserved molecules that feature mimicries with self.

Berent Prakken has immunized Lewis rats with a series of conserved bacterial antigens (superoxide dismutase, aldolase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and HSP70; (Prakken *et al*. 2001)). All antigens appeared highly immunogenic; some differences were observed, however. Immunization with HSP70 was accompanied with a switch in the subclasses of HSP70-specific Abs, suggesting the induction of a Th2-like response. The most striking difference between immunization with HSP70 and all other immuno-dominant antigens was the expression of IL-10 found after immunization with HSP70. Even more, while immunization with HSP70 led to antigen-induced production of IL-10 and IL-4, immunization with aldolase led to increased production of IFN-gamma and TNF-alpha. Previous studies had already shown that HSP70 immunization protected against arthritis in a manner apparently similar to HSP60. The other conserved and immunogenic proteins did not. The protective effect of conserved immuno-dominant proteins in experimental arthritis therefore seemed to be a specific feature of heat-shock proteins (HSPs), which could have been noted already from a changed quality of the HSP-induced immune response.

Irun Cohen has attempted to develop a theoretical basis for the exquisite immunogenicity or functionality of certain molecules in the immune system (Cohen 2014). He has argued that the immune system acts in a selective manner and has no interest in responding to all possible antigens to an equal extent. The immune system has its focus on the so-called "immunological homunculus," similar to the homunculus, once defined for the central nervous system. As stated by Cohen:

> The immunological homunculus is formed by auto-reactive repertoires that form a picture of informative body molecules that can help disclose the state of the body to the immune system. The immunological homunculus

> theory proposes that healthy autoimmune repertoires contribute to healthy immune management of inflammation. Autoantibodies have been noted to enhance wound healing (Nishio *et al*. 2009) and auto-reactive T-cells have been reported to exert a protective function in the central nervous system (Schwartz and Cohen 2000). Some contribution of benign autoimmunity to health is supported by the fact that healthy human babies are born with a shared repertoire of IgM and IgA autoantibodies produced by the developing fetus *in utero* and directed to a defined set of homuncular self-antigens; babies also receive a repertoire of IgG autoantibodies transferred from mother (Merbl *et al*. 2007). It is reasonable to suppose that if every human is born with a shared autoantibody repertoire, such autoimmunity must be beneficial.

In addition to this possibility of a homunculus as an in-built property in the hardware of the immune system, the role of microbial HSP in triggering the innate immune response could contribute to their immunogenicity. There is evidence that HSP60 triggers TLR2 and TLR4 (Vabulas *et al*. 2001). Even so, and also for HSP70, it has been proposed that it has the capacity to trigger a number of innate receptors (Binder 2009). Such a feature of HSPs would enable them to prepare the grounds for an enthusiastic and functionally skewed immune response upon their encounter with B- and T-cells of the adaptive immune system.

Lastly, the more down-to-earth explanation is exposure. As the author has argued before (van Eden *et al*. 2012):

> … the immune system is in continuous contact with conserved microbial HSPs. The symbiotic relationship between the gut and intestinal microbiota ensures the regular immunological surveillance of our resident flora. Be it benign colonization by commensals or true invasion by pathogens, it will impose stress on every intruder, which will lead to an up-regulation of microbial HSP and provide ample and repetitive opportunity for our immune system to respond to HSP. And logically, the immune response would most frequently encounter the most conserved epitopes. In this response, the expansion of T cells specific for conserved epitopes increases the likelihood of cross-reactivity with self-HSP.

HSP are frequently described as being so-called DAMPS (damage-associated molecular patterns). The logic here is that cell stress associated with tissue damage leads to the production of HSPs, and that the resulting response to such tissue damage might therefore be a result from the immunogenic character of HSP. However, there are a number of arguments to be made against their functioning as DAMPS. Kono and Rock (2008) have proposed four criteria in terms of biological outcomes that must be fulfilled for a molecule to be classified as a DAMP, as follows.

1) A DAMP should be active as a highly purified molecule.
2) The biological effect should not be due to contamination with microbial molecules. Caution is particularly warranted if the putative DAMP is found to work through receptors for PAMPs such as toll-like receptors (TLRs).

3) The DAMP should be active at concentrations present in pathophysiological situations.
4) Selective elimination or inactivation of the DAMP should ideally inhibit the biological activity of dead cells in *in vitro* or *in vivo* assays.

When thinking of HSPs, the first two criteria are problematic. Being molecular chaperones, HSPs easily interact with other substances; from experience, we know that recombinant HSPs are frequently contaminated with microbial impurities following production in microbial expression systems. This may have caused a bias in the interpretation of experimental findings due to stimulatory components present as contaminants in recombinant HSP. A fairly balanced discussion on this matter can be found in a recent review-type paper by workers in this field (Henderson *et al*. 2010). Number 4 seems an ideal and a well-discriminating criterion. In most cases, proposed DAMPs do not fulfil this criterion; in the case of HSPs, this experimental maneuver has certainly not been carried out. Given the anti-inflammatory qualities that HSP have shown in various different models, HSP would possibly better be called DAMPERs than DAMPS.

4.3 HSP Co-induction, Food, Microbiota, and T-cell Regulation

According to the immunological homunculus theory as proposed by Irun Cohen, and mentioned here above: "healthy autoimmune repertoires contribute to healthy immune management of inflammation." Our experimental findings obtained for the autoimmune repertoire directed to HSP70 may possibly illustrate the reality of this proposal. We have seen that *in vivo* the endogenous upregulation of HSP70, with a novel HSP co-inducer, was inducing an anti-inflammatory T-cell response directed against HSP70 that suppressed experimental arthritis. This HSP co-inducer was carvacrol, which is one of the essential oils present in Oregano species. Carvacrol was already known to have bactericidal activity and to induce HSPs in bacteria as the result of the bacterial stress response. Carvacrol did not induce stress proteins in mammalian cells however, but under a raised temperature or in the presence of low amounts of arsenite, carvacrol was seen to significantly improve the production of HSP70 (Wieten *et al*. 2010). In other words, carvacrol acted as a typical HSP co-inducer; it did not impose stress by itself, but enhanced the production of HSP when the stress response was triggered. When administered orally in mice, carvacrol was found to upregulate the presence of HSP70 in Peyer's patches and some parts of the lamina propia, the lymphoid organs of the intestinal tract. This is compatible with the fact that the gut tissues are already experiencing stress from peristaltic movements and possibly its unique level of continuous tissue regeneration. The latter co-induced upregulation of HSP70 in Peyer's patches provided a stimulus to HSP70-specific T-cells, as reflected for instance by the greatly enhanced lymphocyte responses upon *in vitro* re-stimulation with HSP70 that were found only in the carvacrol-treated animals. Responses to control antigens, such as ovalbumin, were not affected. In addition, increased expression of Foxp3 in these Peyer's

patches was observed. When oral carvacrol was given prior to the induction of PGIA no arthritis was seen over a period of more than three weeks, whereas, over the same time frame, controls without carvacrol suffered from arthritis. After that period of time, arthritis developed in some animals but with less severity in comparison with the non-treated group. This protection was T-cell mediated, since transfer of T-cells obtained from splenocytes of carvacrol-treated mice transferred full protection against arthritis in recipient animals (Wieten *et al*. 2010). Very much in line with the proposal of the homunculus theory, responses directed towards endogenous homuncular antigens such as HSPs may therefore contribute to the control and proper management of inflammation. While the substantiating evidence for the homunculus theory was mostly derived from the analysis of antibody reactivities, the carvacrol co-induction has indicated that the same may well be true for T reactivities.

There is evidence that, apart from HSP co-inducers present in food, the microbiota also has a direct impact on inflammation-controlling levels of HSP in the gut. Microbial triggering of TLR receptors on the epithelium has been shown to cause the endogenous upregulation of HSP70 (Rakoff-Nahoum *et al*. 2004). In the presence of disrupted TLR triggering, the consequential absence of HSP70 upregulation was seen to become associated with spontaneous development of colitis. In addition to this, a recent analysis of gut-region-specific presence of microbiota in the pig showed that colonization with *Lactobacillus* spp. and clostridia was associated with HSP70 expression in the gut (Liu *et al*. 2014).

4.3.1 HSP as Targets for T-Cell Regulation

Autoimmune diseases are thought to result from failing mechanisms of immunological tolerance. Of these mechanisms, defective suppressive activities of a specialized subset of T-cells, called regulatory T-cells (Treg), is assumed to be the main factor in the loss of self-tolerance. Herewith, the restoration of the faulty regulation of self-reactivity is currently the ultimate goal of novel therapeutic interventions for autoimmune diseases. However, a problem is the lack of well-identified causal auto-antigens for most of the diseases that are candidates for therapeutic triggering of auto-antigen-specific Treg. In addition to this, since these diseases were already the likely result of the deficient activity of Tregs directed to these antigens, the chances of using these particular antigens for reversing the lost tolerance seem not to be optimal. This, together with the failed attempts of reaching a therapeutic effect with self-antigens such as insulin in the case of type I diabetes and collagens in the case of RA (Miller *et al*. 2007), made investigators doubt the potential of single auto-antigens for triggering a relevant inflammation-dampening effect. It could well be that the abundance of single auto-antigens present at the site of inflammation is too low to activate sufficient Tregs for eliciting an effective suppression of ongoing inflammation (Shevach 2009). Heat-shock proteins such as HSP60 and HSP70 could however be present at the needed level of abundance. They are produced in every cell of all tissues and are preferentially uploaded into the antigen-presenting MHC molecules, especially under conditions of stress. Tissue inflammation is also a known producer of stress, leading to upregulation of stress proteins. Under stress, cells

revert to mechanisms of autophagy which include the loading of endogenous proteins into the MHC class II pathway of antigen presentation. HSP70 is involved with so-called chaperone-mediated autophagy, and this may explain why fragments of HSP70 are easily detected in the MHCII molecules of stressed cells (Dengjel *et al*. 2005). There is evidence that heat-shock proteins are the major contributors to the MHCII ligandome (van Eden *et al*. 2013). Based on relative abundance, HSP could therefore be the default source of antigens seen by CD4+ T-cells and therefore ideal targets for the default mechanisms of self-recognition, leading to tolerance and healthy immune management of inflammation.

The existence of a default connection between cells that regulate and HSP would possibly explain why HSP70 was triggering IL10, whereas the other conserved and immunogenic proteins tested by Prakken in rats, as discussed above, did not (Prakken *et al*. 2001). In other experimental studies with HSP, the induction of regulatory cytokines such as IL10 was also reported. In patients with juvenile idiopathic arthritis, the occurrence of T-cell responses with specificity for HSP60 was observed to correlate with phases of spontaneous disease remission, an observation which was compatible with the activity of HSP60-specific Tregs (Prakken *et al*. 1996). When analyzed with the help of a selected set of conserved HSP60 peptides, patient peripheral blood mononuclear cells were found to produce IL10 in the presence of these peptides (Kamphuis *et al*. 2005). Even in patients with an inflammatory track record, the CD4+ T-cells responding to HSP60 were tuned in the direction of a regulatory function.

Recently we have obtained direct proof for the fact that HSP-specific Tregs can be efficient suppressors of inflammation. For this, van Herwijnen and co-workers used adoptive transfers of CD4 + CD25+ positive T-cells obtained from mice that were immunized with a dominant HSP70 peptide called B29 (van Herwijnen *et al*. 2012). This B29 peptide was defined on the basis of a very conserved sequence of mycobacterial HSP70, which was a dominant T-cell epitope in Balb/c mice. The peptide sequence has its homologs, which differ in only one or two amino acids, in multiple members of the HSP70 family, both constitutively and stress-dependently expressed. The Tregs selected by cell sorting from the B29-immunized mice protected against proteoglycan-induced arthritis (PGIA) at low cell numbers in the recipients. Interestingly, using CD90.1 or CD90.2 as congenic markers, it was possible to track the transferred cells in organs, including the joints. The transferred cells kept their suppressive phenotypes for at least 50 days after transfer. Further, when given during active and overt arthritis, disease was suppressed; when they were blocked in their activity *in vivo* by a depleting CD90 antibody, the control of arthritis was lost and the disease flared. When Tregs were selected from B29 immunized donor mice on the basis of CD4 + CD25 + Foxp3+ and also LAG3 positivity, the astonishingly low number of 4000 Tregs was seen to suppress disease. Everything in these experiments depended on the immunization with B29. When donor animals were immunized with OVA as a control antigen induction of functional Tregs was observed, but upon transfer the cells did not interfere with arthritis. That makes sense since OVA is not a possible target for active Tregs *in vivo*, whereas HSP70 naturally is such a target, especially in the inflamed tissues.

Interestingly, also through completely other mechanisms, HSP70 mediates anti-inflammatory effects (Tanaka *et al*. 2014). One of the critical subunits of NF-κB, a transcription factor that induces the expression of inflammation-related genes such as the proinflammatory cytokines, was shown recently to become degraded under the influence of HSP70. In this process HSP70 acts as a bridge between a ubiquitin E3 ligase and the proteasome, leading to degradation of the p65 subunit of NF-κB, herewith inhibiting inflammatory signaling. In addition to this, also by effects on the natural NF-κB inhibitor, I-κBα, HSP co-inducing compounds have been observed to inhibit NF-κB (Wieten *et al*. 2007).

4.4 The Contribution of Moonlighting Virulence Factors to Immunological Tolerance

Exposure to microbial antigens is essential for a developing and functioning immune system. The immune systems of germ-free animals remain deficient and are characterized by an imbalance between the different phenotypic T-cell subsets (Alam *et al*. 2011). In particular, the gut mucosal lymphoid systems remain underdeveloped (Cebra 1999). The confrontation of the naïve immune system with the microbiota early after birth is a decisive step for the immune system to create a balance between, among others, Th1 and Th2 T-cells. In theory, the innate Th2 dominance, when not properly corrected by the microbiota-stimulated Th1 cells, could lead to an enhanced tendency to develop allergies later in life. On the same bandwagon, the so-called hygiene hypothesis states that reduced exposure to infectious organisms leads to the raised prevalence of allergies that is seen in Western countries these days (Strachan 1989). More recently, the hygiene hypothesis was revised and proposed to include also the rising prevalence of autoimmune diseases, such as type I diabetes, multiple sclerosis, inflammatory bowel disease, etc. (Bach 2002). The mechanism behind the new variant of the hypothesis was shifted from Th1/Th2 balances to a deficient function of Tregs, due to reduced exposure to infection (Guarner *et al*. 2006).

Despite the ongoing debate about the validity of the hygiene hypothesis, the fact is that diseases mediated by the immune system are on the rise in Western developed countries and that exposure to infection is reduced as compared to the previous times when worms, mites, saprophytic mycobacteria, bifidobacteria, lactobacilli, and many other organisms were constantly infesting our bodies. Some clinicians have seen in this an excuse to re-infest patients suffering from allergies or inflammatory bowel diseases with helminths as a cure (Summers *et al*. 2005). Despite the fact that it seems to work and produce positive effects in certain groups of patients, it may not be the best medical practice. Of all the possible underlying mechanisms, microbial virulence factors could well have a critical contribution. An illuminating aspect of a moonlighting function of virulence factors could be in the signaling of the innate immune system. Organisms recognized as harmless by the innate immune system, through pattern-recognition receptors such as TLRs, cause dendritic cells to mature into regulatory dendritic cells that drive regulatory-T-cell polarization (Guarner *et al*. 2006). Examples can be found in Bilharzia worms that trigger TLR2-producing regulatory DC

which activates IL10-producing T-cells (van der Kleij *et al*. 2002), *Mycobacterium vaccae* which induces a population of pulmonary CD11c + cells with regulatory potential (Adams *et al*. 2004), and probiotic bacteria which induce IL10-producing Treg through DC-SIGN-mediated DC modulation (Smits *et al*. 2005). Further, recognition of HSP means that it is highly possible that they provide a molecular basis for the hygiene hypothesis. If so, the recognition of such molecules or virulence factors would be an essential driver behind the maintenance of tolerance for self and herewith a stimulus for regulatory T-cell activities. An example of a role of microbial HSP60 in this sense is the Treg induction by an HSP60-derived peptide SJMHE1 from *Schistosoma japonicum* through TLR2 (Wang *et al*. 2009). Along similar lines, this observation was made for mammalian HSP60 and the enhancement of CD4+ CD25+ regulatory T-cell function via innate TLR2 signaling by others (Zanin-Zhorov *et al*. 2006).

The immune system has evolved in the presence of microbes; it is therefore logical that the system adapted itself to acceptance of the microbiota and that it developed essential means to discriminate between safe microbiota and real pathogens. The virulence factors of such pathogens may have become engaged in the resulting host–microbe interactions and may have developed mechanisms that helped to avoid unnecessary collateral damage in the inflammatory process. In such a manner, virulence factors may have adopted their moonlighting functions in their interactions with receptors of both the innate and adaptive immune system.

References

Adams, V.C., Hunt, J.R., Martinelli, R., Palmer, R., Rook, G.A., Brunet, L.R. 2004. Mycobacterium vaccae induces a population of pulmonary CD11c+ cells with regulatory potential in allergic mice. *European Journal of Immunology*, 34(3), 631–638.

Alam, C., Bittoun, E., Bhagwat, D., Valkonen, S., Saari, A., Jaakkola, U., Eerola, E., Huovinen, P., Hanninen, A. 2011. Effects of a germ-free environment on gut immune regulation and diabetes progression in non-obese diabetic (NOD) mice. *Diabetologia*, 54(6), 1398–1406.

Anderton, S.M., Van Der Zee, R., Prakken, B., Noordzij, A., Van Eden, W. 1995. Activation of T cells recognizing self 60-kD heat shock protein can protect against experimental arthritis. *The Journal of Experimental Medicine*, 181(3), 943–952.

Bach, J.F. 2002. The effect of infections on susceptibility to autoimmune and allergic diseases. *The New England Journal of Medicine*, 347(12), 911–920.

Bernini, L., Manzini, C.U., Giuggioli, D., Sebastiani, M., Ferri, C. 2013. Reactive arthritis induced by intravesical BCG therapy for bladder cancer: our clinical experience and systematic review of the literature. *Autoimmunity Reviews*, 12(12), 1150–1159.

Binder, R.J. 2009. Hsp receptors: the cases of identity and mistaken identity. *Current Opinion in Molecular Therapeutics*, 11(1), 62–71.

Cebra, J.J. 1999. Influences of microbiota on intestinal immune system development. *The American Journal of Clinical Nutrition*, 69(5), 1046S–1051S.

Cohen, I.R. 2014. Activation of benign autoimmunity as both tumor and autoimmune disease immunotherapy: a comprehensive review. *Journal of Autoimmunity*, 54, 112–117.

Del Giudice, G., Gervaix, A., Costantino, P., Wyler, C.A., Tougne, C., De Graeff-Meeder, E.R., Van Embden, J., Van Der Zee, R., Nencioni, L., Rappuoli, R. 1993. Priming to heat shock proteins in infants vaccinated against pertussis. *Journal of Immunology (Baltimore, MD: 1950)*, 150(5), 2025–2032.

Dengjel, J., Schoor, O., Fischer, R., Reich, M., Kraus, M., Muller, M., Kreymborg, K., Altenberend, F., Brandenburg, J., Kalbacher, H., Brock, R., Driessen, C., Rammensee, H.G., Stevanovic, S. 2005. Autophagy promotes MHC class II presentation of peptides from intracellular source proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 102(22), 7922–7927.

Guarner, F., Bourdet-Sicard, R., Brandtzaeg, P., Gill, H.S., Mcguirk, P., Van Eden, W., Versalovic, J., Weinstock, J.V., Rook, G.A. 2006. Mechanisms of disease: the hygiene hypothesis revisited. *Nature Clinical Practice: Gastroenterology & Hepatology*, 3(5), 275–284.

Henderson, B., Calderwood, S.K., Coates, A.R., Cohen, I., Van Eden, W., Lehner, T., Pockley, A.G. 2010. Caught with their PAMPs down? The extracellular signalling actions of molecular chaperones are not due to microbial contaminants. *Cell Stress & Chaperones*, 15(2), 123–141.

Kamphuis, S., Kuis, W., De Jager, W., Teklenburg, G., Massa, M., Gordon, G., Boerhof, M., Rijkers, G.T., Uiterwaal, C.S., Otten, H.G., Sette, A., Albani, S., Prakken, B.J. 2005. Tolerogenic immune responses to novel T-cell epitopes from heat-shock protein 60 in juvenile idiopathic arthritis. *Lancet*, 366(9479), 50–56.

Kono, H., Rock, K.L. 2008. How dying cells alert the immune system to danger. *Nature Reviews: Immunology*, 8(4), 279–289.

Liu, H., Dicksved, J., Lundh, T., Lindberg, J.E. 2014. Heat shock proteins: intestinal gatekeepers that are influenced by dietary components and the gut microbiota. *Pathogens (Basel, Switzerland)*, 3(1), 187–210.

Merbl, Y., Zucker-Toledano, M., Quintana, F.J., Cohen, I.R. 2007. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. *The Journal of Clinical Investigation*, 117(3), 712–718.

Miller, S.D., Turley, D.M., Podojil, J.R. 2007. Antigen-specific tolerance strategies for the prevention and treatment of autoimmune disease. *Nature Reviews: Immunology*, 7(9), 665–677.

Nishio, N., Ito, S., Suzuki, H., Isobe, K. 2009. Antibodies to wounded tissue enhance cutaneous wound healing. *Immunology*, 128(3), 369–380.

Prakken, A.B., Van Eden, W., Rijkers, G.T., Kuis, W., Toebes, E.A., De Graeff-Meeder, E.R., Van Der Zee, R., Zegers, B.J. 1996. Autoreactivity to human heat-shock protein 60 predicts disease remission in oligoarticular juvenile rheumatoid arthritis. *Arthritis and Rheumatism*, 39(11), 1826–1832.

Prakken, B.J., Wendling, U., Van Der Zee, R., Rutten, V.P., Kuis, W., Van Eden, W. 2001. Induction of IL-10 and inhibition of experimental arthritis are specific

features of microbial heat shock proteins that are absent for other evolutionarily conserved immunodominant proteins. *Journal of Immunology (Baltimore, MD: 1950)*, 167(8), 4147–4153.

Rakoff-Nahoum, S., Paglino, J., Eslami-Varzaneh, F., Edberg, S., Medzhitov, R. 2004. Recognition of commensal microflora by toll-like receptors is required for intestinal homeostasis. *Cell*, 118(2), 229–241.

Schwartz, M., Cohen, I.R. 2000. Autoimmunity can benefit self-maintenance. *Immunology Today*, 21(6), 265–268.

Shevach, E.M. 2009. Mechanisms of foxp3+ T regulatory cell-mediated suppression. *Immunity*, 30(5), 636–645.

Smits, H.H., Engering, A., Van Der Kleij, D., De Jong, E.C., Schipper, K., Van Capel, T.M., Zaat, B.A., Yazdanbakhsh, M., Wierenga, E.A., Van Kooyk, Y., Kapsenberg, M.L. 2005. Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin. *The Journal of Allergy and Clinical Immunology*, 115(6), 1260–1267.

Strachan, D.P. 1989. Hay fever, hygiene, and household size. *BMJ (Clinical Research ed.)*, 299(6710), 1259–1260.

Summers, R.W., Elliott, D.E., Urban, J.F., Jr, Thompson, R., Weinstock, J.V. 2005. Trichuris suis therapy in Crohn's disease. *Gut*, 54(1), 87–90.

Tanaka, T., Shibazaki, A., Ono, R., Kaisho, T. 2014. HSP70 mediates degradation of the p65 subunit of nuclear factor kappaB to inhibit inflammatory signaling. *Science Signaling*, 7(356), ra119.

Thole, J.E., Hindersson, P., De Bruyn, J., Cremers, F., Van Der Zee, J., De Cock, H., Tommassen, J., Van Eden, W., Van Embden, J.D. 1988. Antigenic relatedness of a strongly immunogenic 65 kDA mycobacterial protein antigen with a similarly sized ubiquitous bacterial common antigen. *Microbial Pathogenesis*, 4(1), 71–83.

Vabulas, R.M., Ahmad-Nejad, P., Da Costa, C., Miethke, T., Kirschning, C.J., Hacker, H., Wagner, H. 2001. Endocytosed HSP60s use toll-like receptor 2 (TLR2) and TLR4 to activate the toll/interleukin-1 receptor signaling pathway in innate immune cells. *The Journal of Biological Chemistry*, 276(33), 31332–31339.

van der Kleij, D., Latz, E., Brouwers, J.F., Kruize, Y.C., Schmitz, M., Kurt-Jones, E.A., Espevik, T., De Jong, E.C., Kapsenberg, M.L., Golenbock, D.T., Tielens, A.G., Yazdanbakhsh, M. 2002. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *The Journal of Biological Chemistry*, 277(50), 48122–48129.

van Eden, W., Holoshitz, J., Nevo, Z., Frenkel, A., Klajman, A., Cohen, I.R. 1985. Arthritis induced by a T-lymphocyte clone that responds to Mycobacterium tuberculosis and to cartilage proteoglycans. *Proceedings of the National Academy of Sciences of the United States of America*, 82(15), 5117–5120.

van Eden, W., Van Der Zee, R., Prakken, B. 2005. Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nature Reviews Immunology*, 5(4), 318–330.

van Eden, W., Spiering, R., Broere, F., Van Der Zee, R. 2012. A case of mistaken identity: HSPs are no DAMPs but DAMPERs. *Cell Stress & Chaperones*, 17(3), 281–292.

van Eden, W., Van Herwijnen, M., Wagenaar, J., Van Kooten, P., Broere, F., Van Der Zee, R. 2013. Stress proteins are used by the immune system for cognate

interactions with anti-inflammatory regulatory T cells. *FEBS Letters*, 587(13), 1951–1958.

van Herwijnen, M.J., Wieten, L., Van Der Zee, R., Van Kooten, P.J., Wagenaar-Hilbers, J.P., Hoek, A., Den Braber, I., Anderton, S.M., Singh, M., Meiring, H.D., Van Els, C.A., van Eden, W., Broere, F. 2012. Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. *Proceedings of the National Academy of Sciences of the United States of America*, 109(35), 14134–14139.

Wang, X., Zhou, S., Chi, Y., Wen, X., Hoellwarth, J., He, L., Liu, F., Wu, C., Dhesi, S., Zhao, J., Hu, W., Su, C. 2009. CD4+CD25+ Treg induction by an HSP60-derived peptide SJMHE1 from Schistosoma japonicum is TLR2 dependent. *European Journal of Immunology*, 39(11), 3052–3065.

Whitehouse, M.W. 2007. Adjuvant arthritis 50 years on: The impact of the 1956 article by C. M. Pearson, 'Development of arthritis, periarthritis and periostitis in rats given adjuvants'. *Inflammation Research: Official Journal of the European Histamine Research Society*, 56(4), 133–138.

Wieten, L., Broere, F., van der Zee, R., Koerkamp, E.K., Wagenaar, J., van Eden, W. 2007. Cell stress induced HSP are targets of regulatory T cells: a role for HSP inducing compounds as anti-inflammatory immuno-modulators? *FEBS Letters*, 581(19), 3716–3722.

Wieten, L., van der Zee, R., Spiering, R., Wagenaar-Hilbers, J., van Kooten, P., Broere, F., van Eden, W. 2010. A novel heat-shock protein coinducer boosts stress protein Hsp70 to activate T cell regulation of inflammation in autoimmune arthritis. *Arthritis and Rheumatism*, 62(4), 1026–1035.

Zanin-Zhorov, A., Cahalon, L., Tal, G., Margalit, R., Lider, O., Cohen, I.R. 2006. Heat shock protein 60 enhances CD4+ CD25+ regulatory T cell function via innate TLR2 signaling. *The Journal of Clinical Investigation*, 116(7), 2022–2032.

Part III

Proteins Moonlighting in Bacterial Virulence

3.1

Chaperonins: A Family of Proteins with Widespread Virulence Properties

5

Chaperonin 60 Paralogs in *Mycobacterium tuberculosis* and Tubercle Formation

Brian Henderson

Department of Microbial Diseases, UCL-Eastman Dental Institute, University College London, UK

5.1 Introduction

As tuberculosis re-emerges as a major health problem worldwide, we need to become aware of the dangers of a disease whose names – consumption, phthisis, white plague, scrofula – clearly show the level of fear that it engendered. Early descriptions of tuberculosis appear in classical Greek literature (in which the term phthisis, meaning "wasting away," originated), and the European artistic scene has many examples of individuals whose works were completed against the background of this disease. Writers such as Keats, Robert Louis Stevenson, the Bronte sisters and, more recently, Eric Blair/George Orwell, all died of tuberculosis (Bynum 2012).

The causative agent of tuberculosis is *Mycobacterium tuberculosis*. In many regards this is an unusual organism, from a genus which is believed to have evolved 150 million years ago (Hayman 1984). Modern strains of *M. tuberculosis* are believed to have originated from a common ancestor some 15–20,000 years ago in the African subcontinent (Sreevatsan *et al*. 1997). However, it was only as recently as 1882 that Koch identified *M. tuberculosis* as the causative agent of tuberculosis, a finding for which he was awarded the Nobel Prize in 1905 (Daniel 2006).

5.2 Tuberculosis and the Tuberculoid Granuloma

Tuberculosis is a chronic infection, due to the inability of the body to get rid of the bacterium. The general pathology induced by inflammatory agents that are persistent is the granuloma (Williams and Williams 1983). Granulomas consist of complex collections of immune cells, with the most notable feature being the presence of unusual forms of the myeloid cells known as macrophages. These include the epithelioid cell (epithelial macrophages), the foam cell, and the multinucleate giant

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

cell (Ramakrishnan 2012). In addition to the apparently distinct activation states that macrophages are now known to exhibit, this is dependent on the microenvironmental signals they encounter. These states exist under the title "alternative macrophage activation," and some workers suggest two distinct states (M1 and M2; see Fig. 5.1) while others suggest there may be a spectrum of activation states (Xue *et al*. 2014). The role of granuloma formation in tuberculosis has a long history, with this structure being proposed by Laennec in 1819 as the causation of this disease (Sakula 1982). Around a century later, Ghon generated data suggesting a role for the granuloma as a marker for latent tuberculosis (Dorhoi *et al*. 2011). An additional hypothesis, which has strong support, suggests that the function of the granuloma is to encapsulate *M. tuberculosis*, thus protecting the host from the bacterium (Lugo-Villarino *et al*. 2013). An alternative explanation emanating from studies in the Zebrafish is that *M. tuberculosis* can manipulate granuloma formation as part of its virulence mechanism, allowing different granuloma "types" to be generated. In this explanation the two major macrophage activation states (M1 and M2) are important in terms of the host response to the mycobacterium (Ramakrishnan 2012). The potential role of the granuloma in killing or protecting the mycobacterial pathogen is shown in Figure 5.1. This is particularly important as *M. tuberculosis* can enter a dormant state in which it is protected from immune attack, and it is estimated that one-third of the world's population are currently infected with this dormant (latent) organism (Rangaka *et al*. 2012).

Clearly, understanding how *M. tuberculosis* interacts with the human immune system to induce and control the perpetuation of the tuberculoid granuloma is vital to be able to control this major bacterial infection.

5.3 Mycobacterial Factors Responsible for Granuloma Formation

Given the central importance of granuloma formation in tuberculosis, it would be assumed that the cell surface or secreted components of *M. tuberculosis* responsible for controlling the dynamic composition of the tuberculoid granuloma would be well established. Surprisingly, this is not the case. The cell-wall glycolipid, trehalose-6,6-dimycolate (TDM), also known as cord factor, was the first mycobacterial component to be shown capable of inducing granuloma formation (Yarkoni and Rapp 1977). There is also limited evidence from an *in vitro* human granuloma assay that proinflammatory lipoarabinomannans from *M. tuberculosis* can induce granuloma formation (Puissegur *et al*. 2007). Other key mycobacterial virulence factors such as lipoproteins, ESAT-6 (early secreted antigenic target-6), and CFP-10 (culture filtrate protein-10) (Xu *et al*. 2007) have major effects on myeloid cells, but have not been shown to be involved in granuloma formation. In contrast, there is emerging evidence that another virulence protein of *M. tuberculosis* – HSP65/chaperonin 60 – has a role to play in granuloma formation.

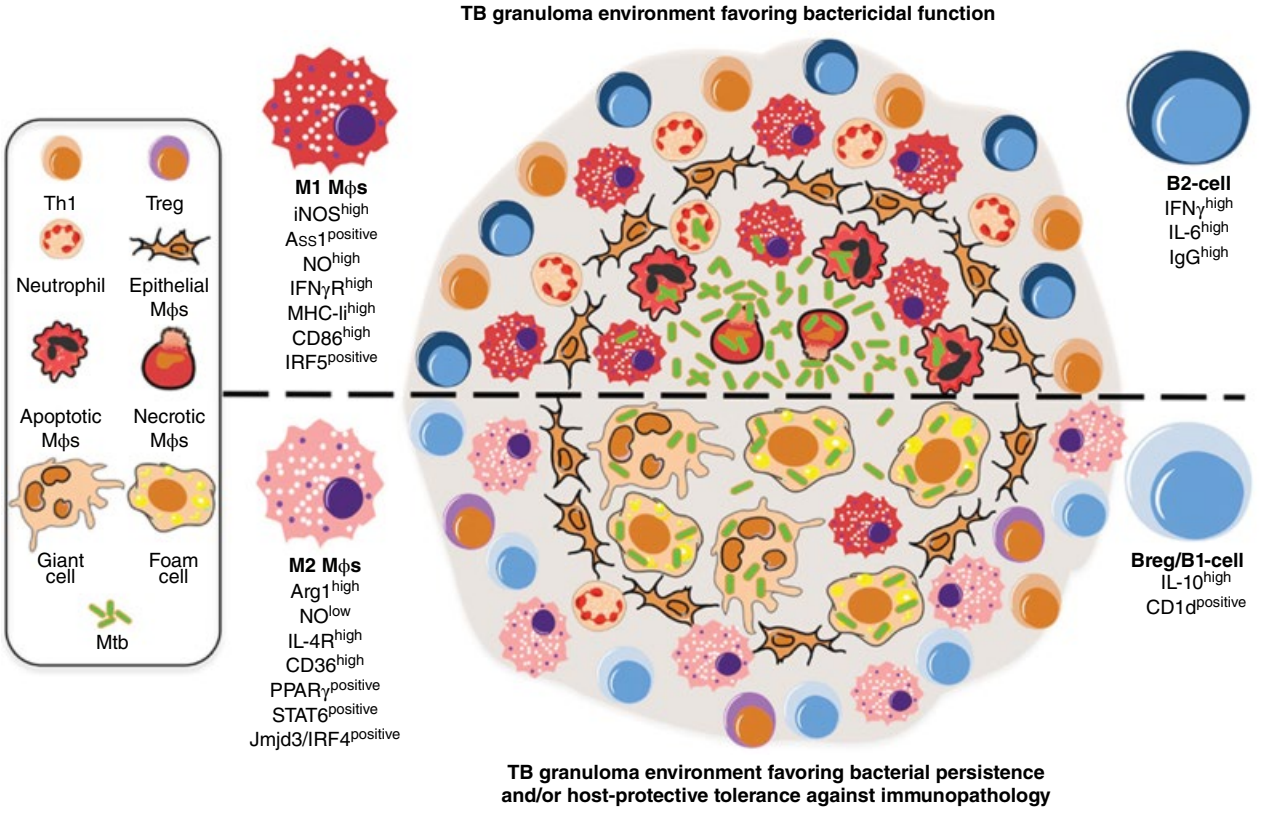

Figure 5.1 A cartoon view of the tuberculoid granuloma with cellular compositions deemed to be associated with bactericidal action or favoring bacterial persistence. *Source*: Lugo-Villarino *et al*. (2013).

5.4 *Mycobacterium tuberculosis* Chaperonin 60 Proteins, Macrophage Function, and Granuloma Formation

Chaperonin (Cpn) 60, also known as heat-shock protein (HSP) 60 and, in the new nomenclature, HSPD1, is a bacterial molecular chaperone which is found within the bacterial cytoplasm and, in eukaryotic cells, within the mitochondria and chloroplast (Saibil *et al*. 2013). This gene family is only found in a small number of the Archaea (Macario *et al*. 2004). Chaperonin 60, as exemplified by the prototypic *E. coli* protein GroEL, has evolved as a folding machine for a variety of cytosolic proteins. To this end, evolution has crafted an oligomeric structure composed of two seven-membered rings (of 60 kDa subunits) stacked back-to-back, generating two cavities lined with a hydrophobic surface which can bind unfolded polypeptide chains. Once bound, the chaperonin 60 protein undergoes significant molecular movement, which forces the unfolded protein to be ejected from the walls of the cavity into the chamber, which is hydrophilic and favors correct folding (Saibil *et al*. 2013). It is surprising that this family of intricate protein-folding oligomeric machines also exhibit a bewildering variety of moonlighting actions (Henderson *et al*. 2013).

In addition to functioning as a molecular chaperone, and having multiple moonlighting actions, chaperonin 60 proteins from microbial pathogens are well-recognized antigenic proteins capable of generating cross-reactive immunity, which is associated with autoimmune disease (van Eden *et al*. 2005). The relationship between protein moonlighting and auto-antigenicity is described in Chapter 4. The significant immunity to the chaperonin 60.2 (HSP65) protein of *M. tuberculosis* in patients with tuberculosis has suggested that this protein could be a vaccine candidate (Doimo *et al*. 2014).

5.4.1 *Mycobacterium tuberculosis* has Two Chaperonin 60 Proteins

The major perceived function of the bacterial chaperonin 60 protein is to fold cytoplasmic proteins. The finding that many bacterial genera have more than one gene encoding chaperonin 60 proteins is therefore unexpected (Lund 2009). The mycobacteria were among the first bacteria to be shown to express two separate chaperonin 60 proteins. These were the well-studied HSP65 protein, which was renamed chaperonin 60.2, and a second gene encoding a protein termed chaperonin 60.1, which was found to be in an operon with the co-chaperone chaperonin 10 (Kong *et al*. 1993). This operon structure is the normal finding in most organisms (Lund 2009) and led to the conclusion that chaperonin 60.1 was the major cell stress protein in *M. tuberculosis* (Kong *et al*. 1993). However, as will be seen, this hypothesis was incorrect.

As described, the prototypic chaperonin 60 protein is the *E. coli* protein GroEL, which forms a tetradecameric oligomeric structure (Saibil *et al*. 2013). However, both *M. tuberculosis* chaperonin 60 proteins failed to show this oligomeric structure (Qamra *et al*. 2004; Tormay *et al*. 2005) and even the crystal structure of the chaperonin 60.2 protein is a dimer (Qamra and Mande 2004). In addition, the ATPase activity of both chaperonin 60 proteins was very low (Qamra *et al*. 2004;

Tormay *et al*. 2005). Surprisingly, given these findings, the gene encoding chaperonin 60.2 could replace the *groEL* gene (in *E. coli*). In contrast, the gene encoding the chaperonin 60.1 protein was unable to generate viable bacteria, suggesting this protein had lost its protein-folding activity (Hu *et al*. 2008). This suggested that the *M. tuberculosis* chaperonin 60.2 protein could form appropriate oligomeric structures within the *E. coli* cytoplasm. It has been shown that oligomerization of the Cpn60.2 proteins of the mycobacteria can be induced in the presence of kosmotropes (compounds which, unlike chaotropes, stabilize protein structure) or ADP or ATP (Fan *et al*. 2012). It is unclear what kosmotropes promote the intracellular stabilization of mycobacterial chaperonin 60.2 proteins. In addition, it is not clear how the oligomeric state of these proteins influences their moonlighting behavior.

5.4.2 Moonlighting Actions of Mycobacterial Chaperonin 60 Proteins

One of the earliest studies that revealed that bacterial and eukaryotic cell stress proteins had cell signaling actions that could render them pro- and/or anti-inflammatory proteins (Henderson and Pockley 2010) was that the *M. tuberculosis* chaperonin 60.2 (HSP65) protein was able to stimulate the human monocyte cell line THP-1 to generate the key early-response proinflammatory cytokine, TNFα (Friedland *et al*. 1993). This finding could either be interpreted as the recombinant protein being contaminated with the major Gram-negative bacterial component, lipopolysaccharide (LPS), or that this protein had actions similar to LPS and was able to induce macrophages to become classically activated. This is similar to the M1 state mentioned above (Martinez and Gordon 2014). This classic activation state is presumed to have evolved to allow macrophages to kill microbes and present microbial antigens to T lymphocytes. This involves multiple changes in the macrophage including upregulation of expression of Fc receptors, MHC class II, and production of oxygen-derived free radicals (Fujiwara and Kobayashi 2005). It would therefore appear that *M. tuberculosis* chaperonin 60.2 is able to induce classic macrophage activation and is part of the process of induction of immunity to *M. tuberculosis*. However, a separate study of this protein found that it did not promote this panoply of changes in macrophages, but only induced the synthesis of cytokines (Peetermans *et al*. 1994). This suggests that *M. tuberculosis* chaperonin 60.2 is inducing a state of alternative activation in these macrophages.

Inflammation involves the trafficking of blood monocytes to sites of bacterial infection. To cross the vascular endothelial barrier, leukocytes need to interact with a range of adhesive proteins on vascular endothelial cells (VECs) including E-selectin and members of the ICAM and VCAM families. These proteins are normally inducible either by LPS or by the proinflammatory cytokines such as IL-1 and TNF – with the LPS being responsible for the synthesis of these cytokines by the VEC – which then acts in a paracrine/autocrine manner (Koizumi *et al*. 2003). *Mycobacterium tuberculosis* chaperonin 60.2 was, like LPS, also able to induce the expression of E-selectin, ICAM-1, and VCAM-1 on cultured human VECs, and to promote the adhesion of human leukocytes to these cells. However, unlike LPS, this induction of adhesion is a direct effect of the

chaperonin 60.2 protein, and was not associated with the synthesis of IL-1 or TNF (Verdegaal *et al*. 1996). The literature reveals that only a small number of bacterial components are able to activate VEC adhesion and this is due to the promotion of cytokine synthesis. This direct activity of *M. tuberculosis* chaperonin 60.2 on human VECs therefore appears to be a unique effect, which is likely to be of significant importance provided that the chaperonin 60.2 protein is secreted. Indeed, as will be described, the chaperonin 60.2 protein of *M. tuberculosis* is secreted and can function as a cell-wall protein (Hickey *et al*. 2009, 2010). Moreover, secretion can be modulated, as in *Mycobacterium bovis*; the inactivation of the gene encoding chaperonin 60.1 increases, by an unknown mechanism, the secretion of the chaperonin 60.2 protein 200-fold (Wang *et al*. 2011). In addition, a cell-surface serine hydrolase of *M. tuberculosis* known as Hip1 (hydrolase important for pathogenesis 1) was found to be a modulator of innate immunity and functioned to control the release of chaperonin 60.2 (Rengarajan *et al*. 2008). Further work has identified that cleavage of *M. tuberculosis* chaperonin 60.2 by Hip1 at position 18 in the N-terminus produces a protein with no oligomeric structure and with significantly less proinflammatory activity than the native protein, so decreasing the host immune response to the bacterium (Naffin-Olivos *et al*. 2014). Interestingly, the nitric oxide-responsive transcription factor WhiB1inhibits transcription from the chaperonin 60.2 promoter, blocking the action of the positively acting CRP family transcription factor Cmr (Stapleton *et al*. 2012).

With the discovery of the second chaperonin 60 gene in *M. tuberculosis*, the obvious question was whether this protein also had proinflammatory activity. The first activity that both proteins were compared for was bone resorption. This arose from the author's finding that the chaperonin 60 protein of *E. coli* was a potent stimulator of bone resorption, which worked by inducing the formation of the multinucleated myeloid cells (known as osteoclasts) which control the normal turnover of bone (Kirby *et al*. 1995; Reddi *et al*. 1998). Surprisingly, both chaperonin 60 proteins of *M. tuberculosis* had no stimulatory effect in the assay used to detect agents able to promote bone destruction (resorption) (Meghji *et al*. 1997). This was one of the first studies to show that moonlighting proteins within a protein family (in this case the chaperonin 60 family of proteins) could have distinct biological actions.

5.4.3 Actions of Mycobacterial Chaperonin 60 Proteins Compatible with the Pathology of Tuberculosis

Since 2000, a small but growing number of studies of the chaperonin 60 proteins of the mycobacteria have revealed their role in the relationship between these bacteria and their host. The two *M. tuberculosis* chaperonin 60 proteins exhibit 76% amino acid sequence similarity, which would suggest these proteins have identical biological actions. However, the chaperonin 60.1 protein is a significantly more active monocyte-activating ligand than the chaperonin 60.2 protein. This may be related to the fact that chaperonin 60.2 activity is dependent on binding to CD14 while that of chaperonin 60.1 is only partially CD14-dependent, suggesting these proteins work via different receptor complexes at the cell surface (or within the cell). This was the first evidence that *M. tuberculosis* chaperonin 60.1 could have a role in virulence (Lewthwaite *et al*. 2001).

These findings were of interest, but could be artifacts of the *in vitro* system being used. However, two separate groups have looked at mycobacterial chaperonin 60 proteins in *in vivo* models of the human lung disease, asthma. This is a chronic inflammatory lung disease. Gelfand's group examined the chaperonin 60 proteins from *Streptococcus pneumonia*, *Helicobacter pylori*, and bacillus Calmette-Guerin (BCG) and from *Mycobacterium leprae* and *M. tuberculosis* (both chaperonin 60.2) for their effects in a mouse model of asthma. Only the administration of the *M. leprae* chaperonin 60.2 protein had therapeutic effects, including inhibition of the Th2 cytokines IL-4 and IL-5 (Rha *et al.* 2002). It was notable that administration of as little as 10 μg recombinant chaperonin 60 to mice inhibited asthma. In a subsequent study from this group, the *in vivo* actions of the *M. leprae* chaperonin 60.2 protein in this model was shown to be due to the ability of this protein to upregulate the expression of the Notch receptor ligand, Delta 1, expression on dendritic cells. This appeared to be associated with the chaperonin 60.2-activated dendritic cells being able to skew CD4 T lymphocytes to a Th1 profile of cytokine production (Shin *et al.* 2012). Using the same model to compare the activities of the *M. tuberculosis* chaperonins it was found that the chaperonin 60.1 protein and, to a lesser extent, the chaperonin 10 protein had therapeutic activity, but the chaperonin 60.2 protein had no influence on this experimental lung inflammation model (confirming Gelfand's findings) (Riffo-Vasquez *et al.* 2004). This is an unusual finding, given the results from Gelfand's group with the *M. leprae* chaperonin 60.2 protein. These proteins share 95% sequence identity, suggesting that the active moonlighting site in the *M. leprae* protein is relatively small and has, essentially, been mutated in the *M. tuberculosis* chaperonin 60.2 protein to be biologically inactive. Subsequent studies of the mechanism of action of the *M. tuberculosis* chaperonin 60.1 protein in this model revealed a direct effect of the protein on leukocyte diapedesis which is related to the inhibition of VCAM-1 expression and upregulation of vascular endothelial cadherin expression (Riffo-Vasquez *et al.* 2012).

These studies reveal that the chaperonin 60 proteins of the mycobacteria have biological activity in the intact animal and, of relevance to tuberculosis, in the lung. Moreover, they reveal that these chaperonin 60 proteins are active at picomolar to nanomolar concentrations, based on the molecular mass of the monomeric subunit. If the active protein is oligomeric, then these proteins are even more active. The difference in activities of the chaperonin 60.1 and 60.2 proteins suggests that only minor changes in protein sequence, which is presumably related to protein structure, can switch on or off this anti-inflammatory activity. Of note, the *in vitro* studies of the chaperonin 60.1 and 60.2 proteins would have suggested that both proteins were proinflammatory virulence factors.

One of the complexities in biology is that the actions of individual proteins are context-dependent. These *in vivo* experiments in an asthma model suggest that, where active, the mycobacterial chaperonin 60 proteins can switch off a Th2-mediated inflammation possibly by redirecting lymphocytes into a Th1-type of behavior. However, Mande's group have shown that, in *in vitro* studies, the *M. tuberculosis* chaperonin 60.1 protein can inhibit the actions of the proinflammatory *M. tuberculosis* component known as purified protein derivative (PPD). This appears to be due to the interaction of chaperonin 60.1 with TLR2 and the skewing of lymphocytes to a Th2-type phenotype (Khan *et al.* 2008).

So far, the actions of the mycobacterial chaperonins seem to be compatible with the hypothesis that these proteins are cellular ligands and signaling molecules able to modulate inflammation – provided that the proteins are released by the bacterium. It has also been established that the *M. tuberculosis* chaperonin 60.2 protein is present in substantial amounts on the bacterial surface, where it aids in the interaction of the bacterium with the key cell population in tuberculosis: the macrophage (Hickey *et al*. 2009). This protein functions as another class of bacterial virulence factor: the adhesin. Bacteria adhesins normally bind to a limited number of cell surface or extracellular matrix molecules including fibronectin, various collagens, laminin, and the like. The receptor for *M. tuberculosis* chaperonin 60.2 is unique to bacterial adhesins, being the cell-surface sialylated glycoprotein CD43 (Hickey *et al*. 2010). As CD43 also appears to be important in a range of T-cell functions, including regulating CD4 T lymphocyte trafficking (Cannon *et al*. 2011), it is possible that this chaperonin 60.2/CD43 interaction may have additional roles in controlling granuloma formation. It has recently been reported that common polymorphisms in the CD43 gene region are associated with susceptibility to tuberculosis and disease severity (Campo *et al*. 2015). The interaction of *M. tuberculosis* chaperonin 60.2 with CD43 reveals an important finding that protein moonlighting is able to expand the protein functional repertoire of this bacterium. This use of multifunctional proteins thus allows for greater genetic efficiency.

The two chaperonin 60 proteins of *M. tuberculosis* appear to have distinct biological actions both *in vitro* and *in vivo*, but little is known about the interactions of these proteins with the macrophage cell surface. A recent study has shown that the chaperonin 60.1 protein has different effects on macrophages depending on whether it binds to TLR2 or TLR4 and explains the findings of Mande's group (Khan *et al*. 2008). When chaperonin 60.1 binds to TLR2 the complex is endocytosed via a clathrin-dependent mechanism, which results in the production of the anti-inflammatory cytokine IL-10. Inhibition of the endocytosis of chaperonin 60.1 results in inhibition of IL-10 synthesis and generation of the proinflammatory cytokine, TNFα. In contrast, interaction of chaperonin 60.1 with TLR4 is not associated with significant endocytosis and triggers a proinflammatory response (Parveen *et al*. 2013). This shows that this one moonlighting protein, depending on its interaction, can cause distinct activation states of macrophages, such as has been proposed to occur in the tuberculoid granuloma (Ramakrishnan 2012).

One of the major problems in defining the actions of bacterial moonlighting proteins is that it is rarely possible to inactivate the genes encoding these proteins, as these genes code for essential proteins. An attempt was made to inactivate the two chaperonin 60 genes and the chaperonin 10 gene in *M. tuberculosis* (Hu *et al*. 2008). The chaperonin 10 and chaperonin 60.2 genes could only be inactivated if a plasmid-based copy of the chromosomal inactivated gene was provided, revealing that these proteins were essential and that the chaperonin 60.2 protein was the major 60 kDa cell stress protein in *M. tuberculosis*. In contrast, mutants lacking the activity of the gene coding for chaperonin 60.1: (1) were viable; (2) grew at the normal rate in culture and within macrophages; and (3) responded like the wild-type organism to a range of stresses. This suggested that the chaperonin 60.1 protein had lost its protein-folding actions, confirmed by the finding that

the gene for *M. tuberculosis* chaperonin 60.2 could replace *E. coli* GroEL, but this was not possible with the gene for the chaperonin 60.1 protein (Hu *et al*. 2008). This suggested that the chaperonin 60.1 protein was "surplus to requirement." However, when the chaperonin 60.1 mutant was used to infect mice or guinea pigs it failed to induce a granulomatous inflammatory state in the lungs, even though it grew at the same rate as the wild-type organism. This suggests that the chaperonin 60.1 protein of *M. tuberculosis* is a key virulence factor in inducing the granulomatous inflammatory state that characterizes tuberculosis. Further evidence for this hypothesis comes from the use of a human blood granuloma assay described earlier in this chapter (Puissegur *et al*. 2007). Wild-type *M. tuberculosis* induced the production of multiple multinuclear macrophages from human whole blood. In contrast, the mutant organism lacking the gene for chaperonin 60.1 was virtually unable to generate such cells (Cehovin *et al*. 2010). Further evidence for a role of *M. tuberculosis* chaperonin 60.1 in the multinucleation of myeloid cells has come from a study of the influence of this protein on osteoclast formation. It turns out that this protein is a potent inhibitor of osteoclast formation both *in vitro* and *in vivo*. Administration of *M. tuberculosis* chaperonin 60.1 to rats with adjuvant arthritis therefore fails to inhibit joint inflammation, but virtually abolishes the massive induction of osteoclastogenesis that occurs in this model of human rheumatoid arthritis (Winrow *et al*. 2008). The ability to inhibit osteoclast formation is due to the inhibition of transcription of the gene encoding the major osteoclast transcription factor, NFATc1 (Winrow *et al*. 2008). It would therefore appear that *M. tuberculosis* chaperonin 60.1 is an inducer of multinucleate giant cell formation, but an inhibitor of another form of multinucleate giant cell: the osteoclast. This protein appears to be an interesting ligand which could be used to investigate the pathways leading from the monocyte to the various multinucleate giant cells of the human body.

5.4.4 Identification of the Myeloid-Cell-Activating Site in *M. tuberculosis* Chaperonin 60.1

Key to understanding the mechanism of action of moonlighting proteins is the identification of the active moonlighting sites. An early study generated the three recombinant domains of the *M. tuberculosis* chaperonin 60.1 protein and, using these proteins, showed that only the equatorial domain (which contains both N- and C-terminal residues) had the ability to stimulate monocyte cytokine synthesis (Tormay *et al*. 2005). To refine the search for the active site, a range of protein mutants was generated in which successive 30 residue segments were removed from the C-terminus of the protein. This generated a series of mutants all missing individual 30 residue segments progressing from the C-terminus. This identified residues 460–491 as the active site of this protein. This was confirmed by generating this peptide and a series of sub-peptides. Only the complete 30-residue peptide was active in inducing activation of monocyte cytokine synthesis (Hu *et al*. 2013). Structural modeling of this peptide segment showed that it was present on the surface of the protein and had significant α-helical structure (Hu *et al*. 2013). It is not clear if this particular moonlighting site is also responsible for the other biological actions of this protein.

5.5 Conclusions

Tuberculosis is a disease in which the causative organism invades and evades the actions of the macrophage, and causes the formation of a granuloma in which various differentiated forms of the macrophage are found. How *M. tuberculosis* is able to modulate the macrophage in this way is unclear. However, there is now emerging evidence that molecular chaperones such as the chaperonin 60 proteins of this organism have a range of profound effects on macrophages and their relatives, such as dendritic cells. In *M. tuberculosis*, both chaperonin 60 paralogs have a range of effects on macrophages which appear able to induce alternative macrophage activation and to induce the formation of multinucleate giant cells. It is also possible that chaperonin 60.1 is able to act as an evasin, with chaperonin 60.2 having the actions of an adhesin. The chaperonin 60 proteins of *M. tuberculosis* would therefore seem to be interesting molecular targets for modulating the actions of this bacterium and, potentially, having a major effect on the pathology of tuberculosis.

References

Bynum H (2012) *Spitting Blood: A History of Tuberculosis*. Oxford University Press, Oxford.

Campo M, Randhawa AK, Dunstan S, Farrar J, Caws M, Bang ND, Lan NN, Chau TT, Horne DJ, Thuong NT, Thwaites GE, Hawn TR (2015) Common polymorphisms in the CD43 gene region are associated with tuberculosis disease and mortality. *American Journal of Respiratory Cell and Molecular Biology* 52(3), 342–348.

Cannon JL, Mody PD, Blaine KM, Chen EJ, Nelson AD, Sayles LJ, Moore TV, Clay BS, Dulin NO, Shilling RA, Burkhardt JK, Sperling AI (2011) CD43 interaction with ezrin-radixin-moesin (ERM) proteins regulates T-cell trafficking and CD43 phosphorylation. *Molecular Biology of the Cell* 22: 954–963.

Cehovin A, Coates AR, Hu Y, Riffo-Vasquez Y, Tormay P, Botanch C, Altare F, Henderson B (2010) Comparison of the moonlighting actions of the two highly homologous chaperonin 60 proteins of *Mycobacterium tuberculosis*. *Infection and Immunity* 78: 3196–3206.

Daniel TM (2006) The history of tuberculosis. *Respiratory Medicine* 100: 1862–1870.

Doimo NT, Zárate-Bladés CR, Rodrigues RF, Tefé-Silva C, Trotte MN, Souza PR, Soares LS, Rios WM, Floriano EM, Brandão IT, Masson AP, Coelho V, Ramos SG, Silva CL (2014) Immunotherapy of tuberculosis with *Mycobacterium leprae* Hsp65 as a DNA vaccine triggers cross-reactive antibodies against mammalian Hsp60 but not pathological autoimmunity. *Human Vaccines and Immunotherapeutics* 10: 1238–1243.

Dorhoi A, Reece ST, Kaufmann SH (2011) For better or for worse: the immune response against *Mycobacterium tuberculosis* balances pathology and protection. *Immunological Reviews* 240: 235–251.

Fan M, Rao T, Zacco E, Ahmed MT, Shukla A, Ojha A, Freeke J, Robinson CV, Benesch JL, Lund PA (2012) The unusual mycobacterial chaperonins: evidence for *in vivo* oligomerization and specialization of function. *Molecular Microbiology* 85: 934–944.

Friedland JS, Shattock R, Remick DG, Griffin GE (1993) Mycobacterial 65-kD heat shock protein induces release of proinflammatory cytokines from human monocytic cells. *Clinical and Experimental Immunology* 91: 58–62.

Fujiwara N, Kobayashi K (2005) Macrophages in inflammation. *Current Drug Targets: Inflammation and Allergy* 4: 281–286.

Hayman J (1984) *Mycobacterium ulcerans*: an infection from Jurassic time? *Lancet* 2: 1015–1016.

Henderson B, Pockley AG (2010) Molecular chaperones and protein folding catalysts as intercellular signalling regulators in immunity and inflammation. *Journal of Leukocyte Biology* 88: 445–462.

Henderson B, Fares MA, Lund PA (2013) Chaperonin 60: a paradoxical, evolutionarily conserved protein family with multiple moonlighting functions. *Biological Reviews of the Cambridge Philosophical Society* 88: 955–987.

Hickey TB, Thorson LM, Speert DP, Daffé M, Stokes RW (2009) *Mycobacterium tuberculosis* Cpn60.2 and DnaK are located on the bacterial surface, where Cpn60.2 facilitates efficient bacterial association with macrophages. *Infection and Immunity* 77: 3389–3401.

Hickey TB, Ziltener HJ, Speert DP, Stokes RW (2010) *Mycobacterium tuberculosis* employs Cpn60.2 as an adhesin that binds CD43 on the macrophage surface. *Cell Microbiology* 12: 1634–1647.

Hu Y, Henderson B, Lund PA, Tormay P, Ahmed MT, Gurcha SS, Besra GS, Coates AR (2008) A *Mycobacterium tuberculosis* mutant lacking the groel homologue Cpn60.1 is viable but fails to induce an inflammatory response in animal models of infection. *Infection and Immunity* 76: 1535–1546.

Hu Y, Coates AR, Liu A, Lund PA, Henderson B (2013) Identification of the monocyte activating motif in *Mycobacterium tuberculosis* chaperonin 60.1. *Tuberculosis (Edinb)* 93: 442–447.

Khan N, Alam K, Mande SC, Valluri VL, Hasnain SE, Mukhopadhyay S (2008) *Mycobacterium tuberculosis* heat shock protein 60 modulates immune response to PPD by manipulating the surface expression of TLR2 on macrophages. *Cell Microbiology* 10: 1711–1722.

Kirby AC, Meghji S, Nair SP, White P, Reddi K, Nishihara T, Nakashima K, Willis AC, Sim R, Wilson M, *et al.* (1995) The potent bone-resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone GroEL. *Journal of Clinical Investigations* 96: 1185–1194.

Koizumi K, Poulaki V, Doehmen S, Welsandt G, Radetzky S, Lappas A, Kociok N, Kirchhof B, Joussen AM (2003) Contribution of TNF-alpha to leukocyte adhesion, vascular leakage, and apoptotic cell death in endotoxin-induced uveitis *in vivo*. *Investigative Ophthalmology and Visual Science* 44: 2184–2191.

Kong TH, Coates AR, Butcher PD, Hickman CJ, Shinnick TM (1993) *Mycobacterium tuberculosis* expresses two chaperonin-60 homologs. *Proceedings of the National Academy of Science, USA* 90: 2608–2612.

Lewthwaite JC, Coates AR, Tormay P, Singh M, Mascagni P, Poole S, Roberts M, Sharp L,Henderson B (2001) *Mycobacterium tuberculosis* chaperonin 60.1 is a

more potent cytokine stimulator than chaperonin 60.2 (Hsp 65) and contains a CD14-binding domain. *Infection and Immunity* 69: 7349–7355.

Lugo-Villarino G, Hudrisier A. Benard A, Neyrolles O (2013) Emerging Trends in the Formation and Function of Tuberculosis Granulomas. *Frontiers in Immunology* 3: 405.

Lund PA (2009) Multiple chaperonins in bacteria – why so many? *FEMS Microbiology Reviews* 33: 785–800.

Macario AJ, Malz M, Conway de Macario E (2004) Evolution of assisted protein folding: the distribution of the main chaperoning systems within the phylogenetic domain archaea. *Frontiers in Bioscience* 9: 1318–1332.

Martinez FO, Gordon S (2014) The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep* 6: 13.

Meghji S, White PA, Nair SP, Reddi K, Heron K, Henderson B, Zaliani A, Fossati G, Mascagni P, Hunt JF, Roberts MM, Coates AR (1997) *Mycobacterium tuberculosis* chaperonin 10 stimulates bone resorption: a potential contributory factor in Pott's disease. *Journal of Experimental Medicine* 186: 1241–1246.

Naffin-Olivos JL, Georgieva M, Goldfarb N, Madan-Lala R, Dong L, Bizzell E, Valinetz E, Brandt GS, Yu S, Shabashvili DE, Ringe D, Dunn BM, Petsko GA, Rengarajan J (2014) *Mycobacterium tuberculosis* Hip1 modulates macrophage responses through proteolysis of GroEL2. *PLoS Pathogens* 10: e1004132.

Parveen N, Varman R, Nair S, Das G, Ghosh S, Mukhopadhyay S (2013) Endocytosis of *Mycobacterium tuberculosis* heat shock protein 60 is required to induce interleukin-10 production in macrophages. *Journal of Biological Chemistry* 288: 24956–24971.

Peetermans WE, Raats CJ, Langermans JA, van Furth R (1994) Mycobacterial heat-shock protein 65 induces proinflammatory cytokines but does not activate human mononuclear phagocytes. *Scandinavian Journal of Immunology* 39: 613–617.

Puissegur MP, Lay G, Gilleron M, Botella L, Nigou J, Marrakchi H, Mari B, Duteyrat JL, Guerardel Y, Kremer L, Barbry P, Puzo G, Altare F (2007) Mycobacterial lipomannan induces granuloma macrophage fusion via a TLR2-dependent, ADAM9- and beta1 integrin-mediated pathway. *Journal of Immunology* 178: 3161–3169.

Qamra R, Mande SC (2004) Crystal structure of the 65-kilodalton heat shock protein, chaperonin 60.2, of *Mycobacterium tuberculosis*. *Journal of Bacteriology* 186: 8105–8113.

Qamra R, Srinivas V, Mande SC (2004) *Mycobacterium tuberculosis* GroEL homologues unusually exist as lower oligomers and retain the ability to suppress aggregation of substrate proteins. *Journal of Molecular Biology* 342: 605–617.

Ramakrishnan L (2012) Revisiting the role of the granuloma in tuberculosis. *Nature Reviews Immunology* 12, 352–366.

Rangaka MX, Wilkinson KA, Glynn JR, Ling D, Menzies D, Mwansa-Kambafwile J, Fielding K, Wilkinson RJ, Pai M (2012) Predictive value of interferon-γ release assays for incident active tuberculosis: a systematic review and meta-analysis. *Lancet Infectious Diseases* 12: 45–55.

Reddi K, Meghji S, Nair SP, Arnett TR, Miller AD, Preuss M, Wilson M, Henderson B, Hill P (1998) The *Escherichia coli* chaperonin 60 (groEL) is a potent stimulator of osteoclast formation. *Journal of Bone and Mineral Research* 13: 1260–1266.

Rengarajan J, Murphy E, Park A, Krone CL, Hett EC, Bloom BR, Glimcher LH, Rubin EJ (2008) *Mycobacterium tuberculosis* Rv2224c modulates innate immune responses. *Proceedings of the National Academy of Science, USA* 105: 264–269.

Rha YH, Taube C, Haczku A, Joetham A, Takeda K, Duez C, Siegel M, Aydintug MK, Born WK, Dakhama A, Gelfand EW (2002) Effect of microbial heat shock proteins on airway inflammation and hyperresponsiveness. *Journal of Immunology* 169: 5300–5307.

Riffo-Vasquez Y, Spina D, Page C, Tormay P, Singh M, Henderson B, Coates A (2004) Effect of *Mycobacterium tuberculosis* chaperonins on bronchial eosinophilia and hyper-responsiveness in a murine model of allergic inflammation. *Clinical and Experimental Allergy* 34: 712–719.

Riffo-Vasquez Y, Coates AR, Page CP, Spina D (2012) *Mycobacterium tuberculosis* chaperonin 60.1 inhibits leukocyte diapedesis in a murine model of allergic lung inflammation. *American Journal of Respiratory Cell and Molecular Biology* 47: 245–252.

Saibil HR, Fenton WA, Clare DK, Horwich AL (2013) Structure and allostery of the chaperonin GroEL. *Journal of Molecular Biology* 425: 1476–1487.

Sakula A (1982). Robert Koch: centenary of the discovery of the tubercle bacillus, 1882. *Thorax* 37: 246–251.

Shin YS, Takeda K, Shiraishi Y, Jeong YY, Domenico J, Jia Y, Han J, Spallek R, Singh M, Lucas JJ,Gelfand EW (2012) Microbial heat shock protein 65 attenuates airway hyperresponsiveness and inflammation by modulating the function of dendritic cells. *Journal of Immunology* 189: 3404–3410.

Sreevatsan S, Pan X, Stockbauer KE, Connell ND, Kreswirth BN, Whittam TS, *et al.* (1997) Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. *Proceedings of the National Academy of Science, USA* 94: 9869–9874.

Stapleton MR, Smith LJ, Hunt DM, Buxton RS, Green J (2012) *Mycobacterium tuberculosis* WhiB1 represses transcription of the essential chaperonin GroEL2. *Tuberculosis (Edinb)* 92: 328–332.

Tormay P, Coates AR, Henderson B (2005) The intercellular signaling activity of the *Mycobacterium tuberculosis* chaperonin 60.1 protein resides in the equatorial domain. *Journal of Biological Chemistry* 280: 14272–14277.

van Eden W, van der Zee R, Prakken B (2005) Heat-shock proteins induce T-cell regulation of chronic inflammation. *Nature Reviews Immunology* 5: 318–330.

Verdegaal ME, Zegveld ST, van Furth R (1996) Heat shock protein 65 induces CD62e, CD106, and CD54 on cultured human endothelial cells and increases their adhesiveness for monocytes and granulocytes. *Journal of Immunology* 157: 369–376.

Wang XM, Lu C, Soetaert K, S'Heeren C, Peirs P, Lanéelle MA, Lefèvre P, Bifani P, Content J, Daffé M, Huygen K, De Bruyn J, Wattiez R (2011) Biochemical and immunological characterization of a cpn60.1 knockout mutant of *Mycobacterium bovis* BCG. *Microbiology* 157: 1205–1219.

Williams GT, Williams WJ (1983) Granulomatous inflammation – a review. *Journal of Clinical Pathology* 36: 723–733.

Winrow VR, Mesher J, Meghji S, Morris CJ, Maguire M, Fox S, Coates AR, Tormay P, Blake DR, Henderson B (2008) The two homologous chaperonin 60 proteins of

Mycobacterium tuberculosis have distinct effects on monocyte differentiation into osteoclasts. *Cell Microbiology* 10: 2091–2104.

Xu J, Laine O, Masciocchi M, Manoranjan J, Smith J, Du SJ, Edwards N, Zhu X, Fenselau C, Gao LY (2007) A unique Mycobacterium ESX-1 protein co-secretes with CFP-10/ESAT-6 and is necessary for inhibiting phagosome maturation. *Molecular Microbiology* 66: 787–800.

Xue J, Schmidt SV, Sander J, *et al*. (2014) Transcriptome–based network analysis reveals a spectrum model of human macrophage activation. *Immunity* 40: 274–288.

Yarkoni E, Rapp HJ (1977). Granuloma formation in lungs of mice after intravenous administration of emulsified trehalose-6, 6′-dimycolate (cord factor): reaction intensity depends on size distribution of the oil droplets. *Infection and Immunity* 18: 552–554.

6

Legionella pneumophila Chaperonin 60, an Extra- and Intra-Cellular Moonlighting Virulence-Related Factor

Karla N. Valenzuela-Valderas[1], Angela L. Riveroll[1,2], Peter Robertson[1,3], Lois E. Murray[1], and Rafael A. Garduño[1,3]

[1] *Department of Microbiology and Immunology, Dalhousie University, Halifax, Nova Scotia, Canada*
[2] *Present address: Solarvest BioEnergy Inc., Summerville, PE, Canada*
[3] *Present address: Canadian Food Inspection Agency, Dartmouth Laboratory, Dartmouth, Nova Scotia, Canada*

6.1 Background

The reader is referred to the earlier chapters of this book (in particular Chapters 1 and 5) for expert reference material on moonlighting proteins, the importance of moonlighting, and the need to better understand it. For reference material on chaperonins in general, their primary job as helpers in protein folding, and the relevance of chaperonin moonlighting in bacterial pathogenesis, the comprehensive reviews of Clare and Saibil (2013), England *et al*. (2008), and Henderson *et al*. (2013) are recommended. In relation to the chaperonin of the opportunistic intracellular bacterial pathogen *Legionella pneumophila* (known as HtpB), the reader is referred to our two recent reviews. In the first review, we presented the many known anomalies of HtpB and a historical perspective of the research that led to the establishment of HtpB as a moonlighting chaperonin (Garduño *et al*. 2011). In our second review, we emphasized HtpB's moonlighting functions in all the cellular compartments where this chaperonin is found (Garduño and Chong 2013). Briefly, HtpB is the only chaperonin in *L. pneumophila*, and a portion of it resides in extracytoplasmic bacterial locations either as a membrane-associated, periplasmic, or surface-exposed protein, playing unique protein-folding-independent roles in each of these compartments. In addition, another portion of HtpB is released into the *Legionella*-containing vacuole of infected cells and reaches the cytoplasm of these infected cells, where it potentially interacts with host cell proteins triggering responses that contribute to the intracellular establishment of *L. pneumophila*.

By not having to cover the subjects of protein moonlighting and chaperonins, or re-describe the unique features of HtpB, we will focus this chapter on

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

the work undertaken to study the mechanisms behind HtpB's moonlighting functions and understand its evolution. For clarity in comparing and contrasting differences between chaperonins, in this chapter we refer to the chaperonin 60 of *L. pneumophila* as HtpB, and to that of *Escherichia coli* as GroEL. All other bacterial chaperonins mentioned in this chapter will be generically referred to as Cpn60.

6.2 HtpB is an Essential Chaperonin with Protein-folding Activity

For years, we wondered whether protein-folding was indeed the main day job of HtpB. Although the essentiality of *htpB* strongly suggested that HtpB would have such a day job (Nasrallah *et al*. 2011*a*), in the absence of experimental evidence we could not unequivocally resolve this issue. In a recent study that will be reported in detail elsewhere, it was demonstrated that in the presence of the purified *L. pneumophila* co-chaperonin, HtpA, purified HtpB refolds denatured malate dehydrogenase *in vitro*, according to the method of Hayer-Hartl (2000). HtpA was purified by anion exchange chromatography from a *L. pneumophila* whole cell extract, following the method of Quaite-Randall and Joachimiak (2000). The demonstration that HtpB has protein-folding activity now validates its status as a moonlighting chaperonin, with a primary day job in protein-folding and various secondary night jobs in different cellular compartments (Garduño and Chong 2013).

6.3 Experimental Approaches to Elucidate the Functional Mechanisms of HtpB

Since we cannot study the functional properties of HtpB through deletion of *htpB*, several functional models have been used to study its moonlighting traits. Being able to purify HtpB from *L. pneumophila*, or a mixture of recombinant HtpB and GroEL from *E. coli* carrying a plasmid-borne copy of *htpB*, we have attached these purified products to 1 μm diameter microbeads to check their interaction with mammalian cells (Garduño *et al*. 1998*b*; Chong *et al*. 2009). HtpB exposed on the surface of microbeads is capable of mediating their uptake by non-phagocytic cells (HeLa and Chinese hamster ovary or CHO cell lines), indicating that HtpB interacts with eukaryotic surface receptors and triggers a phagocytic process (Garduño *et al*. 1998*b*; Chong *et al*. 2009). In addition, HtpB-coated beads attracted mitochondria once they were internalized, and induced a rearrangement of the actin cytoskeleton upon interaction with CHO cells (Chong *et al*. 2009; Garduño and Chong 2013). To begin unfolding the mechanism(s) by which HtpB triggers these host cell responses, we are looking for potential protein partners of HtpB by yeast two hybrid screens (see Section 6.3.2. below), and for HtpB-specific host cell receptors (Garduño and Chong 2013).

A second approach has been to express HtpB as a recombinant protein in bacteria, mammalian cells, and yeast. Surprisingly, in each of the above hosts, the recombinant HtpB was able to produce a strong (yet intriguing) phenotype. In Gram-negative bacteria recombinant HtpB, but not GroEL, induces the production of non-septated long filaments (Allan 2002; Garduño and Chong 2013; unpublished results). In CHO cells recombinant HtpB, but not GroEL, induces a rearrangement of the actin cytoskeleton that results in the depolymerization of stress fibers and an accumulation of cortical microfilaments at the periphery of the cell (Chong *et al*. 2009). It is important to note here that this rearrangement of the actin cytoskeleton is morphologically indistinguishable from the rearrangement induced by HtpB-coated beads (see previous paragraph), indicating that this cellular response can be induced by HtpB from without or from within host cells. The mechanism behind this phenotype appears to involve the recruitment of cytoplasmic host cell proteins, either directly (from within) or via membrane receptors (from without). In the budding yeast *Saccharomyces cerevisiae* recombinant HtpB, but not GroEL, induces cell elongation, unipolar budding, and penetration of solid medium, a complex phenotype known as pseudohyphal growth (Nasrallah *et al*. 2011*b*). Since *S. cerevisiae* is a genetically tractable eukaryote, pseudohyphal growth is very well studied in this yeast, and induction of pseudohyphae formation by HtpB is a strong and reproducible phenotype, we attempted to elucidate the signaling mechanism involved.

6.3.1 The Intracellular Signaling Mechanism of HtpB in Yeast

Having access to a number of HtpB expression vectors for *S. cerevisiae* with a variety of selective markers (Nasrallah *et al*. 2011*b*), we expressed recombinant HtpB in a number of yeast mutants with signaling defects related to production of pseudohyphae. From these studies we found that HtpB triggers an intracellular, nitrogen limitation- and glucose limitation-independent signal that is transduced by Ras2p and requires activation of both the Ste-kinase and the cAMP/PKA signaling pathways.

Pseudohyphal growth in *S. cerevisiae* is a tightly regulated natural response to metabolic and environmental signals that activate a number of G-proteins, including the global regulator Ras2p (Fig. 6.1), and is required for mating of haploid cells and (or) survival in nutrient-deficient conditions (Gancedo 2001; Cullen and Sprague 2012). Briefly, Ras2p primarily activates a cAMP-dependent protein kinase A (PKA) pathway, and secondarily through Cdc42 (a conserved mitogen-activated protein kinase or MAPK pathway) the Ste-kinase pathway (Chavel *et al*. 2014). These two pathways, and their respective transcription factors Flo8p and Ste12p, converge in the regulation of *FLO11* expression. Flo11p is a key surface glycoprotein involved in pseudohyphal development and agar invasion (Rupp *et al*. 1999; Lengeler *et al*. 2000). To determine whether HtpB signals through this natural cascade (Fig. 6.1), and to investigate at what level in the cascade HtpB potentially acts, we tested the expression of HtpB in mutants derived from the *S. cerevisiae* haploid and filament-deficient strain W303-1b (Archambault *et al*. 1992), carrying single deletions in genes of the Ste-kinase and (or) cAMP/PKA pathways.

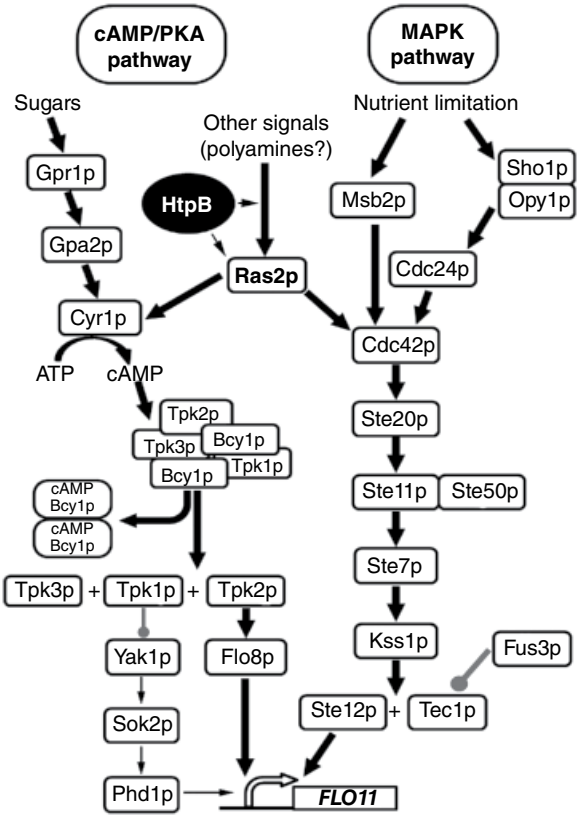

Figure 6.1 Signaling cascades used by HtpB to trigger pseudohyphal growth in *S. cerevisiae*. Diagram showing the natural signaling pathways leading to expression of genes (*FLO11* being a main one) required for pseudohyphae formation. HtpB acts at the level of, or upstream from Ras2, signaling in a nitrogen- and glucose-independent manner. Our data indicate that the Ste-kinase and cAMP/PKA pathways are both critical to, and the only two pathways involved in, the induction of pseudohyphal growth by HtpB. Factors from top to bottom: Gpr1: G protein-coupled receptor/transmembrane sensor; Gpa2: Gα subunit; Msb2: sensor/receptor; Sho1 and Opy2: sensors/G protein adaptors; Cdc24: guanine nucleotide exchange factor for Cdc42; Ras2: G protein global regulator; Cyr1: adenylate cyclase; Cdc42: Rho GTPase; Bcy1: regulatory subunit of the PKA complex; Tpk1/2/3: protein kinases A; Yak1: dual-specificity tyrosine-regulated kinase; Ste20: p21-activated kinase; Ste11: MAPKKK; Ste50: adaptor/scaffold protein; Ste7: MAPKK; Kss1: MAPK of the filamentous growth pathway; Fus3: MAPK of the mating pathway; Flo8/Sok2/Ste12/Tec1/Phd1: transcription factors; *FLO11*: main hub of signal transduction. Notes: Upon binding cAMP, Bcy1 releases the three PKAs Tpk1/2/3, which then become active. Yak1 is active when non-phosphorylated and it mediates low expression of Flo11 (thin arrows). Phosphorylation of Yak1 by Tpk1 inactivates it (gray arrow with circular head). Ste50 is known to specifically bind Ste11, but it could serve as the scaffold for the MAP kinase module (Ste11 + Ste7 + Kss1). Unregulated filamentous growth is detrimental for mating, so activated Fus3 acts as a repressor of Tec1 + Ste12 transcription factors (gray arrow with circular head). Active transcription from the *FLO11* promoter is depicted by the curved white arrow on the left of the *FLO11* gene.

W303-1b mutants carrying a deletion in *ste20*, *ste11*, *ste7*, or *ste12* and expressing HtpB were unable to form filamentous fuzzy colonies and invade solid medium. However, the mutant cells could still elongate and bud in a unipolar fashion (Fig. 6.2). Clearly, HtpB signaling requires the entire Ste-kinase pathway

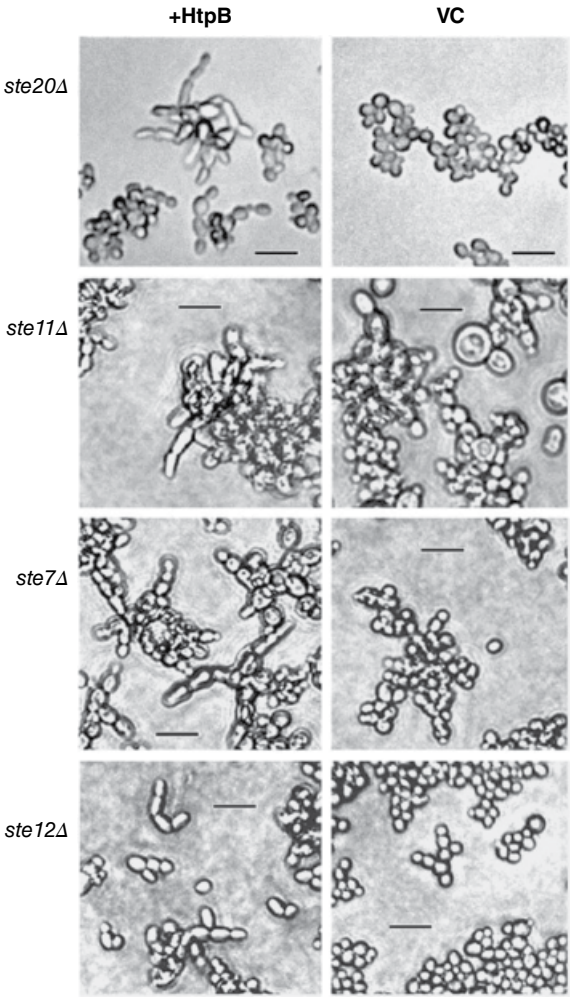

Figure 6.2 HtpB-induced filamentation and agar invasion (but not elongation and unipolar budding) requires the Ste-kinase pathway in *S. cerevisiae*. Light micrographs of microcolonies of *S. cerevisiae* strain W303-1b with the indicated deletions in genes encoding members of the Ste-kinase (MAP-kinase) pathway, and either containing pPP389::*htpB* (Nasrallah *et al*. 2011*b*) for expression of HtpB (+HtpB), or the empty vector control pPP389 (VC). All deletion mutants were grown on inducing galactose solid medium, as HtpB expression is controlled by a galactose-inducible promoter. The *ste* mutants were created by allelic replacement with restriction fragments from plasmids pEL45 (*ste20*Δ::*URA3*) (Leberer *et al*. 1992), pSL1311 (*ste11*Δ::*URA3*), pSL1077 (*ste7*Δ::*URA3*), and pSL1094 (*ste12*Δ::*URA3*) (Stevenson *et al*. 1992). The restriction enzymes used were: pEL45 *Xba*I/*Sal*I, pSL1077 *Kpn*I/*Cla*I, pSL1094 *Bam*HI/*Xho*I, and pSL1311 *Sst*I/*Sph*I. The restriction fragments were transformed as linear DNA into strain W303-1b, and colonies of deletion mutants were then selected on solid medium lacking uracil. Bars represent 12.5 μm.

to induce filamentation and agar invasion. The fact that all the Ste-kinase mutants expressing HtpB had the same phenotype also suggested that HtpB acts upstream of the p21-activated kinase Ste20p, and likely still depends on the cAMP/PKA pathway to mediate cell elongation and unipolar budding.

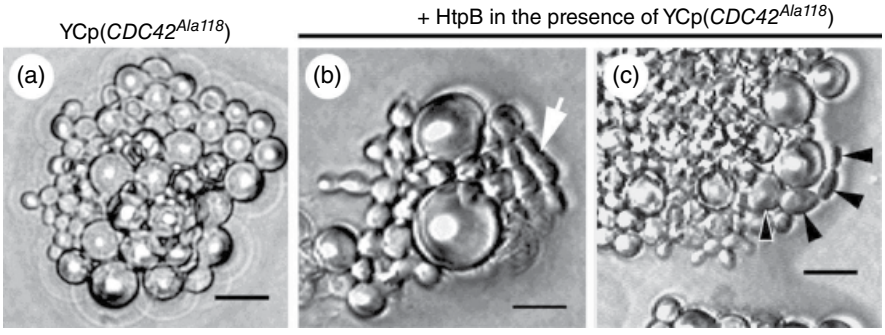

Figure 6.3 HtpB induces cell elongation and unipolar budding in *S. cerevisiae* strain W303-1b carrying the dominant negative *CDC42*$^{Ala\text{-}118}$ allele. Light micrographs of microcolonies (grown on inducing galactose solid medium) of *S. cerevisiae* strain W303-1b bearing: (a) plasmid YCp(*CDC42*$^{Ala\text{-}118}$) for expression of Cdc42p-Ala118 (Ziman *et al*. 1991); or (b, c) plasmids pLM86 (Nasrallah *et al*. 2011*b*) and YCp(*CDC42*$^{Ala\text{-}118}$) for simultaneous expression of HtpB and Cdc42p-Ala118 (+HtpB in the presence of YCp(*CDC42*$^{Ala\text{-}118}$)). Plasmid YCp was generously donated by H.-U. Mösch (Philipps University, Germany). Yeast cells carrying the *CDC42*$^{Ala\text{-}118}$ allele grew large but did not elongate (a). Some large elongated cells are marked with the white arrow in (b), and a chain of elongated cells derived from a single large round cell is marked by the arrowheads in (c). Bars represent 10 μm in all panels.

Immediately upstream of Ste20p lies the Rho GTPase Cdc42p, which serves as a branching point into Ras2p, the cAMP/PKA pathway, and other signaling pathways (Peter *et al*. 1996; Johnson 1999; Gancedo 2001). *CDC42* is an essential gene in *S. cerevisiae* whose function cannot be inferred through gene deletion. We therefore introduced the dominant negative *CDC42*$^{Ala\text{-}118}$ allele into strain W303-1b to explore the involvement of Cdc42p in HtpB-induced signaling. In agreement with the results reported by Ziman *et al*. (1991), expression of Cdc42p-Ala118 caused yeast cells to arrest as large, round, unbudded cells but, importantly, elongated cells were not observed (Fig. 6.3a). Only upon co-expression of Cdc42p-Ala118 and HtpB did we observe cell elongation and chain formation (Fig. 6.3b, c). Although the G1 cell cycle arrest caused by Cdc42p-Ala118 did not allow us to assess the formation of filamentous colonies or agar invasion, we concluded that the signaling by which HtpB induces cell elongation and unipolar budding bypasses Cdc42p. However, the very fact that chains of elongated cells were formed from large arrested cells (Fig. 6.3c) implied a relief of the Cdc42p-Ala118-mediated cell division arrest. One possibility here is that HtpB might directly or indirectly interact with Cdc42p to partially bypass the effects of the dominant Cdc42p-Ala118, thus explaining the complex mixture of large and elongated cells observed in the combined presence of HtpB and Cdc42p-Ala118 (Fig. 6.3).

To test the involvement of the cAMP/PKA pathway in the signaling triggered by HtpB, we had to use the *flo8*Δ *S. cerevisiae* strain BY4741 because we could not delete *FLO8* (encoding the transcription factor Flo8p) in strain W303-1b. Upon expression of HtpB, strain BY4741 showed the same phenotype seen in the *ste12* W303-1b deletion mutant, that is, cells elongated and formed unipolar buds but did not produce agar-invasive filaments (Fig. 6.4a). Genetic complementation of

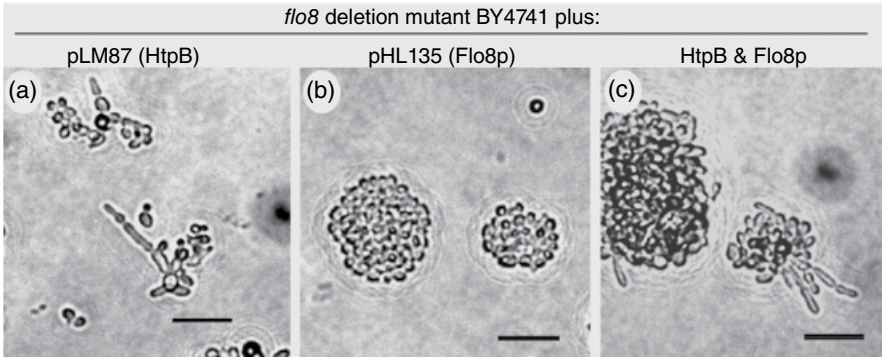

Figure 6.4 HtpB-induced filamentation and agar invasion (but not elongation and unipolar budding) also requires the cAMP/PKA signaling pathway. Light micrographs of microcolonies of haploid cells of *S. cerevisiae* strain BY4741 (*MAT***a** *flo8::kan*^{r} *his3-11 leu2-3,112 met15 ura3-52*), which is a *flo8*Δ derivative of strain S288C (Brachmann *et al*. 1998). Strain BY4741 was either bearing: (a) plasmid pLM87 for expression of HtpB (Nasrallah *et al*. 2011*b*); (b) plasmid pHL135 for expression of Flo8p (Source: Liu *et al*. 1996); or (c) both plasmids pLM87 and pHL135 (HtpB and Flo8p). The elongated cells shown in (a) budded in a unipolar manner, but were not agar-invasive. The *FLO8*-complemented cells shown in (b) are aggregated and did not elongate or penetrate the agar. The cells shown in (c) had penetrated the agar (they were hyper-invasive). Bars represent 10 μm.

BY4741 with wild-type *FLO8* in-trans led to a flocculating phenotype (Fig. 6.4b), likely due to overexpression of Flo8p, but pseudohyphal growth was not observed. However, a *FLO8*-complemented strain expressing HtpB recovered the ability to produce pseudohyphae and became hyper-invasive and hyper-adhesive (Fig. 6.4c). Obtaining the same partial phenotype by independently deleting the transcription factors of either the Ste-kinase pathway or the cAMP/PKA pathway implied that: (1) HtpB simultaneously activates both pathways to fully induce pseudohyphal growth (including formation of long filaments and agar invasion); (2) either the cAMP/PKA or the Ste-kinase pathway can independently mediate elongation and unipolar budding, bypassing each other to induce these phenotypes; and (3) to engage both pathways simultaneously, HtpB must signal at either the branching point of these pathways or upstream of it. Alternatively, HtpB could activate downstream effectors of alternate pseudohyphal growth pathways (Vincent *et al*. 2001; Cullen and Sprague 2012) but this possibility was ruled out by experiments with a double *ste12*Δ *flo8*Δ mutant, as explained next.

To test whether HtpB signaling requires Ras2p, we expressed HtpB in a *ras2* deletion mutant of strain W303-1b, and observed a complete abrogation of pseudohyphal growth phenotypes (Fig. 6.5a). Genetic complementation of the *ras2*Δ mutant with the wild-type *RAS2* gene completely restored pseudohypal growth phenotypes upon expression of HtpB (Fig. 6.5b, c). These results confirmed that the intracellular signal triggered by HtpB in *S. cerevisiae* is either generated at the level of Ras2p, or upstream of this global regulator. Finally, we expressed HtpB in a double *flo8*Δ *ste12*Δ mutant, obtained by mating the *flo8*Δ *S. cerevisiae* strain BY4741 with the W303-1b *ste12*Δ mutant, sporulating the diploid, and screening for haploid double mutants on solid medium containing kanamycin and lacking uracil.

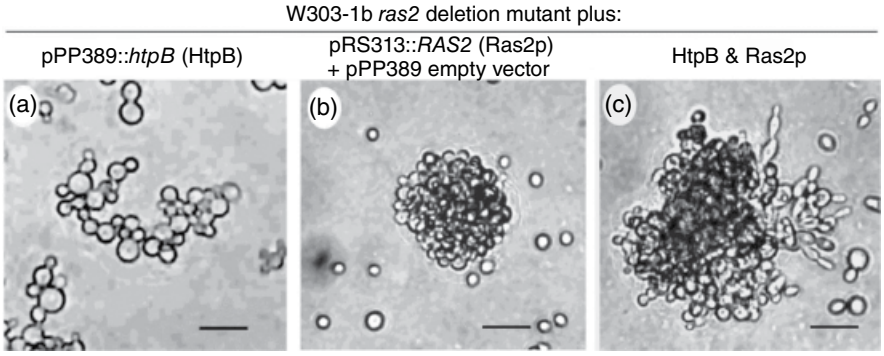

Figure 6.5 HtpB-induced pseudohyphal development in *S. cerevisiae* is dependent on Ras2p. Light micrographs of microcolonies grown on inducing galactose solid medium of a *ras2*Δ mutant strain (W303-1b *ras2* deletion mutant) bearing the following plasmids: (a) pPP389::*htpB* (for expression of HtpB; Nasrallah *et al*. 2011*b*); (b) pRS313::*RAS2* (for expression of Ras2p) plus empty vector pPP389; and (c) pPP389::*htpB* and pRS313::*RAS2* (HtpB and Ras2p). To create the *ras2*Δ mutant, *RAS2* in strain W303-1b was replaced with the allele *ras2*::*URA3* cut with *Eco*RI/*Hin*dIII from plasmid p*ras2*::*URA3* (Kataoka *et al*. 1984) and transformed as linear DNA. Colonies of *ras2*Δ mutant cells were selected on solid medium lacking uracil. For genetic complementation, *RAS2* was amplified by PCR from strain W303-1b using primers *RAS2*full-F (GTGGCCGTATCAATGGATC) and *RAS2*full-R (GGGAAAGAGAAGCTTGTTATTC). The 1967 bp PCR amplification product was digested with *Xba*I, and cloned into the *Xba*I and *Eco*RV sites of the low-copy number yeast plasmid pRS313 (Sikorski and Hieter 1989) to generate pRS313::*RAS2*. Note that complementation of *ras2*Δ cells with wild-type *RAS2* alone did not result in formation of pseudohyphae (b). Bars represent 12.5 µm.

The simultaneous elimination of the Flo8 and Ste12 transcription factors had the same effect as deleting *RAS2*, that is, rendered *S. cerevisiae* completely unable to form pseudophypae in response to HtpB. This indicated that the cAMP/PKA and Ste-kinase pathways are the only two pathways responsible for transducing the Ras2p-mediated HtpB signal, ruling out the activation of any other alternate pathway.

6.3.2 Yeast Two-Hybrid Screens

In an attempt to explain the phenotypes induced by recombinant HtpB in yeast and mammalian cells, we have conducted a number of yeast two-hybrid screenings to identify potential eukaryotic protein partners of HtpB. To specifically address the induction of pseudohyphal growth discussed in the previous section, we screened a *S. cerevisiae* genomic library consisting of random DNA fragments cloned in the three possible translational frames to produce fusion proteins with the activating domain of the GAL4 transcription factor. The bait was HtpB fused to the DNA binding domain of GAL4, which was active at inducing pseudohyphae formation (Nasrallah *et al*. 2011*b*). This screening identified yeast S-adenosyl methionine decarboxylase (SAMDC) as a specific partner of HtpB. SAMDC is a short-lived enzyme involved in the biosynthesis of polyamines, which in turn have been implicated in inducing filamentous growth in fungi (Guevara-Olvera *et al*. 1997; Herrero *et al*. 1999). In fact, the overexpression of

SAMDC in *S. cerevisiae* strain W303-1b induced pseudohyphal growth (Nasrallah *et al*. 2011*b*). The HtpB-SAMDC interaction therefore seemed an obvious explanation for the pseudohyphal growth phenotype and we hypothesized that the underlying mechanism implied the stabilization of SAMDC by HtpB; a subsequent increase in polyamine levels followed by internal metabolic signaling through Ras2p (Fig. 6.1). The Ras2p-mediated signaling mechanism of HtpB discovered in yeast is certainly applicable to the biology of *L. pneumophila*, mainly because by using far-western blots and dot-blots we have shown that HtpB interacts with the SAMDC of *Acanthamoeba castellanii*, L929 mouse cells, and human U937-derived macrophages (Nasrallah *et al*. 2011*b*). It should be noted here that far-western and dot-blots indicated that SAMDC also interacted with purified GroEL, albeit to a lower extent than HtpB (Nasrallah *et al*. 2011*b*). Although it remains to be determined whether the presence of HtpB in the host cell cytoplasm indeed results in increased intracellular levels of polyamines in host cells, we have determined that polyamines are important for the optimal intracellular growth of *L. pneumophila* (Nasrallah *et al*. 2011*b*).

Another yeast two-hybrid screen of a commercially available cDNA library derived from HeLa cells (Matchmaker, BD Biosciences) identified Merlin-associated protein (MAP), recently renamed small G protein signaling modulator 3 (SGSM3, GenBank accession number BC008078.2), as a potential partner of HtpB (Chong *et al*. 2006). SGSM3 contains Rab-GAP, SH3, and RUN signaling domains and was initially identified through a yeast two-hybrid screen using the protein Merlin as bait (Lee *et al*. 2004). The interaction between Merlin and SGSM3 involves the RUN domain of SGSM3, and was confirmed to occur *in vitro* and *in vivo* through biochemical and co-localization studies (Lee *et al*. 2004). If Merlin and HtpB commonly bind to SGSM3, it seems reasonable to speculate that they could either compete (e.g., HtpB could act as an inhibitor of the SGSM3-Merlin interaction) or have a common function (even sharing additional common partners). Although we have not been able to confirm the SGSM3-HtpB interaction by other means or determine whether it depends on the RUN signaling domain of SGSM3, the possibility that HtpB and Merlin have a common function is enticing. This is all the more relevant in relation to the HtpB-mediated rearrangement of cortical actin microfilaments (refer to Section 6.3 above), because Merlin is closely related to the ezrin, radixin, and moesin protein family involved in the rearrangement of cortical actin (Takeuchi *et al*. 1994).

Further screens of the estimated 3.99×10^{11} cDNA clones of the Matchmaker HeLa cell library (BD Biosciences) identified more potential human protein partners of HtpB, including: ribosomal protein L4 (RPL4); cyclin-dependent protein kinase regulatory subunit 1B (CDC28); the purine biosynthetic enzyme phospho-ribosyl-amino-imidazole succino-carboxamide synthetase (Riveroll 2005); and mitochondrial co-chaperonin Hsp10 (Nasrallah 2015). Although the functional relevance of these interactions has not yet been tested, the detected interaction with mitochondrial Hsp10 constitutes a hypothetical mechanism for mitochondrial attraction, where HtpB on the membrane of the *Legionella*-containing vacuole interacts with surface exposed Hsp10 on mitochondria (Garduño and Chong 2013; Nasrallah 2015). In our most recent yeast two-hybrid

screen of a Matchmaker GAL4AD total human cDNA library (Clontech), we identified that the HtpB-GAL4BD bait interacts with a C-terminus fragment of the human homolog of the proteasome-associated yeast protein ECM29 (hECM29, protein accession number NP_001073867.1). This interaction was fully reproducible and robust under different two-hybrid assay conditions, was confirmed *in vitro* by immunoprecipitation and, most importantly, did not occur when GroEL-GAL4BD was used as bait. These characteristics warranted the use of this interaction as our experimental test to evaluate the role of amino acid residues predicted to be functionally important by the evolutionary trace method (see Section 6.5 below). ECM29 is a linker/scaffold protein that helps in the assembly and trafficking of the proteasome particle; the interaction between HtpB and the human homolog of ECM29 (hECM29) could therefore have implications in *L. pneumophila* biology, as an organelle and (or) vesicular traffic modulator.

6.4 Secretion Mechanisms Potentially Responsible for Transporting HtpB to Extracytoplasmic Locations

We determined very early in our research that HtpB could be found in extracytoplasmic locations (Garduño *et al.* 1998*a*), as could other chaperonins then reported (Garduño *et al.* 2011). To date, many extra-cytoplasmic chaperonins have been identified, and it is no longer surprising to find more bacterial species in which chaperonins are associated with the cell envelope, exposed on the bacterial cell surface, or present in culture supernatants, as most recently reported in *Rickettsia* spp. (Qi *et al.* 2013; Gong *et al.* 2014), *Streptococcus gordonii* (Maddi *et al.* 2014), and *Edwardsiella tarda* (Kumar *et al.* 2014). Moreover, at least two reports have shown that the process by which chaperonins reach extra-cytoplasmic locations involves active secretion, as opposed to passive release by cell lysis (Vanet and Labigne 1998; Yang *et al.* 2011). It is therefore accepted now that Cpn60s are secreted by some bacterial species, but the mechanism by which this is accomplished largely remains a mystery. Before discussing the proposed mechanisms of Cpn60 secretion, it seems reasonable to tackle a fundamental question: why are Cpn60s secreted in the first place? Since ATP would not be readily available in extra-cytoplasmic bacterial locations, and protein folding is critically dependent on a supply of ATP (Clare and Saibil 2013), it could be argued that secretion of Cpn60s is not primarily intended to provide protein-folding assistance beyond the cytoplasmic membrane. Although this argument would not be valid for intracellular pathogens and endosymbionts (where their extracellular compartment actually is the ATP-rich intracellular milieu of their host cells), an additional barrier seems to exist. That is, protein folding by Cpn60s happens in large 14-mer complexes that require the participation of a 7-mer co-chaperonin (Cpn10) complex, so that the co-secretion of cognate Cpn10s would need to be ensured. It therefore seems reasonable to surmise that Cpn60 secretion co-evolved with Cpn60 moonlighting rather than with protein-folding. This view is also supported by the many reported moonlighting Cpn60 functions that are compatible with an extra-cytoplasmic location, particularly the bacterial

cell surface (Henderson *et al*. 2013). In conclusion, the evolution of Cpn60 secretion mechanisms seems to be driven by the bacteria-in-question's niche, their particular needs when occupying such niche, and the contribution of extra-cytoplasmic moonlighting Cpn60s to meeting those needs.

One limiting condition in the elucidation of Cpn60-secreting mechanisms is that, by design, these would have to be inefficient. That is, due to the essential role that Cpn60s play in the cytoplasm of bacterial species that possess a single *cpn60* gene (Lund 2009), these bacteria could not afford to have dangerously low cytoplasmic levels of their chaperonin as a consequence of an efficient Cpn60 secretion process. Although this argument would not be valid for the numerous bacterial species with multiple *cpn60* genes and functional Cpn60 diversity (Lund 2009), it could still be argued that an efficient secretion system would be deleterious due to a potential inability to distinguish between the various highly conserved Cpn60s in the cell. We would therefore expect to find only small amounts of secreted Cpn60s at any given time, making it difficult to distinguish their measured levels from the background levels of Cpn60s passively released by lysis. In spite of this experimental limitation, mechanistic hints for chaperonin secretion do exist.

One possible (and favored) mechanism by which chaperonins reach culture supernatants (i.e., the extracellular milieu) is via outer membrane vesicles (OMVs) (Ferrari *et al*. 2006; Joshi *et al*. 2008; McCaig *et al*. 2013; Berleman *et al*. 2014; Li *et al*. 2015). However, it remains to be determined how Cpn60s end up either in the lumen of OMVs or associated with their membrane, as OMVs primarily consist of periplasmic and outer membrane material. HtpB is also found in OMVs (Galka *et al*. 2008), and we have determined that *L. pneumophila* mutants with a defective Dot/Icm system (a type IV secretion system essential for virulence) accumulate periplasmic HtpB (Chong *et al*. 2006), and have increased amounts of OMV-associated HtpB (unpublished results). Based on these observations and other experimental evidence for the periplasmic residence of HtpB (Allan 2002), we have proposed that a portion of the total HtpB in *L. pneumophila* naturally translocates across the cytoplasmic membrane into the *L. pneumophila* periplasm, from where it is then packed as cargo in OMVs. Besides accumulating HtpB in the periplasm, Dot/Icm mutants do not display surface-exposed HtpB, and we have therefore hypothesized that HtpB is also mobilized from the periplasm across the outer membrane by the Dot/Icm system. However, it is not known how HtpB gets into the *L. pneumophila* periplasm.

6.4.1 Ability of GroEL and HtpB to Associate with Membranes

Although GroEL has never been identified as a secreted protein, it interacts with artificial lipid layers and the cytoplasmic membrane in various manners. One manner is via membrane-bound proteins, for example, GroEL specifically interacts with SecA, the ATPase of the SecYEG inner-membrane translocation channel (Bochkareva *et al*. 1998). Interestingly, it was observed that the GroEL-SecA interaction sometimes results in the release of SecA from the membrane (Bochkareva *et al*. 1998). This type of interaction has been confirmed with other

membrane proteins where GroEL is hypothesized to help these proteins solubilize and (or) insert into membranes (Sun *et al.* 2005). A variation of the aforementioned protein–protein interaction is when GroEL acts as a carrier, bringing proteins targeted for secretion to membrane channels, hence assisting in their export (Kusukawa *et al.* 1989). A direct interaction with membranes has also been reported, where the C-terminus of GroEL inserts into artificial lipid monolayers and bilayers to stabilize them through a novel "lipochaperonin" activity (Török *et al.* 1997).

Based on this ability of GroEL to associate with membranes, it is not surprising that HtpB was early recognized as the major cytoplasmic membrane protein of *L. pneumophila* (Blander and Horwitz 1993), and that our immunolocalization studies with HtpB-specific antibodies show a prominent labeling of the cell envelope, particularly after heat shock (Garduño *et al.* 1998*a*). It is enticing to speculate that the mechanism responsible for the translocation of HtpB across the cytoplasmic membrane involves a direct insertion into the membrane, followed by some kind of "wiggling" through it. That is, we have hypothesized that once in close proximity to or after insertion in the cytoplasmic membrane, HtpB could be translocated across the membrane either on its own (similarly to what happens with cell penetration peptides; Di Pisa *et al.* 2015) or with the assistance of membrane proteins, possibly including the inner membrane complexes of any of the secretion systems present in *L. pneumophila*.

6.4.2 Ongoing Mechanistic Investigations on Chaperonins Secretion

To test the aforementioned hypotheses, the Cpn60 of *Bordetella pertussis* (known to be extra-cytoplasmic; Garduño *et al.* 1998*a*), GroEL, and HtpB were swapped between *B. pertussis*, *E. coli*, and *L. pneumophila* in various combinations, followed by immunolocalization and biochemical fractionation (Allan 2002). We already knew that recombinant HtpB expressed in *E. coli* remains largely restricted to the cytoplasm (Garduño *et al.* 1998*a*), but it was interesting to also determine that recombinant Cpn60 and GroEL were mostly cytoplasmic in *E. coli*, recombinant HtpB was found in culture supernatants of *B. pertussis*, and recombinant GroEL was extra-cytoplasmic (found in the periplasm and bacterial cell surface) in *L. pneumophila*. These results clearly indicated that secretion to extra-cytoplasmic locations is more a function of the bacterial species than the chaperonin itself. So, it seems that chaperonin-secreting systems do exist in *L. pneumophila* and *B. pertussis*, but not in *E. coli*. We have therefore ruled out a mechanism that solely depends on the chaperonin for its translocation into the periplasm, across the cytoplasmic membrane. We believe, however, that interaction of chaperonins with the cytoplasmic membrane could still be required for the chaperonin-secretion system to engage. A secondary observation from these chaperonin-swapping experiments was that recombinant HtpB, but not recombinant GroEL or Cpn60, induced filamentation (see second paragraph of Section 6.3 above) in the three bacterial species used.

We have obtained exciting Cpn60 immunolocalization results in *B. pertussis* secretion mutants (generously provided by Alison Weiss, University of Cincinnati College of Medicine). A mutant with a defective Ptl type IV secretion system showed no change in Cpn60 localization in relation to the parent strain Bp388.

However, the Cpn60 of a Bp388 mutant with a defective BvgAS two-component regulatory system (unable to produce or secrete virulence factors) was restricted to the cytoplasm (Fig. 6.6). We therefore concluded that secretion of Cpn60 is mediated by a *B. pertussis* system (not yet identified) that is regulated by BvgAS and independent from the Ptl system. In the absence of a similar mutant in *L. pneumophila*, we are conducting alternative experiments to gain some insights about the mechanism responsible for HtpB translocation.

We previously used CyaA as a translocation reporter to show that HtpB reaches the cytoplasm of *L. pneumophila*-infected CHO and U937-derived human macrophages (Nasrallah *et al*. 2011*b*). To determine whether this is a Dot/Icm-mediated translocation, we are using a new reporter (the GSK tag) fused to HtpB and carried in a *dotA* mutant unable to secrete any type IV secretion effectors. We chose not to continue using CyaA fusions because CyaA is bulky and known to significantly reduce secretion levels of certain proteins (Day *et al*. 2003). The GSK tag is a small 13 amino acid fragment of the glycogen synthase kinase protein that contains a serine residue (Ser-9) exclusively phosphorylated in the cytoplasm of eukaryotic cells (Garcia *et al*. 2006). An additional advantage of this reporter is that commercial antibodies do exist, which can differentially recognize the phosphorylated and non-phosphorylated forms of GSK, making it easily detectable by immunoblot. To test whether the HtpB C-terminus is necessary for translocation across the inner membrane, we have added a 6x-His tag to the C-terminus of HtpB. As histidine is a cationic hydrophilic amino acid, this tag should prevent direct insertion of HtpB into the inner membrane. An added advantage of the 6x-His tag is its easy detection by immunoblot using commercially available antibodies. Finally, to test whether the C-terminus of HtpB is sufficient for translocation, the last 50 and 100 amino acids of HtpB have been

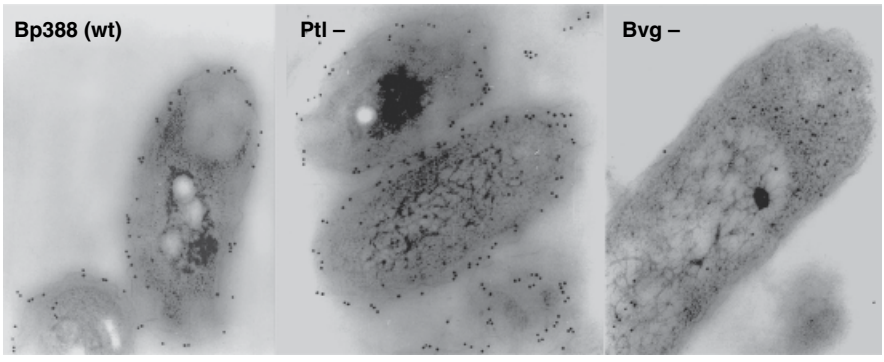

Figure 6.6 A Cpn60-specific secretion mechanism exists in *B. pertussis* and is induced by the BvgAS two-component regulatory system. Electron micrographs of ultrathin sections of specimens fixed with 4% freshly depolymerized paraformaldehyde, and subjected to immunogold labeling using a rabbit polyclonal antibody (Chong *et al*. 2009) that recognizes Cpn60, and a secondary goat anti-rabbit gold conjugate (Sigma Immunochemicals). A wild-type *B. pertussis* parent strain (Bp388 (wt)), a mutant with a defective Ptl type IV secretion system (Ptl –), and a mutant with a non-functional BvgAS two-component regulatory system (Bvg –) are shown. For size reference, the gold spheres that indicate the localization of Cpn60 epitopes are 10 nm in diameter.

fused in frame to the cytoplasmic protein iso-citrate dehydrogenase. Immunolocalization of the tagged proteins and their relative levels in the cytoplasm, membranes and periplasm (after cell fractionation) will indicate the role of the C-terminus of HtpB in translocation.

6.5 Identifying Functionally Important Amino Acid Positions in HtpB

A fundamental concept in basic biology is that the amino acid sequence of a protein (encoded in the DNA sequence of its gene) determines the functional properties of that protein. That is, amino acid positions are linked to functional traits. A few important Cpn60 cases exemplify this fundamental concept. A notorious case is that of the Cpn60 of a symbiotic strain of *Enterobacter aerogenes*, reported to act as an insect toxin (Yoshida *et al*. 2001). This toxic Cpn60 (comprising 545 amino acids) differs from the non-toxic GroEL (comprising 548 amino acids) in only 11 amino acid positions, of which 4 turned out to be critical for toxicity. When the non-toxic GroEL was engineered at the 4 critical residues to resemble the *E. aerogenes* Cpn60, it too became a potent insect toxin (Yoshida *et al*. 2001). In the case of the *Mycobacterium leprae*'s Cpn60, which acts as a protease, only three amino acids (Thr-375, Lys-409, and Ser-502) form a threonine calalytic group responsible for protease activity (Portaro *et al*. 2002). Finally, in the *Buchnera aphidicola* Cpn60 (also known as symbionin) the histidine in position 133 is essential for its unique histidine kinase activity (Morioka *et al*. 1994). It therefore seems reasonable to surmise that differences between the amino acid sequences of Cpn60s constitute the ultimate basis for functional diversity.

However, current biochemical knowledge is not sufficiently advanced to allow us to predict all the functional traits of a protein based solely on amino acid sequence. Nonetheless, as more protein sequences and crystal structures become available, the more accurate our functional predictions will become. Finally, inroads have been made in the development of bioinformatics tools for the study of structure–function relationships and protein evolution, so that the analysis of amino acid sequence differences, their role in evolution, and their potential impact on protein function are now within reach.

HtpB and GroEL differ in a total of 137 amino acid positions scattered throughout the entire protein; it is therefore not easy to discern which of these residues would confer HtpB its unique moonlighting properties. We have used a three-step bioinformatics approach primarily based on the Evolutionary Trace (ET) method (Madabushi *et al*. 2004; Rajagopalan *et al*. 2006; Bonde *et al*. 2010) to predict the functional importance of amino acid positions that are different between HtpB and GroEL.

1) First we identified HtpB orthologs in the non-redundant protein sequence database of the National Center for Biotechnology Information (NCBI) using BLAST (Basic Local Alignment Search Tool). Orthologs (arbitrarily chosen to have an E-value less than 1×10^{-6}) were retrieved and aligned using ClustalOmega (ClustalO) to produce a multiple sequence alignment (MSA),

which was then used as input into the ET code available at http://mammoth.bcm.tmc.edu/. We performed the ET analysis developed by Lichtarge *et al*. (1996), as reported by Wilkins *et al*. (2012). The ET code output was a relative ranking of evolutionary importance for each amino acid position in the MSA. Lower ranks indicated high conservation of the amino acid position, and therefore a likely important role in functionality.

2) A 3D structure was then predicted for HtpB using the ModWeb server available online at https://modbase.compbio.ucsf.edu/modweb/, so that the ranking of amino acids obtained in the first step could be color-coded and mapped to the 3D structure using PyMol and the PyETV plugin at http://mammoth.bcm.tmc.edu/traceview/HelpDocs/PyETVHelp/pyInstructions.html.
3) Finally, we used the BLOSUM 62 matrix as a tool to focus only on the amino acid substitutions between HtpB and GroEL. Negative BLOSUM 62 scores are given to the less likely substitutions.

A combination of low ET rank and a negative BLOSUM 62 score were therefore the criteria that we used to predict the "most functionally relevant" HtpB amino acids (i.e., amino acids in evolutionarily/functionally important positions, but harboring unlikely substitutions). The results, which will be reported in detail elsewhere, pointed at 41 amino acid positions, which we now will be systematically testing with our developed functional models.

6.5.1 Site-Directed Mutagenesis

Our first attempt to define a functionally important amino acid in HtpB was based on amino acid sequence comparisons between HtpB, symbionin (the Cpn60 of *Buchnera aphidicola*), and other Cpn60s. Considering these comparisons in addition to structural predictions modeled after the 3D crystal structure of GroEL, we hypothesized that HtpB's His-400 could be functionally equivalent to symbionin's His-133, providing kinase activity and a potential direct implication in pseudohyphal growth signaling in *S. cerevisiae* (see Section 6.3.1 above). His-400 is located near the ATP-binding pocket of HtpB, and therefore meets the structural requirement for His residues involved in kinase activity to be near an ATP-binding site (Alex and Simon 1994). However, site-directed mutagenesis performed to change His-400 to a glycine residue had no effect on HtpB's ability to induce pseudohyphal growth in yeast (unpublished results).

Our most recent site-directed mutagenesis experiment was based on the bioinformatics predictions derived from our ET analysis, and was met with success. Of the 41 amino acid positions identified as functionally important for the moonlighting activities of HtpB (see above), we chose 10 (based on their exposure on HtpB's surface) to evaluate their role in mediating the interaction of HtpB and hECM29 (used as our structure–function experimental model; see last two sentences of Section 6.3.2 above). Site-directed mutagenesis of the codons encoding the selected 10 amino acids were performed in HtpB and GroEL using the commercially available QuickChange® mutagenesis kit (Stratagene). All mutations were verified by DNA sequencing, and the mutated chaperonin genes were fused to the DNA-binding domain of *GAL4* to make the bait constructs for yeast two-hybrid experiments with hECM29 fused to the *GAL4*-activating domain.

In a first screening, we found that alanine substitutions in five of the ten selected HtpB residues (M68A, M212A, S236A, K298A, and N507A) affected the interaction of HtpB with hECM29. When all these five residues were changed at once for the amino acids found in GroEL, the ability of HtpB to interact with hECM29 was also significantly reduced but not abolished. Finally, when all ten chosen amino acids (at the aforementioned positions 68, 212, 236, 298, and 507, plus a cluster of five amino acids in positions 471–475) were substituted at once in GroEL by the corresponding residues found in HtpB, GroEL became partially proficient at interacting with hECM29. It should be remembered here that hECM29 was chosen as our functional model because it does not interact with wild-type GroEL. These results are indeed very exciting, and strongly suggest that the ability of HtpB to interact with eukaryotic host cell proteins evolved through substitutions in few specific amino acid positions.

6.6 Functional Evolution of HtpB

The functional diversity of Cpn60s, as well as the diversity of host responses that they trigger (Ranford *et al*. 2000; Ranford and Henderson 2002) seems to be linked to the natural history of its bacterial host. On the one hand, the Cpn60s of *Actinobacillus* (now *Aggregatibacter*) *actinomycetemcomitans* and *E. coli* (which are mammalian commensals and opportunistic extracellular pathogens) are extremely active stimulators of bone resorption in a mouse model, whereas the Cpn60 of *Mycobacterium leprae* and one of the two Cpn60s of *Mycobacterium tuberculosis* (which are human intracellular pathogens) showed no activity (Kirby *et al*. 1995). On the other hand, functional novelty of Cpn60s seems to primarily originate in endosymbionts and intracellular pathogens, perhaps because proteomes of intracellular bacteria experience accelerated rates of amino acid substitution in relation to free-living bacteria (Buades *et al*. 1999). In the case of bacteria that have a single Cpn60 essential for protein folding, conserved regions involved in this function must be spared from genetic drift and maintained in the population by purifying selection (Fares *et al*. 2002). Concurrently, advantageous changes in amino acid residues (e.g., those conferring toxic properties to the Cpn60 of the endosymbiotic *E. aerogenes* strain; Yoshida *et al*. 2001) can be maintained under positive selection pressures, suggesting that they have become essential for the intracellular lifestyle of the organism. Investigating the molecular and functional evolution of HtpB may therefore prove a fruitful area of study, as there may exist regions of HtpB that have become essential for the intracellular lifestyle of *L. pneumophila*.

To begin exploring the functional evolution of HtpB we retrieved 231 Cpn60 protein sequences (belonging to 181 microorganisms) available from Pfam 28.0 at the Wellcome-Trust Sanger Institute (Finn *et al*. 2014), and aligned them using a frame with 534 amino acid positions. The PROTEST tool was then used to select the best-fit amino acid substitution model for the alignment. To evaluate phylogenetic relationships, the Maximum Likelihood (ML) analysis was performed using both the PhyML tool (Guindon *et al*. 2010) and the LG substitution model (Le and Gascuel 2008). The LG model was selected, as it had the smallest

Akaike Information Criterion. In general, the phylogeny of Cpn60s correlated well with that of the 16S rRNA, with exceptions for those bacteria with multiple Cpn60s where some were highly divergent. It seems reasonable to hypothesize that these highly divergent Cpn60s have been kept (in spite of the fact that they might have lost their protein-folding function) because newly acquired functions might support the host bacterium's lifestyle.

The phylogenetic relationships of a smaller group of Cpn60s reported to have moonlighting functions (48 protein sequences from 35 bacterial species) were also evaluated (Fig. 6.7). In this case, it was clearly shown that some Cpn60s have evolved rapidly in bacteria with multiple chaperonins. Notably, our results suggested that gene duplications occurred in the *Chlamydiae* clade before speciation, and then their Cpn60.2 and Cpn60.3 greatly diverged (purple triangle marked with the asterisk) from Cpn60.1, which remained more closely related to the other Cpn60s in the phylogenetic tree (small purple triangle marked with the black arrowhead). Although not experimentally proven, we speculate that the Cpn60.1 of chlamydiales would be essential because it has kept its protein-folding activity. Another interesting observation from the tree shown in Figure 6.7a is about the Firmicutes clade: the Cpn60s of *Mycoplasma* spp. (*pneumoniae*, *genitalium*, and *gallisepticum*) clustered within the same group (yellow triangle marked with the open triangle), but the Cpn60 of *Mycoplasma penetrans* grouped with *Helicobacter pylori*'s Cpn60 (black branch marked with the pound sign), strongly suggesting a case of horizontal gene transfer from *H. pylori* to *M. penetrans*. Cpn60 is absent or is not essential in many species of *Mycoplasma*, indicating that these bacteria may have another stress regulatory system and (or) protein-folding machinery. Interestingly, the Cpn60 of *M. penetrants* has been implicated in the distinctive ability of this bacterium to invade human cells (Clark and Tillier 2010). Finally, the grouping of HtpB within these 48 moonlighting chaperonins (Fig. 6.7b) was in agreement with the 16S rRNA phylogeny. This suggested that HtpB has co-evolved only with *L. pneumophila* (i.e., was not horizontally acquired), developing functions that support the lifestyle and unique biology of *Legionella pneumophila* while maintaining its protein-folding ability (see Section 6.2 above), likely after the complex evolutionary model recently proposed by Fares (2014).

6.7 Concluding Remarks

HtpB has clearly emerged as a moonlighting Cpn60 with unique functions that are compatible with the intracellular lifestyle of *L. pneumophila*. Our emphasis in this chapter was on our studies of the mechanisms behind these unique moonlighting functions, and we hope to have convinced readers of the usefulness of our experimental approaches. The use of surrogate hosts for the expression of recombinant HtpB have provided us with numerous possible directions for our investigative efforts. In addition, when we were not sure of how to dissect the marked functional differences between HtpB and GroEL, yeast two-hybrid screens and the ET bioinformatics tool came to our rescue. Now we have a plethora of potential amino acid positions to mechanistically test for functional relevance.

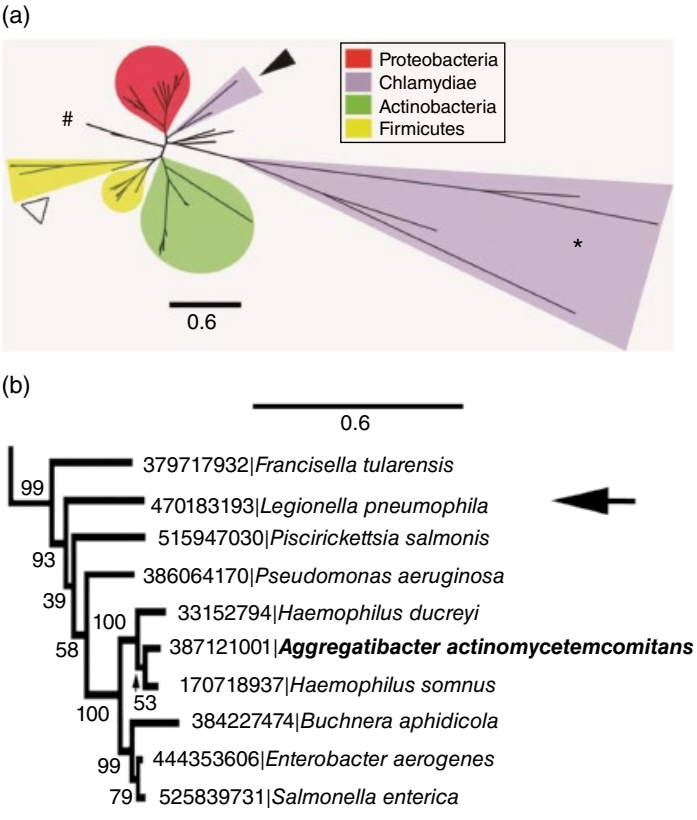

Figure 6.7 Phylogenetic relationships between 48 chaperonins (belonging to 35 bacterial species) known to have moonlighting functions and (or) extra-cytoplasmic locations. (a) Diagram of an unrooted maximum likelihood phylogenetic tree of the 48 chaperonins. Colors indicate phylum as per legend. Asterisk marks the highly divergent Cpn60.2 and Cpn60.3 of *Chlamydia trachomatis* and *Chlamydophila pneumoniae*, whereas the black arrowhead shows the positions of the Cpn60.1 of these two chlamydiales, which are more closely related to the Cpn60 of *Borrelia burgdorferi*, *Porphyromonas gingivalis*, and *Leptospira interrogans* (black branches between the two purple triangles). The open triangle marks the group formed by the three Cpn60s of *Mycoplasma gallisepticum*, *Mycoplasma genitalium*, and *Mycoplasma pneumoniae*, whereas the pound sign marks the position of the *Mycoplasma penetrans* Cpn60, which closely groups with the *Helicobacter pylori*'s Cpn60. Branch lengths are proportional to the number of amino acid substitutions per site (scale shown at the bottom of the diagram). (b) Diagram of part of the rooted tree of the same 48 chaperonins shown in (a). The part shown would be equivalent to the central part of the Proteobacteria branch (red bubble) depicted in (a), and includes HtpB (marked with the black arrow) and its closest moonlighting Cpn60s from the intracellular pathogens *Piscirickettsia salmonis* and *Francisella tularensis*. The tip labels include the amino acid sequence identification number (Gis for the Pfam database) followed by the bacterial taxonomic name. Branch lengths are proportional to the number of substitutions per site (scale given at the top of the panel). Bootstrap values (%) based on 100 replications are given for every branch. (*See color plate section for the color representation of this figure*.)

These methods are therefore highly recommended to explore and elucidate chaperonin moonlighting functional mechanisms.

With respect to the intracellular signaling mechanism of HtpB discovered in the model eukaryote *S. cerevisiae* we recognize some potential broad implications,

one being the ability of some Cpn60s to engage signaling molecules (e.g., Ras) from within, in the eukaryotic cytoplasm. Although our results presented here cannot unequivocally support a direct interaction between HtpB and Ras, the interaction of Ras and chaperonins (albeit not necessarily for signaling purposes) has been previously reported in mammalian cells (Ikawa and Weinberg 1992). Now HtpB could also be used by yeast geneticists and physiologists as an experimental tool to explore the signaling complexities of pseudohyphal growth.

One final word on secretion is that we undoubtedly believe in the existence of specific mechanisms for the translocation of Cpn60s to extra-cytoplasmic locations, but these would be absent from bacteria unable to cope with a strong immune response, such as commensal *E. coli*, which according to our observations (refer to Section 6.4.2 above) prefers to "hide" Cpn60s in the cytoplasm. In fact, forcing *E. coli* to express GroEL on its surface leads to enhanced recognition and clearance by macrophages (Zhu *et al*. 2013). One clue to elucidate the woefully understudied mechanisms of Cpn60 secretion would therefore be to focus on identifying secretion commonalities among bacteria that, through evolution, have come to thrive in locations where possessing surface exposed Cpn60s is more an advantage than a disadvantage. That is, we should turn our focus to intracellular pathogens and endosymbionts which seem to flagrantly display their Cpn60s and benefit from the advantages these might offer once they are secreted, as is the case with the fascinating intracellular pathogen *L. pneumophila* and HtpB.

References

Alex LA, Simon MI (1994) Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. *Trends in Genetics* 10: 133–138.

Allan, DS (2002) Secretion of Hsp60 chaperonin (GroEL) homologs by *Legionella pneumophila*. M.Sc. thesis, Dalhousie University. Nova Scotia, Canada.

Archambault J, Drebot MA, Stone JC, Friesen JD (1992) Isolation and phenotypic analysis of conditional-lethal, linker-insertion mutations in the gene encoding the largest subunit of RNA polymerase II in *Saccharomyces cerevisiae*. *Molecular and General Genetics* 232: 408–414.

Berleman JE, Allen S, Danielewicz MA, Remis JP, Gorur A, Cunha J, Hadi MZ, Zusman DR, Northen TR, Witkowska HE, Auer M (2014) The lethal cargo of *Myxococcus xanthus* outer membrane vesicles. *Frontiers in Microbiology* 5: 474, doi:10.3389/fmicb.2014.00474.

Blander SJ, Horwitz MA (1993) Major cytoplasmic membrane protein of *Legionella pneumophila*, a genus common antigen and member of the hsp60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease. *Journal of Clinical Investigation* 91: 717–723.

Bochkareva ES, Solovieva ME, Girshovich AS (1998) Targeting of GroEL to SecA on the cytoplasmic membrane of *Escherichia coli*. *Proceedings of the National Academy of Science USA* 95: 478–483.

Bonde MM, Yao R, Ma J-N, Madabushi S, Haunsø S, Burstein ES, Whistler JL, Sheikh SP, Lichtarge O, Hansen JL (2010) An angiotensin II type 1 receptor activation switch patch revealed through evolutionary trace analysis. *Biochemical Pharmacology* 80: 86–94.

Brachmann CB, Davies A, Cost GJ, Caputo E, Li J, Hieter P, Boeke JD (1998) Designer deletion strains derived from *Saccharomyces cerevisiae* S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* 14: 115–132.

Buades C, Michelena JM, Latorre A, Moya A (1999) Accelerated evolution in bacterial endosymbionts of aphids. *International Microbiology* 2: 11–14.

Chavel CA, Caccamise LM, Li B, Cullen PJ (2014) Global regulation of a differentiation MAPK pathway in yeast. *Genetics* 198: 1309–1328.

Chong A, Riveroll A, Allan DS, Garduño E, Garduño RA (2006) The Hsp60 chaperonin of *Legionella pneumophila*: an intriguing player in infection of host cells. In *Legionella: State of the Art 30 Years After its Recognition* (eds NP Cianciotto, Y Abu Kwaik, PH Edelstein, BS Fields, DF Geary, TG Harrison, CA Joseph, RM Ratcliff, JE Stout, MS Swanson), pp. 255–260. ASM Press, Washington.

Chong A, Lima CA, Allan DS, Nasrallah GK, Garduño RA (2009) The purified and recombinant *Legionella pneumophila* chaperonin alters mitochondrial trafficking and microfilament organization. *Infection and Immunity* 77: 4724–4739.

Clare DK, Saibil HR (2013) ATP-driven molecular chaperone machines. *Biopolymers* 99: 846–859.

Clark GW, Tillier ER (2010) Loss and gain of GroEL in the mollicutes. *Biochemistry and Cell Biology* 88: 185–194.

Cullen PJ, Sprague GFJr (2012) The regulation of filamentous growth in yeast. *Genetics* 190: 23–49.

Day JB, Ferracci F, Plano GV (2003) Translocation of YopE and YopN into eukaryotic cells by *Yersinia pestis yopN, tyeA, sycN, yscB* and *lcrG* deletion mutants measured using a phosphorylatable peptide tag and phosphospecific antibodies. *Molecular Microbiology* 47: 807–823.

Di Pisa M, Chassaing G, Swiecicki J-M (2015) Translocation mechanism(s) of cell penetrating peptides: Biophysical studies using artificial membrane bilayers. *Biochemistry* 54: 194–207.

England J, Lucent D, Pande V (2008) Rattling the cage: computational models of chaperonin-mediated protein folding. *Current Opinion in Structural Biology* 18: 163–169.

Fares MA (2014) The evolution of protein moonlighting: adaptive traps and promiscuity in the chaperonins. *Biochemical Society Transactions* 42: 1709–1714.

Fares MA, Barrio E, Sabater-Muñoz B, Moya A (2002) The evolution of the heat–shock protein GroEL from *Buchnera*, the primary endosymbiont of aphids, is governed by positive selection. *Molecular Biology and Evolution* 19: 1162–1170.

Ferrari G, Garaguso, Adu-Bobie J, Doro F, Taddei AR, Biolchi A, Brunelli B, Giuliani MM, Pizza M, Norais N, Grandi G (2006) Outer membrane vesicles from group B *Neisseria meningitidis* Δ*gna33* mutant: Proteomic and immunological comparison with detergent-derived outer membrane vesicles. *Proteomics* 6: 1856–1866.

Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, Heger A, Hetherington K, Holm L, Mistry J, Sonnhammer ELL, Tate J, Punta M (2014) Pfam: the protein families database. *Nucleid Acids Research* 42: D222–D230.

Galka F, Wai SN, Kusch H, Engelmann S, Hecker M, Schmeck B, Hippenstiel S, Uhlin BE, Steinert M (2008) Proteomic characterization of the whole secretome of *Legionella pneumophila* and functional analysis of outer membrane vesicles. *Infection and Immunity* 76: 1825–1836.

Gancedo JM (2001) Control of pseudohyphae formation in *Saccharomyces cerevisiae*. *FEMS Microbiology Reviews* 25: 107–123.

Garcia JT, Ferracci F, Jackson MW, Joseph SS, Pattis I, Plano LRW, Fischer W, Plano GV (2006) Measurement of effector protein injection by type III and type IV secretion systems using a 13-residue phosphorylatable glycogen synthase kinase tag. *Infection and Immunity* 74: 5645–5657.

Garduño RA, Chong A (2013) The *Legionella pneumophila* chaperonin 60 and the art of keeping several moonlighting jobs. In *Moonlighting Cell Stress Proteins in Microbial Infections Heat Shock Proteins* Vol. 7, Chapter 9 (ed. B. Henderson), pp. 143–160. Springer Science + Business Media, Dordrecht.

Garduño RA, Faulkner G, Trevors MA, Vats N, Hoffman PS (1998*a*) Immunolocalization of Hsp60 in *Legionella pneumophila*. *Journal of Bacteriology* 180: 505–513.

Garduño RA, Garduño E, Hoffman PS (1998*b*) Surface-associated Hsp60 chaperonin of *Legionella pneumophila* mediates invasion in a HeLa cell model. *Infection and Immunity* 66: 4602–4610.

Garduño RA, Chong A, Nasrallah GK, Allan DS (2011) The *Legionella pneumophila* chaperonin: an unusual multifunctional protein in unusual locations. *Frontiers in Microbiology* 2: 122, doi: 10.3389/fmicb.2011.00122.

Gong W, Xiong X, Qi Y, Jiao J, Duan C, Wen B (2014) Identification of novel surface-exposed proteins of *Rickettsia rickettsii* by affinity purification and proteomics. *PLoS ONE* 9(6): e100253, doi: 10.1371/journal.pone.0100253

Guevara-Olvera L, Xoconostle-Cazares B, Ruiz-Herrera J (1997) Cloning and disruption of the ornithine decarboxylase gene of *Ustilago maydis*: evidence for a role of polyamines in its dimorphic transition. *Microbiology* 143: 2237–2245.

Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O (2010) New algorithms and methods to estimate maximum-likelihood phylogenies: Assessing the performance of PhyML 3.0. *Systematic Biology* 59: 307–21.

Hayer-Hartl M (2000) Assay of malate dehydrogenase. A substrate for the *E. coli* chaperonins GroEL and GroES. In *Chaperonin Protocols* (ed. C. Schneider), pp. 127–132. Humana Press Inc., Methods in Molecular Biology 140.

Henderson B, Fares MA, Lund PA (2013) Chaperonin 60: a paradoxical, evolutionarily conserved protein family with multiple moonlighting functions. *Biological Reviews of the Cambridge Philosophical Society* 88: 955–987.

Herrero AB, Lopez MC, Garcia S, Schmidt A, Spaltmann F, Ruiz-Herrera J, Dominguez A (1999) Control of filament formation in *Candida albicans* by polyamine levels. *Infection and Immunity* 67: 4870–4878.

Ikawa S, Weinberg RA (1992) An interaction between $p21^{ras}$ and heat shock protein hsp60, a chaperonin. *Proceedings of the National Academy of Science, USA* 89: 2012–2016.

Johnson DI (1999) Cdc42: An essential Rho-type GTPase controlling eukaryotic cell polarity. *Microbiology and Molecular Biology Reviews* 63: 54–105.

Joshi MC, Sharma A, Kant S, Birah A, Gupta GP, Khan SR, Bhatnagar R, Banerjee N (2008) An insecticidal GroEL protein with chitin binding activity from *Xenorhabdus nematophila*. *Journal of Biological Chemistry* 283: 28287–28296.

Kataoka T, Powers S, McGill C, Fasano O, Strathern J, Broach J, Wigler M (1984) Genetic analysis of yeast *RAS1* and *RAS2* genes. *Cell* 37: 437–445.

Kirby AC, Meghji S, Nair SP, White P, Reddi K, Nishihara T, Nakashima K, Willis AC, Sim R, Wilson M, Henderson B (1995) The potent bone-resorbing mediator of *Actinobacillus actinomycetemcomitans* is homologous to the molecular chaperone GroEL. *Journal of Clinical Investigation* 96: 1185–1194.

Kumar G, Rathore G, El-Matbouli M (2014) Outer membrane protein assembly factor YaeT (omp85) and GroEL proteins of *Edwardsiella tarda* are immunogenic antigens for *Labeo rohita* (Hamilton). *Journal of Fish Diseases* 37: 1055–1059.

Kusukawa N, Yura T, Chiharu U, Akiyama Y, Ito K (1989) Effects of mutations in heat–shock genes *groES* and *groEL* on protein export in *Escherichia coli*. *EMBO Journal* 8: 3517–3521.

Le SQ, Gascuel O (2008) An improved general amino acid replacement matrix. *Molecular Biology and Evolution* 25: 1307–1320.

Leberer E, Dignard D, Harcus D, Thomas DY, Whiteway M (1992) The protein kinase homologue Ste20p is required to link the yeast pheromone response G-protein beta gamma subunits to downstream signalling components. *EMBO Journal* 11: 4815–4824.

Lee IK, Kim KS, Kim H, Lee JY, Ryu CH, Chun, HJ, Lee KU, Lim Y, Kim YH, Huh PW, Lee KH, Han SI, Jun TY, Rha HK (2004) MAP, a protein interacting with a tumor suppressor, merlin, through the run domain. *Biochemical and Biophysical Research Communications* 325: 774–783.

Lengeler KB, Davidson RC, D'Souza C, Harashima T, Shen WC, Wang P, Pan X, Waugh M, Heitman J (2000) Signal transduction cascades regulating fungal development and virulence. *Microbiology and Molecular Biology Reviews* 64: 746–785.

Li Z-T, Zhang R-L, Bi X-G, Xu L, Fan M, Xie D, Xian Y, Wang Y, Li X-J, Wu Z-D, Zhang K-X (2015) Outer membrane vesicles isolated from two clinical *Acinetobacter baumannii* strains exhibit different toxicity and proteome characteristics. *Microbial Pathogenesis* 81: 46–52.

Lichtarge O, Bourne HR, Cohen FE (1996) An evolutionary trace method defines binding surfaces common to protein families. *Journal of Molecular Biology* 257: 342–358.

Liu H, Styles CA, Fink GR (1996) *Saccharomyces cerevisiae* S288C has a mutation in *FLO8*, a gene required for filamentous growth. *Genetics* 144: 967–978.

Lund PA (2009) Multiple chaperonins in bacteria – why so many? *FEMS Microbiology Reviews* 33: 785–800.

Madabushi S, Gross AK, Philippi A, Meng EC, Wensel TG, Lichtarge O (2004) Evolutionary trace of G protein-coupled receptors reveals clusters of residues that determine global and class-specific functions. *Journal of Biological Chemistry* 279: 8126–8132.

Maddi A, Haase EM, Scannapieco FA (2014) Mass spectrometric analysis of whole secretome and amylase-precipitated secretome proteins from *Streptococcus gordonii*. *Journal of Proteomics and Bioinformatics* 7: 287–295.

McCaig WD, Koller A, Thanassi DG (2013) Production of outer membrane vesicles and outer membrane tubes by *Francisella novicida*. *Journal of Bacteriology* 195: 1120–1132.

Morioka M, Muraoka H, Yamamoto K, Ishikawa H (1994) An endosymbiont chaperonin is a novel type of histidine protein kinase. *Journal of Biochemistry (Tokyo)* 116: 1075–1081.

Nasrallah GK (2015) A yeast two-hybrid screen reveals a strong interaction between the *Legionella* chaperonin Hsp60 and the host cell small heat shock protein Hsp10. *Acta Microbiologica et Immunologica Hungarica* 62: 121–135.

Nasrallah GK, Gagnon E, Orton DJ, Garduño RA (2011*a*) The *htpAB* operon of *Legionella pneumophila* cannot be deleted in the presence of the *groE* chaperonin operon of *Escherichia coli*. *Canadian Journal of Microbiology* 57: 943–952.

Nasrallah GK, Riveroll AL, Chong A, Murray LE, Lewis PJ, Garduño RA (2011*b*) *Legionella pneumophila* requires polyamines for optimal intracellular growth. *Journal of Bacteriology* 193: 4346–4360.

Peter M, Neiman AM, Park HO, van Lohuizen M, Herskowitz I (1996) Functional analysis of the interaction between the small GTP binding protein Cdc42 and the Ste20 protein kinase in yeast. *EMBO Journal* 15: 7046–7059.

Portaro FC, Hayashi MA, De Arauz LJ, Palma MS, Assakura MT, Silva CL, de Camargo AC (2002) The *Mycobacterium leprae* hsp65 displays proteolytic activity. Mutagenesis studies indicate that the *M. leprae* hsp65 proteolytic activity is catalytically related to the HslVU protease. *Biochemistry* 41: 7400–7406.

Qi Y, Xiong X, Wang X, Duan C, Jia Y, Jiao J, Gong W, Wen B (2013) Proteome analysis and serological characterization of surface–exposed proteins of *Rickettsia heilongjiangensis*. *PLoS ONE* 8 (7): e7040, doi: 10.1371/journal.pone.0070440.

Quaite-Randall E, Joachimiak A (2000) Purification of GroES from an overproducing *E. coli* strain. In Chaperonin Protocols (ed. C. Schneider), pp. 41–49. Humana Press Inc., Methods in Molecular Biology 140.

Rajagopalan L, Patel N, Madabushi S, Goddard JA, Anjan V, Lin F, Shope C, Farrell B, Lichtarge O, Davidson AL, Brownell WE, Pereira FA (2006) Essential helix interactions in the anion transporter domain of prestin revealed by evolutionary trace analysis. *Journal of Neuroscience* 26: 12727–12734.

Ranford JC, Henderson B (2002) Chaperonins in disease: mechanisms, models, and treatments. *Journal of Clinical Pathology: Molecular Pathology* 55: 209–213.

Ranford JC, Anthony RM, Henderson B (2000) Chaperonins are cell-signalling proteins: the unfolding biology of molecular chaperones. *Expert Reviews in Molecular Medicine* 2: 1–17.

Riveroll AL (2005) The *Legionella pneumophila* chaperonin – An investigation of virulence-related roles in a yeast model. Ph.D. thesis, Dalhousie University, Nova Scotia, Canada.

Rupp S, Summers E, Lo HJ, Madhani H, Fink G (1999) MAP kinase and cAMP filamentation signalling pathways converge on the unusually large promoter of the yeast *FLO11* gene. *EMBO Journal* 18: 1257–1269.

Sikorski RS, Hieter P (1989) A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122: 19–27.

Stevenson BJ, Rhodes N, Errede B, Sprague GF Jr (1992) Constitutive mutants of the protein kinase *STE11* activate the yeast pheromone response pathway in the absence of the G protein. *Genes and Development* 6: 1293–1304.

Sun J, Savva CG, Deaton J, Kaback HR, Svrakic M, Young R, Holzenburg A (2005) Asymmetric binding of membrane proteins to GroEL. *Archives of Biochemistry and Biophysics* 434: 352–357.

Takeuchi K, Kawashima A, Nagafuchi A, Tsukita S (1994) Structural diversity of band 4.1 superfamily members. *Journal of Cell Science* 107: 1921–1928.

Török Z, Horváth I, Goloubinoff P, Kovács E, Glatz A, Balogh G, Vigh L (1997) Evidence for a lipochaperonin: association of active protein-folding GroESL oligomers with lipids can stabilize membranes under heat shock. *Proceedings of the National Academy of Science, USA* 94: 2192–2197.

Vanet A, Labigne A (1998) Evidence for specific secretion rather than autolysis in the release of some *Helicobacter pylori* proteins. *Infection and Immunity* 66: 1023–1027.

Vincent O, Townley R, Kuchin S, Carlson M (2001) Subcellular localization of the Snf1 kinase is regulated by specific beta subunits and a novel glucose signaling mechanism. *Genes and Development* 15: 1104–1114.

Wilkins A, Erdin S, Lua R, Lichtarge O (2012) Evolutionary trace for prediction and redesign of protein functional sites. *Methods in Molecular Biology* 819: 29–42.

Yang C-K, Ewis HE, Zhang XZ, Lu C-D, Hu H-J, Pan Y, Abdelal AT, Tai PC (2011) Nonclassical protein secretion by *Bacillus subtilis* in the stationary phase is not due to cell lysis. *Journal of Bacteriology* 193: 5607–5615.

Yoshida N, Oeda K, Watanabe E, Mikami T, Fukita Y, Nishimura K, Komai K, Matsuda K (2001) Protein function. Chaperonin turned insect toxin. *Nature* 411: 44.

Zhu H, Lee C, Zhang D, Wu W, Wang L, Fang X, Xu X, Song D, Xie J, Ren S, Gu J (2013) Surface-associated GroEL facilitates the adhesion of *Escherichia coli* to macrophages through lectin-like oxidized low-density lipoprotein receptor-1. *Microbes and Infection* 15: 172–180.

Ziman M, O'Brien JM, Ouellette LA, Church WR, Johnson DL (1991) Mutational analysis of *CDC42Sc*, a *Saccharomyces cerevisiae* gene that encodes a putative GTP-binding protein involved in the control of cell polarity. *Molecular and Cellular Biology* 11: 3537–3544.

3.2

Peptidylprolyl Isomerases, Bacterial Virulence, and Targets for Therapy

7

An Overview of Peptidylprolyl Isomerases (PPIs) in Bacterial Virulence

Brian Henderson

Department of Microbial Diseases, UCL-Eastman Dental Institute, University College London, UK

7.1 Introduction

This chapter introduces the reader to individual examples of related families of proteins known as the peptidylprolyl isomerases (PPIs). There is rapidly emerging evidence that human PPIs, such as the cyclophilins, are important secreted biomarkers of inflammation and of various human disease states (Satoh *et al*. 2010; Bukrinsky *et al*. 2013; Nigro *et al*. 2013). For this reason, the cyclophilins and their receptor CD147 are now seen as important therapeutic targets (Bukrinsky 2015). One important question is whether the secreted PPIs emanating from pathogenic bacteria also bind to CD147, or compete with host PPIs for binding to this receptor. *Neissera meningitidis* does bind to this receptor, but through its PilE and PilV components (Bernard *et al*. 2014). Evidence has emerged over the past decades to support the hypothesis that bacteria utilize PPIs in their interactions with the host and that this interaction is actually a two-way process. This chapter will present an abbreviated review of bacterial PPIs (mainly from *Legionella pneumophila*) as virulence factors, as this topic has been the subject of a number of recent reviews (Rasch *et al*. 2014; Ünal and Steinert 2014, 2015).

7.2 Proline and PPIs

With one exception, the common amino acids that populate proteins form linear structures (through the generation of peptide bonds) when binding with other residues. The exception is proline in which the amine nitrogen is bound not to a single alkyl group, but to two alkyl groups (in a ring structure), generating a secondary amine. The cyclic nature of proline's side-chain confers conformational rigidity, thus affecting the secondary structure of proteins near this residue. Proline residues in proteins have roughly equal numbers of *cis/trans* conformations. However, proline is exclusively generated in the ribosome as the

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

trans isomer. Isomerization is a slow process and can effect correct protein folding; the process of evolution has resulted in the generation of enzymes, known as peptidyl prolyl isomerases (PPIs), to accelerate this process. A number of families of PPIs have been identified including the cyclophilins, FK506-binding proteins (FKBs), and the parvulins (Mueller and Bayer 2008; Schiene-Fischer *et al*. 2013). The cyclophilins and the FKBs, although having unrelated primary sequences, bind T cell immunosuppressive compounds such as cyclosporine, FK506, and rapamycin (Schiene-Fischer *et al*. 2013).

7.3 Host PPIs and Responses to Bacteria and Bacterial Toxins

While the focus of this chapter is the moonlighting role of PPIs in bacterial virulence, it is important to recognize that bacterial infection is part of a two-way network of interactions between the infectious bacterium and its host. Indeed, the first indication that PPIs could moonlight was the report that human monocytes exposed to lipopolysaccharide (LPS) secreted cyclophilin A, and that this secreted PPI was a potent proinflammatory ligand (Sherry *et al*. 1992). In 2016, it is now well recognized that human cyclophilin A is a therapeutic target in many human disease states (Bukrinsky 2014). There is now an emerging story of the role of host cyclophilins and FKBs in the interaction with, and action of, a number of bacterial toxins. This has been recently reviewed by Barth (2013) and so will only be briefly described. ADP-ribosylating bacterial toxins are the major client proteins for host PPIs, and this was first encountered with *Pseudomonas aeruginosa* exoenzyme S which has an anti-phagocyte mode of action (DiNovo *et al*. 2006). Since then, and largely through the work of Holger Barth and colleagues, it has been shown that host cyclophilins and FKBs are involved in the uptake/translocation of the C2 toxin of *Clostridium botulinum* (Kaiser *et al*. 2009), the actin ADP-ribosylating toxins from *Clostridium difficile* and *Clostridium perfringens* (Kaiser *et al*. 2011), the *Bacillus anthracis* protective toxin (Dmochewitz *et al*. 2011), and the *Photorhabdus luminescens* ADP-ribosyltransferases (Lang *et al*. 2014). This role of the immunophilins in bacterial toxin translocation suggests that inhibitors of these PPIs could have anti-toxin/antibacterial effects (Barth 2013; Ernst *et al*. 2014). This has been shown with the C2 toxin of *C. botulinum*, which interacts with FK506-binding protein 51. Toxin translocation is blocked by the action of the immunosuppressant and FKB-binding peptide, FK506 (Kaiser *et al*. 2012). It is therefore interesting that the immunosuppressive compounds such as cyclosporine, FK506/tacrolimus, and rapamycin may have future potential as antibacterial agents.

7.4 Bacterial PPIs as Virulence Factors

The three families of PPIs were discovered between the late 1980s and the early 1990s. The bacterial homolog of eukaryotic cyclophilin, termed rotamase, was identified in 1990 (Liu and Walsh 1990). Our understanding of the moonlighting

roles of bacterial PPIs in virulence behavior started with the discovery of the first virulence factor of the recently emerged human lung pathogen, *Legionella pneumophila*. This was a 24 kDa protein chosen from a panel of recombinant bacterial surface proteins. The gene encoding this protein was inactivated and the resultant isogenic mutant had a significant reduction in its ability to infect macrophages (Cianciotto *et al*. 1989). The gene encoding this protein was termed *mip* for macrophage infectivity potentiator. It was further shown that this *mip* isogenic mutant was less virulent in animals, confirming this protein as a clinically relevant virulence factor (Cianciotto *et al*. 1990). A similar protein was then found in the intracellular pathogen, *Chlamydia trachomatis* (Lundemose *et al*. 1991). The genes in two species of Legionella were then compared and it was suggested that they had homology with eukaryotic FKBs (Bangsborg *et al*. 1991). This was confirmed when it was shown that recombinant *L. pneumophila* Mip had PPIase activity, being inhibited by FK506, but not by cyclosporine (Fischer *et al*. 1992). Further evidence for the widespread use of FKBs in bacterial infection was the finding that the Mip protein of *C. trachomatis* was also a PPI and that its activity was blocked by FK506 and rapamycin, and that these drugs inhibited cell infection by this bacterium (Lundemose *et al*. 1993). It is now known that a range of human bacterial pathogens have cell-surface Mips (PPIs) which are likely to be involved in virulence (Table 7.1).

Legionella pneumophila is a recently described human pathogen which normally resides within freshwater protozoa; it has only recently interacted with humanity because of our need for water for purposes such as air conditioning. This bacterium which can interact with us, lives in such waters. This raised the question: was this Mip protein responsible for the ability of this bacterium to invade protozoa? Using the *mip* isogenic mutant it was found that it was 1000-fold less invasive than the wild-type organism. Another apparently distantly related Legionella strain, *L. micdadei*, which causes Legionnaire's disease in the immunocompromised, also utilizes a Mip-like PPI to infect macrophages and

Table 7.1 Bacteria with cell-surface Mip-like PPIs.

Bacterium	Is Mip involved in virulence?	Reference
Burkholderia pseudomallei	Yes	Norville *et al*. 2011*b*
Chlamydia psittaci	Unknown	Rockey *et al*. 1996
Chlamydia trachomatis	Probably	Lundemose *et al*. 1991
Chlamydophila pneumoniae	Probably	Herrmann *et al*. 2006
Coxiella burnetti	Unknown	Mo *et al*. 1995
Helicobacter pylori	Yes	Basak *et al*. 2005
Legionella pneumophila	Yes	e.g., Cianciotto and Fields 1992
Mycoplasma pneumoniae	Unknown	Reddy *et al*. 1996
Neisseria gonorrhoea	Probably	Leuzzi *et al*. 2005
Salmonella typhimurium	In *in vitro* studies	Horne *et al*. 1997

protozoa (O'Connell *et al*. 1995). The role of this PPI protein was therefore not some artifact of interactions with human macrophages, but was a genuine invasion strategy used in the normal interactions of the bacterium in its usual environment (Cianciotto and Fields 1992).

Macrophages are only one cellular component in the human lung, and it has been shown that *L. pneumophila* is also found in lung epithelial cells (Blackmon *et al*. 1980). It was found that Mip was also involved in the invasion of these epithelial cells, so this PPI seems to have a wider mechanism of action that just aiding the invasion of myeloid cells (Cianciotto *et al*. 1995).

7.4.1 Proposed Mechanism of Virulence of *Legionella pneumophila* Mip

As briefly described, there is good evidence that the *L. pneumophila* Mip protein is directly involved in the virulence of this organism. A surprising finding was that Mip bound to a number of collagens, including types I through VI, and that interaction of Mip with collagen and with a serine protease was responsible for the invasiveness of this bacterium across tissue barriers (Wagner *et al*. 2007). Further analysis revealed that the Mip protein bound specifically to basement membrane type IV collagen, and a type IV collagen peptide binding site for Mip was identified. This peptide was found to bind to Mip and to also compete with the Mip for binding to type IV collagen (Ünal *et al*. 2011). This has led to the search for pharmacophores to block the binding of Mip to type IV collagen. The first such compounds described were pipecolic acids which had some inhibitory function *in vitro* (Juli *et al*. 2011). More recently, cyclohexamide derivatives with adamantyl derivatives identified as novel FKBP ligands have been shown to block Mip PPIase activity and to inhibit the growth of *L. pneumophila* and, significantly, to inhibit macrophage infection with this organism (Rasch *et al*. 2015). These are promising beginnings towards the development of PPIase inhibitors to block bacterial pathogenicity.

7.5 Other Bacterial PPIs Involved in Virulence

Less persuasive evidence exists for the role of PPIs in the virulence of a small number of human pathogenic bacteria, and these organisms are listed in Table 7.1 with some explanation of the nature of the evidence. The genera Chlamydia and Chlamydyophila – obligately intracellular bacteria – have therefore been shown, by use of the inhibitor FK506, to involve this enzyme in their virulence behavior (Lundemose *et al*. 1991; Herrmann *et al*. 2006).

The stomach-colonizing organism, *Helicobacter pylori*, is both the cause of gastric ulcers and the one bacterium that has been associated with the causation of a human cancer (gastric adenocarcinoma). This curious bacterium has been found to utilize a cell-surface/-secreted PPI (termed HP0175) in its virulence behavior. This secreted protein (Kim *et al*. 2002) was first identified as a potential vaccine candidate (McAtee *et al*. 1998). In 2005 this protein was reported to promote the apoptosis of gastric epithelial cells by a mechanism controlled by the signaling through the TLR4 protein, and also involving

apoptosis signal-regulating kinase 1-activated MAPK p38-induced intracellular activation of mitochondrial cytochrome c release and of caspases 9 and 3 (Basak *et al*. 2005). A decade later HP0175 was found to promote the process of autophagy, a complex intracellular degradation process which ultimately delivers cellular constituents to the lysosome and precedes apoptosis. HP0175 induces autophagy by promoting the key intracellular unfolded protein response (UPR). This is a novel moonlighting action of this PPI (Halder *et al*. 2015). It should be noted that homeostatic autophagy is a prominent barrier to the malignant transformation of cells and the modulation of autophagy is associated with cellular transformation (Galluzzi *et al*. 2015). The *Helicobacter pylori*-induced gastroduodenal diseases ulcer formation and cancer are linked to processes including activation of the epidermal factor receptor (EGFR) and synthesis of vascular endothelial growth factor (VEGF) (e.g., Chaturvedi *et al*. 2014). Established *H. pylori* virulence factors such as VacA are known to regulate these processes (Caputo *et al*. 2003). It has also been established that HP0175 has similar actions through activation of a TLR4 activation pathway (Basu *et al*. 2008).

Synthesis of IL-6 synthesis by local macrophages is a prominent event at the margins of gastric ulceration. HP0175 is potentially responsible, in part, for this synthesis. The purified protein can induce macrophage IL-6 synthesis, and an isogenic mutant lacking the *hp0175* gene is attenuated in its ability to induce synthesis of this cytokine. Activation of this cytokine gene is dependent on HP0175 interacting with the extracellular domain of TLR4 (Pathak *et al*. 2006). Later studies found that HP0175 could induce the tumor-infiltrating lymphocytes (TILs) obtained from patients with gastric adenocarcinoma to produce the neutrophil chemoattractant cytokine IL-17, which may account for the dominance of T cells and neutrophils in the infiltrates of this form of cancer (Amedei *et al*. 2014).

The sexually transmitted bacterium, *Neisseria gonorrhoea*, has also been reported to have a surface-exposed PPI, inhibitible by FK506 and rapamycin, and using an isogenic strain missing the gene encoding this protein has been found to promote the persistence of infection (Leuzi *et al*. 2005). A recent report has suggested the potential of this neisserial protein as a vaccine candidate (Humbert *et al*. 2015).

It has recently been reported that the intracellular bacterium, *Burkholderia pseudomallei*, the causative agent of the potentially fatal tropical disease melioidosis, has an enzymically inactive FKB-type PPI (gene encoded by BPSL0918) involved in virulence. Inactivation of the gene encoding this protein results in impaired ability to cope with life within the macrophage *in vitro* and the isogenic mutant is significantly attenuated in a murine infection model (Norville *et al*. 2011*a*). Surprisingly this same group has reported that another PPI, BPSS1823, which is a Mip-like protein inhibitible by rapamycin, is also important for the virulence of *B. pseudomallei*. Again, gene deletion results in a mutant with a reduced ability to survive within the macrophage and with reduced virulence *in vivo* (Norville *et al*. 2011*b*). Inhibitors of this PPI have been developed and shown to inhibit the virulence of this organism in *in vitro* experiments (Begley *et al*. 2014).

7.6 Conclusions

Yet again, this chapter introduces the finding that cosmic time and the evolutionary mechanism can work on particular proteins to provide additional functional activity that contributes to the virulence phenotype of the bacterium. A growing number of bacterial pathogens have evolved the ability to secrete and surface-associate particular PPIs, and use these cell-surface proteins for the interaction of the bacterium with the human host. It is not yet clear if the number of examples of moonlighting PPIs all function through the same mechanism. Judging by the bacteria involved and the details of the moonlighting actions of these PPIs, it is likely that there is a diversity of mechanistic function which suggests that there might be a much wider spectrum of biological actions to be ascribed to these PPIs. What is particularly inspiring is that two groups have already focused on these moonlighting PPIs as therapeutic targets and have actually developed compounds with *in vitro* antibacterial activity (e.g., Rasch *et al*. 2015). In this context it is interesting to read the recent report that Theileria parasites, the only human parasite known to transform leukocytes, does so by using a secreted PPI (Marsolier *et al*. 2015). This suggests there may be a rich seam of PPI moonlighting actions that are important in host–pathogen interactions.

References

Amedei A, Munari F, Bella CD, Niccolai E, Benagiano M, Bencini L, Cianchi F, Farsi M, Emmi G, Zanotti G, de Bernard M, Kundu M, D'Elios MM (2014) *Helicobacter pylori* secreted peptidyl prolyl cis, trans-isomerase drives Th17 inflammation in gastric adenocarcinoma. *Internal and Emergency Medicine* 9: 303–309.

Bangsborg JM, Cianciotto NP, Hindersson P (1991) Nucleotide sequence analysis of the *Legionella micdadei mip* gene, encoding a 30-kilodalton analog of the *Legionella pneumophila* Mip protein. *Infection and Immunity* 59: 3836–3840.

Barth H (2013) Role of peptidyl-prolyl *cis/trans* isomerases in cellular uptake of bacterial protein toxins. In *Moonlighting Cell Stress Protens in Microbial Infections* (ed. B. Henderson), pp. 251–265. Springer Science: Dordrecht, Heidelberg, New York, London.

Basak C, Pathak SK, Bhattacharyya A, Pathak S, Basu J, Kundu M (2005) The secreted peptidyl prolyl cis,trans-isomerase HP0175 of *Helicobacter pylori* induces apoptosis of gastric epithelial cells in a TLR4- and apoptosis signal-regulating kinase 1-dependent manner. *Journal of Immunology* 174: 5672–5680.

Basu S, Pathak SK, Chatterjee G, Pathak S, Basu J, Kundu M (2008) *Helicobacter pylori* protein HP0175 transactivates epidermal growth factor receptor through TLR4 in gastric epithelial cells. *Journal of Biological Chemistry* 283: 32369–3276.

Begley DW, Fox D 3rd, Jenner D, Juli C, Pierce PG, Abendroth J, Muruthi M, Safford K, Anderson V, Atkins K, Barnes SR, Moen SO, Raymond AC, Stacy R, Myler PJ, Staker BL, Harmer NJ, Norville IH, Holzgrabe U, Sarkar-Tyson M, Edwards TE, Lorimer DD (2014) A structural biology approach enables the development of

antimicrobials targeting bacterial immunophilins. *Antimicrobial Agents and Chemotherapy* 58: 1458–1467.

Bernard SC, Simpson N, Join-Lambert O, Federici C, Laran-Chich MP, Maïssa N, Bouzinba-Ségard H, Morand PC, Chretien F, Taouji S, Chevet E, Janel S, Lafont F, Coureuil M, Segura A, Niedergang F, Marullo S, Couraud PO, Nassif X, Bourdoulous S (2014) Pathogenic *Neisseria meningitidis* utilizes CD147 for vascular colonization. *Nature Medicine* 20: 725–731.

Blackmon JA, Hicklin MD, Chandler FW (1980) Legionnaires' disease. Pathological and historical aspects of a 'new' disease. *Archives of Pathology and Laboratory Medicine* 102: 337–343.

Bukrinsky M (2015) Extracellular cyclophilins in health and disease. *Biochimica et Biophysica Acta* 1850(10): 2087–2095.

Bukrinsky M, Orekhov A, Ditiatkovski M, Sviridov D (2013) Cyclophilins in atherosclerosis: a new therapeutic target? *Current Pharmaceutical Design* 19: 5904–5908.

Caputo R, Tuccillo C, Manzo BA, Zarrilli R, Tortora G, Blanco Cdel V, Ricci V, Ciardiello F, 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. *Clinical Cancer Research* 9: 2015–2021.

Chaturvedi R, Asim M, Piazuelo MB, Yan F, Barry DP, Sierra JC, Delgado AG, Hill S, Casero RA Jr, Bravo LE, Dominguez RL, Correa P, Polk DB, Washington MK, Rose KL, Schey KL, Morgan DR, Peek RM Jr, Wilson KT (2014) Activation of EGFR and ERBB2 by *Helicobacter pylori* results in survival of gastric epithelial cells with DNA damage. *Gastroenterology* 146: 1739–1751.

Cianciotto NP, Fields BS (1992) *Legionella pneumophila* mip gene potentiates intracellular infection of protozoa and human macrophages. *Proceedings of the National Academy of Science, USA* 89: 5188–5191.

Cianciotto NP, Eisenstein BI, Mody CH, Toews GB, Engleberg NC (1989) A *Legionella pneumophila* gene encoding a species-specific surface protein potentiates initiation of intracellular infection. *Infection and Immunity* 57: 1255–1262.

Cianciotto NP, Eisenstein BI, Mody CH, Engleberg NC (1990) A mutation in the *mip* gene results in an attenuation of *Legionella pneumophila* virulence. *Journal of Infectious Diseases* 162: 121–126.

Cianciotto NP, Stamos JK, Kamp DW (1995) Infectivity of *Legionella pneumophila mip* mutant for alveolar epithelial cells. *Current Microbiology* 30: 247–250.

DiNovo AA, Schey KL, Vachon WS, McGuffie EM, Olson JC, Vincent TS (2006) ADP-ribosylation of cyclophilin A by *Pseudomonas aeruginosa* exoenzyme S. *Biochemistry* 45: 4664–4673.

Dmochewitz L, Lillich M, Kaiser E, Jennings LD, Lang AE, Buchner J, Fischer G, Aktories K, Collier RJ, Barth H (2011) Role of CypA and Hsp90 in membrane translocation mediated by anthrax protective antigen. *Cell Microbiology* 13: 359–373.

Ernst K, Langer S, Kaiser E, Osseforth C, Michaelis J, Popoff MR, Schwan C, Aktories K, Kahlert V, Malesevic M, Schiene-Fischer C, Barth H (2014) Cyclophilin-facilitated membrane translocation as pharmacological target to

prevent intoxication of mammalian cells by binary clostridial actin ADP-ribosylated toxins. *Journal of Molecular Biology* pii: S0022-2836(14)00360-X.

Fischer G, Bang H, Ludwig B, Mann K, Hacker J (1992) Mip protein of *Legionella pneumophila* exhibits peptidyl-prolyl-cis/trans isomerase (PPIase) activity. *Molecular Microbiology* 6: 1375–1383.

Galluzzi L, Pietrocola F, Bravo-San Pedro JM, Amaravadi RK, Baehrecke EH, Cecconi F, Codogno P, Debnath J, Gewirtz DA, Karantza V, Kimmelman A, Kumar S, Levine B, Maiuri MC, Martin SJ, Penninger J, Piacentini M, Rubinsztein DC, Simon HU, Simonsen A, Thorburn AM, Velasco G, Ryan KM, Kroemer G (2015) Autophagy in malignant transformation and cancer progression. *EMBO Journal* 34: 856–880.

Halder P, Datta C, Kumar R, Sharma AK, Basu J, Kundu M (2015) The secreted antigen, HP0175, of *Helicobacter pylori* links the unfolded protein response (UPR) to autophagy in gastric epithelial cells. *Cell Microbiology* 17: 714–729.

Herrmann M, Schuhmacher A, Mühldorfer I, Melchers K, Prothmann C, Dammeier S (2006) Identification and characterization of secreted effector proteins of *Chlamydophila pneumoniae* TW183. *Research in Microbiology* 157: 513–524.

Horne SM, Kottom TJ, Nolan LK, Young KD (1997) Decreased intracellular survival of an fkpA mutant of *Salmonella typhimurium Copenhagen*. *Infection and Immunity* 65: 806–810.

Humbert MV, Almonacid Mendoza HL, Jackson AC, Hung MC, Bielecka MK, Heckels JE, Christodoulides M (2015) Vaccine potential of bacterial macrophage infectivity potentiator (MIP)-like peptidyl prolyl cis/trans isomerase (PPIase) proteins. *Expert Review of Vaccines* 14(12): 1633–1649.

Juli C, Sippel M, Jäger J, Thiele A, Weiwad M, Schweimer K, Rösch P, Steinert M, Sotriffer CA, Holzgrabe U (2011) Pipecolic acid derivatives as small-molecule inhibitors of the Legionella MIP protein. *Journal of Medicinal Chemistry* 54: 277–283.

Kaiser E, Pust S, Kroll C, Barth H (2009) Cyclophilin A facilitates translocation of the *Clostridium botulinum* C2 toxin across membranes of acidified endosomes into the cytosol of mammalian cells. *Cell Microbiology* 11: 780–795.

Kaiser E, Kroll C, Ernst K, Schwan C, Popoff M, Fischer G, Buchner J, Aktories K, Barth H (2011) Membrane translocation of binary actin-ADP-ribosylating toxins from *Clostridium difficile* and *Clostridium perfringens* is facilitated by cyclophilin A and Hsp90. *Infection and Immunity* 79: 3913–3921.

Kaiser E, Böhm N, Ernst K, Langer S, Schwan C, Aktories K, Popoff M, Fischer G, Barth H (2012) FK506-binding protein 51 interacts with *Clostridium botulinum* C2 toxin and FK506 inhibits membrane translocation of the toxin in mammalian cells. *Cell Microbiology* 14: 1193–1205.

Kim N, Weeks DL, Shin JM, Scott DR, Young MK, Sachs G (2002) Proteins released by *Helicobacter pylori in vitro*. *Journal of Bacteriology* 184: 6155–6162.

Lang AE, Ernst K, Lee H, Papatheodorou P, Schwan C, Barth H, Aktories K (2014) The chaperone Hsp90 and PPIases of the cyclophilin and FKBP families facilitate membrane translocation of *Photorhabdus luminescens* ADP-ribosyltransferases. *Cell Microbiology* 16: 490–503.

Leuzzi R, Serino L, Scarselli M, Savino S, Fontana MR, Monaci E, Taddei A, Fischer G, Rappuoli R, Pizza M (2005) Ng-MIP, a surface-exposed lipoprotein of

Neisseria gonorrhoeae, has a peptidyl-prolyl cis/trans isomerase (PPIase) activity and is involved in persistence in macrophages. *Molecular Microbiology* 58: 669–681.

Liu J, Walsh CT (1990) Peptidyl-prolyl cis-trans-isomerase from *Escherichia col*i: a periplasmic homolog of cyclophilin that is not inhibited by cyclosporin A. *Proceedings of the National Academy of Science, USA* 87: 4028–4032.

Lundemose AG, Birkelund S, Fey SJ, Larsen PM, Christiansen G (1991) *Chlamydia trachomatis* contains a protein similar to the *Legionella pneumophila* mip gene product. *Molecular Microbiology* 5: 109–115.

Lundemose AG, Kay JE, Pearce JH (1993) *Chlamydia trachomatis* Mip-like protein has peptidyl-prolyl cis/trans isomerase activity that is inhibited by FK506 and rapamycin and is implicated in initiation of chlamydial infection. *Molecular Microbiology* 7: 777–783.

Marsolier J, Perichon M, DeBarry JD, Villoutreix BO, Chluba J, Lopez T, Garrido C, Zhou XZ, Lu KP, Fritsch L, Ait-Si-Ali S, Mhadhbi M, Medjkane S, Weitzman JB (2015) Theileria parasites secrete a prolyl isomerase to maintain host leukocyte transformation. *Nature* 520: 378–382.

McAtee CP, Lim MY, Fung K, Velligan M, Fry K, Chow TP, Berg DE (1998) Characterization of a Helicobacter pylori vaccine candidate by proteome techniques. *Journal of Chromatography B: Biomedical Sciences and Applications* 714: 325–333.

Mo YY, Cianciotto NP, Mallavia LP (1995) Molecular cloning of a *Coxiella burnetii* gene encoding a macrophage infectivity potentiator (Mip) analogue. *Microbiology* 141: 2861–2871.

Mueller JW, Bayer P (2008) Small Family with Key Contacts: Par14 and Par17 Parvulin Proteins, Relatives of Pin1, Now Emerge in Biomedical Research. *Perspectives in Medicinal Chemistry* 2: 11–20.

Nigro P, Pompilio G, Capogrossi MC (2013) Cyclophilin A: a key player for human disease. *Cell Death and Disease* 4: e888.

Norville IH, Breitbach K, Eske-Pogodda K, Harmer NJ, Sarkar-Tyson M, Titball RW, Steinmetz I (2011*a*) A novel FK-506-binding-like protein that lacks peptidyl-prolyl isomerase activity is involved in intracellular infection and *in vivo* virulence of *Burkholderia pseudomallei*. *Microbiology* 157: 2629–2638.

Norville IH, Harmer NJ, Harding SV, Fischer G, Keith KE, Brown KA, Sarkar-Tyson M, Titball RW (2011*b*) A *Burkholderia pseudomallei* macrophage infectivity potentiator-like protein has rapamycin-inhibitable peptidylprolyl isomerase activity and pleiotropic effects on virulence. *Infection and Immunity* 79: 4299–4307.

O'Connell WA, Bangsborg JM, Ciancotto NP (1995) Characterization of a *Legionella micdadei mip* mutant. *Infection and Immunity* 63: 2840–2845.

Pathak SK, Basu S, Bhattacharyya A, Pathak S, Banerjee A, Basu J, Kundu M (2006) TLR4-dependent NF-kappaB activation and mitogen- and stress-activated protein kinase 1-triggered phosphorylation events are central to *Helicobacter pylori* peptidyl prolyl cis-,trans-isomerase (HP0175)-mediated induction of IL–6 release from macrophages. *Journal of Immunology* 177: 7950–7958.

Rasch J, Ünal CM, Steinert M (2014) Peptidylprolyl cis-trans isomerases of *Legionella pneumophila*: virulence, moonlighting and novel therapeutic targets. *Biochemical Society Transactions* 42: 1728–1733.

Rasch J, Theuerkorn M, Ünal C, Heinsohn N, Tran S, Fischer G, Weiwad M, Steinert M (2015) Novel Cycloheximide Derivatives Targeting the Moonlighting Protein Mip Exhibit Specific Antimicrobial Activity Against *Legionella pneumophila*. *Frontiers in Bioengineering and Biotechnology* 3: 41.

Reddy SP, Rasmussen WG, Baseman JB (1996) Correlations between *Mycoplasma pneumoniae* sensitivity to cyclosporin A and cyclophilin-mediated regulation of mycoplasma cytadherence. *Microbial Pathogenesis* 20: 155–169.

Rockey DD, Chesebro BB, Heinzen RA, Hackstadt T (1996) A 28 kDa major immunogen of Chlamydia psittaci shares identity with Mip proteins of *Legionella spp*. and *Chlamydia trachomatis*-cloning and characterization of the *C. psittaci mip*-like gene. *Microbiology* 142: 945–953.

Satoh K, Shimokawa H, Berk BC (2010) Cyclophilin A: promising new target in cardiovascular therapy. *Circulation Journal* 74: 2249–2256.

Schiene-Fischer C, Aumüller T, Fischer G (2013) Peptide bond cis/trans isomerases: a biocatalysis perspective of conformational dynamics in proteins. *Topics in Current Chemistry* 328: 35–67.

Sherry B, Yarlett N, Strupp A, Cerami A (1992) Identification of cyclophilin as a proinflammatory secretory product of lipopolysaccharide-activated macrophages. *Proceedings of the National Academy of Science, USA* 89: 3511–3515.

Ünal CM, Steinert M (2014) Microbial peptidyl-prolyl cis/trans isomerases (PPIases): virulence factors and potential alternative drug targets. *Microbiology and Molecular Biology Reviews* 78: 544–571.

Ünal CM, Steinert M (2015) FKBPs in bacterial infections. *Biochimica et Biophysica Acta* 1850: 2096–2102.

Ünal C, Schwedhelm KF, Thiele A, Weiwad M, Schweimer K, Frese F, Fischer G, Hacker J, Faber C, Steinert M (2011) Collagen IV-derived peptide binds hydrophobic cavity of Legionella pneumophila Mip and interferes with bacterial epithelial transmigration. *Cell Microbiology* 13: 1558–1572.

Wagner C, Khan AS, Kamphausen T, Schmausser B, Unal C, Lorenz U, Fischer G, Hacker J, Steinert M (2007) Collagen binding protein Mip enables *Legionella pneumophila* to transmigrate through a barrier of NCI-H292 lung epithelial cells and extracellular matrix. *Cell Microbiology* 9: 450–462.

3.3

Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH): A Multifunctional Virulence Factor

8

GAPDH: A Multifunctional Moonlighting Protein in Eukaryotes and Prokaryotes

Michael A. Sirover

Department of Pharmacology, Temple University, School of Medicine, Philadelphia, USA

8.1 Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is perhaps the archetypical example of a moonlighting protein. Although there were early indications of its functional diversity, it is only over the last two decades that its role as a premier multidimensional protein has become evident (Sirover 1999, 2005, 2011, 2014; Tristan *et al.* 2012). In eukaryotes, its diverse activities range in normal cells from nuclear tRNA export and the maintenance of genomic integrity, to cytoplasmic post-transcriptional control of gene expression and receptor mediated cell signaling, to membrane facilitation of iron metabolism, trafficking, and fusion (Fig. 8.1). The role of GAPDH in eukaryotic pathology extends from its fundamental role in apoptosis to its association with age-related neurodegenerative proteins to its distinctive expression during tumorigenesis. In both normal cell function and in cell pathology, eukaryotic GAPDH function requires either a defined subcellular localization or a dynamic change in its intracellular distribution. Lastly, its paradoxical functions in cell susceptibility as well as in cellular responses to oxidative stress indicate its physiological importance. In contrast, in prokaryotes such investigations focus primarily on its role in bacterial virulence which are based on its cell adhesion properties, its facilitation of iron metabolism in bacterial pathogenesis, and its targeted destruction of host cells. The first requires a specific membrane localization, the second involves not only membrane function but also, perhaps, its new role as an "iron scavenger," and the third would, of necessity, involve the regulation of the complex pathway known as apoptosis. That being said, recent evidence identifies GAPDH not only as a bacterial virulence factor but also as a requirement for fungal, protozoal, or viral virulence. In each instance, selective GAPDH moonlighting functions are utilized as virulence mechanisms.

The functional diversity role of GAPDH begs a fundamental question. In the vernacular, what makes this moonlighting protein tick, what makes it what it is, and what permits it to perform such diverse functions in distinct parts of the

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

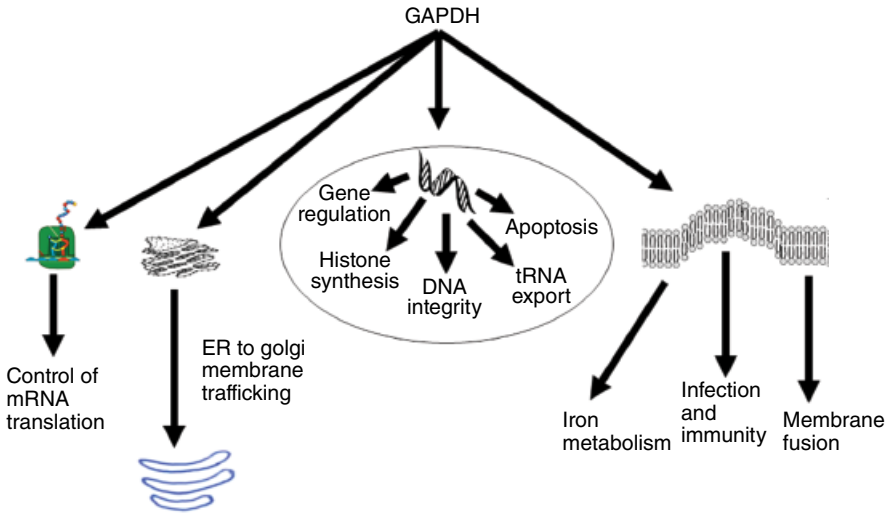

Figure 8.1 Functional diversity of GAPDH.

eukaryotic or prokaryotic cell? Or, using scientific terminology, what permits one protein containing a single active site to perform such diverse functions in disparate subcellular locales? In this chapter we will consider the interrelationship between GADPH as a moonlighting protein and its role in bacterial virulence. As such, it is intended as an introduction to subsequent chapters which discuss in detail salient aspects of GAPDH-mediated bacterial pathogenesis. As such, it is hoped that the reader will gain insight into the mechanisms through which pathogenic bacteria have utilized GAPDH moonlighting properties to facilitate their successful infection and propagation. We shall also highlight areas in which the role of GAPDH in bacterial virulence has not been established, indicating the potential for future productive investigations.

8.2 GAPDH Membrane Function and Bacterial Virulence

Reports of the membrane localization of GAPDH were among the earliest which indicated its functional diversity in eukaryotes (Tanner and Gray 1971; Kant and Steck 1973; McDaniel *et al*. 1974). Recent studies validated those early findings and have revealed not only the details of those structural interactions but also their importance with respect to both normal cell function and to pathogen virulence. In eukaryotes, membrane-bound GAPDH is required for nuclear membrane assembly for iron metabolism as well as for interorganelle trafficking (Fig. 8.2; Sirover 1999, 2011, 2014). Each of these activities may require separate GAPDH isoforms, each of which may have a different post-translational structure as determined by pI as well as by different rates of catalysis (Glaser and Gross 1995; Sheokand *et al*. 2014).

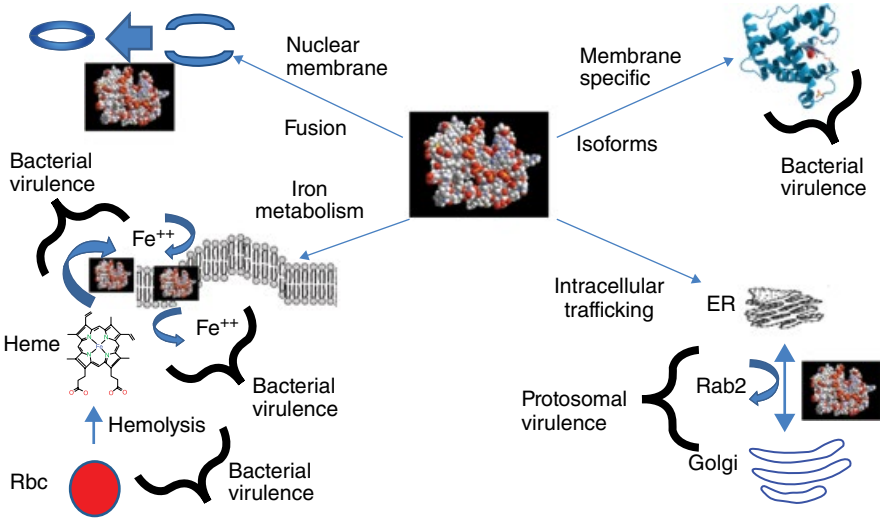

Figure 8.2 Membrane-associated GAPDH: structure and function. (*See color plate section for the color representation of this figure.*)

8.2.1 Bacterial GAPDH Virulence

Bacterial GAPDH was identified similarly as a membrane constituent (Pancholi and Fischetti 1992). Subsequent studies indicated that the functional diversity of membrane GAPDH has been well utilized by bacterial, protozoal fungal, and parasitic pathogens, causing a variety of pathologies, a non-exclusive list which includes periodontal disease, pneumonia, MRSA, sepsis, tuberculosis, and malaria. As introduced in Chapter 5, moonlighting proteins including GAPDH may function not only as adhesion factors to facilitate bacterial infection, but also as a means to evade immune responses as well as nutritional magnets, especially for Fe^{++} metabolism. Table 8.1 includes a partial list of bacterial GAPDH membrane functions. In addition, GAPDH membrane localization and activity is required for protozoal (*T. vaginalis*), fungal (*C. Albicans*), and parasitic pathogenicity (Gil-Navarro *et al.* 1997; Daubenberger *et al.* 2003; Fugier *et al.* 2009; Lama *et al.* 2009; de Bolle *et al.* 2012). With respect to the latter, instead of surface membrane localization GAPDH is required for intracellular membrane trafficking (Tisdale 2001).

Bacterial GAPDH virulence may involve a co-infection with two pathogens. As indicated in Table 8.1, *S. oralis* and *P. gingivalis* act synergistically in the development of periodontal disease. Apart from the subsequent pathology, this bacterial interaction may provide a means to understand the structure–function relationships which underlie the role of GAPDH in bacterial virulence. Analyses of both GAPDH structure and that of the *S. oralis* GAPDH binding site for *P. gingivalis* major fimbriae reveals a phosphatidyl binding site (amino acids 70–94 in the GAPDH NAD^+ domain; Kaneda *et al.* 1997) on the former and a binding site for the latter (amino acids 166–183 in the GAPDH glyceraldehyde-3-phosphate domain; Nagata *et al.* 2009).

Table 8.1 Membrane-bound bacterial GAPDH as a virulence factor.

Bacteria (alphabetical order)	Pathology	GAPDH Activity	GAPDH Function	Reference
E. coli	Food poisoning, acute hemorrhagic diarrhea	Plasminogen, fibrinogen binding	Cell adhesion	Egea *et al*. 2007; Aguilera *et al*. 2012
M. tuberculin	Tuberculosis	Transferrin binding	Iron metabolism	Boradia *et al*. 2014
P. oris	Peridontal disease	ND	ND	Sato *et al*. 2012
S. agalactiae	Sepsis, meningitis, pneumonia	Surface protein interactions, B cell activation	Immunomodulation, induction of apoptosis	Seifert *et al*. 2003; Madureira *et al*. 2007; Oliveira *et al*. 2012
S. aureus	MRSA	Enzyme catalysis	Glycolysis, gluconeogenesis	Purves *et al*. 2010
S. oralis	Periodontal disease	Binding to *P. gingivalis proteins*	*P. gingivalis* colonization	Maeda *et al*. 2004*a*, *b*, 2013
S. pneumoniae	Pneumonia	Hemoglobin, heme binding; binding to human C1q protein, plasminogen	Release of Fe^{++}, evasion of immune defense	Bergmann *et al*. 2004; Terrasse *et al*. 2012; Vázquez-Zamorano *et al*. 2014
S. pyogenes	Pharyngitis, sepsis, toxic shock syndrome	Binding to host proteins	Adhesion, antiphagocytic activity, evasion of immune defense	Boël *et al*. 2005; Jin *et al*. 2005, 2011; Terao *et al*. 2006

As phosphatidyl serine is membrane associated, it is tempting to speculate a two-step, sequential mechanism through which *S. oralis* GAPDH binds to *P. gingivalis* fimbriae as a mechanism for bacterial GAPDH virulence. This may be tested experimentally, perhaps by competition analysis using peptides which contain the respective GAPDH sequences. This has been used previously in determining the GAPDH phosphatidyl binding site (Kaneda *et al.* 1997).

8.2.2 GAPDH and Iron Metabolism in Bacterial Virulence

Iron is a required nutrient, an *a priori* requirement for cell and organism viability. Nowhere is this more evident than in the role of iron in oxygen/carbon dioxide transport through the role of hemoglobin as well as ATP production through the electron transport chain. As such, deficiencies in iron metabolism may pose an existential threat to survival not only to normal cells but also to pathogens. Recent studies indicate a new and intriguing role for GAPDH in iron transport and uptake (Modun *et al.* 2000; Raje *et al.* 2007; Kumar *et al.* 2012). Specifically, not only can membrane GAPDH bind transferring but, in the case of iron scarcity, GAPDH can also be secreted extracellularly as an iron scavenger (Sheokand *et al.* 2014). Further, those investigations indicate the use of GAPDH as a bacterial virulence factor to recruit iron from the host to the pathogen (Boradia *et al.* 2014). These studies are described in detail in Chapter 14.

As indicated in Figure 8.2, current investigations indicate that GAPDH-mediated iron metabolism in bacterial virulence may be a complex three-step process involving hemolysis of host erythrocytes liberating both GAPDH and hemoglobin, binding of GAPDH to heme, followed by transport of liberated iron into the bacterial pathogen. The evidence for the first step of this pathway is the study by Sato *et al.* (2012) indicating the release of glyceraldehyde-3-phosphate dehydrogenase from human erythrocyte membranes mediated by *Prevotella oris* hemolysin. This has the effect of releasing what is presumed to be significant GAPDH concentrations as well as free hemoglobin. The evidence for the second step of this pathway is the observation by Vázquez-Zamorano *et al.* (2014) indicating that *Streptococcus pneumonia* secretes GAPDH, which subsequently binds hemoglobin and heme. The third step is the transport of liberated iron into the bacterial pathogen (Boradia *et al.* 2014). The transition from step two to step three requires binding of GAPDH to heme followed by the release of Fe^{++} to transferring, or the formation of a ternary complex with GAPDH-heme-transferrin such that the Fe^{++} is transferred to transferrin. Support for the latter is provided by recent studies indicating that GAPDH regulates the incorporation of Fe^{++} into hemoglobin (Chakravarti *et al.* 2010; Hannibal *et al.* 2012).

8.3 Role of Nitric Oxide in GAPDH Bacterial Virulence

Nitric oxide (NO) is, simultaneously, the quintessential inter- and intracellular signaling molecule, an immunoprotective defense mechanism as well as a toxic agent capable of causing cell damage and inducing apoptosis. Initially isolated as endothelium-derived relaxing factor (Furchgott 1999), its characterization

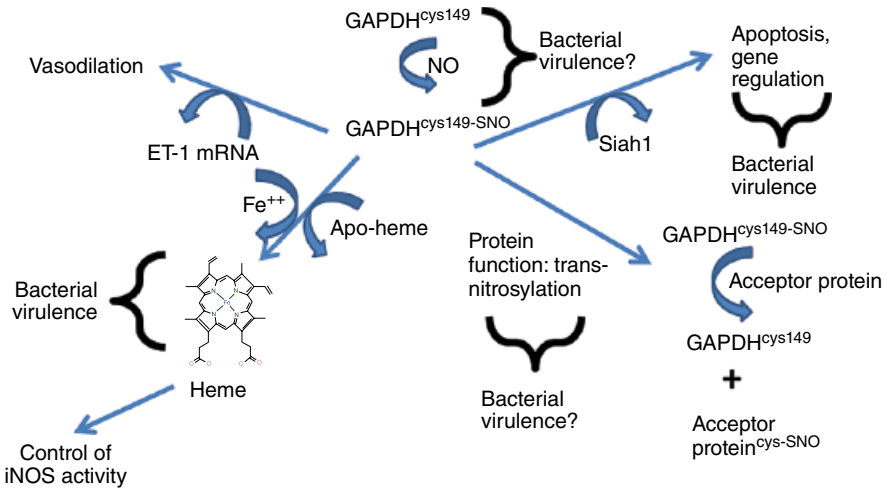

Figure 8.3 GAPDH and nitric oxide: multipotential effects.

revealed fundamental mechanisms of cell information transfer through a complex series of biochemical pathways. Its induction by macrophage nitric oxide synthetases as a function of infection represents one of the earliest cellular responses to virulent pathogens. Lastly, its reaction with molecular oxygen results in the production of peroxynitrite, one of the most potent cellular damaging agents, and its role in the Fenton reaction results in the production of reactive oxygen species.

As illustrated in Figure 8.3, GAPDH may be a unique intracellular target for NO. GAPDH is nitrosylated at its active site cysteine ($GAPDH^{cys\text{-}NO}$). Apart from inhibition of catalytic activity, formation of $GAPDH^{cys\text{-}NO}$ results in a complex series of cellular events (Sirover 2011, 2012, 2014). These include the induction of apoptosis, the regulation of gene expression (both through transcriptional and post-translational mechanisms), the control of heme synthesis through Fe^{++} metabolism, and, intriguingly, the ability of $GAPDH^{cys\text{-}NO}$ to act as a transnitrosylase, transferring its nitroso group to acceptor proteins. Significantly, several of these functions require the dynamic movement of $GAPDH^{cys\text{-}NO}$ from one subcellular locale to another (Sirover 2012; Tristan *et al*. 2012). *En toto*, these cumulative findings indicate the significance of $GAPDH^{cys\text{-}NO}$ formation as the production of this single, "simple" molecule results in a series of pleiotropic changes in cell and organism function.

8.3.1 Nitric Oxide in Bacterial Virulence: Evasion of the Immune Response

As indicated, macrophages present a proverbial "first line of defense" against bacterial virulence, using NO as a primary weapon. Accordingly, it may seem counterintuitive that bacterial pathogens may contain their own nitric oxide synthases whose functions are to produce NO upon infection. Nevertheless, recent studies demonstrate the presence and function of NO synthases in *B. subtilis*

(Adak *et al*. 2002; Pant *et al*. 2002), *S. aureus* (Chartier and Couture 2007), and *B. anthracis* (Shatalin *et al*. 2008). Each appears to be used as a weapon in a "counterattack" to forestall macrophage-mediated bacterial cell damage and destruction. Primarily, bacterial NO may function to induce catalase activity and to diminish the Fenton reaction to diminish bacterial DNA damage as a means to prevent cytotoxicity (Gusarov and Nudler 2005; Shatalin *et al*. 2008). This will be considered in detail in Chapter 13.

8.3.2 Formation of GAPDH$^{cys\text{-}NO}$ by Bacterial NO Synthases

Although conventional wisdom suggests a specific role of bacterial-produced nitric oxide in catalase and Fenton reaction inhibition, the pathways described in Figure 8.3 indicate an alternative fate for that prokaryotic molecule. In particular, as GAPDH comprises approximately 10–15% of total cell protein, it is reasonable to suggest that GAPDH may provide an attractive target for bacterial produced NO. Should that be the case, bacterial produced GAPDH$^{cys\text{-}NO}$ could initiate pleiotropic changes in infected cells.

Support for this supposition is a previous study demonstrating oxidative stress-induced modification of active site GAPDH^{cys} in *S. aureus* (Weber *et al*. 2004). Exposure to 100 μM H_2O_2 for 5 minutes resulted in a change in GAPDH pI from alkaline to acidic. Analysis of GAPDH in treated cells indicated formation of a sulfonated active site cysteine. These findings may be notable for several reasons. First, the effect is rapid in accord with the temporal sequence observed for catalase and Fenton reaction inhibition. Second, the effect is total. Only a single acidic protein species was observed. No alkaline species was detected. This total change in protein structure is in sharp contrast to the formation of small amounts of GAPDH$^{cys\text{-}NO}$ in eukaryotes (Benhar and Stamler 2005; Hara *et al*. 2005) which nevertheless results in substantial changes in eukaryotic cells. Third, the effect was reversible; within 30 minutes after H_2O_2 exposure bacterial proliferation is observed. *En toto*, this suggests the possibility that the formation of bacterial GAPDH$^{cys\text{-}NO}$ represents a protective measure to counter host production of nitric oxide as part of the immune response.

8.3.3 GAPDH$^{cys\text{-}NO}$ in Bacterial Virulence: Induction of Macrophage Apoptosis

Nowhere perhaps is the significance of GAPDH as a moonlighting protein illustrated as well as with its role in apoptosis, including its "activation" by nitric oxide forming GAPDH$^{cys\text{-}NO}$ (Hara *et al*. 2006). Most notably, this is highlighted by the role of GAPDH$^{cys\text{-}NO}$ in the complex intracellular pathways through which cells program their own destruction. These biochemical pathways exhibit defined protein: protein and protein: nucleic acid interactions, affinity-mediated changes in protein binding, changes in intracellular localization, uncovering of dormant protein activities, sequential post-translational modifications, induction of gene expression, and, lastly, the role of GAPDH$^{cys\text{-}NO}$ as a transnitrosylase. All of the above are initiated subsequent to a single event, that is, NO modification of GAPDH at its active site cysteine. To initiate apoptosis, cytoplasmic GAPDH$^{cys\text{-}NO}$ binds to Siah1, an E3 ubiquitin ligase which catalyzes protein degradation

(Hara *et al*. 2005; Hara and Snyder 2006). This protein complex translocates to the nucleus where not only does Siah1 catalyze the degradation of nuclear proteins, but also, through the formation of a second nuclear protein complex $GAPDH^{cys\text{-}NO}$: Siah1: P300/CBP, induces a cell death program of gene involving the regulation of p53, PUMA, Bax, and p21 (Sen *et al*. 2008). Further analysis indicated that $GAPDH^{cys\text{-}NO}$ may act a as a transnitrosylase, shifting enzymatically its NO moiety to an acceptor protein which now may assume new functions (Kornberg *et al*. 2010; Kohr *et al*. 2014; Rodriguez-Ortigosa *et al*. 2014).

As if these were not, by themselves, a series of complex cellular events, other studies indicate the presence of a competing mechanism which may be intended to protect cells from the apoptotic effects of $GAPDH^{cys\text{-}NO}$. These investigations identified a novel protein, GOSPEL (GADPH's competitor of Siah Protein Enhances Life), which binds $GAPDH^{cys\text{-}NO}$ thereby preventing the formation of the $GAPDH^{cys\text{-}NO}$: Siah1 complex (Sen *et al*. 2009). This protein: protein interaction vitiates Siah1 nuclear translocation and all the subsequent nuclear apopotic effects. The kinetic relationship between the two protein: protein complexes ($GAPDH^{cys\text{-}NO}$: Siah1 and $GAPDH^{cys\text{-}NO}$: GOSPEL) is unknown. However, it was suggested that a ternary intermediate complex may be formed by interaction of Siah1 with $GAPDH^{cys\text{-}NO}$: GOSPEL, resulting in the formation of $GAPDH^{cys\text{-}NO}$: Siah1 and the dissociation of GOSPEL (Sirover 2011).

Bacterial virulence may be maintained by inducing apoptosis in host macrophages. In this manner, the respective pathogen may evade immunoprotective defenses by destroying those cells responsible for that protection. Three separate studies identified that both nitric oxide and GAPDH were required for bacteria to induce macrophage-programmed cell death. First, Marriott *et al*. (2004) demonstrated using iNOS inhibitors that *S. pneumonia*-induced human macrophage apoptosis was dependent on its ability to produce nitric oxide. In those studies, infection resulted in stimulation of iNOS activity and rates of apoptosis. The addition of now-classical iNOS inhibitors diminished both the former and the latter in a coordinated manner.

Second, Ulett and Adderson (2005) reported that *S. agalactiae* infection of murine macrophages induced apoptosis. Using inhibitor analysis, they observed that iNOS expression was required. Third, in both *S. agalactiae* and in *S. aureus* infection of murine macrophages, Oliveira *et al*. (2012) demonstrated that extracellular bacterial GAPDH was required for the induction of apoptosis by each pathogen. In particular, depletion of bacterial GAPDH from culture supernatants diminished programmed cell death. Cumulatively, these results suggest, but do not prove that, each pathogen forms bacterial $GAPDH^{cys\text{-}NO}$ as the active molecule which induces macrophage apoptosis, thereby providing an immunoevasive pathway. This may be tested experimentally.

8.3.4 $GAPDH^{cys\text{-}NO}$ in Bacterial Virulence: Inhibition of Macrophage iNOS Activity

New investigations provide evidence identifying $GAPDH^{cys\text{-}NO}$ as a mechanism which regulates iNOS activity. The latter is a heme-containing protein whose activation from its apo-iNOS form is dependent on heme insertion as

the rate-limiting activation event. Of note, preliminary studies demonstrated that NO blocked heme insertion not only in iNOS but in a series of cellular proteins (Waheed *et al*. 2010). GAPDH was identified as a heme-binding protein by affinity chromatography, co-immunoprecipitation *in vivo* and by *in vitro* binding studies (Chakravarti *et al*. 2010). Its role as a major heme transport and transfer protein responsible for its insertion into apo-iNOS was defined by GAPDH knockdown and overexpression analyses. The former diminished heme insertion and reduced iNOS activity while the latter facilitated heme insertion and stimulated iNOS activity.

S-nitrosylation of GAPDH at its active site cysteine forming $GAPDH^{cys\text{-}NO}$ abolished GAPDH transfer activity and diminished iNOS activity. Mutation of the active site cysteine abolished both the formation of $GAPDH^{cys\text{-}NO}$ and its regulation of iNOS activity. Intriguingly, denitrosylation of $GAPDH^{cys\text{-}NO}$ by thioredoxin 1 restored both heme insertion and iNOS activity (Chakravarti and Stuehr 2012).

There is no demonstrable evidence indicating that the effect of $GAPDH^{cys\text{-}NO}$ on iNOS activity presents a bacterial pathogenesis mechanism. That being said, the active participation of bacterial NO synthetases in pathogenesis, the rapidity through which bacterially produced NO is involved as an immunoevasive protocol, the significance of membrane GAPDH in bacterial virulence, the significant amount of GAPDH active site modification during oxidative stress, as well as the diverse methods through which bacterial pathogens seek and sequester "free" Fe^{++} suggest that the examination of this possibility may present a fertile area of investigation.

8.3.5 $GAPDH^{cys\text{-}NO}$ in Bacterial Virulence: Transnitrosylation to Acceptor Proteins

The diversity of $GAPDH^{cys\text{-}NO}$ function is indicated further by recent studies demonstrating a new, unique catalytic activity observed as function of GAPDH S-nitrosylation (Table 8.2). Originally, Kornberg *et al*. (2010) made the salient observation that $GAPDH^{cys\text{-}NO}$ could act as a transnitrosylase during apoptosis. The acceptor proteins, deacetylating enzyme sirtuin-1 (SIRT1), histone deacetylase-2 (HDAC2), and DNA-activating protein kinase (DNA-PK) are significant in that their post-translational modification has pleiotropic downstream effects on gene regulation.

More recently, two other groups using other cell systems demonstrated the potential significance of this new $GAPDH^{cys\text{-}NO}$ function. Rodriguez-Ortigosa *et al*. (2014) reported that, in hepatocytes, the nitroso group of nuclear $GAPDH^{cys\text{-}NO}$ was transferred to SIRT1 and to HDAC2. This indicates that the effect first observed by Kornberg *et al*. (2010) may be a general phenomenon. However, instead of inducing gene expression, in this instance GAPDH transnitrosylation may function as a feedback mechanism of bile salt synthesis.

Third, in mouse heart mitochondria, Kohr *et al*. (2014) demonstrated that $GAPDH^{cys\text{-}NO}$ could transfer its nitroso group to a series of important mitochondrial proteins. The importance of this report resides not only in its demonstration of a second GAPDH transnitrosylation pathway, but also that this effect

Table 8.2 GAPDH-mediated transnitrosylation.

Cell type	Subcellular localization	Acceptor proteins	Reference
HEK293	Nuclear	SIRT1, HDAC2, DNA-PK	Kornberg *et al.* 2010
Heart	Mitochondria	Heat-shock protein 60, voltage-dependent anion channel 1, acetyl CoA transferase	Kohr *et al.* 2014
Hepatocytes	Nuclear	SIRT1, HDAC2	Rodriguez-Ortigosa *et al.* 2014

takes place in the mitochondria. In addition, there is an intriguing study suggesting that GAPDH can catalyze nitrite generation from nitroglycerin (Seabra *et al.* 2013). The latter is important in the treatment of cardiovascular disease as, taken sublingually, it provides for the rapid release of nitric oxide in patients thereby promoting vascular dilation. The relationship between these two studies is unknown at the present time. Given the ability of $GAPDH^{cys\text{-}NO}$ to transfer its nitroso group to a number of important cell proteins, there is no evidence at the present time that this function plays a role in bacterial virulence. That being said, as evidenced by the ability of bacterial pathogens to produce nitric oxide and the diverse roles of $GAPDH^{cys\text{-}NO}$, examination of transnitrosylation as a factor in bacterial pathogenesis may be a fertile area of investigation.

8.4 GAPDH Control of Gene Expression and Bacterial Virulence

Identification of the role of GADPH in transcription and post-transcriptional gene expression is one of the perfect examples in which an investigator sought to examine a fundamental problem in basic cell function and, after significant effort, reached an unexpected, perplexing conclusion. In this instance, the question asked was: how is a particular gene regulated and what factors influence that regulation? Using elegant, albeit now classical, protocols these investigators identified an unknown protein as being involved in the respective gene's expression. Subsequent to performing all the requisite controls to validate the role of the unknown protein, its characterization revealed it to be GAPDH. Apart from the inherent surprise (and the re-performance of many controls), Sherlock Holmes's classic comment to Dr Watson in *The Sign of the Four* was self-evident: "How often have I said to you that when you have eliminated the impossible, whatever remains, however improbable, must be the truth?" On a personal note, in the early days of GAPDH studies the author was the recipient of many a telephone call (there were no cell phones and the internet was in its infancy) from individuals who had made such discoveries and were seeking either advice or solace.

At this stage, some two to three decades later, it is clear that GAPDH may play a fundamental role in gene regulation and that its participation may be a focal

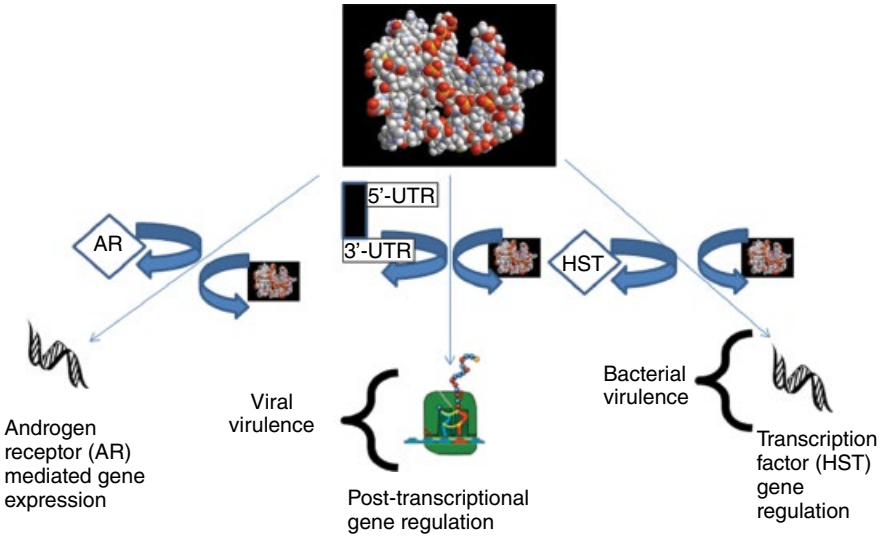

Figure 8.4 GAPDH and gene regulation.

point for pathogen virulence. As indicated in Figure 8.4, this may take two forms: transcriptional and post-transcriptional. The former is involved in bacterial virulence and will be discussed in this chapter. The latter is involved in viral virulence and will not be considered. The reader is referred to its previous consideration (Sirover 1999).

With respect to transcriptional expression, detailed studies indicated the requirement for GAPDH as a trans activator for the androgen receptor, inducing its nuclear translocation upon ligand binding followed by the transcriptional activation of prostate specific genes (Harada *et al.* 2007). Similarly, analyses of histone gene regulation identified a cytoplasmic complex, termed OCA-S, which upon formation entered the nucleus and initiated histone gene synthesis. GAPDH was identified as an integral part of this coactivator protein complex which controls histone gene expression (McKnight 2003; Zheng *et al.* 2003).

8.4.1 Bacterial GAPDH Virulence

NF-κB is a well-recognized transcription factor which mediates the cellular immune response. Its activation is a defense mechanism against bacterial infection, while its inhibition would facilitate successful pathogenesis. Recent evidence demonstrates that GAPDH is a required component of the signaling pathway through which NF-κB activation is achieved. This is accomplished through its interaction with TRAF2, representing an early event in this activation pathway. This finding is similar to that observed with both GAPDH: androgen receptor and GAPDH: OCA-S interactions, that is, GAPDH acts at an early stage in such regulation. This represents perhaps not only a general GAPDH transcriptional activation mechanism but also its use in transcriptional control.

Recent evidence indicates that enterohemorrhagic and enteropathic *E. coli* utilize NieB as an effector to prevent NF-κB activation, thereby facilitating

infection (Gao *et al*. 2013). Detailed examination reveals that, although NieB acts by preventing TRAF2 polyubiquitination, it does not interact with TRAF2. A search for NieB-interacting proteins identified GAPDH as a binding partner. Significantly, further studies indicated that NieB is an O-linked N-acetyl glucosamine transferase which post-translationally modifies GAPDH. As previous studies demonstrate that this post-translational modification disrupts GAPDH structure (Park *et al*. 2009), this NieB-mediated perturbation of GAPDH structure would prevent its binding to TRAF2, thereby forestalling NF-κB transcriptional activity and facilitating *E. coli* virulence.

8.5 Discussion

The evolutionary origin of moonlighting proteins remains poorly understood and presents an unresolved, fundamental problem. As indicated in Figure 8.5, it may be hypothesized that multifunctional proteins may have been developed to provide a mechanism to help resolve a central paradox with respect to DNA structure and function, that is, cellular gene content may be insufficient to provide the requisite information needed to perform all necessary activities for cell, tissue, and organism viability. Accordingly, as with alternate splicing and post-translational protein modification, moonlighting proteins may serve as a means to potentiate exponentially the utilization of the limited amount of genetic information contained within individual structural genes.

In this regard, understanding the role of GAPDH as a moonlighting protein may represent a particular challenge. With respect to its genetic structure, in higher organisms it is encoded by a single structural gene in somatic cells (Bruns and Gerald 1976; Bruns *et al*. 1979) although a unique species is detected in spermatozoa (Miki *et al*. 2004). Alternative transcription does not appear to occur

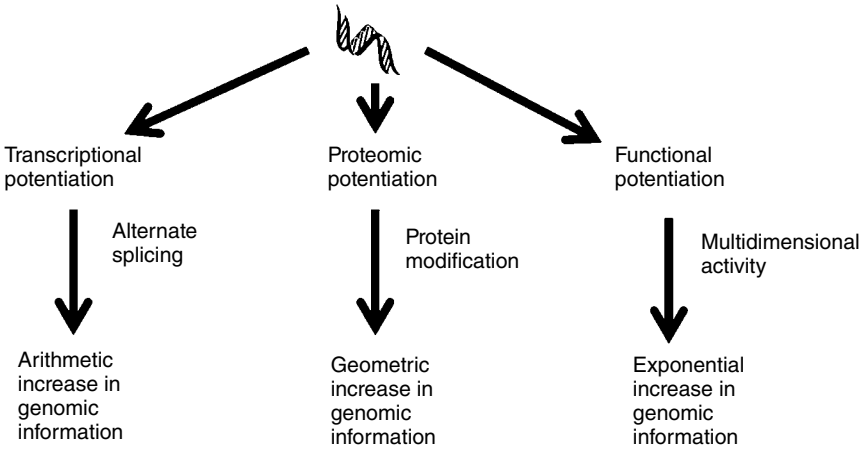

Figure 8.5 Mechanisms of genomic potentiation.

(Mezquita *et al*. 1998). In prokaryotes, although multiple genes are present, their genetic structure may be similarly straightforward (Branlant *et al*. 1983).

Accordingly, the unique mechanisms through which individual GAPDH molecules are "chosen" for their respective moonlighting functions and intracellular localizations are unclear. This may be particularly relevant as GAPDH is an abundant protein, at times comprising 10–15% of total cell protein. In higher organisms, it was postulated that the high degree of GAPDH post-translational modification may provide a mechanism through which such GAPDH molecules are "selected" and its diverse activities are mediated (Sirover 2014). In contrast, the nature and degree of prokaryotic GAPDH post-translational modifications remains uncertain (Aguilera *et al*. 2009). In that regard, the latter may represent a productive area of investigation most notable with respect to the role of membrane GAPDH as an adhesion molecule, given that it appears to lack "typical" secretory or membrane localization signals.

Further, as discussed here two areas of GAPDH function in bacterial virulence appear ripe for the initiation of detailed analysis, that is, the role of GAPDH in bacterial pathogen Fe^{++} metabolism and the consequences of nitric oxide production as a function of bacterial virulence. With respect to the former, a number of studies have already been performed, providing the proverbial road map for analysis (Sato *et al*. 2012; Boradia *et al*. 2014; Vázquez-Zamorano *et al*. 2014). That being said, each represents an independent study in three separate pathogens. Accordingly, whether they are linked is an open question.

With respect to bacterial-produced nitric oxide and the formation of $GAPDH^{cys\text{-}NO}$ as a virulence mechanism, bacterial NO is clearly defined with a reasonable mechanism independent of $GAPDH^{cys\text{-}NO}$ formation. In particular, diminution of catalase activity and inhibition of the Fenton reaction presents a clear pathway to mitigate basic mechanisms intrinsic to the immune response. However, unless it is directed intracellularly by an unknown means, it is reasonable to suggest that bacterial produced NO would clearly interact with either bacterial or host GAPDH. This would initiate the complex series of reactions indicated in Figure 8.3. The general concept underlying this chapter, and indeed this book, is the contribution of moonlighting proteins to bacterial virulence. Given the proclivity of cells to utilize GAPDH, it may also be possible that target cells employ this moonlighting protein in their self-defense. Two such studies suggest that possibility. Xie *et al*. (2006) demonstrated that GAPDH was upregulated in rat liver and rat lung following exposure to bacterial endotoxin lipopolysaccharide (LPS). The latter may be involved as a mediator of sepsis by its facilitation of inflammatory gene expression. In their study, Xie *et al*. (2006) demonstrated that exposure to LPS selectively increased GAPDH transcription and translation. Although they postulated that this may be virulence related, a further study by Takaoka *et al*. (2014) demonstrated an anti-inflammatory role for GAPDH subsequent to LPS treatment. In those studies, pre-injection of GAPDH mitigated sepsis related mortality and inhibited LPS related increases in inflammatory gene transcription. Accordingly, GAPDH may present a proverbial two-edged sword, that is, in one case it is used as a means to facilitate bacterial virulence, while in the other case it may be used to protect cells against bacterial pathogenesis.

Acknowledgements

Work in the author's laboratory was funded by an award from the National Institutes of Health (CA 119285).

References

Adak S, Aulak KS, Stuehr DJ (2002) Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from *Bacillus subtilis*. *Journal of Biological Chemistry* 277: 16167–16171.

Aguilera L, Giménez R, Badia J, Aguilera J, Baldomá L (2009) NAD^+-dependent post-translational modification of *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase. *International Journal of Microbiology* 12: 187–192.

Aguilera L, Ferreira E, Giménez R, Fernámdez FJ, Taulés M, Aguilar J, Vega MC, Badia J, Baldomá L (2012) Secretion of the housekeeping protein glyceraldehyde-3-phosphate dehydrogenase by the LEE-encoded type III secretion system in enteropathogenic *Escherichia coli*. *International Journal of Biochemistry and Cell Biology* 44: 955–962.

Benhar M, Stamler JS (2005) A central role for S-nitrosylation in apoptosis. *Nature Cell Biology* 7: 645–646.

Bergmann S, Rohde M, Sammerschmidt S (2004) Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. *Infection and Immunity* 72: 2416–2419.

Boël G, Jin H, Pancholi V (2005) Inhibition of cell surface export of Group A Streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties. *Infection and Immunity* 73: 6237–6248.

Boradia VM, Malhotra H, Thakkar JS, Tillu VA, Vuppala B, Patil P, Sheokand N, Sharma P, Chauhan AS, Raje M, Raje CI (2014) *Mycobacterium tuberculosis* acquires iron by cell-surface sequestration and internalization of human holo-transferrin. *Nature Communications* 5: 4730.

Branlant G, Flesch G, Branlant C (1983) Molecular cloning of the glyceraldehyde-3-phosphate dehydrogenase genes of *Bacillus stearothermophilus* and *Escherichia coli*, and their expression in *Escherichia coli*. *Gene* 25: 1–7.

Bruns GAP, Gerald PS (1976) Human glyceraldehyde-3-phosphate dehydrogenase in man-rodent somatic cell hybrids. *Science* 192: 54–56.

Bruns G, Gerald PS, Lalley P, Francke U, Minna J (1979) Gene mapping of the mouse by somatic cell hybridization. *Cytogenetics and Cell Genetics* 25: 139.

Chakravarti R, Stuehr DJ (2012) Thioredoxin-1 regulates cellular heme insertion by controlling S-nitrosation of glyceraldehyde-3-phosphate dehydrogenase. *Journal of Biological Chemistry* 287: 16179–16186.

Chakravarti RR, Aulak KS, Fox PL, Stueher DJ (2010) GAPDH regulates cellular heme insertion into inducible nitric oxide synthetase. *Proceedings of the National Academy of Science, USA* 104: 18004–18009.

Chartier FJM, Couture M (2007) Interactions between substrates and the haem-bound nitric oxide of ferric and ferrous bacterial nitric oxide synthases. *Biochemical Journal* 401: 235–245.

Daubenberger CA, Tisdale EJ, Curcic M, Diaz D, Silvie O, Mazier D, Eling W, Bohrmann B, Matile H, Pluschke G (2003) The N'terminal domain of glyceraldehyde-3-phosphate dehydrogenase of the apicomplexan *Plasmodium falciparum* mediates GTPase Rab2-dependent recruitment to membranes. *Traffic* 384: 1227–1237.

de Bolle X, Letesson JJ, Gorvel JP (2012) Small GTPases and *Brucella* entry into the endoplasmic reticulum. *Biochemistry Society Transactions* 40: 1348–1352.

Egea L, Aguilera L, Giménez R, Sorolla MA, Aguilar J, Badía Baldoma, L (2007) Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathic *Escherichia coli*: Interaction of the extracellular enzyme with human plasminogen and fibrinogen. *International Journal of Biochemistry and Cell Biology* 39: 1190–1203.

Fugier E, Salcedo SP, de Chastellier C, Pophillat M, Muller A, Arce-Gorvel V, Fourquet P, Gorvel JP (2009) The glyceraldehyde-3-phosphate dehydrogenase and the small GTPase Rab 2 are crucial for *Brucella* replication. *PLoS Pathogens* 5: e1000487.

Furchgott RF (1999) Endothelium-derived relaxing factor: discovery, early studies, and identification as nitric oxide. *Bioscience Reports* 19: 235–251.

Gao X, Wang X, Pham TH, Feuerbacher LA, Lubos ML, Huang M, Olsen R, Mushegian A, Slawson C, Hardwidge PR (2013) NleB, a bacterial effector with glycosyltransferase activity, targets GAPDH function to inhibit NF-κB Activation. *Cell Host and Microbe* 13: 87–99.

Gil-Navarro I, Gil ML, Casonova M, O'Connor JE, Martinez JP, Gozalbo D (1997) The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is a surface antigen. *Journal of Bacteriology* 179: 4992–4999.

Glaser PE, Gross RW (1995) Rapid plasmenylethanolamine-selective fusion of membrane bilayers catalyzed by an isoform of glyceraldehyde-3 phosphate dehydrogenase: discrimination between glycolytic and fusogenic roles of individual isoforms. *Biochemistry* 34: 12194–12203.

Gusarov I, Nudler E (2005) NO-mediated cytoprotection: Instant adaption to oxidative stress in bacteria. *Proceedings of the National Academy of Science, USA* 102: 13855–13860.

Hannibal L, Collins D, Brassard J, Chakravarti R, Vemapti R, Dorlet P, Santolini J, Dawson J, Stuehr D (2012) Heme binding properties of glyceraldehyde-3-phosphate dehydrogenase. *Biochemistry* 51: 8514–8529.

Hara MR, Snyder SH (2006) Nitric oxide-GAPDH-Siah: a novel cell death cascade. *Cell and Molecular Neurobiology* 26: 525–536.

Hara MR, Agrawal N, Kim SF, Cascio MB, Fujimuro M, Ozeki Y, Takahashi M, Cheah JH, Tankou SK, Hester LD, Ferris CD, Hayward SH, Snyder SH, Sawa A (2005) S-nitrosylated GAPDH initiates apoptotic cell death by nuclear translocation following Siah1 Binding. *Nature Cell Biology* 7: 665–674.

Hara MR, Cascio MB, Sawa A (2006) GAPDH as a sensor of NO stress *Biochimica et Biophysica Acta* 1762: 502–509.

Harada N, Yasunaga R, Higashimura Y, Yamaji R, Fujimoto K, Moss J, Inui H, Nakano Y (2007) Glyceraldehyde-3-phosphate dehydrogenase enhances transcriptional activity of androgen receptor in prostate cancer cells. *Journal of Biological Chemistry* 282: 22651–22661.

Jin H, Song YP, Boel G, Kochar J, Pancholi V (2005) Group A Streptococcal GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *Journal of Molecular Biology* 350: 27–41.

Jin H, Agarwal S, Agarwal S, Pancholi V (2011) Surface export of GAPDH/SDH, a glycolytic enzyme is essential for *Streptococcus pyogenes* virulence. *MBio* 2: e00068-11.

Kaneda M, Takeuchi KI, Inoue K, Umeda M (1997) Localization of the phosphatidylserine-binding site of glyceraldehyde-3-phosphate dehydrogenase responsible for membrane fusion. *Journal of Biochemistry* 122: 1233–1240.

Kant JA, Steck TL (1973) Specificity in the association of glyceraldehyde 3-phosphate dehydrogenase with isolated human erythrocyte membranes. *Journal of Biological Chemistry* 248: 8457–8464.

Kohr MJ, Murphy E, Steenbergen C (2014) Glyceraldehyde-3-phosphate dehydrogenase acts as a mitochondrial trans-S-nitrosylase in the heart. *PLOS One* 9: e111448.

Kornberg MD, Sen N, Hara VMR, Juluri KR, Nguyen JVK, Snowman SM, Law L, Hester LD, Snyder SH (2010) GAPDH mediates nitrosylation of nuclear proteins. *Nature Cell Biology* 12: 1094–1100.

Kumar S, Sheokand N, Mhadeshwar M, Raje C, Raje M (2012) Characterization of glyceraldehyde-3-phosphate dehydrogenase as a novel transferrin receptor. *International Journal of Biochemistry and Cell Biology* 44: 189–199.

Lama A, Kucknoor A, Mundodi V, Alderete JF (2009) Glyceraldehyde-3-phosphate dehydrogenase is a surface-associated fibronectin-binding protein of *Trichomonas vaginalis*. *Infection and Immunity* 77: 2703–2711.

Madureira P, Baptista M, Vieira M, Magalhães V, Camelo A, Oliveira L, Ribeiro A, Tavares D, Trieu-Cuot P, Vilanova M, Ferreira P (2007) *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. *Journal of Immunology* 178: 1379–1387.

Maeda K, Nagata H, Yamamoto Y, Tanaka M, Tanaka J, Minamino N, Shizukuishi S (2004*a*) Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesion for *Porphyromonas gingivalis* major fimbriae. *Infection and Immunity* 72: 1341–1348.

Maeda K, Nagata H, Nonaka A, Kataoka K, Tanaka M, Shizukuishi S (2004*b*) Oral streptococcal glyceraldehyde-3-phosphate dehydrogenase mediates interaction with *Porphyromonas gingivalis* fimbriae. *Microbes and Infection* 6: 1163–1170.

Maeda K, Nagata H, Kuboniwa M, Ojima M, Osaki T, Minamino N, Amano A (2013) Identification and characterization of *Porphyromonas gingivalis* client protein proteins that bind to *Streptococcus oralis* glyceraldehyde-3-phosphate dehydrogenase. *Infection and Immunity* 81: 753–763.

Marriott HM, Ali F, Read RC, Mitchell TJ, Whyte MKB, Dockrell DH (2004) Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infections. *Faseb Journal* 18: 1126–1128.

McDaniel CF, Kirtley ME, Tanner MJA (1974) The interaction of glyceraldehyde 3-phosphate dehydrogenase with human erythrocyte membranes. *Journal of Biological Chemistry* 249: 6478–6485.

McKnight S (2003) Gene switching by metabolic enzymes — how did you get on the invitation list? *Cell* 114: 150–152.

Mezquita J, Pau M, Mezquita C (1998) Several novel transcripts of glyceraldehyde-3-phosphate dehydrogenase expressed in adult chicken testis. *Journal of Cellular Biochemistry* 71: 127–139.

Miki K, Qu W, Goulding EH, Willis WD, Bunch DO, Strader LF, Perreault SD, Eddy EM, O'Brien DA (2004) Glyceraldehyde-3-phosphate dehydrogenase-S, a sperm specific glycolytic enzyme, is required for sperm motility and male fertility. *Proceedings of the National Academy of Science, USA* 101: 16501–16506.

Modun B, Morrissey J, Williams P (2000) The staphylococcal transferrin receptor: a glycolytic enzyme with novel functions. *Trends in Microbiology* 8: 231–237.

Nagata H, Iwasaki M, Maeda K, Kuboniwa M, Hashino E, Toe M, Minamino N, Kuwahara H, Shizukuishi S (2009) Identification of the binding domain of *Streptococcus oralis* glyceraldehyde-3-phosphate dehydrogenase for *Porphyromonas gingivalis* major fibmbriae. *Infection and Immunity* 77: 5130–5138.

Oliveira L, Madureira P, Andrade EB, Bouaboud A, Morello E, Ferreira P, Poyart C, Trieu-Cuot P, Dramsi S (2012) Group B Streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. *PLoS One* 7: e29963.

Pancholi V, Fischetti VA (1992) A major surface protein on Group A Streptococci is a glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity. *Journal of Experimental Medicine* 176: 415–426.

Pant K, Bilwes AM, Adak S, Stuehr DJ, Crane BR (2002) Structure of a nitric oxide synthase heme protein from *Bacillus subtilis*. *Biochemistry* 41: 11071–11079.

Park J, Han D, Kim K, Kang Y, Kim Y (2009) O-GlcNAcylation disrupts glyceraldehyde-3-phosphate dehydrogenase homo-tetramer formation and mediates its nuclear translocation. *Biochimica et Biophysica Acta* 1794: 254–262.

Purves J, Cockayne A, Moody PCE, Morrissey JA (2010) Comparison of the regulation, metabolic functions and roles in virulence of the glyceraldehyde-3-phosphate dehydrogenase homologues *gap*A and *gap*B in *Staphylococcus aureus*. *Infection and Immunity* 78: 5223–5232.

Raje C, Kumar S, Harle A, Nanda J, Raje M (2007) The macrophage cell surface glyceraldehyde-3-phosphate dehydrogenase is a novel transferrin receptor. *Journal of Biological Chemistry* 282: 3252–3261.

Rodriguez-Ortigosa CM, Celay J, Olivas I, Juanarena N, Arcelius S, Uriarte I, Marin JJG, Avila MA, Medina JF, Parieto J (2014) A GAPDH-mediated trans-nitrosylation pathway is required for feedback inhibition of bile salt synthesis in rat liver. *Gastroenterology* 147: 1084–1093.

Sato T, Kamaguchi A, Nakazawa F (2012) The release of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from human erythrocyte membranes lysed by hemolysin of *Prevotella oris*. *Anaerobe* 18: 553–555.

Seabra AB, Oueller M, Antonic M, Chrétien MN, English AM (2013) Catalysis of nitrite generation from nitroglycerin by glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Nitric Oxide* 35: 118–122.

Seifert KN, McArthur WP, Bleiweis AS, Brady LJ (2003) Characterization of group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization,

enzymatic activity, and protein-protein interactions. *Canadian Journal of Microbiology* 49: 350–356.

Sen N, Hara MR, Kornberg MD, Cascio MB, Bae BI, Shahani N, Thomas B, Dawson TM, Dawson VL, Snyder SH, Sawa A (2008) Nitric oxide-induced nuclear GAPDH activates p300/CBP and mediates apoptosis. *Nature Cell Biology* 10: 866–873.

Sen N, Hara MR, Ahmad AS, Cascio MB, Kamiya A, Ehmsen JT, Aggrawal N, Hester L, Dore S, Snyder SH, Sawa A (2009) GOSPEL: a neuroprotective protein that binds to GAPDH upon s-nitrosylation. *Neuron* 63: 81–91.

Shatalin K, Gusarov I, Avetissova E, Shatalina Y, McQuade LE, Lippard SJ, Nudler E (2008) *Bacillus anthracis*-derived nitric oxide is essential for pathogen virulence and survival in macrophages. *Proceedings of National Academy of Science, USA* 105: 1009–1013.

Sheokand N, Malhotra H, Kumar S, Tillu VA, Chauhan AS, Raje CI, Raje M (2014) Moolighting cell-surface GAPDH recruits apotransferrin to effect iron egress from mammalian cells. *Journal of Cell Science* 127: 4279–4291.

Sirover M (1999) New insight into an old protein: The functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochimica et Biophysica Acta* 1432: 159–184.

Sirover M (2005) New nuclear functions of the glycolytic protein glyceraldehyde-3-phosphate dehydrogenase. *Journal of Cellular Biochemistry* 95: 45–52.

Sirover M (2011) On the functional diversity of glyceraldehyde-3-phosphate dehydrogenase: Biochemical mechanisms and regulatory control. *Biochimica et Biophysica Acta* 1810: 741–751.

Sirover M (2012) Subcellular dynamics of multifunctional protein regulation: Mechanisms of GAPDH intracellular translocation. *Journal of Cellular Biochemistry* 113: 2193–2200.

Sirover M (2014) Structural analysis of glyceraldehyde-3-phosphate dehydrogenase functional diversity. *International Journal of Biochemistry and Cell Biology* 57: 20–26.

Takaoka Y, Goto S, Nakano T, Tseng H-P, Yang S-M, Kawamoto S, Ono K, Chen C-L (2014) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) prevents lipopolysaccharide (LPS)-induced sepsis-related severe acute lung injury in mice. *Science Reports* 4: 5204.

Tanner MJA, Gray WR (1971) The isolation and functional identification of a protein from the human erythrocyte 'ghost'. *Biochemical Journal* 125: 1109–1117.

Terao Y, Yamaguchi M, Hamada S, Kawabata S (2006) Multifunctional glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pyogenes* is essential for evasion from neutrophils. *Journal of Biological Chemistry* 281: 14215–14223.

Terrasse R, Tacnet-Delorme P, Moriscot C, Pérard J, Schoehn G, Vernet T, Thielens NM, Di Guilmi AM, Frachet P (2012) Human and pneumococcal cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins are both ligands of human C1q protein. *Journal of Biological Chemistry* 287: 42620–42633.

Tisdale E (2001) Glyceraldehyde-3-phosphate dehydrogenase is required for vesicular transport in the early secretory pathway. *Journal of Biological Chemistry* 276: 2480–2486.

Tristan C, Shahani N, Sedlak T, Sawa A (2011) The diverse functions of GAPDH: Views from different subcellular compartments. *Cell Signalling Technology* 23: 317–323.

Ulett GC, Adderson EE (2005) Nitric oxide is a key determinant of Group B Streptococcus-induced murine macrophage apoptosis. *Journal of Infectious Diseases* 191: 1761–1670.

Vázquez-Zamorano ZE, González-López MA, Romero-Espejel ME, Azuara-Liceaga EI, López-Casamichana M, Olivares-Trejo JdJ (2014) *Stretococcus pneumonia* secretes a glyceraldehyde-3-phosphate dehydrogenase, which binds haemoglobin and haem. *Biometals* 27: 683–693.

Waheed SM, Ghosh A, Chakravarti R, Biswas A, Haque MM, Panda K, Stueher DJ (2010) Nitric oxide blocks cellular heme insertion into a broad range of heme proteins. *Free Radical Biology and Medicine* 48: 1548–1558.

Weber H, Engelmann S, Becher D, Hecker M (2004) Oxidative stress triggers thiol oxidation in the glyceraldehyde-3-phosphate dehydrogenase of *Staphylococcus aureus*. *Molecular Microbology* 52: 133–140.

Xie W, Shao N, Ma X, Ling B, Wei Y, Ding Q, Yang G, Liu N, Wang H, Chen K (2006) Bacterial endotoxin lipopolysaccharide induces up-regulation of glyceraldehyde-3-phosphate dehydrogenase in rat liver and lungs. *Life Science* 79: 1820–1827.

Zheng I, Roeder RG, Luo Y (2003) S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell* 114: 255–266.

9

Streptococcus pyogenes GAPDH: A Cell-Surface Major Virulence Determinant

Vijay Pancholi

Department of Pathology, The Ohio State University, College of Medicine and Wexner Medical Center, Columbus, Ohio, USA

9.1 Introduction and Early Discovery

Streptococcus pyogenes (Group A *Streptococcus* (GAS)) is one of the most successful pathogens, and is recognized as one of the first microorganisms to cause contagious disease by Louis Pasteur in 1879 (Pasteur, 1880). Worldwide, GAS is responsible for more than 600 million infections each year with more than half a million deaths (Carapetis *et al*. 2005). GAS causes many severe and often fatal invasive diseases, such as necrotizing fasciitis (NF) or flesh-eating syndrome and streptococcal toxic- or septic-shock syndrome (STSS) (Stevens 1992, 1995; Wong and Stevens 2013). However, the typical spectrum of GAS diseases is limited to mild, noninvasive, and non-life-threatening pharyngitis and impetigo. In the late 1980s, a single GAS bacterium acquired a phage that contained a gene encoding the streptococcal pyrogenic exotoxin A (Nasser *et al*. 2014; Musser and Shelburne 2009) superantigen SpeA2, and the frequency and severity of invasive GAS infection suddenly increased worldwide (Musser and DeLeo 2005). GAS infection-associated severe morbidity is also associated with post-streptococcal autoimmune sequelae, such as rheumatic fever, rheumatic heart diseases, acute glomerulonephritis, and chorea affecting the human heart (Carapetis and Currie 1996; Carapetis 2008; Ralph and Carapetis 2013), kidney (Rodriguez-Iturbe and Musser 2008), and nervous system (Swedo *et al*. 2010).

Despite important advances in hygiene and modern modes of prevention and therapy, the severity, success, and prevalence of GAS infections in humans persist. The severity of GAS infections is attributed to the ability of GAS to express a variety of secreted and cell-wall-associated virulence factors (Cunningham 2000). The expression of these proteins is dynamically regulated by several external and hostile host tissue environmental factors (Kreikemeyer *et al*. 2003). Until the early 1990s, secreted proteins were classified as proteins that possess a signal sequence and are excreted through the Sec pathway (Akimaru *et al*. 1991; Driessen and Nouwen 2008). Genomic analyses of several genes that encode

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

cell-wall-associated surface proteins of Gram-positive bacteria, including the major streptococcal virulence factor, the M protein, indicated that these are putative transmembrane proteins characterized by a hydrophobic tail that is located in the membrane, a charged tail that extends into the cytoplasm, and a proline-glycine-rich region that associates with the cell wall (Fischetti 2006; Navarre and Schneewind 1999). It was not known until 1988 that mature cell-wall-associated surface proteins, including the M protein, do not contain the hydrophobic tail or the charged tail (Pancholi and Fischetti 1988). The putative cleavage site "LPXTGE" (Pancholi and Fischetti 1989), which is located between the pro-gly-rich domain and the hydrophobic charged tail of the M protein, was later found to be a target for the transpeptidase sortase enzyme (Fischetti *et al*. 1990; Schneewind *et al*. 1992; Mazmanian *et al*. 2001). A sortase-mediated transpeptidation reaction allows the cleaved surface protein to be covalently linked to peptidoglycan (Navarre and Schneewind 1994, 1999; Scott and Barnett 2006). Sortase-mediated targeting of the LPXTGX-motif therefore became an important finding related to the surface protein biology of Gram-positive bacteria (Schneewind *et al*. 1992). The hexapeptide LPXTGX was then established as a signature motif for all Gram-positive cell-wall-associated surface proteins (Navarre and Schneewind 1999).

To understand the nature of cell-wall-associated proteins, Fischetti's group used the group C phage lysin (amidase enzyme) in an osmotically balanced buffer to obtain cell-wall fragments associated with proteins and intact protoplasts with their cytoplasmic contents (Fischetti *et al*. 1971). Using this type of cell-wall extract, Pancholi and Fischetti (1992) detected a major protein that migrated at *c*. 40 kDa in SDS-PAGE resolved gels. Surprisingly, biochemical as well as mass-spectrometry analyses revealed that this protein was the cytoplasmic protein, glyceraldehyde-3 phosphate dehydrogenase (GAPDH) (Pancholi and Fischetti 1992). Because GAS GAPDH lacks a signal sequence and an LPXTGX cell-surface-sorting motif, this finding raised an intriguing debate concerning how and why a cytoplasmic protein is exported to the surface without these essential attributes. This report on GAS GAPDH and the subsequent identification of the major plasminogen-binding protein enolase on the surface of GAS (Pancholi and Fischetti 1998) established the existence of a new class of anchorless surface proteins in bacteria (Pancholi and Chhatwal 2003). While several reviews, including the preceding Chapter 8, have highlighted the multifunctional nature of GAPDH and its implications for eukaryotic biology (Sirover 2005, 2011; Henderson and Martin 2011; Seidler 2013), in this chapter various aspects of GAS GAPDH functions that are related to its biological properties and its interaction with host cells are described in detail. Finally, this chapter also ponders the debatable and burgeoning interest in understanding whether the surface export of anchorless surface proteins in general and the surface export of GAS GAPDH in particular are a cause or an effect.

9.2 GAS GAPDH: A Major Surface Protein with Multiple Binding Activities

The cell surface of GAS is decorated with numerous proteins which typically constitute the outer electron-dense fuzz during visualization by transmission electron microscopy (Fischetti and Fazio-Zanakis 1985). Because the M protein

is a major virulence factor due to its antiphagocytic nature (Fischetti 1989), GAS pathology has been attributed primarily to the M protein. The lack of importance of several other proteins that were extracted via cell-wall digest and found in much higher relative quantities than the M protein led to the identification of a 35.8 kDa protein as an enzymatically active GAS GAPDH or streptococcal surface dehydrogenase (SDH) (Pancholi and Fischetti 1992). Pancholi and Fischetti further confirmed this finding by demonstrating that intact group A streptococci also possess enzymatically active GAPDH. Other contemporary findings concerning the same protein revealed that GAS GAPDH binds to plasminogen (Lottenberg *et al*. 1992), which is an important component of the host fibrinolytic system (Boyle and Lottenberg 1997). However, the direct binding of plasminogen to the cell-wall extract obtained from GAS failed to reveal strong binding activity to the 35.8 kDa protein. Instead, a higher-molecular-weight 43–45 kDa protein was found to be a stronger plasminogen-binding protein; that protein was later identified enzymatically as α-enolase (Pancholi and Fischetti 1998). On the other hand, GAS GAPDH exhibits a strong binding activity to extracellular matrix proteins, such as fibrinogen and fibronectin, and other mammalian proteins, such as myosin, tropomyosin, actin, and lysozyme (Pancholi and Fischetti 1992). The M protein of GAS, tropomyosin and myosin share a similar alpha-helical coiled-coil structure (Fischetti 1989). However, SDH does not bind to the M protein, indicating that the binding of SDH to tropomyosin is physiologically relevant and likely amino acid sequence-specific.

In contrast, the findings of D'Costa *et al*. (2000) demonstrated that the anchoring of GAPDH/SDH/Plr (plasminogen-binding receptor) to the streptococcal cell surface is associated with the expression of the Mga-regulated protein or M protein-related proteins and binding to IgM. However, their study did not report the level of GAPDH expression in the Mga (multiple gene regulator of GAS) (–) mutant or the specificity of IgM for GAPDH on the GAS surface. As recently revealed, it is possible that the expression of GAPDH is co-regulated with that of Mga (Jin *et al*. 2011). The association of SDH with the M protein could also be mediated by the common binding protein fibrinogen (Herwald *et al*. 2004).

The discovery of the cytosolic enzyme GAPDH on the surface of bacteria intrigued many researchers. It is now well-documented that SDH and its homologs are found in either a secreted or cell-wall-/membrane-associated form in a variety of microorganisms, including many Gram-positive bacteria (Modun and Williams 1999; Nelson *et al*. 2001; Bergmann *et al*. 2004; Terao *et al*. 2006; Madureira *et al*. 2007; Kinoshita *et al*. 2008*a*, *b*; Matta *et al*. 2010; Maeda *et al*. 2013), Gram-negative bacteria (Kenney and Finlay 1995; Espinosa-Urgel and Kolter 1998; Grifantini *et al*. 2002; Egea *et al*. 2007), mycoplasma (Alvarez *et al*. 2003), mycobacteria (Bermudez *et al*. 1996; Boradia *et al*. 2014), fungi/*Candida* (Gil-Navarro *et al*. 1997; Gozalbo *et al*. 1998) and parasites (Goudot-Crozel *et al*. 1989; Shoemaker *et al*. 1992; Delgado-Corona *et al*. 2002; Alvarez *et al*. 2007). The list of proteins that are targeted by bacterial surface-exported GAPDHs now extends beyond plasminogen and fibronectin and includes transferrin (Bermudez *et al*. 1996; Boradia *et al*. 2014; see also Chapter 11), mucin (Kinoshita *et al*. 2008*a*; see also Chapter 12), complement factor C5a (Terao *et al*. 2006), and even the human ABO blood groups (Kinoshita *et al*. 2008*b*).

9.3 AutoADP-Ribosylation of SDH and Other Post-Translational Modifications

Nitric oxide enhances the post-translational ADP-ribosylation of GAPDH in a variety of human tissues, such as the liver, intestine, heart, lung (Brune and Lapetina 1989), brain (Brune and Lapetina 1989; Zhang and Snyder 1992), and erythrocytes (Kots *et al*. 1993, 1992). ADP-ribosylation is also an important mechanism of action of many bacterial toxins (Ueda and Hayaishi 1985). Based on these reports, Pancholi and Fischetti (1993) examined whether SDH is ADP-ribosylated and whether such a modification affects the function of SDH. Their findings revealed that in the presence of NAD, purified SDH is covalently modified at its catalytic cysteine 152 residue by the formation of a thioglycosidic (thiozolidone) linkage between the amino-group of the cysteine and the ADP-ribose moiety of NAD. Further, in the crude streptococcal cell-wall extract, only SDH is ADP-ribosylated. Interestingly, the findings reported by those authors revealed that the GAS cytoplasm contained a large quantity of GAPDH that was not ADP-ribosylated, possibly due to the presence of NAD-glycohydrolase (Bricker *et al*. 2002, 2005), as the GAPDH purified from the cytoplasm is ADP-ribosylated. The ADP-ribosylation of SDH is abrogated by $HgCl_2$ or free L-cysteine but not by hydroxylamine, arginine, glutamic acid, or histidine. SDH therefore functions as a cysteine-specific autoADP-ribosyl transferase.

Importantly, and consistent with other studies (Brune and Lapetina 1989; Zhang and Snyder 1992; Kots *et al*. 1993), spontaneously generated nitric oxide also enhances the ADP-ribosylation of SDH. Both S-nitrosylation and NO-induced ADP-ribosylation abrogate the GAPDH activity of SDH. This finding raises important questions about the physiological relevance of this modification *in vivo*, including: (1) why GAS should inactivate its essential enzyme activity; (2) what the benefit of such a post-translational modification may be; and (3) whether SDH-mediated ADP-ribosylation occurs only in the host.

Cysteine residue-directed ADP-ribosylation has been reported for the pertussis toxin; this modification targets the membrane-associated Gi protein and disrupts signal transduction, affecting cytotoxicity (Moss and Vaughan 1988; Tanuma *et al*. 1988; Jacobson *et al*. 1990; Tanuma and Endo 1990). SDH has been demonstrated to ADP-ribosylate its previously reported binding proteins (Pancholi and Fischetti 1992). More recently, another cell-wall-associated ADP-ribosyl transferase enzyme was shown to target arginine residues in host actin filaments (Icenogle *et al*. 2012; Korotkova *et al*. 2012). However, this enzyme is distinct from SDH, which also binds to actin (Pancholi and Fischetti 1992).

Similar to GAS SDH, enterohemorrhagic *Escherichia coli* GAPDH (Aguilera *et al*. 2009) and the GAPDH of the parasite *Enteroamoeba histolytica* (Delgado-Corona *et al*. 2002; Alvarez *et al*. 2007) were recently shown to possess ADP-ribosylation activity. Intracellular *Listeria monocytogenes* also exhibits ADP-ribosylation activity for GAPDH (Alvarez-Dominguez *et al*. 2008), which in turn ADP-ribosylates membrane-bound prenylated Rab5a-GDP. The latter protein is resistant to the host GDP–GTP exchange factor; this protein is not converted to Rab5a-GTP, and the maturation of the phagosome is inhibited. *Listeria* is therefore able to survive intracellularly (Table 9.1; Fig. 9.1).

Table 9.1 Post-translational modifications of SDH and resulting moonlighting functions.

Post-translational modifications	Targeted site	Functions	Reference
ADP-ribosylation	Cysteine	Transcriptional regulation/signaling	Pancholi and Fischetti 1993; Jin *et al*. 2011
Phosphorylation	Ser and Thr	Surface export (?), transcription (?)	Jin *et al*. 2011; Pancholi and Caparan 2016
Ubiquitinylation	unknown	Lysosomal entry	Ito *et al*. 2013
S-guanylation	unknown	Lysosomal entry	Ito *et al*. 2013

The post-translational reversible Ser/Thr-phosphorylation of many glycolytic enzymes, including GAPDH, by eukaryote-type Ser/Thr kinases (STKs) and phosphatase (STPs) was recently reported (Lomas-Lopez *et al*. 2007). SDH was also found to be reversibly phosphorylated by *S. pyogenes* SP-STK and SP-STP (Jin and Pancholi 2006; Agarwal *et al*. 2011). The implications of the *in vitro* modification of SDH for surface export, as revealed for *E. coli* enolase (Doran *et al*. 1999) or for interactions with other streptococcal proteins within the cytoplasm, on the cell surface or with eukaryotic proteins are presently unknown. Recently, during the co-culture of GAS with host cells, the SDH present on the surface of the intracellular population of GAS was found to be ubiquitinated (Ito *et al*. 2013). However, the physiological implications of this modification remain unclear (Table 9.1; Fig. 9.1).

9.4 Implications of the Binding of SDH to Mammalian Proteins for Cell Signaling and Virulence Mechanisms

The ability of SDH to strongly bind to several important mammalian proteins, cytoskeletal proteins, and innate immunity proteins (Pancholi and Fischetti 1992) provides evidence that the interaction between GAS and the host is dynamic. In particular, the ability of SDH to bind specifically to the ATP-binding segment of myosin that is involved in the hydrolysis of ATP indicates that SDH may play a significant role in invasion. During active infection, such cytoskeletal proteins become exposed in injured cells/tissues. SDH-acquired plasmin from the tissues or bodily fluids can also cause tissue injury due to its non-specific protease activity (Table 9.2; Fig. 9.1).

The ADP-ribosylation activity of many bacterial toxins contributes significantly to the hijacking of key intracellular signaling events in the host (Ueda and Hayaishi 1985; Moss and Vaughan 1988, 1990). The role of SDH in the regulation of intracellular signaling events through ADP-ribosylation has not yet been demonstrated. However, upon direct contact between SDH and polarized or non-polarized human pharyngeal cells, SDH causes DNA condensation, which is a landmark feature of apoptosis (Pancholi and Fischetti 1997). This study also shows that SDH contributes significantly to the GAS-induced phosphorylation

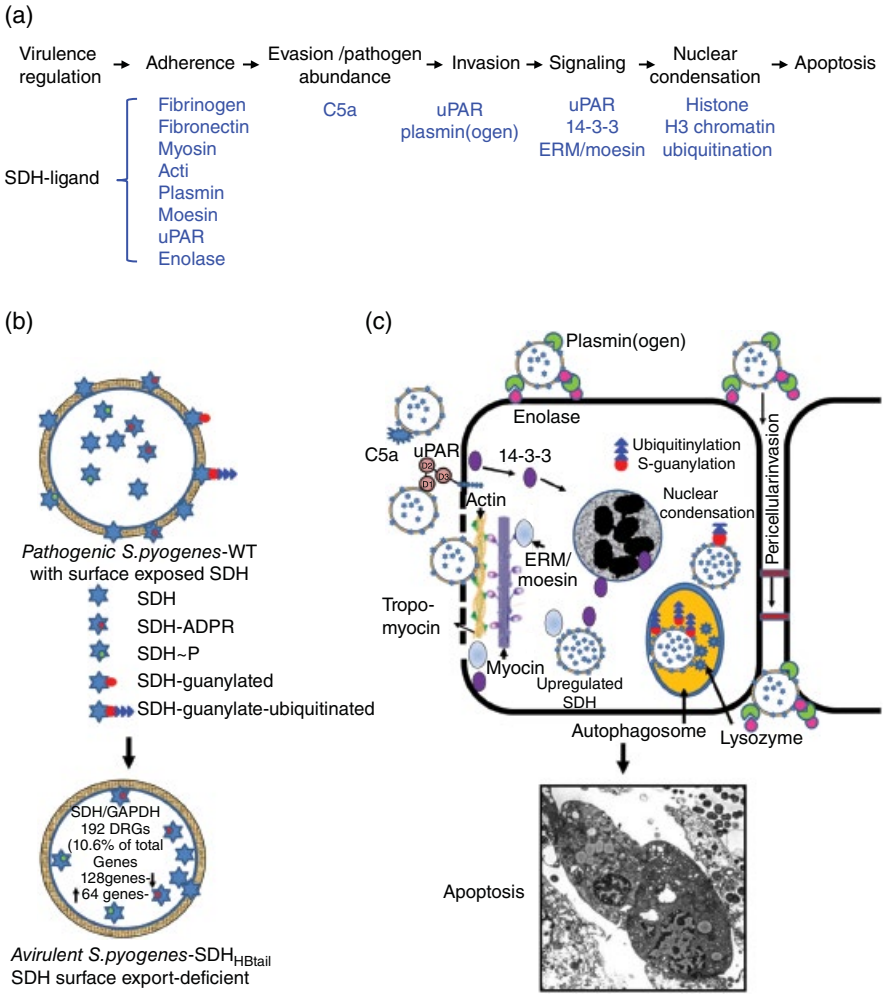

Figure 9.1 Schematic diagram showing the role of SDH in the *S. pyogenes* pathogenesis. (a) Various stages of *S. pyogenes* infection. (b) During infection, as a successful pathogen *S. pyogenes* adheres to and invades host cells. Subsequently, it hides and proliferates within the host tissues by evading host innate immune responses and maintains its abundance in a local microenvironment. *S. pyogenes* also causes apoptosis of host cells. In all these different stages of the infection, SDH participates in almost all stages by binding through different receptor ligands as illustrated. (c) The SDH surface export is also important to maintain GAS virulence as, by retaining SDH within cytoplasm, 128 genes, including 25 major virulence genes, are downregulated. SDH is therefore a quintessential important virulence regulator. The surface export of the cytoplasmic SDH and its role in the *S. pyogenes* virulence regulation may be mediated via several post-translational modifications including reported phosphorylation, ADP-ribosylation, S-guanylation, and ubiquitination. The mechanism underlying this possible and predicted regulation is however unknown. (*See color plate section for the color representation of this figure.*)

of pharyngeal proteins, including the 17 kDa histone H3 protein, which is associated with nuclear chromatin (Pancholi and Fischetti 1997). Mass spectrometry analysis of the SDH-binding human pharyngeal cell membrane-associated proteins identified four potential receptor proteins. These putative receptors

Table 9.2 SDH binding to mammalian proteins and resulting moonlighting functions.

Binding partner	Functions	Reference
Adherence		
Fibrinogen	Extracellular matrix	Pancholi and Fischetti 1992
Fironectin	Extracellular matrix	
uPAR	Epithelial cells and hematopoietic cells	Jin *et al*. 2005
Invasion		
Plasmin(ogen)	Epithelial cells and hematopoietic cells and the fibrinolytic system	Lottenberg *et al*. 1992; Pancholi *et al*. 2003; Boel *et al*. 2005; Jin *et al*. 2005
Myosin	Epithelial cells and open wound or injured cells	Pancholi and Fischetti 1992
Tropomyosin		
Actin		
Enolase	Epithelial cells and hematopoietic cells	Pancholi *et al*. 2003; Jin *et al*. 2005
Evasion		
Lysozyme	Cell-wall digesting muramidase, lysosome	Pancholi and Fischetti 1992
C5a	Complement-mediated phagocytosis	Terao *et al*. 2006
M-protein	Antiphagocytic/ factor H-binding	D'Costa *et al*. 2000
Signaling		
14-3-3	Intracellular signaling	Jin *et al*. 2005
Moesin	Intracellular signaling	Jin *et al*. 2005
uPAR	Intracellular signaling	Jin *et al*. 2005; Pancholi 2006
Pathogen abundance		
Moesin	No phagocytosis accumulation at the infection site	Jin *et al*. 2005
C5a		Tarao *et al*. 2006

include the 30–32 kDa protein 14-3-3, the 47 kDa enolase, the 55 kDa protein urokinase plasminogen activator (uPAR), and the 80 kDa protein moesin (Jin *et al*. 2005). 14-3-3 is an important regulatory protein that plays a diverse regulatory role in cell signaling (Fu *et al*. 2000; Nomura *et al*. 2003; Rosenquist 2003) and serves as a gatekeeper of phosphorylation (Yaffe 2002).

While the role of SDH and 14-3-3 in GAS infection has not been explored, the 14-3-3 protein has been shown to play a determining role in parasitic and chlamydial infections (Scidmore and Hackstadt 2001; Siles-Lucas and Gottstein 2003).

Moesin is a member of the ezrin, radixin, moesin (ERM) protein family and plays a key role in membrane cortex integration and epithelial organization; this protein is therefore involved in endothelial barrier integrity and neutrophil functions (Ivetic and Ridley 2004; Fievet *et al*. 2007; Neisch and Fehon 2011; Futosi *et al*. 2013; Garcia-Ponce *et al*. 2015). Similar to SDH, the GAS streptococcal inhibitor of complement (Sic) protein has also been shown to bind to moesin (Hoe *et al*. 2002). By binding to moesin, the Sic protein enhances bacterial survival by enabling GAS to avoid the intracellular environment, resulting in pathogen abundance at the site of infection (Hoe *et al*. 2002). Like Sic, SDH may contribute to GAS abundance during human infection and the subsequent dissemination of GAS to the community. Similar to secreted Sic, the cell-associated streptococcal C5a peptidase (ScpA) protects *S. pyogenes* from phagocytosis by enzymatically cleaving complement C5a and thus interrupting host defenses (Wexler *et al*. 1985; Cleary *et al*. 1992). Terao *et al*. (2006) reported that unlike ScpA, which binds C5a with low affinity ($K_D = 7$ mM) and cleaves C5a, SDH binds to C5a on neutrophils with very high affinity ($K_D = 0.4$ nM) and inhibits its activation, thus contributing to GAS evasion from human neutrophils. The ability of ScpA to cleave C5a may contribute to its low binding affinity. Both ScpA and SDH are therefore required for the inactivation of C5a. On the other hand, the extracellular released GAPDH from *S. pneumoniae* binds to C5a with a similar high affinity ($K_D = 0.34$ nM); however, this binding activates C5a (Terrasse *et al*. 2012). It is not clear whether this contrasting finding is caused by the absence of ScpA in *Pneumococcus* and is associated only with the secreted form of pneumococcal GAPDH (Terao *et al*. 2006). The mechanism that underlies the pneumococcal GAPDH-mediated activation of C5a is currently unknown (Table 9.2; Fig. 9.1).

The binding of SDH to the receptor uPAR/CD87 revealed that SDH binds to the D1-domain of uPAR (Jin *et al*. 2005). Based on its unique pattern of cysteine residues, uPAR possesses three Kringle-type domains (Ploug *et al*. 1993). Urokinase plasminogen activator recognizes and binds to only glycosylated uPAR. SDH binds to D1-uPAR independent of glycosylation. Further, computer modeling of SDH and competitive inhibition of SDH binding in the presence of several truncated SDH molecules revealed that the D1-urokinase domain targets the lysine residue cluster of SDH that is constituted by K336, K116, and K117. GAS mutants expressing mutated SDH_{K336L} or $SDH\Delta_{K336}$ adhere poorly to pharyngeal cells, indicating that this interaction is crucial for GAS adherence and subsequent uPAR-mediated signaling. Interestingly, Jin *et al*. (2005) demonstrated that uPAR is associated with cell membranes in a periodic fashion, indicating that uPAR is internalized into the cytoplasm and subsequently recycled prior to re-appearing on the surface. uPAR is a GPI-anchored protein that is internalized via endocytosis in a clathrin-independent manner (Nykjaer *et al*. 1997; Sturge *et al*. 2003; Cortese *et al*. 2008). It is likely that the binding of SDH to uPAR may contribute to the induction of downstream signaling events (Koshelnick *et al*. 1997; Tarui *et al*. 2003), the activation of the fibrinolytic system and GAS internalization and invasion (Table 9.2; Fig. 9.1).

The fact that the direct interaction of SDH with human pharyngeal cells results in nuclear condensation indicates that SDH has the potential to play a role in

GAS-mediated apoptosis (Pancholi and Fischetti 1997). Further, this interaction results in the *de novo* phosphorylation of the histone H3 protein. This protein and the other three histone proteins (i.e., H2A, H2B, and H4) are important components of the highly ordered octameric structure of DNA supercoils (Luger *et al*. 1997). Reversible histone Ser/Thr phosphorylation plays a decisive role in the activation and repression of gene transcription that occurs during growth and development (Wolffe *et al*. 1997; Ciccarelli and Giustetto 2014; Sawicka and Seiser 2014; Tessarz and Kouzarides 2014). H3 phosphorylation has been shown to be associated with cell apoptosis (Ajiro and Nishimoto 1985; Tikoo *et al*. 2001; Huang *et al*. 2006).

Consistent with SDH-induced H3 phosphorylation and nuclear condensation, a preliminary microarray-based study by Pancholi (2001*b*) demonstrated that the treatment of Detroit 562 cells with SDH for 12 h results in the apoptosis of 30–40% of the cells. This study also revealed significant up- and downregulation of genes that encode key pro-apoptotic proteins and anti-apoptotic proteins, respectively. The ability of SDH to induce apoptosis in pharyngeal cells is not surprising, as eukaryotic GAPDH is known to localize to the nuclear compartment and cause apoptosis (Shashidharan *et al*. 1999; Ishitani *et al*. 2003; Kusner *et al*. 2004; Sirover 2005). A recent report also provided evidence of the ability of intracellularly released GAPDH from group B *Streptococcus* to induce apoptosis in murine macrophages (Oliveira *et al*. 2012). It is not clear whether streptococcal GAPDH/SDH, like eukaryotic GAPDH (Shashidharan *et al*. 1999; Sirover 2005) and the GAS Ser/Thr phosphatase (SP-STP) (Agarwal *et al*. 2012), enters the nucleus and causes apoptosis.

Autophagy has been shown to be an essential event in GAS-mediated apoptosis (Agarwal *et al*. 2012; Nakagawa *et al*. 2004). Autophagy is an innate immune response that is characterized by a self-catabolic process that sequesters invading microbes in autophagosomes with other macromolecules and organelles. The autophagosomes eventually fuse with lysosomes and kill the intracellular bacteria (Spallek *et al*. 2009; Sorbara and Girardin 2014). Depending on the host cell, GAS appears to evade or succumb to autophagy when interacting with normal cells (Barnett *et al*. 2013; O'Seaghdha and Wessels 2013) or established immortalized cells (Ito *et al*. 2013). In the latter case, intracellular GAS is modified extensively by S-guanylate, and only modified bacteria undergo polyubiquitination. Autphagosomes then selectively internalize the ubiquitinated intracellular GAS. Interestingly, one of the surface proteins of intracellular GAS that is S-guanylated and ubiquitinated is SDH (Ito *et al*. 2013). Mono- and polyubiquitinated proteins are deubiquitinated by CYLD (cylandromatosis) deubiquitinase enzymes (Alameda *et al*. 2013; Bhattacharya and Ghosh 2014). These enzymes are expressed in higher quantities in normal cells than in cancer cells or established cell lines. The observed differences in the susceptibility of GAS to the innate autophagic response and the ability of GAS to evade such a response can therefore be partly attributed to the intracellular expression level of deubiquitinase enzymes. GAS also expresses several proteinases/hydrolases. These enzymes can also degrade ubiquitin proteins (Alameda *et al*. 2013) and allow GAS to evade host innate immune responses (Fig. 9.1).

The above description of the seemingly simple metabolic glycolytic enzyme SDH firmly establishes that, once exported to the GAS surface, SDH performs a variety of virulence-related functions and contributes to nearly every stage of GAS infection. As a successful pathogen, GAS utilizes one or more of the following war strategies when it interfaces with the host: (1) if you want to fight, come out; (2) if you want to invade, stick to it; (3) if you do not want to get killed, bring your own weapon and fight; (4) if you do not have weapons, beg, borrow, and steal them; (5) if you cannot borrow, commit suicide and make way for others; and (6) if you cannot commit suicide, behave like a host and terrorize. Correlating these strategies with SDH functions it is clear that, once exported to the GAS surface, SDH behaves like an adhesin by binding to extracellular matrix proteins. SDH also behaves like an invasin by binding to uPAR. In addition, SDH exploits and hijacks host innate immune responses by binding to C5a and possibly via ADP-ribosylation, allowing GAS to evade protective host immune responses. SDH also acquires plasminogen and enhances GAS invasion. SDH also plays an important role in the ability of GAS to modify the histone H3 protein, cause apoptosis and possibly disrupt phagolysosomal fusion. During GAS infection, changes in histone phosphorylation status can induce epigenetic changes in the host, which can adversely affect growth and development (Fig. 9.1c). Opsonic antibodies against SDH (Boel *et al*. 2005) provide *c*. 50% protection against experimental challenge with the homologous M1 or M6 GAS strains in mice. Interestingly, Fontan *et al*. (2000) revealed that the titers of anti-SDH antibodies are not elevated in the sera of patients with rheumatic fever and rheumatic heart diseases. The apparent absence of cross-reacting anti-GAPDH antibodies in patients with post-streptococcal autoimmune diseases supports the notion that SDH could serve as an important vaccine candidate. More investigations are required to validate SDH as a promising vaccine candidate, as a high-titer anti-SDH/GAPDH antibody has been reported to be associated with post-streptococcal acute glomerulonephritis (Yamakami *et al*. 2000).

9.5 Surface Export of SDH/GAPDH: A Cause or Effect?

In addition to the role of GAPDH as a multifunctional (i.e., moonlighting) protein (Sirover 2005, 2011), its surface exportation in prokaryotes is the sole reason that this protein has attained overwhelming pathophysiological importance in prokaryotic biology. The question of how this protein is able to traverse the membrane barrier without the necessary transport machinery and subsequently expressed on the bacterial surface has therefore been a major point of interest. However, it is equally important to understand why bacteria export this protein to the cell surface. This question has not received as much attention as the question of how this multifunctional protein is exported, as it is presumed that GAS and other pathogens have devised this function as a useful strategy to be more pathogenic. Evolutionarily, this hypothesis is not viable; long before the origin of humans, the *Streptococcus* species were already equipped with the unique properties of a multifunctional GAPDH and surface exportation. Rather, one can

state that *Streptococcus* species or other bacteria are pathogenic because they are endowed with a unique property that allows the surface exportation of a GAPDH with multiple functions.

In eukaryotes, many proteins including enolase (Miles *et al*. 1991; Fontan *et al*. 2000; Pancholi 2001*a*), cytokines such as IL-1β (Rubartelli *et al*. 1990, 1993*a*, *b*), and nuclear proteins such as the histone and HMGB1 proteins (Rakowicz-Szulczynska *et al*. 1991; Koutouzov *et al*. 1996; Venticinque and Meruelo 2012), are exported to the surface. In eukaryotes, such proteins are classified as proteins that are surface exported via a "non-classical or unconventional protein export" pathway (Muesch *et al*. 1990; Nickel and Seedorf 2008). This type of unconventional protein export in eukaryotes is carried out through endocytosis, caveolae, lipid rafts, and post-translational modifications.

However, defining surface export in prokaryotes, which have either two outer membranes or a membrane and a thick cell wall, is not simple. The established prokaryotic transport systems, such as the sortase A-mediated export system (Navarre and Schneewind 1999), the signal peptide-dependent (Von Heijne 1981) sec translocation system (Driessen and Nouwen 2008; Natale *et al*. 2008; Kusters and Driessen 2011; Lycklama and Driessen 2012) and twin-arginine translocation system (Natale *et al*. 2008), the Holin-antiholin system (Rice and Bayles 2003), and the mycobacterial ESX/ESAT-6 or Type VII secretion systems (Houben *et al*. 2012), do not explain the surface export of many anchorless cytoplasmic proteins, including SDH/GAPDH. Similarly, certain ABC transporters are responsible for the export of anchorless small peptides such as lantibiotics/bacteriocin (Jack *et al*. 1995; Chatterjee *et al*. 2005), antimicrobial peptides (Herbert *et al*. 2007), staphylococcal phenol-soluble modulins (Wang *et al*. 2007), and PTS-transporters for the transport of carbohydrates (Deutscher *et al*. 2006). Whether such transport systems can export larger proteins like GAPDH remains unclear.

The Caparon group identified a unique exportal system in the *S. pyogenes* membrane (Rosch and Caparon 2005; Rosch *et al*. 2007). The Exportal system is a distinct membrane lipid microdomain that contains a high concentration of translocons of the general secretory pathway and serves as the cellular site for protein secretion. In particular, Rosch and Caparon (2005) demonstrated that the heat temperature requirement A protein (HtrA) that is required for the maturation of the SpeB toxin/cysteine protease localizes exclusively to the Exportal system. Because HtrA (Clausen *et al*. 2011) is typically required for the proper folding and maturation of proteins, the co-localization of SpeB and HtrA to a single lipid microdomain indicates that the Exportal system plays a significant role in the maturation and secretion of proteins. Interestingly, immuno-electron microscopy-based experiments in GAS that revealed the location of plasminogen-binding SDH and SEN also revealed a similar clustered pattern at a specific area, particularly near the septum (Pancholi and Fischetti 1998; Rosch and Caparon 2005). Whether SDH and SEN of *S. pyogenes* localize to the Exportal system is currently unknown.

However, in the "gold rush" of this search, few export mechanisms for anchorless surface proteins have been proposed. Currently, the quick and easy explanation is that cell lysis releases cytosolic proteins. Interestingly, this mechanism is

proposed only for organisms that are lysed by their own autolysin enzymes, such as *Pneumococcus* lytA (Bergmann *et al*. 2001; Terrasse *et al*. 2012) and *S. aureus* AtlA (Pasztor *et al*. 2010). If this mechanism is accurate, one may question whether the surface export of anchorless cytoplasmic proteins is a physiological phenomenon or simply an aftereffect of lysis. Indeed, proving that a genuine export protein mechanism for anchorless surface proteins exists in fragile bacteria, such as *Pneumococcus*, would be extremely difficult. The phenomenon of lysis followed by the rebinding of proteins to the bacteria does not explain the selective surface export of certain anchorless proteins, including GAPDH, in non-lysed bacteria (Boel *et al*. 2005). Because the anchorless proteins remain associated with the cell wall/membranes and the bacterial cells are not autolysing, it is reasonable to ask why a bacterium would kill itself using its own enzymes to export and decorate its surface with cytoplasmic proteins. This altruistic hypothesis is not evolutionarily viable and is physiologically incomprehensible. A successful pathogen must develop an evolutionarily viable mechanism that allows it to survive in the most hostile environment. Despite the continuing interest in knowing this mechanism for more than two decades since the first discovery of the GAPDH protein on the surface of S. *pyogenes* (Pancholi and Fischetti 1992), a plausible, physiologically relevant, and universally acceptable answer is still not available.

This knowledge gap is caused partly because of the fact that an important question (i.e., whether the surface export of GAPDH or another anchorless protein is a cause or an effect) has not been addressed effectively. If one agrees with the above argument that surface export is an effect or an outcome of lysis, one concedes that the surface export of GAPDH/SDH is a non-physiological phenomenon. On the other hand, if surface export is a cause, we need to know why the bacterium exports this protein to the cell surface. By addressing surface export as a cause, the precise mechanism that underlies the surface export of anchorless surface proteins can be identified.

9.6 SDH: The GAS Virulence Factor-Regulating Virulence Factor

Consistent with the above argument, Boel *et al*. (2005) explained the constraints of making an SDH-negative mutant and the necessity of creating a surface export-deficient GAS mutant. SDH is encoded by a single gene and is therefore essential for GAS survival. Boel *et al*. (2005) argued that by retaining SDH essentially in the cytoplasm and concomitantly preventing its surface export, it is possible to determine why GAS exports SDH to the surface. By genetically inserting a 12-amino acid hydrophobic tail (IVLVGLVMLLLS) at the C-terminal end of SDH, those authors demonstrated that the export of SDH to the surface could be prevented, with SDH retained in the membrane. The so-called hydrophobic tail-containing SDH mutant SDH_{HBtail} displays substantially decreased plasminogen-binding and surface-associated GAPDH activity (Boel *et al*. 2005).

A similar attempt has been made to create a GAPDH export-deficient pneumococcal mutant to determine whether surface-expressed GAPDH also binds

to C5a complement factor (Terrasse *et al*. 2012). The pneumococcal surface export-deficient GAPDH mutant also exhibits decreased C5a binding, indicating that such a strategy may answer the important questions related to the pathogenesis mechanisms associated with SDH/GAPDH. Most interestingly, Boel *et al*. (2005) reported that, after preventing the surface export of SDH, the SDH_{HBtail} GAS mutant becomes defective in adherence to host cells and evasion of neutrophil-mediated phagocytosis. That study clearly demonstrated that the loss of anti-phagocytic activity occurs due to the downregulation of the major anti-phagocytic M protein. Additionally, the opsonic nature of the anti-SDH antibody that enhances the phagocytosis of the wild-type strain indicates that the antiphagocytic nature of SDH is mediated by the inactivation of C5a, as recently demonstrated by Terao *et al*. (2006). The study of Boel *et al*. (2005) therefore provided an important first step toward determining why GAS exports SDH to the surface.

Extending this study, Jin *et al*. (2011) (who are from the same group) performed a microarray analysis of this mutant and subsequently demonstrated that preventing the surface export of SDH completely attenuates GAS virulence. However, if the mutant is complemented to express the wild-type SDH, the virulence returns to the level of wild-type GAS. A microarray analysis of the SDH_{HBtail} mutant also revealed the downregulation of two-thirds of the 192 differentially regulated genes, including 24 virulence genes, in addition to the previously reported M protein (Boel *et al*. 2005; Table 9.3; Fig. 9.1). The downregulated genes included genes that encode exotoxins (SpeB), Mga and Mga-regulated genes (Jin *et al*. 2011). Another important consequence of the prevention of SDH export is significantly increased expression levels of genes responsible for the biosynthesis of saturated fatty acids with a chain length of 12–18 carbons.

The studies of Boel *et al*. (2005) and Jin *et al*. (2011) that are described above do not explain how SDH is exported to the surface. However, those studies do establish that the surface export of SDH in *S. pyogenes* is essential for maintaining bacterial virulence and is a physiologically controlled phenomenon, rather than an artifact that occurs as a result of bacterial lysis. Further, those studies also indicated that the extra SDH that could not be exported is likely to be responsible for gene regulation. These results can therefore be interpreted in two ways. Because GAPDH is known to interact with DNA (Mansur *et al*. 1993; Sirover 2005) and has been found to be an essential component of the eukaryotic transcription machinery (Zheng *et al*. 2003), the non-exported excess SDH may serve as a transcription factor. Alternatively, the differential gene regulation observed in the mutant could be an outcome of excess glycolytic activity. Because the growth of the wild-type strain and the SDH_{HBtail} GAS mutant in CDM-media supplemented with glucose, sucrose, or fructose remains unaltered (Jin *et al*. 2011), it is unlikely that the inability of the mutant to utilize complex carbohydrate is due to increased glycolytic activity. Future genomic, proteomic, and metabolomic studies may address some of these unanswered questions to reveal the mechanisms that underlie SDH-mediated gene regulation. The intracellular GAS population and the GAS biofilm community express significantly greater amounts of GAPDH (Agarwal *et al*. 2012). An increased expression level of GAPDH is therefore a marker of increased pathogenic potential in *S. pyogenes*.

Table 9.3 SDH-mediated *S. pyogenes* virulence gene regulation (Jin *et al*. 2011).

M1-GAS gene ORF#	Gene	Annotation	Fold-change GEO database reference GSE15231
Downregulation (–) out of 128 genes			
SPy_0165	*nga*	NAD-glycohydrolase	5.1
SPy_0167	*slo*	Streptolysin-O	4.76
SPy_0428	*spyA*	C3-family ADP-ribosyl transferase	2.44
SPy_0436	*speJ*	Superantigen/exotoxin-J	2.36
SPy_0738–SPy_0746	*sagA–sagI*	Streptolysin–S operon	4.75–18
SPy_1302	*amyA*	Cyclomaltodextrin glucanotransferase	10.85
SPy_1972	*pulA*	Pullulanase	2.23
SPy_1979	*ska*	Streptokinase	2.82
SPy_1983	*scl*	Collagen-like protein	4.8
SPy_1985	*rgfB*	Exodeoxyribonuclease III	3.9
SPy_2016	*sic*	Inhibitor of complement mediated lysis	4.3
SPy_2018	*emm1*	M-protein Type 1	2.2
SPy_2039	*speB*	Pyogenic exotoxin B	7.0
SPy_2043	*mf*	Mitogenic factor	3.6
SPy_2200–SPy_2202	*hasA–hasC*	Hyluronate synthase	20–22
SPy_0148 SPy_0157	*ntpI–ntpD*	V_0V_1-Na^+–ATP synthase	5.58–20.7
36 genes	*Various*	Carbohydrate metabolism	2.4–45.0
Upregulation (+) out of 64 genes			
SPy_0711	*speC*	Phage-associated pyrogenic exotoxin C	2.9
SPy_0712	*mf2*	DNase/mitogenic factor	3.22
SPy_0737	*epf*	Extracellular matrix-binding protein	10.9
SPy_1357	*grab*	Protein-related α2M-binding protein	5.35
SPy_1743–SPy_1745	*accA, D, C*	AcetylCo-A carboxylase	2.34–4.6
SPy_1746–SPy_1758	*fabZ, accB, fabF, acpP fabH, phaB*	Fatty acid biosynthesis	2.8–4.1

GAPDH is not simply a housekeeping enzyme, and the export of SDH/GAPDH is an essential process for maintaining virulence and other important functions in GAS and other bacteria. The prevention of SDH export is therefore a novel strategy for attenuating the virulence of *S. pyogenes*.

9.7 Concluding Remarks and Future Perspectives

Since the first report of the multifunctional nature of the surface-exported *S. pyogenes* GAPDH/SDH, there has been an explosion of interest in understanding the novel role of GAPDH and other anchorless surface proteins that are associated with virulence in many bacteria, especially pathogens. Together, the contributions of these reports have established that GAPDH plays a critical role in the regulation of virulence. Several attributes of SDH have demonstrated that the surface export of SDH helps GAS adhere to, invade, hide within, and kill the host cell. Surprisingly, SDH also regulates the expression of GAS virulence factors. This "double-edged" sword effect indicates that SDH is a virulence-factor-regulating virulence factor.

It is at the same time intriguing that SDH/GAPDH is endowed with numerous functions and perplexing that the surface export mechanism of SDH/GAPDH has remained unidentified. One explanation could be that GAPDH is a quintessential protein. The multifunctional nature of GAPDH has allowed many primordial organisms to evolve successfully by performing a variety of new functions until a dedicated protein/enzyme evolves to perform a specific function. Because many cellular functions are environmentally regulated, SDH/GAPDH may be playing a "proxy role" to maintain uninterrupted cellular function and promote bacterial survival and successful proliferation. In the future, we may identify more functions that are performed by SDH/GAPDH, including its role in epigenetics. For some bacteria, including *S. pyogenes*, GAPDH appears to be a protective antigen. However, the association of this protein with autoimmune diseases may raise questions. Despite these caveats, SDH/GAPDH remains an amazing candidate molecule for prokaryotic and eukaryotic fundamental, applied, and translational research.

References

Agarwal, S., Agarwal, S., Pancholi, P., and Pancholi, V. (2011) Role of Serine/threonine phosphatase (SP-STP) in *Streptococcus pyogenes* physiology and virulence. *Journal of Biological Chemistry* 286: 41368–41380.

Agarwal, S., Agarwal, S., Jin, H., Pancholi, P., and Pancholi, V. (2012) Serine/threonine phosphatase (SP-STP), secreted from *Streptococcus pyogenes*, is a proapoptotic protein. *Journal of Biological Chemistry* 287: 9147–9167.

Aguilera, L., Gimenez, R., Badia, J., Aguilar, J., and Baldoma, L. (2009) NAD+-dependent post-translational modification of *Escherichia coli* glyceraldehyde-3-phosphate dehydrogenase. *International Microbiology* 12: 187–192.

Ajiro, K. and Nishimoto, T. (1985) Specific site of histone H3 phosphorylation related to the maintenance of premature chromosome condensation. *Journal of Biological Chemistry* 260: 15379–15381.

Akimaru, J., Matsuyama, S.-I., Tokuda, H., and Mizushima, S. (1991) Reconstitution of a protein translocation system containing purified SecY, SecE, and SecA from *Escherichia coli*. *Proceedings of the National Academy of Science, USA* 88: 6545–6549.

Alameda, J.P., Fernandez-Acenero, M.J., Quintana, R.M., Page, A., Ramirez, A., Navarro, M., and Casanova, M.L. (2013) Functional inactivation of CYLD promotes the metastatic potential of tumor epidermal cells. *Journal of Investigative Dermatology* 133: 1870–1878.

Alvarez, A.H., Martinez-Cadena, G., Silva, M.E., Saavedra, E., and Avila, E.E. (2007) *Entamoeba histolytica*: ADP-ribosylation of secreted glyceraldehyde-3-phosphate dehydrogenase. *Experimental Parasitology* 117: 349–356.

Alvarez, R.A., Blaylock, M.W., and Baseman, J.B. (2003) Surface localized glyceraldehyde-3-phosphate dehydrogenase of *Mycoplasma genitalium* binds mucin. *Molecular Microbiology* 48: 1417–1425.

Alvarez-Dominguez, C., Madrazo-Toca, F., Fernandez-Prieto, L., Vandekerckhove, J., Pareja, E., Tobes, R. *et al*. (2008) Characterization of a *Listeria monocytogenes* protein interfering with Rab5a. *Traffic* 9: 325–337.

Barnett, T.C., Liebl, D., Seymour, L.M., Gillen, C.M., Lim, J.Y., Larock, C.N. *et al*. (2013) The globally disseminated M1T1 clone of group A *Streptococcus* evades autophagy for intracellular replication. *Cell Host and Microbe* 14: 675–682.

Bergmann, S., Rohde, M., Chhatwal, G.S., and Hammerschmidt, S. (2001) a-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Molecular Microbiology* 40: 1273–1287.

Bergmann, S., Rohde, M., and Hammerschmidt, S. (2004) Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein 16. *Infection and Immunity* 72: 2416–2419.

Bermudez, L.E., Petrofsky, M., and Shelton, K. (1996) Epidermal growth factor-binding protein in *Mycobacterium avium* and *Mycobacterium tuberculosis*: a possible role in the mechanism of infection. *Infection and Immunity* 64: 2917–2922.

Bhattacharya, S. and Ghosh, M.K. (2014) Cell death and deubiquitinases: perspectives in cancer. *BioMed Research International* 2014: 435197.

Boel, G., Jin, H., and Pancholi, V. (2005) Inhibition of cell surface export of group A streptococcal anchorless surface dehydrogenase affects bacterial adherence and antiphagocytic properties. *Infection and Immunity* 73: 6237–6248.

Boradia, V.M., Malhotra, H., Thakkar, J.S., Tillu, V.A., Vuppala, B., Patil, P. *et al*. (2014) *Mycobacterium tuberculosis* acquires iron by cell-surface sequestration and internalization of human holo-transferrin. *Nature Communications* 5: 4730.

Boyle, M.D.P. and Lottenberg, R. (1997) Plasminogen activation by invasive human pathogens. *Thromb Haemost* 77: 1–10.

Bricker, A.L., Cywes, C., Ashbaugh, C.D., and Wessels, M.R. (2002) NAD+-glycohydrolase acts as an intracellular toxin to enhance the extracellular survival of group A streptococci. *Molecular Microbiology* 44: 257–269.

Bricker, A.L., Carey, V.J., and Wessels, M.R. (2005) Role of NADase in virulence in experimental invasive group A streptococcal infection. *Infection and Immunity* 73: 6562–6566.

Brune, B. and Lapetina, E.G. (1989) Activation of a cytosolic ADP-ribosyltrnsferase by nitric oxide-generating agents. *Journal of Biological Chemistry* 264: 8455–8458.

Carapetis, J.R. (2008) Rheumatic heart diseases in Asia. *Circulation* 118: 2748–2753.

Carapetis, J.R. and Currie, B.J. (1996) Group A streptococcus, pyoderma, and rheumatic fever. *Lancet* 347: 1271–1272.

Carapetis, J.R., Steer, A.C., Mulholland, E.K., and Weber, M. (2005) The global burden of group A streptococcal diseases. *Lancet Infectious Diseases* 5: 685–694.

Chatterjee, C., Paul, M., Xie, L., and van der Donk, W.A. (2005) Biosynthesis and mode of action of lantibiotics. *Chemical Reviews* 105: 633–684.

Ciccarelli, A. and Giustetto, M. (2014) Role of ERK signaling in activity-dependent modifications of histone proteins. *Neuropharmacology* 80: 34–44.

Clausen, T., Kaiser, M., Huber, R., and Ehrmann, M. (2011) HTRA proteases: regulated proteolysis in protein quality control. *Natre Reviews Molecular Cell Biology* 12: 152–162.

Cleary, P.P., Prahbu, U., Dale, J.B., Wexler, D.E., and Handley, J. (1992) Streptococcal C5a peptidase is a highly specific endopeptidase. *Infection and Immunity* 60: 5219–5223.

Cortese, K., Sahores, M., Madsen, C.D., Tacchetti, C., and Blasi, F. (2008) Clathrin and LRP-1-independent constitutive endocytosis and recycling of uPAR. *PLoS One* 3: e3730.

Cunningham, M.W. (2000) Pathogenesis of group A streptococcal infections. *Clinical Microbiology Reviews* 13: 470–511.

D'Costa, S.S., Romer, T.G., and Boyle, M.D.P. (2000) Analysis of expression of a cytosolic enzyme on the surface of *Streptococcus pyogenes*. *Biochemical and Biophysical Research Communications* 278: 826–832.

Delgado-Corona, P., Martinez-Cadena, G., Alvarez, A.H., Torres-Calzada, H.E., and Avila, E.E. (2002) An extracellular monoADP-ribosyl transferase activity in Entamoeba histolytica trophozoites. *Journal of Eukaryotic Microbiology* 49: 454–459.

Deutscher, J., Francke, C., and Postma, P.W. (2006) How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiology and Molecular Biology Reviews* 70: 939–1031.

Doran, J.D., Nomizu, M., Takebe, S., Menard, R., Griffith, D., and Ziomek, E. (1999) Autocatalytic processing of the streptococcal cysteine protease zymogen: processing mechanism and characterization of the autoproteolytic cleavage sites. *European Journal of Biochemistry* 263: 145–151.

Driessen, A.J. and Nouwen, N. (2008) Protein translocation across the bacterial cytoplasmic membrane. *Annual Review of Biochemistry* 77: 643–667.

Egea, L., Aguilera, L., Gimenez, R., Sorolla, M.A., Aguilar, J., Badia, J., and Baldoma, L. (2007) Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathogenic *Escherichia coli*: interaction of the extracellular enzyme with human plasminogen and fibrinogen. *International Journal of Biochemistry and Cell Biology* 39: 1190–1203.

Espinosa-Urgel, M. and Kolter, R. (1998) *Escherichia coli* genes expressed preferentially in an aquatic environment. *Molecular Microbiology* 28: 325–332.

Fievet, B., Louvard, D., and Arpin, M. (2007) ERM proteins in epithelial cell organization and functions 76. *Biochimica et Biophysica Acta* 1773: 653–660.

Fischetti, V.A. (1989) Streptococcal M protein: Molecular design and biological behavior. *Clinical Microbiology Reviews* 2: 285–314.

Fischetti, V.A. (2006) Surface proteins on Gram-positive bacteria. In *Gram-positive Pathogens* (eds Fischetti, V.A., Novick, R.P., Ferretti, J.J., Portnoy, D.A., and Rood, J.I.), pp. 12–25. ASM Press, Washington, DC.

Fischetti, V.A. and Fazio-Zanakis, M. (1985) Electron microscopy of the group A streptococcal cell surface. In *Recent Advances in Streptococci and Streptococcal Diseases* (eds Kimura, Y., Kotani, S., and Shiokawa, Y.), pp. 180–183. Reedbooks, Ltd., Chertsey, UK.

Fischetti, V.A., Gotschlich, E.C., and Bernheimer, A.W. (1971) Purification and physical properties of group C streptococcal phage-associated lysin. *Journal of Experimental Medicine* 133: 1105–1117.

Fischetti, V.A., Pancholi, V., and Schneewind, O. (1990) Conservation of a hexapeptide sequence in the anchor region of surface proteins of Gram-positive cocci. *Molecular Microbiology* 4: 1603–1605.

Fontan, P.A., Pancholi, V., Nociari, M.M., and Fischetti, V.A. (2000) Antibodies to streptococcal surface enolase react with human a-enolase:Implications in poststreptococcal sequelae. *Journal of Infectious Diseases* 182: 1712–1721.

Fu, H., Subramanian, R.R., and Masters, S.C. (2000) 14-3-3 Proteins: Structure, function, and regulation. *Annual Review of Pharmacology and Toxicology* 40: 617–647.

Futosi, K., Fodor, S., and Mocsai, A. (2013) Neutrophil cell surface receptors and their intracellular signal transduction pathways. *International Immunopharmacology* 17: 638–650.

Garcia-Ponce, A., Citalan-Madrid, A.F., Velazquez-Avila, M., Vargas-Robles, H., and Schnoor, M. (2015) The role of actin-binding proteins in the control of endothelial barrier integrity. *Thromb Haemost* 113: 20–36.

Gil-Navarro, I., Gil, M.L., Casanova, M., O'Connor, J.E., Martinez, J.P., and Gozalbo, D. (1997) The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is a surface antigen. *Journal of Bacteriology* 179: 4992–4999.

Goudot-Crozel, V., Caillol, D., Djabali, M., and Dessein, A.J. (1989) The major parasite surface antigen associated with human resistance to schistosomiasis is a 37-kD glyceraldehyde-3P-dehydrogenase. *Journal of Experimental Medicine* 170: 2065–2080.

Gozalbo, D., Gil-Navarro, I., Azorin, I., Renau-Piqueras, J., Martinez, J.P., and Gil, M.L. (1998) The cell wall-associated glycerladehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein. *Infection and Immunity* 66: 2052–2059.

Grifantini, R., Bartolini, E., Muzzi, A., Draghi, M., Frigimelica, E., Berger, J. *et al.* (2002) Gene expression profile in Neisseria memingitidis and Neisseria lactamica upon host-cell contact: from basic research to vaccine development. *Annals of the NY Academy of Sciences* 975: 202–216.

Henderson, B. and Martin, A. (2011) Bacterial virulence in the moonlight:Multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity* 79: 3476–3491.

Herbert, S., Bera, A., Nerz, C., Kraus, D., Peschel, A., Gerke, C. *et al.* (2007) Molecular basis of resistance to muramidase and cationic antimicrobialpeptide activity of lysozyme in staphylococci. *PLoS Pathogens* 3: 981–994.

Herwald, H., Cramer, H., Morgelin, M., Russell, W., Sollenberg, U., Norrby-Teglund, A. *et al*. (2004) M protein, a classical bacterial virulence determinant, forms complexes with fibrinogen that induce vascular leakage. *Cell* 116: 367–379.

Hoe, N.P., Ireland, R.M., DeLeo F.R., Gowen, B.B., Dorward, D.W., Voyich, J.M. *et al*. (2002) Insight into the molecular basis of pathogen abundance: Group A streptococcus inhibitor of complement in hibits bacterial adherence and internalization into human cells. *Proceedings of the National Academy of Science, USA* 99: 7646–7651.

Houben, D., Demangel, C., van, I.J., Perez, J., Baldeon, L., Abdallah, A.M. *et al*. (2012) ESX-1-mediated translocation to the cytosol controls virulence of mycobacteria. *Cell Microbiology* 14: 1287–1298.

Huang, X., Kurose, A., Tanaka, T., Traganos, F., Dai, W., and Darzynkiewicz, Z. (2006) Sequential phosphorylation of Ser-10 on histone H3 and ser-139 on histone H2AX and ATM activation during premature chromosome condensation: relationship to cell-cycle phase and apoptosis. *Cytometry A* 69: 222–229.

Icenogle, L.M., Hengel, S.M., Coye, L.H., Streifel, A., Collins, C.M., Goodlett, D.R., and Moseley, S.L. (2012) Molecular and biological characterization of Streptococcal SpyA-mediated ADP-ribosylation of intermediate filament protein vimentin. *Journal of Biological Chemistry* 287: 21481–21491.

Ishitani, R., Tajima, H., Takata, H., Tsuchiya, K., Kuwae, T., Yamada, M. *et al*. (2003) Proapoptotic protein glyceraldehyde-3-phosphate dehydrogenase: a possible site of action of antiapoptotic drugs. *Progress in Neuro-psychopharmacology and Biological Psychiatry* 27: 291–301.

Ito, C., Saito, Y., Nozawa, T., Fujii, S., Sawa, T., Inoue, H. *et al*. (2013) Endogenous nitrated nucleotide is a key mediator of autophagy and innate defense against bacteria. *Molecular Cell* 52: 794–804.

Ivetic, A. and Ridley, A.J. (2004) Ezrin/radixin/moesin proteins and Rho GTPase signalling in leucocytes. *Immunology* 112: 165–176.

Jack, R.W., Tagg, J.R., and Ray, B. (1995) Bacteriocins of gram-positive bacteria. *Microbiology Reviews* 59: 171–200.

Jacobson, M.K., Loflin, P.T., Aboul-Ela, N., Mingmuang, M., Moss, J., and Jobson, E.L. (1990) Modification of plasma membrane protein cysteine residues by ADP-ribose *in vivo*. *Journal of Biological Chemistry* 265: 10825–10828.

Jin, H. and Pancholi, V. (2006) Identification and biochemical characterization of a eukaryotic-type serine/threonine kinase and its cognate phosphatase in *Streptococcus pyogenes*: Their biological functions and substrate identification. *Journal of Molecular Biology* 357: 1351–1372.

Jin, H., Song, Y.P., Boel, G., Kochar, J., and Pancholi, V. (2005) Group A streptococcal surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *Journal of Molecular Biology* 350: 27–41.

Jin, H., Agarwal, S., Agarwal, S., and Pancholi, V. (2011) Surface export of GAPDH/SDH, a glycolytic enzyme, is essential for *Streptococcus pyogenes* virulence. *mBio* 2: e00068–11.

Kenney, B. and Finlay, B.B. (1995) Protein secretion by enteropathogenic *Escherichia coli* is essential for transducting signals to epithelial cells. *Proceedings of the National Academy of Science, USA* 92: 7791–7795.

Kinoshita, H., Uchida, H., Kawai, Y., Kawasaki, T., Wakahara, N., Matsuo, H. *et al*. (2008*a*) Cell surface Lactobacillus plantarum LA 318 glyceraldehyde-3-phosphate dehydrogenase (GAPDH) adheres to human colonic mucin. *Journal of Applied Microbiology* 104: 1667–1674.

Kinoshita, H., Wakahara, N., Watanabe, M., Kawasaki, T., Matsuo, H., Kawai, Y. *et al*. (2008*b*) Cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of Lactobacillus plantarum LA 318 recognizes human A and B blood group antigens. *Research in Microbiology* 159: 685–691.

Korotkova, N., Hoff, J.S., Becker, D.M., Quinn, J.K., Icenogle, L.M., and Moseley, S.L. (2012) SpyA is a membrane-bound ADP-ribosyltransferase of Streptococcus pyogenes which modifies a streptococcal peptide, SpyB. *Molecular Microbiology* 83: 936–952.

Koshelnick, Y., Ehart, M., Hufnagl, P., Heinrich, P.C., and Binder, B.R. (1997) Urokinase receptor is associated with the components of the JAK1/STAT1 singaling pathway and leads to activation of this pathway upon receptor clustering in the human kindey epithelial tumor cell line TCL-598. *Journal of Biological Chemistry* 272: 28563–28567.

Kots, A.Y., Skurat, A.V., Sergienko, E.A., Bulargina, T.V., and Severin, E.S. (1992) Nitroprusside stimulates the cysteine-specific mono(ADP- ribosylation) of glyceraldehyde-3-phosphate dehydrogenase from human erythrocytes. *FEBS Letters* 300: 9–12.

Kots, A.Y., Sergienk, E.A., Bulargina, T.V., and Severin, E.S. (1993) Glyceraldehyde-3-phosphate activates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *FEBS Letters* 1: 33–36.

Koutouzov, S., Cabrespines, A., Amoura, Z., Chabre, H., Lotton, C., and Bach, J.F. (1996) Binding of nucleosomes to a cell surface receptor: redistribution and endocytosis in the presence of lupus antibodies. *European Journal of Immunology* 26: 472–486.

Kreikemeyer, B., McIver, K.S., and Podbielski, A. (2003) Virulence factors regulation and regulatory networks in *Streptococcus pyogenes* and their impact on pathogen-host interactions. *Trends in Microbiology* 11: 224–232.

Kusner, L.L., Sarthy, V.P., and Mohr, S. (2004) Nuclear translocation of glyceraldehyde-3-phosphate dehydrogenase: a role in high glucose-induced apoptosis in retinal Muller cells. *Investigative Ophthalmology and Visual Science* 45: 1553–1561.

Kusters, I. and Driessen, A.J. (2011) SecA, a remarkable nanomachine. *Cell and Molecular Life Sciences* 68: 2053–2066.

Lomas-Lopez, R., Paracuellos, P., Riberty, M., Cozzone, A.J., and Duclos, B. (2007) Several enzymes of the central metabolism are phosphorylated in *Staphylococcus aureus*. *FEMS Microbiol Letters* 272: 35–42.

Lottenberg, R., Broder, C.C., Boyle, M.D.P., Kain, S.J., Schroeder, B.L., and Curtiss III, R. (1992) Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. *Journal of Bacteriology* 174: 5204–5210.

Luger, K., Mader, A.W., Richmond, R.K., Sargent, D.F., and Richmond, T.J. (1997) Crystal structure of the nucleosome core particle at 2.8A resolution. *Nature* 389: 251–260.

Lycklama, A.N.J. and Driessen, A.J. (2012) The bacterial Sec-translocase: structure and mechanism. *Philosophical Transactions of the Royal Society of London B: Biological Science* 367: 1016–1028.

Madureira, P., Baptista, M., Vieira, M., Magalhaes, V., Camelo, A., Oliveira, L. *et al.* (2007) *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. *Journal of Immunology* 178: 1379–1387.

Maeda, K., Nagata, H., Kuboniwa, M., Ojima, M., Osaki, T., Minamino, N., and Amano, A. (2013) Identification and characterization of Porphyromonas gingivalis client proteins that bind to Streptococcus oralis glyceraldehyde-3-phosphate dehydrogenase. *Infection and Immunity* 81: 753–763.

Mansur, N.R., Meyer-Siegler, K., Wurzer, J.C., and Sirover, M.A. (1993) Cell cycle regulation of the glyceraldehyde-3-phosphate dehyrogenase/uracil DNA glycosylase gene in normal human cells. *Nucleic Acids Research* 21(4): 993–998.

Matta, S.K., Agarwal, S., and Bhatnagar, R. (2010) Surface localized and extracellular Glyceraldehyde-3-phosphate dehydrogenase of Bacillus anthracis is a plasminogen binding protein. *Biochemica et Biophysica Acta* 1804: 2111–2120.

Mazmanian, S.K., Ton-That, H., and Schneewind, O. (2001) Sortase-catalysed anchoring of surface proteins to the cell wall of Staphylococcus aureus. *Molecular Microbiology* 40: 1049–1057.

Miles, L.A., Dahlberg, C.M., Plescia, J., Felez, J., Kato, K., and Plow, E.F. (1991) Role of cell-surface lysines in plasminogen binding to cells:Identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry* 30: 1682–1691.

Modun, B. and Williams, P. (1999) The staphylococcal transferin-binding protein is a cell wall glycerladehyde-3-phospahte dehydrogenase. *Infection and Immunity* 67: 1086–1092.

Moss, J. and Vaughan, M. (1988) ADP-ribosylation of guanyl nucleotide-binding regulatory proteins by bacterial toxins. *Advances in Enzymology* 61: 303–379.

Moss, J. and Vaughan, M. (1990) I. Bacterial ADP-ribosyltransferases:toxins and related proteins. In *ADP-ribosylating Toxins and G Proteins* (eds Moss, J. Vaughan M.), pp. 3–155. American Society for Microbiology, Washington, DC.

Muesch, A., Hartmann, E., Rhode, K., Rubartelli, A., Sitia, R., and Rapoport, T.A. (1990) A novel pathway for secretory proteins ? *Trends in Biochemical Science* 15: 86–88.

Musser, J.M. and DeLeo, F.R. (2005) Toward a genome-wide systems biology analysis of host-pathogen interactions in group A Streptococcus. *American Journal of Pathology* 167: 1461–1472.

Musser, J.M. and Shelburne, S.A., III (2009) A decade of molecular pathogenomic analysis of group A Streptococcus. *Journal of Clinical Investigations* 119: 2455–2463.

Nakagawa, I., Amano, A., Mizushima, N., Yamamoto, A., Yamaguchi, H., Kamimoto, T. *et al.* (2004) Autophagy defends cells against invading group A Streptococcus. *Science* 306: 1037–1040.

Nasser, W., Beres, S.B., Olsen, R.J., Dean, M.A., Rice, K.A., Long, S.W. *et al.* (2014) Evolutionary pathway to increased virulence and epidemic group A Streptococcus disease derived from 3, 615 genome sequences. *Proceedings of the National Academy of Science, USA* 111: E1768–E1776.

Natale, P., Bruser, T., and Driessen, A.J. (2008) Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane-distinct translocases and mechanisms. *Biochimica et Biophysica Acta* 1778: 1735–1756.

Navarre, W.W. and Schneewind, O. (1994) Proteolytic cleavage and cell wall anchoring at the LPXTG motif of surface proteins in gram-positive bacteria. *Molecular Microbiology* 14: 115–121.

Navarre, W.W. and Schneewind, O. (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiology and Molecular Biology Reviews* 63: 174–229.

Neisch, A.L. and Fehon, R.G. (2011) Ezrin, Radixin and Moesin: key regulators of membrane-cortex interactions and signaling. *Current Opinion in Cell Biology* 23: 377–382.

Nelson, D., Goldstein, J.M., Boatright, K., Harty, D.W.S., Cook, S.L., Hickman, P.J. *et al*. (2001) pH-regulatedsecretion of a glycerladehyde-3-phosphate dehydrogenase from *Streptococcus gordonii* FSS2: Purification, characterization, and cloning of the gene encoding this enzyme. *Journal of Dental Research* 80: 371–377.

Nickel, W. and Seedorf, M. (2008) Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. *Annual Review of Cell and Developmental Biology* 24: 287–308.

Nomura, M., Shimuzu, S., Sugiyama, T., Narita, M., Ito, T., Matsuda, H., and Tsujimoto, Y. (2003) 14-3-3 interacts directly with and negatively regulates pro-apoptotic Bax. *Journal of Biological Chemistry* 278: 2058–2005.

Nykjaer, A., Conese, M., Christensen, E.I., Olson, D., Cremona, O., Gliemann, J., and Blasi, F. (1997) Recycling of the urokinase receptor upon internalization of the uPA:serpin complexes. *EMBO Journal* 16: 2610–2620.

O'Seaghdha, M. and Wessels, M.R. (2013) Streptolysin O and its co-toxin NAD-glycohydrolase protect group A *Streptococcus* from xenophagic killing. *PLoS Pathogens* 9: e1003394.

Oliveira, L., Madureira, P., Adrade, E.B., Bouaboud, A., Morello, E., Ferreira, P. *et al*. (2012) Group B *Streptococcus* GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. *PLoS One* 7: e29963.

Pancholi, V. (2001*a*) Multifunctional a-enolase: its role in diseases. *Cell and Molecular Life Sciences* 58: 902–920.

Pancholi, V. (2001*b*) The regulatory role of streptococcal Surface dehydrogenase (SDH) in the expression of cytokines and apoptosis related genes in group A streptococci infected human pharyngeal cells. 101st ASM General Meeting, Orlando, Florida May 20–24, B-177-p. 80.

Pancholi, V. (2006) Streptococcus-mediated host cell signaling, In *Gram-Positive Pathogens* (eds Fischetti, V., Novick, R.P., Ferretti, J.J., Portnoy, D.A., and Rood, J.I.), pp. 100–112. ASM Press: Washington, DC.

Pancholi, V. and Caparon, M. (2016) *Streptococcus pyogenes* metabolism. In *Streptococcus pyogenes*: *Basic Biology to Clinical Manifestations [Internet]* (eds Ferretti, J.J., Stevens, D.L., and Fischetti, V.A.). NCBI Bookshelf, University of Oklahoma Health Sciences Center: Oklahoma City (OK).

Pancholi, V. and Chhatwal, G.S. (2003) Housekeeping enzymes as virulence factors for pathogens. *International Journal of Medical Microbiology* 293: 1–11.

Pancholi, V. and Fischetti, V.A. (1988) Isolation and characterization of the cell-associated region of group A streptococcal M6 protein. *Journal of Bacteriology* 170: 2618–2624.

Pancholi, V. and Fischetti, V.A. (1989) Identification of an endogeneous membrane anchor-cleaving enzyme for group A streptococcal M protein. *Journal of Experimental Medicine* 170: 2119–2133.

Pancholi, V. and Fischetti, V.A. (1992) A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate dehydrogenase with multiple binding activity. *Journal of Experimental Medicine* 176: 415–426.

Pancholi, V. and Fischetti, V.A. (1993) Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. *Proceedings of the National Academy of Science, USA* 90: 8154–8158.

Pancholi, V. and Fischetti, V.A. (1997) Regulation of the phosphorylation of human pharyngeal cell proteins by group A streptococcal surface dehydrogenase(SDH): Signal transduction between streptococci and pharyngeal cells. *Journal of Experimental Medicine* 186: 1633–1643.

Pancholi, V. and Fischetti, V.A. (1998) a-Enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. *Journal of Biological Chemistry* 273: 14503–14515.

Pancholi, V., Fontan, H. and Jin, H. (2003) Plasminogen-mediated group A streptococcal adherence to and pericellular invasion of human pharyngeal cells. *Microbial Pathogenesis* 35: 293–303.

Pasteur, L. (1880) On the extension of the germ theory to the etiology of certain common diseases. *Compte Rendu de l'Academie des Science* XC: 1033–1044.

Pasztor, L., Ziebandt, A.K., Nega, M., Schlag, M., Haase, S., Franz-Wachtel, M. *et al*. (2010) Staphylococcal major autolysin (Atl) is involved in excretion of cytoplasmic proteins. *Journal of Biological Chemistry* 285: 36794–36803.

Ploug, M., Kjalke, M., Ronne, E., Weidle, U.H., Hoyer-Hansen, G., and Dano, K. (1993) Localization of the disulfide bonds in the NH_2-terminal domain of the cellular receptor for human urokinase plasminogen activator. A domain structure belonging to a novel superfamily of glycolipid-anchored proteins.*Journal of Biological Chemistry* 268: 17539–17546.

Rakowicz-Szulczynska, E.M., Reddy, U., Vorbrodt, A., Herlyn, D., and Koprowski, H. (1991) Chromatin and cell surface receptors mediate melanoma cell growth response to nerve growth factor. *Molecular Carcinogenesis* 4: 388–396.

Ralph, A.P. and Carapetis, J.R. (2013) Group a streptococcal diseases and their global burden. *Current Topics in Microbiology and Immunology* 368: 1–27.

Rice, K.C. and Bayles, K.W. (2003) Death's toolbox: examining the molecular components of bacterial programmed cell death. *Molecular Microbiology* 50: 729–738.

Rodriguez-Iturbe, B. and Musser, J.M. (2008) The current state of post-streptococcal glomerulonehritis. *Journal of the American Society of Nephrology* 19: 1855–1864.

Rosch, J.W. and Caparon, M.G. (2005) The ExPortal: an organelle dedicated to the biogenesis of secreted proteins in *Streptococcus pyogenes*. *Molecular Microbiology* 58: 959–968.

Rosch, J.W., Hsu, F.F., and Caparon, M.G. (2007) Anionic lipids enriched at the ExPortal of *Streptococcus pyogenes*. *Journal of Bacteriology* 189: 801–806.

Rosenquist, M. (2003) 14-3-3 proteins in apoptosis. *Brazilian Journal of Medical and Biological Research* 36: 403–408.

Rubartelli, A., Cozzolino, F., Talio, M., and Sitia, R. (1990) A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO Journal* 9: 1503–1510.

Rubartelli, A., Bajetto, A., Allavena, G., Cozzolino, F., and Sitia, R. (1993*a*) Post-translational regulation of interleukin 1 beta secretion. *Cytokine* 5: 117–124.

Rubartelli, A., Bajetto, A., Bonifaci, N., Di, B.E., Solito, E., and Sitia, R. (1993*b*) A novel way to get out of the cell. *Cytotechnology* 11 Suppl 1: S37–S40.

Sawicka, A. and Seiser, C. (2014) Sensing core histone phosphorylation - a matter of perfect timing. *Biochimica et Biophysica Acta* 1839: 711–718.

Schneewind, O., Model, P., and Fischetti, V.A. (1992) Sorting of protein A to the staphylococcal cell wall. *Cell* 70: 267–281.

Scidmore, M.A. and Hackstadt, T. (2001) Mammalian 14-3-3b associates with the Chlamydia tarchomatoisis inclusion membrane via its interaction with IncG. *Molecular Microbiology* 39: 1638–1650.

Scott, J.R. and Barnett, T.C. (2006) Surface proteins of gram-positive bacteria and how they get there. *Annual Review of Microbiology* 60: 397–423.

Seidler, N.W. (2013) GAPDH, as a virulence factor. *Advances in Experimental Medicine and Biology* 985: 149–178.

Shashidharan, P., Chalmers-Redman, R.M., Carlile, G.W., Rodic, V., Gurvich, N., Yuen, T. *et al*. (1999) Nuclear translocation of GAPDH-GFP fusion protein during apoptosis. *Neuroreport* 10: 1149–1153.

Shoemaker, C., Gross, A., Gebremichael, A., and Harn, D. (1992) cDNA cloning and functional expression of the *Schistosoma mansoni* protective antigen triose-phosphate isomerase. *Proceedings of the National Academy of Science* 89: 1842–1846.

Siles-Lucas, M.d.M. and Gottstein, B. (2003) The 14-3-3 protein: a key molecule in parasitesas in other organisms. *Trends in Parasitology* 19: 575–581.

Sirover, M.A. (2005) New nuclear functions of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in mammalian cells. *Journal of Cell Biochemistry* 95: 45–52.

Sirover, M.A. (2011) On the functional diversity of glyceraldehyde-3-phosphate dehydrogenase: biochemical mechanisms and regulatory control. *Biochimica et Biophysica Acta* 1810: 741–751.

Sorbara, M.T. and Girardin, S.E. (2014) Emerging themes in bacterial autophagy. *Current Opinion in Microbiology* 23C: 163–170.

Spallek, T., Robatzek, S., and Gohre, V. (2009) How microbes utilize host ubiquitination. *Cell Microbiology* 11: 1425–1434.

Stevens, D.L. (1992) Invasive group A *Streptococcus* infections. *Clinical Infectious Diseases* 14: 2–13.

Stevens, D.L. (1995) Streptococcal toxic-shock syndrome: specturm of disease, pathogenesis, and new concepts in treatment. *Emerging Infectious Diseases* 1: 69–78.

Sturge, J., Wienke, D., East, L., Jones, G.E., and Isacke, C.M. (2003) GPI-anchored uPAR requires Endo180 for rapid directional sensing during chemotaxis. *Journal of Cell Biology* 162: 789–794.

Swedo, S.E., Schrag, A., Gilbert, R., Giovannoni, G., Robertson, M.M., Metcalfe, C. *et al*. (2010) Streptococcal infection, Tourette syndrome, and OCD: is there a connection? PANDAS: horse or zebra? *Neurology* 74: 1397–1398.

Tanuma, S. and Endo, H. (1990) Identification in human erythrocytes of mono(ADP-risbosyl) protein hydrolase that cleaves a mono(ADP-ribosyl)Gi linkage. *FEBS Letters* 261: 381–384.

Tanuma, S., Kawashima, K., and Endo, H. (1988) Eukaryotic mono (ADP-ribosyl) transferase that ADP-ribosylates GTP-binding regulatory Gi protein. *Journal of Biological Chemistry* 263: 5485–5489.

Tarui, T., Andromicos, N., Czekay, R.-P., Mazar, A.P., Bdeir, K., Parry, G.C. *et al*. (2003) Critical role of integrin a5b1 in urokinase (uPA) urokinase receptor (uPAR, CD87) signaling. *Journal of Biological Chemistry* 278: 29863–29872.

Terao, Y., Yamaguchi, M., Hamada, S., and Kawabata, S. (2006) Multifunctional glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pyogenes is essential for evasion from neutrophils. *Journal of Biological Chemistry* 281: 14215–14223.

Terrasse, R., Tacnet-Delorme, P., Moriscot, C., Perard, J., Schoehn, G., Vernet, T. *et al*. (2012) Human and pneumococcal cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) proteins are both ligands of human C1q protein. *Journal of Biological Chemistry* 287: 42620–42633.

Tessarz, P. and Kouzarides, T. (2014) Histone core modifications regulating nucleosome structure and dynamics. *Natre Reviews Molecular Cell Biology* 15: 703–708.

Tikoo, K., Lau, S.S., and Monks, T.J. (2001) Histone H3 phosphorylation is coupled to poly-(ADP-ribosylation) during reactive oxygen species-induced cell death in renal proximal tubular epithelial cells. *Molecular Pharmacology* 60: 394–402.

Ueda, K. and Hayaishi, O. (1985) ADP-ribosylation. *Annual Review of Biochemistry* 54: 73–100.

Venticinque, L. and Meruelo, D. (2012) Comprehensive proteomic analysis of nonintegrin laminin receptor interacting proteins. *Journal of Proteome Research* 11: 4863–4872.

Von Heijne, G. (1981) On the hydrophobic nature of signal sequences. *European Journal of Biochemistry* 116: 419–422.

Wang, R., Braughton, K.R., Kretschner, D., Bach, T.-H.L., Queck, S.Y., Li.M. *et al*. (2007) Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA. *Nature Medicine* 13: 1510–1514.

Wexler, D.E., Chenoweth, D.E., and Cleary, P.P. (1985) Mechanism of action of the group A streptococcal C5a inactivator. *Proceedings of the National Academy of Science, USA* 82: 8144–8148.

Wolffe, A., Wong, J., and Pruss, D. (1997) Activators and repressors: making use of chromatin to regulate transcription. *Genes to Cells* 2: 291–302.

Wong, C.J. and Stevens, D.L. (2013) Serious group a streptococcal infections. *Medical Clinics of North America* 97: 721–736, xi–xii.

Yaffe, M.B. (2002) How do 14-3-3 proteins work?-Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Letters* 513: 53–57.

Yamakami, K., Yoshizawa, N., Wakabayashi, K., Takeushi, A., Tadakuma, T., and Boyle, M.D. (2000) The potential role for nephritis-associated plasmin receptor in acute poststreptococcal glomerulonephritis. *Methods* 21: 185–197.

Zhang, J. and Snyder, S.H. (1992) Nitric oxide stiumlates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Proceedings of the National Academy of Science, USA* 89: 9382–9385.

Zheng, L., Roeder, R.G., and Luo, Y. (2003) S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. *Cell* 114: 255–266.

10

Group B *Streptococcus* GAPDH and Immune Evasion

Paula Ferreira[1,2] *and Patrick Trieu-Cuot*[3]

[1] *ICBAS, Instituto de Ciências Biomédicas de Abel Salazar, Universidade do Porto, Porto, Portugal*
[2] *Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal*
[3] *Unité de Biologie des Bactéries Pathogènes à Gram-positif, Institut Pasteur, CNRS ERL 3526, 28 rue du Dr ROUX, Paris, France*

10.1 The Bacterium GBS

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), is a major neonatal pathogen causing pneumonia, sepsis, and meningitis (Edwards and Baker 2005; Johri *et al*. 2006; Phares *et al*. 2008; Verani *et al*. 2010). GBS is also responsible for significant morbidity in pregnant women and the elderly, and a cause of mortality in immunocompromised adults (Dermer *et al*. 2004; Edwards and Baker 2005). This Gram-positive encapsulated bacterium is a commensal of the gastrointestinal and genitourinary tracts and colonizes the birth canal of up to 30% of healthy pregnant women (Johri *et al*. 2006; Verani *et al*. 2010). Vaginal colonization during pregnancy increases the incidence of premature delivery and perinatal transmission of the bacterium (Ferrieri *et al*. 1977; Schuchat 2000). Neonates likely acquire part of the mother vaginal microflora, including GBS, by vertical transmission during labor through aspiration of the infected amniotic fluid. Despite early antimicrobial treatment and improvement in neonatal intensive care, up to 10% of neonatal invasive GBS infections are lethal and 25–35% of surviving infants with meningitis experience permanent neurological sequelae (Edwards and Baker 2005). As with most extracellular pathogens, GBS survival depends on its ability to evade the host immune system; neonates, particularly those born prematurely, are of course highly vulnerable to life-threatening infections.

10.2 Neonates are More Susceptible to GBS Infection than Adults

It is known that neonates are more susceptible to infections than adults (Hostetter 2012) but this susceptibility cannot be solely ascribed to the immaturity of their immune system (Elahi *et al*. 2013). The fetus, being semi-allogenic, needs to be

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

tolerated by the mother during the pregnancy and its immune response has evolved to prevent potentially damaging inflammation that will lead to abortions or preterm delivery (Koch and Platt 2003; Levy 2007). To avoid rejection by the mother, a maternal-fetal tolerance/immunosuppression must therefore be developed (Williams 2012). In fact, during pregnancy, the immune response of the mother and the developing immune system of the fetus must tolerate each other (Koch and Platt 2003; Burt 2013). Neonatal immunotolerance is therefore essential for species survival but, on the other hand, this makes neonates extremely vulnerable to infections. Moreover, the immunosuppressive/tolerant state of the neonate is an active strategy that allows the colonization of the intestine by commensals in a non-inflammatory manner (Elahi *et al*. 2013).

The human microbial colonization begins at birth and continues to develop for about 3 years, until the microbiota becomes adult-like (Arrieta *et al*. 2014). The development of fetal tolerance toward the microbiome of the mother during pregnancy is the major factor for a successful acquisition of a normal microbiome (Zaura *et al*. 2014). Several mechanisms, including the production of IL-10, have been described as responsible for maintaining immune tolerance in neonates (Pettengill *et al*. 2014; Belderbos *et al*. 2013). The interleukin-10 (IL-10) is an anti-inflammatory cytokine that plays an important role in the induction of immune tolerance (Pettengill *et al*. 2014) and in microbiome-associated immune modulation (Levast *et al*. 2015). In mice, IL-10 deficiency results in colitis after microbial colonization (Kühn *et al*. 1993; Izcue *et al*. 2009). In humans, mutations in the genes encoding either IL-10 or its receptors results in an autosomal recessive disease characterized by early-onset severe inflammatory bowel disease (Glocker *et al*. 2009, 2011). The immune system of the newborn infant is therefore adapted for early postnatal life (Dowling and Levy 2014), but pathogens such as GBS can benefit from IL-10 anti-inflammatory activity.

10.3 IL-10 Production Facilitates Bacterial Infection

IL-10 is a cytokine that limits the immune response, thereby playing a vital role in preventing the damage induced by an over-activation of immune cells. Originally described as a product of Th2 cells, IL-10 is now recognized to be produced by a wide array of cells including immune and non-immune cells (Kalkunte *et al*. 2011; Ouyang *et al*. 2011). This cytokine binds to a specific receptor complex that activates JAK-STAT and PI3K-Akt signaling pathways and inhibits the NF-kB signaling pathway (Donnelly *et al*. 1999; Ouyang *et al*. 2011), and so restraining the cellular inflammatory process. Indeed, IL-10 affects the inflammatory function of monocytes, macrophages, and dendritic cells by inhibiting the production of proinflammatory cytokines and chemokines (Moore *et al*. 2001; Saraiva and O'Garra 2010; Chatterjee *et al*. 2014), and the expression of co-stimulatory molecules and major histocompatibility complex molecules (Moore *et al*. 2001; Thaxton and Sharma 2010). To reinforce its regulatory/suppressor properties, IL-10 also induces the upregulation of anti-inflammatory molecules, such as IL-1 receptor antagonist (IL-1RA) and the soluble TNF

receptor (Moore *et al*. 2001). Moreover, IL-10 production by a Th cell subset was observed when dendritic cells were exposed to IL-10, thereby enhancing the control of the inflammatory response (Carosella *et al*. 2011).

IL-10 therefore plays a central role in controlling the inflammatory response, an ability that can be used by pathogens to evade the host immune response. As mentioned above, neonates express a unique immune system rendering them vulnerable to infections. Several reports indicate that neonatal innate immune cells are more competent than adult cells in producing IL-10 upon Toll-like receptor (TLR) engagement by microbial products (Sun *et al*. 2005; Chelvarajan *et al*. 2007; Belderbos *et al*. 2009; Kollmann *et al*. 2009; Andrade *et al*. 2013). Moreover, leukocytes of newborn mice are highly committed to produce increased amounts of IL-10 (Genovese *et al*. 1999; Sun *et al*. 2005; Madureira *et al*. 2011) and this was also demonstrated in human neonates (Rainsford and Reen 2002; Belderbos *et al*. 2009; Kollmann *et al*. 2009).

10.4 GBS Glyceraldehyde-3-Phosphate Dehydrogenase Induces IL-10 Production

GBS is an extracellular pathogen that uses its glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to evade the host immune system by interacting with the IL-10 pathway (Madureira *et al*. 2007). GAPDH, an ubiquitous glycolytic enzyme found in large amounts in the cytoplasm of prokaryotic and eukaryotic cells, converts glyceraldehyde-3-phosphate into 1, 3-bisphosphoglycerate (Polgar 1964; Kim and Dang 2005). For more details on GAPDH see Chapters 8 and 9. However, this protein has been detected at the surface of both prokaryotic and eukaryotic cells (see also Chapter 11) and is known to exert multiple unrelated functions, making it a fascinating moonlighting protein. In particular, it constitutes an important surface-associated virulence factor of many pathogens (Alvarez *et al*. 2003; Ling *et al*. 2004; Maeda *et al*. 2004) due to its ability to bind several host proteins (Pancholi and Fischetti 1992; D'Costa *et al*. 2000; Alvarez *et al*. 2003; Bergmann *et al*. 2004). It also confers resistance against reactive oxygen species produced by host phagocytic cells (Holzmuller *et al*. 2006).

In Group A and B Streptococcus, surface-localized GADPH bind plasminogen and plasmin (Winram and Lottenberg 1996; Seifert *et al*. 2003). Although devoid of signal peptide, these streptococcal GAPDHs have been detected in their surface proteome among many other abundant cytoplasmic proteins such as the enolase, DnaK, and SodA (Nelson *et al*. 2001; Hughes *et al*. 2002; Fluegge *et al*. 2004). To explain their presence at the bacterial surface the involvement of specific export processes has been suggested, in particular that of the SecA2 secretion system (Braunstein *et al*. 2003; Lenz *et al*. 2003). However, there is now compelling evidence that these cytoplasmic proteins are released upon cell lysis (Pasztor *et al*. 2010; Oliveira *et al*. 2012). In particular, it was recently demonstrated that the presence of GAPDH at the surface of GBS was correlated with the level of bacterial lysis and that the released enzyme can re-associate with surface of living bacteria (Fig. 10.1; Oliveira *et al*. 2012).

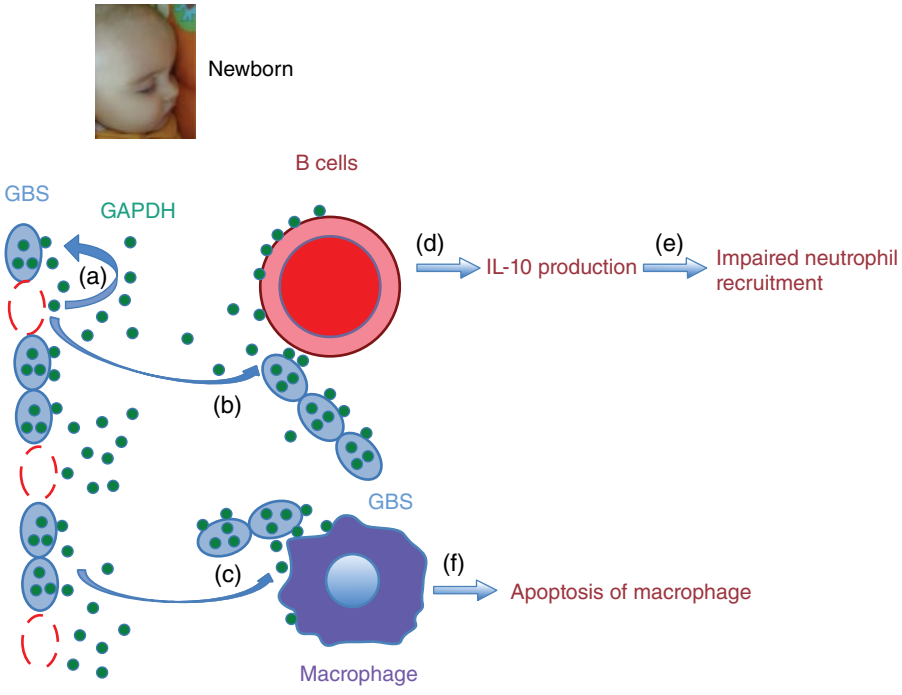

Figure 10.1 Group B *Streptococcus* GAPDH modulates the neonatal inflammatory response. (a) GBS cocci growing in chains may spontaneously lyse (dashed red cocci) and the released cytosolic GAPDH (green dot) can then re-associate with living bacteria. Free or GBS-bound GAPDH can interact with (b) B cells or with (c) macrophages. Upon interaction with B cells, GAPDH (d) triggers IL-10 production and (e) impairs neutrophil recruitment. (f) GAPDH can also interact with macrophages to induce their apoptosis. (*See color plate section for the color representation of this figure.*)

GBS GAPDH was detected at the surface of unrelated GBS isolates belonging to different serotypes and responsible for severe neonatal infections (Madureira *et al.* 2011). The extracellular form of this enzyme induces the production of IL-10 by newborn mouse cells, very early after infection (Madureira *et al.* 2011). *In vitro*, a recombinant GBS GAPDH induced an upregulation of CD69 expression on B cells from mice and also promoted macrophage apoptosis (Madureira *et al.* 2007; Oliveira *et al.* 2012). A GBS strain overexpressing GAPDH showed increased virulence in mice as compared with the wild-type strain, and the bacterial virulence was markedly reduced in IL-10-deficient and anti-rGAPDH antiserum-treated mice (Madureira *et al.* 2007). As observed with other pathogens (Reed *et al.* 1994; Genovese *et al.* 1999; Belkaid *et al.* 2001; Murphy *et al.* 2001; Silva and Appelberg 2001; Roque *et al.* 2007), GBS virulence therefore benefits from the tolerogenic IL-10-secreting phenotype induced by GAPDH, a mechanism highly adapted to the neonatal environment (Fig. 10.1).

The induction of IL-10 production rapidly following GBS infection allows bacterial colonization and survival within the host by preventing the development of a protective immune response. Neutrophil recruitment is a crucial event in the host effector immune response to GBS infections (Schuit and DeBiasio

1980; Cleat *et al.* 1984) and IL-10 production induced by GAPDH or by an inflammatory stimuli prevents the recruitment of neutrophils in infected/inflamed organs (Madureira *et al.* 2011; Andrade *et al.* 2013). Accordingly, the absence of neutrophil recruitment into infected organs was associated with neonatal susceptibility against GBS infections (Quirante *et al.* 1974; Hemming *et al.* 1976; Hill *et al.* 1988; Liu and Nizet 2004). It is therefore likely that IL-10 production induced by GBS GAPDH indirectly inhibits leukocyte trafficking to inflamed tissues and therefore prevents bacterial clearance. Moreover, it was shown that GBS GAPDH is able to induce host IL-10 production upon exposure to an oxidative agent, indicating that this mechanism still operates within the highly oxidative environment generated by the host inflammatory response (Madureira *et al.* 2011).

By inducing IL-10 production very early after infection, GAPDH should play an important role in determining host susceptibility to GBS infection. Accordingly, GBS-associated pathology can be counteracted either by GAPDH vaccination or IL-10 neutralization (Madureira *et al.* 2011). Both treatments lead to an enhanced recruitment of neutrophils to infected organs that controls the infection and allows the survival of the newborns mice (Madureira *et al.* 2011). These results indicate that the reduced ability of neonates to develop a protective response to GBS infection is not due to a deficiency/immaturity of neutrophils population but, instead, to a GAPDH-induced IL-10 production that suppresses the recruitment of these immune cells. Given the high level of identity between their GAPDH, these results could also apply to other human neonatal pathogens phylogenetically related to GBS such as GAS, pneumococci, and staphylococci.

10.5 Summary

It is remarkable that a cytosolic glycolytic enzyme, once released and complexed to the bacterial surface, behaves as a critical virulence factor that modulates the host immune response by triggering the production of the anti-inflammatory cytokine IL-10. Production of this immunosuppressive cytokine very early after infection prevents the recruitment of neutrophils, the effector cells essential for bacterial clearance. The GBS GAPDH allows the bacterium to evade the host immune system and this selective advantage may be crucial in the neonatal context where IL-10 controls the innate immune responses. In mammals, cytosolic GAPDH also interacts with diadenosine phosphates to induce neutrophil apoptosis (Baxi and Vishwanatha 1995; Gasmi *et al.* 1996), whereas a GAPDH-transferrin complex supposedly involved in iron acquisition was detected at the surface of macrophages (Raje *et al.* 2007; see Chapter 11 for more details). Importantly, intraperitoneal injection of GAPDH prevents LPS-induced sepsis in an acute lung injury mouse model, a protection associated with decreased levels of serum proinflammatory cytokines and an impaired neutrophil recruitment in the lung (Takaoka *et al.* 2014). Overall, these results suggest that mammalian GAPDH are involved in the control of the inflammatory response and that their bacterial counterparts, at least the GBS GAPDH, have retained this activity.

References

Alvarez RA, Blaylock MW, Baseman JB (2003) Surface localized glyceraldehydes-3-phosphate dehydrogenase of *Mycoplasma genitalium* binds mucin. *Molecular Microbiology* 48: 1417–1425.

Andrade EB, Alves J, Madureira P, Oliveira L, Ribeiro A, Cordeiro-da-Silva A, Correia-Neves M, Trieu-Cuot P, Ferreira P (2013) TLR2-induced IL-10 production impairs neutrophil recruitment to infected tissues during neonatal bacterial sepsis. *Journal of Immunology* 191: 4759–1768.

Arrieta MC, Stiemsma LT, Amenyogbe N, Brown EM, Finlay B (2014) The intestinal microbiome in early life: health and disease. *Frontiers in Immunology* 5: 427.

Baxi MD, Vishwanatha JK (1995) Uracil DNA-glycosylase/glyceraldehydes-3-phosphate dehydrogenase is an Ap4A binding protein. *Biochemistry* 34: 9700–9707.

Belderbos ME, van Bleek GM, Levy O, Blanken MO, Houben ML, Schuijff L, Kimpen JL, Bont L (2009) Skewed pattern of Toll-like receptor 4-mediated cytokine production in human neonatal blood: low LPS-induced IL-12p70 and high IL-10 persist throughout the first month of life. *Clinical Immunology* 133: 228–237.

Belderbos ME, Levy O, Meyaard L, Bont L (2013) Plasma-mediated immune suppression: a neonatal perspective. *Pediatric Allergy and Immunology* 24: 102–13.

Belkaid Y, Hoffmann KF, Mendez S, Kamhawi S, Udey MC, Wynn TA, Sacks DL (2001) The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. *Journal of Experimental Medicine* 194: 1497–1506

Bergmann S, Rohde M, Hammerschmidt S (2004) Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. *Infection and Immunity* 72: 2416–2419.

Braunstein M, Espinosa BJ, Chan J, Belisle JT, Jacobs WR, Jr (2003) SecA2 functions in the secretion of superoxide dismutase A and in the virulence of *Mycobacterium tuberculosis*. *Molecular Microbiology* 48: 453–464.

Burt TD (2013) Fetal Regulatory T cells and peripheral immune tolerance in utero: implications for development and disease. *American Journal of Reproductive Immunology* 69: 346–358.

Carosella ED, Gregori S, LeMaoult J (2011) The tolerogenic interplay(s) among HLA-G, myeloid APCs, and regulatory cells. *Blood* 118: 6499–5505.

Chatterjee P, Chiasson VL, Bounds KR, Mitchell BM (2014) Regulation of the anti-inflammatory cytokines interleukin-4 and interleukin-10 during pregnancy. *Frontiers in Immunology* 5: 253.

Chelvarajan L, Popa D, Liu Y, Getchell TV, Stromberg AJ, Bondada S (2007). Molecular mechanisms underlying anti-inflammatory phenotype of neonatal splenic macrophages. *Journal of Leukocyte Biology* 82: 403–416.

Cleat PH, Wells C, Coid CR (1984) Electron microscopic evidence of antibody entry into neutrophils after phagocytosis of highly virulent group B streptococci. *Journal of General Microbiology* 130: 3059–3061.

D'Costa SS, Romer TG, Boyle MD (2000) Analysis of expression of a cytosolic enzyme on the surface of *Streptococcus pyogenes*. *Biochemical and Biophysical Research Communications* 278: 826–832.

Dermer P, Lee C, Eggert J, Few B (2004) A history of neonatal group B streptococcus with its related morbidity and mortality rates in the United States. *Journal of Pediatric Nursing* 19: 357–363.

Donnelly RP, Dickensheets H, Finbloom DS (1999) The interleukin-10 signal transduction pathway and regulation of gene expression in mononuclear phagocytes. *Journal of Interferon and Cytokine Research* 19: 563–573.

Dowling DJ, Levy O (2014) Ontogeny of early life immunity. *Trends in Immunology* 35: 299–310.

Edwards MS, Baker CJ (2005) Group B Streptococcal infections in elderly adults. *Clinical Infectious Diseases* 41: 839–847.

Elahi S, Ertelt JM, Kinder JM, Jiang TT, Zhang X, Xin L, Chaturvedi V, Strong BS, Qualls JE, Steinbrecher KA, Kalfa TA, Shaaban AF, Way SS (2013) Immunosuppressive CD71+ erythroid cells compromise neonatal host defence against infection. *Nature* 504: 158–162.

Ferrieri P, Cleary PP, Seeds AE (1977) Epidemiology of group B streptococcal carriage in pregnant women and newborn infants. *Journal of Medical Microbiology* 10: 103–114.

Fluegge K, Schweier O, Schiltz E, Batsford S, Berner R (2004) Identification and immunoreactivity of proteins released from *Streptococcus agalactiae*. *European Journal of Clinical Microbiology and Infectious Diseases* 23: 818–824.

Gasmi L, McLennan AG, Edwards SW (1996) Regulation of neutrophil apoptosis by diadenosine pentaphosphate and GM-CSF. *Biochemical Society Transactions* 24: 491S.

Genovese F, Mancuso G, Cuzzola M, Biondo C, Beninati C, Delfino D, Teti G (1999) Role of IL-10 in a neonatal mouse listeriosis model. *Journal of Immunology* 163: 2777–2782.

Glocker EO, Kotlarz D, Boztug K, Gertz EM, Schäffer AA, Noyan F, Perro M, Diestelhorst J, Allroth A, Murugan D, Hätscher N, Pfeifer D, Sykora KW, Sauer M, Kreipe H, Lacher M, Nustede R, Woellner C, Baumann U, Salzer U, Koletzko S, Shah N, Segal AW, Sauerbrey A, Buderus S, Snapper SB, Grimbacher B, Klein C (2009) Inflammatory bowel disease and mutations affecting the interleukin-10 receptor. *New England Journal of Medicine* 361: 2033–2045.

Glocker EO, Kotlarz D, Klein C, Shah N, Grimbacher B (2011) IL-10 and IL-10 receptor defects in humans. *Annals of NY Academy of Sciences* 1246: 102–107.

Hemming VG, McCloskey DW, Hill HR (1976) Pneumonia in the neonate associated with group B streptococcal septicemia. *American Journal of Diseases of Children* 130: 1231–1233.

Hill HR, Bohnsack JF, Morris EZ, Augustine NH, Parker CJ, Cleary PP, Wu JT (1988) Group B streptococci inhibit the chemotactic activity of the fifth component of complement. *Journal of Immunology* 141: 3551–3556.

Holzmuller P, Hide M, Sereno D, Lemesre JL (2006) *Leishmania infantum* amastigotes resistant to nitric oxide cytotoxicity: impact on in vitro parasite developmental cycle and metabolic enzyme activities. *Infection, Genetics and Evolution* 6: 187–197.

Hostetter MK (2012) What we don't see. *New England Journal of Medicine* 366: 1328–1334.

Hughes MJ, Moore JC, Lane JD, Wilson R, Pribul PK, Younes ZN, Dobson RJ, Everest P, Reason AJ, Redfern JM, Greer FM, Paxton T, Panico M, Morris HR, Feldman RG, Santangelo JD (2002) Identification of major outer surface proteins of Streptococcus agalactiae. *Infection and Immunity* 70: 1254–1259.

Izcue A, Coombes JL, Powrie F (2009) Regulatory lymphocytes and intestinal inflammation. *Annual Review of Immunology* 27: 313–38.

Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuolli R (2006) Group B Streptococcus: global incidence and vaccine development. *Nature Reviews Microbiology* 4: 932–942.

Kalkunte S, Nevers T, Norris WE, Sharma S (2011) Vascular IL-10: a protective role in preeclampsia. *Journal of Reproductive Immunology* 88: 165–169.

Kim JW, Dang CV (2005) Multifaceted roles of glycolytic enzymes. *Trends in Biochemical Sciences* 30: 142–150.

Koch CA, Platt JL (2003) Natural mechanisms for evading graft rejection: the fetus as an allograft. *Springer Seminars in Immunopathology* 25: 95–117.

Kollmann TR, Crabtree J, Rein-Weston A, Blimkie D, Thommai F, Wang XY, Lavoie PM, Furlong J, Fortuno ES 3rd, Hajjar AM, Hawkins NR, Self SG, Wilson CB (2009) Neonatal innate TLR-mediated responses are distinct from those of adults. *Journal of Immunology* 183: 7150–1760.

Kühn R, Löhler J, Rennick D, Rajewsky K, Müller W (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.

Lenz LL, Mohammadi S, Geissler A, Portnoy DA (2003) SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. *Proceedings of the National Academy of Science, USA* 100: 12432–12437.

Levast B, Li Z, Madrenas J (2015) The role of IL10 in microbiome associated immune modulation and disease tolerance. *Cytokine* 75(2): 291–301.

Levy O (2007) Innate immunity of the newborn: basic mechanisms and clinical correlates. *Nature Reviews Immunology* 7: 379–390.

Ling E, Feldman G, Portnoi M, Dagan R, Overweg K, Mulholland F, Chalifa-Caspi V, Wells J, Mizrachi-Nebenzahl Y (2004) Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. *Clinical and Experimental Immunology* 138: 290–298.

Liu GY, Nizet V (2004) Extracellular virulence factors of group B Streptococci. *Frontiers in Bioscience* 9: 1794–1802.

Madureira P, Baptista M, Vieira M, Magalhães V, Camelo A, Oliveira L, Ribeiro A, Tavares D, Trieu-Cuot P, Vilanova M, Ferreira P (2007) *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. *Journal of Immunology* 178: 1379–1387.

Madureira P, Andrade EB, Gama B, Oliveira L, Moreira S, Ribeiro A, Correia-Neves M, Trieu-Cuot P, Vilanova M, Ferreira P (2011) Inhibition of IL-10 production by maternal antibodies against Group B Streptococcus GAPDH confers immunity to offspring by favoring neutrophil recruitment. *PLoS Pathogens* 7: e1002363.

Maeda K, Nagata H, Kuboniwa M, Kataoka K, Nishida N, Tanaka M, Shizukuishi S (2004). Characterization of binding of *Streptococcus oralis* glyceraldehydes-

3-phosphate dehydrogenase to Porphyromonas gingivalis major fimbriae. *Infection and Immunity* 72: 5475–5477.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annual Review of Immunology* 19: 683–765.

Murphy ML, Wille U, Villegas EN, Hunter CA, Farrell JP (2001) IL-10 mediates susceptibility to *Leishmania donovani* infection. *European Journal of Immunology* 31: 2848–2856.

Nelson D, Goldstein JM, Boatright K, Harty DW, Cook SL, Hickman PJ, Potempa J, Travis J, Mayo JA (2001) pH-regulated secretion of a glyceraldehydes-3-phosphate dehydrogenase from *Streptococcus gordonii* FSS2: purification, characterization, and cloning of the gene encoding this enzyme. *Journal of Dental Research* 80: 371–377.

Oliveira L, Madureira P, Andrade EB, Bouaboud A, Morello E, Ferreira P, Poyart C, Trieu-Cuot P, Dramsi S (2012) Group B streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. *PLoS One* 7: e29963.

Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG (2011) Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annual Review of Immunology* 29: 71–109.

Pancholi V, Fischetti VA (1992) A major surface protein on group A streptococci is a glyceraldehydes-3-phosphate-dehydrogenase with multiple binding activity. *Journal of Experimental Medicine* 176: 415–426.

Pasztor L, Ziebandt AK, Nega M, Schlag M, Haase S, Franz-Wachtel M, Madlung J, Nordheim A, Heinrichs DE, Götz F (2010) *Staphylococcal major* autolysin (Atl) is involved in excretion of cytoplasmic proteins. *Journal of Biological Chemistry* 285: 36794–803.

Pettengill MA, van Haren SD, Levy O (2014) Soluble mediators regulating immunity in early life. *Frontiers in Immunology* 5: 457.

Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ; Active Bacterial Core surveillance/Emerging Infections Program Network (2008) Epidemiology of invasive group B streptococcal disease in the United States 1999–2005. *JAMA* 299: 2056–65.

Polgar L (1964) The mechanism of action of glyceraldehydes-3-phosphate dehydrogenase. *Experientia* 20: 408–413.

Quirante J, Ceballos R, Cassady G (1974) Group B beta-hemolytic streptococcal infection in the newborn. I. Early onset infection. *American Journal of Diseases of Children* 128: 659–665.

Rainsford E, Reen DJ (2002) Interleukin 10, produced in abundance by human newborn T cells, may be the regulator of increased tolerance associated with cord blood stem cell transplantation. *British Journal of Haematology* 116: 702–709.

Raje CI, Kumar S, Harle A, Nanda JS, Raje M (2007) The macrophage cell surface glyceraldehydes-3-phosphate dehydrogenase is a novel transferrin receptor. *Journal of Biological Chemistry* 282: 3252–3261.

Reed SG, Brownell CE, Russo DM, Silva JS, Grabstein KH, Morrissey PJ (1994) IL-10 mediates susceptibility to *Trypanosoma cruzi* infection. *Journal of Immunology* 153: 3135–3140.

Roque S, Nobrega C, Appelberg R, Correia-Neves M (2007) IL-10 underlies distinct susceptibility of BALB/c and C57BL/6 mice to *Mycobacterium avium* infection and influences efficacy of antibiotic therapy. *Journal of Immunology* 178: 8028–8035.

Saraiva M, O'Garra A (2010) The regulation of IL–10 production by immune cells. *Nature Reviews Immunology* 10: 170–181.

Schuchat A, Zywicki SS, Dinsmoor MJ, Mercer B, Romaguera J, O'Sullivan MJ, Patel D, Peters MT, Stoll B, Levine OS (2000) Risk factors and opportunities for prevention of early-onset neonatal sepsis: a multicenter case-control study. *Pediatrics* 105: 21–26.

Schuit KE, DeBiasio R (1980) Kinetics of phagocyte response to group B streptococcal infections in newborn rats. *Infection and Immunity* 28: 319–324.

Seifert KN, McArthur WP, Bleiweis AS, Brady LJ (2003) Characterization of group B streptococcal glyceraldehydes-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein-protein interactions. *Canadian Journal of Microbiology* 49: 350–356.

Silva RA, Appelberg R (2001) Blocking the receptor for interleukin 10 protects mice from lethal listeriosis. *Antimicrobial Agents and Chemotherapy* 45: 1312–1314.

Sun CM, Deriaud E, Leclerc C, Lo-Man R (2005) Upon TLR9 signaling, CD5+ B cells control the IL-12-dependent Th1-priming capacity of neonatal DCs. *Immunity* 22: 467–477.

Takaoka Y, Goto S, Nakano T, Tseng H-P, Yang S-M, Kawamoto S, Ono K, Chen C-L (2014) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) prevents lipopolysaccharide (LPS)-induced, sepsis-related severe acute lung injury in mice. *Science Reports* 4: 5204.

Thaxton JE, Sharma S (2010) Interleukin-10: a multi-faceted agent of pregnancy. *Americal Journal of Reproductive Immunology* 2010 63: 482–491.

Verani JR1, McGee L, Schrag SJ; Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (2010) Prevention of perinatal group B streptococcal disease-revised guidelines from CDC. *MMWR Recommendations and Reports* 59 (RR-10): 1–36.

Williams Z (2012) Inducing tolerance to pregnancy. *New England Journal of Medicine* 367: 1159–1161.

Winram SB, Lottenberg R (1996) The plasmin-binding protein Plr of group A streptococci is identified as glyceraldehydes-3-phosphate dehydrogenase. *Microbiology* 142: 2311–2320.

Zaura E, Nicu EA, Krom BP, Keijser BJ (2014) Acquiring and maintaining a normal oral microbiome: current perspective. *Frontiers in Cellular and Infection Microbiology* 4: 85.

11

Mycobacterium tuberculosis Cell-Surface GAPDH Functions as a Transferrin Receptor

Vishant M. Boradia[1,2], Manoj Raje[3], and Chaaya Iyengar Raje[1]

[1] *Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), SAS Nagar, Punjab, India*
[2] *Present address: Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland, USA*
[3] *Council of Scientific and Industrial Research (CSIR), Institute of Microbial Technology (CSIR-IMTECH), Chandigarh, India*

11.1 Introduction

Tuberculosis (TB) is one of the leading causes of death worldwide due to infectious diseases. It is estimated that in the year 2013 alone, 9 million individuals were infected with TB and 1.5 million persons succumbed to the disease (WHO 2014). *Mycobacterium tuberculosis (M. tb)* is the causative agent of the disease where infection is usually initiated by inhalation of aerosols carrying the bacilli. Although primary infection occurs in the lungs, extrapulmonary TB can affect almost any other organ of the body including the brain, digestive system, spine, and urinogenital system. Infection is initiated when alveolar macrophages engulf the bacilli which can then replicate and survive for long periods within a specialized intracellular compartment known as the phagosome.

Iron is a necessity for the survival of both prokaryotes and eukaryotes, due to its importance as a cofactor in several enzymes. However, since excess iron can be toxic due to the synthesis of free radicals by the Fenton reaction, most organisms have evolved intricate mechanisms to acquire and store iron. Several studies have documented the close correlation between iron availability and pathogenesis. During an infection, pathogens utilize multiple mechanisms to obtain iron from host resources such as macromolecules involved in iron storage and transport. At the same time the host attempts to withhold available iron in order to limit infection, a process known as nutritional immunity. The availability and successful acquisition of surplus iron can enhance virulence and in fact skew the physiological outcome in favor of the pathogen (Andrews *et al.* 2003; Skaar 2010). This tug-of-war for iron between invading pathogen and mammalian host is similar to the circumstances in which an invading army attempts to seize the strategic resources

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

of a territory under their occupation, for their own growth and sustenance, while the invaded nation attempts to deny the availability of the same to the aggressor.

As with other pathogens, iron is critical for the survival of *M. tb*; the bacilli are known to acquire iron from heme, which is readily available in the macrophage due to its role in the breakdown of effete RBCs (red blood cells) for recycling of heme iron (Tullius *et al*. 2011). They also synthesize small iron-chelating molecules (siderophores) that withdraw iron from host sources such as transferrin, ferritin, and lactoferrin (Ratledge 2004). Very recently a novel mechanism for iron uptake has been identified in *M. tb* wherein iron-replete transferrin binds on the cell surface to specific receptors, followed by internalization of the receptor–ligand complex into the bacterial cell effecting iron delivery into the microorganism (Boradia *et al*. 2014). The glycolytic enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a well-known pleiotropic molecule, was identified as one of the receptors involved in this process. It is pertinent that, in macrophages, the host GAPDH also functions as a receptor for transferrin and is involved in the influx as well as the efflux of transferrin iron (Raje *et al*. 2007; Sheokand *et al*. 2014).

The other *M. tb* transferrin receptors to have been identified are: iron-regulated elongation factor tu (Rv0685); L-Lactate dehydrogenase (Rv1872c); Acyl-carrier protein desaturase (Rv0824c); and the 50S ribosomal proteins L1 (Rv0641) and L2 (Rv0704) (Boradia *et al*. 2014). Interestingly, homologs of some of these conserved proteins are known to possess alternate functions in other organisms (Wool 1996; Piatigorsky 2009; Fu *et al*. 2012).

11.2 Iron Acquisition by Bacteria

11.2.1 Heme Uptake

Since heme is one the most abundant sources of iron in any mammalian host, many microorganisms have also evolved mechanisms to utilize heme, hemoglobin, or the hemopexin-heme complex as a source of iron. Pathogens can acquire heme either by direct uptake or by heme-scavenging proteins known as hemophores.

Direct uptake involves the binding of heme or heme proteins to specific outer membrane (OM) receptors. Subsequently, heme is removed from the bacterial receptor and transported into the cell by a process that is energy dependent. The TonB-ExbB-ExbD system generates the proton-motive force required for transport in both Gram-positive and Gram-negative bacteria (Genco and Dixon 2001). Alternatively, uptake may occur by means of secreted heme-binding proteins known as hemophores. These molecules function in a manner analogous to siderophores where heme is withdrawn from host proteins and the hemophore-heme complex is docked back onto the bacterial surface. Heme is trafficked into the cell by specific transporters and heme-associated iron is then released in the cytoplasm. Gram-negative organisms can utilize either one or both of the above mechanisms to obtain iron (Cassat and Skaar 2013). For example, *P. aeruginosa* can obtain heme by direct uptake as well as hemophore-mediated uptake (Cornelis 2010). On the other hand, Gram-positive bacteria commonly acquire

iron by the direct uptake of heme; an example is the human pathogen *S. aureus* which preferentially acquires iron by the direct uptake of heme (Skaar *et al*. 2004). In addition, certain Gram-negative species such as *P. gingivalis* and *E. coli* also utilize proteases that degrade hemoglobin, resulting in the release and uptake of heme (Otto *et al*. 1998; DeCarlo *et al*. 1999; Lewis *et al*. 1999).

11.2.2 Siderophore-Mediated Uptake

A wide spectrum of bacterial species including *E.coli*, *M. tuberculosis*, *P. aeruginosa*, *S. tymphimurium*, *Bordetella pertusis*, *Y. pestis*, *Vibrio cholera* and *S. aureus* are known to synthesize a variety of high-affinity metal chelators that withdraw and acquire iron from host resources (Ratledge and Dover 2000). Siderophores possess an exceedingly high affinity for iron of the order 10^{30}, which is significantly more than that of transferrin (10^{23}); they therefore steal iron from host resources. Based on their chemical nature, siderophores are classified as phenolic ring-based structures where hydroxyl or dihydroxybenzoic acid chelates Fe^{3+}, hydroxamates, or mixed ligands of the previous two categories (Ratledge 2004). Biosynthesis of these molecules is initiated in response to iron deficiency and is tightly regulated.

11.2.3 Transferrin Iron Acquisition

Transferrin is an abundantly present serum protein; it has a bi-lobed structure with a single Fe^{3+} ion binding to each lobe (Gomme *et al*. 2005). Several pathogenic organisms such as *N. gonorrhoea*, *N. meningitidis*, *A. pleuropneumoniae* and *H. influenzae* directly capture host transferrin or lactoferrin at their surface. The specific transferrin-binding proteins TbpA and TbpB and the equivalent lactoferrin-binding proteins LbpA and LbpB are responsible for cell-surface sequestration of host iron carrier proteins. Iron is then released from the mammalian host carrier protein and transported across the microbial membrane in a TonB-dependent manner (Gray-Owen and Schyvers 1996).

In the Gram-positive bacteria *S. aureus* and *S. epidermidis*, cell-surface GAPDH (along with other proteins) have been reported to function as receptors for transferrin (Modun and Williams 1999; Taylor and Heinrichs 2002). It has been suggested that the reductase activity of GAPDH causes the release and internalization of iron from surface-sequestered transferrin (Ratledge and Dover 2000). Lastly, a unique survival strategy is adopted by *Borrelia burgdorferi* (the causative agent of Lyme disease), where it completely circumvents the requirement of iron uptake. This organism has evolved to utilize manganese as a cofactor in metalloenzymes instead of iron (Posey and Gherardini 2000).

11.3 Iron Acquisition by Intracellular Pathogens

Pathogens that survive within cells are faced with the challenge of obtaining iron from the highly restrictive conditions within the host cell. Intracellular pathogens such as *Salmonella typhimurium*, *Coxiella burnetti*, *Legionella pneumophila*,

Francisella tularensis, and *Mycobacterium tuberculosis* all demonstrate a diminution in their pathogenicity upon iron deprivation (Collins 2003; Chlosta *et al*. 2006; Paradkar *et al*. 2008; Pan *et al*. 2010). Such pathogens survive intracellularly by occupying specific niches within the host cell and by hijacking available host iron. This is achieved by one or more mechanisms that can include: use of siderophores; expression of surface ferric reductases; heme uptake; and trafficking of transferrin (Schaible and Kaufmann 2004).

M. tb survives within the phagosome of macrophages, cells that are central to iron homeostasis. Numerous studies have demonstrated that *M. tb* has a very high requirement for iron, and iron overload (in the host) can cause a relapse of the disease (Trousseau 1872). An abundance of iron due to high dietary iron or iron supplementation has been shown to exacerbate the disease in both mouse models (Schaible *et al*. 2002) as well as human patients (Boelaert *et al*. 2007; Shemisa *et al*. 2014). In studies conducted on African patients, high dietary serum iron levels were found to be associated with greater mortality and morbidity due to tuberculosis (Gangaidzo *et al*. 2001; Isanaka *et al*. 2012).

11.4 Iron Acquisition by *M. tb*

Macrophages acquire iron primarily from two sources. One source is from the heme released due to the recycling of effete erythrocytes. In addition, they also acquire iron via the receptor-mediated endocytosis of transferrin (Gkouvatsos *et al*. 2012) and lactoferrin (Adlerova *et al*. 2008). Transferrin iron uptake by receptor-mediated endocytosis is a well-defined mechanism in mammalian cells. Two transmembrane transferrin receptors (TfR1 and TfR2) had previously been identified in mammalian cells (Jandl *et al*. 1959; Kawabata *et al*. 1999; West *et al*. 2000; Mayle *et al*. 2012). In this process, holo-transferrin binds to cell-surface transferrin receptors followed by subsequent internalization into the cell. Acidification of the early endosome results in the release of iron and the residual apo-transferrin complex re-cycles back to the cell surface.

In order to contain an infection, macrophages downregulate the expression of transferrin receptors to limit the entry of iron into the cell (Olakanmi *et al*. 2002). The host may also synthesize molecules known as siderocalins that sequester the bacterial siderophores and thereby inhibit the uptake of iron by the pathogen (Holmes *et al*. 2005). To evade the withholding strategies of the host iron, *M. tb* utilizes multiple mechanisms to appropriate host iron. These include use of siderophores (Ratledge 2004), heme capture (Tullius *et al*. 2011), and the recruitment of transferrin and lactoferrin to the phagosome (Olakanmi *et al*. 2002, 2004).

11.4.1 Heme Uptake

Macrophages are responsible for the breakdown of erythrocytes, thereby making heme abundantly available within the cell. Recent findings have identified that, in addition to the siderophore pathway, *M. tb* uses free heme and

heme from hemoglobin as a source of iron. The heme carrier protein Rv0203 scavenges heme from host proteins and transports it to the bacterium, while two other proteins Mmp3 and Mmp11 transport heme across the bacterial membrane (Tullius *et al*. 2011).

11.4.2 Siderophore-Mediated Iron Acquisition

Along with other species such as *Nocardia* and *Rhodococci*, *Mycobacteria* are unique in the fact that they synthesize both a membrane-bound and secreted siderophores. *M. tb* synthesizes mycobactin T, a membrane-bound siderophore, and carboxymycobactin, the secreted molecule. These siderophores belong to the hydroxamate and mixed ligand types. Mycobactins were first identified in the 1950s as a growth factor for *M. paratuberculosis* (Francis *et al*. 1953). Structurally, mycobactin T possesses short methyl and ethyl side-chains on the core and a long alkyl chain that renders it extremely insoluble in aqueous media. In addition to iron acquisition, it is also proposed to function as a temporary store for iron.

Carboxymycobactin is a variant of mycobactin, where the long alkyl chain is replaced by a shorter chain terminating in a carboxylic group (Ratledge 2004). Knockout studies have revealed that siderophores are essential for *M. tb* virulence and replication (De Voss *et al*. 2000). Enzymes involved in the biosynthesis of these siderophores are tightly regulated by the iron-dependent regulator (IdeR) (Gold *et al*. 2001).

As with other bacterial siderophores, carboxymycobactin withdraws iron from host proteins such as transferrin and lactoferrin and traffics it back to the bacterial surface (Olakanmi *et al*. 2004). It is proposed that iron may then be taken into the bacterium by transfer to membrane-bound mycobactin (Gobin and Horwitz 1996). It is known that carboxymycobactin is also directly internalized by a specific transporter known as IrtAB (Farhana *et al*. 2008; Ryndak *et al*. 2010). Intracellular ferric reductases then convert the bound Fe^{3+} to Fe^{2+} within the cell. Recent evidence also suggests that mycobactin-loaded membrane vesicles are secreted from bacilli during iron starvation. These vesicles allow the capture and delivery of iron from the environment into the bacterium (Prados-Rosales *et al*. 2014).

11.4.3 Transferrin-Mediated Iron Acquisition

M. tb is capable of obtaining iron from exogenous and endogenous sources within the host cell (Olakanmi *et al*. 2002). Several studies have demonstrated that both transferrin and lactoferrin are recruited to the phagosome (Clemens and Horwitz 1996). It is known that exogenously added transferrin and lactoferrin can deliver iron to bacilli within the phagosome, and acquisition from lactoferrin is reported to be 30-fold greater as compared to transferrin (Olakanmi *et al*. 2004). While it has been demonstrated that siderophores withdraw iron from host proteins (Clemens and Horwitz 1996), little is known about the detailed events within the phagosome related to the transport of iron carrier proteins, iron release and acquisition, or the involvement of bacterial components in this process (Schaible and Kaufmann 2004).

11.5 Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

As an enzyme, GAPDH plays a central role in glycolysis where it catalyzes the sixth step of glycolysis. The functionally active molecule is a homo tetramer that catalyzes the conversion of glyceraldehyde-3-phosphate in the presence of NAD^+ to 1, 3 bisphosphoglycerate and NADH. In addition to its role in glycolysis, it has numerous alternate functions in both prokaryotes and eukaryotes. This protein is present in different intracellular compartments, on the cell surface, and is also secreted into the extracellular milieu. The alternate functions include: vesicular trafficking; cytoskeletal reorganization; apoptosis; transcriptional and post-transcriptional gene regulation; and transferrin iron uptake (Sirover 2011). Some of these moonlighting roles of human GAPDH have been implicated in pathological conditions as diverse as cancer, neurodegenerative disorders, and diabetes and also infectious diseases such as malaria and brucellosis (Sirover 1999). Likewise, GAPDH from many bacterial species has been reported to possess alternate functions that include adhesion, immune evasion, and plasminogen binding (Pancholi and Chhatwal 2003; Maeda *et al*. 2004; Jin *et al*. 2005; Terao *et al*. 2006). GAPDH of both mammalian and bacterial origin have not been found to contain specific secretory sequences; like many other secreted multifunctional glycolytic enzymes, GAPDH is therefore broadly grouped as a "non-classically" secreted protein (Cleves *et al*. 1996; Nombela *et al*. 2006). Until recently, very little was known as to how these proteins are secreted. Recent reports now suggest that the alternate localization and multiple functions of mammalian GAPDH may depend upon the presence of specific post-translational modifications, rather than typical signal sequences (Sirover 2012).

11.6 Macrophage GAPDH and Iron Uptake

Mammalian glyceraldehyde-3-phosphate dehydrogenase was identified as a dual receptor for both transferrin and the related iron transport protein lactoferrin on macrophages, as well as several other cell types (Raje *et al*. 2007; Kumar *et al*. 2011; Rawat *et al*. 2012). The finding was somewhat surprising given that GAPDH bears no structural similarity to the two well-characterized receptors for transferrin (i.e., TfR1 and TfR2) or with the known Lf receptors. The GAPDH-Tf pathway differs from the TfR-Tf pathway in several aspects including: membrane localization of the receptor; affinity of binding; internalization pathway; kinetics of uptake; and regulation and fate of endocytosed transferrin (Kumar *et al*. 2011). In addition, GAPDH carries out its iron delivery functions not only as a membrane-bound receptor but is also secreted into the extracellular milieu, where it functions as a soluble receptor for transferrin effecting autocrine and paracrine delivery of transferrin and iron into cells (Sheokand *et al*. 2013).

11.6.1 Regulation

Uptake of iron and its metabolism are tightly controlled within mammalian cells. This regulation occurs at the post-transcriptional level, through specific iron response elements that are located at the 3' and 5' untranslated regions of mRNA.

Upon iron depletion, iron response proteins (IRP) bind to the 3' iron responsive elements (IRE) sequences and stabilize the mRNA of transferrin receptor 1. The IRP simultaneously binds at the 5'-IRE sequence present in the mRNA of the iron storage protein ferritin. The net consequence is an enhanced translation of TfR, resulting in an increased uptake of iron by the transferrin–transferrin receptor pathway. At the same time, the decrease in translation of ferritin reduces iron storage capacity. Conversely, in the presence of excess iron, IRP dissociates from the 3' IRE sequences in the transferrin receptor mRNA, thereby causing rapid degradation of the transcript and hence reduced protein translation. At the same time the release of IRP also initiates the transcription and translation of ferritin, making it possible to sequester any excess iron (Gkouvatsos *et al*. 2012). Unlike these iron-regulated proteins, sequence analysis did not reveal the presence of any specific IRE sequences in the mRNA transcript of GAPDH. Despite this, iron depletion was observed to result in a marked increase in the recruitment of the protein at the cell surface (Raje *et al*. 2007).

11.6.2 Mechanism of Iron Uptake and Efflux

The Tf-TfR pathway delivers transferrin iron into cells solely via clathrin-pit-mediated endocytosis. Distinct from this, GAPDH-mediated trafficking also involves two other mechanisms: lipid-raft endocytosis and micropinocytosis (Kumar *et al*. 2011). The affinity of interaction for Tf-TfR1 and Tf-TfR2 is extremely high (K_D = 1.0 nM and 25 nM, respectively) as compared to the lower affinity for Tf-GAPDH (K_D = 120 nM *in vitro* and 60 nM *in vivo*) (Kawabata *et al*. 1999; Raje *et al*. 2007). In addition, GAPDH has also been identified to play a role in transferrin-mediated efflux of iron from macrophages. In this role, a distinct form of GAPDH is re-localized to the surface of cells which captures apo-transferrin (K_D 1.11 nM) in close proximity to the iron transporter ferroportin to remove excess iron from cells (Sheokand *et al*. 2014).

11.6.3 Role of Post-Translational Modifications

Numerous reports have indicated that GAPDH can switch between its alternate functions depending on the pattern of multiple post-translational modifications (Seidler 2013; Sirover 2014). Recent studies have indicated that post-translational modifications are responsible for altered membrane localization and transferrin-binding ability of GAPDH in response to cellular iron levels (Sheokand *et al*. 2014). The discovery of differential binding by GAPDH to either holo or apo forms of transferrin during conditions of iron depletion or excess, respectively, has shown that this switch in ligand specificity occurs due to the presence of different isoforms of GAPDH. An abundance of post-translational modifications (PTMs) including oxidation, dimethylation, acetylation, nitrosylation, and phosphorylation were evident in membrane GAPDH from iron-depleted cells as compared to GAPDH isolated from iron-loaded cells (Sheokand *et al*. 2014). These results indicate that, in addition to post-transcriptional regulation, post-translational modifications are an important regulatory mechanism for proteins involved in iron homeostasis.

11.7 Mycobacterial GAPDH and Iron Uptake

Several previous studies have indicated the existence of some alternate siderophore-independent pathways for mycobacterial iron acquisition. Support for this has come from the observation that mutant strains unable to synthesize siderophores can continue to acquire transferrin iron and survive both *in vivo* and *in vitro*. The siderophore negative strain *M. tb* H37RvO1A survives within macrophages and acquires transferrin iron during the early stages of infection (Wagner *et al*. 2005). Similarly, the recombinant siderophore-deficient strain BCG(*mbtB*)30 continues to acquire transferrin iron and survives *in vivo* (Tullius *et al*. 2008). Some researchers have also failed to detect siderophores in *M. tb* infected tissues, thereby supporting the presence of siderophore-independent iron uptake mechanisms (Lambrecht and Collins 1993).

Biochemical studies combined with proteomic analysis have identified that GAPDH is localized to the cell envelope of *M. tb* cells (Malen *et al*. 2007, 2010; Bell *et al*. 2012). This surface localization has also been associated with its alternate function as an EGF receptor that promotes bacterial growth (Bermudez *et al*. 1996). In comparison to other species, there is relatively limited information available regarding its other alternate functions.

A preliminary analysis using flow cytometry and transmission electron microscopy revealed that *M. tb* sequesters holo-transferrin at its surface (Fig. 11.1). Detailed proteomic analysis identified six transferrin-binding proteins, one of which was found to be GAPDH (Table 11.1; Boradia *et al*. 2014). Sequence analysis revealed significant homology with human GAPDH and *S. aureus* GAPDH (*c*. 50 % identity), two organisms where it has previously been identified as a transferrin receptor (Modun and Williams 1999; Raje *et al*. 2007). GAPDH from cytosol, cell membrane and cell-wall fractions of virulent *M. tb* H37Rv, and the non-pathogenic strain *M.tb* H37Ra and *M. smegmatis* were found to be enzymatically active. The interaction of GAPDH-Tf at the cell surface was confirmed by co-immunoprecipitation assay, confocal-microscopy-based foster resonance energy transfer studies and transmission-electron-microscopy-based analysis (Fig. 11.2).

To estimate the affinity of interaction with transferrin, recombinant *M. tb* GAPDH (rGAPDH) was expressed and purified. The K_D was determined to be 160 ± 24 nM, which is comparable to the affinity of mammalian GAPDH with transferrin. Transferrin binding to GAPDH on the surface of *M. tb* was found to be saturable and could be inhibited (up to 80% inhibition could be achieved) with increasing molar concentrations of rGAPDH, a characteristic feature of receptor binding. The total number of transferrin receptors (including GAPDH as well as other identified proteins) was estimated to be approximately 7136 ± 255 receptors per bacterial cell (Boradia *et al*. 2014).

11.7.1 Regulation

In bacteria, iron levels regulate the synthesis or degradation of proteins at the transcriptional level via specific iron-dependent regulators. So far, two families of regulators are known: the ferric uptake regulator (Fur) and the diphtheria toxin repressor (DtxR) families. *M. tb* contains two Fur-like proteins (Fur A and Fur B)

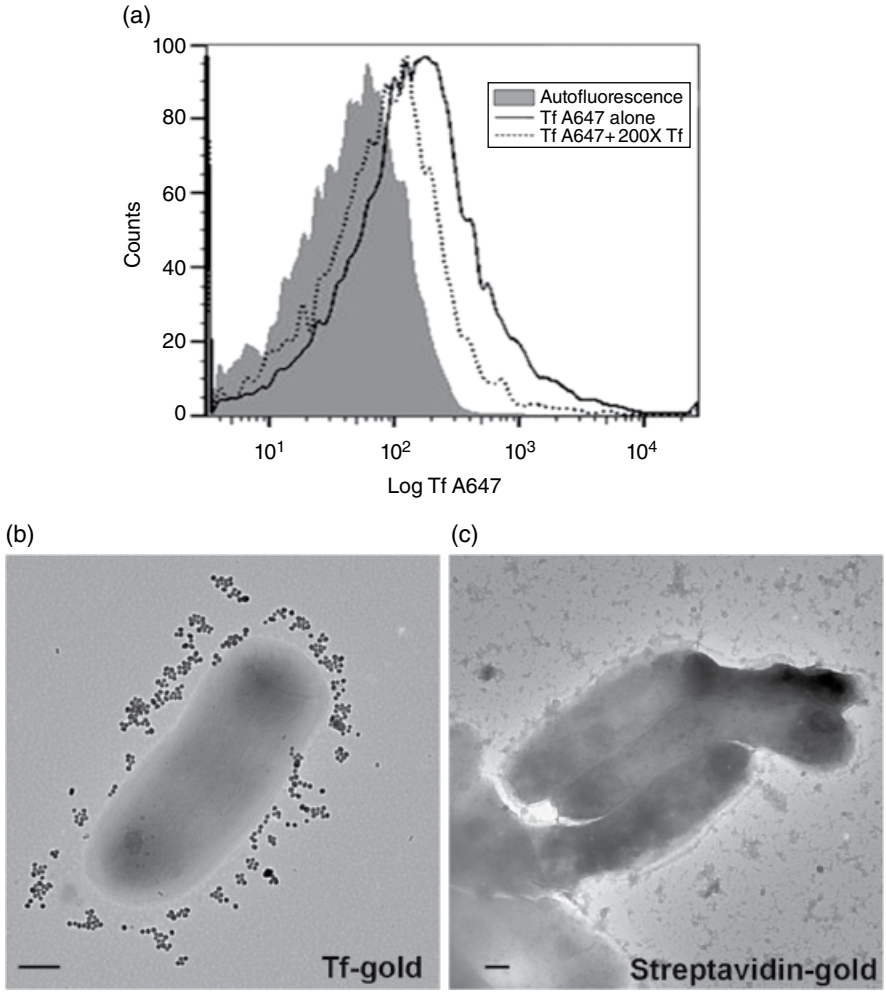

Figure 11.1 *M. tuberculosis* expresses cell-surface transferrin-binding proteins. (a) FACS analysis indicates specific binding of Tf-Alexa647 which is competitively inhibited in the presence of 200X unlabeled transferrin. (b)Transferrin conjugated gold particles localize on the surface of intact *M. tb* H37Ra. (c) No surface labeling is observed in controls (Streptavidin-gold conjugate). Scale bar represents 0.2 µm. *Source*: Boradia *et al*. (2014).

and two members of the DtxR family (IdeR and SirR). The regulation of siderophore synthesis and the iron storage protein bacterioferritin is tightly regulated by IdeR. Upon iron depletion, iron is unavailable to bind to IdeR. This renders it incapable of binding to the promoter elements of siderophore genes, which in turn permits transcription of genes necessary for siderophore synthesis. Conversely, the lack of promoter binding inhibits the synthesis of bacterioferritin, an iron storage protein. On the other hand, when iron is abundant, it associates with IdeR. The iron-bound IdeR binds to promoter sequences and causes the downregulation of siderophore synthesis, while at the same time upregulating the synthesis of bacterioferritin (Banerjee *et al*. 2011).

Table 11.1 Identified *M. tb* H37Rv transferrin-binding proteins (Boradia *et al*. 2014).

Serial no.	Identity	MOWSE score	Coverage	No. peptides matched	Theoretical size (Daltons)
1.	Iron regulated elongation factor tu (Rv 0685)	107	25%	10	43561.5
2.	L-Lactate dehydrogenase (Rv 1872 c)	83	38%	14	45341.6
3.	GAPDH (Rv 1436)	552	32%	8	35923.9
4.	Acyl desaturase (Rv 0824c)	166	16%	5	38770.2
5.	50 S ribosomal protein L2 rplB (Rv 0704)	100	46%	10	30576.8
6.	50S ribosomal protein L1 rplA (Rv 0641)	269	37%	6	24756.4

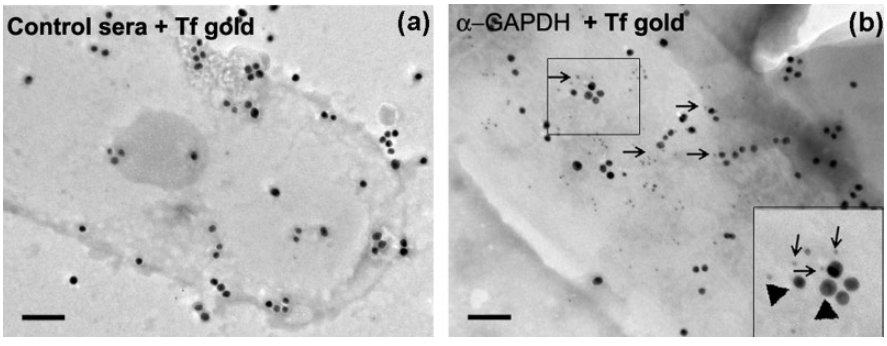

Figure 11.2 Transferrin and GAPDH co-localize at the cell surface. (b) Co-localization of GAPDH and transferrin by immunogold labeling transmission electron microscopy of *M. tb* H37Ra strain overexpressing GAPDH. GAPDH was detected using 1:100 polyclonal rabbit α-GAPDH, followed by secondary antibody conjugated with 5 nm gold particles. Cells were simultaneously labeled with transferrin-20 nm gold conjugate. Inset: magnified image of highlighted area showing co-localization of GAPDH and transferrin. Arrows indicate 5 nm particles (GAPDH); arrowheads indicate the presence of transferrin-gold. (a) Cells labeled with an equivalent amount of rabbit pre-immune sera (controls). Scale bar represents 100 nm. *Source*: Boradia *et al*. (2014).

As in the case of mammalian GAPDH, an analysis of the *M. tb* GAPDH sequence did not indicate the presence of any iron regulatory elements. Despite this, *M. tb* H37Ra cells in iron-depleted media demonstrated a marked enhancement of GAPDH on their cell surface associated with a corresponding increase in transferrin binding. The enhanced transferrin binding in turn resulted in a significant increase in iron uptake as confirmed by the uptake of radiolabeled Tf-^{55}Fe (Boradia *et al*. 2014).

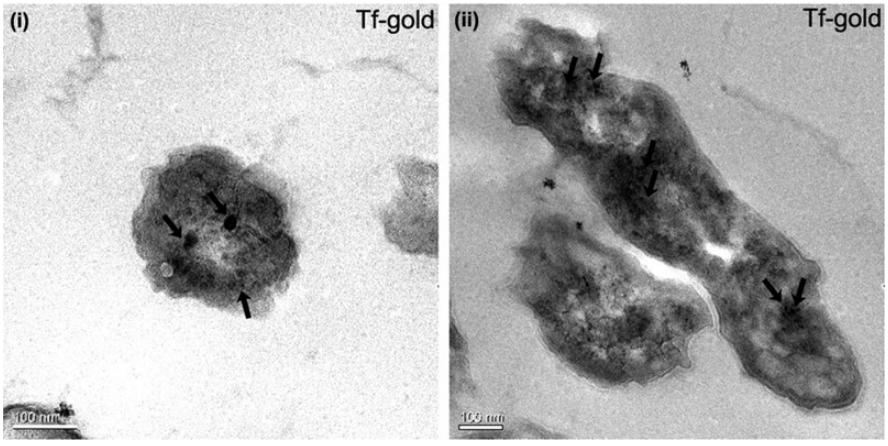

Figure 11.3 Transferrin-iron uptake is mediated by the internalization of transferrin. Presence of transferrin gold particles within the cytoplasm of *M. tb* H37Ra cells after incubation at 37°C for 1 hr. Scale bar represents 100 nm. *Source*: Boradia *et al*. (2014).

11.7.2 Mechanism of Iron Uptake

In vitro studies were undertaken to characterize this mechanism. A significant increase in transferrin capture along with an elevated iron uptake by recombinant strains overexpressing GAPDH as compared to control strains was evident. Further studies confirmed that transferrin-iron uptake is a relatively rapid process (occurring within 6 hours) as compared to siderophore iron acquisition where, after 24 hours of co-incubation with transferrin, only 30% of mycobactin is converted to ferric-mycobactin (Luo *et al*. 2005; Boradia *et al*. 2014). Although it has been suggested that the reductase activity of GAPDH may drive the release of iron at the cell surface, followed by its capture and internalization by siderophores (Ratledge and Dover 2000), that mechanism could not account for the extremely rapid uptake of transferrin-iron observed in these recent studies (Boradia *et al*. 2014). Moreover, the *M. smegmatis Δesx-3* strain that is unable to utilize mycobactins (Siegrist *et al*. 2009) was also able to acquire transferrin iron to an extent comparable with that of the wild-type strain, indicating that iron was not being released at the surface for recapture by mycobactin (Boradia *et al*. 2014).

Transmission electron microscopy (TEM) and TEM-based tomography studies revealed an extremely unusual process (for prokaryotes) whereby the entire GAPDH-Tf complex is internalized into the bacterium. Tf-gold particles were internalized to the cytoplasm of *M. tb* cells within one hour of incubation at 37°C (Fig. 11.3). Co-immunoprecipitation assays confirmed that biotinylated cell-surface GAPDH is also internalized along with transferrin by this process. Presumably, iron is then released within the cell by the action of cytosolic ferri-reductases (Boradia *et al*. 2014). It is still unclear whether the internalized GAPDH-transferrin is recycled or degraded within the bacterium after iron release.

This internalization of the GAPDH-transferrin complex for intracellular delivery of iron in *M. tb* reveals a mechanism that is reminiscent of receptor-mediated endocytosis in eukaryotic cells. An endocytosis-like process has previously been reported only in case of a single bacterial species (i.e., *Gemmata obscuriglobus*; Lonhienne *et al*. 2010). At present the components and detailed events that permit internalization of a large protein complex composed of GAPDH-Tf (150–80 kD) are unknown. It would be of interest to further characterize this mechanism, particularly in view of the fact that *M. tb* has a waxy and almost impermeable cell wall that makes it resistant to many anti-tubercular drugs (Nguyen and Jacobs 2012).

11.7.3 Uptake by Intraphagosomal *M. tb*

Additional studies analyzed the significance of the GAPDH-Tf uptake process during infection using an *in vitro* THP-1 cell culture model using co-localization and biochemical assays. Cells were infected with *M. tb* followed by incubation with biotinylated transferrin. Intraphagosomal bacilli were then isolated and cytosolic fractions were assessed for the presence of internalized transferrin. Biotinylated transferrin was detected in the cytosolic fraction of these bacilli. Confocal microscopy confirmed the co-localization of GAPDH and transferrin on intracellular bacilli. The *M. tb* H37Ra strain overexpressing GAPDH demonstrated an almost two-fold increase of transferrin internalization as compared to control strains. Both *M. smegmatis* wild-type and *M. smeg*matis Δesx-3 strains also demonstrated an internalization of transferrin, indicating that uptake of transferrin is independent of the siderophore-mediated iron uptake process. This indicated that not only is transferrin transported to the phagosome during infection, but it is also internalized into the bacilli via specific receptors (Boradia *et al*. 2014).

At present it is not known whether *M. tb* GAPDH exits the phagosome and localizes at the cell surface to capture transferrin or, alternatively, whether macrophage GAPDH/TfRs traffic transferrin to the phagosome where subsequent internalization is mediated by *M. tb* GAPDH.

In summary, so far there appear to be three pathways for transferrin-iron uptake: (1) withdrawal of iron by carboxymycobactin and transfer to membrane-bound mycobactin; (2) internalization of iron-loaded carboxymycobactin via IrtA; and (3) the receptor-mediated uptake by GAPDH and other identified receptors (Fig. 11.4).

The parallels between mammalian GAPDH and *M. tb* GAPDH in the critical process of iron acquisition suggest that further investigation of this mycobacterial protein may reveal a diverse array of functions that are central to virulence and host–pathogen interaction in tuberculosis. In the broader context, the presence of this endocytosis-like process could be important not only in terms of iron acquisition, but perhaps acquisition of other nutrients by bacteria.

11.8 Conclusions and Future Perspectives

During the last several years there has been an increase in the incidence of extremely drug-resistant (XDR) and multidrug-resistant tuberculosis (MDR). Due to its indispensable nature, the iron uptake pathways have been identified as

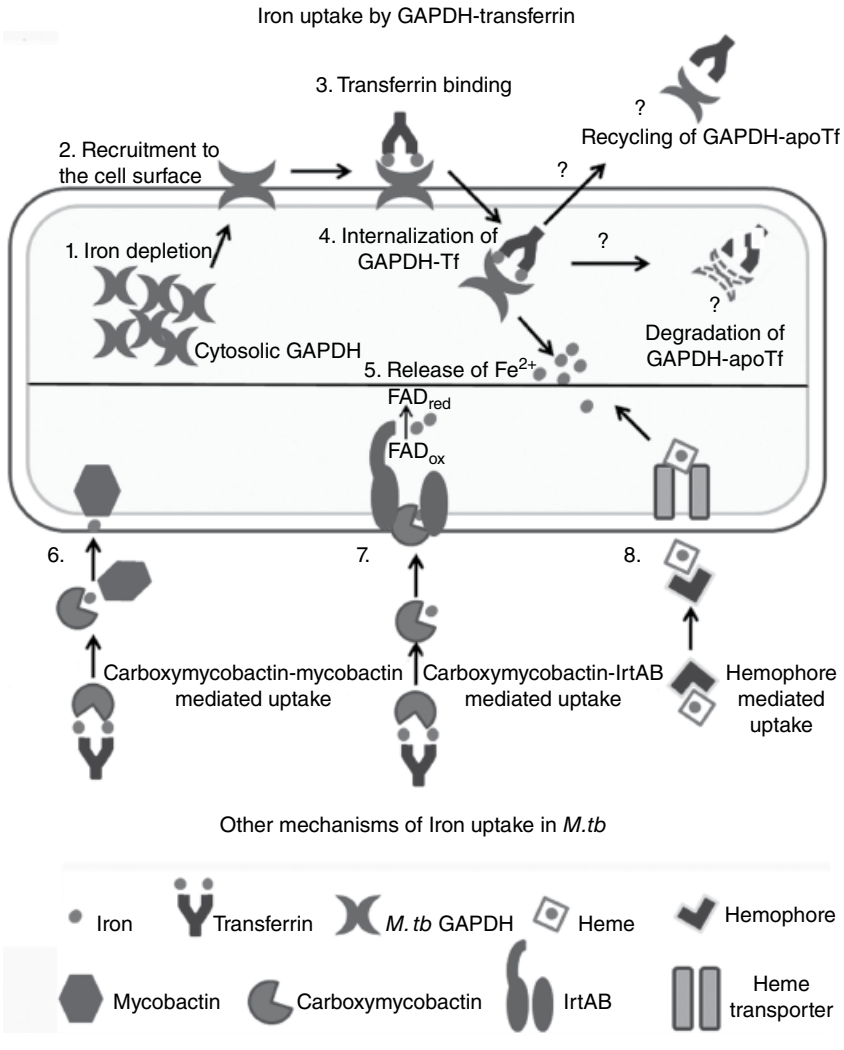

Figure 11.4 Mechanisms for the uptake of transferrin iron and Heme by *M. tb*. 1, 2. Iron depletion stimulates the recruitment of *M. tb* GAPDH to the cell surface. 3, 4. Surface GAPDH captures holo-transferrin and the entire complex is internalized within the bacilli. 5, Iron is released from transferrin; its subsequent fate regarding degradation or recycling out of the bacterium is not known. 6, Carboxymycobactin acquires transferrin iron, which is then delivered to the bacilli via mycobactin. 7, Carboxymycobactin directly utilizes the high affinity importer Irt AB to deliver transferrin iron. 8, Heme utilization by *M. tb*. Source: Boradia *et al*. (2014).

an important target for therapeutic intervention. The acquisition of iron by the GAPDH-Tf pathway suggests that it is a primitive mechanism retained by both pathogen and host. The fact that GAPDH is conserved across diverse species offers the possibility of it being a functional iron acquisition mechanism in other pathogenic organisms as well.

The obvious advantage of this pathway is the ability to sense the need for iron and immediately recruit an abundantly available protein (GAPDH) to the surface for iron acquisition, thereby avoiding the immediate need to translate multiple enzymes required for siderophore synthesis (Quadri *et al*. 1998; Krithika *et al*. 2006). In addition, since both host and pathogen proteins are conserved, *M. tb* GAPDH is likely to be camouflaged from the host response, unlike siderophores which are detected and sequestered by host siderocalins to limit infection (Holmes *et al*. 2005).

The rapid cell-surface recruitment could perhaps be explained by considering the changes in post-translational modification that have been well-characterized in mammalian cells. In this respect, as compared to the well-defined roles of PTMs in the localization and multifunctionality of mammalian GAPDH, there are only a few such instances reported for bacterial GAPDH. In fact, until recently it was thought that only a limited number of post-translational modifications occur in bacterial proteins. However, several recent studies have revealed that bacterial proteins also exhibit a vast array of modifications that can alter their functions (Cain *et al*. 2014); such modifications have also been reported to occur in *M. tb* proteins (Van Els *et al*. 2014). The modifications and their associated roles include O-glycosylation (pathogenesis), phosphorylation (regulation), methylation (protease resistance), acetylation (protein stability and compartmentalization), lipidation (compartmentalization) deamidation (regulation of protein–ligand interactions), and pupylation (degradation) (Cain *et al*. 2014; Van Els *et al*. 2014).

With reference to bacterial GAPDH, *E. coli* GAPDH is reported to be ADP-ribosylated and is thought to promote virulence (Aguilera *et al*. 2009). At present there are two reported PTMs for cell-surface *M. tb* GAPDH – phosphorylation (Parandhaman *et al*. 2014) and N-acetyl glucosamine (Bell *et al*. 2012) – but the relevance of these modifications is as yet unknown. Given that the GAPDH is highly conserved both structurally and functionally, one could conjecture that PTMs may be responsible for the surface localization of *M. tb* GAPDH and its ability to bind transferrin. However, further studies would be essential to identify and individually assess these alternate species of GAPDH and elaborate their associated functions.

Finally, it remains to be ascertained whether *M. tb* GAPDH, mammalian GAPDH, or both are responsible for transferrin-iron uptake during infection. Other aspects that are of interest involve the detailed sequence of events leading to the internalization of transferrin into *M. tb*. This would be relevant for iron uptake as well as the uptake of other nutrients from the environment. In addition, it would be of interest to elaborate the relative importance of the other multifunctional proteins identified as transferrin receptors in this iron-acquisition pathway.

Acknowledgements

We acknowledge Nature publishing group (NPG) for permitting the reuse of images (Figs 11.1–11.3) and Table 11.1 originally published in *Nature Communications* (Boradia *et al*. 2014). VMB is a recipient of research fellowship

provided by NIPER, SAS Nagar. The financial assistance received from Department of Biotechnology (DBT), Department of Science and Technology (DST) and Council of Scientific and Industrial Research (CSIR), Government of India is acknowledged.

References

Adlerova L, Bartoskova A, Faldyna M (2008) Lactoferrin: a review. *VET MED–CZECH* 53, 457–468.

Aguilera L, Gimenez R, Badia J, Aguilar J, Baldoma L (2009) NAD^+-dependent post-translational modification of *Escherichia coli* glyceraldehydes-3-phosphate dehydrogenase. *International Microbiology* 12, 187–192.

Andrews SC, Robinson AK, Rodríguez-Quiñones F (2003) Bacterial iron homeostasis. *FEMS Microbiology Reviews* 27, 215–237.

Banerjee S, Farhana A, Ehtesham NZ, Hasnain SE (2011) Iron acquisition, assimilation and regulation in mycobacteria. *Infection, Genetics and Evolution* 11, 825–838.

Bell C, Smith GT, Sweredoski MJ, Hess S (2012) Characterization of the *Mycobacterium tuberculosis* proteome by liquid chromatography mass spectrometry-based proteomics techniques: a comprehensive resource for tuberculosis research. *Journal of Proteome Research* 11, 119–130.

Bermudez LE, Petrofsky M, Shelton K (1996) Epidermal growth factor-binding protein in *Mycobacterium avium* and *Mycobacterium tuberculosis*: a possible role in the mechanism of infection. *Infection and Immunity* 64, 2917–2922.

Boelaert JR, Vandecasteele SJ, Appelberg R, Gordeuk VR (2007) The effect of the host's iron status on tuberculosis. *Journal of Infectious Diseases* 195, 1745–1753.

Boradia VM, Malhotra H, Thakkar JS, Tillu VA, Vuppala B, Patil P, Sheokand N, Sharma P, Chauhan AS, Raje M, Raje CI (2014) *Mycobacterium tuberculosis* acquires iron by cell surface sequestration and internalization of human holo-transferrin. *Nature Communications* 5, 5730.

Cain JA, Solis N, Cordwell SJ (2014) Beyond gene expression: the impact of protein post–translational modifications in bacteria. *Journal of Proteomics* 97, 265–286.

Cassat JE, Skaar EP (2013) Iron in infection and immunity. *Cell Host and Microbe* 13, 509–519.

Chlosta S, Fishman DS, Harrington L, Johnson EE, Knutson MD, Wessling-Resnick M, Cherayil BJ (2006) The iron efflux protein ferroportin regulates the intracellular growth of *Salmonella enterica*. *Infection and Immunity* 74, 3065–3067.

Clemens DL, Horwitz MA (1996) The *Mycobacterium tuberculosis* phagosome interacts with early endosomes and is accessible to exogenously administered transferrin. *Journal of Experimental Medicine* 184, 1349–1355.

Cleves AE, Cooper DN, Barondes SH, Kelly RB (1996) A new pathway for protein export in *Saccharomyces cerevisiae*. *Journal of Cell Biology* 133, 1017–1026.

Collins HL (2003) The role of iron in infections with intracellular bacteria. *Immunology Letters* 85, 193–195.

Cornelis P (2010) Iron uptake and metabolism in pseudomonads. *Applied Microbiology and Biotechnology* 86, 1637–1645.

De Voss JJ, Rutter K, Schroeder BG, Su H, Zhu Y, Barry III CE (2000) The salicylate-derived mycobactin siderophores of *Mycobacterium tuberculosis* are essential for growth in macrophages. *Proceedings of the National Academy of Science, USA* 97, 1252–1257.

DeCarlo AA, Paramaesvaran M, Yun PLW, Collyer C, Hunter N (1999) Porphyrin-mediated binding to hemoglobin by the HA2 domain of cysteine proteinases (gingipains) and hemagglutinins from the periodontal pathogen *Porphyromonas gingivalis*. *Journal of Bacteriology* 181, 3784–3791.

Farhana A, Kumar S, Rathore SS, Ghosh PC, Ehtesham NZ, Tyagi AK, Hasnain SE (2008) Mechanistic insights into a novel exporter–importer system of *Mycobacterium tuberculosis* unravel its role in trafficking of iron. *PloS One* 3, e2087.

Francis J, Macturk HM, Madinaveitia J, Snow GA (1953) Mycobactin, a growth factor for *Mycobacterium johnei*. 1. Isolation from *Mycobacterium phlei*. *Biochemical Journal* 55, 596.

Fu J, Momčilović I, Prasad PV (2012) Roles of protein synthesis elongation factor EF-Tu in heat tolerance in plants. *Journal of Botany* 2012, article ID 835836.

Gangaidzo IT, Moyo VM, Mvundura E, Aggrey G, Murphree NL, Khumalo H, Saungweme T, Kasvosve I, Gomo ZAR, Rouault T (2001) Association of pulmonary tuberculosis with increased dietary iron. *Journal of Infectious Diseases* 184, 936–939.

Genco CA, Dixon DW (2001) Emerging strategies in microbial haem capture. *Molecular Microbiology* 39, 1–11.

Gkouvatsos K, Papanikolaou G, Pantopoulos K (2012) Regulation of iron transport and the role of transferrin. *Biochimica et Biophysica Acta – General Subjects* 1820, 188–202.

Gobin J, Horwitz MA (1996) Exochelins of *Mycobacterium tuberculosis* remove iron from human iron-binding proteins and donate iron to mycobactins in the *M. tuberculosis* cell wall. *Journal of Experimental Medicine* 183, 1527–1532.

Gold B, Rodriguez GM, Marras SAE, Pentecost M, Smith I (2001) The *Mycobacterium tuberculosis* IdeR is a dual functional regulator that controls transcription of genes involved in iron acquisition, iron storage and survival in macrophages. *Molecular Microbiology* 42, 851–865.

Gomme PT, McCann KB, Bertolini J (2005) Transferrin: structure, function and potential therapeutic actions. *Drug Discovery Today* 10, 267–273.

Gray-Owen SD, Schyvers AB (1996) Bacterial transferrin and lactoferrin receptors. *Trends in Microbiology* 4, 185–191.

Holmes MA, Paulsene W, Jide X, Ratledge C, Strong RK (2005) Siderocalin (Lcn 2) also binds carboxymycobactins, potentially defending against mycobacterial infections through iron sequestration. *Structure* 13, 29–41.

Isanaka S, Aboud S, Mugusi F, Bosch RJ, Willett WC, Spiegelman D, Duggan C, Fawzi WW (2012) Iron status predicts treatment failure and mortality in tuberculosis patients: A prospective cohort study from Dar es Salaam, Tanzania. *PloS One* 7, e37350.

Jandl JH, Inman JK, Simmons RL, Allen DW (1959) Transfer of iron from serum iron-binding protein to human reticulocytes. *Journal of Clinical Investigations* 38, 161.

Jin H, Song YP, Boel G, Kochar J, Pancholi V (2005) Group A *streptococcal* surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *Journal of Molecular Biology* 350, 27–41.

Kawabata H, Yang R, Hirama T, Vuong PT, Kawano S, Gombart AF, Koeffler HP (1999) Molecular cloning of transferrin receptor 2: A new member of the transferrin receptor-like family. *Journal of Biological Chemistry* 274, 20826–20832.

Krithika R, Marathe U, Saxena P, Ansari MZ, Mohanty D, Gokhale RS (2006) A genetic locus required for iron acquisition in *Mycobacterium tuberculosis*. *Proceedings of the National Academy of Science, USA* 103, 2069–2074.

Kumar S, Sheokand N, Mhadeshwar MA, Raje CI, Raje M (2011) Characterization of Glyceraldehyde-3-phosphate dehydrogenase as a novel transferrin receptor. *International Journal of Biochemistry and Cell Biology* 44, 189–199.

Lambrecht RS, Collins MT (1993) Inability to detect mycobactin in mycobacteria-infected tissues suggests an alternative iron acquisition mechanism by mycobacteria *in vivo*. *Microbial Pathogenesis* 14, 229–238.

Lewis JP, Dawson JA, Hannis JC, Muddiman D, Macrina FL (1999) Hemoglobinase activity of the lysine gingipain protease (Kgp) of *Porphyromonas gingivalis* W83. *Journal of Bacteriology* 181, 4905–4913.

Lonhienne TGA, Sagulenko E, Webb RI, Lee K-C, Franke J, Devos DP, Nouwens A, Carroll BJ, Fuerst JA (2010) Endocytosis-like protein uptake in the bacterium *Gemmata obscuriglobus*. *Proceedings of the National Academy of Science, USA* 107, 12883–12888.

Luo M, Fadeev EA, Groves JT (2005) Mycobactin-mediated iron acquisition within macrophages. *Nature Chemical Biology* 1, 149–153.

Maeda K, Nagata H, Yamamoto Y, Tanaka M, Tanaka J, Minamino N, Shizukuishi S (2004) Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus oralis* functions as a coadhesin for *Porphyromonas gingivalis* major fimbriae. *Infection and Immunity* 72, 1341–1348.

Malen H, Berven FS, Fladmark KE, Wiker HG (2007) Comprehensive analysis of exported proteins from *Mycobacterium tuberculosis* H37Rv. *Proteomics* 7, 1702–1718.

Malen H, Pathak S, Softeland T, de Souza G, Wiker H (2010) Definition of novel cell envelope associated proteins in Triton X-114 extracts of *Mycobacterium tuberculosis* H37Rv. *BMC Microbiology* 10, 132–142.

Mayle KM, Le AM, Kamei DT (2012) The intracellular trafficking pathway of transferrin. *Biochimica et Biophysica Acta – General Subjects* 1820, 264–281.

Modun B, Williams P (1999) The staphylococcal transferrin-binding protein is a cell wall glyceraldehydes-3-phosphate dehydrogenase. *Infection and Immunity* 67, 1086–1092.

Nguyen L, Jacobs MR (2012) Counterattacking drug–resistant tuberculosis: molecular strategies and future directions. *Expert Review of Anti-Infective Therapy* 10, 959.

Nombela C, Gil C, Chaffin WLJ (2006) Non-conventional protein secretion in yeast. *Trends in Microbiology* 14, 15–21.

Olakanmi O, Schlesinger LS, Ahmed A, Britigan BE (2002) Intraphagosomal *Mycobacterium tuberculosis* acquires iron from both extracellular transferrin and intracellular iron pools. *Journal of Biological Chemistry* 277, 49727–49734.

Olakanmi O, Schlesinger LS, Ahmed A, Britigan BE (2004) The nature of extracellular iron influences iron acquisition by *Mycobacterium tuberculosis* residing within human macrophages. *Infection and Immunity* 72, 2022–2028.

Otto BR, Van Dooren SJM, Nuijens JH, Luirink J, Oudega B (1998) Characterization of a hemoglobin protease secreted by the pathogenic *Escherichia coli strain* EB1. *Journal of Experimental Medicine* 188, 1091–1103.

Pan X, Tamilselvam B, Hansen EJ, Daefler S (2010) Modulation of iron homeostasis in macrophages by bacterial intracellular pathogens. *BMC Microbiology* 10, 64.

Pancholi V, Chhatwal GS (2003) Housekeeping enzymes as virulence factors for pathogens. *International Journal of Medical Microbiology* 293, 391–401.

Paradkar PN, De Domenico I, Durchfort N, Zohn I, Kaplan J, Ward DM (2008) Iron depletion limits intracellular bacterial growth in macrophages. *Blood* 112, 866–874.

Parandhaman DK, Sharma P, Bisht D, Narayanan S (2014) Proteome and phosphoproteome analysis of the serine/threonine protein kinase E mutant of *Mycobacterium tuberculosis*. *Life Science* 109, 116–126.

Piatigorsky J (2009). *Gene Sharing and Evolution: The Diversity of Protein Functions*. Harvard University Press, Harvard.

Posey JE, Gherardini FC (2000) Lack of a role for iron in the Lyme disease pathogen. *Science* 288, 1651–1653.

Prados-Rosales R, Weinrick BC, Piqué DG, Jacobs WR, Casadevall A, Rodriguez GM (2014) Role for *Mycobacterium tuberculosis* membrane vesicles in iron acquisition. *Journal of Bacteriology* 196, 1250–1256.

Quadri LEN, Sello J, Keating TA, Weinreb PH, Walsh CT (1998) Identification of a *Mycobacterium tuberculosis* gene cluster encoding the biosynthetic enzymes for assembly of the virulence-conferring siderophore mycobactin. *Chemistry and Biology* 5, 631–645.

Raje CI, Kumar S, Harle A, Nanda JS, Raje M (2007) The macrophage cell surface glyceraldehydes-3-phosphate dehydrogenase is a novel transferrin receptor. *Journal of Biological Chemistry* 282, 3252–3261.

Ratledge C (2004) Iron, mycobacteria and tuberculosis. *Tuberculosis* 84, 110–130.

Ratledge C, Dover LG (2000) Iron metabolism in pathogenic bacteria. *Annual Review of Microbiology* 54, 881–941.

Rawat P, Kumar S, Sheokand N, Raje CI, Raje M (2012) The multifunctional glycolytic protein glyceraldehydes-3-phosphate dehydrogenase (GAPDH) is a novel macrophage lactoferrin receptor. *Biochemistry and Cell Biology* 90, 329–338.

Ryndak MB, Wang S, Smith I, Rodriguez GM (2010) The *Mycobacterium tuberculosis* high-affinity iron importer, IrtA, contains an FAD-binding domain. *Journal of Bacteriology* 192, 861–869.

Schaible UE, Kaufmann SHE (2004) Iron and microbial infection. *Nature Reviews Microbiology* 2, 946–953.

Schaible UE, Collins HL, Priem F, Kaufmann SHE (2002) Correction of the iron overload defect in β-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. *Journal of Experimental Medicine* 196, 1507–1513.

Seidler NW (ed.) (2013) Target for diverse chemical modifications. In *GAPDH: Biological Properties and Diversity*. Netherlands, Springer, pp. 179–206.

Shemisa K, Jafferjee N, Thomas D, Jacobs G, Meyerson HJ (2014) *Mycobacterium avium* complex infection in a patient with sickle cell disease and severe iron overload. *Case Reports in Infectious Diseases* 2014, 405323.

Sheokand N, Kumar S, Malhotra H, Tillu V, Raje CI, Raje M (2013) Secreted glyceraldehye-3-phosphate dehydrogenase is a multifunctional autocrine transferrin receptor for cellular iron acquisition. *Biochimica et Biophysica Acta – General Subjects* 1830, 3816–3827.

Sheokand N, Malhotra H, Kumar S, Tillu VA, Chauhan AS, Raje CI, Raje M (2014) Moonlighting cell-surface GAPDH recruits apotransferrin to effect iron egress from mammalian cells. *Journal of Cell Science* 127, 4279–4291.

Siegrist MS, Unnikrishnan M, McConnell MJ, Borowsky M, Cheng T-Y, Siddiqi N, Fortune SM, Moody DB, Rubin EJ (2009) Mycobacterial Esx-3 is required for mycobactin-mediated iron acquisition. *Proceedings of the National Academy of Science, USA* 106, 18792–18797.

Sirover MA (1999) New insights into an old protein: the functional diversity of mammalian glyceraldehydes-3-phosphate dehydrogenase. *Biochimica et Biophysica Acta (BBA)–Protein Structure and Molecular Enzymology* 1432, 159–184.

Sirover MA (2011) On the functional diversity of glyceraldehydes-3-phosphate dehydrogenase: biochemical mechanisms and regulatory control. *Biochimica et Biophysica Acta – General Subjects* 1810, 741–751.

Sirover MA (2012) Subcellular dynamics of multifunctional protein regulation: mechanisms of GAPDH intracellular translocation. *Journal of Cellular Biochemistry* 113, 2193–2200.

Sirover MA (2014) Structural analysis of glyceraldehydes-3-phosphate dehydrogenase functional diversity. *International Journal of Biochemistry and Cell Biology* 57, 20–26.

Skaar EP (2010) The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathogens* 6, e1000949.

Skaar EP, Humayun M, Bae T, DeBord KL, Schneewind O (2004) Iron-source preference of *Staphylococcus aureus* infections. *Science* 305, 1626–1628.

Taylor JM, Heinrichs DE (2002) Transferrin binding in *Staphylococcus aureus*: involvement of a cell wall-anchored protein. *Molecular Microbiology* 43, 1603–1614.

Terao Y, Yamaguchi M, Hamada S, Kawabata S (2006) Multifunctional glyceraldehydes-3-phosphate dehydrogenase of *Streptococcus pyogenes* is essential for evasion from neutrophils. *Journal of Biological Chemistry* 281, 14215–14223.

Trousseau A (1872). True and false chlorosis. In *Lectures on Clinical Medicine*, Vol. 5. The New Sydenham Society, London, pp. 95–117. Also published by Lindsay and Blakiston: Philadelphia.

Tullius MV, Harth G, Masleša-Galić S, Dillon BJ, Horwitz MA (2008) A replication-limited recombinant *Mycobacterium bovis* BCG vaccine against tuberculosis designed for human immunodeficiency virus-positive persons is safer and more efficacious than BCG. *Infection and Immunity* 76, 5200–5214.

Tullius MV, Harmston CA, Owens CP, Chim N, Morse RP, McMath LM, Iniguez A, Kimmey JM, Sawaya MR, Whitelegge JP (2011) Discovery and characterization of a unique mycobacterial heme acquisition system. *Proceedings of the National Academy of Science, USA* 108, 5051–5056.

Van Els CACM, Corbiere V, Smits K, van Gaans-van den Brink JAM, Poelen MCM, Mascart F, Meiring HD, Locht C (2014) Towards understanding the essence of post-translational modifications for the *Mycobacterium tuberculosis* immunoproteome. *Frontiers in Immunology* 5, 361.

Wagner D, Maser J, Lai B, Cai Z, Barry 3rd CE, Hönor Zu Bentrup K, Russell DG, Bermudez LE (2005) Elemental analysis of *Mycobacterium avium, Mycobacterium tuberculosis*, and *Mycobacterium smegmatis* containing phagosomes indicates pathogen–induced microenvironments within the host cell's endosomal system. *Journal of Immunology* 174, 1491–1500.

West AP, Bennett MJ, Sellers VM, Andrews NC, Enns CA, Bjorkman PJ (2000) Comparison of the interactions of transferrin receptor and transferrin receptor 2 with transferrin and the hereditary hemochromatosis protein HFE. *Journal of Biological Chemistry* 275, 38135–38138.

WHO (2014) WHO Report 2014. Global Tuberculosis Report. Available at: http://apps.who.int/iris/bitstream/10665/137094/1/9789241564809_eng.pdf (accessed September 2016).

Wool IG (1996) Extraribosomal functions of ribosomal proteins. *Trends in Biochemical Sciences* 21, 164–165.

12

GAPDH and Probiotic Organisms

Hideki Kinoshita

Department of Food Management, School of Food, Agricultural and Environmental Sciences, Miyagi University, Miyagi, Japan
Present address: Laboratory of Food Biochemistry, Department of Bioscience, School of Agriculture, Tokai University, Kumamoto, Japan

12.1 Introduction

Recently, it has been reported that many moonlighting proteins such as glyceraldehydes-3-phosphate dehydrogenase (GAPDH), enolase, glutamine synthetase (GS), elongation factor Tu (EF-Tu), GroEL, and DnaK exist on bacterial cell surfaces even in probiotics (Antikainen *et al*. 2007*b*; Hurmalainen *et al*. 2007; Kinoshita *et al*. 2008*a*; Katakura *et al*. 2010; Glenting *et al*. 2013; Nishiyama *et al*. 2013; Kainulainen and Korhonen 2014). It has been confirmed that these moonlighting proteins express on bacterial cell surface while the functionalities are not well understood. As far as is known, it seems that the main "moonlighting jobs" in these moonlighting proteins are plasminogen-binding and enhancement of its activation, and adhesion to the intestinal tract of the host.

In this chapter, the role of GAPDH in probiotics is discussed while also referring to other moonlighting proteins as the need arises. Interested readers should refer to some excellent reviews of other moonlighting proteins (Sánchez *et al*. 2008; Henderson and Martín 2011; Wang *et al*. 2013; Kainulainen and Korhonen 2014).

12.2 Probiotics and Safety

The original definition of probiotics is "live microbial feed supplements which beneficially affect the host animal by improving its intestinal microbial balance" (Fuller 1989). The redefinition by FAO/WHO for probiotics is "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Joint FAO/WHO Working Group 2002). *Lactobacillus*, *Bifidobacterium*

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

Table 12.1 Representative probiotic strains.

Genus	Species	Strain
Lactobacillus	*acidophilus*	SBT-2062, L92, NCFM
	gasseri	OLL2716 (LG21), SP
	casei	Shirota
	fermentum	KLD, RC-14
	johnsonii	La1
	paracasei	KW3110, CRL431, F19
	plantarum	299v, LA318
	reuteri	SD2112, DSM 17938
	rhamnosus	GG, 271, LB21
	salivarius	UCC118
Bifidobacterium	*breve*	Yakult
	longum	BB536, SBT2928
	lactis	Bb12, HNO19(DR10)

species, and non-pathogenic yeast such as *Saccharomyces* are common microbes used as probiotics. Table 12.1 lists the representative probiotic strains. Probiotics demonstrate many beneficial effects, for example managing lactose intolerance (Savaiano and Kotz 1989), lowering cholesterol (Danielson *et al*. 1989), improving immune function (Perdigón *et al*. 2002), and prevention of colon cancer (Lim *et al*. 2002). Many probiotics are GRAS-certified (generally recognized as safe) by the United States Food and Drug Administration (FDA) (Stiles and Holzapfel 1997; Feord 2002). We can find them in various foods such as a yogurt, cheese, kimchi, and pickles, and foods containing probiotics have long been eaten by people.

Streptococcus thermophilus is widely used for the manufacture of yogurt and cheese. This dairy species of major economic importance is phylogenetically close to pathogenic streptococci, raising the possibility that it has a potential for virulence. Interestingly, most streptococcal virulence-related genes that are not involved in basic cellular processes are either inactivated or absent from the dairy *Streptococcus* (Bolotin *et al*. 2004). Probiotics do not produce toxic substances such as ammonia, indole, phenol, and nitrosamine, while probiotics can assimilate ammonia, inhibit the growth of toxic-substance-producing bacteria such as *Escherichia coli*, and adsorb and eliminate these substances. Recently, some reports show biosorption of heavy metals such as cadmium, mercury, and lead by probiotics (Tian *et al*. 2012; Kinoshita *et al*. 2013*b*). These facts mean that probiotics can be used for detoxification tools. Furthermore, probiotics do not appear to pose any safety concerns for pregnant and lactating women (Elias *et al*. 2011). The authors wrote that systemic absorption is rare when probiotics are used by healthy individuals, and the current literature does not indicate an increase in adverse outcomes.

12.3 Potential Risk of Probiotics

In very rare cases, *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Pediococcus*, *Enterococcus* and *Leuconostoc* are isolated from the focus of attention (Aguirre and Collins 1993; Brook 1996; Ishibashi and Yamazaki 2001). *L. rhamnosus* was isolated from the liver tumor of a patient who ingested fermented milk made from *L. rhamnosus* GG (LGG) (Rautio *et al*. 1999). It was reported that this isolated *L. rhamnosus* could not be distinguished from LGG by pulsed-field gel electrophoresis. Furthermore, *L. rhamnosus* has been isolated from patients with pathology and compromised hosts (Mackay *et al*. 1999; Avlami *et al*. 2001; Kunz *et al*. 2004; Land *et al*. 2005). Recently, it was reported that LGG has pili that act as an adhesin and bind to intestinal mucus (Kankainen *et al*. 2009; von Ossowski *et al*. 2010). This may relate to a bacterial translocation. LGG is, however, one of the best-studied probiotic strains and many beneficial effects have been reported (Hatakka *et al*. 2001; Kalliomäki *et al*. 2001; Kawase *et al*. 2009, 2010). LGG has been commercialized and consumed in a wide variety of food products and dietary supplements throughout the world for long time.

In *Bifidobacterium*, when *B. longum* BB536 is administered orally to germ-free mice, the bacteria colonize the intestinal tract and reach a concentration of 10^9–10^{10}/g intestinal content in 2–3 days (Ishibashi and Yamazaki 2001). Translocation of the colonized *B. longum* to the liver, mesenteric lymph nodes (MLN), and kidney occurs between 1 and 2 weeks after the association, but the translocated *B. longum* causes neither infection nor any harmful effect. Furthermore, the translocated *B. longum* disappears after 4 weeks, clearly showing inhibition of translocation. In immunodeficient nude mice that cannot generate mature T lymphocytes, translocation of the colonized *B. longum* continued for 12 weeks without causing infection or any harmful effect. By contrast, in the case of monoassociation of *E. coli* C25 to mice, translocation occurs and the systemic immunity is subsequently impaired (Deitch *et al*. 1991). When *E. coli* O111 was administered orally to germ-free mice, translocation to various organs occurred and the mice died by endotoxin shock or organ failure (Yamazaki *et al*. 1982). However, when *B. longum*-monoassociated mice were challenged with *E. coli* O111 at a lethal dose, death was avoided at 18 hours after injection. Antilethal activity was not elicited by the feeding of heat-killed *B. longum*. When at a sublethal dose, translocation of *E. coli* O111 was observed in the beginning but became totally undetectable after 7 days in *B. longum*-monoassociated mice; translocation in germ-free mice (*B. longum*-unassociated) was observed after 2 weeks (Ishibashi and Yamazaki 2001).

The same protection effect against *E. coli* is also reported in *B. pseudocatenulatum* CECT7765. When *B. pseudocatenulatum* CECT7765 is administrated with *E. coli* to carbon tetrachloride-induced liver-damaged mice, *B. pseudocatenulatum* CECT7765 showed no significant effect on structural liver damage. However, bacterial DNA translocation and serum endotoxin levels were significantly decreased and gut barrier integrity markers and gene expression levels of several anti-inflammatory mediators were upregulated compared with placebo (Moratalla *et al*. 2014).

Probiotics are never the same as pathogenic bacteria, even if are isolated from the focus. It would be a rash decision to make them the pathogens with the infectibility. These cases are not common but may occur randomly, so should be thoroughly investigated. LGG and *B. longum* BB536 are also GRAS-certified and isolation from the focus is also rare. Endogenous infection as a result of translocation of intestinal bacteria is one cause of opportunistic infection in immunocompromised hosts. A compromised host may need to check whether the use of probiotics would be appropriate, but there would be no problems for a healthy person basically.

12.4 Plasminogen Binding and Enhancement of its Activation

The cell-surface GAPDH has been found in many pathogenic bacteria such as group A *Streptococcus* (Pancholi and Fischetti 1992; Jin *et al*. 2005), group B *Streptococcus* (Seifert *et al*. 2003), *Staphylococcus* (Modun and Williams 1999), *Candida albicans* (Gozalbo *et al*. 1998), and *E. coli* (Egea *et al*. 2007). It has been reported that GAPDH binds to plasmin(ogen) and fibrinogen, and relates to fibrinolysis.

There exists limited knowledge of GAPDH in probiotics. Hurmalainen *et al*. (2007) report that GAPDH is expressed on cell surfaces with enolase in *L. crispatus* ST1. The proteins bound plasminogen (Plg) and enhanced its activation using the tissue-type Plg activator (tPA). Furthermore, GAPDH and enolase were also detected from PBS extracts in seven lactobacilli and one lactococci – group A1 (*L. acidophilus* E507), A3 (*L. amylovorus* JCM 5807), A4 (*L. gallinarum* T-50), B1 (*L. gasseri* JCM 1130/ATCC 19992) and B2 (*L. johnsonii* F133) – as well as three strains in probiotic or dairy use (*L. rhamnosus* GG, *L. paracasei* E506, and *Lactococcus lactis* E523). When the extracellular proteins were screened for enhancement of tPA- and uPA (urokinase-type Plg activator) -catalyzed Plg activation and analyzed by Western blotting for the possible presence of enolase and GAPDH, the extracellular preparations exhibited variable capacity to enhance tPA- and uPA-catalyzed Plg activation; *L. gallinarum* T-50 (A4) and *L. johnsonii* F133 (B2) demonstrated the highest activity, and *L. amylovorus* JCM 5807 (A3), *L. gasseri* JCM 1130/ATCC 19992 (B1), and *L. rhamnosus* GG the lowest activity. The preparations from *L. acidophilus* E507, *L. paracasei* E506, and *Lc. lactis* E523 yielded similar activity to that of the preparation from *L. crispatus* ST1.

Further, Antikainen *et al*. (2007*a*) describe plasminogen binding and enhancement of tPA- and uPA-mediated plasminogen activation by the His_6-enolases of *L. crispatus* and *L. johnsonii*. GS also shows plasminogen binding and an effect on tPA-mediated plasminogen activation (Kainulainen *et al*. 2012).

In bifidobacteria, it had been reported that *B. lactis*, *B. bifidum*, and *B. longum* also bind to human plasmin(ogen) (Candela *et al*. 2007, 2008, 2011). Plg-binding proteins of *B. lactis* BI07 were identified as DnaK, GS, enolase, bile salt hydrolase, and phosphoglycerate mutase, but GAPDH was not mentioned.

In this way, some moonlighting proteins such as GAPDH and enolase of probiotics show plasminogen binding and plasminogen activation as well as

that of pathogen. This suggests these moonlighting proteins may cause a potential health risk in opportunistic infections.

12.5 GAPDH as an Adhesin

Most reports about the multiple moonlighting functions of surface GAPDH in probiotics refer to adhesins. Table 12.2 lists probiotic strains which express GAPDH on the cell surface and their targeting ligands. GAPDH in the form of adhesin are reported in relatively large numbers in *Lactobacillus*, especially *L. plantarum*.

Table 12.2 Probiotics strains expressing GAPDH on cell surface and its targeting ligands.

Genus	Species	Strain	Targeting ligands	References
Enterococcus	*faecalis*	Symbioflor®	Actin	Peng *et al.* 2014
Lactobacillus	*gasseri*	Lv19	Porcine gastric mucin	Martín *et al.* 2012
	casei	BL23	Fibronectin	Muñoz-Provencio *et al.* 2011
			Collagen	Muñoz-Provencio *et al.* 2011
	crispatus	ST1	Plasminogen	Hurmalainen *et al.* 2007
			Lipoteichoic acid	Antikainen *et al.* 2007*b*
	jensenii	CECT 4306	Unknown	Martín *et al.* 2015
	plantarum	423	Caco-2 cells	Ramiah *et al.* 2008
		LA 318	Human colonic mucin	Kinoshita *et al.* 2008*a*
			ABO blood type antigens	Kinoshita *et al.* 2008*b*
		BMCM12	Fibronectin	Sánchez *et al.* 2009*a*
			Mucin	Sánchez *et al.* 2009*a*
		Li69	Porcine gastric mucin	Martín *et al.* 2012
		Li70	Porcine gastric mucin	Martín *et al.* 2012
		299v	Mucin	Sánchez *et al.* 2009*a*
			Plasminogen	Glenting *et al.* 2013
			Fibronectin	Sánchez *et al.* 2009*a*; Glenting *et al.* 2013
			Porcine mucin	Glenting *et al.* 2013
			Caco-2 cells	Glenting *et al.* 2013
	rhamnosus	GG	Unknown	Sánchez *et al.* 2009*b*
Lactococcus	*lactis*	IL1403	Invertase (mannoprotein)	Katakura *et al.* 2010
Oenococcus	*oeni*		Unknown	Carreté *et al.* 2005

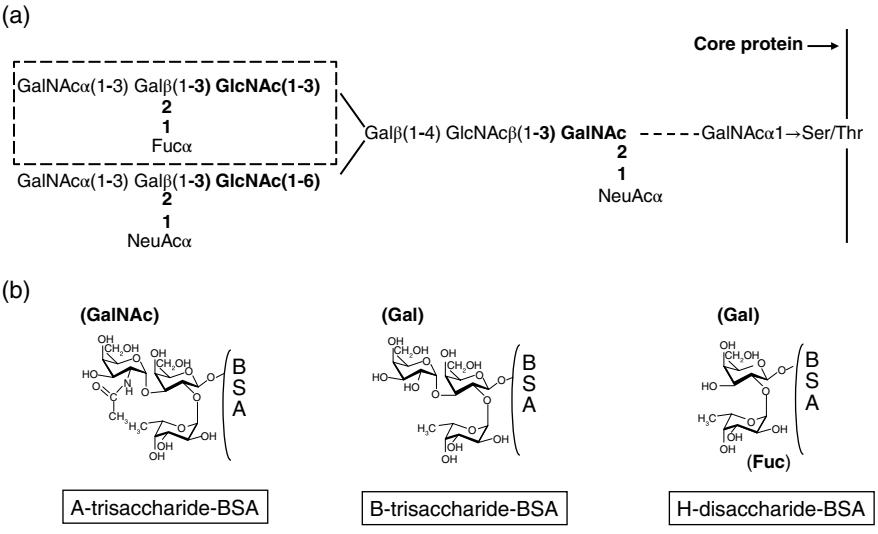

Figure 12.1 (a) A representative structure of human intestinal mucus. A dashed square indicates expression of A-type blood group antigen. (b) Chemical structure of three kinds of human ABO blood group antigens conjugated-BSA probes. GalNAc: N-acetylgalactosamine; Gal: galactose; Fuc: fucose; GlcNAc: N-acetylglucosamine; NeuAc: N-acetylneuraminic acid (sialic acid); Ser/Thr: serine or threonine.

L. plantarum LA 318 is a potential probiotic strain isolated from normal human intestinal tissue that shows high adhesion to human colonic mucin (HCM) (Fig. 12.1a; Kinoshita *et al*. 2007). Although distilled water-washed bacterial cells (control) showed high adhesion to HCM, the adhesive activity drastically decreased by about 80% after only washing with PBS. The PBS wash fraction and the purified GAPDH strongly adhered to HCM (Kinoshita *et al*. 2008a). Further, in BIACORE binding assay of the GAPDH to the blood group antigen-conjugated BSA probe (Fig. 12.1b), high binding was observed to the A and B group antigens while binding to the H (O) group antigen was lower (about one sixth) (Kinoshita *et al*. 2008*b*). There are no significant differences between the A and B antigens. This suggests GAPDH may have weak recognition between GalNAc and Gal or, at the molecular level, GAPDH might not distinguish the difference between the hydroxyl group and the N-acetyl group at the C2 position of each sugar. Further, no interaction was observed between GAPDH and various monosaccharides, and the GAPDH binding to B-trisaccharide (Galα1-3 (Fucα1-2) Gal-) biotinyl polymer (BP)-probe was significantly higher as compared to B-disaccharide (Galα1-3Gal-), H type 1 (Fucα1-2Galβ1-3GlcNAc-), 3-fucosyl-N-acetylglucosamine (Fucα1-3GlcNAc-), and α-N-acetylneuraminic acid (NueNAc-) BP-probes. These suggest the trisaccharide structure is important in binding to the blood group antigens and GAPDH may be a GalNAc and Gal recognition lectin-like protein. Some pathogens such as *Helicobacter pylori* (Aspholm-Hurtig *et al*. 2004) and Norwalk virus (Hutson *et al*. 2002) and enterotoxin of *E. coli* (Holmner *et al*. 2007) also bind to blood group antigens as well as LAB (Uchida *et al*. 2006; Kinoshita *et al*. 2008*b*; Watanabe *et al*. 2010). The blood

group antigen recognition LAB might inhibit pathogenic infections because these LAB can competitively inhibit the blood group antigen recognition pathogens to HCM (Saito 2013).

Sánchez *et al*. (2009*a*) reported GAPDH of *L. plantarum* strains, 299v and BMCM12, adhered to mucin and fibronectin. Glenting *et al*. (2013) also report that GAPDH and enolase could play a role in the adhesion of *L. plantarum* 299v to extracellular matrix proteins and to mucin. These proteins showed a highly specific binding to plasminogen and fibronectin, whereas GAPDH but not enolase showed weak binding to mucin. Castaldo *et al*. (2009) report *L. plantarum* LM3 expressed enolase on its cell surface and bound to fibronectin. *L. plantarum* 423 has GAPDH, EF-Tu, and triosephosphate isomerase (TPI) on cell surface and plays a role in adhesion of to Caco-2 cells (Ramiah *et al*. 2008). This strain prevented *Clostridium sporogenes* and *Enterococcus faecalis* from adhering to Caco-2 cells. Martín *et al*. (2012) reported that the cell-surface GAPDH of *L. plantarum* Li69, *L. plantarum* Li70, and *L. gasseri* Lv19, which shows high adhesion to HT-29 or HeLa cells, bound to porcine gastric mucin.

Cell-surface GAPDH is often reported to the probiotic strain as well as *L. plantarum* and, in many cases, a cell-surface GAPDH remains its enzymatic activities. Glenting *et al*. (2013) reported that when the extracellular GAPDH enzyme activities were measured in 23 lactobacilli, eight out of nine tested *L. plantarum* strains, two out of five *L. rhamnosus*, two out of five *L. gasseri*, and one out of three *L. paracasei*, whereas no GAPDH activity was found in the *L. casei* ATCC 334. When GAPDH enzymatic activities were measured in 30 lactobacilli isolated from human intestinal tissues, they were detected in 21 out of 30 samples from 12 hour cultures and in all samples from 18 hour cultures (Kinoshita *et al*. 2013*a*). This suggests that the accumulated growth of GAPDH is time dependent. Saad *et al*. (2009) also reported that the growth of GAPDH concentration on cell walls was time dependent in *L. plantarum* 299v. *L. gasseri* LA 305, *L. gasseri* LA 313, and *L. casei* LA 316 showed high adhesion values and correlated with high GAPDH activities, while low GAPDH activity strains (*L. salivarius* LA 301, *L. fermentum* LA 304, *L. salivarius* LA 310, and *L. fermentum* LA 311) showed significantly low adhesion values compared with the positive control strain, LA 318 (Kinoshita *et al*. 2013*a*). The correlation coefficient between GAPDH activity and the adhesion value was 0.69. Surprisingly, when the same test was performed using 47 lactobacilli derived from plants such as pickles, all strains had GAPDH activity although there was no correlation between the GAPDH activity and adhesion. It is considered that lactobacilli may adjust to the environment and change the structure of the cell-surface protein, even if GAPDH is universally expressed on cell surface of lactobacilli.

Spurbeck and Arvidson (2010) report that surface-associated proteins of *L. jensenii* ATCC 25258 and its cell-surface proteins inhibit the adherence to epithelial cells of the sexually transmitted pathogen *Neisseria gonorrhoeae*. When a soluble fibronectin was added, the inhibitory activity against *N. gonorrhoeae* adherence by the proteins was reduced, suggesting the proteins bound fibronectin. The cell-surface proteins were separated into two fractions by anion-exchange column chromatography. Both two fractions also inhibit

N. gonorrhoeae adherence. The main proteins containing the two fractions were identified as GAPDH and enolase, and recombinant enolase also inhibited *N. gonorrhoeae* adherence.

Cell-wall fractions of *L. casei* BL23 enriched in fibronectin and collagen-binding proteins were isolated (Muñoz-Provencio *et al*. 2011). Their protein content revealed the presence of stress-related proteins (GroEL, ClpL), translational elongation factors (EF-Tu, EF-G), oligopeptide solute-binding proteins, and the glycolytic enzymes enolase and GAPDH. Sánchez *et al*. (2009*b*) also report GAPDH and phosphoglycerate kinase (PGK) are expressed on the cell surface of *L. rhamnosus* GG. It is not certain that GAPDH behave as an adhesin in *L. rhamnosus* GG, however.

Peng *et al*. (2014) report four proteins show actin binding in the different lifestyles of *Enterococcus faecalis*, that is, commensal (OG1RF), probiotic (Symbioflora®), and pathogen (clinical isolate from severe infections V583). The actin-binding proteins are identified by LC–MS/MS as GAPDH, pyruvate formate lyase, enolase, and GroEL. This suggests *E. faecalis* has same adhesins to bind actin.

Katakura *et al*. (2010) report many moonlighting proteins that show affinity to invertase, a hyperglycosylated mannoprotein from *S. cerevisiae*, were found in *Lc. lactis* subsp. *lactis* IL1403. Fifteen of the sixteen spots detected in two-dimensional electrophoresis of affinity-purified cell-wall proteins were identified to be cytosolic proteins, including molecular chaperones DnaK, GroEL, and GroE, and enzymes in the central metabolic pathway such as GAPDH, pyruvate kinase (PK), and 6-phosphofructokinase. Further, it is reported that DnaK shows the highest adhesion to LAB and yeast at pH 4, and is an aggregation factor between LAB and yeast. When almost the same number of LAB cells and yeast cells were mixed, aggregation of the yeast cells was observed in the presence of the recombinant DnaK at above 0.1 mg mL^{-1}. In contrast, auto-agglutination of LAB or yeast was not observed, even in the presence of 1.0 mg mL^{-1} DnaK. Furthermore, when an excess number of LAB cells was added to yeast cells, the yeast cells did not aggregate because they were surrounded by LAB cells. Authors suggest these results show that DnaK has two independent binding sites: one specific to LAB and another specific to yeast.

It is known that moonlighting proteins show pH-dependent binding. Binding of GAPDH and enolase of *L. plantarum* 299v to intestinal epithelial Caco-2 cells was observed at pH 5, but not at pH 7. The enolase showed higher affinity to Caco-2 cells than GAPDH (Glenting *et al*. 2013). EF-Tu and GroEL of *L. johnsonii* NCC 533 (La1) show mucin and epithelial cells at pH 5.0, whereas no specific binding was observed at natural pH (Granato *et al*. 2004; Bergonzelli *et al*. 2006). Nishiyama *et al*. (2013) report similar pH-dependent binding of EF-Tu to sulfated carbohydrate of porcine gastric mucin.

12.6 Binding Regions

GAPDH has multi-binding properties as mentioned above, although the mechanism is poorly understood. GAPDH consists of NAD-binding domain and C-terminal domain. Figure 12.2 depicts the predicted three-dimensional structure of GAPDH of *L. plantarum* LA 318 by SWISS-MODEL. SMART (simple modular

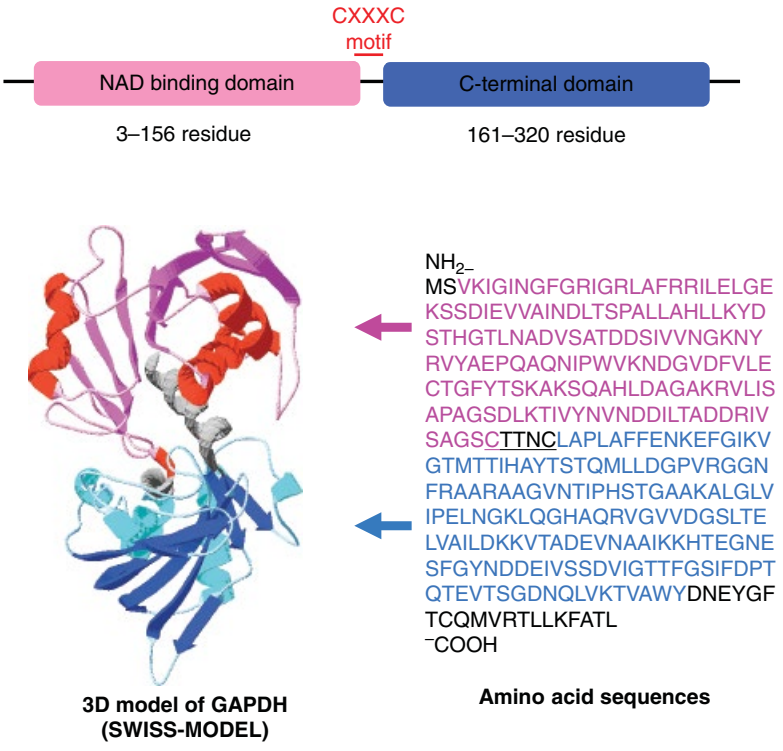

Figure 12.2 The three-dimensional structure and amino acid sequences of GAPDH of *L. plantarum* LA 318. The underline indicates CXXXC motif. (*See color plate section for the color representation of this figure.*)

architecture research tool) domain analysis shows that residues 3–156 is NAD-binding domain and residues 161–320 is C-terminal domain in GAPDH of *L. plantarum* LA 318 (Fig. 12.2). When NAD^+ was added to GAPDH, the binding of GAPDH to HCM was significantly decreased (Kinoshita *et al.* 2008*b*). This suggests that the NAD-binding domain of GAPDH may be related to binding to HCM; however, further investigation is needed.

It has been known that plasminogen has kringle domains containing four or five lysine-binding sites (LBS). It is reported that the lysines of the C-terminal end of GAPDH and enolase are related, allowing the binding of LBS of plasminogen in the group A streptococci (Winram and Lottenberg 1998; Derbise *et al.* 2004). Enolase and GAPDH of *L. crispatus* ST1 as well as GAPDH of *L. plantarum* LA 318 (Fig. 12.2) lack the C-terminal lysine residues (Hurmalainen *et al.* 2007; Kinoshita *et al.* 2008*a*). However, the sequence of the enolase of *L. crispatus* ST1 contains a similar internal Plg binding sequence, ^{248}FYNKDDHKY, in the same position as in the pneumococcal enolase (Ehinger *et al.* 2004).

Jin *et al.* (2005) showed that GAPDH of *S. pyogenes* ATCC 700294 bound to uPAR (uPA receptor)/CD87 on human pharyngeal cells. In ligand-binding assays, GAPDH binds to only the N-terminal domain (D1) of uPAR. Further, it is revealed the C-terminal alpha-helix and two immediate flanking regions of the S-loop of the GAPDH molecule more specifically bind to uPAR-D1.

When the multi-binding of GAPDH is considered, it is unsurprising that there is more than one binding site. Further in-depth studies are required before a full understanding can be achieved.

12.7 Mechanisms of Secretion and Surface Localization

It is thought that the mechanism of surface localization of moonlighting proteins is ionic-bound, whereas secretion to the cell surface is not yet well understood.

It is thought that pH is low around lactic acid bacteria (LAB) due to lactic acid, whereas intestinal tract has a neutral pH. Antikainen *et al*. (2007*b*) report GAPDH bound to extracellular negative-charged substances such as lipoteichoic acid (LTA) on the bacterial cell surface using an ionic bond. Enolase- and GAPDH-coated beads bound more efficiently to LTA than to peptidoglycan or BSA at pH 4.4, whereas only poor binding was detected at pH 7.0. The authors concluded that enolase and GAPDH interact with LTA under their isoelectric point, but not at pH values higher than it. Many report that moonlighting proteins are easily extracted with a basic solution and a salt-contained solution such as PBS from the cell surface, which they are bound to ionic bound (Kinoshita *et al*. 2008*a*; Katakura *et al*. 2010; Glenting *et al*. 2013).

Moonlighting proteins have been called anchorless surface proteins because their sequences do not contain known sequence motifs for surface anchoring, nor do the protein sequences contain identified secretion signals. The translocation mechanisms of cell surfaces are poorly understood and remains an open issue.

Saad *et al*. (2009) reported an interesting study about translocation of GAPDH onto a cell surface. The membrane permeabilization of *L. plantarum* 299v was estimated by labeling cells with propidium iodide (PI). The amount of cell-surface GAPDH correlated with stationary growth phase and increased cell permeability. Moreover, when glucose was added to the growth medium, the PI value and cell-surface GAPDH decreased, indicating that GAPDH is eluted when cells are injured. Similar results were reported by Kainulainen *et al*. (2012). The pH shift was associated with a rapid and transient increase in cell-wall permeability, as measured by *L. crispatus* ST1 cells staining with PI at pH 8. A gradual increase in the release of the four moonlighting proteins – GS, glucose-6-phosphate isomerase (GPI), enolase, and GAPDH – was also observed after the treatment of *L. crispatus* ST1 cells with increasing concentrations of the antimicrobial cationic peptide LL-37, which kills bacteria by disturbing membrane integrity. The level of expression of moonlighting proteins on the cell surface corresponds to the permeability of the cell wall in *L. crispatus* ST1, suggesting that moonlighting proteins are eluted due to cell injury. Furthermore, the recombinant four moonlighting proteins from *L. crispatus* ST1 were added to *L. crispatus* ST1 or *L. rhamnosus* GG after washing at pH 8, and all proteins bound to both lactobacilli. This means that the moonlighting proteins can associate with the cell surface of the same *Lactbacillus* species where the proteins were originally

released, but also with the cell surface of other *Lactbacillus* species. However, the binding is not evenly distributed around the cells, but is concentrated around cell division areas as well as at the cell poles. By contrast, when recombinant GPI from *L. crispatus* ST1 or *L. rhamnosus* GG was added, no binding to *L. rhamnosus* GG was observed. This suggests extracellular components are different among bacterial strains.

On other hand, Lenz *et al.* (2003) report SecA2-dependent secretion of some moonlighting proteins in *Listeria monocytogenes*. When comparing the profiles of secreted proteins and surface extracts of exponentially grown wild-type and ΔSecA2 strains, 19 proteins were either undetectable or markedly reduced in ΔSecA2 mutant. The moonlighting proteins, RNA polymerase, pyruvate dehydrogenase, DnaK, GroEL, EF-Tu, Enolase, phosphomannose isomerase, and ribosomal protein, are identified as SecA2-dependent secreted proteins. It is conceivable that moonlighting proteins of probiotics are secreted SecA2-dependently, since the *SecA2* gene has been reported in LAB (Kleerebezem *et al.* 2003; Johansson *et al.* 2011); however, no reports of transporter-dependent secretion of moonlighting proteins in probiotics have yet been published.

12.8 Other Functions

In probiotic strains, studies of GAPDH are almost mentioned above. GAPDH, however, may relate to stress response because some reports show expression of moonlighting proteins such as GAPDH increase under various stress conditions. Prasad *et al.* (2003) observed that when pre-stressed with either heat (50°C) or salt (0.6 M NaCl), *L. rhamnosus* HN001 (DR20) showed significant improvement in viability compared with the non-stressed control culture after storage at 30°C in the dried form. Several proteins were up- or downregulated after either heat or osmotic shock treatments. The expression levels of 10 proteins identified as GroEL, DnaK, lactate dehydrogenase (LDH), enolase, PGK, TPI, tagatose 1,6-diphosphate aldolase (TBPA), ABC transporter protein, histidine-containing protein (HPr), and GAPDH were measured during different stages of growth and stress treatments. GAPDH expression level marginally increased when heat shock was applied after the log phase, while levels of the other enzymes, TPI and PGK, remained relatively unchanged. Heat shock after the stationary phase increased the expression levels of GAPDH and TPI by a factor of 2.5 and 5, respectively, while the levels of GAPDH, PGK, and TPI decreased after osmotic stress. In bifidobacteria, DnaK from *B. animalis* subsp. *lactis* is also upregulated in response to bile salts (Sánchez *et al.* 2007; Candela *et al.* 2010). Carreté *et al.* (2005) reported that the profiles of membrane proteins of *Oenococcus oeni* change under particular stress conditions of wine. GAPDH was overexpressed in the presence of SO_2 but the protein showed no GAPDH activity. Moonlighting proteins can therefore be overexpressed as a result of various environmental stresses. It is thought that GAPDH may protect itself from such stresses, although the exact mechanisms are unclear, or that GAPDH is simply eluted by cell trauma and binds to the cell surface. It is anticipated that this will be better understood in the future.

Further, GAPDH may be involved in immunoresponse. Martín *et al*. (2015) also report that Fe^{3+} enhances the adhesion of *L. jensenii* CECT 4306 to mucin and to HT-29 and HT-29 MTX cells, and improves the anti-inflammatory profile as judged by an increase in the ratio of interleukin (IL)-10/IL-12p70 that was secreted by macrophages. When cultured in the presence of Fe^{3+}, *L. jensenii* overproduced 6 proteins and underproduced 14. The most upregulated proteins were identified as GADPH isoforms, ATP synthase β subunit, and glutamyl-tRNA glutamine amidotransferase β subunit. This suggests GAPDH may be an adhesin of *L. jensenii* CECT 4306 and may be related to immunological response. Furthermore, it is reported that GroEL and EF-Tu stimulate expression of IL-8 in La1 strain. Recombinant GroEL of La1 stimulates IL-8 secretion in macrophages and HT29 cells in a CD14-dependent mechanism (Bergonzelli *et al*. 2006). This property is common to rGroEL from other Gram-positive bacteria but not to the rGroEL of *H. pylori*. In addition, rGroEL of La1 mediates the aggregation of *H. pylori* but not that of other intestinal pathogens such as *Salmonella enterica* serovar Typhimurium and *E. coli*. Granato *et al*. (2004) reported that recombinant EF-Tu also stimulated expression of IL-8 on HT29 cells in the presence of soluble CD14. These facts indicate that moonlighting proteins of probiotics are involved in immunomodulation in the host. Although the moonlighting proteins from pathogens and probiotics can elicit host immune responses, it seems that the proteins involved and the degree of immune responsiveness are different (Wang *et al*. 2013).

GAPDH is thiol enzyme and Cys-X-X-X-Cys motif (X is any amino acid) exists between the 156–160 end of the NAD-binding domain and C-terminal domain in *L. plantarum* LA 318 (Fig. 12.2). It is known that a CXXXC motif is a metal-binding site (Borrelly *et al*. 2004; López-Serrano *et al*. 2007; Blundell *et al*. 2013). Recently, some reports have demonstrated the biosorption of harmful heavy metals such as cadmium, mercury, and lead in LAB (Kinoshita *et al*. 2013*b*; Zhai *et al*. 2013). An oral ingestion of LAB showing high biosorption of heavy metals can prevent the absorption of heavy metals into the body, and can discharge them via feces from the body efficiently and be used as a biosorbent for detoxification. GAPDH may be able to bind heavy metals via CXXXC motif. However, experimental evidence is needed.

12.9 Conclusion

Figure 12.3 shows overview diagram of GAPDH in probiotics mentioned above. GAPDH has many functions, such as: adhesion to host and other bacteria; Pln binding and enhancement of its activation; and stress- and immuno-response. Moonlighting proteins may be a potential risk factor in opportunistic infections in probiotics. Considering the overall safety of probiotics, this is however not a major concern; the overexpression of moonlighting proteins in probiotics imparts more positive than negative effects to the host.

It seems that GAPDH is universally expressed on the bacterial cell surfaces from many probiotic strains. Probiotics which are prokaryote and have low genetic information may use proteins for many occasions. The multi-functionality of

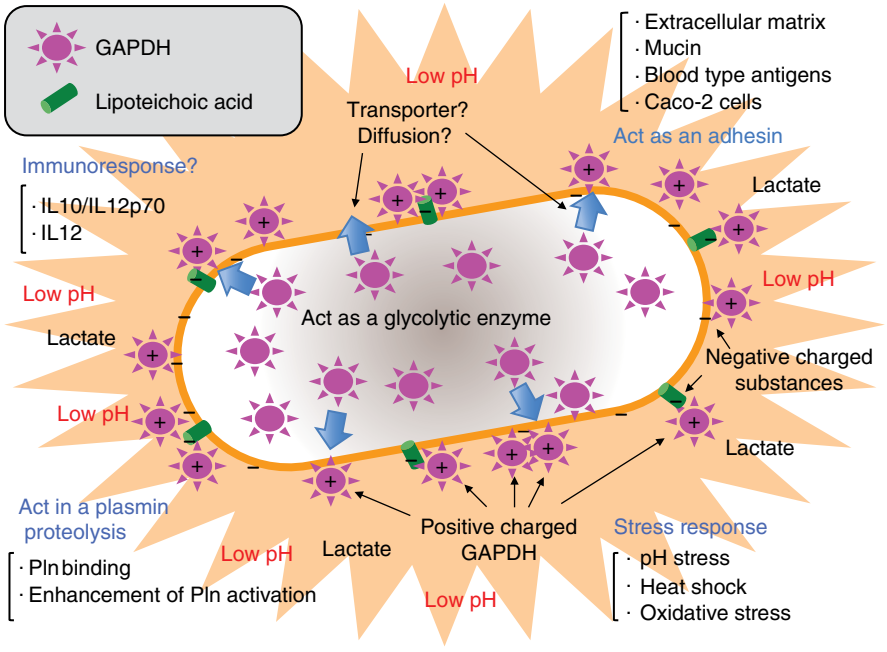

Figure 12.3 Overview diagram of GAPDH in probiotics. (*See color plate section for the color representation of this figure.*)

moonlighting proteins such as GAPDH is not yet understood sufficiently, but this will become clearer with further experiments.

References

Aguirre M, Collins MD (1993) Lactic acid bacteria and human clinical infection. *Journal of Applied Bacteriology* 75: 95–107.

Antikainen J, Kuparinen V, Lähteenmäki K, Korhonen TK (2007*a*) Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunology and Medical Microbiology* 51: 526–534.

Antikainen J, Kuparinen V, Lähteenmäki K, and Korhonen TK (2007*b*) pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of *Lactobacillus crispatus* with the cell wall and lipoteichoic acids. *Journal of Bacteriology* 189: 4539–4543.

Aspholm-Hurtig M, Dailide G, Lahmann M, Kalia A, Ilver D, Roche N, Vikström S, Sjöström R, Lindén S, Bäckström A, Lundberg C, Arnqvist A, Mahdavi J, Nilsson UJ, Velapatiño B, Gilman RH, Gerhard M, Alarcon T, López-Brea M, Nakazawa T, Fox JG, Correa P, Dominguez-Bello MG, Perez-Perez GI, Blaser MJ, Normark S, Carlstedt I, Oscarson S, Teneberg S, Berg DE, Borén T (2004) Functional adaptation of BabA, the *H. pylori* ABO blood group antigen binding adhesin. *Science* 305: 519–522.

Avlami A, Kordossis T, Vrizidis N, Sipsas NV (2001) *Lactobacillus rhamnosus* endocarditis complicating colonoscopy. *Journal of Infection* 42: 283–285.

Bergonzelli GE, Granato D, Pridmore RD, Marvin-Guy LF, Donnicola D, Corthésy-Theulaz IE (2006) GroEL of *Lactobacillus johnsonii* La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gastric pathogen *Helicobacter pylori*. *Infection and Immunity* 74: 425–434.

Blundell KL, Wilson MT, Vijgenboom E, Worrall JA (2013) The role of the Cys-X-X-X-Cys motif on the kinetics of cupric ion loading to the *Streptomyces lividans* Sco protein. *Dalton Transactions* 42: 10608–10616.

Bolotin A, Quinquis B, Renault P, Sorokin A, Ehrlich SD, Kulakauskas S, Lapidus A, Goltsman E, Mazur M, Pusch GD, Fonstein M, Overbeek R, Kyprides N, Purnelle B, Prozzi D, Ngui K, Masuy D, Hancy F, Burteau S, Boutry M, Delcour J, Goffeau A, Hols P (2004) Complete sequence and comparative genome analysis of the dairy bacterium *Streptococcus thermophilus*. *Nature Biotechnology* 22: 1554–1558.

Borrelly GP, Blindauer CA, Schmid R, Butler CS, Cooper CE, Harvey I, Sadler PJ, Robinson NJ (2004) A novel copper site in a cyanobacterial metallochaperone. *Biochemical Journal* 378: 293–297.

Brook I (1996) Isolation of non-sporing anaerobic rods from infections in children. *Journal of Medical Microbiology* 45: 21–26.

Candela M, Bergmann S, Vici M, Vitali B, Turroni S, Eikmanns BJ, Hammerschmidt S, Brigidi P (2007) Binding of human plasminogen to *Bifidobacterium*. *Journal of Bacteriology* 189: 5929–5936.

Candela M, Fiori J, Dipalo S, Naldi M, Gotti R, Brigidi P (2008) Rapid MALDI-TOF-MS analysis in the study of interaction between whole bacterial cells and human target molecules: binding of *Bifidobacterium* to human plasminogen. *Journal of Microbiological Methods* 73: 276–278.

Candela M, Centanni M, Fiori J, Biagi E, Turroni S, Orrico C, Bergmann S, Hammerschmidt S, Brigidi P (2010) DnaK from *Bifidobacterium animalis* subsp. *lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts. *Microbiology* 156: 1609–1618.

Candela M, Turroni S, Centanni M, Fiori J, Bergmann S, Hammerschmidt S, Brigidi P (2011) Relevance of *Bifidobacterium animalis* subsp. *lactis* plasminogen binding activity in the human gastrointestinal microenvironment. *Applied and Environmental Microbiology* 77: 7072–7076.

Carreté R, Reguant C, Bordons A, Constantí M (2005) Relationship between a stress membrane protein of *Oenococcus oeni* and glyceraldehyde-3-phosphate dehydrogenases. *Applied Biochemistry and Biotechnology* 127: 43–51.

Castaldo C, Vastano V, Siciliano RA, Candela M, Vici M, Muscariello L, Marasco R, Sacco M (2009) Surface displaced alfa-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. *Microbial Cell Factories* 8: 14.

Danielson AD, Peo Jr ER, Shahani KM, Lewis AJ, Whalen PJ, Amer MA (1989) Anticholesteremic property of *Lactobacillus acidophilus* yogurt fed to mature boars. *Journal of Animal Science* 67: 966–974.

Deitch EA, Xu DZ, Qi L, Berg RD (1991) Bacterial translocation from the gut impairs systemic immunity. *Surgery* 109: 269–276.

Derbise A, Song YP, Parikh S, Fischetti VA, Pancholi V (2004) Role of the C-terminal lysine residues of streptococcal surface enolase in Glu- and Lys-plasminogen-binding activities of group A streptococci. *Infection and Immunity* 72: 94–105.

Egea L, Aguilera L, Gimenez R, Sorolla MA, Aguilar J, Badía J, Baldoma L (2007) Role of secreted glyceraldehyde-3-phosphate dehydrogenase in the infection mechanism of enterohemorrhagic and enteropathogenic *Escherichia coli*: interaction of the extracellular enzyme with human plasminogen and fibrinogen. *International Journal of Biochemistry and Cell Biology* 39: 1190–1203.

Ehinger S, Schubert WD, Bergmann S, Hammerschmidt S, Heinz DW (2004) Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. *Journal of Molecular Biology* 343: 997–1005.

Elias J, Bozzo P, Einarson A (2011) Are probiotics safe for use during pregnancy and lactation? *Canadian Family Physician* 57: 299–301.

Feord J (2002) Lactic acid bacteria in a changing legislative environment. *Antonie Van Leeuwenhoek* 82: 353–360.

Fuller R (1989) Probiotics in man and animals. *Journal of Applied Bacteriology* 66: 365–378.

Glenting J, Beck HC, Vrang A, Riemann H, Ravn P, Hansen AM, Antonsson M, Ahrné S, Israelsen H, Madsen S (2013) Anchorless surface associated glycolytic enzymes from *Lactobacillus plantarum* 299v bind to epithelial cells and extracellular matrix proteins. *Microbiological Research* 168: 245–253.

Gozalbo D, Gil-Navarro I, Azorín I, Renau-Piqueras J, Martínez JP, Gil. ML (1998) The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein. *Infection and Immunity* 66: 2052–2059.

Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Corthésy-Theulaz IE (2004) Cell surface-associated elongation factor Tu mediates the attachment of *Lactobacillus johnsonii* NCC533 (La1) to human intestinal cells and mucins. *Infection and Immunity* 72: 2160–2169.

Hatakka K, Savilahti E, Pönkä A, Meurman JH, Poussa T, Näse L, Saxelin M, Korpela R (2001) Effect of long term consumption of probiotic milk on infections in children attending day care centres: double blind, randomised trial. *BMJ* 322: 1327.

Henderson B, Martin A (2011) Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity* 79: 3476–3491.

Holmner A, Askarieh G, Okvist M, Krengel U (2007) Blood group antigen recognition by *Escherichia coli* heat-labile enterotoxin. *Journal of Molecular Biology* 371: 754–764.

Hurmalainen V, Edelman S, Antikainen J, Baumann M, Lähteenmäki K, Korhonen TK (2007) Extracellular proteins of *Lactobacillus crispatus* enhance activation of human plasminogen. *Microbiology* 153: 1112–1122.

Hutson AM, Atmar RL, Graham DY, Estes MK (2002) Norwalk virus infection and disease is associated with ABO histo–blood group type. *Journal of Infectious Diseases* 185: 1335–1337.

Ishibashi N, Yamazaki S (2001) Probiotics and safety. *American Journal of Clinical Nutrition* 73: 465S–470S.

Jin H, Song YP, Boel G, Kochar J, Pancholi V (2005) Group A streptococcal surface GAPDH, SDH, recognizes uPAR/CD87 as its receptor on the human pharyngeal cell and mediates bacterial adherence to host cells. *Journal of Molecular Biology* 350: 27–41.

Johansson P, Paulin L, Säde E, Salovuori N, Alatalo ER, Björkroth KJ, Auvinen P (2011) Genome sequence of a food spoilage lactic acid bacterium, *Leuconostoc gasicomitatum* LMG 18811T, in association with specific spoilage reactions. *Applied and Environmental Microbiology* 77: 4344–4351.

Joint FAO/WHO Working Group (2002) Report on Drafting Guidelines for the Evaluation of Probiotics in Food. London, Ontario, Canada.

Kainulainen V, Korhonen TK (2014) Dancing to another tune-adhesive moonlighting proteins in bacteria. *Biology (Basel)* 3: 178–204.

Kainulainen V, Loimaranta V, Pekkala A, Edelman S, Antikainen J, Kylväjä R, Laaksonen M, Laakkonen L, Finne J, Korhonen TK (2012) Glutamine synthetase and glucose-6-phosphate isomerase are adhesive moonlighting proteins of *Lactobacillus crispatus* released by epithelial cathelicidin LL-37. *Journal of Bacteriology* 194: 2509–2519.

Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P, Isolauri E (2001) Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* 357: 1076–1079.

Kankainen M, Paulin L, Tynkkynen S, von Ossowski I, Reunanen J, Partanen P, Satokari R, Vesterlund S, Hendrickx AP, Lebeer S, De Keersmaecker SC, Vanderleyden J, Hämäläinen T, Laukkanen S, Salovuori N, Ritari J, Alatalo E, Korpela R, Mattila-Sandholm T, Lassig A, Hatakka K, Kinnunen KT, Karjalainen H, Saxelin M, Laakso K, Surakka A, Palva A, Salusjärvi T, Auvinen P, de Vos WM (2009) Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human-mucus binding protein. *Proceedings of the National Academy of Science, USA* 106: 17193–17198.

Katakura Y, Sano R, Hashimoto T, Ninomiya K, Shioya S (2010) Lactic acid bacteria display on the cell surface cytosolic proteins that recognize yeast mannan. *Applied Microbiology and Biotechnology* 86: 319–326.

Kawase M, He F, Kubota A, Hiramatsu M, Saito H, Ishii T, Yasueda H, Akiyama K (2009) Effect of fermented milk prepared with two probiotic strains on Japanese cedar pollinosis in a double-blind placebo-controlled clinical study. *International Journal of Food Microbiology* 128: 429–434.

Kawase M, He F, Kubota A, Harata G, Hiramatsu M (2010) Oral administration of lactobacilli from human intestinal tract protects mice against influenza virus infection. *Letters in Applied Microbiology* 51: 6–10.

Kinoshita H, Uchida H, Kawai Y, Kitazawa H, Miura K, Shiiba K, Horii A, Saito T (2007) Quantitative evaluation of adhesion of lactobacilli isolated from human intestinal tissues to human colonic mucin using surface plasmon resonance (BIACORE assay). *Journal of Applied Microbiology* 102: 116–123.

Kinoshita H, Uchida H, Kawai Y, Kawasaki T, Wakahara N, Matsuo H, Watanabe M, Kitazawa H, Ohnuma S, Miura K, Horii A, Saito T (2008*a*) Cell surface *Lactobacillus plantarum* LA 318 glyceraldehyde-3-phosphate dehydrogenase

(GAPDH) adheres to human colonic mucin. *Journal of Applied Microbiology* 104: 1667–1674.

Kinoshita H, Wakahara N, Watanabe M, Kawasaki T, Matsuo H, Kawai Y, Kitazawa H, Ohnuma S, Miura K, Horii A, Saito T (2008*b*) Cell surface glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of *Lactobacillus plantarum* LA 318 recognizes human A and B blood group antigens. *Research in Microbiology* 159: 685–691.

Kinoshita H, Imoto S, Suda Y, Ishida M, Watanabe M, Kawai Y, Kitazawa H, Miura K, Horii A, Saito T (2013*a*) Proposal of screening method for intestinal mucus adhesive lactobacilli using the enzymatic activity of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). *Journal of Animal Science* 84: 150–158.

Kinoshita H, Sohma Y, Ohtake F, Ishida M, Kawai Y, Kitazawa H, Saito T, Kimura K (2013*b*) Biosorption of heavy metals by lactic acid bacteria and identification of mercury binding protein. *Research in Microbiology* 164: 701–9.

Kleerebezem M, Boekhorst J, van Kranenburg R, Molenaar D, Kuipers OP, Leer R, Tarchini R, Peters SA, Sandbrink HM, Fiers MW, Stiekema W, Lankhorst RM, Bron PA, Hoffer SM, Groot MN, Kerkhoven R, de Vries M, Ursing B, de Vos WM, Siezen RJ (2003) Complete genome sequence of *Lactobacillus plantarum* WCFS1. *Proceedings of the National Academy of Science, USA* 100: 1990–1995.

Kunz AN, Noel JM, Fairchok MP (2004) Two cases of *Lactobacillus bacteremia* during probiotic treatment of short gut syndrome. *Journal of Pediatric Gastroenterology and Nutrition* 38: 457–458.

Land MH, Rouster-Stevens K, Woods CR, Cannon ML, Cnota J, Shetty AK (2005) *Lactobacillus* sepsis associated with probiotic therapy. *Pediatrics* 115: 178–181.

Lenz LL, Mohammadi S, Geissler A, Portnoy DA. (2003) SecA2-dependent secretion of autolytic enzymes promotes *Listeria monocytogenes* pathogenesis. *Proceedings of the National Academy of Science, USA* 100: 12432–12437.

Lim BK, Mahendran R, Lee YK, Bay BH (2002) Chemopreventive effect of *Lactobacillus rhamnosus* on growth of a subcutaneously implanted bladder cancer cell line in the mouse. *Japanese Journal of Cancer Research* 93: 36–41.

López-Serrano D, Solano F, Sanchez-Amat A (2007) Involvement of a novel copper chaperone in tyrosinase activity and melanin synthesis in *Marinomonas mediterranea*. *Microbiology* 153: 2241–2249.

Mackay AD, Taylor MB, Kibbler CC, Hamilton-Miller JM (1999) *Lactobacillus endocarditis* caused by a probiotic organism. *Clinical Microbiology and Infection* 5: 290–292.

Martín R, Sánchez B, Suárez JE, Urdaci MC (2012) Characterization of the adherence properties of human Lactobacilli strains to be used as vaginal probiotics. *FEMS Microbiology Letters* 328: 166–173.

Martín R, Sánchez B, Urdaci MC, Langella P, Suárez JE, Bermudez-Humaran L (2015) Effect of iron on the probiotic properties of the vaginal isolate *Lactobacillus jensenii* CECT 4306. *Microbiology* 161(4): 708–718.

Modun B, Williams P (1999) The staphylococcal transferrin-binding protein is a cell wall glyceraldehyde-3-phosphate dehydrogenase. *Infection and Immunity* 67: 1086–1092.

Moratalla A, Gómez-Hurtado I, Santacruz A, Moya Á, Peiró G, Zapater P, González-Navajas JM, Giménez P, Such J, Sanz Y, Francés R (2014) Protective

effect of *Bifidobacterium pseudocatenulatum* CECT7765 against induced bacterial antigen translocation in experimental cirrhosis. *Liver International* 34: 850–858.

Muñoz-Provencio D, Pérez-Martínez G, Monedero V (2011) Identification of surface proteins from *Lactobacillus casei* BL23 able to bind fibronectin and collagen. *Probiotics and Antimicrobial Proteins* 3: 15–20.

Nishiyama K, Ochiai A, Tsubokawa D, Ishihara K, Yamamoto Y, Mukai T (2013) Identification and characterization of sulfated carbohydrate-binding protein from *Lactobacillus reuteri*. *PLoS One* 8: e83703.

Pancholi V, Fischetti VA (1992) A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate–dehydrogenase with multiple binding activity. *Journal of Experimental Medicine* 176: 415–426.

Peng Z, Krey V, Wei H, Tan Q, Vogelmann R, Ehrmann MA, Vogel RF (2014) Impact of actin on adhesion and translocation of *Enterococcus faecalis*. *Archives of Microbiology* 196: 109–17.

Perdigón G, Maldonado-Galdeano C, Valdez JC, Medici M (2002) Interaction of lactic acid bacteria with the gut immune system. *European Journal of Clinical Nutrition* 56 Suppl 4: S21–26.

Prasad J, McJarrow P, Gopal P (2003) Heat and osmotic stress responses of probiotic *Lactobacillus rhamnosus* HN001 (DR20) in relation to viability after drying. *Applied and Environmental Microbiology* 69: 917–925.

Ramiah K, van Reenen CA, Dicks LM (2008) Surface-bound proteins of *Lactobacillus plantarum* 423 that contribute to adhesion of Caco-2 cells and their role in competitive exclusion and displacement of *Clostridium sporogenes* and *Enterococcus faecalis*. *Research in Microbiology* 159: 470–475.

Rautio M, Jousimies-Somer H, Kauma H, Pietarinen I, Saxelin M, Tynkkynen S, Koskela M (1999) Liver abscess due to a *Lactobacillus rhamnosus* strain indistinguishable from *L. rhamnosus* strain GG. *Clinical Infectious Diseases* 28: 1159–1160.

Saad N, Urdaci M, Vignoles C, Chaignepain S, Tallon R, Schmitter JM, Bressollier P (2009) *Lactobacillus plantarum* 299v surface-bound GAPDH: a new insight into enzyme cell walls location. *Journal of Microbiology and Biotechnology* 19: 1635–1643.

Saito T (2013) New Japanese yogurts using functional probiotic lactic acid bacteria and future strategies to protect human gut. *Current Topics in LAB Probiotics* 1: 20–27.

Sánchez B, Champomier-Vergès MC, Stuer-Lauridsen B, Ruas-Madiedo P, Anglade P, Baraige F, de los Reyes-Gavilán CG, Johansen E, Zagorec M, Margolles A (2007) Adaptation and response of *Bifidobacterium animalis* subsp. *lactis* to bile: a proteomic and physiological approach. *Applied and Environmental Microbiology* 73: 6757–6767.

Sánchez B, Bressollier P, Urdaci MC (2008) Exported proteins in probiotic bacteria: adhesion to intestinal surfaces, host immunomodulation and molecular cross-talking with the host. *FEMS Immunology and Medical Microbiology* 54: 1–17.

Sánchez B, Schmitter JM, Urdaci MC (2009*a*) Identification of novel proteins secreted by *Lactobacillus plantarum* that bind to mucin and fibronectin. *Journal of Molecular Microbiology and Biotechnology* 17: 158–162.

Sánchez B, Schmitter JM, Urdaci MC (2009*b*) Identification of novel proteins secreted by *Lactobacillus rhamnosus* GG grown in de Mann-Rogosa-Sharpe broth. *Letters in Applied Microbiology* 48: 618–622.

Savaiano DA, Kotz C (1989) Recent advances in the management of lactose intolerance. *ASDC Journal of Dentistry for Children* 56: 228–233.

Seifert KN, McArthur WP, Bleiweis AS, Brady LJ (2003) Characterization of group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein–protein interactions. *Canadian Journal of Microbiology* 49: 350–356.

Spurbeck RR, Arvidson CG (2010) *Lactobacillus jensenii* surface-associated proteins inhibit *Neisseria gonorrhoeae* adherence to epithelial cells. *Infection and Immunity* 78: 3103–3111.

Stiles ME, Holzapfel WH (1997) Lactic acid bacteria of foods and their current taxonomy. *International Journal of Food Microbiology* 36: 1–29.

Tian F, Zhai Q, Zhao J, Liu X, Wang G, Zhang H, Chen W (2012) *Lactobacillus plantarum* CCFM8661 alleviates lead toxicity in mice. *Biological Trace Element Research* 150: 264–271.

Uchida H, Kinoshita H, Kawai Y, Kitazawa H, Miura K, Shiiba K, Horii A, Kimura K, Taketomo N, Oda M, Yajima T, Saito T (2006) Lactobacilli binding human A-antigen expressed in intestinal mucosa. *Research in Microbiology* 157: 659–665.

von Ossowski I, Reunanen J, Satokari R, Vesterlund S, Kankainen M, Huhtinen H, Tynkkynen S, Salminen S, de Vos WM, Palva A (2010) Mucosal adhesion properties of the probiotic *Lactobacillus rhamnosus* GG SpaCBA and SpaFED pilin subunits. *Applied and Environmental Microbiology* 76: 2049–2057.

Wang G, Xia Y, Cui J, Gu Z, Song Y, Chen YQ, Chen H, Zhang H, Chen W (2013) The Roles of Moonlighting Proteins in Bacteria. *Current Issues in Molecular Biology* 16: 15–22.

Watanabe M, Kinoshita H, Nitta M, Yukishita R, Kawai Y, Kimura K, Taketomo N, Yamazaki Y, Tateno Y, Miura K, Horii A, Kitazawa H, Saito T (2010) Identification of a new adhesin-like protein from *Lactobacillus mucosae* ME-340 with specific affinity to the human blood group A and B antigens. *Journal of Applied Microbiology* 109: 927–935.

Winram SB, Lottenberg R (1998) Site-directed mutagenesis of streptococcal plasmin receptor protein (Plr) identifies the C-terminal Lys334 as essential for plasmin binding, but mutation of the plr gene does not reduce plasmin binding to group A streptococci. *Microbiology* 144: 2025–2035.

Yamazaki S, Kamimura H, Momose H, Kawashima T, Ueda K (1982) Protective effect of *Bifidobacterium*-monoassociation against lethal activity of *Escherichia coli*. *Bifidobacteria Microflora* 1: 55–59.

Zhai Q, Wang G, Zhao J, Liu X, Tian F, Zhang H, Chen W (2013) Protective effects of *Lactobacillus plantarum* CCFM8610 against acute cadmium toxicity in mice. *Applied and Environmental Microbiology* 79: 1508–1515.

3.4

Cell-Surface Enolase: A Complex Virulence Factor

13

Impact of Streptococcal Enolase in Virulence

Marcus Fulde[1,2] *and Simone Bergmann*[3]

[1] *Department of Infectious Diseases, Institute for Microbiology, University of Veterinary Medicine Hannover, Hannover, Germany*
[2] *Present address: Centre for Infection Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, Berlin, Germany*
[3] *Department of Infection Biology, Institute of Microbiology, Technische Universität Braunschweig, Braunschweig, Germany*

13.1 Introduction

Enolases represent typical examples of moonlighting proteins, exhibiting multiple functions as a consequence of changes in cellular localization, cell type, oligomeric state, or the cellular concentration of a ligand, substrate, cofactor, or product (Jeffery 1999). Usually these proteins are produced in the cytoplasm and act as key glycolytic enzymes of the Embden Mayerhoff Parnas catabolic pathway and the first step of gluconeogenesis (Wold *et al*. 1971). Biochemically, enolases display hydrolyzing activity, thereby reversibly converting 2-phospho glycerate (2-PG) to phosphoenol pyruvate (PEP).

Although first mentioned as early as in 1934 (Lohmann and Meyerhof 1934), surface localization as well as the plasminogen-binding activity of eukaryotic enolase was not described fully until around 60 years later (Miles *et al*. 1991). The first experimental evidence characterizing the streptococcal enolase as a moonlighting protein took an additional seven years, and was not published until 1998 (Pancholi and Fischetti 1998). As key metabolic enzymes, enolases are widely distributed among all branches of life including archaea, bacteria, and eukaryotes (Wold *et al*. 1971). During the last decade, the amount of publications presenting additional functions of surface-displayed streptococcal enolases has increased massively and led to the definition of enolase as a protein with multiple functions.

This chapter provides an overview of the diversity of streptococcal enolases in virulence (Fig. 13.1). Interaction with the host's extracellular matrix, but also adhesion to and invasion of eukaryotic cells, is discussed.

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

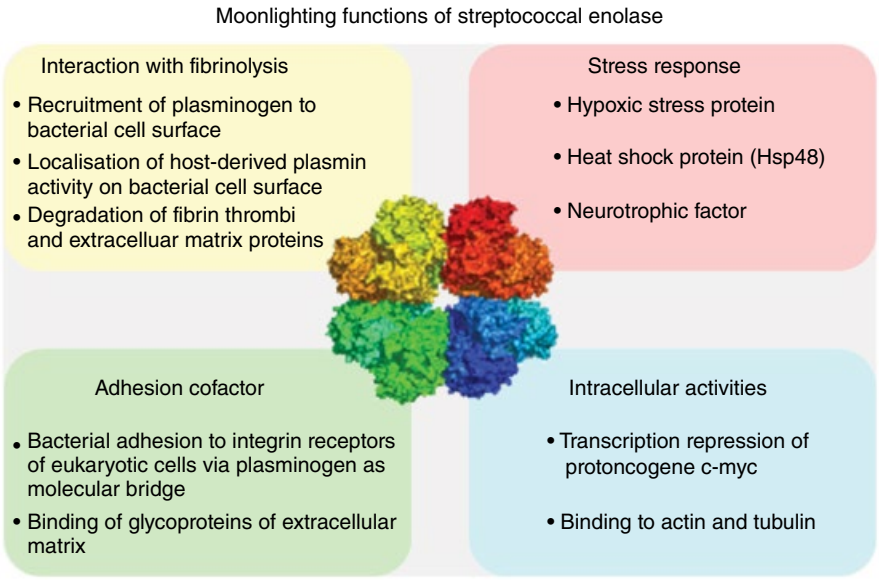

Figure 13.1 Scheme of moonlighting functions of streptococcal enolase in four categories: interaction with fibrinolysis; stress response; intracellular activities; and function as adhesion cofactor. Enolase structure *source*: Ehinger *et al*. (2004). Reproduced with kind permission of Elsevier.

13.2 General Characteristics

Structure and sequence homology of eukaryotic enolases have been widely characterized. Depending on their tissue-specific localization, enolases of vertebrates are expressed in up to three different isoforms: alpha, beta, and gamma. Based on X-ray crystallographic structure analyses, it was shown that eukaryotic enolases comprise homo- or heterodimers of the three different isoforms (excluding beta-gamma heterodimers; Malmström 1961; Fletcher *et al*. 1976; Kato *et al*. 1983; Lebioda and Stec 1988). In addition to human enolases, several eubacterial alpha-enolases, for example from *Bacillus subtilis* (Brown *et al*. 1998), *Thermatoga maritima* (Schurig *et al*. 1995), and *Zymomonas mobilis* (Pawluk *et al*. 1986), have been superimposed and by contrast are quaternary molecules composed of eight subunits. The dimeric yeast alpha-enolase had previously been used to model an alpha-enolase octamer (Brown *et al*. 1998). This model indicated that three to five residues inserted at the end of the helix 4 prevent octamerization. In yeast, expression of two isoforms depends on carbon source (McAlister and Holland 1982) and in *Toxoplasma gondii*, stage of life-cycle determines expression of isoform 1 (linked to encystation in anaerobic glycolysis) or isofom two (in actively dividing and invasive tachozyte) (Bolten *et al*. 2008).

Shortly after discovery of the *E. coli* enolase structure in 2001, the first crystal structure of a Gram-positive enolase was reported for *Enterococus hirae*, which crystallizes in a tetragonal space group (Kühnel and Luisi 2001; Hosaka *et al*. 2003). It shares high similarity to the crystallization and X-ray analysis of the

alpha enolase of *S. pneumoniae* (Ehinger *et al*. 2004). Comparative crystal structure analysis of *E. hirae* as well as *S. pneumoniae* enolase revealed an octameric ring of monomers and dimer–dimer contacts involving identical regions of the protein. In detail, the pneumococcal enolase could be visualized as homo-octamer forming a flattened, ring-like structure of 153 Å across a central pore of about 19 Å in diameter (Ehinger *et al*. 2004). The N-terminal domain of each monomer is located towards the center of the octamer, whereas the C-terminal domains form the outer ring. A similar arrangement of enolase monomers was later identified for the *S. suis* enolase, confirming the latter results (Lu *et al*. 2012). In contrast to the conserved active center catalyzing identical substrate conversion in all enolases described, differences in oligomeric state have a significant impact on adhesion to other ligands, thus determining the relevance of the enolase as virulence factor. Lastly, the 3D structure of the enolase might influence the immunogenicity as described for *S. suis*. In this case, the enolase induces strong humoral antibody response, and was observed to protect mice from fatal *S. suis* infection (Feng *et al*. 2009).

13.3 Expression and Surface Exposition of Enolase

It has been concurrently reported that the expression of enolase-specific messenger RNA (mRNA) substantially increases in exponentially growing cells, but remains almost undetectable during stationary growth phase (Holland *et al*. 1983). In yeast, expression levels are under metabolic and/or developmental control (McAlister and Holland 1982). Enolase is expressed on the surface of a variety of eukaryotic cells as a strong plasminogen-binding protein (Miles *et al*. 1991; Dudani *et al*. 1993; Nakajima *et al*. 1994; Redlitz *et al*. 1995). Interestingly, the expression of eukaryotic α-enolase depends on the pathophysiological state of these cells (Fontan *et al*. 2000).

Surface exposition of enolase on prokaryotic cells as well as plasminogen-binding activity was first described for streptococci, in particular for *S. pyogenes* (Pancholi and Fischetti 1998; Table 13.1). A plethora of studies reported surface expression and virulence function of enolase on pathogenic and commensal bacteria, irrespective of cell-wall constitution or number of cell membranes (Table 13.2).

Surface expression of other glycolytic enzymes, such as glyceraldehyde-3-phospho-dehdrogenase (GAPDH) and phosphoglycerate kinase (PGK), has similarly been reported for various bacteria and fungi (Lottenberg *et al*. 1992; Pancholi and Fischetti 1992, 1997; Alloush *et al*. 1997; Bergmann *et al*. 2001, 2004; Fulde *et al*. 2014). Although the localization of intracellular enolase on the bacterial surface is experimentally evidenced by a variety of biochemical techniques, the mode of surface transfer and anchorage still remains elusive. Bacterial enolases described so far do not possess one of the conserved classical secretion signal and hydrophobic transmembrane domains (von Heijne and Blomberg 1979; Blobel 1980) or the LPXTG-containing cell-wall anchorage motifs of Gram-positive bacterial surface proteins (Fischetti *et al*. 1990; Navarre and Schneewind 1999). Glycolytic enzymes located on the microbial cell surface of

Table 13.1 Streptococcus enolases.

Species	Surface expression	Function	Reference
S agalactiae	Surface		Hughes *et al*. 2002
	Released	Immunogenic	Fluegge *et al*. 2004
S. gordonii	Surface	Mucin binding	Kesimer *et al*. 2009
S. canis	Surface	Plasminogen binding	Fulde *et al*. 2013*a*, *b*
S. mutans	Surface	Plasminogen binding	Ge *et al*. 2004
S. pneumoniae	Surface	Plasminogen binding	Bergmann *et al*. 2001
		Immunogenic properties	Whiting *et al*. 2002
		Fibrinolysis	Bergmann and Hammerschmidt 2007
		Induction of neutrophil extracellular traps	Mori *et al*. 2012
		Cell adherence	Bergmann *et al*. 2013
S. pyogenes	Surface	Plasminogen binding	Pancholi and Fischetti 1998
		Plasminogen-mediated cell adherence	Pancholi *et al*. 2003
S. suis	Surface	Fibronectin+plasminogen binding	Esgleas *et al*. 2008
		Protective antigen	Feng *et al*. 2009
		Structure (octamer)	Lu *et al*. 2012

both Gram-positive and Gram-negative bacteria warrants their assignment to a special class of "non-classical" surface proteins (Bergmann and Hammerschmidt 2006). Whether or not the hydrophobic domain in human enolases (33-AAVPSGASTGIY44-), the post-translational acylation (Bottalico *et al*. 1993), or phosphorylation (Cooper *et al*. 1984) plays a role in membrane association and surface expression of cytoplasmic enolase is not yet clear. Interestingly, a recent publication demonstrated localization of eukaryotic ENO-1 in a specialized subset of lipid rafts, called caveolae, via interaction of enolase with caveolin 1 and with annexin 2. Knockdown-analyses confirmed a direct contribution between caveolae-generation and enolase surface expression, indicating that this type of vesicular trafficking is directly involved in enolase surface exposition of eukaryotic cells (Zakrzewicz *et al*. 2014).

Proteome studies repeatedly detected prokaryotic enolase in the cell-wall fraction and the secretome of various Gram-positive and Gram-negative bacteria, including *Bacillus* species (Gohar *et al*. 2005), *Clostridium thermocellum* (Yu *et al*. 2012), *Bifidobacterium longum* and *B. animalis* (Ruiz *et al*. 2009; Candela *et al*. 2010*b*), *Staphylococcus aureus* (Glowalla *et al*. 2009), *Borrelia burgdorferi* (Nowalk *et al*. 2006), *E. coli* (Stefanopoulou *et al*. 2011), and *Listeria monocytogenes* (Schaumburg *et al*. 2004), and streptococci such as *S. agalactiae* (Hughes

Table 13.2 Bacterial enolases.

Species	Surface expression	Function	Reference
Actinobacillus actinomycetemcomitans	Surface -associated		Hara *et al.* 2000
Aeromonas hydrophila	Surface	Virulence factor	Sha *et al.* 2009
Aeromonas salmonicida	Major outer membrane protein		Ebanks *et al.* 2005
Bacillus anthracis	Surface	Plasminogen binding	Agarwal *et al.* 2008
Bacillus cereus, *Bacillus thuringiensis*	Extracellular proteome		Gohar *et al.* 2005
Bidobacterium spp.	Cell surface	Plasminogen binding	Candela *et al.* 2009
Borrelia burgdorferi	Surface	Plasminogen binding	Floden *et al.* 2011
	Eno in outer membrane vesicles		Toledo *et al.* 2012
Lactobacillus plantarum		Cell adhesion, ECM binding	Glenting *et al.* 2013
		Fibronectin binding	Castaldo *et al.* 2009
Leptospira interrogans	Secreted	Plasminogen binding	Nogueira *et al.* 2013
Leuconostoc mesenteriodes	Surface		Lee *et al.* 2006
Listeria monocytogenes	In cell wall		Schaumburg *et al.* 2004
Mycoplasma bovis		Adhesion-related factor	Song *et al.* 2012
Mycoplasma fermentans	Surface	Plasminogen binding	Yavlovich *et al.* 2007
Mycoplasma gallisepticum	Surface	Plasminogen-adherence	Chen *et al.* 2011
Mycoplasma pneumonia		Plasminogen binding	Thomas *et al.* 2013
Mycoplasma suis	Surface	Adhesion	Schreiner *et al.* 2011
Neisseria meningitides	Surface	Plasminogen binding	Knaust *et al.* 2007
Paenibacillus larvae		Virulence factor	Antúnez *et al.* 2011
Pseudomonas aeruginosa		Enolase-like, plasminogen binding	Ceremuga *et al.* 2014
Staphylococcus aureus	Surface		Glowalla *et al.* 2009
		Laminin binding	Carneiro *et al.* 2004
Vibrio parahaemolyticus	Secreted, outer membrane	Plasminogen binding	Jiang *et al.* 2014

et al. 2002), *S. pneumoniae* (Saba *et al*. 2007), and *S. suis* (Chen *et al*. 2011). Moreover, by using enolase-specific antibodies, the presence of enolase on the surface of *B. anthracis* spores and as a component of outer membrane vesicles generated by *Borrelia burgdorferi* was confirmed (Chitlaru *et al*. 2007; Toledo *et al*. 2012). It has further been supposed that the presence of enolase as immunogenic plasminogen receptor in outer membrane vesicles contributes to the induction of external proteolysis in the pericellular environment, thereby promoting bacterial dissemination within the host (Toledo *et al*. 2012).

Interestingly, surface expression of streptococcal enolase is regulated by the protease HtrA, which is mainly involved in maturation of extracellular and degradation of abnormal or misfolded proteins. Inactivation of *htr*A massively affects the tolerance of *S. mutans* to thermal and environmental stress and reduces its virulence. In detail, the lack of HtrA resulted in an up to three-fold-enhanced protein expression and accumulation of several surface proteins including enolase and GAPDH in the supernatant of stationary *S. mutans* cultures (Biswas and Biswas 2005).

13.4 Streptococcal Enolase as Adhesion Cofactor

13.4.1 Enolase as Plasminogen-Binding Protein

Plasminogen circulates as a mono-chained multidomain protein in the blood of vertebrates (Pollanen *et al*. 1991). The 92 kDa glycoprotein consists of five homologous kringle domains containing binding sites for lysine-exposing ligands followed by a serine protease domain (Miyashita *et al*. 1988). Even without activation, recruitment of plasminogen to the bacterial surface has been reported as pivotal pathogenicity mechanism promoting bacterial attachment to eukaryotic cells (Lottenberg *et al*. 1994). An interaction of enolase with plasminogen has been reported for many species of the genus *Streptococcus* (Pancholi and Fischetti 1992; Bergmann *et al*. 2001; Itzek *et al*. 2010; Fulde *et al*. 2013*b*) and its role in pathogenicity has been analyzed using a variety of *in vivo* and *ex vivo* infection models (Pancholi and Fischetti *et al*. 1998; Pancholi and Chhatwal 2003; Bergmann *et al*. 2005, 2013; Agarwal *et al*. 2012). Remarkably, streptococcal surface enolases (SEN and Eno) harbor much stronger affinities for direct plasminogen binding compared to streptococcal surface plasminogen-binding proteins (e.g., PAM protein, SDH/Plr; Pancholi and Fischetti 1992; Bergmann *et al*. 2003; Lottenberg *et al*. 1992; Berge and Sjobring 1993).

In addition to enolase, other surface-exposed glycolytic enzymes such as GAPDH, PGK, and the endopeptidase O (PepO) have been identified as plasminogen receptors in *S. pneumoniae*. These non-classical surface proteins similarly subvert plasminogen to interact with host cells, but are also involved in tissue degradation and complement evasion (Bergmann *et al*. 2001, 2003, 2013; Bergmann and Hammerschmidt 2007; Bernardo-Garcia *et al*. 2011; Agarwal *et al*. 2012, 2013, 2014; Blom *et al*. 2014; Fulde *et al*. 2014). Lastly, plasminogen binding and subsequent cell attachment is also mediated by the fibronectin

receptors PavB and PfbA, as well as by the esterase Pce (Bergmann *et al*. 2001, 2003; Attali *et al*. 2008*a*, *b*; Yamaguchi *et al*. 2008; Jensch *et al*. 2010; Agarwal *et al*. 2013; Fulde *et al*. 2013*b*).

13.4.1.1 Plasminogen-Binding Sites of Streptococcal Enolases

For a long time, C-terminally located lysine residues of host proteins such as fibrin(ogen), alpha2-antiplasmin, and eukaryotic enolase, but also of bacterial receptors, were assumed to constitute the exclusive binding sites significantly participating in the interaction with plasminogen (Miles *et al*. 1991; Ponting *et al*. 1992). However, site-specific amino acid exchange of the terminal located two lysines followed by plasminogen-binding analysis resulted in up to 43% reduction of binding activity (Derbise *et al*. 2004). Residual binding activity as well as *in silico* analyses strongly indicated the contribution of an additional internal plasminogen-binding motif (Cork *et al*. 2009). In *S. pneumoniae* enolase the internal plasminogen-binding site was first identified and mapped to the "FYDKERKVY" loop which is exposed on the surface of each of the eight enolase monomers, mediating binding to plasminogen (Bergmann *et al*. 2003). A primary sequence comparison published by Pancholi (2001) including enolase from 50 distinct species revealed 40–90% sequence identity. Thereby, the enzymatic domain comprised an identity hotspot. In addition to all three eukaryotic enolase isoforms, enolases of Gram-positives, Gram-negatives, fungi, and parasites were included in this analysis. Interestingly, only 15 out of 50 enolases harbor at least one of the C-terminally located lysines, whereas the flanking amino acids (**FY** DKERKV **Y**) of the internal plasminogen-binding nonapeptide were detected in every enolase. In general, a stretch of alternating charged and non-charged amino acids flanked by hydrophobic residues is a central feature of internal plasminogen-binding motifs (Pancholi 2001).

The high relevance of internal plasminogen-binding motifs in octameric enolases is also supported by crystal structure analysis of pneumococcal enolase. In contrast to the dimeric eukaryotic enolase, C-terminal lysine residues are located within the interdimer grooves connecting the monomers in the complex globular structure of the octameric molecule, thereby maintaining structural integrity (Ehinger *et al*. 2004; Lu *et al*. 2012). Intramolecular localization of C- terminal lysines in intact octameric enolases might therefore hamper their accessibility for ligands such as plasminogen.

Crystal structure analysis and characterization of the plasminogen-binding activity of *S. suis* enolase, which shares 88% primary sequence identity to *S. pneumoniae* enolase, revealed not only an octameric structure composition but also the presence of both C- terminal lysines and surface-displayed plasminogen-binding nonapeptide (Lu *et al*. 2012). Interestingly, whereas site-directed mutagenesis of the internal motif or C-terminal lysine residues significantly affects the plasminogen-binding activity of *S. pyogenes* and *S. pneumoniae*, respectively, similar experiments with the enolase protein of *S. suis* remained without any changes (Lu *et al*. 2012). These results strongly indicate the presence of additional binding sites in *S. suis* enolase, which may be located in surface-exposed protein regions comprising differences in amino acid sequence compared to the other streptococcal enolases.

Similarly, additional internal positively charged amino acid motifs had been identified as important binding sites for the dimeric PGK of *S. agalactiae* and of *S. pneumoniae* (Boone and Tyrrell 2012; Fulde *et al*. 2014), the pneumococcal endopeptidase O (PepO; Agarwal *et al*. 2013) and also for the M-protein and M-like proteins PAM of *S. pyogenes* and SCM of *S. canis* (Sanderson-Smith *et al*. 2006*a*, *b*, 2007, 2012; Fu *et al*. 2008; Fulde *et al*. 2011). The interaction of the M-like protein PAM with plasminogen is mediated by two N-terminally located repeat sequences designated a1 and a2. Interestingly, in contrast to what is known for other binding motifs, an arginine and histidine but not lysine represent the essential amino acids-mediating protein–protein interaction (Walker *et al*. 2005; Sanderson-Smith *et al*. 2007). In conclusion, despite the variations in structure and amino acid composition of plasminogen-binding motifs, the presence of positively charged amino acids in a hydrophobic surrounding seems to constitute the principal requirement for binding to plasminogen.

13.4.2 Role of Enolase in Plasminogen-Mediated Bacterial-Host Cell Adhesion and Internalization

In addition to colonization, recruitment of ECM-proteins to the bacterial surface has been shown to promote activation of fibronectin-specific integrin receptors, which induces bacterial uptake (Hoffmann *et al*. 2010; Jensch *et al*. 2010). Similar to fibronectin, plasminogen mediates adherence of GAS to integrin receptors and triggers bacterial internalization into the cells (Siemens *et al*. 2011). Both α1β5 integrins and α1β1 integrins have been identified as receptors for bacterial-bound plasminogen (Siemens *et al*. 2011). Comparably, plasminogen mediates adherence of *S. pneumoniae* to epithelial and endothelial cells. This interaction is based on an enolase-mediated plasminogen binding susceptible to inhibition by the lysine analogon ε-aminocaproic acid (EACA) (Bergmann *et al*. 2013). In contrast to what has been shown for internalization of GAS, pneumococcal uptake in epithelial cells was not promoted by non-activated plasminogen. This strongly indicates the involvement of additional, as yet unknown, cofactors in cell entry mechanisms of GAS (Siemens *et al*. 2011).

Increased plasminogen binding to thrombin-stimulated platelet cells is due to increased expression of integrins and the recruitment of additional fibrin, which serves as the receptor for plasminogen (Miles and Plow 1985). However, it was not known how other blood cells which do not express fibrin-binding integrins could bind to plasminogen with great affinity. Using the U937-monocytoid cells as a model system, the same group subsequently found that alpha-enolase was a candidate plasminogen receptor on nucleated blood cells (Miles *et al*. 1991). Using alpha-enolase-specific monoclonal antibody 9C12, the researchers identified its surface-exposed epitope on neutrophils and U937 cells using the phage display method (Arza *et al*. 1997). This surface-exposed epitope, which spans a stretch of 16 amino acids (257-DLDFKSPDDPSRYISP-272), is located in the central loop of human enolase (Giallongo *et al*. 1986; Arza *et al*. 1997). It is, however, worth noting that this loop is not found in any of the reported prokaryotic enolase. Enolase is now known to be present on the surface of hematopoietic cells such as monocytes, T-cells and B-cells, neuronal cells, and endothelial cells (Miles *et al*. 1991; Dudani *et al*. 1993; Redlitz *et al*. 1995). Enolase (both *aa* and

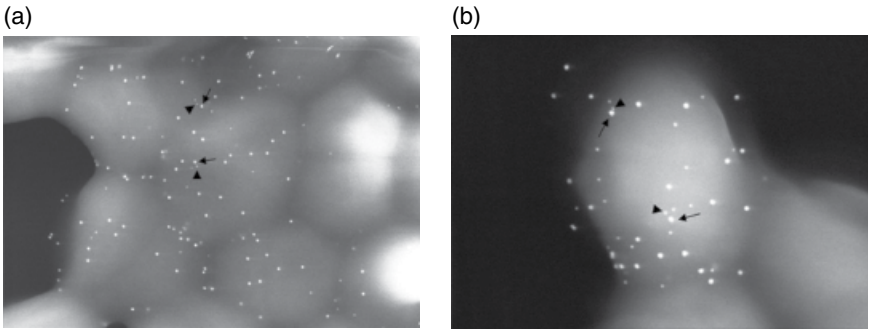

Figure 13.2 (a) Electron microscopic co-localization of enolase and GAPDH on the surface of *Streptococcus canis* G2 by immune-gold labeling performed by Manfred Rohde, Helmholtz Center for Infection Research (HZI), Braunschweig. (b) FESEM-visualization at 50,000× magnification and at 100,000× magnification. Arrows point to protein A gold particle (15 nm) detecting GAPDH and arrow heads mark protein A gold (10 nm) detecting enolase.

bb isoforms) from muscle has been shown to bind to other glycolytic enzymes such as phosphoglycerate mutase, muscle creatine kinase, pyruvate kinase, and muscle troponin with high affinity (Merkulova *et al*. 1997). The high affinity of these three enzymes suggests that they may make a functional glycolytic segment in the muscle where ATP production is required for muscle contraction (Merkulova *et al*. 1997). Electron microscopy visualization demonstrated a co-localization of ENO and GAPDH on the surface of streptococci (Fig. 13.2), indicating a functional cooperation in moonlighting activity.

A co-localization between glycolytic enzymes and other plasminogen receptors expressed on streptococcal surfaces are conceivable, and had already been demonstrated for the enolase and the M protein SCM of zoonotic *S. canis* (Fulde *et al*. 2011, 2013*a*). Interestingly, in contrast to the *S. canis* enolase which interacts with the N-terminal part of plasminogen representing angiostatin, the M-like protein of *S. canis* specifically binds to mini-plasminogen comprising the fifth kringle domain as well as the enzymatic domain. Our data therefore demonstrated that both enolase and SCM exhibit a cooperative plasminogen-binding activity which facilitates anti-phagocytic properties (Fulde *et al*. 2013*a*).

Considering the conformation of plasminogen, two further interesting observations with respect to cell adhesion have been reported. While coating of pneumococci with Glu-plasminogen decreases attachment, pre-incubation of M-protein expressing *S. dysgalactiae* sub*. equisimilis* with N-terminally truncated Lys-plasminogen resulted in enhanced bacterial adherence to nasopharyngeal cells (Bergmann *et al*. 2011). Since Lys-plasminogen possesses an open molecule form the adhesive capacity may reflect a conformation-dependent effect, as already mentioned in the literature (Marshall *et al*. 1994; Lähteenmaki *et al*. 2005).

13.4.3 Enolase as Plasminogen-Binding Protein in Non-Pathogenic Bacteria

Notably, the subversion of plasminogen or other extracellular matrix proteins to promote adherence is not only restricted to pathogenic or facultative pathogenic species. Several reports demonstrate the impact of plasminogen recruitment by

commensals such as *Bifidobacteria* spp. and probiotic *Lactobacilli* (Candela *et al*. 2009; Glenting *et al*. 2013; Wei *et al*. 2014). Lactic acid bacteria of the genus *Lactobacillus* and *Bifidobacterium* are part of the intestinal microbiota. They are therefore important for a variety of beneficial effects, such as colonization resistance, processing of dietary compounds and, finally, maintaining the local immune homeostasis. An important step in generating and maintaining a stable microbiota is ability of the bacteria to effectively colonize the intestine. For example, *Lactobacillus plantarum* uses ENO and GAPDH to recruit fibronectin and is assumed to mediate bacterial adhesion to gastrointestinal epithelial cells (Castaldo *et al*. 2009; Glenting *et al*. 2013). *Bifidobacterium*, on the other hand, interacts with plasminogen via moonlighting proteins enolase, DNAK, and EfTu (Candela *et al*. 2009, 2010*a*; Wei *et al*. 2014). Various *in vitro* analysis provided evidence for a substantial impact of plasminogen recruitment to colonize the gastrointestinal tract (Candela *et al*. 2009; Wei *et al*. 2014). Interestingly, the ENO of lactobacilli directly interacts with Caco-2 cells without using plasminogen as a bridging molecule (Glenting *et al*. 2013). Although the impact of plasminogen as a co-adhesin represents an accepted fact in bacterial virulence, the abovementioned examples highlight the important role of enolase in promoting colonization of beneficial bacteria in special host niches. Even more remarkable are data recently reported from *Lactobacillus gasseri*: the enolase of *L. gasseri* has been shown to impede the ability of *Neisseria gonorrhoeae* to adhere to epithelial cells (Raghunathan *et al*. 2014). In addition to adhesive properties, enolases of some probiotic bacteria display their beneficial role in outcompeting pathogenic species. In staphylococci, enolase has been reported as binding protein for the extracellular matrix component laminin (Carneiro *et al*. 2004). However, in contrast to the enolases of *Staphylococcus* and *Lactobacillus*, neither enolase nor SEN, which are structurally very similar to staphylococcal enolase, bind to laminin (Pancholi 2001).

13.5 Enolase as Pro-Fibrinolytic Cofactor

Bacterial interaction with the host fibrinolytic system represents a double-edged sword. While plasminogen binding to some commensals enhances bacterial colonization and protects tissues against access by pathogens, subversion of plasmin-mediated proteolysis by pathogens promotes infection with occasionally severe pathophysiological consequences.

A remarkable common strategy of all streptococcal species tested so far is the recruitment of host-derived plasminogen to the bacterial surface followed by conversion into proteolytic active plasmin (Bergmann *et al*. 2013). Cleavage of plasminogen by urokinase-type plasminogen activator (uPA) or tissue-type plasminogen activator (tPA) converted the zymogen into proteolytic active plasmin. Plasmin plays a key role in both extrinsic and intrinsic fibrinolysis and serves as an essential component in vascular patency (Castellino and Powell 1981; Dano *et al*. 1985). Plasminogen recruitment and its conversion to plasmin has been recognized as common virulence mechanism displayed by pathogenic bacteria,

parasites, fungi, and several symbiotic organisms (Lähteenmäki *et al*. 2001, 2005; Bergmann and Hammerschmidt 2007).

The vast majority of reports characterize bacterial plasminogen recruitment to endow the bacteria with host-derived proteolytic activity. In general, immobilization of plasminogen on cellular surfaces causes conformational opening of plasminogen, which in turn enhances its activation by uPA and tPA (Miles and Plow 1985; Plow *et al*. 1986, 1991, 1995). Interestingly, the moonlighting protein PGK has recently been identified as plasminogen and tPA-binding protein on the surface of *S. pneumoniae* (Fulde *et al*. 2014). It has been suggested that the recruitment of a plasminogen activator might enhance the efficiency of plasmin conversion at the side of infection. In contrast, uPA specifically binds to the eukaryotic receptor uPAR (urokinase-type plasminogen activator receptor) and is mainly involved in processes of cellular adhesion and migration, tissue reconstruction, and protection against apoptosis (Lijnen and Collen 1995; Plow *et al*. 1999; Crippa 2007). UPAR is a glyosylphosphatidyl inositol-(GPI)-anchored glycoprotein (Ploug *et al*. 1991) which is present on many leukocytic blood cells (Cubellis *et al*. 1986; Miles and Plow 1987; Nykjaer *et al*. 1994). Binding of the uPA-plasminogen complex to uPAR concentrates plasmin activity on cell surfaces and promotes cell migration. In addition, the immobilized from of plasmin is protected against the serine protease inhibitor alpha2-antiplasmin (Rouy and Angles-Cano 1990).

13.5.1 Degradation of Fibrin Thrombi and Components of the Extracellular Matrix

Severe systemic streptococcal infections are frequently accompanied by enhanced vascular coagulation, which entraps bacteria in fibrinous thrombi (Abraham 2000; Gunther *et al*. 2000). Utilization of proteolytic activity has therefore been shown to promote bacterial escape from these entrapments and enable transmigration through ECM and vascular barriers (Lähteenmaki *et al*. 2001). In this regard, incubation of semi-synthetic fibrin thrombi with plasmin-coated pneumococci or *S. canis* resulted in a complete dissolution of the fibrin bundles (Bergmann *et al*. 2005; Fulde *et al*. 2011). Fibrinogen serves as a major target for plasmin proteolysis. The reported fibrinogen binding of group A and B streptococci has been shown to improve the efficiency of fibrin clot degradation by plasmin independent of bacterial intrinsic plasminogen acitivators, such as streptokinase (Seifert *et al*. 2003; Olsen *et al*. 2009). In addition to fibrin degradation, recruitment of plasmin results in enhanced degradation of ECM glycoproteins, like fibronectin and laminin, and therefore weakens the matrix integrity and might provide a benefit for dissemination of non-motile streptococci (Liotta *et al*. 1981; Bergmann *et al*. 2005; Attali *et al*. 2008*a*; Fulde *et al*. 2013*b*). Moreover, plasmin-mediated cleavage of cellular junction proteins is supposed to promote subsequent pericellular transmigration of bacteria, as has been proposed for *S. pyogenes* and *S. pneumoniae*, respectively (Pancholi *et al*. 2003; Attali *et al*. 2008*b*). The entirety of reported functional effects of surface-exposed plasmin activity highlights this mechanism as a key strategy of streptococci to facilitate their transmigration through tissue barriers, thereby establishing an infection.

13.6 Streptococcal Enolase as Cariogenic Factor in Dental Disease

In 2004, Ge and co-workers identified the enolase of the oral streptococcal pathogen *S. mutans* as surface-displayed plasminogen-binding protein (Ge *et al*. 2004). Additionally, a specific interaction with the salivary glycoprotein mucin was demonstrated. Based on these findings, the authors assumed a contribution of *S. mutans* enolase in bacterial attachment and colonization of the oral cavity. Although many studies have failed to directly demonstrate that fluoride reduces the solubility of enamel, it is proposed that inhibition of caries formation by fluoride could be due to its ability to cause an altered growth rate and hence the changes in metabolism of cariogenic microorganisms such as *S. mutans* (Kaufmann and Bartholmes 1992). Fluoride is known to inhibit Mg++-dependent enolase in the presence of phosphate (Spencer and Brewer 1982; Hamilton 1990; Lebioda *et al*. 1991; Kaufmann and Bartholmes 1992). Some *S. mutans* strains are sensitive to fluoride-inhibition, while others display weak or no fluoride sensitivity. Likewise, Van Loveren and co-workers demonstrated that enolases, which are expressed by fluoride-sensitive or non-sensitive *S. mutans* strains, display differences in susceptibility depending on kind of fluorides and pH, although the enolase genes of both strains share identical nucleotide seuqences (van Loveren *et al*. 2008). The same report describes the susceptibility of the *S. mutans* ATPase for fluoride, indicating that fluoride inhibits metabolic activities of *S. mutans* via significant reduction of enolase and ATPase activity (Konings and Otto 1983). Inhibition of enolase by fluoride may result in phosphoenolpyruvate (PEP) deficiency, which is essential for the PEP-dependent phosphotransferase system, a major glucose uptake mechanism in Gram-positive bacteria (Schachtele 1975; Roberts and Ruddle 1980; Konings and Otto 1983). Fluoride treatment of supragingival plaques has been shown to reduce lactate production from 10% glucose by up to 46% along with an increase in 3-phosphoglycerate and a decrease in PEP (Takahashi and Washio 2011). These data provided further confirmation that active enolase contributes in a fluoride-sensitive manner to cariogenesis; further studies have to elucidate whether the observed differences in strain-dependent fluorid susceptibility based on structure-related differences of enolases or depend on susceptibility of further metabolic factors.

13.7 Conclusion

Although the transport of enolases to the bacterial surface is still an open question, their role as moonlighting proteins involved in the onset and progression of disease is widely accepted. The protein sequences of enolases from Streptococci and other bacteria are highly conserved, which is mainly attributed to their central role in glucose metabolism. In fact, the moonlighting functions of enolases vary tremendously. Enolases have been described as bacterial receptors for ECM and plasma proteins, such as plasminogen, and subvert their activity for bacterial colonization and dissemination. In the oral cavity, some pathogenic streptococci

use enolase to promote their cariogenic properties. The moonlighting function of enolases is also used to actively inhibit the establishment of pathogenic species and is therefore involved in maintaining homeostatic conditions on mucosal surfaces. Enolase, although genetically similar, displays a versatile tool for bacteria to ensure their biological fitness and pathogenic potential. Further work is required to completely understand the synergistic moonlighting function of this particular protein.

Acknowledgement

We thank Manfred Rohde for electron microscopic visualization and Ina Schleicher for technical assistance. Research of SB is funded by a grant from the Deutsche Forschungsgemeinschaft (DFG BE 4570/4-1). MF received support by the Freie Universität Berlin within the Excellence Initiative of the German Research Foundation.

References

Abraham E (2000) Coagulation abnormalities in acute lung injury and sepsis. *American Journal of Respiratory Cell and Molecular Biology* 22: 401–404.

Agarwal S, Kulshreshtha P, Bambah Mukku D, Bhatnagar R (2008) alpha-Enolase binds to human plasminogen on the surface of *Bacillus anthracis*. *Biochimica et Biophysica Acta* 1784(7–8): 986–94.

Agarwal V, Hammerschmidt S, Malm S, Bergmann S, Riesbeck K, *et al.* (2012) Enolase of *Streptococcus pneumoniae* binds human complement inhibitor C4b-binding protein and contributes to complement evasion. *Journal of Immunology* 189: 3575–3584.

Agarwal V, Kuchipudi A, Fulde M, Riesbeck K, Bergmann S, *et al.* (2013) *Streptococcus pneumoniae* endopeptidase O (PepO) is a multifunctional plasminogen- and fibronectin-binding protein, facilitating evasion of innate immunity and invasion of host cells. *Journal of Biological Chemistry* 288: 6849–6863.

Agarwal V, Sroka M, Fulde M, Bergmann S, Riesbeck K, *et al.* (2014) Binding of *Streptococcus pneumoniae* Endopeptidase O, PepO) to Complement Component C1q Modulates the Complement Attack and Promotes Host Cell Adherence. *Journal of Biological Chemistry* 289: 15833–15844.

Alloush HM, Lopez-Ribot JL, Masten BJ, Chaffin WL (1997) 3-phosphoglycerate kinase: a glycolytic enzyme protein present in the cell wall of *Candida albicans*. *Microbiology* 143 (Pt 2): 321–330.

Antúnez K, Anido M, Arredondo D, Evans JD, Zunino P (2011) *Paenibacillus larvae* enolase as a virulence factor in honeybee larvae infection. *Vetinary Microbiology* 147(1–2): 83–89.

Arza B, Felez J, Lopez-Alemany R, Miles LA, Munoz-Canoves P (1997) Identification of an epitope of alpha-enolase (a candidate plasminogen receptor) by phage display. *Thrombosis and Haemostasis* 78: 1097–1103.

Attali C, Durmort C, Vernet T, Di Guilmi AM (2008*a*) The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across endothelial and epithelial monolayers by intercellular junction cleavage. *Infection and Immunity* 76: 5350–5356.

Attali C, Frolet C, Durmort C, Offant J, Vernet T, *et al.* (2008*b*) *Streptococcus pneumoniae* choline-binding protein E interaction with plasminogen/plasmin stimulates migration across the extracellular matrix. *Infection and Immunity* 76: 466–476.

Berge A, Sjobring U (1993) PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *Journal of Biological Chemistry* 268: 25417–25424.

Bergmann R, Dinkla K, Nitsche-Schmitz DP, Graham RM, Lüttge M, Sanderson-Smith ML, Nerlich A, Rohde M, Chhatwal GS (2011) Biological functions of GCS3, a novel plasminogen-binding protein of *Streptococcus dysgalactiae* ssp. equisimilis. *International Journal of Medical Microbiology* 301(2): 157–164.

Bergmann S, Hammerschmidt S (2006) Versatility of pneumococcal surface proteins. *Microbiology* 152: 295–303.

Bergmann S, Hammerschmidt S (2007) Fibrinolysis and host response in bacterial infections. *Thrombosis and Haemostasis* 98: 512–520.

Bergmann S, Rohde M, Chhatwal GS, Hammerschmidt S (2001) alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Molecular Microbiology* 40: 1273–1287.

Bergmann S, Wild D, Diekmann O, Frank R, Bracht D, *et al.* (2003) Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. *Molecular Microbiology* 49: 411–423.

Bergmann S, Rohde M, Hammerschmidt S (2004) Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. *Infection and Immunity* 72: 2416–2419.

Bergmann S, Rohde M, Preissner KT, Hammerschmidt S (2005) The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration. *Thrombosis and Haemostasis* 94: 304–311.

Bergmann S, Schoenen H, Hammerschmidt S (2013) The interaction between bacterial enolase and plasminogen promotes adherence of *Streptococcus pneumoniae* to epithelial and endothelial cells. *International Journal of Medical Microbiology* 303: 452–462.

Bernardo-Garcia N, Bartual SG, Fulde M, Bergmann S, Hermoso JA (2011) Crystallization and preliminary X-ray diffraction analysis of phosphoglycerate kinase from *Streptococcus pneumoniae*. *Acta Crystallograhica Section F: Structural Biology and Crystallization Communications* 67: 1285–1289.

Biswas S, Biswas I (2005) Role of HtrA in surface protein expression and biofilm formation by *Streptococcus mutans*. *Infection and Immunity* 73: 6923–6934.

Blobel G (1980) Intracellular protein topogenesis. *Proceedings of the National Academy of Science, USA* 77: 1496–1500.

Blom AM, Bergmann S, Fulde M, Riesbeck K, Agarwal V (2014) *Streptococcus pneumoniae* phosphoglycerate kinase is a novel complement inhibitor affecting the membrane attack complex formation. *Journal of Biological Chemistry* 289(47): 32499–32511.

Bolten KE, Marsh AE, Reed SM, Dubey JP, Toribio RE, *et al.* (2008) *Sarcocystis neurona*: molecular characterization of enolase domain I region and a comparison to other protozoa. *Experimental Parasitology* 120: 108–112.

Boone TJ, Tyrrell GJ (2012) Identification of the actin and plasminogen binding regions of group B streptococcal phosphoglycerate kinase. *Journal of Biological Chemistry* 287: 29035–29044.

Bottalico LA, Kendrick NC, Keller A, Li Y, Tabas I (1993) Cholesteryl ester loading of mouse peritoneal macrophages is associated with changes in the expression or modification of specific cellular proteins, including increase in an alpha-enolase isoform. *Arteriosclerosis and Thrombosis* 13: 264–275.

Brown CK, Kuhlman PL, Mattingly S, Slates K, Calie PJ, *et al.* (1998) A model of the quaternary structure of enolases, based on structural and evolutionary analysis of the octameric enolase from *Bacillus subtilis*. *Journal of Protein Chemistry* 17: 855–866.

Candela M, Biagi E, Centanni M, Turroni S, Vici M, *et al.* (2009) Bifidobacterial enolase, a cell surface receptor for human plasminogen involved in the interaction with the host. *Microbiology* 155: 3294–3303.

Candela M, Centanni M, Fiori J, Biagi E, Turroni S, *et al.* (2010*a*) DnaK from *Bifidobacterium animalis subsp. lactis* is a surface-exposed human plasminogen receptor upregulated in response to bile salts. *Microbiology* 156: 1609–1618.

Candela M, Fiori J, Dipalo S, Brigidi P (2010*b*) Development of a high-performance affinity chromatography-based method to study the biological interaction between whole micro-organisms and target proteins. *Letters in Applied Microbiology* 51: 678–682.

Carneiro CR, Postol E, Nomizo R, Reis LF, Brentani RR (2004) Identification of enolase as a laminin-binding protein on the surface of *Staphylococcus aureus*. *Microbes and Infection* 6: 604–608.

Castaldo C, Vastano V, Siciliano RA, Candela M, Vici M, *et al.* (2009) Surface displaced alfa-enolase of *Lactobacillus plantarum* is a fibronectin binding protein. *Microbial Cell Factories* 8: 14.

Castellino FJ, Powell JR (1981) Human plasminogen. *Methods in Enzymology* 80 Pt C: 365–378.

Ceremuga I, Seweryn E, Bednarz-Misa I, Pietkiewicz J, Jermakow K, *et al.* (2014) Enolase-like protein present on the outer membrane of *Pseudomonas aeruginosa* binds plasminogen. *Folia Microbiol (Praha)* 59: 391–397.

Chen B, Zhang A, Xu Z, Li R, Chen H, *et al.* (2011) Large-scale identification of bacteria-host crosstalk by affinity chromatography: capturing the interactions of *Streptococcus suis* proteins with host cells. *Journal of Proteome Research* 10: 5163–5174.

Chitlaru T, Gat O, Grosfeld H, Inbar I, Gozlan Y, *et al.* (2007) Identification of *in vivo*-expressed immunogenic proteins by serological proteome analysis of the *Bacillus anthracis* secretome. *Infection and Immunity* 75: 2841–2852.

Cooper JA, Esch FS, Taylor SS, Hunter T (1984) Phosphorylation sites in enolase and lactate dehydrogenase utilized by tyrosine protein kinases in vivo and in vitro. *Journal of Biological Chemistry* 259: 7835–7841.

Cork AJ, Jergic S, Hammerschmidt S, Kobe B, Pancholi V, *et al.* (2009) Defining the structural basis of human plasminogen binding by streptococcal surface enolase. *Journal of Biological Chemistry* 284: 17129–7137.

Crippa MP (2007) Urokinase-type plasminogen activator. *International Journal of Biochemistry and Cell Biology* 39: 690–694.

Cubellis MV, Nolli ML, Cassani G, Blasi F (1986) Binding of single-chain prourokinase to the urokinase receptor of human U937 cells. *Journal of Biological Chemistry* 261: 15819–15822.

Dano K, Andreasen PA, Grondahl-Hansen J, Kristensen P, Nielsen LS, *et al.* (1985) Plasminogen activators, tissue degradation, and cancer. *Advances in Cancer Research* 44: 139–266.

Derbise A, Song YP, Parikh S, Fischetti VA, Pancholi V (2004) Role of the C-terminal lysine residues of streptococcal surface enolase in Glu- and Lys-plasminogen-binding activities of group A streptococci. *Infection and Immunity* 72: 94–105.

Dudani AK, Cummings C, Hashemi S, Ganz PR (1993) Isolation of a novel 45 kDa plasminogen receptor from human endothelial cells. *Thrombosis Research* 69: 185–196.

Ebanks RO, Goguen M, McKinnon S, Pinto DM, Ross NW (2005) Identification of the major outer membrane proteins of *Aeromonas salmonicida*. *Diseases of Aquatic Organisms* 68(1): 29–38.

Ehinger S, Schubert WD, Bergmann S, Hammerschmidt S, Heinz DW (2004) Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. *Journal of Molecular Biology* 343: 997–1005.

Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD, *et al.* (2008) Isolation and characterization of alpha-enolase, a novel fibronectin-binding protein from *Streptococcus suis*. *Microbiology* 154: 2668–2679.

Feng Y, Pan X, Sun W, Wang C, Zhang H, *et al.* (2009) *Streptococcus suis* enolase functions as a protective antigen displayed on the bacterial cell surface. *Journal of Infectious Diseases* 200: 1583–1592.

Fischetti VA, Pancholi V, Schneewind O (1990) Conservation of a hexapeptide sequence in the anchor region of surface proteins from gram-positive cocci. *Molecular Microbiology* 4: 1603–1605.

Fletcher L, Rider CC, Taylor CB (1976) Enolase isoenzymes. III. Chromatographic and immunological characteristics of rat brain enolase. *Biochimica et Biophysica Acta* 452: 245–252.

Floden AM, Watt JA, Brissette CA. (2011) *Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein. *PLoS One* 6(11): e27502.

Fluegge K, Schweier O, Schiltz E, Batsford S, Berner R (2004) Identification and immunoreactivity of proteins released from *Streptococcus agalactiae*. *European Journal of Clinical Microbiology and Infectious Diseases* 23(11): 818–824.

Fontan PA, Pancholi V, Nociari MM, Fischetti VA (2000) Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. *Journal of Infectious Diseases* 182: 1712–1721.

Fu Q, Figuera-Losada M, Ploplis VA, Cnudde S, Geiger JH, *et al.* (2008) The lack of binding of VEK-30, an internal peptide from the group A streptococcal M-like protein, PAM, to murine plasminogen is due to two amino acid replacements in the plasminogen kringle-2 domain. *Journal of Biological Chemistry* 283: 1580–1587.

Fulde M, Rohde M, Hitzmann A, Preissner KT, Nitsche-Schmitz DP, *et al.* (2011) SCM, a novel M-like protein from *Streptococcus canis*, binds (mini)-plasminogen with high affinity and facilitates bacterial transmigration. *Biochemical Journal* 434: 523–535.

Fulde M, Rohde M, Polok A, Preissner KT, Chhatwal GS, *et al.* (2013*a*) Cooperative plasminogen recruitment to the surface of *Streptococcus canis* via M protein and enolase enhances bacterial survival. *MBio* 4: e00629-00612.

Fulde M, Steinert M, Bergmann S (2013*b*) Interaction of streptococcal plasminogen binding proteins with the host fibrinolytic system. *Frontiers in Cellular and Infection Microbiology* 3: 85.

Fulde M, Bernardo-Garcia N, Rohde M, Nachtigall N, Frank R, *et al.* (2014) Pneumococcal phosphoglycerate kinase interacts with plasminogen and its tissue activator. *Thrombosis and Haemostasis* 111: 401–416.

Ge J, Catt DM, Gregory RL (2004) *Streptococcus mutans* surface alpha-enolase binds salivary mucin MG2 and human plasminogen. *Infection and Immunity* 72: 6748–6752.

Giallongo A, Feo S, Moore R, Croce CM, Showe LC (1986) Molecular cloning and nucleotide sequence of a full-length cDNA for human alpha enolase. *Proceedings of the National Academy of Science, USA* 83: 6741–6745.

Glenting J, Beck HC, Vrang A, Riemann H, Ravn P, *et al.* (2013) Anchorless surface associated glycolytic enzymes from *Lactobacillus plantarum* 299v bind to epithelial cells and extracellular matrix proteins. *Microbiological Research* 168: 245–253.

Glowalla E, Tosetti B, Kronke M, Krut O (2009) Proteomics-based identification of anchorless cell wall proteins as vaccine candidates against *Staphylococcus aureus*. *Infection and Immunity* 77: 2719–2729.

Gohar M, Gilois N, Graveline R, Garreau C, Sanchis V, *et al.* (2005) A comparative study of Bacillus cereus, Bacillus thuringiensis and Bacillus anthracis extracellular proteomes. *Proteomics* 5: 3696–3711.

Gunther A, Mosavi P, Heinemann S, Ruppert C, Muth H, *et al.* (2000) Alveolar fibrin formation caused by enhanced procoagulant and depressed fibrinolytic capacities in severe pneumonia. Comparison with the acute respiratory distress syndrome. *American Journal of Respiratory and Critical Care Medicine* 161: 454–462.

Hamilton IR (1990) Biochemical effects of fluoride on oral bacteria. *Journal of Dental Research* 69: 660–667; discussion 682–663.

Hara H, Ohta H, Inoue T, Ohashi T, Takashiba S, Murayama Y, Fukui K. (2000) Cell surface-associated enolase in *Actinobacillus actinomycetemcomitans*. *Microbiology and Immunology* 44(5): 349–56.

Hoffmann C, Berking A, Agerer F, Buntru A, Neske F, *et al.* (2010) Caveolin limits membrane microdomain mobility and integrin-mediated uptake of fibronectin-binding pathogens. *Journal of Cell Science* 123: 4280–4291.

Holland JP, Labieniec L, Swimmer C, Holland MJ (1983) Homologous nucleotide sequences at the 5' termini of messenger RNAs synthesized from the *yeast* enolase and glyceraldehyde-3-phosphate dehydrogenase gene families. The primary structure of a third yeast glyceraldehyde-3-phosphate dehydrogenase gene. *Journal of Biological Chemistry* 258: 5291–5299.

Hosaka T, Meguro T, Yamato I, Shirakihara Y (2003) Crystal structure of *Enterococcus hirae* enolase at 2.8 A resolution. *Journal of Biochemistry* 133: 817–823.

Hughes MJ, Moore JC, Lane JD, Wilson R, Pribul PK, *et al.* (2002) Identification of major outer surface proteins of *Streptococcus agalactiae*. *Infection and Immunity* 70: 1254–1259.

Itzek A, Gillen CM, Fulde M, Friedrichs C, Rodloff AC, *et al.* (2010) Contribution of plasminogen activation towards the pathogenic potential of oral streptococci. *PLoS One* 5: e13826.

Jeffery CJ (1999) Moonlighting proteins. *Trends in Biochemical Science* 24: 8–11.

Jensch I, Gamez G, Rothe M, Ebert S, Fulde M, *et al.* (2010) PavB is a surface-exposed adhesin of *Streptococcus pneumoniae* contributing to nasopharyngeal colonization and airways infections. *Molecular Microbiology* 77: 22–43.

Jiang W, Han X, Wang Q, Li X, Yi L, Liu Y, Ding C. (2014) *Vibrio parahaemolyticus* enolase is an adhesion-related factor that binds plasminogen and functions as a protective antigen. *Applied Microbiology and Biotechnology* 98(11): 4937–4948.

Kato K, Asai R, Shimizu A, Suzuki F, Ariyoshi Y (1983) Immunoassay of three enolase isozymes in human serum and in blood cells. *Clinica Chimica Acta* 127: 353–363.

Kaufmann M, Bartholmes P (1992) Purification, characterization and inhibition by fluoride of enolase from *Streptococcus mutans* DSM 320523. *Caries Research* 26: 110–116.

Kesimer M, Kiliç N, Mehrotra R, Thornton DJ, Sheehan JK (2009) Identification of salivary mucin MUC7 binding proteins from *Streptococcus gordonii*. *BMC Microbiology* 11(9): 163.

Knaust A, Weber MV, Hammerschmidt S, Bergmann S, Frosch M, Kurzai O (2007) Cytosolic proteins contribute to surface plasminogen recruitment of *Neisseria meningitidis*. *Journal of Bacteriology* 189(8): 3246–3255.

Konings WN, Otto R (1983) Energy transduction and solute transport in streptococci. *Antonie Van Leeuwenhoek* 49: 247–257.

Kühnel K, Luisi BF (2001) Crystal structure of the Escherichia coli RNA degradosome component enolase. *Journal of Molecular Biology* 313: 583–592.

Lähteenmaki K, Kuusela P, Korhonen TK (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiology Reviews* 25: 531–552.

Lähteenmaki K, Edelman S, Korhonen TK (2005) Bacterial metastasis: the host plasminogen system in bacterial invasion. *Trends in Microbiology* 13: 79–85.

Lebioda L, Stec B (1988) Crystal structure of enolase indicates that enolase and pyruvate kinase evolved from a common ancestor. *Nature* 333: 683–686.

Lebioda L, Stec B, Brewer JM, Tykarska E (1991) Inhibition of enolase: the crystal structures of enolase-Ca2(+)- 2-phosphoglycerate and enolase-Zn2(+)-phosphoglycolate complexes at 2.2-A resolution. *Biochemistry* 30: 2823–2827.

Lee JH, Kang HK, Moon YH, Cho DL, Kim D, Choe JY, Honzatko R, Robyt JF (2006) Cloning, expression and characterization of an extracellular enolase from *Leuconostoc mesenteroides*. *FEMS Microbiology Letters* 259(2): 240–248.

Lijnen HR, Collen D (1995) Fibrinolytic agents: mechanisms of activity and pharmacology. *Thrombosis and Haemostasis* 74: 387–390.

Liotta LA, Goldfarb RH, Terranova VP (1981) Cleavage of laminin by thrombin and plasmin: alpha thrombin selectively cleaves the beta chain of laminin. *Thrombosis Research* 21: 663–673.

Lohman K and Meyerhof O (1934) Enzymatic transformation of phosphoglyceric acid into pyruvic and phosphoric acid. *Biochemische Zeitschrift* 273: 60–72.

Lottenberg R, Broder CC, Boyle MD, Kain SJ, Schroeder BL, *et al*. (1992) Cloning, sequence analysis, and expression in *Escherichia coli* of a streptococcal plasmin receptor. *Journal of Bacteriology* 174: 5204–5210.

Lottenberg R, Minning-Wenz D, Boyle MD (1994) Capturing host plasmin(ogen): a common mechanism for invasive pathogens? *Trends in Microbiology* 2: 20–24.

Lu Q, Lu H, Qi J, Lu G, Gao GF (2012) An octamer of enolase from *Streptococcus suis*. *Protein Cell* 3: 769–780.

Malmström BG (1961) Enolase. In *The Enzymes*, 2nd edition, vol. 5 (eds Boyer PD, Lardy H and Myrbäck K), pp. 471–494. Academic Press, New York.

Marshall JM, Brown AJ, Ponting CP (1994) Conformational studies of human plasminogen and plasminogen fragments: evidence for a novel third conformation of plasminogen. *Biochemistry* 33: 3599–3606.

McAlister L, Holland MJ (1982) Targeted deletion of a *yeast* enolase structural gene. Identification and isolation of *yeast* enolase isozymes. *Journal of Biological Chemistry* 257: 7181–7188.

Merkulova T, Lucas M, Jabet C, Lamande N, Rouzeau JD, *et al*. (1997) Biochemical characterization of the mouse muscle-specific enolase: developmental changes in electrophoretic variants and selective binding to other proteins. *Biochemical Journal* 323 (Pt 3): 791–800.

Miles LA, Plow EF (1985) Binding and activation of plasminogen on the platelet surface. *Journal of Biological Chemistry* 260: 4303–4311.

Miles LA, Plow EF (1987) Receptor mediated binding of the fibrinolytic components, plasminogen and urokinase, to peripheral blood cells. *Thrombosis and Haemostasis* 58: 936–942.

Miles LA, Dahlberg CM, Plescia J, Felez J, Kato K, *et al*. (1991) Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry* 30: 1682–1691.

Miyashita C, Wenzel E, Heiden M (1988) Plasminogen: a brief introduction into its biochemistry and function. *Haemostasis* 18 Suppl 1: 7–13.

Mori Y, Yamaguchi M, Terao Y, Hamada S, Ooshima T, Kawabata S (2012) α-Enolase of *Streptococcus pneumoniae* induces formation of neutrophil extracellular traps. *Journal of Biological Chemistry* 287(13): 10472–10481.

Nakajima K, Hamanoue M, Takemoto N, Hattori T, Kato K, *et al*. (1994) Plasminogen binds specifically to alpha-enolase on rat neuronal plasma membrane. *Journal of Neurochemistry* 63: 2048–2057.

Navarre WW, Schneewind O (1999) Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiology and Molecular Biology Reviews* 63: 174–229.

Nogueira SV, Backstedt BT, Smith AA, Qin JH, Wunder EA Jr, Ko A, Pal U (2013) *Leptospira interrogans* enolase is secreted extracellularly and interacts with plasminogen. *PLoS One* 8(10): e78150.

Nowalk AJ, Nolder C, Clifton DR, Carroll JA (2006) Comparative proteome analysis of subcellular fractions from *Borrelia burgdorferi* by NEPHGE and IPG. *Proteomics* 6: 2121–2134.

Nykjaer A, Moller B, Todd RF, 3rd, Christensen T, Andreasen PA, *et al*. (1994) Urokinase receptor. An activation antigen in human T lymphocytes. *Journal of Immunology* 152: 505–516.

Olsen RJ, Shelburne SA, Musser JM (2009) Molecular mechanisms underlying group A streptococcal pathogenesis. *Cell Microbiology* 11(1): 1–12.

Pancholi V (2001) Multifunctional alpha-enolase: its role in diseases. *Cellular and Molecular Life Sciences* 58: 902–920.

Pancholi V, Fischetti VA (1992) A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. *Journal of Experimental Medicine* 176: 415–426.

Pancholi V, Fischetti VA (1997) A novel plasminogen/plasmin binding protein on the surface of group A streptococci. *Advances in Experimental Medicine and Biology* 418: 597–599.

Pancholi V, Fischetti VA (1998) alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. *Journal of Biological Chemistry* 273: 14503–14515.

Pancholi V, Chhatwal GS (2003) Housekeeping enzymes as virulence factors for pathogens. *International Journal of Medical Microbiology* 293: 391–401.

Pancholi V, Fontan P, Jin H (2003) Plasminogen-mediated group A streptococcal adherence to and pericellular invasion of human pharyngeal cells. *Microbial Pathogenesis* 35: 293–303.

Pawluk A, Scopes RK, Griffiths-Smith K (1986) Isolation and properties of the glycolytic enzymes from Zymomonas mobilis. The five enzymes from glyceraldehyde-3-phosphate dehydrogenase through to pyruvate kinase. *Biochemical Journal* 238: 275–281.

Ploug M, Rønne E, Behrendt N, Jensen AL, Blasi F, Danø K (1991) Cellular receptor for urokinase plasminogen activator. Carboxyl-terminal processing and membrane anchoring by glycosyl-phosphatidylinositol. *Journal of Biological Chemistry* 266(3): 1926–1933.

Plow EF, Freaney DE, Plescia J, Miles LA (1986) The plasminogen system and cell surfaces: evidence for plasminogen and urokinase receptors on the same cell type. *Journal of Cell Biology* 103: 2411–2420.

Plow EF, Felez J, Miles LA (1991) Cellular regulation of fibrinolysis. *Thrombosis and Haemostasis* 66: 32–36.

Plow EF, Herren T, Redlitz A, Miles LA, Hoover-Plow JL (1995) The cell biology of the plasminogen system. *Faseb Journal* 9: 939–945.

Plow EF, Ploplis VA, Busuttil S, Carmeliet P, Collen D (1999) A role of plasminogen in atherosclerosis and restenosis models in mice. *Thrombosis and Haemostasis* 82 Suppl 1: 4–7.

Pollanen J, Stephens RW, Vaheri A (1991) Directed plasminogen activation at the surface of normal and malignant cells. *Advances in Cancer Research* 57: 273–328.

Ponting CP, Marshall JM, Cederholm-Williams SA (1992) Plasminogen: a structural review. *Blood Coagulation and Fibrinolysis* 3: 605–614.

Raghunathan K, Harris PT, Spurbeck RR, Arvidson CG, Arvidson DN (2014) Crystal structure of an efficacious gonococcal adherence inhibitor: an enolase from *Lactobacillus gasseri*. *FEBS Letters* 588: 2212–2216.

Redlitz A, Fowler BJ, Plow EF, Miles LA (1995) The role of an enolase-related molecule in plasminogen binding to cells. *European Journal of Biochemistry* 227: 407–415.

Roberts M, Ruddle FH (1980) The Chinese hamster gene map. Assignment of four genes (DTS, PGM2, 6PGD, EnoI) to chromosome 2. *Experimental Cell Research* 127: 47–54.

Rouy D, Angles-Cano E (1990) The mechanism of activation of plasminogen at the fibrin surface by tissue-type plasminogen activator in a plasma milieu in vitro. Role of alpha 2-antiplasmin. *Biochemical Journal* 271: 51–57.

Ruiz L, Coute Y, Sanchez B, de los Reyes-Gavilan CG, Sanchez JC, *et al.* (2009) The cell-envelope proteome of *Bifidobacterium longum* in an *in vitro* bile environment. *Microbiology* 155: 957–967.

Saba JA, McComb ME, Potts DL, Costello CE, Amar S (2007) Proteomic mapping of stimulus-specific signaling pathways involved in THP-1 cells exposed to *Porphyromonas gingivalis* or its purified components. *Journal of Proteome Research* 6: 2211–2221.

Sanderson-Smith M, Batzloff M, Sriprakash KS, Dowton M, Ranson M, *et al.* (2006*a*) Divergence in the plasminogen-binding group a streptococcal M protein family: functional conservation of binding site and potential role for immune selection of variants. *Journal of Biological Chemistry* 281: 3217–3226.

Sanderson-Smith ML, Walker MJ, Ranson M (2006*b*) The maintenance of high affinity plasminogen binding by group A streptococcal plasminogen-binding M-like protein is mediated by arginine and histidine residues within the a1 and a2 repeat domains. *Journal of Biological Chemistry* 281: 25965–25971.

Sanderson-Smith ML, Dowton M, Ranson M, Walker MJ (2007) The plasminogen-binding group A streptococcal M protein-related protein Prp binds plasminogen via arginine and histidine residues. *Journal of Bacteriology* 189: 1435–1440.

Sanderson-Smith ML, De Oliveira DM, Ranson M, McArthur JD (2012) Bacterial plasminogen receptors: mediators of a multifaceted relationship. *Journal of Biomedicine and Biotechnology* 2012: 272148.

Schachtele CF (1975) Glucose transport in *Streptococcus mutans*: preparation of cytoplasmic membranes and characteristics of phosphotransferase activity. *Journal of Dental Research* 54: 330–338.

Schaumburg J, Diekmann O, Hagendorff P, Bergmann S, Rohde M, *et al.* (2004) The cell wall subproteome of *Listeria monocytogenes*. *Proteomics* 4: 2991–3006.

Schreiner SA, Sokoli A, Felder KM, Wittenbrink MM, Schwarzenbach S, Guhl B, Hoelzle K, Hoelzle LE (2011) The surface-localised α-enolase of *Mycoplasma suis* is an adhesion protein. *Vetinary Microbiology* 156(1–2): 88–95.

Schurig H, Rutkat K, Rachel R, Jaenicke R (1995) Octameric enolase from the hyperthermophilic bacterium *Thermotoga maritima*: purification, characterization, and image processing. *Protein Science* 4: 228–236.

Seifert KN, McArthur WP, Bleiweis AS, Brady LJ (2003) Characterization of group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein-protein interactions. *Canadian Journal of Microbiology* 49: 350–356.

Sha J, Erova TE, Alyea RA, Wang S, Olano JP, Pancholi V, Chopra AK (2009) Surface-expressed enolase contributes to the pathogenesis of clinical isolate SSU of *Aeromonas hydrophila*. *Journal of Bacteriology* 191(9): 3095–3107.

Siemens N, Patenge N, Otto J, Fiedler T, Kreikemeyer B (2011) *Streptococcus pyogenes* M49 plasminogen/plasmin binding facilitates keratinocyte invasion via

integrin-integrin-linked kinase (ILK) pathways and protects from macrophage killing. *Journal of Biological Chemistry* 286: 21612–21622.

Song Z, Li Y, Liu Y, Xin J, Zou X, Sun W (2012) α-Enolase, an adhesion-related factor of *Mycoplasma bovis*. *PLoS One* 7(6): e38836.

Spencer SG, Brewer JM (1982) Substrate-dependent inhibition of yeast enolase by fluoride. *Biochemical and Biophysical Research Communications* 106: 553–558.

Stefanopoulou M, Kokoschka M, Sheldrick WS, Wolters DA (2011) Cell response of Escherichia coli to cisplatin-induced stress. *Proteomics* 11: 4174–4188.

Takahashi N, Washio J (2011) Metabolomic effects of xylitol and fluoride on plaque biofilm *in vivo*. *Journal of Dental Research* 90: 1463–1468.

Thomas C, Jacobs E, Dumke R (2013) Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*. *Microbiology* 159: 352–365.

Toledo A, Coleman JL, Kuhlow CJ, Crowley JT, Benach JL (2012) The enolase of *Borrelia burgdorferi* is a plasminogen receptor released in outer membrane vesicles. *Infection and Immunity* 80: 359–368.

van Loveren C, Hoogenkamp MA, Deng DM, ten Cate JM (2008) Effects of different kinds of fluorides on enolase and ATPase activity of a fluoride-sensitive and fluoride-resistant *Streptococcus mutans* strain. *Caries Research* 42: 429–434.

von Heijne G, Blomberg C (1979) Trans-membrane translocation of proteins. The direct transfer model. *European Journal of Biochemistry* 97: 175–181.

Walker MJ, McArthur JD, McKay F, Ranson M (2005) Is plasminogen deployed as a *Streptococcus pyogenes* virulence factor? *Trends in Microbiology* 13: 308–313.

Wei X, Yan X, Chen X, Yang Z, Li H, *et al.* (2014) Proteomic analysis of the interaction of Bifidobacterium longum NCC2705 with the intestine cells Caco-2 and identification of plasminogen receptors. *Journal of Proteomics* 108: 89–98.

Whiting GC, Evans JT, Patel S, Gillespie SH (2002) Purification of native alpha-enolase from *Streptococcus pneumoniae* that binds plasminogen and is immunogenic. *Journal of Medical Microbiology* 51(10): 837–843.

Wold JS, Longnecker DS, Fischer LJ (1971) Species dependent pancreatic islet toxicity produced by cyproheptadine: alterations in beta cell structure and function. *Toxicology and Applied Pharmacology* 19: 188–201.

Yamaguchi M, Terao Y, Mori Y, Hamada S, Kawabata S (2008) PfbA, a novel plasmin- and fibronectin-binding protein of *Streptococcus pneumoniae*, contributes to fibronectin-dependent adhesion and antiphagocytosis. *Journal of Biological Chemistry* 283: 36272–36279.

Yavlovich A, Rechnitzer H, Rottem S (2007)Alpha-enolase resides on the cell surface of *Mycoplasma fermentans* and binds plasminogen. *Infection and Immunity* 75(12): 5716–5719.

Yu T, Xu X, Peng Y, Luo Y, Yang K (2012) Cell wall proteome of *Clostridium thermocellum* and detection of glycoproteins. *Microbiological Research* 167: 364–371.

Zakrzewicz D, Didiasova M, Zakrzewicz A, Hocke AC, Uhle F, *et al.* (2014) The interaction of enolase-1 with caveolae-associated proteins regulates its subcellular localization. *Biochemical Journal* 460: 295–307.

14

Streptococcal Enolase and Immune Evasion

Masaya Yamaguchi and Shigetada Kawabata

Department of Oral and Molecular Microbiology, Osaka University Graduate School of Dentistry, Suita, Osaka, Japan

14.1 Introduction

Enolase is known to be a glycolytic enzyme that exists in the cytoplasm of many eukaryotic and prokaryotic organisms (Postma *et al*. 1993; Pancholi 2001; Terrier *et al*. 2007). This protein is a metalloenzyme and requires magnesium ion for its activity. Most eukaryotic enolases are composed of three isoforms: α-enolase (*eno1*), β-enolase (*eno3*), and γ-enolase (*eno2*). α-enolase is expressed in nearly all types of tissues, β-enolase is predominantly expressed in muscle tissues, and γ-enolase is expressed in neuron and neuroendocrine tissues. Each shows a high level of identity (>80%) in regard to their amino acid sequence, and forms a homo- or hetero-dimer composed of two subunits facing each other in an antiparallel fashion (Pancholi 2001). Recently, Nakamura *et al*. (2013) identified the novel *eno4* gene encoding Enolase 4 in mouse testis and sperm specimens, and showed that it contributes to normal assembly of the fibrous sheath in the sperm flagellum and functions in the major portion of enolase activity in sperm. Nearly all Gram-positive and -negative bacteria, including *Streptococcus* species, express only α-enolase; bacterial α-enolases have a 40–100% identity between different bacterial species (Pancholi 2001). Enolases play a common important role as enzymes catalyzing the dehydration of 2-phosphoglycerate to phosphoenolpyruvate in the Embden Meyerhof Parnas (EMP) pathway. Interestingly, eukaryotic and prokaryotic α-enolase on cell surfaces, as well as those secreted into extracellular space, function as multifunctional proteins, whereas α-enolase was initially reported to be a glycolytic enzyme in cytoplasm (Lohman and Meyerhof 1934).

Streptococcus is a Gram-positive bacterium that forms chained or paired spheres, and *Streptococcus* species are classified by their hemolytic activities on blood agar plates. α-hemolytic *Streptococcus* organisms, such as oral streptococci and *Streptococcus pneumoniae*, show green incomplete hemolytic plaque on

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

blood agar. Oral streptococci are therefore also called viridans streptococci. On the other hand, β-hemolytic *Streptococcus* species, such as *Streptococcus pyogenes* and *Streptococcus agalactiae*, show clear complete hemolytic plaque, while γ-hemolytic *Streptococcus* does not show any hemolytic plaque. *Streptococcus* species are classified by Lancefield grouping based on the antigenicity of their cell-wall carbohydrates (Lancefield 1933). *S. pyogenes* belongs to Lancefield serogroup A, also known as group A *Streptococcus* (GAS), and *S. agalactiae* is a member of group B *Streptococcus* (GBS). In addition, 16S rRNA sequencing classifies *Streptococcus* species into pyogenic, mitis, anginosus, salivarius, bovis, and mutans groups, with GAS and GBS belonging to the pyogenic group and *S. pneumoniae* to the mitis group (Kawamura *et al*. 1995). Streptococcal α-enolase shows a high similarity in regard to amino acid sequence, crystal structure, and functions. However, several different phenotypes (differences probably due to small structural differences) have been reported in studies of each species.

Streptococcus species largely colonize as commensal bacteria in the host environment, although some are associated with a wide spectrum of human diseases ranging from pharyngitis to severe invasive conditions. Notably, GAS, GBS, and *S. pneumoniae* are medically important human pathogens that can cause fatal diseases. For example, the annual burden of GAS infection was calculated to include 616 million cases of pharyngitis and 111 million cases of impetigo, while invasive GAS infection is now estimated to occur in over 650,000 cases and causes 150,000 deaths worldwide each year (Carapetis *et al*. 2005). Economic loss is another serious result of GAS infectious diseases, with the total cost of GAS pharyngitis among children in the United States estimated to range from $224 to $539 million per year (Pfoh *et al*. 2008). Although common outcomes of GAS infection include transient asymptomatic colonization or self-limited mucosal infection, some invasive GAS strains produce systemic infections in otherwise healthy children and adults. In particular, the fatality of toxic shock-like syndrome caused by GAS has been reported to be greater than 40% (Lamagni *et al*. 2005; Lappin and Ferguson 2009). GBS is a leading pathogen related to newborn infant meningitis, while approximately 25% of healthy adults carry GBS asymptomatically (Edwards and Baker 2005; Maisey *et al*. 2008). The mortality rate associated with GBS infection is estimated to fall within the range 3–15% in Europe and the United States, and up to 50% of GBS meningitis patients suffer neurodevelopmental impairment (Le Doare and Heath 2013). *S. pneumoniae* is a commensal bacterium harbored in the human nasopharynx that colonizes in approximately 20% of children without causing clinical symptoms (Bogaert *et al*. 2004). However, it is also known as a major pathogen of invasive diseases such as pneumonia, sepsis, and meningitis. *S. pneumoniae* is the most common cause of severe community-acquired pneumonia and has been estimated to cause about 14.5 million cases of serious disease, with 826,000 deaths among children each year (O'Brien *et al*. 2009). These invasive pathogens invade into deeper tissues and evade the host immune systems, in addition to colonization. Interestingly, α-enolase contributes to bacterial colonization and the distribution of host immunity through interactions with various host factors, such as plasminogen, while the protein is also present in commensal and pathogenic bacteria. In this chapter, we describe the roles of α-enolase in streptococcal infection.

14.2 Localization and Crystal Structure

Streptococcal α-enolase is essential for bacteria, since the protein plays an important role as an enzyme in the EMP pathway. It is therefore considered impossible to construct a conventional knockout mutant with the *eno* gene encoding α-enolase (Bergmann *et al*. 2001; Feng *et al*. 2009; Boleij *et al*. 2011), which hampers studies of its possible role in streptococcal virulence. The amino acid sequences in streptococcal species share a 90–100% identity. Streptococcal α-enolase localizes in the cytoplasm as well as mammalian cells. In addition, α-enolase is expressed on the surface of bacterial cells and released into culture supernatant, although it lacks a signal sequence and the cell-wall-anchoring motif LPXTG (Pancholi and Fischetti 1998; Bergmann *et al*. 2001; Hughes *et al*. 2002; Ge *et al*. 2004; Mori *et al*. 2012). A binding experiment with radio-labeled recombinant α-enolase and *S. pneumoniae* clarified that secreted α-enolase can be re-associated onto the pneumococcal cell surface (Bergmann *et al*. 2001). Furthermore, ELISA findings obtained using an autolysin-deficient mutant strain showed that approximately 24% of pneumococcal α-enolase is released into supernatant in an autolysin-dependent manner (Mori *et al*. 2012). However, the secretion system and cell-surface localization mechanism require further study.

The crystal structures of α-enolase have been identified in many eukaryotic and prokaryotic organisms and shown to form dimers or octamers (Pancholi 2001; Ehinger *et al*. 2004). Biological multimeric structures are necessary for these enzyme activities. The monomers of yeast enolase formed by hydrostatic pressure are enzymatically inactive (Kornblatt *et al*. 1998). Although most organisms including the Gram-negative bacterium *Escherichia coli* express α-enolase as a dimeric structure, at least some Gram-positive organisms such as *Bacillus subtilis* and several *Streptococcus* species express octameric α-enolase (Brown *et al*. 1998; Ehinger *et al*. 2004). Human and pneumococcal multimeric structures are shown in Figure 14.1. There is a possibility that ancestral enolase was an octamer and the evolutionary process has independently altered enolase multiple times to form a dimeric structure (Fig. 14.2; Brown *et al*. 1998). It is interesting to note that there is a conformational difference between the α-enolase of the γ-proteobacterium such as *E. coli* and firmicutes such as *B. subtilis* and *Streptococcus* species. The divergence point for these groups of bacteria is estimated to have occurred 2.8–3.6 billion years ago (Battistuzzi *et al*. 2004). The dimeric form of GAS α-enolase is unstable regardless of the fact that enolases from most organisms are dimers. No significant dimer or multimer amounts were formed in dissociation experiments using mutations such as F137L and E363G to destabilize the octameric structure (Karbassi *et al*. 2010). Cork *et al*. demonstrated that a lysine residue at position 344 in GAS α-enolase plays an important role in maintaining the integrity of the octameric structure using site-directed mutagenesis of surface-located lysine residues. In addition, findings from a surface plasmon resonance (SPR) assay with immobilized plasminogen showed that the mutation K344E lacked enzyme activity and monomers of yeast enolase, and also showed increased plasminogen-binding ability. On the other hand, K304A and K334A mutations did have altered plasminogen-binding ability as compared to the wild type, whereas other lysine residue mutations in

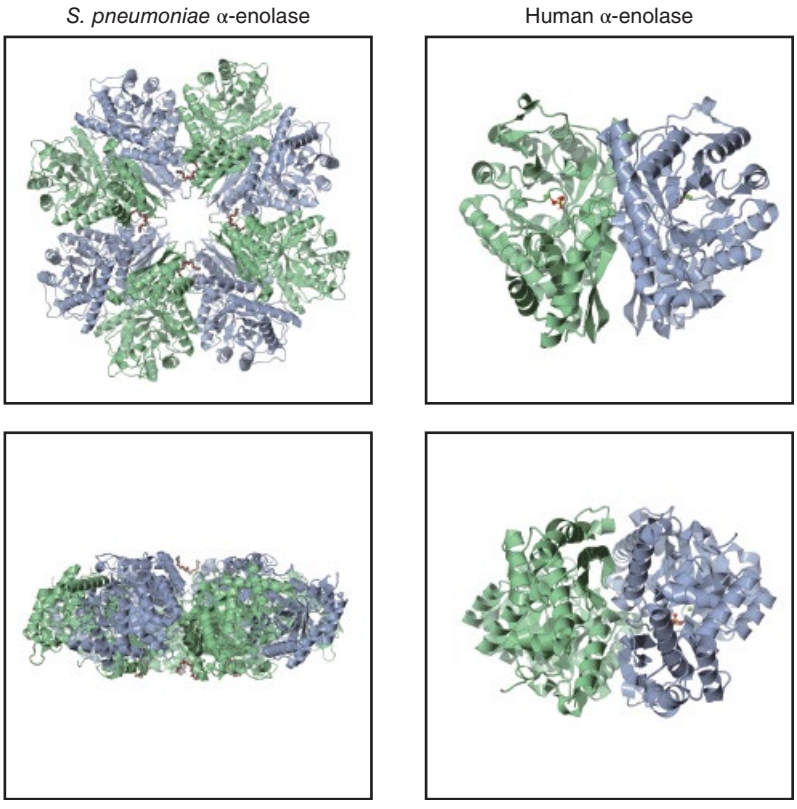

Figure 14.1 Multimeric crystal structure of *S. pneumoniae* and human α-enolase. *S. pneumoniae* α-enolase (PDBID: 1w6t) forms an octamer and the human version (PDBID: 3B97) is a dimer. This figure was produced using Yorodumi (run by PDBj) with Jmol, available at http://pdbj.org/emnavi/viewtop.php.

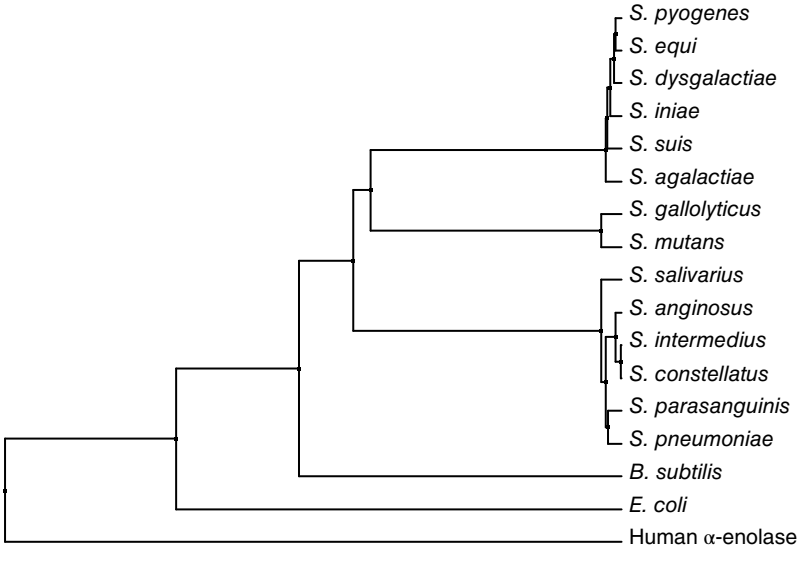

Figure 14.2 Phylogenetic tree of *Streptococcus* α-enolase. The tree is calculated and drawn by "Average Distance using BLOSUM62" on Jalview 2.8.2. The amino acid sequences were obtained from each genome referential strains.

K252 + 255A, K435L, and Δ434–435 resulted in greatly diminished binding ability (Cork *et al*. 2009). These results indicate that the presence of lower multimeric forms may interact more efficiently with plasminogen. Kornblatt *et al*. reported that the native structure of octameric α-enolase of GAS is not able to interact with human plasminogen based on findings obtained with multi-prolonged methods, including fluorescence polarization, isothermal titration calorimetry, dynamic light scattering, analytical ultracentrifugation, pull-down reactions, and SPR. Interestingly, the non-native structure of monomeric or multimeric enolase is capable of binding human plasminogen, while the non-native structure of plasminogen can also bind native enolase (Kornblatt *et al*. 2011). Recently, another group that included Kornblatt used a different set of multi-prolonged methods, including dual polarization interferometry, atomic force microscopy observations, isothermal titration calorimetry, dynamic light scattering, and fluorescence resonance energy transfer, and showed that native GAS α-enolase and plasminogen can bind with each other in the presence of a sticky surface (Balhara *et al*. 2014).

14.3 Multiple Binding Activities of α-Enolase

α-enolase can interact with various host proteins and plays multiple roles in bacterial pathogenesis via binding. Notably, plasminogen-binding ability has been widely reported for eukaryotic and prokaryotic organisms. Several pathogenic bacteria that cause invasive diseases activate and utilize host plasminogen for the invasion of deeper tissues and host immune evasion. Plasminogen plays an important role in the *in vivo* change from non-invasive GAS to the invasive phenotype. Recent studies have shown that mutations in the regulator genes have effects on the expression of virulence factors and generate hyper-virulent GAS variants (Cole *et al*. 2006, 2011; Walker *et al*. 2007; Ato *et al*. 2008; Hollands *et al*. 2010; Ikebe *et al*. 2010; Shea *et al*. 2011; Liang *et al*. 2013). Ikebe *et al*. (2010) sequenced 164 GAS strains isolated in Japan since 1992 and found that mutations in the genes encoding CovR/S or Rgg (also known as RopB) were detected in 57.3% of invasive isolates, but in only 1.7% of the non-invasive isolates. Shea *et al*. (2011) sequenced 301 GAS serotype M3 strains isolated in Canada between 2003 and 2009, and found that 37% of the invasive strains contained mutations in the *covR/S* genes as compared to only 4.6% of the pharyngitis strains. In another study, mutations in the *covR/S* genes of an M1T1 serotype GAS strain resulted in strong transcriptional upregulation of multiple virulence-associated genes including streptokinase, which activates plasminogen via a different conformational change as compared to host-derived plasminogen activators (Walker *et al*. 2007; Cole *et al*. 2011). M1T1 is a clinically and epidemiologically important GAS serotype frequently associated with severe invasive disease throughout the world (Aziz and Kotb 2008; Maamary *et al*. 2012). In addition, the *covR/S* mutation decreases GAS cysteine protease SpeB expression and loss of SpeB prevents auto-degradation of streptokinase, M1 protein, and host plasminogen. M1 protein is a multifunctional cell-surface protein capable of binding to plasminogen. In a previous study, GAS was found to have plasmin activity on the cell surface,

and showed an increased ability to evade host immunity and cause invasive infection (Cole *et al*. 2006; Walker *et al*. 2007). GBS also utilizes host plasminogen for invasive disease development. It was recently reported that GBS treated with plasminogen and tissue plasminogen activator (t-PA) showed increased adherence to, invasion of, and transmigration across a human brain microvascular endothelial cell line, hBMEC, while GBS with plasmin activated by t-PA showed enhanced invasion of the central nervous system of mice (Magalhaes *et al*. 2013).

In addition to GAS, groups C and G *Streptococcus* contain streptokinase to convert plasminogen into an active form (Lähteenmaki *et al*. 2001). This activation by streptokinase is not regulated by host plasminogen activator inhibitor-1 and -2, or inhibited by host factors including α2-antiplasmin and α2-macroglobulin (Parry *et al*. 2000). Streptokinase-activated plasminogen can degrade antimicrobial peptide LL-37, thus promoting GAS resistance to the peptide (Hollands *et al*. 2012). Interestingly, *Streptococcus mitis*, *Streptococcus oralis*, and *Streptococcus salivarius* lacking streptokinase are also capable of activating human plasminogen in human plasma (Itzek *et al*. 2010). To activate plasminogen, those strains as well as *S. pneumoniae* require host plasminogen activators, such as urokinase-type plasminogen activator (u-PA) or t-PA (Bergmann *et al*. 2005; Bergmann and Hammerschmidt 2007; Itzek *et al*. 2010; Fulde *et al*. 2013*b*). t-PA also enhances activation of plasminogen on the surface of groups A, C, and G *Streptococcus* in PBS or human plasma (Kuusela *et al*. 1992). Furthermore, plasminogen was found to enhance the virulence of a streptokinase-deficient mutant GAS strain in systemic and skin infection cases (Khil *et al*. 2003). These results indicate that streptokinase and the host plasminogen activator contribute to increase plasmin activity and bacterial virulence in a synergetic manner.

Pneumococcal enolase binds human plasminogen on the bacterial cell surface. Furthermore, C-terminal lysine residues of both eukaryotic and prokaryotic α-enolase have been found in plasminogen-binding sites (Miles *et al*. 1991; Bergmann *et al*. 2001; Derbise *et al*. 2004). Bergmann *et al*. (2003) identified a novel internal plasminogen-binding site, termed 248FYDKERKVY256, in pneumococcal α-enolase using spot-synthesized peptide analysis, while crystal structure analysis indicated that C-terminal residues are inaccessible in an octamer form. In contrast, the internal binding site is exposed on the surface. In that study, α-enolase was constructed to form a mutation with three residues replaced, 248FYDLGRLVY256, in the internal binding site (Eno_{int}). Eno_{int} showed 48% plasminogen binding as compared to the wild-type pneumococcal α-enolase (Ehinger *et al*. 2004). In addition, a mutation of the internal binding site showed significantly reduced degradation of the extracellular matrix and transmigration across the fibrin matrix (Bergmann *et al*. 2005).

Western and dot-blot analysis results revealed that α-enolase of *Streptococcus canis*, a zoonotic group G *Streptococcus*, binds to both human and canine plasminogen. That interaction facilitates degradation of the fibrin matrix through plasmin activated by u-PA. Also, exogenous addition of plasminogen enhanced *S. canis* survival after incubation with human neutrophils (Fulde *et al*. 2013*a*).

Plasminogen also mediates streptococcal translocation via a human epithelial or endothelial paracellular route. Serine protease inhibitor aprotinin-treated

plasminogen significantly enhanced GAS adhesion to a human pharyngeal cell line, Detroit 562, as compared to GAS alone and plasmin. However, plasminogen and plasmin on the GAS cell surface increased GAS paracellular translocation across the epithelial cell monolayer, while the proteins did not affect the GAS invasion index, calculated as the percentage of adherent GAS organisms (Pancholi *et al*. 2003). *S. pneumoniae* also showed similar results. Furthermore, plasminogen and aprotinin-inactivated plasmin increased pneumococcal adherence to the human lung alveolar carcinoma cell line A549 and human vascular endothelial cell line EaHy, while active plasmin decreased the level of that adherence. Finally, active plasmin mediated transmigration across epithelial A549 and endothelial EaHy cell layers by degrading intercellular tight junctions (Attali *et al*. 2008).

In addition to plasminogen, bacterial α-enolase is reported to bind several host proteins such as fibronectin (Fn), laminin, collagen-I, mucin, lactoferrin, and cytokeratin-8 (Ge *et al*. 2004; Antikainen *et al*. 2007; Esgleas *et al*. 2008; Boleij *et al*. 2011; Garbe *et al*. 2014). Fn is an extracellular matrix protein assembled by cells in the fibrillar matrix and then circulates in body fluids as a soluble dimer (Singh *et al*. 2010). Many bacteria bind and utilize Fn on host Fn-receptor $\alpha_5\beta_1$-integrins, leading to the rearrangement of cytoskeletal actin in host cells and invasion of those cells (Henderson *et al*. 2011; Yamaguchi *et al*. 2013). Using ELISA, Esgleas *et al*. showed that recombinant α-enolase of *Streptococcus suis*, an important swine pathogen, binds immobilized plasminogen and Fn. In addition, their SPR analysis showed high-affinity interactions between *S. suis* enolase and plasminogen ($K_D = 14$ nM) or Fn ($K_D = 21$ nM) (Esgleas *et al*. 2008). Another group reported that recombinant α-enolase of GAS and *S. pneumoniae* did not bind Fn, as shown by ELISA (Antikainen *et al*. 2007). Also, *S. suis* α-enolase was identified as one of three surface-interacting proteins by investigating the surface molecules of HEp-2 (HeLa) cells and using results of affinity chromatography-based surface proteomics. In the presence of recombinant α-enolase or anti-α-enolase antiserum, *S. suis* adhesion to HEp-2 (HeLa) cells was inhibited (Chen *et al*. 2011). These results are consistent with the Fn-binding ability of *S. suis* α-enolase. HEp-2 cells were previously thought to be derived from squamous cell laryngeal cancer, though the cell line has been shown to be HeLa cells due to contamination (Lacroix 2008). Several additional interactions have been reported, although their roles are unknown. Immobilized α-enolase of *Streptococcus mutans*, a major pathogen of human dental caries, binds human plasminogen and salivary mucin MUC7 (previously called MG2) (Ge *et al*. 2004). α-enolase of *Streptococcus gordonii* also binds MUC7 (Kesimer *et al*. 2009) and immobilized α-enolase of *Enterococcus faecalis* binds human lactoferrin (Garbe *et al*. 2014). Lactoferrin is a non-heme iron binding protein and a member of the family of transferrins that regulate iron homeostasis. *E. faecalis* is a Gram-positive bacterium formerly classified as group D *Streptococcus*. Lactoferrin is considered to function as an antimicrobial substance in blood and on mucosal surfaces. *E. faecalis* de-glycosylates and inhibits lactoferrin using its glycosidase, EndoE. However, it remains unknown whether α-enolase functions as a lactoferrin-binding site on the bacterial cell surface or if α-enolase supports de-glycosylation of lactoferrin. α-enolase of *Streptococcus gallolyticus* interacts with cytokeratin-8,

and that interaction was reported to have potential to contribute to bacterial adhesion of host epithelial cells (Boleij *et al*. 2011).

Streptococcal α-enolase is capable of disrupting the host immune system. *Streptococcus sobrinus* as well as *S. mutans* are human cariogenic bacteria. Intraperitoneal injection of *S. sobrinus* α-enolase suppressed primary immune response against T-cell-dependent antigens in mice, whereas rabbit enolase did not. In addition, injection of recombinant enolase induced interleukin-10 production in mice sera (Veiga-Malta *et al*. 2004). Furthermore, α-enolase is involved in inhibition of the host complement system. Complement C3b opsonizes bacteria as well as host immunoglobulins for enhanced phagocytosis, then recruited phagocytes can easily engulf and digest the opsonized pathogens. In addition, the complement system plays a major role in innate immunity and can be activated through classical, alternative, and lectin pathways. C3b is a key component of: the classical and alternative pathways, which share a common terminal pathway; and formation of the membrane attack complex (C5b-9), which disrupts the phospholipid bilayer of target cells and lyses bacteria (Laarman *et al*. 2010). α-enolase of *S. pneumoniae* binds to the complement inhibitor, C4b-binding protein (C4BP), and the α-enolase-C4BP complex can degrade C4b. C4BP inhibits C3 convertase, which activates conversion of C3 into C3b. α-enolase enhances the binding of C4BP and deposition of opsonin C3b on pneumococcal surfaces (Agarwal *et al*. 2012), while it also induces neutrophil cell death, called NETosis. In 2004, it was shown that activated neutrophils release DNA fibers that include antimicrobial proteins and peptides, which form neutrophil extracellular traps (NETs) to bind, disarm, and kill pathogens in an extracellular manner (Brinkmann *et al*. 2004). Extracellular traps are not formed exclusively by neutrophils, but also by other immune cells including mast cells (von Kockritz-Blickwede *et al*. 2008), eosinophils (Yousefi *et al*. 2008), and macrophages/monocytes (Chow *et al*. 2010). GAS, *S. sanguinis*, and *S. pneumoniae* evade NET killing by degrading DNA and resistance to antimicrobial peptides on NETs. Those bacteria have genes encoding DNases, such as Sda1, EndA, and the cell-wall-anchored protein SpnA, which degrade DNA as the backbone of NETs (Beiter *et al*. 2006; Buchanan *et al*. 2006; Chang *et al*. 2011; Morita *et al*. 2014). α-enolase of *S. pneumoniae* induces neutrophil cell death, NET formation, and NET-dependent bacterial killing in human blood. A pull-down assay showed that α-enolase binds the neutrophil cell-surface protein, myoblast antigen 24.1D5, although it remains unclear whether the interaction induces formation of NETs. NET formation induced by α-enolase may destroy host tissue or disseminate intravascular coagulation *in vivo* (Mori *et al*. 2012).

14.4 Involvement of α-Enolase in Gene Expression Regulation

α-enolase is involved in the regulation of gene expression. It has been established that nuclear enolases of eukaryotes, including those of mammalians and plants, regulate gene expression through direct binding to a TATA motif in their target gene promoters (Feo *et al*. 2000; Subramanian and Miller 2000; Lee *et al*. 2002).

Recently, the Apicomplexa parasites *Toxoplasma gondii* Eno1 and Eno2 were reported to specifically bind the putative gene promoter motif TTTTCT, while there was no binding to the TATA motif observed (Mouveaux *et al*. 2014). Eukaryotic α-enolase binds not only to DNA, but also to RNA. Yeast enolase binds to transfer RNA (tRNA) and functions as a cofactor in mitochondrial tRNA import (Entelis *et al*. 2006). Magnetic bead affinity and electrophoretic mobility shift assay results showed that rat α-enolase binds RNA through a $(CUG)_n$ triplet repeat, while there was no binding to *E. coli* α-enolase (Hernandez-Perez *et al*. 2011). Another protozoan parasite, *Entamoeba histolytica* enolase, was found to inhibit tRNA methyltransferase activity through interaction with Cytosine-5 methyltransferase 2 (Tovy *et al*. 2010).

Although there are no reports showing a direct interaction between bacterial α-enolase and DNA/RNA, α-enolase is known to be a component of the bacterial RNA degradosome. Messenger RNA (mRNA) must be precisely degraded for control of gene expression in bacteria. The RNA degradosome is a multi-enzyme complex that degrades mRNA. *E. coli* endoribonuclease RNase E assembles RNA helicase RhlB, phosphorolytic exoribonuclease PNPase, and α-enolase in its C-terminal regions, and forms an RNA degradosome. Although the role of α-enolase in the RNA degradosome remains unclear, it is speculated to function as a linker between metabolic status and post-transcriptional gene regulation (Chandran and Luisi 2006). Several different bacteria have an RNase Y ortholog with or without another RNase J instead of an RNase E type enzyme. In *B. subtilis*, RNase Y is speculated to form a degradosome complex by assembling α-enolase, phosphofructokinase, RNA helicase CshA, PNPase, and paralogous RNase J1 and J2 (Laalami *et al*. 2014). Kang *et al*. suggested that GAS α-enolase also regulates virulence-related gene expression by modifying RNA degradation. Western blotting following *in vivo* cross-linking showed that α-enolase forms a complex with the GAS RNase Y ortholog CvfA. Their transcriptome analysis also indicated that the CvfA-Eno complex represses GAS virulence genes encoding M protein, streptokinase, and CAMP factor in a carbohydrate starvation condition, and also upregulates the *speB* gene transcription in a peptide starvation condition (Kang *et al*. 2010). Furthermore, GAS express RNase J1 and J2 homologs, which can degrade mRNA (Bugrysheva and Scott 2010). There seems to be a difference between γ-proteobacteria and firmicutes regarding the RNA degradosome, as well as the multimeric structure of α-enolase.

14.5 Role of Anti-α-Enolase Antibodies in Host Immunity

Anti-α-enolase auto-antibodies have been reported in a wide range of autoimmune diseases, such as systemic lupus erythematosus, rheumatoid sclerosis, Behçet's disease, and others, while 0–6% of healthy individuals have been found to possess antibodies against α-enolase (Terrier *et al*. 2007). In addition, expression of α-enolase on human cell surfaces is increased by inflammatory stimulation. It is possible that the upregulated cell-surface expression exacerbates

autoimmune inflammation. *S. sanguinis* and sera from patients with Behçet's disease were shown to stimulate expression of α-enolase on the membrane of human dermal microvascular endothelial cells (Cho *et al.* 2013). In addition, anti-α-enolase antibodies have been detected in infants with biliary atresia, a life-threatening condition. In examinations of a Rhesus rotavirus-induced mouse model of biliary atresia as well as sera from biliary atresia patients, anti-enolase IgM and IgG were shown to be increased as compared to healthy controls. Anti-enolase antibodies cross-react with ~102 kDa proteins of rhesus rotavirus, including viral protein (VP)6, VP2, and VP4. These viral proteins may induce development of anti-enolase auto-antibodies (Lu *et al.* 2010).

Antibodies against streptococcal α-enolase often cross-react with human enolase and may be involved in autoimmune diseases. Streptococcal α-enolase is an immunogenic protein. In GAS, α-enolase is 1 of 33 known proteins that are immunoreactive against GAS-reactive human sera (Cole *et al.* 2005). α-enolase of GBS is one of the most immunogenic proteins and showed reactions with sera from patients infected with 60 different GBS strains (Brzychczy-Wloch *et al.* 2013). A cohort study of 329 Finnish children under 2 years old showed that 99% (292/294) of serum samples from those children contained anti-pneumococcal α-enolase antibodies, while there was no correlation between colonization of and infection with *S. pneumoniae* and antibody titers (Adrian *et al.* 2004). Interestingly, anti-human β-enolase antibodies and sera from patients with Buerger disease and atherosclerosis showed cross-reactions with enolase-like proteins on the surfaces of various Gram-negative bacteria, including *Shigella sonnei*, *Shigella flexneri*, *Klebsiella pneumoniae*, *E. coli*, *Salmonella* Typhimurium, *Citrobacter freundii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Hafnia alvei* (Witkowska *et al.* 2005). GAS infection sometimes generates antibodies that cross-react with auto-antigens causing autoimmune sequelae, such as acute rheumatic fever (ARF) and acute glomerulonephritis. Also, anti-GAS α-enolase and polyclonal anti-human α-enolase antibodies cross-reacted with recombinant human and GAS α-enolase, respectively, in dot-blot and ELISA findings. Furthermore, PMA or lipopolysaccharide stimulation upregulated α-enolase expression on the cell surfaces of monocytes, neutrophils, T cells, and B cells. Sera from ARF patients showed significantly higher anti-GAS α-enolase levels as compared to healthy subjects, while uncomplicated GAS pharyngitis patients also showed higher anti-GAS α-enolase levels as compared to healthy controls, though those were significantly lower as compared to ARF patients. There were no differences in anti-GAS GAPDH levels between the subjects. Together, these results indicated that GAS α-enolase plays important roles in autoimmune complications following infection (Fontan *et al.* 2000).

Streptococcal α-enolase has received focus as a possible vaccine antigen in parallel with its role as an autoimmune antigen. As previously described, α-enolase is one of the most abundant and immunogenic proteins on the surface of streptococcal cells (Cole *et al.* 2005; Zhang *et al.* 2008; Brzychczy-Wloch *et al.* 2013). However, α-enolase vaccination produced varied results in several different studies. Oral immunization with recombinant *S. sobrinus* α-enolase increased anti-α-enolase IgA and IgG in rat saliva, and also inhibited dental caries development in rats. In addition, anti-α-enolase IgG induced by subcutaneous

immunization did not cross-react with human enolase, even though *S. sobrinus* α-enolase has a greater than 46% homology with human or rat enolase (Dinis *et al*. 2009). The same group showed that maternal intranasal immunization with *S. sobrinus* α-enolase before pregnancy protected rat pups from dental caries. Furthermore, results of an infection assay using swapped Eno- or PBS-immunized rat mothers and pups indicated that the most effective maternal antibodies were transmitted via the placenta (Dinis *et al*. 2011). Subcutaneous immunization with recombinant *S. suis* α-enolase provided complete protection from lethal intraperitoneal (i.p.) infection of *S. suis* serotype 2 in mice (Feng *et al*. 2009). Another Chinese group also reported that i.p. immunization with recombinant *S. suis* α-enolase protected mice against lethal i.p. infection with *S. suis* serotype 2 and 7 (Zhang *et al*. 2009). α-enolase has also been shown to function as a protective antigen against fish pathogens. *Streptococcus iniae* is one of the most economically important bacteria in the aquaculture industry, and effective commercial vaccines are important for prevention. α-enolase was identified as one of three immunoreactive proteins involved in vaccine protection (LaFrentz *et al*. 2011). Wang *et al*. constructed *B. subtilis* spores expressing *Clonorchis sinensis* α-enolase as an oral vaccine for freshwater fish, which showed protective efficiency against *C. sinens* infection in fish (Wang *et al*. 2014). On the other hand, subcutaneous immunization of mice with recombinant α-enolase of *S. zooepidemicus* did not result in significant protection against a lethal i.p. challenge with the bacterium (Velineni and Timoney 2013). When evaluating the effects of vaccine antigens, it is important to note that negative results are more difficult to publish. Additional studies are required to develop α-enolase as a vaccine antigen.

14.6 α-Enolase as Potential Therapeutic Target

Several groups have evaluated the effects of compounds against α-enolase activities in the EMP pathway or its transcription level in oral streptococci. An antimicrobial component of tea polyphenols, epigallocatechin gallate, inhibited >50% of *S. mutans eno* gene transcription at the sub-MIC level (15.6 $\mu g\,mL^{-1}$). However, the component did not affect α-enolase enzyme activity even at 62.5 $\mu g\,mL^{-1}$ (Xu *et al*. 2011). Takahashi and Washio evaluated two major caries-preventive reagents, fluoride and xylitol. Fluoride-glucose rinses (fluoride at 225 and 900 ppm) inhibited α-enolase enzyme activity in dental plaque biofilm, whereas 10% xylitol-glucose rinse did not show any effect (Takahashi and Washio 2011). In another study, fluoride-ion induced conformational change of enolase resulting from formation of a magnesium-fluoride-phosphate complex, which inhibited substrate-binding to enolase and related enzyme activity (Qin *et al*. 2006). Purified plant-based compounds, quercitrin and deoxynojirimycin, were shown to inhibit the α-enolase activity *of S. mutans* synergistically at the sub-MIC level (32 and 8 $\mu g\,mL^{-1}$, respectively) *in vitro* (Hasan *et al*. 2014). On the other hand, penicillin, vancomycin, and linezolid at sub-MIC levels upregulated the *eno* and *gapdh* genes of *S. oralis* organisms isolated from infective endocarditis patients, while antibiotics did not affect gene transcriptions of *eno* and *gapdh* in *S. mitis*

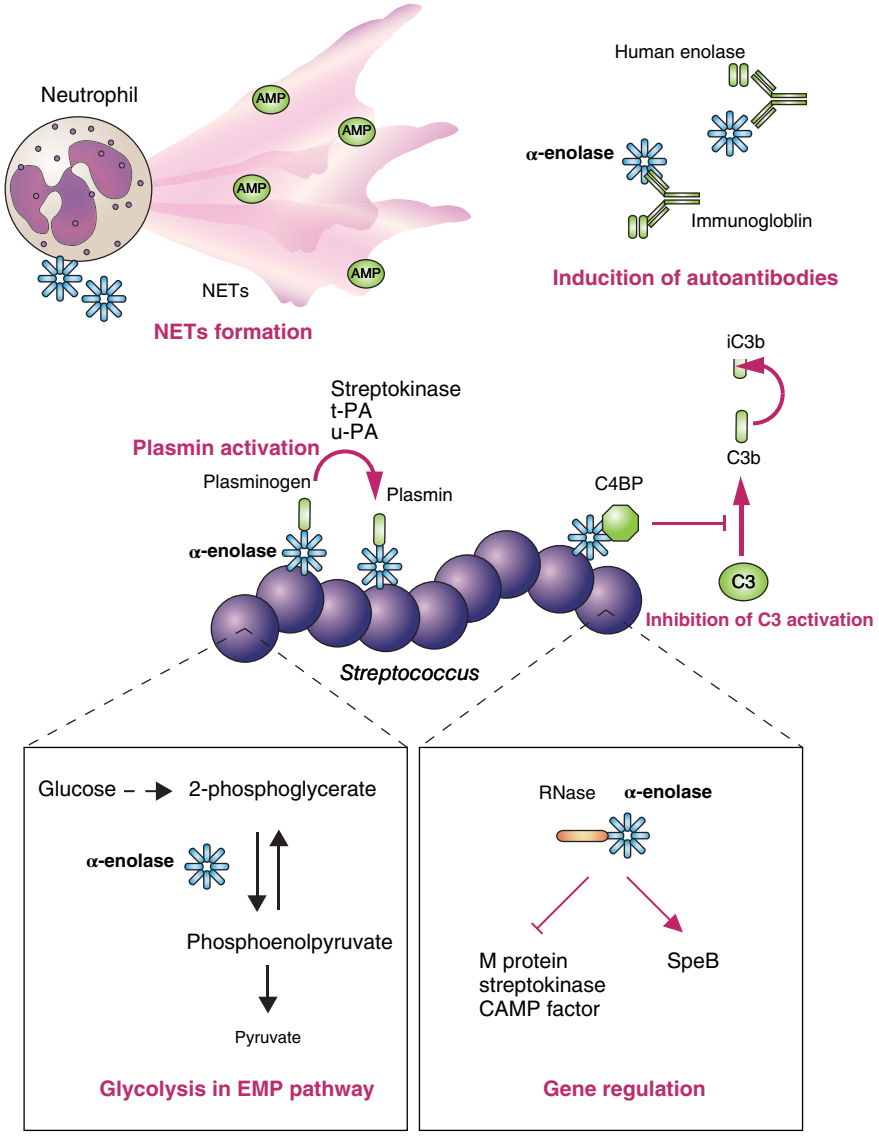

Figure 14.3 Multiple roles of α-enolase in *Streptococcus*. α-enolase has been shown to play various roles in bacterial cell cytoplasm and on the cell surface, as well as in culture supernatant. In the cytoplasm, α-enolase functions as a glycolytic enzyme in energy metabolism and is also involved in gene regulation. α-enolase interrupts the host immune system by binding host factors, such as plasminogen and C4b-binding protein. α-enolase is an immunogenic protein and antibodies cross-react with human enolase. In addition, α-enolase interacts with neutrophils and induces formation of NETs.

and *S. sanguinis* isolated from patients with infective endocarditis (Teles *et al.* 2012). Jung *et al.* identified a small molecule compound, termed ENOblock, as an enolase inhibitor, which bound purified human enolase and inhibited enolase enzyme activity. ENOblock also inhibited invasion and migration by the colon carcinoma cell line HCT116 *in vitro*, and induced glucose uptake in the human

embryonic kidney cell line HEK, human hepatocellular carcinoma cell line Huh7, and zebrafish. This novel compound may have potential as a useful tool to examine the role of α-enolase in bacterial infection (Jung *et al*. 2013).

14.7 Questions Concerning α-Enolase

We have described the various functions of streptococcal α-enolase in this chapter (Fig. 14.3). The studies cited indicate that α-enolases have common functions as well as organism-specific functions derived from evolutionary diversity. On the other hand, species specificity regarding biological functions remains unclear and further studies are needed to identify the mechanisms of functional difference.

α-enolase is a unique enzyme converting 2-phosphoglycerate into phosphoenolpyruvate in the EMP pathway of *Streptococci*. However, other functions are not irreplaceable. For example, pathogenic bacteria express several alternative plasminogen- and/or Fn-binding proteins (Fulde *et al*. 2013*b*; Yamaguchi *et al*. 2013). Construction of conditional *eno* gene mutant strains is needed to clarify the role and importance of each function in streptococcal infections. A few groups constructed conditional GAS mutants using the tetracycline-inducible promoter P_{tet} and a conditional *S. pneumoniae* mutant using the zinc ion-inducible promoter P_{czcD} (Eberhardt *et al*. 2009; Bugrysheva and Scott 2010; Peters *et al*. 2014). Additional studies using such techniques will greatly contribute to better understanding of various infectious diseases and autoimmune disorders.

References

Adrian PV, Bogaert D, Oprins M, Rapola S, Lahdenkari M, Kilpi T, de Groot R, Kayhty H, Hermans PW (2004) Development of antibodies against pneumococcal proteins alpha-enolase, immunoglobulin A1 protease, streptococcal lipoprotein rotamase A, and putative proteinase maturation protein A in relation to pneumococcal carriage and Otitis Media. *Vaccine* 22: 2737–2742.

Agarwal V, Hammerschmidt S, Malm S, Bergmann S, Riesbeck K, Blom AM (2012) Enolase of *Streptococcus pneumoniae* binds human complement inhibitor C4b-binding protein and contributes to complement evasion. *Journal of Immunology* 189: 3575–3584.

Antikainen J, Kuparinen V, Lahteenmaki K, Korhonen TK (2007) Enolases from Gram-positive bacterial pathogens and commensal lactobacilli share functional similarity in virulence-associated traits. *FEMS Immunology and Medical Microbiology* 51: 526–534.

Ato M, Ikebe T, Kawabata H, Takemori T, Watanabe H (2008) Incompetence of neutrophils to invasive group A *streptococcus* is attributed to induction of plural virulence factors by dysfunction of a regulator. *PLoS One* 3: e3455.

Attali C, Durmort C, Vernet T, Di Guilmi AM (2008) The interaction of *Streptococcus pneumoniae* with plasmin mediates transmigration across

endothelial and epithelial monolayers by intercellular junction cleavage. *Infection and Immunity* 76: 5350–5356.

Aziz RK, Kotb M (2008) Rise and persistence of global M1T1 clone of *Streptococcus pyogenes*. *Emerging Infectious Diseases* 14: 1511–1517.

Balhara V, Deshmukh SS, Kalman L, Kornblatt JA (2014) The interaction of streptococcal enolase with canine plasminogen: the role of surfaces in complex formation. *PLoS One* 9: e88395.

Battistuzzi FU, Feijao A, Hedges SB (2004) A genomic timescale of prokaryote evolution: insights into the origin of methanogenesis, phototrophy, and the colonization of land. *BMC Evolutionary Biology* 4: 44.

Beiter K, Wartha F, Albiger B, Normark S, Zychlinsky A, Henriques-Normark B (2006) An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Current Biology* 16: 401–407.

Bergmann S, Hammerschmidt S (2007) Fibrinolysis and host response in bacterial infections. *Thrombosis and Haemostasis* 98: 512–520.

Bergmann S, Rohde M, Chhatwal GS, Hammerschmidt S (2001) alpha-Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Molecular Microbiology* 40: 1273–1287.

Bergmann S, Wild D, Diekmann O, Frank R, Bracht D, Chhatwal GS, Hammerschmidt S (2003) Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. *Molecular Microbiology* 49: 411–423.

Bergmann S, Rohde M, Preissner KT, Hammerschmidt S (2005) The nine residue plasminogen-binding motif of the pneumococcal enolase is the major cofactor of plasmin-mediated degradation of extracellular matrix, dissolution of fibrin and transmigration. *Thrombosis and Haemostasis* 94: 304–311.

Bogaert D, van Belkum A, Sluijter M, Luijendijk A, de Groot R, Rumke HC, Verbrugh HA, Hermans PW (2004) Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet* 363: 1871–1872.

Boleij A, Laarakkers CM, Gloerich J, Swinkels DW, Tjalsma H (2011) Surface-affinity profiling to identify host-pathogen interactions. *Infection and Immunity* 79: 4777–4783.

Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, Weinrauch Y, Zychlinsky A (2004) Neutrophil extracellular traps kill bacteria. *Science* 303: 1532–1535.

Brown CK, Kuhlman PL, Mattingly S, Slates K, Calie PJ, Farrar WW (1998) A model of the quaternary structure of enolases, based on structural and evolutionary analysis of the octameric enolase from *Bacillus subtilis*. *Journal of Protein Chemistry* 17: 855–866.

Brzychczy-Wloch M, Gorska S, Brzozowska E, Gamian A, Heczko PB, Bulanda M (2013) Identification of high immunoreactive proteins from *Streptococcus agalactiae* isolates recognized by human serum antibodies. *FEMS Microbiology Letters* 349: 61–70.

Buchanan JT, Simpson AJ, Aziz RK, Liu GY, Kristian SA, Kotb M, Feramisco J, Nizet V (2006) DNase expression allows the pathogen group A *Streptococcus* to escape killing in neutrophil extracellular traps. *Current Biology* 16: 396–400.

Bugrysheva JV, Scott JR (2010) The ribonucleases J1 and J2 are essential for growth and have independent roles in mRNA decay in *Streptococcus pyogenes*. *Molecular Microbiology* 75: 731–743.

Carapetis JR, Steer AC, Mulholland EK, Weber M (2005) The global burden of group A streptococcal diseases. *Lancet Infectious Diseases* 5: 685–694.

Chandran V, Luisi BF (2006) Recognition of enolase in the Escherichia coli RNA degradosome. *Journal of Molecular Biology* 358: 8–15.

Chang A, Khemlani A, Kang H, Proft T (2011) Functional analysis of *Streptococcus pyogenes* nuclease A (SpnA), a novel group A streptococcal virulence factor. *Molecular Microbiology* 79: 1629–1642.

Chen B, Zhang A, Xu Z, Li R, Chen H, Jin M (2011) Large-scale identification of bacteria-host crosstalk by affinity chromatography: capturing the interactions of *Streptococcus suis* proteins with host cells. *Journal of Proteome Research* 10: 5163–5174.

Cho S, Zheng Z, Cho SB, Choi MJ, Lee KH, Bang D (2013) *Streptococcus sanguinis* and the sera of patients with Behcet's disease stimulate membrane expression of alpha-enolase in human dermal microvascular endothelial cells. *Archives of Dermatological Research* 305: 223–232.

Chow OA, von Kockritz-Blickwede M, Bright AT, Hensler ME, Zinkernagel AS, Cogen AL, Gallo RL, Monestier M, Wang Y, Glass CK, Nizet V (2010) Statins enhance formation of phagocyte extracellular traps. *Cell Host and Microbe* 8: 445–454.

Cole JN, Ramirez RD, Currie BJ, Cordwell SJ, Djordjevic SP, Walker MJ (2005) Surface analyses and immune reactivities of major cell wall-associated proteins of Group A *Streptococcus*. *Infection and Immunity* 73: 3137–3146.

Cole JN, McArthur JD, McKay FC, Sanderson-Smith ML, Cork AJ, Ranson M, Rohde M, Itzek A, Sun H, Ginsburg D, Kotb M, Nizet V, Chhatwal GS, Walker MJ (2006) Trigger for group A streptococcal M1T1 invasive disease. *FASEB JOURNAL* 20: 1745–1747.

Cole JN, Barnett TC, Nizet V, Walker MJ (2011) Molecular insight into invasive group A streptococcal disease. *Nature Reviews Microbiology* 9: 724–736.

Cork AJ, Jergic S, Hammerschmidt S, Kobe B, Pancholi V, Benesch JL, Robinson CV, Dixon NE, Aquilina JA, Walker MJ (2009) Defining the structural basis of human plasminogen binding by streptococcal surface enolase. *Journal of Biological Chemistry* 284: 17129–17137.

Derbise A, Song YP, Parikh S, Fischetti VA, Pancholi V (2004) Role of the C-terminal lysine residues of streptococcal surface enolase in Glu- and Lys-plasminogen-binding activities of Group A streptococci. *Infection and Immunity* 72: 94–105.

Dinis M, Tavares D, Veiga-Malta I, Fonseca AJ, Andrade EB, Trigo G, Ribeiro A, Videira A, Cabrita AM, Ferreira P (2009) Oral therapeutic vaccination with *Streptococcus sobrinus* recombinant enolase confers protection against dental caries in rats. *Journal of Infectious Diseases* 199: 116–123.

Dinis M, Trigo G, Chaves N, Fonseca AJ, Ribeiro A, Tavares D, Cabrita AM, Ferreira P (2011) rEnolase maternal immunization confers caries protection on offspring. *Journal of Dental Research* 90: 325–330.

Eberhardt A, Wu LJ, Errington J, Vollmer W, Veening JW (2009) Cellular localization of choline-utilization proteins in *Streptococcus pneumoniae* using novel fluorescent reporter systems. *Molecular Microbiology* 74: 395–408.

Edwards MS, Baker CJ (2005) Group B streptococcal infections in elderly adults. *Clinical Infectious Diseases* 41: 839–847.

Ehinger S, Schubert WD, Bergmann S, Hammerschmidt S, Heinz DW (2004) Plasmin(ogen)-binding alpha-enolase from *Streptococcus pneumoniae*: crystal structure and evaluation of plasmin(ogen)-binding sites. *Journal of Molecular Biology* 343: 997–1005.

Entelis N, Brandina I, Kamenski P, Krasheninnikov IA, Martin RP, Tarassov I (2006) A glycolytic enzyme, enolase, is recruited as a cofactor of tRNA targeting toward mitochondria in *Saccharomyces cerevisiae*. *Genes and Development* 20: 1609–1620.

Esgleas M, Li Y, Hancock MA, Harel J, Dubreuil JD, Gottschalk M (2008) Isolation and characterization of alpha-enolase, a novel fibronectin-binding protein from *Streptococcus suis*. *Microbiology* 154: 2668–2679.

Feng Y, Pan X, Sun W, Wang C, Zhang H, Li X, Ma Y, Shao Z, Ge J, Zheng F, Gao GF, Tang J (2009) *Streptococcus suis* enolase functions as a protective antigen displayed on the bacterial cell surface. *Journal of Infectious Diseases* 200: 1583–1592.

Feo S, Arcuri D, Piddini E, Passantino R, Giallongo A (2000) ENO1 gene product binds to the c-*myc* promoter and acts as a transcriptional repressor: relationship with Myc promoter-binding protein 1 (MBP-1). *FEBS Letters* 473: 47–52.

Fontan PA, Pancholi V, Nociari MM, Fischetti VA (2000) Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. *Journal of Infectious Diseases* 182: 1712–1721.

Fulde M, Rohde M, Polok A, Preissner KT, Chhatwal GS, Bergmann S (2013*a*) Cooperative plasminogen recruitment to the surface of Streptococcus canis via M protein and enolase enhances bacterial survival. *MBio* 4: e00629-00612.

Fulde M, Steinert M, Bergmann S (2013*b*) Interaction of streptococcal plasminogen binding proteins with the host fibrinolytic system. *Frontiers in Cellular and Infection Microbiology* 3: 85.

Garbe J, Sjogren J, Cosgrave EF, Struwe WB, Bober M, Olin AI, Rudd PM, Collin M (2014) EndoE from *Enterococcus faecalis* hydrolyzes the glycans of the biofilm inhibiting protein lactoferrin and mediates growth. *PLoS One* 9: e91035.

Ge J, Catt DM, Gregory RL (2004) *Streptococcus mutans* surface alpha-enolase binds salivary mucin MG2 and human plasminogen. *Infection and Immunity* 72: 6748–6752.

Hasan S, Singh K, Danisuddin M, Verma PK, Khan AU (2014) Inhibition of major virulence pathways of *Streptococcus mutans* by quercitrin and deoxynojirimycin: a synergistic approach of infection control. *PLoS One* 9: e91736.

Henderson B, Nair S, Pallas J, Williams MA (2011) Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. *FEMS Microbiology Reviews* 35: 147–200.

Hernandez-Perez L, Depardon F, Fernandez-Ramirez F, Sanchez-Trujillo A, Bermudez-Cruz RM, Dangott L, Montanez C (2011) alpha-Enolase binds to RNA. *Biochimie* 93: 1520–1528.

Hollands A, Pence MA, Timmer AM, Osvath SR, Turnbull L, Whitchurch CB, Walker MJ, Nizet V (2010) Genetic switch to hypervirulence reduces colonization phenotypes of the globally disseminated group A *Streptococcus* M1T1 clone. *Journal of Infectious Diseases* 202: 11–19.

Hollands A, Gonzalez D, Leire E, Donald C, Gallo RL, Sanderson-Smith M, Dorrestein PC, Nizet V (2012) A bacterial pathogen co-opts host plasmin to resist killing by cathelicidin antimicrobial peptides. *Journal of Biological Chemistry* 287: 40891–40897.

Hughes MJ, Moore JC, Lane JD, Wilson R, Pribul PK, Younes ZN, Dobson RJ, Everest P, Reason AJ, Redfern JM, Greer FM, Paxton T, Panico M, Morris HR, Feldman RG, Santangelo JD (2002) Identification of major outer surface proteins of *Streptococcus agalactiae*. *Infection and Immunity* 70: 1254–1259.

Ikebe T, Ato M, Matsumura T, Hasegawa H, Sata T, Kobayashi K, Watanabe H (2010) Highly frequent mutations in negative regulators of multiple virulence genes in group A streptococcal toxic shock syndrome isolates. *PLoS Pathog* 6: e1000832.

Itzek A, Gillen CM, Fulde M, Friedrichs C, Rodloff AC, Chhatwal GS, Nitsche-Schmitz DP (2010) Contribution of plasminogen activation towards the pathogenic potential of oral streptococci. *PLoS One* 5: e13826.

Jung DW, Kim WH, Park SH, Lee J, Kim J, Su D, Ha HH, Chang YT, Williams DR (2013) A unique small molecule inhibitor of enolase clarifies its role in fundamental biological processes. *ACS Chemical Biology* 8: 1271–1282.

Kang SO, Caparon MG, Cho KH (2010) Virulence gene regulation by CvfA, a putative RNase: the CvfA-enolase complex in *Streptococcus pyogenes* links nutritional stress, growth-phase control, and virulence gene expression. *Infection and Immunity* 78: 2754–2767.

Karbassi F, Quiros V, Pancholi V, Kornblatt MJ (2010) Dissociation of the octameric enolase from *S. pyogenes*: one interface stabilizes another. *PLoS One* 5: e8810.

Kawamura Y, Hou XG, Sultana F, Miura H, Ezaki T (1995) Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. *International Journal of Systemic Bacteriology* 45: 406–408.

Kesimer M, Kilic N, Mehrotra R, Thornton DJ, Sheehan JK (2009) Identification of salivary mucin MUC7 binding proteins from *Streptococcus gordonii*. *BMC Microbiology* 9: 163.

Khil J, Im M, Heath A, Ringdahl U, Mundada L, Cary Engleberg N, Fay WP (2003) Plasminogen enhances virulence of group A streptococci by streptokinase-dependent and streptokinase-independent mechanisms. *Journal of Infectious Diseases* 188: 497–505.

Kornblatt MJ, Lange R, Balny C (1998) Can monomers of yeast enolase have enzymatic activity? *European Journal of Biochemistry* 251: 775–780.

Kornblatt MJ, Kornblatt JA, Hancock MA (2011) The interaction of canine plasminogen with *Streptococcus pyogenes* enolase: they bind to one another but what is the nature of the structures involved? *PLoS One* 6: e28481.

Kuusela P, Ullberg M, Saksela O, Kronvall G (1992) Tissue-type plasminogen activator-mediated activation of plasminogen on the surface of group A, C, and G streptococci. *Infection and Immunity* 60: 196–201.

Laalami S, Zig L, Putzer H (2014) Initiation of mRNA decay in bacteria. *Cellular and Molecular Life Sciences* 71: 1799–1828.

Laarman A, Milder F, van Strijp J, Rooijakkers S (2010) Complement inhibition by gram-positive pathogens: molecular mechanisms and therapeutic implications. *Journal of Molecular Medicine (Berl)* 88: 115–120.

Lacroix M (2008) Persistent use of "false" cell lines. *International Journal of Cancer* 122: 1–4.

LaFrentz BR, Shoemaker CA, Klesius PH (2011) Immunoproteomic analysis of the antibody response obtained in Nile tilapia following vaccination with a *Streptococcus iniae* vaccine. *Vetinary Microbiology* 152: 346–352.

Lähteenmaki K, Kuusela P, Korhonen TK (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiology Reviews* 25: 531–552.

Lamagni TL, Efstratiou A, Vuopio-Varkila J, Jasir A, Schalen C (2005) The epidemiology of severe *Streptococcus pyogenes* associated disease in Europe. *Eurosurveillance* 10: 179–184.

Lancefield RC (1933) A serological differentiation of human and other groups of hemolytic streptococci. *Journal of Experimental Medicine* 57: 571–595.

Lappin E, Ferguson AJ (2009) Gram-positive toxic shock syndromes. *Lancet Infectious Diseases* 9: 281–290.

Le Doare K, Heath PT (2013) An overview of global GBS epidemiology. *Vaccine* 31 Suppl 4: D7–12.

Lee H, Guo Y, Ohta M, Xiong L, Stevenson B, Zhu JK (2002) LOS2, a genetic locus required for cold-responsive gene transcription encodes a bi-functional enolase. *EMBO Journal* 21: 2692–2702.

Liang Z, Zhang Y, Agrahari G, Chandrahas V, Glinton K, Donahue DL, Balsara RD, Ploplis VA, Castellino FJ (2013) A natural inactivating mutation in the CovS component of the CovRS regulatory operon in a pattern D *Streptococcal pyogenes* strain influences virulence-associated genes. *Journal of Biological Chemistry* 288: 6561–6573.

Lohman K, Meyerhof O (1934) Über die enzymatische Umwandlung von Phosphoglyzerinsäure in Brenztrauben- säure und Phosphorsäure. *Biochemische Zeitschrift* 273: 60–72.

Lu BR, Brindley SM, Tucker RM, Lambert CL, Mack CL (2010) alpha-enolase autoantibodies cross-reactive to viral proteins in a mouse model of biliary atresia. *Gastroenterology* 139: 1753–1761.

Maamary PG, Ben Zakour NL, Cole JN, Hollands A, Aziz RK, Barnett TC, Cork AJ, Henningham A, Sanderson-Smith M, McArthur JD, Venturini C, Gillen CM, Kirk JK, Johnson DR, Taylor WL, Kaplan EL, Kotb M, Nizet V, Beatson SA, Walker MJ (2012) Tracing the evolutionary history of the pandemic group A streptococcal M1T1 clone. *FASEB Journal* 26: 4675–4684.

Magalhaes V, Andrade EB, Alves J, Ribeiro A, Kim KS, Lima M, Trieu-Cuot P, Ferreira P (2013) Group B *Streptococcus* hijacks the host plasminogen system to promote brain endothelial cell invasion. *PLoS One* 8: e63244.

Maisey HC, Doran KS, Nizet V (2008) Recent advances in understanding the molecular basis of group B *Streptococcus* virulence. *Expert Reviews in Molecular Medicine* 10: e27.

Miles LA, Dahlberg CM, Plescia J, Felez J, Kato K, Plow EF (1991) Role of cell-surface lysines in plasminogen binding to cells: identification of alpha-enolase as a candidate plasminogen receptor. *Biochemistry* 30: 1682–1691.

Mori Y, Yamaguchi M, Terao Y, Hamada S, Ooshima T, Kawabata S (2012) alpha-Enolase of *Streptococcus pneumoniae* induces formation of neutrophil extracellular traps. *Journal of Biological Chemistry* 287: 10472–10481.

Morita C, Sumioka R, Nakata M, Okahashi N, Wada S, Yamashiro T, Hayashi M, Hamada S, Sumitomo T, Kawabata S (2014) Cell wall-anchored nuclease of *Streptococcus sanguinis* contributes to escape from neutrophil extracellular trap-mediated bacteriocidal activity. *PLoS One* 9: e103125.

Mouveaux T, Oria G, Werkmeister E, Slomianny C, Fox BA, Bzik DJ, Tomavo S (2014) Nuclear glycolytic enzyme enolase of Toxoplasma gondii functions as a transcriptional regulator. *PLoS One* 9: e105820.

Nakamura N, Dai Q, Williams J, Goulding EH, Willis WD, Brown PR, Eddy EM (2013) Disruption of a spermatogenic cell-specific mouse enolase 4 (eno4) gene causes sperm structural defects and male infertility. *Biology of Reproduction* 88: 90.

O'Brien KL, Wolfson LJ, Watt JP, Henkle E, Deloria-Knoll M, McCall N, Lee E, Mulholland K, Levine OS, Cherian T (2009) Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 374: 893–902.

Pancholi V (2001) Multifunctional alpha-enolase: its role in diseases. *Cellular and Molecular Life Sciences* 58: 902–920.

Pancholi V, Fischetti VA (1998) alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. *Journal of Biological Chemistry* 273: 14503–14515.

Pancholi V, Fontan P, Jin H (2003) Plasminogen-mediated group A streptococcal adherence to and pericellular invasion of human pharyngeal cells. *Microbial Pathogenesis* 35: 293–303.

Parry MA, Zhang XC, Bode I (2000) Molecular mechanisms of plasminogen activation: bacterial cofactors provide clues. *Trends in Biochemical Science* 25: 53–59.

Peters K, Schweizer I, Beilharz K, Stahlmann C, Veening JW, Hakenbeck R, Denapaite D (2014) *Streptococcus pneumoniae* PBP2x mid-cell localization requires the C-terminal PASTA domains and is essential for cell shape maintenance. *Molecular Microbiology* 92: 733–755.

Pfoh E, Wessels MR, Goldmann D, Lee GM (2008) Burden and economic cost of group A streptococcal pharyngitis. *Pediatrics* 121: 229–234.

Postma PW, Lengeler JW, Jacobson GR (1993) Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. *Microbiology Reviews* 57: 543–594.

Qin J, Chai G, Brewer JM, Lovelace LL, Lebioda L (2006) Fluoride inhibition of enolase: crystal structure and thermodynamics. *Biochemistry* 45: 793–800.

Shea PR, Beres SB, Flores AR, Ewbank AL, Gonzalez-Lugo JH, Martagon-Rosado AJ, Martinez-Gutierrez JC, Rehman HA, Serrano-Gonzalez M, Fittipaldi N, Ayers SD, Webb P, Willey BM, Low DE, Musser JM (2011) Distinct signatures of diversifying selection revealed by genome analysis of respiratory tract and invasive bacterial populations. *Proceedings of the National Academy of Science, USA* 108: 5039–5044.

Singh P, Carraher C, Schwarzbauer JE (2010) Assembly of fibronectin extracellular matrix. *Annual Review of Cell and Developmental Biology* 26: 397–419.

Subramanian A, Miller DM (2000) Structural analysis of alpha-enolase. Mapping the functional domains involved in down-regulation of the c-*myc* protooncogene. *Journal of Biological Chemistry* 275: 5958–5965.

Takahashi N, Washio J (2011) Metabolomic effects of xylitol and fluoride on plaque biofilm in vivo. *Journal of Dental Research* 90: 1463–1468.

Teles C, Smith A, Lang S (2012) Antibiotic modulation of the plasminogen binding ability of viridans group streptococci. *Antimicrobial Agents and Chemotherapy* 56: 458–463.

Terrier B, Degand N, Guilpain P, Servettaz A, Guillevin L, Mouthon L (2007) Alpha-enolase: a target of antibodies in infectious and autoimmune diseases. *Autoimmunity Reviews* 6: 176–82.

Tovy A, Siman Tov R, Gaentzsch R, Helm M, Ankri S (2010) A new nuclear function of the *Entamoeba histolytica* glycolytic enzyme enolase: the metabolic regulation of cytosine-5 methyltransferase 2 (Dnmt2) activity. *PLoS Pathogens* 6: e1000775.

Veiga-Malta I, Duarte M, Dinis M, Tavares D, Videira A, Ferreira P (2004) Enolase from *Streptococcus sobrinus* is an immunosuppressive protein. *Cell Microbiology* 6: 79–88.

Velineni S, Timoney JF (2013) Identification of novel immunoreactive proteins of *Streptococcus zooepidemicus* with potential as vaccine components. *Vaccine* 31: 4129–4135.

von Kockritz-Blickwede M, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, Medina E (2008) Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* 111: 3070–3080.

Walker MJ, Hollands A, Sanderson-Smith ML, Cole JN, Kirk JK, Henningham A, McArthur JD, Dinkla K, Aziz RK, Kansal RG, Simpson AJ, Buchanan JT, Chhatwal GS, Kotb M, Nizet V (2007) DNase Sda1 provides selection pressure for a switch to invasive group A streptococcal infection. *Nature Medicine* 13: 981–985.

Wang X, Chen W, Tian Y, Mao Q, Lv X, Shang M, Li X, Yu X, Huang Y (2014) Surface display of *Clonorchis sinensis* enolase on *Bacillus subtilis* spores potentializes an oral vaccine candidate. *Vaccine* 32: 1338–1345.

Witkowska D, Pietkiewicz J, Szostko B, Danielewicz R, Maslowski L, Gamian A (2005) Antibodies against human muscle enolase recognize a 45-kDa bacterial cell wall outer membrane enolase-like protein. *FEMS Immunology and Medical Microbiology* 45: 53–62.

Xu X, Zhou XD, Wu CD (2011) The tea catechin epigallocatechin gallate suppresses cariogenic virulence factors of *Streptococcus mutans*. *Antimicrobial Agents and Chemotherapy* 55: 1229–1236.

Yamaguchi M, Terao Y, Kawabata S (2013) Pleiotropic virulence factor - *Streptococcus pyogenes* fibronectin-binding proteins. *Cell Microbiology* 15: 503–511.

Yousefi S, Gold JA, Andina N, Lee JJ, Kelly AM, Kozlowski E, Schmid I, Straumann A, Reichenbach J, Gleich GJ, Simon HU (2008) Catapult-like release of mitochondrial DNA by eosinophils contributes to antibacterial defense. *Nature Medicine* 14: 949–953.

Zhang A, Xie C, Chen H, Jin M (2008) Identification of immunogenic cell wall-associated proteins of *Streptococcus suis* serotype 2. *Proteomics* 8: 3506–3515.

Zhang A, Chen B, Mu X, Li R, Zheng P, Zhao Y, Chen H, Jin M (2009) Identification and characterization of a novel protective antigen, Enolase of *Streptococcus suis* serotype 2. *Vaccine* 27: 1348–1353.

15

Borrelia burgdorferi Enolase and Plasminogen Binding

Catherine A. Brissette

Department of Microbiology, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota, USA

15.1 Introduction to Lyme Disease

Bacteria of the Order Spirochaetales include human and animal pathogens that cause diseases including syphilis, leptospirosis, periodontal disease, relapsing fever, and Lyme disease. Spirochetes are unique among bacteria in their extraordinary ability to invade, disseminate, and persist in their hosts. The Lyme disease spirochete, *B. burgdorferi*, is the cause of more than 90% of all arthropod-borne diseases affecting humans in the United States (Bacon *et al*. 2008; Radolf *et al*. 2012). Roughly 30,000 cases are reported to the Centers for Disease Control and Prevention (CDC) each year, although the actual number is thought to be much greater (Campbell *et al*. 1998; CDC 2011). Indeed, new estimates released by the CDC in 2013 indicate that the actual number of Americans affected by Lyme disease each year is around 300,000 (CDC 2013*a*; Hinckley *et al*. 2014). The disease is endemic to the US, Europe, and parts of Asia. The prevalence of Lyme disease is likely to escalate as a result of the expanding habitat of vector ticks (Rand *et al*. 2007; Ogden *et al*. 2008, 2009, 2010); indeed, in North America, Lyme disease has established footholds north and west of the traditional hot zones of New England/Mid-Atlantic and Upper Midwest regions (Rand *et al*. 2007; Russart *et al*. 2014; Simon *et al*. 2014; Stone *et al*. 2015). Lyme disease can affect many tissues and organs, with symptoms that include skin rashes, arthritis, and meningitis. Sudden death due to Lyme carditis, although rare, can occur (CDC 2013*b*). Failure to treat this infection promptly can result in long-term debilitating effects on the patient's health (Steere 2001; Stanek and Strle 2003; Stanek *et al*. 2012). A preventative vaccine was approved for human use in 1998 but production was halted in early 2002, although a vaccine for dogs remains available.

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

15.2 Life Cycle

B. burgdorferi is maintained in nature in an enzootic infectious cycle involving vertebrate hosts and hard ticks of the genus *Ixodes* (reviewed in Radolf *et al*. 2012). Uninfected larvae hatch from eggs and acquire *B. burgdorferi* by feeding on an infected animal, usually a small mammal or bird. After feeding, the larva drops to the ground and molts into the next life stage called a nymph. Nymphs feed on a second host, again usually a small mammal or bird, thus transmitting *B. burgdorferi* from the tick to a new reservoir. In this way, the bacterium is maintained in the environment. Occasionally, a nymph will feed on an incidental host, such as a human or dog. Humans are at most risk of being bitten during the late spring and summer, when nymphs are active. Replete nymphs drop to the ground and molt into the adult life stage. Adult ticks feed on larger animals, such as deer. After feeding, a mated adult female will lay eggs and die, and the cycle continues.

Remarkably, *B. burgdorferi* survives inside the tick midgut throughout the molts, when massive tissue reorganization occurs. As a result of this complex life cycle, *B. burgdorferi* adapts to wildly different environments (cold-blooded arthropod vector and warm-blooded vertebrate host) through its ability to persist, evade immune responses, disseminate, and spread to new hosts.

15.3 Borrelia Virulence Factors

Unlike many pathogenic bacteria, *B. burgdorferi* lacks many traditional virulence factors. While possessing two membranes like Gram-negative bacteria, *B. burgdorferi* lacks endotoxin (lipopolysaccharide) (Fraser *et al*. 1997; Casjens *et al*. 2000). In addition, *B. burgdorferi* makes no known exotoxins (Fraser *et al*. 1997; Casjens *et al*. 2000). Many pathogens express specialized secretion systems that allow for injection of effectors directly into host cells; *B. burgdorferi* lacks Type III or IV secretion systems (Fraser *et al*. 1997; Casjens *et al*. 2000). While *B. burgdorferi* has been detected inside mammalian cells, there is little evidence that *B. burgdorferi* is a true intracellular pathogen with the ability to create a niche within a host cell vacuole to evade host immune responses (Comstock and Thomas 1989; Ma *et al*. 1991; Klempner *et al*. 1993; Girschick *et al*. 1996; Fraser *et al*. 1997; Casjens *et al*. 2000; Livengood and Gilmore 2006; Wu *et al*. 2011). Many bacterial pathogens have sophisticated strategies to sequester iron, an essential and limiting nutrient. *B. burgdorferi*, however, has no need for iron (Posey and Gherardini 2000). *B. burgdorferi* does have an impressive array of adhesins to facilitate binding to host tissues (Antonara *et al*. 2011; Brissette and Gaultney 2014). *B. burgdorferi* also has the ability to evade host complement through the binding of host complement regulators (Kraiczy and Stevenson 2013). Complement evasion is especially important, as hematogenous spread is a critical component of the bacterium's ability to disseminate to a wide variety of tissues. Despite spreading throughout a host, the bacterium's strategy is one of evasion rather than confrontation. The vector host, the *Ixodes* tick, and the mammalian reservoir hosts, such as white-footed mice, do not seem to suffer any ill effects from *B. burgdorferi* infection (Hersh *et al*. 2014; Herrmann and

Gern 2015). Humans are accidental and dead-end hosts, and the bulk of disease pathology is a result of our immune responses to the bacterium rather than an effect of the bacterium itself.

15.4 Plasminogen Binding by Bacteria

Some bacteria, including *B. burgdorferi*, degrade the host's extracellular matrix (ECM) by adhering and activating the host's own proteases, for example plasminogen/plasmin (Lähteenmaki *et al*. 2005). Proteolysis is a common mechanism used by bacteria to evade host defenses and ultimately disseminate throughout the infected host. The mammalian ECM is a complex structure composed of many polysaccharides and proteins that serve as structural scaffolding for cells. The structure consists of glycosaminoglycans, which are repeating disaccharide units, and fibrous proteins, such as collagens, elastin (which is important in recoil after tensile stress), and fibronectin. The ECM is continuously maintained, yet it must also be quickly degraded in order to allow for the passage of cells, nutrients, and hormones. The breakdown of the ECM by proteases is tightly controlled and localized, as the ECM must be quickly regenerated. There are many proteases that can degrade mammalian ECM. However, the plasminogen-plasmin system is essential to the host's ability to allow for cellular migration, and is susceptible to the use of bacteria for dissemination.

Plasminogen is a 810 amino acid, 92 kDa zymogen, or proenzyme that is essential in the mammalian clotting cascade and can be converted into plasmin, a serine protease (Syrovets *et al*. 2012) by two specific serine proteases, tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA). A key feature of plasminogen is the Kringle domains. Plasminogen has five Kringle domains, triple loop structures that facilitate the binding of substrates, inhibitors, and cell receptors (Syrovets *et al*. 2012). These loops have an extremely high affinity for lysine residues. When bound to a eukaryotic or bacterial cell, plasminogen is converted into an immobilized form that allows for more efficient activation into plasmin, which is protected structurally from inactivation (Lähteenmaki *et al*. 2005).

Plasmin retains the five Kringle domains, and is therefore able to serve many functions (Lähteenmaki *et al*. 2005). Plasmin does not directly degrade collagen and elastin, but it does activate procollagenases and stromolysin that can break down those proteins (Lähteenmaki *et al*. 2001). Plasmin also has the ability to cleave many different proteins including coagulation factors Factor V and VIII, and metalloproteinases. Such a potent protease must be controlled, and unbound plasmin has a short half-life due to the inactivation of the protease α_2-antiplasmin. When plasmin is bound to a cell surface it is protected from inactivation, allowing for more degradation of the ECM and the continual activation of more plasminogen. By exploiting this mechanism, many bacteria have been shown to disseminate through a host by binding to plasminogen and activating and utilizing the plasmin, which is resistant against inactivation. In addition to dissemination, bacteria bind plasminogen to aid in adherence to cell surfaces and protection against the host immune system (Fulde *et al*. 2013).

Most mechanisms by which bacteria utilize the plasminogen-plasmin system are through the immobilization of plasminogen to an outer surface protein of the bacterium, followed by the activation of the plasminogen to plasmin by the host's plasminogen activator. Immobilization of plasminogen on the bacterium is mediated through the C-terminal lysines on surface proteins of the pathogen. However, some bacterial plasminogen-binding proteins possess both internal and charged amino acid motifs, instead of C-terminal lysines that facilitate interaction (Walker *et al*. 2005; Sanderson-Smith *et al*. 2012).

15.5 *B. burgdorferi* and Plasminogen Binding

The first demonstration that *B. burgdorferi* could bind host plasminogen came in 1994. Fuchs *et al*. demonstrated that strain ZS7 binds plasminogen, which could subsequently be activated to plasmin by exogenous uPA or tPA. This cell-bound plasmin was able to degrade the host ECM component fibronectin (Fuchs *et al*. 1994). The authors identified the plasminogen-binding protein as outer surface protein A (OspA), an outer surface protein that is required for colonization and survival within the tick. Plasmin bound by OspA could not be inactivated by host anti-plasmin. Another research group identified a second plasminogen-binding protein of 70 kDa with homology to the OppA family of transporters (Hu *et al*. 1995, 1997). Subsequent research identified several other plasminogen-binding proteins, including outer surface protein C (OspC; Lagal *et al*. 2006; Önder *et al*. 2012); OspE-related proteins (Erp) ErpA, ErpC, and ErpP (Brissette *et al*. 2009); and the complement regulator-acquiring surface proteins, CRASP-1 and CRASP-2 (Hallstrom *et al*. 2010). Plasminogen binding has been demonstrated among various Lyme borreliae as well as relapsing fever borreliae (Klempner *et al*. 1995; Seling *et al*. 2010). Plasminogen binding by whole-cell *B. burgdorferi* occurs in a dose-dependent manner (Nogueira *et al*. 2012) and could be inhibited by the presence of unlabeled plasminogen and a lysine analog, aminocaproic acid, implicating lysines in *B. burgdorferi*-plasminogen interaction (Coleman *et al*. 1995; Hu *et al*. 1995). Plasmin-coated *B. burgdorferi* could penetrate endothelial cell monolayers and an artificial ECM substrate (Coleman *et al*. 1995). These results hinted at the importance of plasminogen binding by *B. burgdorferi* as a way to facilitate dissemination through host cell tissues. Indeed, plasminogen was required for efficient dissemination within the tick vector (where *B. burgdorferi* acquires plasminogen as ticks take a blood meal); plasminogen assimilation also enhanced dissemination in a mouse model (Coleman *et al*. 1997). Later studies found a correlation between the strength of the interaction between plasminogen and OspC, and the invasiveness of a particular strain. Those more invasive strains caused an increased bacterial burden in mouse tissues distal to the site of infection, and resulted in more severe arthritis than strains that bound plasminogen less tightly (Lagal *et al*. 2006).

Active plasmin on the surface of *B. burgdorferi* facilitates degradation of fibronectin (Fuchs *et al*. 1994; Coleman *et al*. 1999), laminin, vitronectin, native ECM, and matrigel (Coleman *et al*. 1999; Nogueira *et al*. 2012) but not collagen (Coleman *et al*. 1999). Plasmin on the surface of *B. burgdorferi* also activates

matrix metalloprotease-9 (MMP-9), which facilitates *B. burgdorferi* penetration through the ECM *in vitro* (Gebbia *et al*. 2001). Plasminogen must be cleaved by tPA or uPA to active plasmin, and *B. burgdorferi* induces uPA expression from monocytes (Fuchs *et al*. 1996; Haile *et al*. 2006). Indeed, *B. burgdorferi* induces the expression of several components of the clot-busting fibrinolytic system, including the uPA receptor (uPAR) on monocytes (Coleman *et al*. 2001; Coleman and Benach 2003; Hovius *et al*. 2009), pro-MMP-9 and MMP-1 from various cell types (Gebbia *et al*. 2001), and the plasminogen inhibitor PAI-2 (Haile *et al*. 2006). Interestingly, PAI-2 did not affect *B. burgdorferi* transmigration across a fibronectin barrier, in line with the observation that plasmin bound to the surface of *B. burgdorferi* is not inhibited by PAI-2 (Fuchs *et al*. 1994; Haile *et al*. 2006). Taken together, the preponderance of evidence suggests that upregulation of components of the fibrinolytic cascade and subsequent plasminogen binding is an important virulence strategy for *B. burgdorferi*.

An additional characteristic of *B. burgdorferi*'s suite of plasminogen-binding proteins is their propensity to bind multiple ligands. The proteins CRASP-1, CRASP-2, ErpA, ErpC, and ErpP all interact with host plasminogen as well as host complement regulators (Kraiczy and Stevenson 2013). CRASP-1, for example, binds Factor H as well as Factor H-like protein 1 (FHL1). In most cases, the binding sites for specific ligands are physically separated, maximizing the function of each protein (Brissette *et al*. 2009; Seling *et al*. 2010). This feature of plasminogen-binding proteins is shared with other host-interaction proteins of *B. burgdorferi*, including many extracellular matrix adhesins (reviewed by Antonara *et al*. 2011; Brissette and Gaultney 2014).

15.6 Enolase

Enolase is an enzyme that catalyzes the penultimate step in the conversion of glucose to pyruvate (Pancholi 2001). Because of its critical metabolic function, enolase is highly conserved across species (Fig. 15.1). Enolase is generally cytoplasmic and enolases in bacteria, yeast, parasites, and mammals lack N-terminal sorting sequences that would target them to the outer surface of a cell (Pancholi 2001). Potential export routes have been postulated in mammals, including membrane blebbing, membrane flipping, endosomal recycling, or an unknown plasma membrane transporter (Capello *et al*. 2011). Post-translational modifications including phosphorylation, acetylation, and methylation have been identified in human enolases (Zhou *et al*. 2010). In the protozoan parasite *Leishmania*, proteins lacking endoplasmic reticulum secretory signal sequences, transmembrane spanning domains, or glycosylphosphatidylinositol-anchor consensus sequences translocate onto the cell surface via a non-classical mechanism involving N-myristoylation and/or palmitoylation (Denny *et al*. 2000). These modifications could potentially change how the protein interacts with the membrane, or how it interacts with a chaperone, transporter, or flippase. Whether any of the post-translational modifications are necessary or sufficient for surface exposure is not clear. Interaction of enolase with scaffold proteins, such as the interaction of human gamma-enolase with syntrophin, may also be responsible for surface

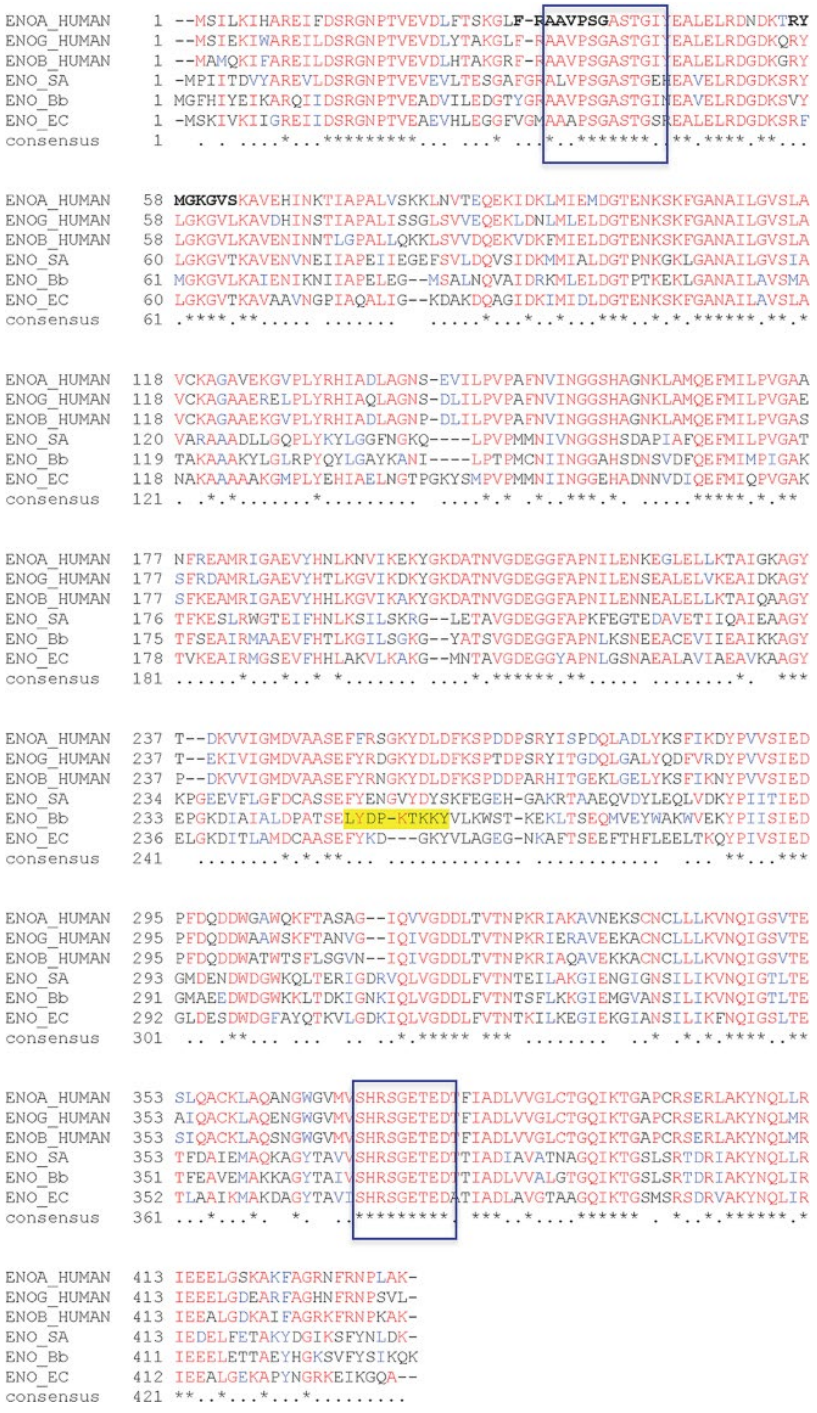

Figure 15.1 Alignment of enolase protein sequences from human (three isoforms), *Staphylococcus aureus*, *Escherichia coli*, and *B. burgdorferi*. Identical amino acids are in red; similar amino acids are in blue font. A consensus line underneath the alignment indicates identity (*) or similarity (.). The box at the top of the alignment indicates a hydrophobic domain thought to play a role in membrane association (Pancholi 2001). The box at the bottom of the alignment indicates the catalytic site of the enzyme. Yellow highlighted text indicates an internal plasminogen-binding motif (Noguiera *et al*. 2012). Bold black text indicates cross-reactive epitopes associated with autoimmunity in cancer (Adamus *et al*. 1998). (*See color plate section for the color representation of this figure.*)

localization (Hafner *et al*. 2010). Enolases on the surface of mammalian cells are of interest due to their expression on tumor cells of breast, lung, and pancreatic cancers (Capello *et al*. 2011). In these instances, enolase can not only bind plasminogen, but also facilitate activation of matrix metalloproteinases and degradation of the ECM, actions which promote metastases. Surface-exposed enolase can also promote auto-antibody production, leading to deleterious consequences for the host.

Enolases have been localized to the surface of lower eukaryotes such as yeast (Lopez-Villar *et al*. 2006), various parasites, and numerous bacterial species, particularly Gram-positive bacteria (Henderson and Martin 2011). As in eukaryotes, how these proteins are exported to the surface in bacteria is unknown. In some instances, surface expression is dependent on pH (Antikainen *et al*. 2007). Recently, an internal hydrophobic helical domain was identified as essential but not sufficient for cell-surface localization of the *Bacillus subtilis* enolase (Yang *et al*. 2014). Interaction with a holding chaperone along with unfolding during transport is another possibility. Autolysis has been suggested as a mechanism for outer surface exposure of enolase in Gram-positive bacteria; bacteria lyse and cytoplasmic contents associate with the surfaces of other bacteria in the environment (Jedrzejas 2001; Guiral *et al*. 2005). A growing number of surface-exposed enolases have been identified in Gram-negative bacteria, including *Aeromonas hydrophilia* (Sha *et al*. 2009) and *Pseudomonas aeruginosa* (Witkowska *et al*. 2005; Ceremuga *et al*. 2014). Potential mechanisms of surface export in bacteria with two membranes may involve mechanisms similar to those postulated in mammals, including membrane blebbing and re-association with the outer surface, membrane flipping, or post-translational modifications such as acetylation. Recently, enolase was discovered on the surface of the didermic spirochete, *B. burgdorferi* (Floden *et al*. 2011; Nogueira *et al*. 2012; Toledo *et al*. 2012).

15.7 *B. burgdorferi* Enolase and Plasminogen Binding

Glycolysis is the only energy-generating pathway in *B. burgdorferi* (Fraser *et al*. 1997), and the enolase gene *bb0337* is essential to *B. burgdorferi* survival. Attempts to delete the gene were not successful, and *bb0337* was not picked up in a signature-tagged mutagenesis screen (Lin *et al*. 2012; Nogueira *et al*. 2012). Nowalk and colleagues analyzed borrelial proteins after subcellular fractionation into membrane and soluble fractions by non-equilibrium pH gel electrophoresis (NEPHGE; Nowalk *et al*. 2006); enolase was detected in both soluble and cytoplasmic fractions. This suggested that, as in other bacteria, enolase in *B. burgdorferi* might be a surface-exposed moonlighting protein. Indeed, a genome-wide proteome array revealed a limited set of *B. burgdorferi* antigens recognized by humans and mice, including the borrelial enolase. Antibodies recognizing *B. burgdorferi* enolase were detected in 5 out of 39 patients with late Lyme disease, indicating that the host comes into contact with enolase at some point during the infection (Barbour *et al*. 2008). A separate study identified antibodies against *B. burgdorferi* enolase in infected mice, rabbits, and human patients (Toledo *et al*. 2012). Surface exposure of enolase was also suggested by the ability of anti-enolase antibodies to bind intact *B. burgdorferi* cells (Nogueira *et al*. 2012).

A common way to determine surface exposure of *B. burgdorferi* proteins is the protease protection assay. Whole cells are incubated with various proteases, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotted with specific antibodies. As a spirochete, *B. burgdorferi* has periplasmic flagella sandwiched between the inner and outer membranes that are not surface exposed. The flagellin antigen therefore serves as a convenient control for the intactness of the bacterium. Using this assay, two independent research groups demonstrated that enolase was degraded by proteases, indicating surface exposure (Floden *et al*. 2011; Nogueira *et al*. 2012). Surface exposure was also confirmed by immunogold electron microscopy (Floden *et al*. 2011). In contrast, Toledo *et al*. (2012) did not detect enolase on the outer surface by protease protection assay or electron microscopy. *B. burgdorferi* enolase was detected by this group as one of a small group of proteins present in outer membrane vesicles that included other plasminogen-binding proteins, for example OspA, glyceraldehyde-3-phosphate dehydrogenase (GADPH), and CRASP-1 (Toledo *et al*. 2012). The bulk of the evidence suggests that, at the very least, enolase is associated with the outer membrane, may be surface exposed under certain conditions, and is seen by the host immune system (Nowalk *et al*. 2006; Barbour *et al*. 2008; Floden *et al*. 2011; Nogueira *et al*. 2012; Toledo *et al*. 2012).

How enolase gets to the outer membrane remains a mystery; protein export to the outer surface is still incompletely understood in *B. burgdorferi* (Zückert 2013, 2014). Most lipoproteins in *Borrelia* have a 19 amino acid leader and a cysteine residue that becomes lipidated, and enolase lacks this sequence. It is unlikely that *B. burgdorferi* undergoes autolysis with free cytoplasmic contents attaching to neighboring bacteria, as has been suggested as a mechanism for surface acquisition of enolase in other organisms (Jedrzejas 2001; Guiral *et al*. 2005). When whole *B. burgdorferi* cells are incubated with exogenous enolase no increase in the amount of protein is detected, suggesting *B. burgdorferi* does not bind its own enolase (Floden *et al*. 2011). There is a hydrophilic domain in human enolase that has been implicated in membrane association (Pancholi 2001); a similar motif exists in the *B. burgdorferi* enolase (Fig. 15.1), suggesting a possible mechanism for its localization to the membrane.

Other enolases have been identified as plasminogen-binding proteins, and *B. burgdorferi* enolase is no exception. Antibodies compete against enolase for *B. burgdorferi*-plasminogen interaction (Nogueira *et al*. 2012). Recombinant *B. burgdorferi* enolase binds plasminogen in a dose-dependent manner with a K_D of 125 nM (Floden *et al*. 2011; Nogueira *et al*. 2012; Toledo *et al*. 2012). This binding is inhibited by the presence of the lysine analog aminocaproic acid (Floden *et al*. 2011; Nogueira *et al*. 2012; Toledo *et al*. 2012). Ionic charges are not involved in the plasminogen-enolase binding, as excess sodium chloride or heparin (anionic salt) did not inhibit this interaction (Floden *et al*. 2011; Toledo *et al*. 2012). Plasminogen bound to enolase can be converted to active plasmin (Floden *et al*. 2011; Toledo *et al*. 2012). The recombinant enolase retains enzymatic activity, including the catalysis of NADH2 to NAD+, and enolase activity can be detected on intact *B. burgdorferi* (Nogueira *et al*. 2012). Whether there is some benefit to the bacteria retaining enzymatic activity of this moonlighting protein on its surface is unclear.

B. burgdorferi enolase is expressed throughout its natural life cycle, in both vector ticks and reservoir host mammals (Nogueira *et al*. 2012). However, expression appears to be enhanced in both fed and unfed nymphal ticks (Nogueira *et al*. 2012), as well as *in vitro* at 34 °C, pH 6.4, conditions that mimic the blood meal (Toledo *et al*. 2012). This increased expression appears to be post-translational, as transcript levels of enolase were not enhanced in response to blood or a temperature shift in culture (Tokarz *et al*. 2004). These data make sense in the context of plasminogen binding. The bacterium would encounter plasminogen as the tick begins to take a blood meal, and would have an opportunity to acquire this protease before moving into the mammal. Thus, the bacterium is preloaded and ready to disseminate from the bite site. Using a mouse model, Nogueira *et al*. (2012) found that *bb0337*, the *B. burgdorferi* enolase gene, was expressed in multiple tissues including skin, joints, and heart. Its essential nature and heightened expression during infection suggested that enolase might be a good vaccine candidate. Unfortunately, antibodies against enolase were not bactericidal and did not prevent infection. Immunization also had no effect on arthritis development in the mouse model (Nogueira *et al*. 2012). However, immunization of mice did interfere with *B. burgdorferi* acquisition by nymphal ticks, suggesting a possible strategy to interrupt the enzootic life cycle in nature. Indeed, many field trials are currently underway to immunize mice in the wild against *B. burgdorferi* (Tsao *et al*. 2004; Bensaci *et al*. 2012; Voordouw *et al*. 2013; Gomes-Solecki 2014).

Önder *et al*. (2012) showed that OspC is most likely the predominant plasminogen-binding protein in *B. burgdorferi*. *B. burgdorferi* lacking OspC bound minimal plasminogen to their outer surface; OspC and plasminogen also co-localized on the surface of *B. burgdorferi*. Why would *B. burgdorferi*, with OspC likely being the predominant plasminogen-binding protein, need to utilize enolase? One idea is that enolase is cheap for the cell to produce, unlike secreted proteins or lipoproteins such as OspC (Smith and Chapman 2010). For instance, the borrelial enolase is low in "expensive" amino acids such as phenylalanine and tryptophan, and rich in "cheap" amino acids such as alanine and glycine. Under times of stress (e.g., faced with a hostile immune response), *B. burgdorferi* might conserve resources by using a moonlighting protein like enolase rather than a more expensive lipoprotein like OspC. *B. burgdorferi* enolase has an internal plasminogen-binding motif (LYDPKTKKY) that does not overlap with the catalytic motif (SHRSGETED), so the bacterium loses no metabolic capability by moonlighting with its enolase (Fig. 15.1).

Plasminogen binding may serve different purposes in different niches. Different plasminogen-binding proteins have different dissociation constants and affinities; *B. burgdorferi* enolase has a K_D for plasminogen of 125 nM, while ErpP has a K_D of 25 nM. Many other bacteria express both high- and low-affinity plasminogen-binding proteins (Ullberg *et al*. 1990; Berge and Sjobring 1993). Binding tightly to a substrate is not always an advantage, and bacteria need to constantly fine tune and adjust their associations with cells, ECM, and tissues. For instance, in a particular environment plasminogen binding might facilitate adhesion to cell surfaces. Indeed, surface-exposed enolase was identified as a mediator for plasminogen-dependent attachment of the bacterium *Streptococcus pneumoniae*

to endothelial and epithelial cells (Bergmann *et al*. 2013). Alternatively, different plasminogen-binding proteins could facilitate immune evasion. The plasminogen-binding protein staphylokinase from *Staphylococcus aureus* acts as an anti-opsonic agent (Rooijakkers *et al*. 2005). A plasminogen-binding protein could also influence inflammation by inducing anti-inflammatory cytokines in the local milieu, as is seen with *Streptococcus sobrinus* enolase (Veiga-Malta *et al*. 2004). Toledo *et al*. (2012) found the *B. burgdorferi* enolase not expressed on the surface, but rather packaged in outer membrane vesicles along with other plasminogen-binding proteins including OspA, OspC, and GAPDH. Packaging enolase in outer membrane vesicles may therefore serve as a way to degrade ECM in the peribacterial environment, an advance assault before the arrival of the main bacterial force. Many didermic and Gram-negative bacteria shed outer membrane vesicles, which can carry numerous virulence factors including adhesins and proteases (Ellis and Kuehn 2010). These outer membrane vesicles can then deliver virulence factors in advance, or act as decoys for the immune system. *B. burgdorferi* is known to produce outer membrane vesicles or blebs under a number of conditions *in vitro* and *in vivo* (Shoberg and Thomas 1993; Whitmire and Garon 1993; Radolf *et al*. 1995; Dunham-Ems *et al*. 2009; Floden *et al*. 2011). The importance of these vesicles in *B. burgdorferi* pathogenesis, and their fate *in vivo*, is an open question.

What could surface expression of enolase mean for Lyme disease? Enolase is clearly an immunogenic protein, yet antibodies against it are not bactericidal unlike antibodies to other surface exposed or membrane-associated proteins (LaRocca *et al*. 2010; Nogueira *et al*. 2012; Floden *et al*. 2013). There is an obvious benefit to the bacterium in acquiring a host cell protease to facilitate dissemination, which may explain why *B. burgdorferi* has such pronounced redundancy in plasminogen-binding proteins. Mice that are deficient in plasminogen can still be infected with *B. burgdorferi*, so acquisition of this protease is not essential; however, plasminogen clearly enhances bacterial dissemination (Coleman *et al*. 1997). By binding plasminogen, enolase could contribute to tissue-specific pathology; although immunization against *B. burgdorferi* enolase had no effect on arthritis in a mouse model (Nogueira *et al*. 2012), the effect of blocking enolase in other tissues and organs such as the heart is unknown. Surface expression of *B. burgdorferi* enolase could also contribute to autoimmunity. Enolases from streptococcal species are cross-reactive with human enolase (not surprising given the essential nature of glycolysis and the conservation of these proteins) and are thought to contribute to sequelae such as rheumatic heart disease (Fontan *et al*. 2000). Human enolases are also associated with autoimmune conditions, particularly during cancer (Adamus *et al*. 1998). As in prokaryotes, human enolases are normally cytoplasmic proteins but have been known to moonlight under certain (usually pathologic) conditions (Capello *et al*. 2011). Interestingly, the cross-reactive epitopes of enolase in cancer-associated retinopathy are nearly identical in the *B. burgdorferi* enolase (Adamus *et al*. 1998). Ultimately, the ability of *B. burgdorferi* enolase to associate with the outer surface and bind plasminogen is likely just one piece of the bacterium's armamentarium.

15.8 Concluding Thoughts

Until recently, it was assumed that *B. burgdorferi* had to acquire host proteases as the bacterium lacked any native proteolytic activity. However, in 2013 Coleman and colleagues characterized a protein, BB0104, that has 41% amino acid identity to DegP, a potent protease of *Escherichia coli* (Coleman *et al*. 2013). The borrelial protein was able to degrade casein and maintained its proteolytic activity unless the catalytic site, S198, was mutated to alanine. The protease forms a trimer and is present in membrane-bound and soluble forms. Despite its similarity to DegP, BB0104 was unable to complement *E. coli* DegP (Coleman *et al*. 2013). BB0104 was also able to cleave and process the borrelial proteins basic membrane protein D (BmpD), chemotaxis protein X (CheX), as well as an uncharacterized protein, BB0323 (Kariu *et al*. 2013), suggesting an innate function of borrelial protein processing that has nothing to do with virulence. However, Russell and Johnson subsequently demonstrated that BB0104 is surface exposed and binds host aggrecan, an important constituent of the extracellular matrix in joints (Russell and Johnson 2013). This protease degrades aggrecan, fibronectin, and proteoglycans. In addition, it induces cytokines and chemokines interleukin 6 (IL-6), soluble cell adhesion molecule (sICAM), C-X-C chemokine 1 (CXCL-1), and C-C chemokines 1, 2, and 5 (CCL1, 2, 5) in chondrocytes, suggesting a role in both inducing inflammation and causing joint damage in Lyme arthritis (Russell *et al*. 2013). Indeed, the *bb0104* gene is expressed during human disease and conserved across the Lyme borreliae. Interestingly, like enolase, the mechanism for transport beyond the periplasm is unknown.

With its small genome and complex life cycle, *B. burgdorferi* has likely evolved to cram as much information into each protein-encoding gene as possible. We should consider that *B. burgdorferi* produces other potential moonlighters, such as GAPDH. Indeed, Toledo *et al*. (2012) recovered GAPDH, in conjunction with other plasminogen-binding proteins, in outer membrane vesicles from *B. burgdorferi*. We should not be surprised if other moonlighting surprises await us as we learn more about this fascinating spirochete.

Acknowledgements

The author thanks Brandee Stone for critical reading and review of this chapter.

References

Adamus G, Amundson D, Seigel GM, Machnicki M (1998) Anti-enolase-alpha autoantibodies in cancer-associated retinopathy: epitope mapping and cytotoxicity on retinal cells. *Journal of Autoimmunity* 11: 671–677.

Antikainen J, Kuparinen V, Lähteenmaki K, Korhonen TK (2007) pH-dependent association of enolase and glyceraldehyde-3-phosphate dehydrogenase of

Lactobacillus crispatus with the cell wall and lipoteichoic acids. *Journal of Bacteriology* 189: 4539–4543.

Antonara S, Ristow L, Coburn J (2011) Adhesion mechanisms of *B. burgdorferi*. *Advances in Experimental Medicine and Biology* 715: 35–49.

Bacon RM, Kugeler KJ, Mead PS (2008) Surveillance for Lyme disease: United States, 1992-2006. *MMWR Surveillance Summaries* 57: 1–9.

Barbour AG, Jasinskas A, Kayala MA, Davies, DH, Steere AC, Baldi P, Felgner PL (2008) A genome-wide proteome array reveals a limited set of immunogens in natural infections of humans and white-footed mice with *B. burgdorferi*. *Infection and Immunity* 76: 3374–3389.

Bensaci M, Bhattacharya D, Clark R, Hu LT (2012) Oral vaccination with vaccinia virus expressing the tick antigen subolesin inhibits tick feeding and transmission of *B. burgdorferi*. *Vaccine* 30: 6040–6046.

Berge A, Sjobring U (1993) PAM, a novel plasminogen-binding protein from *Streptococcus pyogenes*. *Journal of Biological Chemistry* 268: 25417–25424.

Bergmann, S, Schoenen, H, Hammerschmidt, S (2013) The interaction between bacterial enolase and plasminogen promotes adherence of *Streptococcus pneumoniae* to epithelial and endothelial cells. *International Journal of Medical Microbiology* 303: 452–462.

Brissette CA, Gaultney RA (2014) That's my story, and I'm sticking to it – an update on *B. burgdorferi* adhesins. *Frontiers in Cell and Infection Microbiology* 4: 41.

Brissette CA, Haupt K, Barthel D, Cooley AE, Bowman A, Skerka C, Wallich R, Zipfel PF, Kraiczy P, Stevenson B (2009) The *B. burgdorferi* infection-associated surface proteins ErpP, ErpA, and ErpC bind human plasminogen. *Infection and Immunity* 77: 300–306.

Campbell GL, Fritz CL, Fish D, Nowakowski J, Nadelman RB, Wormser GP (1998) Estimation of the incidence of Lyme disease. *American Journal of Epidemiology* 148: 1018–1026.

Capello M, Ferri-Borgogno S, Cappello P, Novelli F (2011) alpha-Enolase: a promising therapeutic and diagnostic tumor target. *FEBS Journal* 278: 1064–1074.

Casjens S, Palmer N, Van Vugt R, Huang WM, Stevenson B, Rosa P, Lathigra R, Sutton G, Peterson J, Dodson RJ, Haft D, Hickey E, Gwinn M, White O, Fraser C (2000) A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs of an infectious isolate of the Lyme disease spirochete *B. burgdorferi*. *Molecular Microbiology* 35: 490–516.

CDC (2011) Summary of notifiable diseases – United States, 2009. *Morbidity and Mortality Weekly Report* 58: 1–100.

CDC (2013*a*) Revised Lyme disease estimate. Available at http://www.cdc.gov/media/releases/2013/p0819-lyme-disease.html (accessed September 2016).

CDC (2013*b*) Three sudden cardiac deaths associated with Lyme carditis – United States, November 2012–July 2013. *MMWR Morbidity and Mortality Weekly Report* 62: 993–996.

Ceremuga I, Seweryn E, Bednarz-Misa I, Pietkiewicz J, Jermakow K, Banas T, Gamian A (2014) Enolase-like protein present on the outer membrane of *Pseudomonas aeruginosa* binds plasminogen. *Folia Microbiologica (Praha)* 59: 391–397.

Coleman JL, Benach JL (2003) The urokinase receptor can be induced by *B. burgdorferi* through receptors of the innate immune system. *Infection and Immunity* 71: 5556–5564.

Coleman JL, Sellati TJ, Testa JE, Kew RR, Furie MB, Benach JL (1995) *B. burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infection and Immunity* 63: 2478–2484.

Coleman JL, Gebbia JA, Piesman J, Degen JL, Bugge TH, Benach JL (1997) Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell* 89: 1111–1119.

Coleman JL, Roemer EJ, Benach JL (1999) Plasmin-coated *B. burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix. *Infection and Immunity* 67: 3929–3936.

Coleman JL, Gebbia JA, Benach JL (2001) *B. burgdorferi* and other bacterial products induce expression and release of the urokinase receptor (CD87). *Journal of Immunology* 166: 473–480.

Coleman JL, Crowley JT, Toledo AM, Benach JL (2013) The HtrA protease of *B. burgdorferi* degrades outer membrane protein BmpD and chemotaxis phosphatase CheX. *Molecular Microbiology* 88: 619–633.

Comstock LE, Thomas DD (1989) Penetration of endothelial cell monolayers by *B. burgdorferi*. *Infection and Immunity* 57: 1626–1628.

Denny PW, Gokool S, Russell, DG, Field MC, Smith DF (2000) Acylation-dependent protein export in *Leishmania*. *Journal of Biological Chemistry* 275: 11017–11025.

Dunham-Ems SM, Caimano MJ, Pal U, Wolgemuth CW, Eggers CH, Balic A, Radolf JD (2009) Live imaging reveals a biphasic mode of dissemination of *B. burgdorferi* within ticks. *Journal of Clinical Investigations* 119: 3652–3665.

Ellis TN, Kuehn MJ (2010) Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiology and Molecular Biology Reviews* 74: 81–94.

Floden AM, Watt JA, Brissette CA (2011) *B. burgdorferi* enolase is a surface-exposed plasminogen binding protein. *PLoS One*, 6: e27502.

Floden AM, Gonzalez T, Gaultney RA, Brissette CA (2013) Evaluation of RevA, a fibronectin-binding protein of *B. burgdorferi*, as a potential vaccine candidate for Lyme disease. *Clinical Vaccine Immunology* 20: 892–899.

Fontan PA, Pancholi V, Nociari MM, Fischetti VA (2000) Antibodies to streptococcal surface enolase react with human alpha-enolase: implications in poststreptococcal sequelae. *Journal of Infectious Diseases* 182: 1712–1721.

Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb J-F, Fleischmann RD, Richardson D, Peterson J, Kerlavage AR, Quackenbush J, Salzberg S, Hanson M, Van Vugt R, Palmer, N, Adams, M. D, Gocayne, J, Weidmann, J, Utterback, T, Watthey, L, Mcdonald, L, Artiach P, Bowman C, Garland S, Fujii C, Cotton MD, Horst K, Roberts K, Hatch B, Smith HO, Venter JC (1997) Genomic sequence of a Lyme disease spirochaete, *B. burgdorferi*. *Nature* 390: 580–586.

Fuchs H, Wallich R, Simon MM, Kramer MD (1994) The outer surface protein A of the spirochete *B. burgdorferi* is a plasmin(ogen) receptor. *Proceedings of the National Academy of Science, USA* 91: 12594–12598.

Fuchs H, Simon MM, Wallich R, Bechtel M, Kramer MD (1996) *B. burgdorferi* induces secretion of pro-urokinase-type plasminogen activator by human monocytes. *Infection and Immunity* 64: 4307–4312.

Fulde M, Steinert M, Bergmann S (2013) Interaction of streptococcal plasminogen binding proteins with the host fibrinolytic system. *Frontiers in Cell and Infection Microbiology* 3: 85.

Gebbia JA, Coleman JL, Benach JL (2001) *Borrelia* spirochetes upregulate release and activation of matrix metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells. *Infection and Immunity* 69: 456–462.

Girschick HJ, Huppertz HI, Russmann H, Krenn V, Karch H (1996) Intracellular persistence of *B. burgdorferi* in human synovial cells. *Rheumatology International* 16: 125–132.

Gomes-Solecki, M (2014) Blocking pathogen transmission at the source: reservoir targeted OspA-Based Vaccines Against *B. Burgdorferi*. *Frontiers in Cell and Infection Microbiology* 4: 136.

Guiral S, Mitchell TJ, Martin B, Claverys JP (2005) Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements. *Proceedings of the National Academy of Science, USA* 102: 8710–8715.

Hafner A, Obermajer N, Kos J (2010) Gamma-1-syntrophin mediates trafficking of gamma-enolase towards the plasma membrane and enhances its neurotrophic activity. *Neuro-Signals* 18: 246–258.

Haile WB, Coleman JL, Benach JL (2006) Reciprocal upregulation of urokinase plasminogen activator and its inhibitor, PAI-2, by *B. burgdorferi* affects bacterial penetration and host-inflammatory response. *Cell Microbiology* 8: 1349–1360.

Hallstrom T, Haupt K, Kraiczy P, Hortschansky P, Wallich R, Skerka C, Zipfel PF (2010) Complement regulator-acquiring surface protein 1 of *B. burgdorferi* binds to human bone morphogenic protein 2, several extracellular matrix proteins, and plasminogen. *Journal of Infectious Diseases* 202: 490–498.

Henderson B, Martin A (2011) Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity* 79: 3476–3491.

Herrmann C, Gern L (2015) Search for blood or water is influenced by *B. burgdorferi* in *Ixodes ricinus*. *Parasites and Vectors* 8: 6.

Hersh MH, Ladeau SL, Previtali MA, Ostfeld RS (2014) When is a parasite not a parasite? Effects of larval tick burdens on white-footed mouse survivaL *Ecology* 95: 1360–1369.

Hinckley AF, Connally NP, Meek JI, Johnson BJ, Kemperman MM, Feldman KA, White Jl, Mead PS (2014) Lyme disease testing by large commercial laboratories in the United States. *Clinical Infectious Diseases* 59: 676–681.

Hovius JW, Bijlsma MF, Van Der Windt GJ, Wiersinga WJ, Boukens BJ, Coumou J, Oei A, De Beer R, De Vos AF, Van 'T Veer C, Van Dam AP, Wang P, Fikrig E, Levi MM, Roelofs JJ, Van Der Poll T (2009) The urokinase receptor (uPAR) facilitates clearance of *B. burgdorferi*. *PLoS Pathog* 5: e1000447.

Hu LT, Perides G, Noring R, Klempner MS (1995) Binding of human plasminogen to *B. burgdorferi*. *Infection and Immunity* 63: 3491–3496.

Hu LT, Pratt SD, Perides G, Katz L, Rogers RA, Klempner MS (1997) Isolation, cloning and expression of a 70-kilodalton plasminogen binding protein of *B. burgdorferi*. *Infection and Immunity* 65: 4989–4995.

Jedrzejas MJ (2001) Pneumococcal virulence factors: structure and function. *Microbiology and Molecular Biology Reviews* 65: 187–207

Kariu T, Yang X, Marks CB, Zhang X, Pal U (2013) Proteolysis of BB0323 results in two polypeptides that impact physiologic and infectious phenotypes in *B. burgdorferi*. *Molecular Microbiology* 88: 510–522.

Klempner MS, Noring R, Rogers RA (1993) Invasion of human skin fibroblasts by the Lyme disease spirochete, *B. burgdorferi*. *Journal of Infectious Diseases* 167: 1074–1081.

Klempner MS, Noring R, Epstein MP, McCloud B, Hu R, Limentani SA, Rogers RA (1995) Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, *B. burgdorferi*. *Journal of Infectious Diseases* 171: 1258–1265.

Kraiczy P, Stevenson B (2013) Complement regulator-acquiring surface proteins of *B. burgdorferi*: Structure, function and regulation of gene expression. *Ticks and Tick-Borne Diseases* 4: 26–34.

Lagal V, Portnoi D, Faure G, Postic D, Baranton G (2006) *B. burgdorferi* sensu stricto invasiveness is correlated with OspC-plasminogen affinity. *Microbes and Infection* 8: 645–652.

Lähteenmaki K, Kuusela P, Korhonen TK (2001) Bacterial plasminogen activators and receptors. *FEMS Microbiology Reviews* 25: 531–552.

Lähteenmaki K, Edelman S, Korhonen TK (2005) Bacterial metastasis: the host plasminogen system in bacterial invasion. *Trends in Microbiology* 13: 79–85.

LaRocca TJ, Crowley JT, Cusack BJ, Pathak P, Benach J, London E, Garcia-Monco JC, Benach JL (2010) Cholesterol lipids of *B. burgdorferi* form lipid rafts and are required for the bactericidal activity of a complement-independent antibody. *Cell Host and Microbe* 8: 331–342.

Lin T, Gao L, Zhang C, Odeh E, Jacobs MB, Coutte L, Chaconas G, Philipp MT, Norris SJ (2012) Analysis of an ordered, comprehensive STM mutant library in infectious *B. burgdorferi:* insights into the genes required for mouse infectivity. *PLoS One* 7: e47532.

Livengood JA, Gilmore RD (2006) Invasion of human neuronal and glial cells by an infectious strain of *B. burgdorferi*. *Microbes and Infection* 8: 2832–2840.

Lopez-Villar E, Monteoliva L, Larsen MR, Sachon E, Shabaz M, Pardo M, Pla J, Gil C, Roepstorff P, Nombela C (2006) Genetic and proteomic evidences support the localization of yeast enolase in the cell surface. *Proteomics* 6 Suppl 1: S107–118.

Ma Y, Sturrock A, Weis JJ (1991) Intracellular localization of *B. burgdorferi* within human endothelial cells. *Infection and Immunity* 59: 671–678.

Nogueira SV, Smith AA, Qin JH, Pal U (2012) A surface enolase participates in *B. burgdorferi*-plasminogen interaction and contributes to pathogen survival within feeding ticks. *Infection and Immunity* 80: 82–90.

Nowalk AJ, Gilmore RD, Carroll JA (2006) Serologic proteome analysis of *B. burgdorferi* membrane-associated proteins. *Infection and Immunity* 74: 3864–3873.

Ogden NH, Lindsay LR, Morshed M, Sockett PN, Artsob H (2008) The rising challenge of Lyme borreliosis in Canada. *Canada Communicable Disease Report* 34: 1–19.

Ogden NH, Lindsay LR, Morshed M, Sockett PN, Artsob (2009) The emergence of Lyme disease in Canada. *Canadian Medical Association Journal* 180: 1221–1224.

Ogden NH, Bouchard C, Kurtenbach K, Margos G, Lindsay LR, Trudel L, Nguon S, Milord F (2010) Active and passive surveillance and phylogenetic analysis of *B. burgdorferi* elucidate the process of Lyme disease risk emergence in Canada. *Environmental Health Perspectives* 118: 909–914.

Önder O, Humphrey PT, McOmber B, Korobova F, Francella N, Greenbaum DC, Brisson D (2012) OspC is potent plasminogen receptor on surface of *B. burgdorferi*. *Journal of Biological Chemistry* 287: 16860–16868.

Pancholi V (2001) Multifunctional alpha-enolase: its role in diseases. *Cellular and Molecular Life Sciences* 58: 902–920.

Posey JE, Gherardini FC (2000) Lack of a role for iron in the Lyme disease pathogen. *Science* 288: 1651–1653.

Radolf JD, Goldberg MS, Bourell K, Baker SI, Jones JD, Norgard MV (1995) Characterization of outer membranes isolated from *B. burgdorferi*, the Lyme disease spirochete. *Infection and Immunity* 63: 2154–2163.

Radolf JD, Caimano MJ, Stevenson B, Hu, LT (2012) Of ticks, mice and men: understanding the dual-host lifestyle of Lyme disease spirochaetes. *Nature Reviews Microbiology* 10: 87–99.

Rand PW, Lacombe EH, Dearborn R, Cahill B, Elias S, Lubelczyk CB, Beckett GA, Smith RP (2007) Passive surveillance in Maine, an area emergent for tick-borne diseases. *Journal of Medical Entomology* 44: 1118–1129.

Rooijakkers SH, Van Wamel WJ, Ruyken M, Van Kessel KP, Van Strijp JA (2005) Anti-opsonic properties of staphylokinase. *Microbes and Infection* 7: 476–484.

Russart NM, Dougherty MW, Vaughan JA (2014) Survey of ticks (Acari: Ixodidae) and tick-borne pathogens in North Dakota. *Journal of Medical Entomology* 51: 1087–1090.

Russell TM, Johnson BJ (2013) Lyme disease spirochaetes possess an aggrecan-binding protease with aggrecanase activity. *Molecular Microbiology* 90: 228–240.

Russell TM, Delorey MJ, Johnson BJ (2013) *B. burgdorferi* BbHtrA degrades host ECM proteins and stimulates release of inflammatory cytokines in vitro. *Molecular Microbiology* 90: 241–251.

Sanderson-Smith Ml, De Oliveira DM, Ranson M, McArthur JD (2012) Bacterial plasminogen receptors: mediators of a multifaceted relationship. *Journal of Biomedicine and Biotechnology* 2012: 272148.

Seling A, Siegel C, Fingerle V, Jutras BL, Brissette CA, Skerka C, Wallich R, Zipfel PF, Stevenson B, Kraiczy P (2010) Functional characterization of *Borrelia spielmanii* outer surface proteins that interact with distinct members of the human factor H protein family and with plasminogen. *Infection and Immunity* 78: 39–48.

Sha J, Erova TE, Alyea RA, Wang S, Olano JP, Pancholi V, Chopra AK (2009) Surface-expressed enolase contributes to the pathogenesis of clinical isolate SSU of *Aeromonas hydrophila*. *Journal of Bacteriology* 191: 3095–3107.

Shoberg RJ, Thomas DD (1993) Specifc adherence of *B. burgdorferi* extracellular vesicles to human endothelial cells in culture. *Infection and Immunity* 61: 3892–3900.

Simon JA, Marrotte RR, Desrosiers N, Fiset J, Gaitan J, Gonzalez A, Koffi JK, Lapointe FJ, Leighton PA, Lindsay LR, Logan T, Milord F, Ogden NH, Rogic A, Roy-Dufresne E, Suter D, Tessier N, Millien V (2014) Climate change and habitat fragmentation drive the occurrence of *B. burgdorferi*, the agent of Lyme disease, at the northeastern limit of its distribution. *Evolutionary Applications* 7: 750–764.

Smith DR, Chapman MR (2010) Economical evolution: microbes reduce the synthetic cost of extracellular proteins. *mBio* 1: 1–9.

Stanek G, Strle F (2003) Lyme borreliosis. *Lancet* 362: 1639–1647.

Stanek G, Wormser GP, Gray J, Strle F (2012) Lyme borreliosis. *Lancet* 379: 461–473.

Steere AC (2001) Lyme disease. *New England Journal of Medicine* 345: 15–125.

Stone BL, Russart NM, Gaultney RA, Floden AM, Vaughan JA, Brissette CA (2015) The western progression of Lyme disease: Infectious and nonclonal *B. burgdorferi* sensu lato populations in Grand Forks County, North Dakota. *Applied and Environmental Microbiology* 81: 48–58.

Syrovets T, Lunov O, Simmet T (2012) Plasmin as a proinflammatory cell activator. *J Leukocyte Biology* 92: 509–519.

Tokarz R, Anderton JM, Katona LI, Benach JL (2004) Combined effects of blood and temperature shift on *B. burgdorferi* gene expression as determined by whole genome array. *Infection and Immunity* 72: 5419–5432.

Toledo A, Coleman JL, Kuhlow CJ, Crowley JT, Benach JL (2012) The enolase of *B. burgdorferi* is a plasminogen receptor released in outer membrane vesicles. *Infection and Immunity* 80: 359–368.

Tsao JL, Wootton JT, Bunikis J, Luna MG, Fish D, Barbour AG (2004) An ecological approach to preventing human infection: vaccinating wild mouse reservoirs intervenes in the Lyme disease cycle. *Proceedings of the National Academy of Science, USA* 101: 18159–18164.

Ullberg M, Kronvall G, Karlsson I, Wiman B (1990) Receptors for human plasminogen on gram-negative bacteria. *Infection and Immunity* 58: 21–25.

Veiga-Malta I, Duarte M, Dinis M, Tavares D, Videira A, Ferreira P (2004) Enolase from *Streptococcus sobrinus* is an immunosuppressive protein. *Cell Microbiology* 6: 79–88.

Voordouw MJ, Tupper H, Önder O, Devevey G, Graves CJ, Kemps BD, Brisson D (2013) Reductions in human Lyme disease risk due to the effects of oral vaccination on tick-to-mouse and mouse-to-tick transmission. *Vector-Borne and Zoonotic Diseases* 13: 203–214.

Walker MJ, McArthur JD, McKay F, Ranson M (2005) Is plasminogen deployed as a *Streptococcus pyogenes* virulence factor? *Trends in Microbiology* 13: 308–313.

Whitmire WM, Garon CF (1993) Specific and nonspecific responses of murine B cells to membrane blebs of *B. burgdorferi*. *Infection and Immunity* 61: 1460–1467.

Witkowska D, Pietkiewicz J, Szostko B, Danielewicz R, Maslowski L, Gamian A (2005) Antibodies against human muscle enolase recognize a 45-kDa bacterial

cell wall outer membrane enolase-like protein. *FEMS Immunology and Medical Microbiology* 45: 53–62.

Wu J, Weening, E. H, Faske, JB, Hook, M, Skare, JT 2011. Invasion of eukaryotic cells by *B. burgdorferi* requires beta(1) integrins and Src kinase activity. *Infection and Immunity* 79: 1338–1348.

Yang CK, Zhang XZ, Lu CD, Tai PC (2014) An internal hydrophobic helical domain of *Bacillus subtilis* enolase is essential but not sufficient as a non-cleavable signal for its secretion. *Biochemical and Biophysical Research Communications* 446: 901–905.

Zhou W, Capello M, Fredolini C, Piemonti L, Liotta LA, Novelli F, Petricoin EF (2010) Mass spectrometry analysis of the post-translational modifications of alpha-enolase from pancreatic ductal adenocarcinoma cells. *Journal of Proteome Research* 9(6): 2929–2936.

Zückert WR (2013) A call to order at the spirochaetal host-pathogen interface. *Molecular Microbiology* 89: 207–211.

Zückert WR (2014) Secretion of bacterial lipoproteins: through the cytoplasmic membrane, the periplasm and beyond. *Biochimica et Biophysica Acta* 1843: 1509–1516.

3.5

Other Glycolytic Enzymes Acting as Virulence Factors

16

Triosephosphate Isomerase from *Staphylococcus aureus* and Plasminogen Receptors on Microbial Pathogens

Reiko Ikeda and Tomoe Ichikawa

Department of Microbial Science and Host Defense, Meiji Pharmaceutical University, Tokyo, Japan

16.1 Introduction

Microbes interact in animals and in the environment through multiple mechanisms, either exclusively or synergistically. To investigate the pathogenic mechanisms of bacteria and fungi, that is, establishment, onset, progression, and persistence of infection, we first focused on the interactions that occur between the microbes. We observed adherence between the cells of *C. neoformans*, a pathogenic fungus, and the bacterium *S. aureus*. Moreover, the death of *C. neoformans* was detected following adherence to the bacterium. During an ongoing investigation of these mechanisms, triosephosphate isomerase, a glycolytic enzyme, was found to play a role in bacterium surface adherence, a role that differed from glycolysis (Saito and Ikeda 2005; Ikeda *et al*. 2007; Ikeda and Sawamura 2008; Furuya and Ikeda 2009; Yamaguchi *et al*. 2010; Ikeda 2011). Additionally, focusing on the interaction between molecules expressed on the bacterial surface and biomolecules in the host, triosephosphate isomerase exhibited an additional function of activating plasminogen (Furuya and Ikeda 2011). Finally, during a search of plasminogen-binding molecules on the opportunistic yeast pathogen *Trichosporon asahii*, heparinase was found to bind and activate plasminogen, suggesting multiple roles of heparinase in addition to its hydrolysis of heparin (Ikeda *et al*. 2014). This chapter describes moonlighting proteins expressed on microbes discovered during the original finding of adherence between *C. neoformans* and *S. aureus*.

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

16.2 Identification of Triosephosphate Isomerase on *S. aureus* as a Molecule that Binds to the Pathogenic Yeast *C. neoformans*

C. neoformans is an encapsulated yeast. Exposure occurs mainly by inhalation and can result in pulmonary cryptococcosis and severe meningitis. The infection is commonly observed in immunosuppressed individuals, including acquired immunodeficiency syndrome patients (Kwon-Chung *et al.* 2011). Nasal colonization of this fungus has been observed experimentally in mouse models (Anderson and Sagha 1988). However, the yeast pathogen may be excluded from the upper respiratory tract of most immunocompetent hosts. Competition among microbes occurs not only in the human body, but also in the environment. To investigate pathogen invasion and colonization, studies therefore require the presence of mucosal saprophytes, including pure cultures. Because *S. aureus* and *S. epidermidis* have been found in the nasal cavities of healthy humans (Lina *et al.* 2003), we attempted to co-cultivate *S. aureus* and *C. neoformans*.

16.2.1 Co-Cultivation of *S. aureus* and *C. neoformans*

When *S. aureus* was co-cultured with *C. neoformans*, the number of live yeast cells decreased in a time-dependent manner. Microscopic examination revealed that *S. aureus* was attached to the surface of the yeast cells, as if the bacterial cells had encircled the yeast cells. Scanning electron microscopy clearly demonstrated the attachment. Attachment was required to induce death of *C. neoformans*, because *C. neoformans* co-cultures separated by a membrane (0.45 μm) grew well. Furthermore, a capsule-deficient mutant of *C. neoformans* cells had difficulty attaching to *S. aureus*. However, when *Candida albicans*, an opportunistic yeast pathogen, was cultured with *S. aureus*, no attachment was observed. It has therefore been postulated that *S. aureus* acts as a barrier against *C. neoformans* entry into the host (Saito and Ikeda 2005; Ikeda *et al.* 2007).

16.2.2 Identification of Adhesins on *S. aureus* and *C. neoformans*

The surface molecules responsible for the adherence of *S. aureus* and *C. neoformans* have been investigated. Experiments using proteases or sodium metaperiodate suggested that carbohydrates and proteins contribute to the adherence of *C. neoformans* and *S. aureus*, respectively. *C. neoformans* is characterized by a capsule mainly comprising the acidic heteropolysaccharide glucuronoxylomannan (GXM). GXM contains a backbone of α-1,3-linked mannan substituted by β-1,2-linked xylose and glucuronic acid residues (Bhattacharjee *et al.* 1984). Experiments were performed using GXM with partially oxidated side chains, α-1,3-linked mannan, and α-1,3-linked mannooligosaccharide of various sizes. The adherence of *S. aureus* to *C. neoformans* and *C. neoformans* mortality were decreased by the addition of oxidized GXM or mannan. Surface plasmon resonance (SPR) analyses indicated that proteins extracted from *S. aureus* bound to GXM. Furthermore, this interaction was inhibited in a dose-dependent manner by mannooligosaccharides larger in size than trisaccharides. This suggested the presence of a lectin protein that recognized mannotriose on the surface of *S. aureus*.

Far-Western blotting was utilized to identify surface proteins of *S. aureus* that facilitated binding, and these studies indicated a role for triosephosphate isomerase, a glycolytic enzyme. The adherence to *C. neoformans* was decreased by triosephosphate isomerase point mutants of *S. aureus*, and binding was subsequently restored by complementation of the gene (Ikeda *et al*. 2007). Furthermore, the binding of GXM to *S. aureus* was visualized by scanning immunoelectron microscopy using 20 nm gold particles (Yamaguchi *et al*. 2010).

We extracted and purified the triosephosphate isomerase protein from *S. aureus* during log-phase growth. SPR analysis using immobilized triosephosphate isomerase showed a reaction with GXM in a dose-dependent manner. When the interactions between staphylococcal triosephosphate isomerase and α-(1 → 3)-mannooligosaccharides derived from GXM were examined, the oligosaccharides exhibited binding with triosephosphate isomerase. Differences in the slopes of the sensorgrams were observed between oligosaccharides with an even versus odd number of residues. A heterogeneous ligand-parallel reaction model revealed the existence of at least two binding sites on triosephosphate isomerase protein. Moreover, it was determined that triosephosphate isomerase had multiple mannotriose binding sites that exhibited different affinities. Docking simulation also suggested that mannotriose could bind to several positions on triosephosphate isomerase, including the substrate binding site. In fact, the enzymatic activity of triosephosphate isomerase was inhibited in a dose-dependent manner by α-(1 → 3)-mannooligosaccharides that were larger than triose (Furuya and Ikeda 2009).

16.2.3 Mechanisms of *C. neoformans* Cell Death

The important question of why *C. neoformans* was killed following adherence to *S. aureus* remains. This mechanism would be helpful in the regulation and development of drugs for cryptococcal infections. We therefore examined: (1) actin dynamics using rhodamine-phalloidin staining; (2) accumulation of reactive oxygen species (ROS) via conversion of 2'2'-dichlorofluorescein diacetate to 2'2'-dichlorofluorescein; and (3) DNA fragmentation via terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). When co-cultured with *S. aureus*, actin exhibited a clumped appearance and the percentage of ROS-positive and TUNEL-positive cells increased. The death of *C. neoformans* was therefore accompanied by a decrease in actin turnover, enhanced ROS accumulation, and DNA fragmentation, indicative of apoptosis-like cell death. Furthermore, increased expression of the voltage-dependent anion channel (VDAC), located in the mitochondrial outer membrane, has been suggested (Ikeda and Sawamura 2008).

Moreover, the potential role of small guanosine triphosphate (GTP)-binding proteins of the Rho subfamily that regulate the actin cytoskeleton was explored. *C. neoformans* was cultured with *S. aureus* in the presence of N-(4-pyridyl)-4-(1-aminoethyl)cyclohexanecarboxamide (Y-27632), an inhibitor of Rho-associated coiled-coil forming kinase (ROCK), a downstream effector of Rho. It was determined that the inhibitor significantly reduced *C. neoformans* death. Concomitantly, Y-27632 prevented the aggregation of actin. We therefore

concluded that the Rho/ROCK pathway is involved in cell death induced by adherence stress. Ruthenium red (RuR), which binds to VDAC and inhibits cytochrome c release, was used to evaluate VDAC involvement in the response to adherence stress caused by *S. aureus*. RuR treatment decreased the mortality of *C. neoformans* co-cultured with *S. aureus*. From these data, Rho-ROCK signaling could be involved, via a mitochondrial pathway, in the apoptosis-like death of *C. neoformans* induced by the adherence of *S. aureus* (Ikeda 2011).

16.3 Binding of Triosephosphate Isomerase with Human Plasminogen

Previous experiments have identified triosephosphate isomerase as an adhesion molecule on *S. aureus* involved in the cell–cell communication with *C. neoformans*. This led us to examine whether triosephosphate isomerase could interact with other eukaryotic molecules. Human biological molecules, including fibronectin, fibrinogen, and thrombin, were evaluated to investigate the role of *S. aureus* triosephosphate isomerase in infection. SPR analyses and far-Western blotting showed that triosephosphate isomerase bound to human plasminogen. Kinetic analysis determined two equilibrium constants, K_{D1} (3.18×10^{-10}) and K_{D2} (3.12×10^{-7}), from the heterogeneous ligand-parallel reaction model. Furthermore, the interaction was inhibited by epsilon aminocaproic acid used as a lysine analog, suggesting that lysine residues contribute to the interaction (Furuya and Ikeda 2011).

Glycolytic enzymes act in the cytoplasm of microbes; however, accumulating evidence suggests that some glycolytic enzymes, such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and enolase, are present on the cell surface where they may perform multiple functions (Pancholi and Chhatwal 2003; Bergmann and Hammerschmidt 2006; Oliveira *et al.* 2012; Mori *et al.* 2012). Several of these glycolytic enzymes interact with proteins in the plasma. For instance, GAPDH of *Streptococcus pneumoniae*, *Streptococcus pyogenes*, and *Streptococcus equisimilis* is a plasminogen-binding protein (Gase *et al.* 1996; Seifert *et al.* 2003; Bergmann *et al.* 2004; Floden *et al.* 2011). GAPDH on the cell wall of *Candida albicans*, an opportunistic yeast pathogen, is able to adhere to fibronectin and laminin (Gozalbo *et al.* 1998).

Triosephosphate isomerase was suggested to be a candidate surface molecule on *S. aureus* that affects plasminogen activation, in addition to enolase and GAPDH. Interestingly, triosephosphate isomerase, enolase, and GAPDH genes are located in the same operon, and could therefore collaborate in invasion, colonization, and dissemination of the pathogen.

16.4 Plasminogen-Binding Proteins on *Trichosporon asahii*

Several bacterial molecules have been shown to bind to plasminogen and affect its activation. Additionally, many of these molecules have roles above and beyond binding to plasminogen; they are therefore considered moonlighting proteins.

Plasminogen receptors on fungal pathogens have been described concerning their role in tissue invasion. Proteins from *Candida albicans*, including enzymes utilized in glycolysis, have been identified as plasminogen-binding proteins (Crowe *et al*. 2003; Polterman *et al*. 2007; Luo *et al*. 2013).

The major focus of our research has been pathogenic basidiomycetous yeasts, mainly *Cryptococcus* and *Trichospron*. Our goal was therefore to ascertain the virulence factors of these species that serve to disseminate, invade, and colonize by utilizing host biomolecules as well as *Candida albicans*, belonging to ascomycetes.

Trichosporon asahii is a yeast-like fungus distributed throughout the environment (Sugita *et al*. 2001; Sugita 2011) and is the major causative agent of deep-seated trichosporonosis, an opportunistic fungal infection with a poor prognosis and high mortality rate in immunocompromised patients and patients with hematological malignancies (Sugita *et al*. 1995, 1999; Erer *et al*. 2000). The virulence factors of *T. asahii* remain uncharacterized, although beta-*N*-acetylhexosaminidase has been suggested as a candidate (Ichikawa *et al*. 2004). To investigate the pathogenicity, we evaluated the interactions between surface molecules on *T. asahii* and host plasminogen using clinical isolates of *T. asahii* (three from blood and one from urine). Live *T. asahii* cells accelerated the conversion of plasminogen to plasmin in a dose-dependent manner in the presence of tissue plasminogen activator. Lithium chloride extracts containing proteins that bind and activate plasminogen were obtained. Based on far-Western blotting analyses using fractions separated by DEAE ion-exchange column chromatography a protein was identified as heparinase, and heparinase activity was detected in the *T. asahii* extract. Furthermore, affinity chromatography using plasminogen as a ligand detected one protein band by SDS-PAGE, which was identified as thioredoxin-dependent peroxide reductase. SPR analyses indicated the existence of molecules on *T. asahii* cells that could bind plasminogen with differing affinities (Ikeda *et al*. 2014).

In our study, heparinase was identified as a plasminogen-binding protein on the surface of *T. asahii* clinical isolates. Heparinase cleaves $\alpha 1 \rightarrow 4$ glycosidic linkages in heparin to oligosaccharides, and some microorganisms have been reported to produce this enzyme, including fungi (Tripathi *et al*. 2012). Heparin has been used as an anticoagulant agent (Liu and Pedersen, 2007) and also regulates cell growth through binding molecules. Heparinase activity may be implicated in angiogenesis, inflammation, and autoimmunity (Vlodavsky *et al*. 2011). The potential anti-cancer activity of low-molecular-weight heparin (LMWH) has been reported, which would be inhibited by heparinase (Tang *et al*. 2014). In tumors, the plasminogen activation system may affect cell growth (Andreasen *et al*. 2000). In our study, the activation of plasminogen to plasmin was promoted in the presence of *T. asahii* plasminogen-binding molecules. The host coagulation and fibrinolysis systems are available during fungal invasion and dissemination. Additional studies using commercially available heparinase were performed. SPR analyses indicated that heparinase II from *Bacteroides eggerthii* bound plasminogen in a dose-dependent manner, and that heparinase II activated the conversion of plasminogen to plasmin.

An adhesin found on the surface of the pathogenic fungus *Blastmyces dermatidis* was identified as a heparin-binding molecule and included the heparin-binding repeat found in tronbospondin-1 that has been shown to inhibit T-cell activation via CD47 (Brandhorst *et al*. 2013). The complex biological activity of heparin has been reported (Whitelock and Iozzo 2005), and the lung and liver are rich sources of heparin, a glycosamino glycan. In cell–cell signaling, a complex formed by heparin located on the cell surface interacting with cytokines or growth factors can bind to receptors on another cell and may induce signal transmission that results in inflammation or other reactions. Heparin plays various roles, preferable or adverse, in lung diseases (Papakonstantinou and Karakiulakis 2009). The heparin-binding proteins on microbial pathogens or the digestion of heparin could play an important role in infectious diseases.

We also detected thioredoxin-dependent peroxide reductase in a fraction prepared by plasminogen-affinity chromatography. We speculated that multiple molecules on the surface of *T. asahii* bind plasminogen with different affinities.

T. asahii may utilize human biomolecules such as plasminogen receptors for invasion. Although the role of these plasminogen-binding molecules in pathogen virulence remains obscure, the findings could identify a target gene to investigate the virulence factors of pathogenic fungi.

16.5 Plasminogen Receptors on *C. neoformans*

Plasminogen-binding proteins on *T. asahii* were suggested as described above. As for *C. neoformans*, a global analysis of plasminogen-binding proteins on the pathogen surface was performed and several candidate proteins were suggested, including heat-shock protein 70, glucose-6 phosphate isomerase, and pyruvate kinase (Stie *et al*. 2009). However, we also found that neutral polysaccharides containing mannose, galactose, glucose, and xylose were able to bind to human plasminogen and affect its activation. The polysaccharide was determined to be different from GXM, the main component of the capsule. A diverse range of biomolecules, including proteins and carbohydrates, can therefore function as plasminogen receptors and play a role in activation (Ikeda and Ichikawa 2014).

16.6 Conclusions

Microbes communicate in animals and in the environment symbiotically, parasitically, or exclusively. Descriptions of the human microbiome survey (Morgan *et al*. 2013) indicate the significance of microbe–microbe and host–microbe interactions in various diseases including diabetes, psychiatric disorders, and infectious diseases. Insulin action was affected by the gut microbial profile (Serino *et al*. 2013), and the participation of a gut–microbiome–brain connection in autism spectrum disorder was suggested (Hsiao *et al*. 2013; Sommer and Bäckhed 2013).

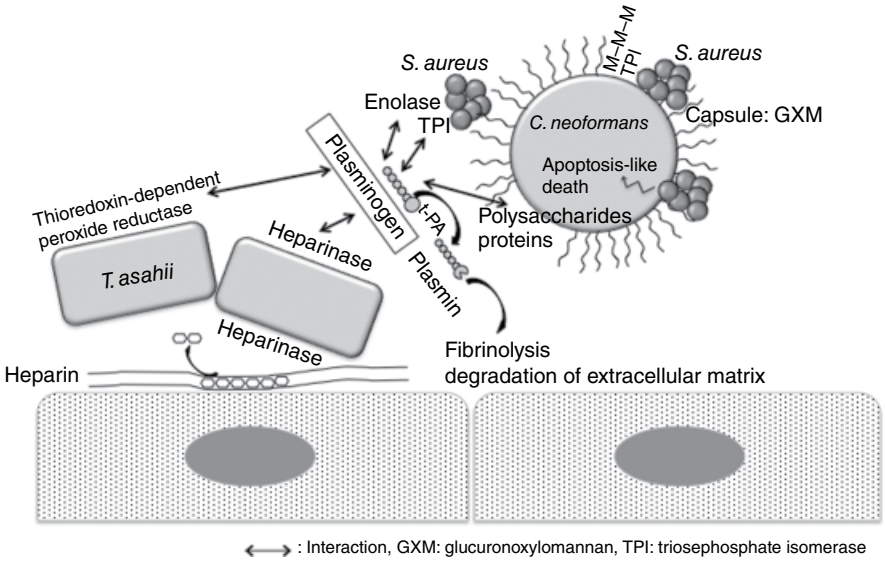

Figure 16.1 Protein–protein and protein–carbohydrate interactions between microbes and human hosts.

Focusing on the interactions between human pathogenic bacteria and fungi we observed an adherence of *S. aureus* and *C. neoformans*, and the resulting *C. neoformans* death was accompanied by the apoptosis-like characteristics. The molecules determined to contribute to the adherence of these species were triosephosphate isomerase and α-(1 → 3) mannooligosaccharides on *S. aureus* and *C. neoformans*, respectively. Furthermore, triosephosphate isomerase of *S. aureus* was able to bind to and activate human plasminogen. Staphylococcal triosephosphate isomerase may therefore be a multifunctional protein that has the ability to communicate with eukaryotes and to play a potential role in tissue adhesion and invasion during the process of infection.

Moreover, additional plasminogen receptors on the yeast pathogen *T. asahii* have been identified as heparinase and thioredoxin-dependent peroxide reductase. The involvement of moonlighting proteins may therefore have the immense potential to predict microbe and human host protein–protein and protein–carbohydrate interactions (Fig. 16.1; Fatoux-Ardore *et al*. 2014).

References

Anderson, D. A., Sagha H. M. (1988) Persistence of infection in mice inoculated intranasally with *Cryptococcus neoformans*. *Mycopathologia* 104: 163–169.

Andreasen P. A., Egelund R., Petersen H. H. (2000) The plasminogen activation system in tumor growth, invasion, and metastasis. *Cellular and Molecular Life Sciences* 57: 25–40.

Bergmann S., Hammerschmidt S. (2006) Versatility of pneumococcal surface proteins. *Microbiology* 152: 295–303.

Bergmann S., Rohde M., Hammerschmidt S. (2004) Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. *Infection and Immunity* 72: 2416–2419.

Bhattacharjee A. K., Bennett J. E., Glaudemans C. P. (1984) Capsular polysaccharides of *Cryptococcus neoformans*. *Reviews of Infectious Diseases* 6: 619–624.

Brandhorst T. T., Roy R., Wuthrich M., Nanjappa S., Filutowicz H., Galles K., Tonelli M., McCaslin D. R., Satyshur K., Klein B. (2013) Structure and function of a fungal adhesin that binds heparin and mimics thrombospondin-1 by blocking T cell activation and effector function. *PLoS Pathogens* 9: e1003464.

Crowe J. D., Sievwright I. K., Auld G. C., Moore N. R., Gow N. A., Booth N. A. (2003) *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. *Molecular Microbiology* 47: 1637–1651.

Erer B., Galimberti M., Lucarelli G., Giardini C., Polchi P., Baronciani D., Gaziev D., Angelucci E., Izzi G. (2000) *Trichosporon beigelii*: a life-threatening pathogen in immunocompromised hosts. *Bone Marrow Transplant* 25: 745–749.

Fatoux-Ardore M., Peysselon F., Weiss, A., Bastienc P., Pratlong F., Ricard-Blum S. (2014) Large-scale investigation of *Leishmania* interaction networks with the host extracellular 1 matrix by surface plasmon resonance imaging. *Infection and Immunity* 82: 594–606.

Floden A. M., Watt J. A., Brissette C. A. (2011) *Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein. *PLoS One* 6: e27502.

Furuya H., Ikeda R. (2009) Interaction of triosephosphate isomerase from the cell surface of *Staphylococcus aureus* and alpha-(1- > 3)-mannooligosaccharides derived from glucuronoxylomannan of *Cryptococcus neoformans*. *Microbiology* 155: 2707–2713.

Furuya H., Ikeda R. (2011) Interaction of triosephosphate isomerase from *Staphylococcus aureus* with plasminogen. *Microbiology and Immunology* 55: 855–862.

Gase K., Gase A., Schirmer H., Malke H. (1996) Cloning, sequencing and functional overexpression of the *Streptococcus equisimilis* H46A gapC gene encoding a glyceraldehyde-3-phosphate dehydrogenase that also functions as a plasmin(ogen)-binding protein. Purification and biochemical characterization of the protein. *European Journal of Biochemistry* 239: 42–51.

Gozalbo D., Gil-Navarro I., Azorin I., Renau-Piqueras J., Martinez J.P., Gil M.L. (1998) The cell wall-associated glyceraldehyde-3-phosphate dehydrogenase of *Candida albicans* is also a fibronectin and laminin binding protein. *Infection and Immunity* 66: 2052–2059.

Hsiao E. Y, McBride S. W, Hsien S., Sharon G., Hyde E. R., McCue T., Codelli J. A., Chow J., Reisman S. E., Petrosino J. F., Patterson P. H., Mazmanian S. K. (2013) Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* 155: 1451–1463.

Ichikawa T., Sugita T., Wang L., Yokoyama K., Nishimura K., Nishikawa A. (2004) Phenotypic switching and beta-N-acetylhexosaminidase activity of the pathogenic yeast *Trichosporon asahii*. *Microbiology and Immunology* 48: 237–242.

Ikeda R. (2011) Possible participation of the Rho/Rho-associated coiled-coil-forming kinase pathway in the cell death of *Cryptococcus neoformans* caused by *Staphylococcus aureus* adherence. *Microbiology and Immunology* 55: 552–557.

Ikeda R., Sawamura K. (2008) Bacterial and H_2O_2 stress-induced apoptosis-like events in *Cryptococcus neoformans*. *Research in Microbiology* 159: 628–634.

Ikeda R., Ichikawa T. (2014) Interaction of surface molecules on *Cryptococcus neoformans* with plasminogen. *FEMS Yeast Research* 14: 445–450.

Ikeda R., Saito F., Matsuo M., Kurokawa K., Sekimizu K., Yamaguchi M., Kawamoto S. (2007) Contribution of the mannan backbone of cryptococcal glucuronoxylomannan and a glycolytic enzyme of *Staphylococcus aureus* to contact-mediated killing of *Cryptococcus neoformans*. *Journal of Bacteriology* 189: 4815–4826.

Ikeda R., Ichikawa T., Miyazaki Y., Shimizu N., Ryoke T., Haru K., Sugita T., Takashima M. (2014) Detection and characterization of plasminogen receptors on clinical isolates of *Trichosporon asahii*. *FEMS Yeast Research* 14: 1186–1195.

Kwon-Chung K. J., Boekhout T., Wickes B. L., Fell J. W. (2011) Systematics of the Genus *Cryptococcus* and its type species *C. neoformans*. In *Cryptococcus: From Human Pathogen to Model Yeast* (eds Heitman J., Kozel T. R., Kwon-Chung K. J., Perfect J. R., Casadevall A), pp. 3–15. ASM Press, Washington DC.

Lina G., Boutite F., Tristan A., Bes M., Etienne J., Vandenesch F. (2003) Bacterial competition for human nasal cavity colonization: role of staphylococcal agr alleles. *Applied and Environmental Microbiology* 69: 18–23.

Liu J., Pedersen L. C. (2007) Anticoagulant heparan sulfate: structural specificity and biosynthesis. *Applied Microbiology and Biotechnology* 74: 263–272.

Luo S., Hoffmann R., Skerka C., Zipfel P. F. (2013) Glycerol-3-phosphate dehydrogenase 2 is a novel factor H-, factor H-like protein 1-, and plasminogen-binding surface protein of *Candida albicans*. *Journal of Infectious Diseases* 207: 594–603.

Morgan X. C., Segata N., Huttenhower C. (2013) Biodiversity and functional genomics in the human microbiome. *Trends in Genetics* 29: 51–58.

Mori Y., Yamaguchi M., Terao Y., Hamada S., Ooshima T., Kawabata S. (2012) α-Enolase of *Streptococcus pneumoniae* induces formation of neutrophil extracellular traps. *Journal of Biological Chemistry* 287: 10472–10481.

Oliveira L., Madureira P., Andrade E. B., Bouaboud A., Morello E., Ferreira P., Poyart C., Trieu-Cuot P., Dramsi S. (2012) Group B streptococcus GAPDH is released upon cell lysis, associates with bacterial surface, and induces apoptosis in murine macrophages. *PLoS One* 7: e29963.

Pancholi V., Chhatwal G. S. (2003) Housekeeping enzymes as virulence factors for pathogens. *International Journal of Medical Microbiology* 293: 391–401.

Papakonstantinou E., Karakiulakis G. (2009) The 'sweet' and 'bitter' involvement of glycosaminoglycans in lung diseases: pharmacotherapeutic relevance. *British Journal of Pharmacology* 157: 1111–1127.

Poltermann S., Kunert A., von der Heide M., Eck R., Hartmann A., Zipfel P. F. (2007) Gpm1p is a factor H-, FHL-1-, and plasminogen-binding surface protein of *Candida albicans*. *Journal of Biological Chemistry* 282: 37537–37544.

Saito F., Ikeda R. (2005) Killing of cryptococcus neoformans by *Staphylococcus aureus*: the role of cryptococcal capsular polysaccharide in the fungal-bacteria interaction. *Medical Mycology* 43: 603–612.

Seifert K. N., McArthur W. P., Bleiweis A. S., Brady L. J. (2003) Characterization of group B streptococcal glyceraldehyde-3-phosphate dehydrogenase: surface localization, enzymatic activity, and protein–protein interactions. *Canadian Journal of Microbiology* 49: 350–356.

Serino M., Fernández-Real J. M., García-Fuentes E., Queipo-Ortuño M., Moreno-Navarrete J. M., Sánchez A., Burcelin R., Tinahones F. (2013) The gut microbiota profile is associated with insulin action in humans. *Acta Diabetologica* 50: 753–761.

Sommer F, Bäckhed F. (2013) The gut microbiota-masters of host development and physiology. *Nature Reviews Microbiology* 11: 227–238.

Stie J., Bruni G., Fox D. (2009) Surface-associated plasminogen binding of *Cryptococcus neoformans* promotes extracellular matrix invasion. *PLoS One* 4: e5780.

Sugita T (2011) *Trichosporon* Behrend (1890). In *The Yeasts, A Taxonomic Study*, 5th edn, vol 3. (eds C. P Kurtzman, J. W. Fell, T. Boekhout), pp. 2015–2061. Elsevier: Amsterdam.

Sugita T., Ichikawa T., Matsukura M., Sueda M., Takashima M., Ikeda R., Sugita T., Nishikawa A., Shinoda T., Kume H. (1995) Taxonomic position of deep-seated, mucosa-associated, and superficial isolates of *Trichosporon cutaneum* from trichosporonosis patients. *Journal of Clinical Microbiology* 33: 1368–1370.

Sugita T., Nishikawa A., Ikeda R., Shinoda T. (1999) Identification of medically relevant *Trichosporon* species based on sequences of internal transcribed spacer regions and construction of a database for *Trichosporon* identification. *Journal of Clinical Microbiology* 37: 1985–1993.

Sugita T., Ichikawa T., Matsukura M., Sueda M., Takashima M., Ikeda R., Nishikawa A., Shinoda T. (2001) Genetic diversity and biochemical characteristics of *Trichosporon asahii* isolated from clinical specimens, houses of patients with summer-type-hypersensitivity pneumonitis, and environmental materials. *Journal of Clinical Microbiology* 39(7): 2405–2411.

Tang X. D., Guo S. L., Wang G. Z., Li N., Wu Y. Y., Fang D. C., Fan Y. H., Yang S. M. (2014) *In vitro* and *ex vivo* evaluation of a multi-epitope heparinase vaccine for various malignancies. *Cancer Science* 105: 9–17.

Tripathi C. K., Banga J., Mishra V. (2012) Microbial heparin/heparan sulphate lyases: potential and applications. *Applied Microbiology and Biotechnology* 94: 307–321.

Vlodavsky I., Elkin M., Ilan N. (2011) Impact of heparanase and the tumor microenvironment on cancer metastasis and angiogenesis: basic aspects and clinical applications. *Rambam Maimonides Medical Journal* 2: e0019.

Whitelock J. M., Iozzo R. V. (2005) Heparan sulfate: a complex polymer charged with biological activity. *Chemical Reviews* 105: 2745–2764.

Yamaguchi M., Ikeda R., Nishimura M., Kawamoto S. (2010) Localization by scanning immunoelectron microscopy of triosephosphate isomerase, the molecules responsible for contact-mediated killing of *Cryptococcus*, on the surface of *Staphylococcus*. *Microbiology and Immunology* 54: 368–370.

17

Moonlighting Functions of Bacterial Fructose 1,6-Bisphosphate Aldolases

Neil J. Oldfield, Fariza Shams, Karl G. Wooldridge, and David P.J. Turner

Molecular Bacteriology and Immunology Group, School of Life Sciences, Centre for Biomolecular Sciences, University of Nottingham, Nottingham, UK

17.1 Introduction

Moonlighting proteins are multifunctional proteins which perform two or more biochemical functions using a single polypeptide chain (Jeffery 1999, 2014; see Chapter 1). Moonlighting proteins have been documented in diverse organisms, including archaea (Jia *et al*. 2013), bacteria (Henderson and Martin 2011), yeasts (Gancedo and Flores 2008), eukaryotic parasites (Ginger 2014), plants (Moore 2004), and vertebrates (Kim and Dang 2005). Pathogenic bacterial species, such as *Mycobacterium tuberculosis*, *Streptococcus pyogenes*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, often employ multiple moonlighting proteins to enhance their pathogenic potential (Henderson 2014). Common examples are cytosolic enzymes or chaperones which moonlight as adhesion receptors, plasminogen-binding proteins, or immunomodulators on the bacterial cell surface (Wang *et al*. 2013). In particular, several enzymes of the Embden–Meyerhof–Parnas (EMP) glycolytic pathway, including fructose 1,6-bisphosphate aldolase (FBA), glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and enolase, have been shown to exhibit moonlighting functions on the bacterial surface (Henderson and Martin 2011).

17.2 Fructose 1,6-bisphosphate Aldolase in Metabolism

Fructose 1,6-bisphosphate aldolase (FBA) (EC 4.1.2.13) catalyzes the reversible cleavage of fructose 1,6-bisphosphate (FBP) into the triose phosphates glyceraldehyde 3-phosphate (GADP) and dihydroxyacetone phosphate (DHAP); the catabolic glycolysis pathway utilizes the forward reaction, while the anabolic gluconeogenesis and Calvin cycle pathways utilize the reverse aldol condensation reaction (Rutter 1964). FBAs can be split into two classes based on the

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

reaction mechanism employed (Perham 1990). Class I FBAs form a Schiff base intermediate between a highly conserved active site lysine and a substrate carbonyl group. Class I FBAs, which often form tetramers, are commonly found in higher animals and plants, but rarely in bacteria (Thomson *et al*. 1998; Sauvé and Sygusch 2001). In bacteria, class II FBAs are more common; these require an active site divalent metal ion, often zinc, for enzymatic activity (Hall *et al*. 1999; Zgiby *et al*. 2000, 2002). Only a few organisms, for example *Escherichia coli*, contain both classes of FBAs (Stribling and Perham 1973). Class II FBAs can be further divided into type A and type B based on their amino acid sequences (Plaumann *et al*. 1997); class IIA enzymes are generally dimeric, while class IIB enzymes may be dimeric, tetrameric, or octameric (Sauvé and Sygusch 2001; Nakahara *et al*. 2003). Both class I and class II aldolases catalyze the same enzymatic reaction and adopt a common folding topology (a triose phosphate isomerase or TIM barrel $(\alpha/\beta)_8$ barrel). However, they do not share significant amino acid sequence homology or conserved catalytic residues and the location of their active sites within the TIM barrel is distinct, suggesting that similarities of the tertiary structures of the two classes of enzyme are a result of convergent evolution (Marsh and Lebherz 1992; Blom *et al*. 1996; Nagano *et al*. 2002). Indeed, aldolases found in some archaeal species which belong to class I (based on their reaction mechanism) lack significant amino acid sequence homology with either class I or class II aldolases (Siebers *et al*. 2001). Bifunctional fructose 1,6-bisphosphate aldolase/phosphatase enzymes, which are a unique class of proteins with both FBP aldolase and FBP phosphatase activity, have also been found in archaea and some rare thermophilic and autotrophic bacteria (Say and Fuchs 2010).

17.3 Surface Localization of Streptococcal Fructose 1,6-bisphosphate Aldolases

Early reports describing the unexpected presence of FBA on the bacterial cell surface resulted from the separation of surface or secreted fractions by gel electrophoresis, and the subsequent identification of proteins by mass spectroscopy or amino-terminal amino acid sequencing. Using these approaches, FBA (often with other cytosolic proteins) was initially identified as a surface-associated or -secreted protein in several species of streptococci, including *S. pyogenes* (Lei *et al*. 2000), *S. oralis* (Wilkins *et al*. 2003), *S. pneumoniae* (Ling *et al*. 2004), *S. agalactiae* (Fluegge *et al*. 2004), and, more recently, *S. suis* (Wu *et al*. 2008).

S. pyogenes (or Group A streptococcus) causes human infections including pharyngitis, impetigo, necrotizing fasciitis, and streptococcal toxic shock syndrome (Walker *et al*. 2014). Lei *et al*. prepared concentrated culture supernatants from representative serotype M1 and M3 strains and identified 44 distinct proteins, which were present in both strains, by two-dimensional (2D) gel electrophoresis and amino-terminal amino acid sequencing (Lei *et al*. 2000). Eight of the identified proteins, including FBA, were glycolytic enzymes and several of these were present in high abundance in mid-log-phase culture supernatants, suggesting specific and active secretion rather than just passive release (Lei *et al*. 2000).

S. oralis is a member of the mitis group of oral streptococci. In addition to being a component of normal dental plaque, it is also associated with extra-oral diseases including endocarditis. Wilkins *et al*. identified 27 proteins, including FBA, from zwittergent-extracted surface preparations of *S. oralis* strain 176 N which had been separated using 2D gel electrophoresis. No recognizable secretion signals anchoring LPXTG- or choline-binding motifs could be identified in these proteins (Wilkins *et al*. 2003). In this study, FBA was found in multiple forms with different isoelectric points, possibly indicating the presence of post-translational modifications (Wilkins *et al*. 2003).

S. agalactiae (or Group B streptococcus) is a leading cause of sepsis and meningitis in neonates (Landwehr-Kenzel and Henneke 2014; see also Chapter 10). To identify proteins released from *S. agalactiae*, supernatants from stationary and logarithmic cultures were concentrated and separated by SDS-PAGE (Fluegge *et al*. 2004). Subsequent amino-terminal amino acid sequencing of proteins identified FBA among the secreted proteins, many of which were immunogenic as they were recognized in immunoblots by sera from infected neonates or healthy adults (Fluegge *et al*. 2004). Similarly in *S. suis* serotype 9, immunoblotting of extracted cell-wall proteins with immune sera identified FBA as one of eight cell-wall-associated immunogenic proteins (Wu *et al*. 2008).

17.4 Pneumococcal FBA Adhesin Binds Flamingo Cadherin Receptor

Although FBA has been found on the cell surface or in secreted fractions of several streptococcal species, it is only in *S. pneumoniae*, a leading cause of otitis media, pneumonia, bacteraemia, and meningitis, that a moonlighting function has been experimentally confirmed (Blau *et al*. 2007). Ling *et al*. initially identified FBA as an immunogenic, pneumococcal cell-wall-associated protein in a study aimed at discovering potential vaccine targets (Ling *et al*. 2004). Cell-wall proteins of *S. pneumoniae* serotype 3 strain WU2 were extracted by mutanolysin treatment and, following separation by 2D gel electrophoresis, were probed with pediatric and adult sera (Ling *et al*. 2004). Using mass spectrometry, 17 immunogenic proteins were identified; 13 of these, including FBA, were increasingly antigenic in children over time (Ling *et al*. 2004). Moreover, recombinant FBA (rFBA) was immunogenic in mice and use of the resulting sera confirmed the surface accessibility of pneumococcal FBA to antibodies using flow cytometry. Significantly, immunization of mice with rFBA conferred partial protection to subsequent respiratory challenge with strain WU2 or a genetically distinct virulent pneumococcal strain (Ling *et al*. 2004).

In a further study by the same research group, Blau *et al*. provided strong evidence that surface FBA acts as an adhesin in *S. pneumoniae* (Blau *et al*. 2007). Both rFBA and anti-rFBA antibody significantly reduced adhesion of strain WU2 and a non-capsulated derivative to cells of the human lung carcinoma cell line A549 (Blau *et al*. 2007). To identify a possible human receptor, a random peptide library was screened for rFBA-binding and a number of peptides corresponding

to Flamingo cadherin receptor 3 (FCR3), a human membrane protein of the cell adhesion molecules (CAM) family, were identified. TMHMM software predicted the rFBA binding site on FCR3 to be localized between amino acids 2891 and 2903, which resides in the extracellular region of FCR3. A synthetic peptide containing this predicted rFBA-binding region (FCRP) was shown to bind rFBA by far-Western immunoblot and anti-FCRP antibodies could detect a protein, presumed to be FCR3, on the surface of A549 cells. Importantly, FCRP significantly reduced adhesion of several *S. pneumoniae* strains to A549 cells in a dose-dependent manner; furthermore, colonization of the nasopharynx and lungs in a mouse model of pneumococcal infection was also inhibited by pretreatment with FCRP (Blau *et al*. 2007).

Humans and mice contain three FCR genes (*CELSR1-3* and *celsr1-3*, respectively), which are orthologs of *Drosophila flamingo*/starry night (*fmi/*stan) (Iwai *et al*. 1997; Chae *et al*. 1999; Usui *et al*. 1999). The FCRs, or cadherin epidermal growth factor (EGF) laminin G (LAG) seven-pass G-type receptors (CELSRs), are expressed in most host tissues (Nollet *et al*. 2000; Wang *et al*. 2014). Although the precise functions of the three FCRs are unclear, mutations in *CELSR1-3* are associated with several pathologies including neural tube defects, coronary heart disease, and some cancers (Wang *et al*. 2014). Amino acid sequences closely matching that of FCRP can be found in FCR1, 2 and 3; it therefore remains unclear which of the three receptors are specifically targeted by pneumococcal FBA.

17.5 FBA is Required for Optimal Meningococcal Adhesion to Human Cells

Neisseria meningitidis is a Gram-negative human nasopharyngeal commensal which can cause rapidly progressing septicemia and meningitis (Stephens 2009). In common with most obligate aerobes, *N. meningitidis* lacks phosphofructokinase which catalyzes the third reaction of the EMP glycolytic pathway, rendering the pathway non-functional (Baart *et al*. 2007). It has been hypothesized that this is due to the limited contribution this pathway makes to ATP generation under aerobic conditions, where energy is more efficiently generated by oxidative phosphorylation (Baart *et al*. 2010). Nevertheless, the meningococcal genome contains apparently functional genes for the remaining glycolytic enzymes and three of these, namely enolase (Knaust *et al*. 2007), GAPDH (Tunio *et al*. 2010*a*), and FBA (Tunio *et al*. 2010*b*), have demonstrated moonlighting functions in the meningococcus.

Based on sequence homology and a characteristic insertion sequence of 21 amino acids (S236-Y256), neisserial FBA is a class IIB aldolase (Tunio *et al*. 2010*b*). Meningococcal FBA (FBA-nm) can be found in the cytoplasm and also in the outer membrane. The latter is surface accessible to anti-FBA antibodies in flow cytometry experiments, even in the presence of the meningococcal polysaccharide capsule (Tunio *et al*. 2010*b*). Unlike in some bacterial species, mutants deficient in FBA expression can be constructed in *N. meningitidis* (Tunio *et al*. 2010*b*). The mutant showed no impairment in growth in standard *in vitro* culture conditions. Importantly, the FBA-nm deficient strain was less adherent to

both human epithelial and endothelial cells, while complementation with an ectopic copy of the gene encoding FBA-nm restored the adherent phenotype (Tunio *et al*. 2010*b*). These data suggest that FBA-nm acts as meningococcal adhesin, although the host ligand(s) remains to be identified. The importance of FBA-nm *in vivo* was underlined in a screen of 2850 insertional mutants of *N. meningitidis* for their capacity to cause systemic infection in an infant rat model (Sun *et al*. 2000). This study identified 73 genes, including the gene encoding for FBA-nm, as being essential for the establishment of septicemia in this model (Sun *et al*. 2000). Presumably this phenotype resulted from loss of the virulence-associated moonlighting function(s) of FBA-nm; nevertheless, a metabolic defect which is only apparent under *in vivo* conditions is feasible.

17.6 *Mycobacterium tuberculosis* FBA Binds Human Plasminogen

The first report of an interaction between *M. tuberculosis* and plasminogen came from Monroy and colleagues who used flow cytometry to demonstrate binding (Monroy *et al*. 2000). Subsequent far-Western blotting of separated soluble protein extracts and cell-wall proteins indicated the presence of four plasminogen-binding proteins of molecular weight 66, 60, 55, and 30 kDa. Plasminogen binding by these proteins was abolished in the presence of the lysine analog ε-aminocaproic acid (EACA), suggesting the involvement of lysine residues in the interactions (Monroy *et al*. 2000). Further work by the same group involved separating the proteins present in soluble extracts and culture filtrates from *M. tuberculosis* by 2D gel electrophoresis. Following transfer to PVDF, membranes were probed with human plasminogen and mass spectrometry and amino-terminal amino acid sequencing identified 15 putative plasminogen-binding proteins, one of which was *M. tuberculosis* FBA (FBA-tb) (Xolalpa *et al*. 2007).

More recent studies have shown the presence of FBA-tb in the cytosol, culture filtrate, cell wall, and cell-membrane fractions (de la Paz Santangelo *et al*. 2011). Flow cytometry and enzymatic activity measurements revealed that FBA-tb was exported to the cell surface and produced under various axenic growth conditions, including oxygen depletion, and hence by non-replicating bacilli (de la Paz Santangelo *et al*. 2011). Importantly, FBA-tb expression was demonstrated *in vivo* in the lungs of experimentally infected guinea pigs and mice (de la Paz Santangelo *et al*. 2011). ELISA experiments confirmed that FBA-tb binds human plasminogen in a dose-dependent manner ($K_D = 6.7 \pm 3$ nM); an interaction which could be abolished by EACA (de la Paz Santangelo *et al*. 2011). In contrast, the presence of TD3, a competitive inhibitor of aldolase activity, had no effect on plasminogen binding, demonstrating that plasminogen binding was independent of aldolase activity (de la Paz Santangelo *et al*. 2011). Tissue plasminogen activator (tPA), but not FBA-tb itself, was able to activate FBA-bound plasminogen to plasmin, while α2-antiplasmin reduced plasmin activity in the absence of FBA-tb. In contrast, the presence of FBA-tb reduced this inhibition, perhaps suggesting competition between FBA-tb and α2-antiplasmin for the same binding site on plasmin. Interestingly, FBA from *M. leprae* (which shares 87% amino acid

identity with FBA-tb) was also detected in cell wall and membrane fractions of the leprosy bacillus purified from chronically infected armadillo tissues, suggesting that the moonlighting properties of FBA in *Mycobacterium* species may not be limited to *M. tuberculosis* (de la Paz Santangelo *et al*. 2011).

Attempts at inactivating the gene encoding FBA in *M. tuberculosis* followed by growth in standard *in vitro* culture media have been unsuccessful, suggesting that FBA is essential for viability in this organism (Griffin *et al*. 2011). However, the use of conditional mutants has shown that strains lacking FBA-tb expression are viable if supplied with an appropriate combination of carbon substrates entering metabolism above and below the FBA-catalyzed reaction (de la Paz Santangelo *et al*. 2011; Puckett *et al*. 2014). Mouse *in vivo* experiments confirmed that *M. tuberculosis* requires FBA activity for growth during acute infections and for persistence during chronic infections, presumably due either to the lack of a growth permissive ratio of carbon sources that can compensate for the lack of FBA-tb and/or the loss of moonlighting functions (Puckett *et al*. 2014). Given the emergence of multidrug-resistant *M. tuberculosis*, and since human (i.e., class I) and bacterial (class II) FBAs are structurally and mechanistically distinct, inhibitors of FBA-tb are currently being developed as potential anti-tuberculosis therapies (Daher *et al*. 2010; Labbé *et al*. 2012).

17.7 Other Examples of FBAs with Possible Roles in Pathogenesis

Xanthomonas oryzae pv. *oryzicola* is the causative agent of bacterial leaf streak, an economically important disease of rice (Nino-Liu *et al*. 2006). As little is known about the virulence of this species, Wang *et al*. screened a Tn*5*-tagged random mutant library of *X. oryzae* pv. *oryzicola* strain RS105 to identify mutants with a reduced ability to cause disease in rice (Wang *et al*. 2007). One of the mutants identified carried a transposon insertion in open reading frame (ORF) *Xoryp_17640* (subsequently renamed *fbaB*) encoding for a class I FBA. This phenotype was subsequently confirmed using a non-polar *fbaB* mutant; virulence could be completely restored by the presence of *fbaB in trans* (Guo *et al*. 2012). Further experiments appeared to suggest that the influence of FBA on pathogenesis in *X. oryzae* pv. *oryzicola* relates to its role in metabolism rather than an additional moonlighting function (Guo *et al*. 2012). Furthermore it remains to be established whether FBA can be detected on the surface of *X. oryzae* pv. *oryzicola*; it therefore remains unclear whether FBA has moonlighting properties in *X. oryzae* pv. *oryzicola*.

Clostridium perfringens causes necrotic enteritis, a severe gastro-intestinal disease in chickens. Kulkarni *et al*. characterized the proteins secreted by a limited number of virulent and avirulent *C. perfringens* strains and identified FBA (FBA-cp) as one of six major proteins apparently only secreted by virulent strains (Kulkarni *et al*. 2006). The proteins were recognized by sera collected from chickens immune to disease (Kulkarni *et al*. 2006). Chickens immunized with either recombinant FBA-cp or an attenuated *Salmonella* vaccine vector expressing FBA-cp were significantly protected against subsequent challenge

(Kulkarni *et al*. 2007, 2008). Collectively, these results suggest that FBA-cp may have a duel role eliciting a protective response against, and in the pathogenesis of, *C. perfringens* disease.

FBA has recently been detected in the cell-wall fraction of *Coxiella burnetii*, the causative agent of Q fever in humans; however, a specific role for *C. burnetii* FBA in pathogenesis has yet to be determined (Flores-Ramirez *et al*. 2014).

In an interesting twist, a surface protein (Chlamydial outer protein N; CopN) of the intracellular pathogen *Chlamydia pneumoniae*, which causes pneumonia in humans, has recently been shown to sequester human FBA to the bacterial surface, leading to enhanced bacterial survival within host cells (Ishida *et al*. 2014). The authors speculate that the sequestration of human FBA might impair its non-canonical role in eliciting apoptosis of infected cells (Shao *et al*. 2007).

17.8 Conclusions

An increasing number of reports have demonstrated the unexpected presence of FBA on the surface of various bacterial species; it remains to be determined how widespread a phenomenon this is. Only in a few organisms have specific virulence-associated moonlighting activities been defined. To date, FBA has been shown to mediate adhesion to mammalian cells and the binding of human plasminogen to the bacterial cell surface; the full repertoire of moonlighting functions mediated by bacterial FBAs is likely to expand in future. FBAs can be divided into one of two classes based on in their mechanism of catalysis and prevalence; higher organisms encode only class I FBAs, whereas bacteria primarily encode class II FBAs. Since the lack of class II FBA expression is often detrimental, these proteins pose an attractive target for the development of novel antimicrobial agents. A non-competitive inhibitor, with specificity to class II FBAs, was recently described which inhibited the growth of *M. tuberculosis* and methicillin-resistant *S. aureus* (MRSA), thus hinting at the potential of aldolase inhibitors (Capodagli *et al*. 2014). Additional aspects of FBA, including their highly conserved nature, surface localization, and immunogenicity, could make this glycolytic enzyme a potentially useful vaccine candidate for a range of pathogens. Finally, translocation of FBA to the bacterial cell surface remains an enigma; elucidation of the pathway(s) of FBA export is likely to have wider significance since export pathways for many other bacterial moonlighting proteins remain unresolved and are likely to be shared.

References

Baart G, Zomer B, de Haan A, van der Pol L, Beuvery EC, Tramper J, Martens D (2007) Modeling *Neisseria meningitidis* metabolism: from genome to metabolic fluxes. *Genome Biology* 8: R136.

Baart GJ, Langenhof M, van de Waterbeemd B, Hamstra HJ, Zomer B, van der Pol LA, Beuvery EC, Tramper J, Martens DE (2010) Expression of phosphofructokinase in *Neisseria meningitidis*. *Microbiology* 156: 530–542.

Blau K, Portnoi M, Shagan M, Kaganovich A, Rom S, Kafka D, Caspi VC, Porgador A, Givon-Lavi N, Gershoni JM, Dagan R, Nebenzahl YM (2007) Flamingo Cadherin: a putative host receptor for *Streptococcus pneumoniae*. *Journal of Infectious Diseases* 195: 1828–1837.

Blom NS, Tetreault S, Coulombe R, Sygusch J (1996) Novel active site in *Escherichia coli* fructose 1,6-bisphosphate aldolase. *Nature and Structural and Molecular Biology* 3: 856–862.

Capodagli GC, Sedhom WG, Jackson M, Ahrendt KA, Pegan SD (2014) A noncompetitive inhibitor for *Mycobacterium tuberculosis*'s class IIa fructose 1,6-bisphosphate aldolase. *Biochemistry* 53: 202–213.

Chae J, Kim MJ, Goo JH, Collier S, Gubb D, Charlton J, Adler PN, Park WJ (1999) The *Drosophila* tissue polarity gene *starry night* encodes a member of the protocadherin family. *Development* 126: 5421–5429.

Daher R, Coincon M, Fonvielle M, Gest PM, Guerin ME, Jackson M, Sygusch J, Therisod M (2010) Rational design, synthesis, and evaluation of new selective inhibitors of microbial class II (zinc dependent) fructose bis-phosphate aldolases. *Journal of Medical Chemistry* 53: 7836–7842.

de la Paz Santangelo M, Gest PM, Guerin ME, Coinçon M, Pham H, Ryan G, Puckett SE, Spencer JS, Gonzalez-Juarrero M, Daher R, Lenaerts AJ, Schnappinger D, Therisod M, Ehrt S, Sygusch J, Jackson M (2011) Glycolytic and non-glycolytic functions of *Mycobacterium tuberculosis* fructose-1,6-bisphosphate aldolase, an essential enzyme produced by replicating and non-replicating bacilli. *Journal of Biological Chemistry* 286: 40219–40231.

Flores-Ramirez G, Jankovicova B, Bilkova Z, Miernyk JA, Skultety L (2014) Identification of *Coxiella burnetii* surface-exposed and cell envelope associated proteins using a combined bioinformatics plus proteomics strategy. *Proteomics* 14: 1868–1881.

Fluegge K, Schweier O, Schiltz E, Batsford S, Berner R (2004) Identification and immunoreactivity of proteins released from *Streptococcus agalactiae*. *European Journal of Clinical Microbiology and Infectious Diseases* 23: 818–824.

Gancedo C, Flores C-L (2008) Moonlighting proteins in yeasts. *Microbiology and Molecular Biology Reviews* 72: 197–210.

Ginger ML (2014) Protein moonlighting in parasitic protists. *Biochemical Society Transactions* 42: 1734–1739.

Griffin JE, Gawronski JD, DeJesus MA, Ioerger TR, Akerley BJ, Sassetti CM (2011) High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. *PLoS Pathogens* 7: e1002251.

Guo W, Zou L-F, Li Y-R, Cui Y-P, Ji Z-Y, Cai L-L, Zou H-S, Hutchins WC, Yang C-H, Chen G-Y (2012) Fructose-bisphosphate aldolase exhibits functional roles between carbon metabolism and the *hrp* system in rice pathogen *Xanthomonas oryzae* pv. *oryzicola*. *PLoS ONE* 7: e31855.

Hall DR, Leonard GA, Reed CD, Watt CI, Berry A, Hunter WN (1999) The crystal structure of *Escherichia coli* class II fructose-1,6-bisphosphate aldolase in complex with phosphoglycolohydroxamate reveals details of mechanism and specificity. *Journal of Molecular Biology* 287: 383–394.

Henderson B (2014) An overview of protein moonlighting in bacterial infection. *Biochemical Society Transactions* 42: 1720–1727.

Henderson B, Martin A (2011) Bacterial virulence in the moonlight: multitasking bacterial moonlighting proteins are virulence determinants in infectious disease. *Infection and Immunity* 79: 3476–3491.

Ishida K, Matsuo J, Yamamoto Y, Yamaguchi H (2014) *Chlamydia pneumoniae* effector chlamydial outer protein N sequesters fructose bisphosphate aldolase A, providing a benefit to bacterial growth. *BMC Microbiology* 14: 330.

Iwai Y, Usui T, Hirano S, Steward R, Takeichi M, Uemura T (1997) Axon patterning requires DN-cadherin, a novel neuronal adhesion receptor, in the *Drosophila* embryonic CNS. *Neuron* 19: 77–89.

Jeffery CJ (1999) Moonlighting proteins. *Trends in Biochemical Science* 24: 8–11.

Jeffery CJ (2014) An introduction to protein moonlighting. *Biochemical Society Transactions* 42: 1679–1683.

Jia B, Cheong GW, Zhang S (2013) Multifunctional enzymes in archaea: promiscuity and moonlight. *Extremophiles* 17: 193–203.

Kim J-W, Dang CV (2005) Multifaceted roles of glycolytic enzymes. *Trends in Biochemical Science* 30: 142–150.

Knaust A, Weber MV, Hammerschmidt S, Bergmann S, Frosch M, Kurzai O (2007) Cytosolic proteins contribute to surface plasminogen recruitment of *Neisseria meningitidis*. *Journal of Bacteriology* 189: 3246–3255.

Kulkarni RR, Parreira VR, Sharif S, Prescott JF (2006) *Clostridium perfringens* antigens recognized by broiler chickens immune to necrotic enteritis. *Clinical and Vaccine Immunology* 13: 1358–1362.

Kulkarni RR, Parreira VR, Sharif S, Prescott JF (2007) Immunization of broiler chickens against *Clostridium perfringens*-induced necrotic enteritis. *Clinical and Vaccine Immunology* 14: 1070–1077.

Kulkarni RR, Parreira VR, Sharif S, Prescott JF (2008) Oral immunization of broiler chickens against necrotic enteritis with an attenuated *Salmonella* vaccine vector expressing *Clostridium perfringens* antigens. *Vaccine* 26: 4194–4203.

Labbé G, Krismanich AP, de Groot S, Rasmusson T, Shang M, Brown MD, Dmitrienko GI, Guillemette JG (2012) Development of metal-chelating inhibitors for the Class II fructose 1,6-bisphosphate (FBP) aldolase. *Journal of Inorganic Biochemistry* 112: 49–58.

Landwehr-Kenzel S, Henneke P (2014) Interaction of *Streptococcus agalactiae* and cellular innate immunity in colonization and disease. *Frontiers in Immunology* 5: 519.

Lei B, Mackie S, Lukomski S, Musser JM (2000) Identification and immunogenicity of group A *Streptococcus* culture supernatant proteins. *Infection and Immunity* 68: 6807–6818.

Ling E, Feldman G, Portnoi M, Dagan R, Overweg K, Mulholland F, Chalifa-Caspi V, Wells J, Mizrachi-Nebenzahl Y (2004) Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. *Clinical and Experimental Immunology* 138: 290–298.

Marsh JJ, Lebherz HG (1992) Fructose-bisphosphate aldolases: an evolutionary history. *Trends in Biochemical Science* 17: 110–113.

Monroy V, Amador A, Ruiz B, Espinoza-Cueto P, Xolalpa W, Mancilla R, Espitia C (2000) Binding and activation of human plasminogen by *Mycobacterium tuberculosis*. *Infection and Immunity* 68: 4327–4330.

Moore B (2004) Bifunctional and moonlighting enzymes: lighting the way to regulatory control. *Trends in Plant Science* 9: 221–228.

Nagano N, Orengo CA, Thornton JM (2002) One fold with many functions: the evolutionary relationships between TIM barrel families based on their sequences, structures and functions. *Journal of Molecular Biology* 321: 741–765.

Nakahara K, Yamamoto H, Miyake C, Yokota A (2003) Purification and characterization of class-I and class-II fructose-1,6-bisphosphate aldolases from the cyanobacterium *Synechocystis* sp. PCC 6803. *Plant and Cell Physiology* 44: 326–333.

Nino-Liu DO, Ronald PC, Bogdanove AJ (2006) *Xanthomonas oryzae* pathovars: model pathogens of a model crop. *Molecular Plant Pathology* 7: 303–324.

Nollet F, Kools P, van Roy F (2000) Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *Journal of Molecular Biology* 299: 551–572.

Perham RN (1990) The fructose-1,6-bisphosphate aldolases: same reaction, different enzymes. *Biochemical Society Transactions* 18: 185–187.

Plaumann M, Pelzer-Reith B, Martin WF, Schnarrenberger C (1997) Multiple recruitment of class-I aldolase to chloroplasts and eubacterial origin of eukaryotic class-II aldolases revealed by cDNAs from *Euglena gracilis*. *Current Genetics* 31: 430–438.

Puckett S, Trujillo C, Eoh H, Marrero J, Spencer J, Jackson M, Schnappinger D, Rhee K, Ehrt S (2014) Inactivation of fructose-1,6-bisphosphate aldolase prevents optimal co-catabolism of glycolytic and gluconeogenic carbon substrates in *Mycobacterium tuberculosis*. *PLoS Pathogens* 10: e1004144.

Rutter WJ (1964) Evolution of aldolase. *Federation Proceedings* 23: 1248–1257.

Sauvé V, Sygusch J (2001) Molecular cloning, expression, purification, and characterization of fructose-1,6-bisphosphate aldolase from *Thermus aquaticus*. *Protein Expression and Purification* 21: 293–302.

Say RF, Fuchs G (2010) Fructose 1,6-bisphosphate aldolase/phosphatase may be an ancestral gluconeogenic enzyme. *Nature* 464: 1077–1081.

Shao W, Yeretssian G, Doiron K, Hussain SN, Saleh M (2007) The caspase-1 digestome identifies the glycolysis pathway as a target during infection and septic shock. *Journal of Biological Chemistry* 282: 36321–36329.

Siebers B, Brinkmann H, Dörr C, Tjaden B, Lilie H, van der Oost J, Verhees CH (2001) Archaeal fructose-1,6-bisphosphate aldolases constitute a new family of archaeal type class I aldolase. *Journal of Biological Chemistry* 276: 28710–28718.

Stephens DS (2009) Biology and pathogenesis of the evolutionarily successful, obligate human bacterium *Neisseria meningitidis*. *Vaccine* 27 Suppl 2: B71–77.

Stribling D, Perham RN (1973) Purification and characterization of two fructose diphosphate aldolases from *Escherichia coli* (Crookes' strain). *Biochemical Journal* 131: 833–841.

Sun Y-H, Bakshi S, Chalmers R, Tang CM (2000) Functional genomics of *Neisseria meningitidis* pathogenesis. *Nature Medicine* 6: 1269–1273.

Thomson GJ, Howlett GJ, Ashcroft AE, Berry A (1998) The *dhnA* gene of *Escherichia coli* encodes a class I fructose bisphosphate aldolase. *Biochemical Journal* 331: 437–445.

Tunio SA, Oldfield NJ, Ala'Aldeen DA, Wooldridge KG, Turner DP (2010*a*) The role of glyceraldehyde 3-phosphate dehydrogenase (GapA-1) in *Neisseria meningitidis* adherence to human cells. *BMC Microbiology* 10: 280.

Tunio SA, Oldfield NJ, Berry A, Ala'Aldeen DA, Wooldridge KG, Turner DP (2010*b*) The moonlighting protein fructose-1, 6-bisphosphate aldolase of *Neisseria meningitidis*: surface localization and role in host cell adhesion. *Molecular Microbiology* 76: 605–615.

Usui T, Shima Y, Shimada Y, Hirano S, Burgess RW, Schwarz TL, Takeichi M, Uemura T (1999) Flamingo, a seven-pass transmembrane cadherin, regulates planar cell polarity under the control of Frizzled. *Cell* 98: 585–595.

Walker MJ, Barnett TC, McArthur JD, Cole JN, Gillen CM, Henningham A, Sriprakash KS, Sanderson-Smith ML, Nizet V (2014) Disease manifestations and pathogenic mechanisms of group A streptococcus. *Clinical Microbiology Reviews* 27: 264–301.

Wang G, Xia Y, Cui J, Gu Z, Song Y, Chen YQ, Chen H, Zhang H, Chen W (2013) The roles of moonlighting proteins in bacteria. *Current Issues in Molecular Biology* 16: 15–22.

Wang L, Makino S, Subedee A, Bogdanove AJ (2007) Novel candidate virulence factors in rice pathogen *Xanthomonas oryzae* pv. *oryzicola* as revealed by mutational analysis. *Applied and Environmental Microbiology* 73: 8023–8027.

Wang XJ, Zhang DL, Xu ZG, Ma ML, Wang WB, Li LL, Han XL, Huo Y, Yu X, Sun JP (2014) Understanding cadherin EGF LAG seven-pass G-type receptors. *Journal of Neurochemistry* 131: 699–711.

Wilkins JC, Beighton D, Homer KA (2003) Effect of acidic pH on expression of surface-associated proteins of *Streptococcus oralis*. *Applied and Environmental Microbiology* 69: 5290–5296.

Wu Z, Zhang W, Lu C (2008) Immunoproteomic assay of surface proteins of *Streptococcus suis* serotype 9. *FEMS Immunology and Medical Microbiology* 53: 52–59.

Xolalpa W, Vallecillo AJ, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, Singh M, Espitia C (2007) Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*. *Proteomics* 7: 3332–3341.

Zgiby SM, Thomson GJ, Qamar S, Berry A (2000) Exploring substrate binding and discrimination in fructose 1,6-bisphosphate and tagatose 1,6-bisphosphate aldolases. *European Journal of Biochemistry* 267: 1858–1868.

Zgiby S, Plater AR, Bates MA, Thomson GJ, Berry A (2002) A functional role for a flexible loop containing Glu182 in the class II fructose-1,6-bisphosphate aldolase from *Escherichia coli*. *Journal of Molecular Biology* 315: 131–140.

3.6

Other Metabolic Enzymes Functioning in Bacterial Virulence

18

Pyruvate Dehydrogenase Subunit B and Plasminogen Binding in *Mycoplasma*

Anne Gründel, Kathleen Friedrich, Melanie Pfeiffer, Enno Jacobs, and Roger Dumke

Technical University Dresden, Dresden, Germany

18.1 Introduction

Species of the class *Mollicutes* are among the smallest self-replicating microorganisms known. The bacteria are discussed to be evolved from Gram-positive ancestors with low G/C content associated with a large reduction in their genomic resources (Citti and Blanchard 2013). The genome sizes of these cell-wall-less bacteria vary between 0.58 and 1.81 Mbp, resulting in a lack of many metabolic pathways and a limited repertoire of virulence factors in the pathogenic species among them (Kühner *et al*. 2009). For instance, the tricarboxylic acid cycle and purine/pyrimidine synthesis are missing. ATP is generated mainly by glycolysis showing all reactions. Nevertheless, mycoplasmas can be found as well-adapted commensals or pathogens of plants, animals, and humans. In the latter, the organisms are successful parasites of the mucosa (urogenital or respiratory), requiring close contact with preferred host tissues. After reaching these sites by unique gliding mechanisms and adherence by specialized structures, mycoplasmas must ensure uptake of essential nutrients and escape the host's immune system. These processes are the first steps in infection and determine whether colonization will be successful or the pathogens are eliminated. The increasing number of sequenced mycoplasma genomes has resulted in deeper insights into the genomics of these microorganisms, but special tools are necessary for extensive investigations of the function of gene products (Renaudin *et al*. 2014). For instance, the analysis of mycoplasma metabolism and virulence is hampered by the use of a TGA codon for tryptophan and not as universal stop codon (Inamine *et al*. 1990), requiring effective mutation steps (Hames *et al*. 2005) for heterologous expression of proteins of interest.

Despite these limitations, increasing efforts have been made to improve our knowledge of the metabolic organization of mycoplasmas and to identify further factors important in the pathogenesis of infections. In this context, the interaction

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

of bacterial proteins with host factors such as extracellular matrix (ECM) components will trigger the colonization process. In addition, the dual function of mycoplasmal proteins in metabolism and in pathogenesis is a way of compensating for the greatly reduced coding capacity of these microorganisms. Investigations in recent years have detected a number of surface-exposed proteins in mycoplasma that function as so-called microbial surface components, recognizing adhesive matrix molecules (MSCRAMMs). These include toxins and cytadhesins which play a primary role as virulence factors (Hopfe and Henrich 2014). Furthermore, glycolytic enzymes with moonlighting functions have been found in various species (Table 18.1). Among them are proteins that interact with different host components as well as particular host proteins that bind to different glycolytic enzymes, suggesting a network of associations between host and mycoplasma factors. Investigation of these associations in more comprehensive studies appears promising in terms of gaining an overall insight into the importance of glycolytic enzymes as surface-displayed proteins.

Due to its importance for public health, *M. pneumoniae* is one of the best investigated among mycoplasma species. *M. pneumoniae* is a common agent in a broad range of human respiratory tract infections ranging from mild forms of tracheobronchitis to severe cases of atypical pneumonia requiring hospitalization of patients (Atkinson *et al.* 2008). In epidemic periods, which occur every three to seven years, up to 30% of all cases of community-acquired pneumonia can be attributed to this pathogen (Dumke *et al.* 2015). In addition, extra-pulmonary manifestations are described, affecting mainly the skin and the central nervous system. Knowledge of further details of the infection and colonization process is key to understanding the particular epidemiology of diseases due to these bacteria. Research activities in the past have focused on virulence factors such as the tip structure in *M. pneumoniae* (Hasselbring *et al.* 2006). This attachment organelle comprises a complex of adhesins and adhesion-related proteins, which targets the cells of the respiratory epithelium.

Table 18.1 Described glycolytic enzymes with multifunction in *Mycoplasma/Spiroplasma*.

Species	Glycolytic enzyme	Host interaction partner	Reference
M. bovis	Enolase	Plasminogen	Song *et al.* (2012)
M. fermentans	Enolase	Plasminogen	Yavlovich *et al.* (2007)
M. gallisepticum	Enolase	Plasminogen	Chen *et al.* (2011)
M. genitalium	GAPDH	Mucin	Alvarez *et al.* (2003)
M. pneumoniae	GAPDH	Fibrinogen	Dumke *et al.* (2011)
	PDHB	Fibronectin	Dallo *et al.* (2002)
		Plasminogen	Thomas *et al.* (2013)
	Enolase	Plasminogen	Thomas *et al.* (2013)
M. suis	GAPDH	Erythrocytes	Schreiner *et al.* (2012)
	Enolase	Erythrocytes	Hoelzle *et al.* (2007)
M. synoviae	Enolase	Fibronectin, plasminogen	Bao *et al.* (2014)
S. citri	PGK*	Actin	Labroussaa *et al.* (2011)

* Phosphoglycerate kinase.

Subsequently, the expression of tissue-damaging substances such as superoxide (Hames *et al*. 2009) and the CARDS toxin (Kannan and Baseman 2006) have been described as important aspects of successful host colonization.

For investigation of proteins with multiple functions in bacteria, species such as *M. pneumoniae* are the simplest models for studying the role of these proteins in host–pathogen interactions. With 693 proposed protein-coding genes and descriptions of the function of about 70% of protein-coding genes, *M. pneumoniae* is a promising candidate for studying the complete proteome of a bacterium (Catrein and Herrmann 2011). Previous proteomic analysis revealed a number of glycolytic enzymes occurring in the fraction of membrane-associated proteins extracted after treatment of *M. pneumoniae* cells with the detergent Triton X-100 (Regula *et al*. 2001). Among these, the subunits A and B of pyruvate dehydrogenase (PDH) were found to belong to the PDH complex (Matic *et al*. 2003). During glycolysis, the enzymes of the complex convert pyruvate to acetyl-CoA. The cluster in *M. pneumoniae* consists of four genes *pdhA* to *D* transcribed in this order (Dandekar *et al*. 2000) and is intermitted by the small MP200 RNA (Göhlmann *et al*. 2000). The subunits of the PDH cluster are phosphorylated like many enzymes in glycolysis (Schmidl *et al*. 2010), allowing regulation of their activity and localization. Furthermore, subunit A (E1 α) of PDH was described as complexed to the major P1 adhesin (Layh-Schmitt *et al*. 2000), indicating that an interaction of this class of proteins with the adherence apparatus of *M. pneumoniae* cannot be excluded.

18.2 Binding of Human Plasminogen to *M. pneumoniae*

Plasminogen has been characterized as a human factor that interacts with bacterial proteins of phylogenetically diverse species (Sanderson-Smith *et al*. 2012), suggesting a general role in the host–pathogen relationship. Plasminogen is a protein that circulates in the serum. It can be cleaved into the broad-spectrum protease plasmin, which degrades host factors such as fibrin and extracellular matrices. The five Kringle domains play a central part in the binding of plasminogen not only to host factors but also to bacterial proteins. Binding and activation of plasminogen are regarded as an effective mechanism to facilitate dissemination of microorganisms.

Despite the fact that *M. pneumoniae* belongs to pathogens without a confirmed tendency of invasiveness into the host body, propagation within the respiratory mucosa is essential for colonization. As described for other bacterial species, several proteins of *M. pneumoniae* are able to interact with human plasminogen. In immunofluorescence experiments with fixed *M. pneumoniae* cells incubated with labeled human plasminogen, strong signals were demonstrated (Fig. 18.1a). In addition, the results of a ligand immunoblot assay using separated whole proteins of the bacterium confirmed at least three plasminogen-binding proteins (Fig. 18.1b). Up to an incubation time of 7 hours, *M. pneumoniae* cells are able to accumulate plasminogen (Fig. 18.2a). During this time, plasminogen added to the medium was not degraded (Fig. 18.2b). In previous studies, we

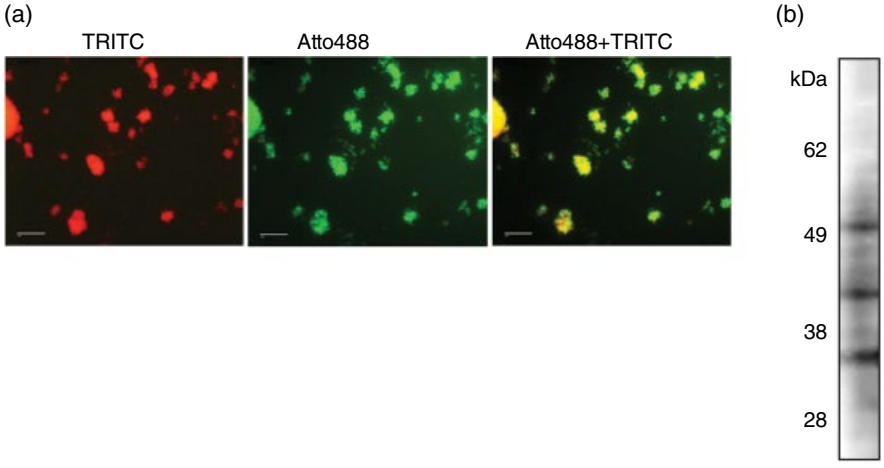

Figure 18.1 Binding of human plasminogen to *M. pneumoniae*. (a) Immunofluorescence of fixed *M. pneumoniae* cells after incubation with Atto488-labeled plasminogen (50 µg mL^{-1}). Antiserum to Triton X-insoluble proteins detected with TRITC-labeled secondary antibody is used as control. Bar: 10 µm. (b) Ligand immunoblotting assay using SDS-PAGE-separated total proteins of *M. pneumoniae* incubated with plasminogen (15 µg mL^{-1}) detected with rabbit anti-plasminogen. (*See color plate section for the color representation of this figure.*)

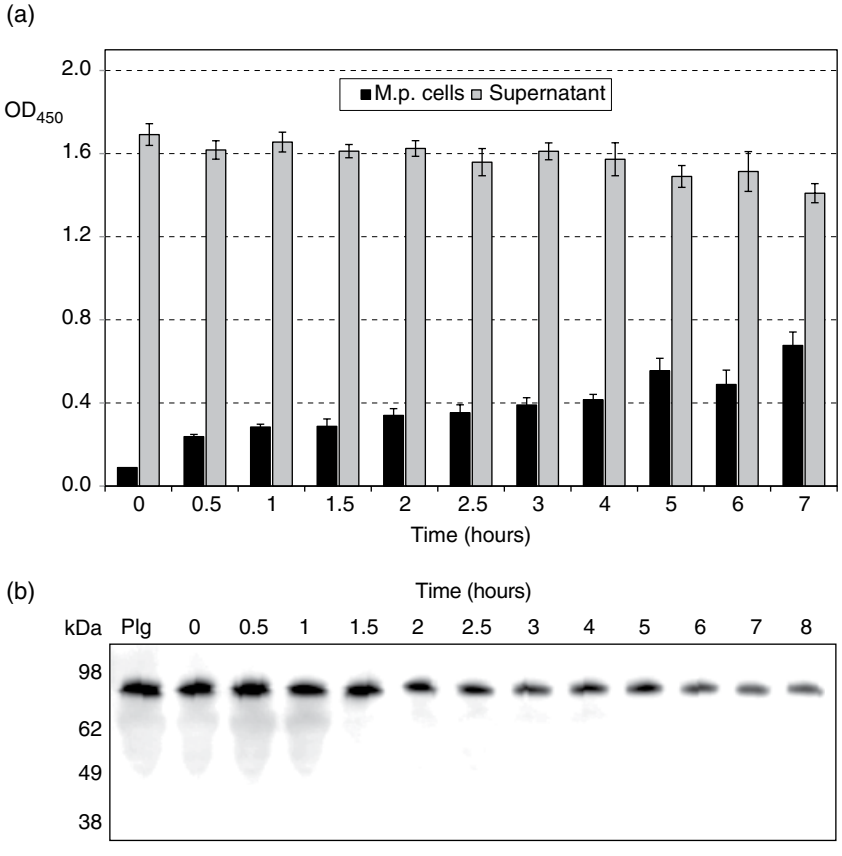

Figure 18.2 (a) Time-dependent binding of human plasminogen (2.5 µg mL^{-1}) after incubation with *M. pneumoniae* cells. (b) Detection of human plasminogen in immunoblotting using rabbit anti-plasminogen.

demonstrated enolase and subunit B of the PDH complex as two of the proteins of the species which were able to interact with plasminogen (Thomas *et al*. 2013). Production of complete enzymes as recombinant proteins and their use as antigens for the production of monospecific polyclonal antisera are preconditions for studying the role of these proteins in the interaction with the host. After exchange of TGA codons, full-length glycolytic enzymes of *M. pneumoniae* were produced successfully. Enolase and PDHB interact concentration-dependently with human plasminogen *in vitro* and monospecific antisera reduce binding between recombinant proteins and plasminogen as well as between *M. pneumoniae* cells and plasminogen. However, the persistent association of bacteria with plasminogen indicates that further bacterial interaction partners can be assumed. PDHB was confirmed as surface-localized and belongs to the class of glycolytic enzymes that can bind to more than one host protein. In addition to plasminogen, an interaction with human fibronectin has been described (Dallo *et al*. 2002).

As reported initially for *Streptococcus pneumoniae*, lysine-rich motifs have been characterized as typical for binding of plasminogen to bacterial proteins (Bergmann *et al*. 2003). The confirmed plasminogen-interacting glycolytic enzymes PDHB and enolase in *M. pneumoniae* feature 6.7% and 5.7% of lysine in the amino acid sequence. The presence of putative plasminogen-binding sites in enolases of different mycoplasma species has been identified (Yavlovich *et al*. 2007), but experimental confirmation is still pending. In contrast, PDHB of *M. pneumoniae* was investigated and binding experiments using overlapping peptides demonstrated a novel region ^{91}FPAMFQIFTHAA^{102} which is responsible for interaction (Thomas *et al*. 2013). This peptide is able to bind plasminogen in a dose-dependent way, indicating that not only lysine-rich domains are able to mediate binding to plasminogen. More interesting for a practical influence on host–pathogen interaction is the question of whether the microbial proteins bound to plasminogen are able to activate plasminogen and induce degradation of further host factors. Incubation of recombinant protein PDHB, human plasminogen, activator uPA, and human fibrinogen resulted in fibrinogen degradation products (Fig. 18.3). It can be concluded that PDHB is the first glycolytic enzyme of *M. pneumoniae* for which binding to human factors such as plasminogen and ECM components, but also an influence on the host's coagulation cascade, was confirmed.

Interestingly, *M. pneumoniae* enolase is a potent plasminogen binder but, in contrast to many other mycoplasma and bacterial species, it does not occur on the surface of the cell. Amino acid sequence identities between *M. pneumoniae* enolase and the surface-located homologs described in *M. bovis*, *M. fermentans*, *M. gallisepticum*, *M. suis*, and *M. synoviae* varied between 55.8 and 65.4%. The influence of these differences on surface localization cannot be excluded. Further studies must show whether enolase is involved in host interaction despite the absence of surface localization, since a recent study confirmed that enolase can be secreted extracellularly in *Leptospira interrogans* and interact with plasminogen (Nogueira *et al*. 2013). Besides *M. pneumoniae*, enolase as plasminogen-binding protein was demonstrated in the human pathogen *M. fermentans* (Yavlovich *et al*. 2007), in *M. bovis* infecting calves (Song *et al*. 2012), and in

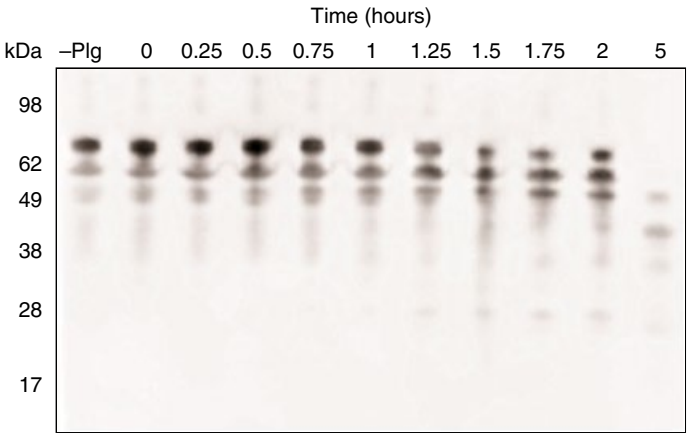

Figure 18.3 Degradation of human fibrinogen (10 µg mL^{-1}) in the presence of plasminogen (Plg; 10 µg/mL^{-1}), recombinant protein PDHB (20 µg mL^{-1}) and activator human uPA (4 ng mL^{-1}). Fibrinogen was separated, blotted, and detected by rabbit anti-fibrinogen.

M. gallisepticum (Chen *et al*. 2011) and *M. synoviae* (Bao *et al*. 2014), both causing infections in poultry. Plasminogen binding to *M. fermentans* influences the adherence of mycoplasmas to human cells, initiates plasminogen activation and increases internalization of microorganisms by eukaryotic cells. Enhancement of the invasiveness of mycoplasmas is caused by activation of bound plasminogen (Yavlovich *et al*. 2004). In *M. bovis*, pretreatment of host cells with plasminogen increased the adherence rates of mycoplasmas and the effect of anti-enolase antibodies on adhesion, indicating that enolase is involved in adherence of *M. bovis* via plasminogen binding. This is in accordance with results from both poultry pathogens in which surface localization of enolases has been confirmed. Anti-enolase sera reduced the adhesion of *M. gallisepticum* and *M. synoviae* cells to chicken embryo fibroblasts. Further studies will have to show whether the enolases of these species are able to activate plasminogen.

18.3 Localization of PDHB on the Surface of *M. pneumoniae* Cells

Subunits of the PDH complex are not among the glycolytic enzymes frequently reported as surface-localized on bacterial cells, such as enolase or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). However, the presence of PDHB in the fraction of membrane-associated proteins was confirmed for the non-pathogenic and non-sterol-requiring *Mollicutes* species *Acholeplasma laidlawii* (Wallbrandt *et al*. 1992). More recently, PDHA-C was detected among the *M. bovis* proteins that induce antibodies in naturally infected animals (Sun *et al*. 2014). In *M. hyopneumoniae*, Pinto *et al*. (2007) identified the PDHB subunit also among the highly antigenic proteins. Outside the *Mollicutes* class, the E1 beta-subunit of PDH (PDHB) was found to be surface-expressed in *Lactobacillus plantarum* and it binds human fibronectin (Vastano *et al*. 2014).

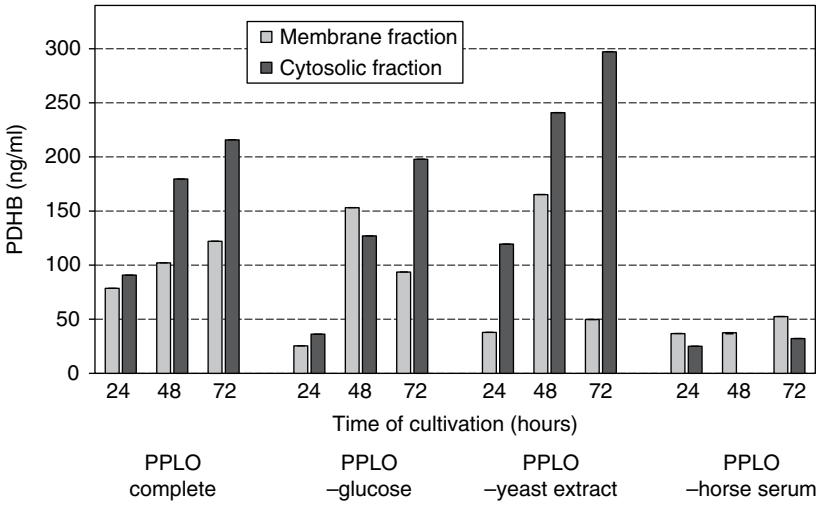

Figure 18.4 Time-dependent quantitative occurrence of PDHB in membrane and cytosolic fraction of total proteins of *M. pneumoniae* M129 under different conditions of incubation as measured by ELISA. Dilutions of recombinant protein PDHB of known concentration were used as standard.

Confirmation of the surface localization of glycolytic enzymes is crucial with respect to the small mycoplasma cells which are 1–2 µm long and 0.1–0.2 µm wide, growing to colonies of diameter <200 µm. Using immunofluorescence, colony blot and mild trypsin treatment of whole cells, the accessibility of PDHB on the surface of *M. pneumoniae* cells was demonstrated (Thomas *et al*. 2013). Further data confirmed that PDHB occurs in high concentrations in the cytoplasm and on the surface of the mycoplasma cell. The amount of surface-localized protein as measured in the fraction of membrane-associated proteins is dependent on the mycoplasma culture conditions (Fig. 18.4). In complete PPLO broth, 36–46% of total PDHB can be found as membrane-associated. A lack of important components of the complex medium for propagation of *M. pneumoniae* such as glucose, yeast extract, or horse serum will cause changes in the overall concentration of PDHB as well as in the proportion of the protein in both protein fractions. However, even major alterations of the nutrient supply with consequences for the metabolism of the bacteria will not prevent the localization of this glycolytic enzyme on the bacterial surface. It can be concluded that the energy-requiring transport of PDHB from cytosol to cell surface is accomplished by the bacteria even under unfavorable nutrient conditions.

In addition, the complex-forming property of PDHA with the main P1 adhesin indicates interaction of this surface-localized glycolytic enzyme with components of the adhesion complex of *M. pneumoniae*. Spontaneous mutants lacking the P1 protein and P40/P90 proteins (products of *mpn142* gene with post-translational cleavage; Sperker *et al*. 1991) are non-adherent and avirulent, confirming the importance of these proteins for survival of the microorganisms under *in vivo* conditions. Using the mutants, the proportion of PDHB in the cytosol and among membrane-associated proteins was determined (Fig. 18.5).

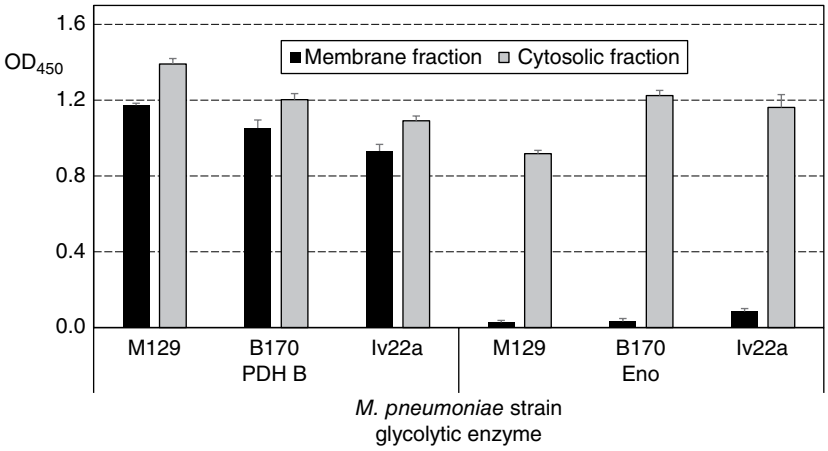

Figure 18.5 Occurrence of subunit B of PDH complex in membrane and cytosolic fraction of total proteins of mutants B170 (lacking adherence-related proteins P40 and P90) and Iv22a (lacking main P1 adhesin) in comparison to type strain M129 of *M. pneumoniae*. Detection of cytosolic enzyme enolase (Eno) was carried out as control.

The relation between the PDHB in both fractions remained nearly constant in the three strains studied. The results show that the integration of PDHB into the complex of membrane-associated proteins of *M. pneumoniae* is independent of the presence of important adherence-related components.

A further aspect of surface-localized proteins of *M. pneumoniae* is a possible role in the pathogenesis of autoimmune diseases characterized by host immune reactions targeting self-proteins. Specific regions of the adhesins P1 and P30 are characterized as cross-reactive with different host factors (Jacobs *et al.* 1995; Dallo *et al.* 1996). Antigenic mimicry of eukaryotic structures may influence the pathogenesis of postinfectious autoimmune phenomena. Extrapulmonary complications of *M. pneumoniae* infections are suggested to be a result of sharing of the antigenic regions of surface proteins of mycoplasmas with those of host factors (Meyer Sauteur *et al.* 2014). Possible interactions between the immune response to *M. pneumoniae* infections and human structures were also described for members of the PDH cluster (Berg *et al.* 2009). A precondition is the induction of specific antibodies in the host after contact with the antigen. In the case of PDHB, strong reactivity of recombinant protein with antisera obtained after s.c. immunization with whole *M. pneumoniae* antigen, Triton X100-insoluble proteins (regarded as membrane-associated proteins), and cytosolic proteins has been demonstrated (Fig. 18.6), confirming the presence of high concentrations of the protein among the total proteins of the bacterium and also the antigenicity of PDHB.

Interestingly, investigation of the serum obtained after intranasal stimulation of animals with viable microorganisms (representing the natural route of infection) showed weak ELISA reactivity of recombinant PDHB. This suggested the presence of a small protein part of PDHB and/or a region with limited antigenicity that is surface-accessible in intact *M. pneumoniae* cells and will be presented to the host immune system. In addition, antibodies to PDHB were investigated in

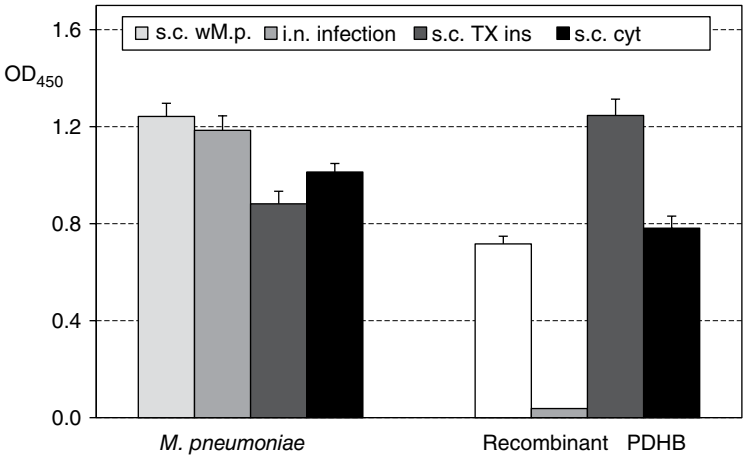

Figure 18.6 ELISA reactivity of total proteins of *M. pneumoniae* and recombinant protein PDHB (10 µg mL^{-1} each) with guinea pig antisera obtained after intranasal (i.n.) infection of animals, after subcutaneous immunization with total antigen (s.c. wM.p.), with fractions of Triton X-100 insoluble proteins (s.c. TX ins) and cytosolic proteins (s.c. cyt) of *M. pneumoniae*.

Table 18.2 Results of reactivity of recombinant protein PDHB with sera ($n = 32$) of patients with symptoms of community-acquired pneumonia and confirmed *M. pneumoniae*-specific antibodies in comparison with total antigen of *M. pneumoniae* (ELISA; secondary antibody: anti-human whole Ig-HRP).

Antigen	Mean OD_{450} ± standard deviation	Minimum–maximum (OD_{450})
Total *M. pneumoniae*	0.887 ± 0.336	0.397–1.663
Recombinant PDHB	0.202 ± 0.238	0.016–1.081

humans, the only natural host (Table 18.2). Sera of patients with confirmed *M. pneumoniae* antibodies reacted with low mean OD values using recombinant PDHB as antigen. However, around 12% of sera demonstrated high reactivity (>OD 0.6). Additional studies are necessary to clarify whether PDHB is a further *M. pneumoniae*-specific antigen involved in the pathogenesis of autoimmune reactions in humans.

18.4 Conclusions

With their greatly reduced genomes, members of the *Mollicutes* class are excellent models for studying moonlighting proteins. Until now, investigation of surface-exposed glycolytic enzymes in these bacteria has resulted in a number of candidates and proteins with a confirmed dual role in cytosol-based and membrane-associated functions. Among them are common multifunctioning proteins such as GAPDH and enolase. In the human pathogenic species *M. pneumoniae*, PDHB has been characterized in different reports as a surface-localized protein that interacts with human plasminogen and fibronectin. The protein is

present in high concentrations in both protein fractions, the cytosolic and the membrane-associated proteins. Surface localization of PDHB is not hampered by changes in culture conditions and absence of some of the most important components of the complex of adherence-associated proteins of *M. pneumoniae*. Despite the fact that PDHB was reported in only a few species as probably surface-localized, this glycolytic enzyme appears interesting for its possible role in pathogenesis. Future studies will have to determine whether additional proteins in *M. pneumoniae* with a function in cytoplasmic metabolism can be identified as involved in host–pathogen interactions. It can be expected that these further data will contribute to the understanding of the long-term survival of the microorganisms in humans, which result not only in respiratory diseases but also in large numbers of asymptomatic carriers and of PCR-positive respiratory samples from patients after convalescence (Nilsson *et al*. 2008; Spuesens *et al*. 2013).

References

Alvarez RA, Blaylock MW, Baseman JB (2003) Surface localized glyceraldehyde-3-phosphate dehydrogenase of *Mycoplasma genitalium* binds mucin. *Molecular Microbiology* 48: 1417–1425.

Atkinson TP, Balish MF, Waites KB (2008) Epidemiology, clinical manifestations, pathogenesis and laboratory detection of *Mycoplasma pneumoniae* infections. *FEMS Microbiology Reviews* 32: 956–973.

Bao S, Guo X, Yu S, Ding J, Tan L, Zhang F, Sun Y, Qiu X, Chen G, Ding C (2014) *Mycoplasma synoviae* enolase is a plasminogen/fibronectin binding protein. *BMC Veterinary Research* 10: 223.

Berg CP, Kannan TR, Klein R, Gregor M, Baseman JB, Wesselborg S, Lauber K, Stein GM (2009) Mycoplasma antigens as a possible trigger for the induction of antimitochondrial antibodies in primary biliary cirrhosis. *Liver International* 29: 797–809.

Bergmann S, Wild D, Diekmann O, Frank R, Bracht D, Chhatwal GS, Hammerschmidt S (2003) Identification of a novel plasmin(ogen)-binding motif in surface displayed alpha-enolase of *Streptococcus pneumoniae*. *Molecular Microbiology* 49: 411–423.

Catrein I, Herrmann R (2011) The proteome of *Mycoplasma pneumoniae*, a supposedly "simple" cell. *Proteomics* 11: 3614–3632.

Chen H, Yu S, Shen X, Chen D, Qiu X, Song C, Ding C (2011) The *Mycoplasma gallisepticum* α-enolase is cell surface-exposed and mediates adherence by binding to chicken plasminogen. *Microbial Pathogenesis* 51: 285–290.

Citti C, Blanchard A (2013) Mycoplasmas and their host: emerging and re-emerging minimal pathogens. *Trends in Microbiology* 21: 196–203.

Dallo SF, Lazzell AL, Chavoya A, Reddy SP, Baseman JB (1996) Biofunctional domains of the *Mycoplasma pneumoniae* P30 adhesin. *Infection and Immunity* 64: 2595–2601.

Dallo SF, Kannan TR, Blaylock MW, Baseman JB (2002) Elongation factor Tu and E1 β subunit of pyruvate dehydrogenase complex act as fibronectin binding proteins in *Mycoplasma pneumoniae*. *Molecular Microbiology* 46: 1041–1051.

Dandekar T, Huynen M, Regula JT, Ueberle B, Zimmermann CU, Andrade MA, Doerks T, Sánchez-Pulido L, Snel B, Suyama M, Yuan YP, Herrmann R, Bork P (2000) Re-annotating the *Mycoplasma pneumoniae* genome sequence: adding value, function and reading frames. *Nucleic Acids Research* 28: 3278–3288.

Dumke R, Hausner M, Jacobs E (2011) Role of *Mycoplasma pneumoniae* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in mediating interactions with the human extracellular matrix. *Microbiology* 157: 2328–2338.

Dumke R, Schnee C, Pletz MW, Rupp J, Jacobs E, Sachse K, Rohde G, CAPNETZ study group (2015) *Mycoplasma pneumoniae* and *Chlamydia* spp. infection in community-acquired pneumonia, Germany, 2011–2012. *Emerging Infectious Diseases* 21: 426–434.

Göhlmann HW, Weiner J 3rd, Schön A, Herrmann R (2000) Identification of a small RNA within the pdh gene cluster of *Mycoplasma pneumoniae* and *Mycoplasma genitalium*. *Journal of Bacteriology* 182: 3281–3284.

Hames C, Halbedel S, Schilling O, Stülke J (2005) Multiple-mutation reaction: a method for simultaneous introduction of multiple mutations into the glpK gene of *Mycoplasma pneumoniae*. *Applied and Environmental Microbiology* 71: 4097–4100.

Hames C, Halbedel S, Hoppert M, Frey J, Stülke J (2009) Glycerol metabolism is important for cytotoxicity of *Mycoplasma pneumoniae*. *Journal of Bacteriology* 191: 747–753.

Hasselbring BM, Jordan JL, Krause RW, Krause DC (2006) Terminal organelle development in the cell wall-less bacterium *Mycoplasma pneumoniae*. *Proceedings of the National Academy of Science, USA* 103: 16478–16483.

Hoelzle LE, Hoelzle K, Helbling M, Aupperle H, Schoon HA, Ritzmann M, Heinritzi K, Felder KM, Wittenbrink MM (2007) MSG1, a surface-localised protein of *Mycoplasma suis* is involved in the adhesion to erythrocytes. *Microbes and Infection* 9: 466–474.

Hopfe M, Henrich B (2014) Multifunctional cytadherence factors. In *Mollicutes Molecular Biology and Pathogenesis* (eds G.F. Browning and C. Citti). Caister Academic Press, Norfolk, England.

Inamine JM, Ho KC, Loechel S, Hu PC (1990) Evidence that UGA is read as a tryptophan codon rather than as a stop codon by *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, and *Mycoplasma gallisepticum*. *Journal of Bacteriology* 172: 504–506.

Jacobs E, Bartl A, Oberle K, Schiltz E (1995) Molecular mimicry by *Mycoplasma pneumoniae* to evade the induction of adherence inhibiting antibodies. *Journal of Medical Microbiology* 43: 422–429.

Kannan TR, Baseman JB (2006) ADP-ribosylating and vacuolating cytotoxin of *Mycoplasma pneumoniae* represents unique virulence determinant among bacterial pathogens. *Proceedings of the National Academy of Science, USA* 103: 6724–6729.

Kühner S, van Noort V, Betts MJ, Leo-Macias A, Batisse C, Rode M, Yamada T, Maier T, Bader S, Beltran-Alvarez P, Castaño-Diez D, Chen WH, Devos D, Güell M, Norambuena T, Racke I, Rybin V, Schmidt A, Yus E, Aebersold R, Herrmann R, Böttcher B, Frangakis AS, Russell RB, Serrano L, Bork P, Gavin AC (2009) Proteome organization in a genome-reduced bacterium. *Science* 326: 1235–1240.

Labroussaa F, Dubrana MP, Arricau-Bouvery N, Beven L, Saillard C (2011) Involvement of a minimal actin-binding region of *Spiroplasma citri* phosphoglycerate kinase in spiroplasma transmission by its leafhopper vector. *PLoS ONE* 6: e17357.

Layh-Schmitt G, Podtelejnikov A, Mann M (2000) Proteins complexed to the P1 adhesin of *Mycoplasma pneumoniae*. *Microbiology* 146: 741–747.

Matic JN, Wilton JL, Towers RJ, Scarman AL, Minion FC, Walker MJ, Djordjevic SP (2003) The pyruvate dehydrogenase complex of *Mycoplasma hyopneumoniae* contains a novel lipoyl domain arrangement. *Gene* 319: 99–106.

Meyer Sauteur PM, Jacobs BC, Spuesens EB, Jacobs E, Nadal D, Vink C, van Rossum AM (2014) Antibody responses to *Mycoplasma pneumoniae*: role in pathogenesis and diagnosis of encephalitis? *PLoS Pathogens* 10: e1003983.

Nilsson AC, Björkman P, Persson K (2008) Polymerase chain reaction is superior to serology for the diagnosis of acute *Mycoplasma pneumoniae* infection and reveals a high rate of persistent infection. *BMC Microbiology* 8: 93–100.

Nogueira SV, Backstedt BT, Smith AA, Qin J, Wunder EA, Ko A, Pal U (2013) *Leptospira interrogans* enolase is secreted extracellularly and interacts with plasminogen. *PLoS ONE* 8: e78150.

Pinto PM, Chemale G, de Castro LA, Costa AP, Kich JD, Vainstein MH, Zaha A, Ferreira HB (2007) Proteomic survey of the pathogenic *Mycoplasma hyopneumoniae* strain 7448 and identification of novel post-translationally modified and antigenic proteins. *Veterinary Microbiology* 121: 83–93.

Regula JT, Boguth G, Görg A, Hegermann J, Mayer F, Frank R, Herrmann R (2001) Defining the mycoplasma "cytoskeleton": the protein composition of the Triton X-100 insoluble fraction of the bacterium *Mycoplasma pneumoniae* determined by 2-D gel electrophoresis and mass spectrometry. *Microbiology* 147: 1045–1057.

Renaudin J, Breton M, Citti C (2014) Molecular genetic tools for Mollicutes. In *Mollicutes Molecular Biology and Pathogenesis* (eds G.F. Browning and C. Citti). Caister Academic Press, Norfolk, England.

Sanderson-Smith ML, De Oliveira DMP, Ranson M, McArthur JD (2012) Bacterial plasminogen receptors: mediators of a multifaceted relationship. *Journal of Biomedicine and Biotechnology* 2012: 272148.

Schmidl SR, Gronau K, Pietack N, Hecker M, Becher D, Stülke J (2010) The phosphoproteome of the minimal bacterium *Mycoplasma pneumoniae*: analysis of the complete known Ser/Thr kinome suggests the existence of novel kinases. *Molecular and Cellular Proteomics* 9: 1228–1242.

Schreiner SA, Sokoli A, Felder KM, Wittenbrink MM, Schwarzenbach S, Guhl B, Hoelzle K, Hoelzle LE (2012) The surface-localised α-enolase of *Mycoplasma suis* is an adhesion protein. *Veterinary Microbiology* 156: 88–95.

Song Z, Li Y, Liu Y, Xin J, Zou X, Sun W (2012) α-Enolase, an adhesion-related factor of *Mycoplasma bovis*. *PLoS ONE* 7: e38836.

Sperker B, Hu P, Herrmann R (1991) Identification of gene products of the P1 operon of *Mycoplasma pneumoniae*. *Molecular Microbiology* 5: 299–306.

Spuesens EBM, Fraaij PLA, Visser EG, Hoogenboezem T, Hop WCJ, van Adrichem LNA, Weber F, Moll HA, Broekman B, Berger MY, van Rijsoort-Vos T, van Belkum A, Schutten M, Pas SD, Osterhaus AD, Hartwig NG, Vink C, van Rossum

AM (2013) Carriage of *Mycoplasma pneumoniae* in the upper respiratory tract of symptomatic and asymptomatic children: an observational study. *PLoS Med* 10: e1001444.

Sun Z, Fu P, Wei K, Zhang H, Zhang Y, Xu J, Jiang F, Liu X, Xu W, Wu W (2014) Identification of novel immunogenic proteins from *Mycoplasma bovis* and establishment of an indirect ELISA based on recombinant E1 beta subunit of the pyruvate dehydrogenase complex. *PLoS ONE* 9: e88328.

Thomas C, Jacobs E, Dumke R (2013) Characterization of pyruvate dehydrogenase subunit B and enolase as plasminogen-binding proteins in *Mycoplasma pneumoniae*. *Microbiology* 159: 352–365.

Vastano V, Salzillo M, Siciliano RA, Muscariello L, Sacco M, Marasco R (2014) The E1 beta-subunit of pyruvate dehydrogenase is surface-expressed in *Lactobacillus plantarum* and binds fibronectin. *Microbiological Research* 169: 121–127.

Wallbrandt P, Tegman V, Jonsson BH, Wieslander A (1992) Identification and analysis of the genes coding for the putative pyruvate dehydrogenase enzyme complex in *Acholeplasma laidlawii*. *Journal of Bacteriology* 174: 1388–1396.

Yavlovich A, Katzenell A, Tarshis M, Higazi AA, Rottem S (2004) *Mycoplasma fermentans* binds to and invades HeLa cells: involvement of plasminogen and urokinase. *Infection and Immunity* 72: 5004–5011.

Yavlovich A, Rechnitzer H, Rottem S (2007) α-Enolase resides on the cell surface of *Mycoplasma fermentans* and binds human plasminogen. *Infection and Immunity* 75: 5716–5719.

3.7

Miscellaneous Bacterial Moonlighting Virulence Proteins

19

Unexpected Interactions of Leptospiral Ef-Tu and Enolase

Natália Salazar and Angela Barbosa

Laboratório de Bacteriologia, Instituto Butantan, São Paulo, Brazil

19.1 *Leptospira* –Host Interactions

Spirochaetes of the genus *Leptospira* may cause leptospirosis, a zoonosis of worldwide distribution. Highly frequent in tropical and subtropical areas, the disease represents an important public health problem. The *Leptospira* genus includes pathogenic and saprophytic species, which are classified into more than 300 serovars (Hartskeerl *et al*. 2011). Pathogenic leptospires have evolved virulence strategies to successfully colonize a variety of hosts. During infection, they express a number of surface-exposed proteins capable of binding to ECM molecules, including laminin and type IV collagen, the major constituents of the basement membrane, as well as cellular and plasma fibronectin, type I collagen, elastin, tropoelastin, and proteoglycans (Barbosa *et al*. 2006; Choy *et al*. 2007; Breiner *et al*. 2009; Lin *et al*. 2009). Binding to host cells has also been reported, notably to kidney epithelial cells, macrophages, fibroblasts, and endothelial cells (Thomas and Higbie 1990; Liu *et al*. 2007). Resistance to the bactericidal effect of host serum is another important attribute of virulent *Leptospira* strains. The ability to escape the natural defense mechanisms of the body relies mainly on their capacity to overcome complement-mediated killing (reviewed in Fraga *et al*. 2011). Among these mechanisms are the acquisition of factor H (FH) and C4b Binding Protein (C4BP), the soluble regulators of the classical, alternative, and lectin pathways of complement (Meri *et al*. 2005; Barbosa *et al*. 2009). Binding to human vitronectin, a negative regulator of the terminal pathway of complement, has recently been reported, indicating that leptospires are able to control both the early and late steps of the complement cascade (Silva *et al*. 2014). Moreover, the secretion of proteases capable of cleaving key complement molecules also contributes to *Leptospira* immune evasion (Fraga *et al*. 2014). By possessing multiple complement escape strategies, pathogenic *Leptospira* efficiently circumvent the host's innate immune responses. Leptospires are also capable of binding plasminogen on their surfaces (Vieira *et al*. 2009; Verma

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

Table 19.1 *Leptospira* proteins exhibiting moonlighting activities.

	Enolase	Ef-Tu
Function associated with cytosolic localization	Glycolytic enzyme: catalysis of 2- phosphoglycerate to phosphoenolpyruvate	Protein synthesis
Functions associated with surface localization	Interaction with plasminogen	Interaction with: ECM components and coagulation cascade molecules; complement FH (bound-FH displays cofactor activity mediating C3b degradation by FI); and plasminogen (bound-plasmin(ogen) cleaves C3b and fibrinogen)

et al. 2010). In the presence of host-specific activators, bound plasminogen is converted to plasmin. This key enzyme of the coagulation system may contribute to bacterial invasion and immune evasion by degrading ECM molecules (Ponting *et al.* 1992), and also by cleaving the central complement components C3b and C5 (Barthel *et al.* 2012).

Various *Leptospira* surface-exposed molecules, most of them lipoproteins, have been shown to have a considerable significance in virulence by playing key roles in adhesion, invasion, and immune evasion of the host complement pathways (reviewed in Fraga *et al.* 2011). As such, some of them seem to be potential vaccine candidates (Silva *et al.* 2007; Faisal *et al.* 2008, 2009; Yan *et al.* 2009). In addition to the panoply of "classical" outer membrane proteins having a presumed role in *Leptospira* pathogenesis described to date, recent studies have reported the involvement of leptospiral Ef-Tu and enolase in plasminogen recruitment and/or adhesion to host ECM and immune evasion (Table 19.1). The moonlighting activities of such proteins are addressed in the following sections.

19.2 *Leptospira* Ef-Tu

In a recent report, Wolff *et al.* (2013) clearly demonstrated the presence of Ef-Tu on the surface of leptospires, which strongly suggested that, besides its classical role in protein synthesis within the cytoplasm, this protein could have additional functions as a bacterial receptor for host proteins. Indeed, *Leptospira* Ef-Tu was shown to interact with multiple ECM components, including type I and type IV collagens, cellular fibronectin, plasma fibronectin, laminin, and elastin (Wolff *et al.* 2013). These interactions may contribute to initial bacterial adhesion. Binding to the coagulation cascade molecules, fibrinogen, and plasminogen has also been described (Wolff *et al.* 2013). Plasminogen, an inactive proenzyme, is converted into the protease plasmin by a variety of enzymes, including tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA). The latter mediates cell-surface-associated plasminogen activation (Castellino and Ploplis 2005). The broad specificity of plasmin enables the enzyme to target different substrates, including fibrin, fibrinogen, complement C3 and C5, vitronectin, osteocalcin,

factors V, VIII, and X, protease-activated receptor 1, injury-induced aggregated proteins, and some collagenases (reviewed in Kuusela *et al*. 1992). Plasminogen bound to *Leptospira* Ef-Tu is converted to functionally active plasmin in the presence of exogenously supplied uPA and is able to degrade fibrinogen, one of its physiological substrates. The fibrinogen α-chain was completely degraded by Ef-Tu-bound plasmin (Wolff *et al*. 2013). A number of microorganisms benefit from plasmin proteolytic activity in order to disseminate within a host (see Chapters 15 and 16). Acquired plasminogen may aid tissue penetration and invasion during infection. A recent study has characterized plasmin as a complement regulator, modulator, and inhibitor (Barthel *et al*. 2012). Plasmin was shown to bind to the central complement protein C3, as well as to C3 activation products and C5. Moreover, it inhibits complement by cleaving C3b and C5. As a proenzyme, plasminogen enhances cofactor activity of FH, the main negative regulator of the alternative pathway of complement (Barthel *et al*. 2012). In this way, plasmin(ogen) affects complement activation in multiple ways. A number of disease-causing pathogens, including pathogenic *Leptospira* strains, acquire complement regulators and plasminogen simultaneously (Meri *et al*. 2005; Barbosa *et al*. 2009; Vieira *et al*. 2009; Castiblanco-Valencia *et al*. 2012). Plasmin bound to *Leptospira* Ef-Tu also presents proteolytic activity against complement C3b (Wolff *et al*. 2013). Another interesting property of *Leptospira* Ef-Tu is the capacity to recruit FH from human serum (Wolff *et al*. 2013). FH, a 150 kDa plasma protein, inhibits the alternative pathway by preventing binding of Factor B to C3b, thus accelerating decay of the C3 convertase C3bBb, and by acting as a cofactor for the serine protease Factor I in the cleavage of C3b (Whaley and Ruddy 1976; Pangburn *et al*. 1977). Interestingly, FH bound to *Leptospira* Ef-Tu remains functionally active, working as a cofactor for FI (Wolff *et al*. 2013). In conclusion, by acquiring both plasminogen and FH, Ef-Tu may contribute to leptospiral immune evasion through inhibition of complement activation.

19.3 *Leptospira* Enolase

Enolases from a number of bacteria have been shown to function as adhesins (see Chapters 13–15). Among its targets are host ECM molecules and cells. In pneumococci, enolase can also function as an immune evasion protein by interacting with the complement inhibitor C4BP, consequently leading to diminished C3b deposition on the bacterial surface (Agarwal *et al*. 2012). This finding is reviewed in Chapter 13. Another well-described moonlighting activity exhibited by enolase is its capacity to recruit host plasminogen (see also Chapter 15). A recent study by Nogueira *et al*. (2013) has demonstrated that *Leptospira interrogans* enolase is secreted extracellularly and then re-associates with the bacterial surface. This interesting phenomenon has been described in Gram-positive bacteria, known to display cell-surface adhesins and invasins devoid of signal sequences for their secretion or a membrane anchor (Chhatwal 2002). The Gram-negative bacterium *Leptospira* also secretes enolase by an as-yet-unknown mechanism. Enolase antibodies recognize intact, non-permeabilized bacteria, suggesting that the protein is exposed at the leptospiral surface. Moreover, a serum antibody response to

enolase following experimental infection of hamsters with pathogenic *Leptospira* was detectable (Nogueira *et al*. 2013). On the spirochetal surface, enolase displays plasminogen-binding activity. *L. interrogans*–plasminogen interactions could be inhibited by recombinant enolase as well as by polyclonal antibodies against this protein (Nogueira *et al*. 2013). It has been demonstrated that *Borrelia burgdorferi*, the Lyme disease spirochete, also recruits host plasminogen through surface-exposed enolase (Floden *et al*. 2011; see also Chapter 15). Plasminogen contributes to efficient dissemination in ticks and enhances spirochetemia in mice (Coleman *et al*. 1997). As a moonlighting protein, enolase may therefore facilitate spirochetal dissemination, contributing to bacterial virulence.

19.4 Conclusions

It is likely that a growing number of proteins that moonlight will be identified in spirochetes in the next few years. Certain bacteria are presently known to benefit from the multifunctionality of up to 20 different moonlighting proteins (reviewed in Chapter 5). Given their importance in virulence, it can be anticipated that the spirochetal "moonlight-ome" will be expanded in the near future.

References

Agarwal V, Hammerschmidt S, Malm S, Bergmann S, Riesbeck K, Blom AM (2012) Enolase of *Streptococcus pneumoniae* binds human complement inhibitor C4b-binding protein and contributes to complement evasion. *Journal of Immunology* 189: 3575–3584.

Barbosa AS, Abreu PA, Neves FO, Atzingen MV, Watanabe MM,Vieira ML, Morais ZM, Vasconcellos SA, Nascimento AL (2006) A newly identified leptospiral adhesin mediates attachment to laminin. *Infection and Immunity* 74: 6356–6364.

Barbosa AS, Abreu PA, Vasconcellos SA, Morais ZM, Gonçales AP, Silva AS, Isaac L (2009) Immune evasion of *Leptospira* species by acquisition of human complement regulator C4BP. *Infection and Immunity* 77: 1137–1143.

Barthel D, Schindler S, Zipfel PF (2012) Plasminogen is a complement inhibitor. *Journal of Biological Chemistry* 287: 18831–18842.

Breiner DD, Fahey M, Salvador R, Novakova J, Coburn J (2009) *Leptospira interrogans* binds to human cell surface receptors including proteoglycans. *Infection and Immunity* 77: 5528–5536.

Castellino FJ, Ploplis VA (2005) Structure and function of the plasminogen/plasmin system. *Thrombosis and Haemostasis* 93: 647–654.

Castiblanco-Valencia MM, Fraga TR, Silva LB, Monaris D, Abreu PA, Strobel S, Józsi M, Isaac L, Barbosa AS (2012) Leptospiral immunoglobulin-like proteins interact with human complement regulators factor H, FHL-1, FHR-1, and C4BP. *Journal of Infectious Diseases* 205: 995–1004.

Chhatwal GS (2002) Anchorless adhesins and invasins of Gram-positive bacteria: a new class of virulence factors. *Trends in Microbiology* 10: 205–208.

Choy HA, Kelley MM, Chen TL, Møller AK, Matsunaga J,Haake DA (2007) Physiological osmotic induction of *Leptospira interrogans* adhesion: LigA and LigB bind extracellular matrix proteins and fibrinogen. *Infection and Immunity* 75: 2441–2450.

Coleman JL, Gebbia JA, Piesman J, Degen JL, Bugge TH, Benach JL (1997) Plasminogen is required for efficient dissemination of *B. burgdorferi* in ticks and for enhancement of spirochetemia in mice. *Cell* 89: 1111–1119.

Faisal SM, Yan W, Chen CS, Palaniappan RU, McDonough SP, Chang YF (2008) Evaluation of protective immunity of *Leptospira* immunoglobulin like protein A (LigA) DNA vaccine against challenge in hamsters. *Vaccine* 26: 277–287.

Faisal SM, Yan W, McDonough SP, Chang YF (2009) *Leptospira* immunoglobulin-like protein A variable region (LigAvar) incorporated in liposomes and PLGA microspheres produces a robust immune response correlating to protective immunity. *Vaccine* 27: 378–387.

Floden AM, Watt JA, Brissette CA (2011) *Borrelia burgdorferi* enolase is a surface-exposed plasminogen binding protein. *PLoS One* 6: e27502.

Fraga TR, Barbosa AS, Isaac L (2011) Leptospirosis: aspects of innate immunity, immunopathogenesis and immune evasion from the complement system. *Scandinavian Journal of Immunology* 73: 408–419.

Fraga TR, dos Santos Courrol D, Castiblanco-Valencia MM, Hirata IY, Vasconcellos SA, Juliano L, Isaac L (2014) Immune evasion by pathogenic *Leptospira* strains: the secretion of proteases that directly cleave complement proteins. *Journal of Infectious Diseases* 209: 876–886.

Hartskeerl RA, Collares-Pereira M, Ellis WA (2011) Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. *Clinical Microbiology and Infection* 17: 494–501.

Kuusela P, Ullberg M, Saksela O, Kronvall G (1992) Tissue-type plasminogen activator-mediated activation of plasminogen on the surface of group A, C, and G streptococci. *Infection and Immunity* 60: 196–201.

Liu Y, Zheng W, Li L, Mao Y, Yan J (2007) Pathogenesis of leptospirosis: interaction of *Leptospira interrogans* with in vitro cultured mammalian cells. *Medical Microbiology and Immunology* 196: 233–239.

Lin YP, Lee DW, McDonough SP, Nicholson LK, Sharma Y, Chang YF (2009) Repeated domains of *Leptospira* immunoglobulin-likeproteins interact with elastin and tropoelastin. *Journal of Biological Chemistry* 284: 19380–19391.

Meri T, Murgia R, Stefanel P, Meri S, Cinco M (2005) Regulation of complement activation at the C3-level by serum resistant leptospires. *Microbial Pathogenesis* 39: 139–147.

Nogueira SV, Backstedt BT, Smith AA, Qin JH, Wunder Jr EA, Ko A, Pal U (2013) *Leptospira interrogans* enolase is secreted extracellularly and interacts with plasminogen. *PloS One 8*: e78150.

Pangburn MK, Schreiber RD, Müller-Eberhard HJ (1977) Human complement C3b inactivator: isolation, characterization, and demonstration of an absolute requirement for the serum protein beta1H for cleavage of C3b and C4b in solution. *Journal of Experimental Medicine* 146: 257–270.

Ponting CP, Marshall JM, Cederholm-Williams SA (1992) Plasminogen: a structural review. *Blood Coagulation and Fibrinolysis* 3: 605–614.

Silva EF, Medeiros MA, McBride AJ, Matsunaga J, Esteves GS, Ramos JG, Santos CS, Croda J, Homma A, Dellagostin OA, Haake DA, Reis MG, Ko AI (2007) The terminal portion of leptospiral immunoglobulin-like protein LigA confers protective immunity against lethal infection in the hamster model of leptospirosis. *Vaccine* 25: 6277–6286.

Silva LB, Miragaia LD, Breda LC, Abe CM, Schmidt MC, Moro AM, Monaris D, Conde JN, Józsi M, Isaac L, Abreu PA, Barbosa AS (2014) Pathogenic *Leptospira* acquire Factor H and vitronectin via the surface protein LcpA. *Infection and Immunity* pii: IAI.02844-14.

Thomas DD, Higbie LM (1990) In vitro association of leptospires with host cells. *Infection and Immunity* 58: 581–585.

Verma A, Brissette CA, Bowman AA, Shah ST, Zipfel PF, Stevenson B (2010) Leptospiral endostatin-like protein A is a bacterial cell surface receptor for human plasminogen. *Infection and Immunity* 78: 2053–2059.

Vieira ML, Vasconcellos SA, Gonçales AP, de Morais ZM, Nascimento AL (2009) Plasminogen acquisition and activation at the surface of *Leptospira* species lead to fibronectin degradation. *Infection and Immunity* 77: 4092–4101.

Whaley K, Ruddy S (1976) Modulation of the alternative complement pathways by beta 1 H globulin. *Journal of Experimental Medicine* 144: 1147–1163.

Wolff DG, Castiblanco-Valencia MM, Abe CM, Monaris D, Morais ZM, Souza GO, Vasconcellos SA, Isaac L, Abreu PA, Barbosa AS (2013) Interaction of *Leptospira* elongation factor tu with plasminogen and complement factor h: a metabolic leptospiral protein with moonlighting activities. *PloS One 8*: e81818.

Yan W, Faisal SM, McDonough SP, Divers TJ, Barr SC, Chang CF, Pan MJ, Chang YF (2009) Immunogenicity and protective efficacy of recombinant *Leptospira* immunoglobulin-like protein B (rLigB) in a hamster challenge model. *Microbes and Infection* 11: 230–237.

20

Mycobacterium tuberculosis Antigen 85 Family Proteins: Mycolyl Transferases and Matrix-Binding Adhesins

Christopher P. Ptak[1], Chih-Jung Kuo[2], and Yung-Fu Chang[1]

[1] *Department of Population Medicine and Diagnostic Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY, USA*
[2] *College of Veterinary Medicine, National Chung Hsing University, Taichung, Taiwan*

20.1 Introduction

The Gram-positive bacteria of the *Corynebacteriales* order contain several notable human and animal pathogens (e.g., *Corynebacterium diphtheria*, *Mycobacterium tuberculosis, M. leprae, M. bovis*, and *M. avium* subsp. paratuberculosis; Gao and Gupta 2012). *Mycobacter* spp., the most widespread and clinically important of these related disease-causing bacteria, are responsible for Johne's disease, leprosy, and tuberculosis (TB) and one of these organisms, *M. avium* subsp. *paratuberculosis*, is also suspected to be a causative agent in Crohn's disease (Greenstein 2003). TB infects about one-third of the world's population with the number of new cases per year continuing to grow (Dye and Williams 2010). Approximately 10% of individuals develop active infections leading to over a million worldwide deaths per year. Acid-fast stain diagnostic screening has been adopted as a common detection method of *M. tuberculosis*, the causative agent of TB (Madison 2001). Positive acid-fast staining results when phenol-based stains are able to permeate into bacterial cells, but acid-alcohols, stain-decolorizing agents, are blocked from efficiently entering the cells. The presence of a dense, lipid-rich outer cell wall has not only been identified as the mechanism of acid-fastness but also provides increased protection against lipophilic antibiotics (Bhatt *et al*. 2007). Mycolic acids, a prominent component of the mycobacterial outer membrane, are required for growth and survival of *Mycobacterium* species (Portevin *et al*. 2004). This chapter is devoted to the class of enzymes that catalyze the final steps of mycolic acid formation but also moonlight as host-binding adhesins.

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

20.2 Identification of Antigen 85

Because of the medical importance of *M. tuberculosis*, much of the early work on proteins at and near the mycobacterial surface was driven by a need to improve TB vaccines. Bacterial surface-associated proteins can be highly accessible to interactions with host macromolecules. The external presentation of proteins is often necessary for a pathogen to sense and respond to external stimuli and may include receptors that stimulate the upregulation of enzymes and transporters required to obtain a nutrient of limited availability. Other surface proteins are directly involved in pathogenic actions such as host adhesion and immune system evasion. Whatever the functional role of host-exposed surface proteins, they offer an opportunity for inducing a targeted immune response as antigens. Characterization of mycobacterial antigens has led to the development of new vaccine therapies. While modern antigen identification requires gene cloning and protein purification, early work to characterize mycobacterial antigens involved hyperimmune antisera reactivity, counter immune electrophoresis (CIE), and polyacrylamide gel electrophoresis (PAGE). Secreted antigens are the most abundant and were the first to be characterized using mycobacterial culture fluid. For the major antigens, multiple competing antigen designations emerged and nomenclature for the most abundant *M. tuberculosis* antigen has included α-antigen, antigen 6 US-Japan, P32 antigen, and antigen 85 (Wiker and Harboe 1992). Eventually, the CIE-derived antigen 85 (Ag85) designation was adopted for the protein species reactive to the HYT27 monoclonal antibody. Despite a substantial increase in our understanding of Ag85's structure and function, this nomenclature has persevered as a testament to its importance as a mycobacterial antigen. Further immunological and sequencing studies have allowed the differentiation of Ag85 into three distinct protein species with similar molecular weights and the consolidation of these species with the PAGE-derived MPT designations: Ag85A (31 kDa, MPT44); Ag85B (30 kDa, MPT59); and Ag85C (31.5 kDa, MPT45) (Matsuo *et al*. 1988; Borremans *et al*. 1989; Dheenadhayalan *et al*. 2002). Although these three Ag85 proteins do not form a higher-order heteromeric structure, they are often referred to as the Ag85 complex in scientific literature (Wiker *et al*. 1990). Occasionally, Ag85 proteins are called fibronectin (Fn) -binding proteins (FbpA, FbpB, and FbpC) because Ag85's first described function was binding to the extracellular host protein, Fn (Abouzeid *et al*. 1988).

The discovery of Ag85 proteins has led to the biotechnological advancement of mycobacterial disease-related vaccines. Currently, the majority of vaccines under development for the treatment of tuberculosis incorporate a component that is intended to enhance the immune response against Ag85 proteins (Principi and Esposito 2015). Ag85A and Ag85B have the strongest Ag85 T-cell response with a high degree of species cross-reactivity, suggesting a wide disease protective potential (Huygen 2014). Multiple delivery methods of Ag85 proteins, including overexpression in non-virulent or attenuated strains, direct incorporation as a recombinant protein adjuvant, and viral vectored vaccines, have undergone clinical trials. While the new generation of Ag85 targeting vaccines have not proven to be more effective than the older versions of vaccination using non-virulent

strains, significant progress is being made towards understanding the complex mechanisms that mycobacteria employ to establish latent infections by balancing the host immune response. Ag85 proteins have also been found to be protective antigens against *M. avium* subsp. *paratuberculosis* challenge in mice, calves, and goats (Kathaperumal *et al*. 2008, 2009; Park *et al*. 2008; Chandra *et al*. 2012).

20.3 Antigen 85 Family Proteins: Mycolyl Transferases

It was not until 1997, almost ten years after its discovery as a Fn-binding protein (Abouzeid *et al*. 1988), that Ag85's role in mycomembrane biogenesis was described (Belisle *et al*. 1997). Interestingly, the enzymatic role of Ag85 as a mycolyl transferase represents its primary function. Antigen 85 proteins catalyze the final trehalose conjugation step in synthesis of several important glycolipids of the mycobacterial cell wall.

20.3.1 Role of the Mycomembrane

A defining characteristic of *M. tuberculosis*, as well as most members of the *Corynebacteriales* order, is the mycomembrane, a mycolic acid-enriched outer cell wall (Marrakchi *et al*. 2014). Mycolic acids, specialized long-chain fatty acids, contribute to the formation of an outer protective coat that is highly impermeable to hydrophobic molecules. In mycobacteria, mycolic acids are composed of two branches of differing length so that the longer C_{42}-C_{62} mero-chain is folded and packed tightly within the interior of the membrane to provide a hydrophobic match with the shorter C_{22}-C_{26} α-branch. The outer leaflets contain free phospholipids and mycolates. Trehalose mycolates exist as both trehalose monomycolate and a fused version, trehalose dimycolate. The inner leaflet also contains phospholipid and mycolates but the majority of the mycolates are covalently attached to the pentaarabinosyl termini of the underlying peptidoglycan–arabinogalactan layer. Modification of the membrane's specific composition confers Mycobacteria with the ability to adapt to a diversity of harsh environments, including those created by host defense systems. The presence of mycolic acids, in particular trehalose dimycolate, increases the mycomembrane's hydrophobic density and contributes to several aspects of tuberculosis infection. In combination with the peptidoglycan–arabinogalactan layer, the thick mycomembrane prevents antibiotics from crossing into the cell. The development of novel mycomembrane inhibitors offers promise in the support of antibiotics that normally fail because of an inability to enter the mycobacteria. Additionally, several physiological properties of mycobacteria including biofilm, cord, and foamy macrophage formation are attributed to the mycolic components of the mycomembrane and can be disrupted by mycomembrane inhibitors.

20.3.2 Ag85 Family of Homologous Proteins

The proteins within the Ag85 family are highly homologous. Through a common catalytic triad, Ag85 proteins are responsible for attaching mycolate species to trehalose to generate trehalose monomycolate, to trehalose monomycolate to

generate trehalose dimycolate, and to arabinogalactan to generate arabinogalactan mycolates (Ronning *et al*. 2000, 2004; Anderson *et al*. 2001). While the Ag85 proteins exhibit the highest degree of sequence homology near the catalytic site, the substrate specificity differs between Ag85A, Ag85B, and Ag85C (Backus *et al*. 2014). Ag85A and Ag85B are more efficient at creating trehalose dimycolate. Conversely, the substrate preference for Ag85C leads to the more efficient production of trehalose monomycolate. In addition, Ag85A preference for short polysaccharide arabinogalans suggests a similar preference in the production of arabinogalactan attached-mycolates. A fourth Ag85 protein family member, Ag85D, has more recently been identified and shown to lack a catalytic site (Wilson *et al*. 2004).

The Ag85 proteins adopt a α/β hydrolase fold characteristic of many lipases, esterases, and dehalogenases (Ronning *et al*. 2000; Lenfant *et al*. 2013). The deviation in the native Ag85 structure of the full set is small, yet several inhibitor bound structures and mutants vary significantly near the active site (Ronning *et al*. 2000, 2004; Anderson *et al*. 2001; Favrot *et al*. 2013, 2014). Two binding pockets can bind to trehalose. One site acts to selectively bind the substrate, then the saccharide is shuttled into the second site which is responsible for catalytical modification. The secondary site controls substrate specificity and also allows more efficient processing of polysaccharides. As proof that the tunnel to the pocket is hydrophobic enough to allow the mycolic acid substrates to enter, Ag85C has been crystallized with a bound detergent molecule (Ronning *et al*. 2004). The mycolated products can then exit the enzyme. The reversible reaction is expected to be limited in the backwards direction due to the sink created by the highly lipophilic products.

20.3.3 Inhibition and Knockouts of Ag85

Anecdotal reports indicate that the triple knockout of the genes encoding Ag85A, Ag85B, and Ag85C is not viable, pointing to the integral importance of the mycolyl transferases to mycobacterial growth and survival. The loss of a single Ag85 gene can be compensated for by other Ag85 family members providing details about the physiological implications of individual Ag85 isoform knockouts; however, the loss of Ag85C significantly impairs the production of arabinogalactan-bound mycolic acids (Jackson *et al*. 1999). Several classes of Ag85 inhibitors have been identified. Inhibitors can bind to the substrate pocket and directly block activity, but also can bind to an allosteric site. Allosteric sites can play an important role in enzyme regulation (Changeux 2012). A novel class of allosteric inhibitors has been shown to form a covalent interaction with a cysteine that is involved in transition state contacts required for enzyme activity (Favrot *et al*. 2013). Mutations to this cysteine also severely impair mycolyl transferase activity. Dynamics of the substrate pocket are essential for enzymatic function and are related to both allosteric inhibition and substrate specificity (Backus *et al*. 2014; Favrot *et al*. 2014). While inhibitors have enhanced the picture of Ag85 function, inhibitor concentrations required to weakly inhibit mycobacterial growth are relatively high and therefore currently offer limited therapeutic application (Belisle *et al*. 1997).

20.4 Antigen 85 Family Proteins: Matrix-Binding Adhesins

The ability of mycobacteria to adhere to extracellular matrix (ECM) and/or plasma proteins plays a crucial role in colonization and dissemination throughout the host. Like other pathogenic bacteria, the initial step in the infection process involves the docking of bacterial surface adhesins (such as MSCRAMMs, microbial surface components recognizing adhesive matrix molecules) to the matrix surrounding host cells. A complex mixture comprising proteoglycans, polysaccharides, proteinaceous fibers, and additional proteins are woven together to impart varying degrees of stiffness and elasticity to specific tissues. Several common ECM proteins, collagens, elastin, Fn, fibrinogen, vitronectin, and laminin function as specific MSCRAMM targets allowing for directed tissue localization (Patti *et al*. 1994; Henderson *et al*. 2011). In addition, MSCRAMM promiscuity for host targets is highly common and increases the opportunity for host attachment. Ag85 proteins were the first mycobacterial proteins identified as mediating macromolecular interactions with host proteins. Positioned on the mycobacterial cell surface for mycomembrane synthesis, Ag85 proteins double their contribution by moonlighting as MSCRAMMs. Ag85 proteins help to establish tuberculosis infections by binding directly to Fn and elastin, both major ECM components of lung and skin tissue.

20.4.1 Abundance and Location

Of the Ag85 proteins Ag85A and Ag85B are particularly abundant, accounting for approximately 40% of the total secreted mycobacterial protein (Fukui *et al*. 1965). After secretion from mycobacteria, Ag85 is not exclusively bound to the mycomembrane surface and Ag85 proteins have been identified in mycobacterial culture fluid. The main catalysis function of Ag85 is the addition of mycolic acids to carbohydrates. Because mycolic acids contribute a large portion of the hydrophobic density to the mycomembrane, these fatty acids are mainly restricted to an association with the bilayer. Ag85 must then also associate with the mycomembrane in order to perform its mycolyl transferase function. The relative degree of mycomembrane localization of secreted Ag85 proteins is thought to be tied to secretion efficiency where Ag85A is the most efficiently secreted and also the most loosely associated Ag85 protein (Wiker *et al*. 1991). Ag85 was also found to associate with detergent extracts as well as solubilized membranes (George and Falkinham 1989; Young and Garbe 1991). Proteomic analysis has further identified Ag85 proteins as components of the mycobacterial cell wall (Wolfe *et al*. 2010).

Using the trehalose-bound Ag85B structure (Anderson *et al*. 2001), the relative orientation of Ag85 to the mycomembrane is likely to allow catalytic site access of substrate molecules that are positioned near the membrane interface, as suggested by Figure 20.1. The relative position is also supported by an Ag85C structure with the detergent octyl-glucoside bound to the active site (Ronning *et al*. 2004). Attachment of Ag85 to the mycobacterial surface has allowed it to adopt

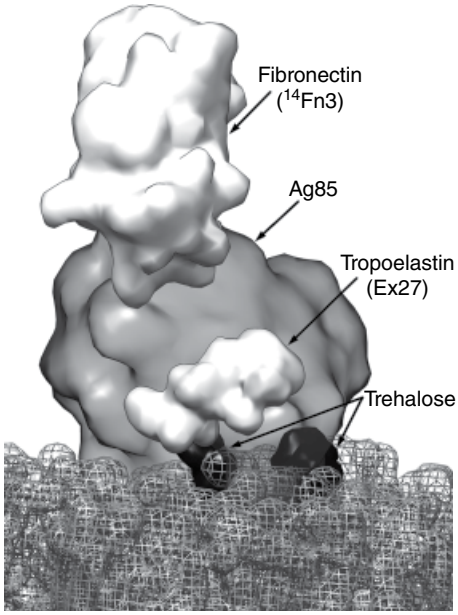

Figure 20.1 An illustrated summary of the multiple roles of Ag85. Ag85 (gray surface) is positioned relative to the mycomembrane (gray mesh) so the trehalose (black surfaces) binding sites are accessible from the membrane surface. The bound locations of host protein domains (white surfaces) are based on experimental models.

the secondary ability of ECM adhesion. Pathogen proteins that moonlight as adhesins generally take advantage of a "geographical" opportunity presented by the locational requirement of their first responsibility. An ideal secondary task, host adhesion, can take advantage of an existing pathogen-attached protein scaffold and remodel a patch to interact with a host ECM protein.

Social psychologists have created the term "residential propinquity" to describe the phenomenon whereby both friendships and romantic relationships tend to form between people living in proximity to each other. In much the same way, a protein's cell-surface localization can evolve into an opportunity for host adhesion and, due to a frequency of shared positioning, can lead to a cycle of reinforcement and strengthening interaction. The mycolyl transferases from pathogenic mycobacteria likely evolved the secondary adhesion function after realizing a niche as obligate pathogens. Indeed, mycolyl transferases from corynebacteria with a high degree of structural similarity to Ag85 proteins have not been shown to exhibit Fn-binding capacity (Huc *et al*. 2013).

20.4.2 Ag85 a Fibronectin-Binding Adhesin

Ag85 binds directly to the ECM glycoprotein Fn with nanomolar binding affinity (Kuo *et al*. 2012). Fn anchors interacting-cell matrix components to cells through an interaction with integrins, while Fn is also an integral part of the matrix assembly process (Potts and Campbell 1994; Vakonakis and Campbell 2007). The 220 kDa Fn monomer is cross-linked by disulfide bonds to form a dimer. Fn is also a highly modular protein composed of 29 domains that can be categorized into three types of module folds: Fn1 (type I); Fn2 (type II); and Fn3 (type III). Multi-module segments of Fn interact with specific proteins in order to direct both higher-order self-assembly and complex assembly of the ECM (Vakonakis

and Campbell 2007). The N-terminal and gelatin-binding segments comprise Fn1 and Fn2 modules ($^{1-6}$Fn1–$^{1-2}$Fn2–$^{7-9}$Fn1) and are followed by 15 consecutive Fn3 modules ($^{1-15}$Fn3) and an additional Fn1-containing terminal segment ($^{10-12}$Fn1). Both integrin-binding and heparin-binding sites are located on the Fn3 module segment. The Ag85-interacting site overlaps with the heparin-binding site, $^{12-14}$Fn3 (Naito *et al.* 2000; Kuo *et al.* 2012).

The Fn3 module is a common immunoglobulin-like, β-sandwich fold composed of about 100 amino acids (Sharma *et al.* 1999). The two β-sheets clasp a core of hydrophobic residues while the surface residues and, particularly, the β-strand connecting loops exhibit a much greater degree of variability (Lappalainen *et al.* 2008). Overall, the Fn3 module shows similarity to the immunoglobulin domains of antibodies with a potential for specific interactions to occur at the loop regions. A combination of ELISA, SPR, and pull-down assays have localized the Ag85-interacting site to the ^{14}Fn3 module which maintains the nanomolar binding affinity of full Fn (Kuo *et al.* 2012). Fn3-based peptides that include the loop region between β-sheets B and C can block the targeted Ag85-Fn interaction. A patch of both charged and hydrophobic residues (QPPR) contribute to the binding surface.

A short surface-exposed helix on Ag85 displays a complimentary patch of charged and hydrophobic residues that bind to the ^{14}Fn3 module (Naito *et al.* 1998; Kuo *et al.* 2012). When Ag85 is positioned with its catalytic trehalose site on a membrane surface, the Fn-binding site is set on the opposite side of the Ag85 molecule and facing the host ECM (Fig. 20.1). Binding of Fn to Ag85 proteins is conserved and the residues involved in the electrostatic interaction are shared for Ag85A, Ag85B, and Ag85C in both *M. tuberculosis* and *M. avium* subsp. *paratuberculosis*. Interestingly, Ag85D also binds to Fn with a motif that is structurally conserved but lacks sequence homology to the other Ag85 proteins (Naito *et al.* 1998; Wilson *et al.* 2004).

20.4.3 Ag85 an Elastin-Binding Adhesin

A second host ECM protein, elastin, is targeted by Ag85 proteins for adhesion. While binding of Ag85 to elastin is not as strong as to Fn, the elastin-Ag85 interaction possesses submicromolar binding affinity (Kuo *et al.* 2013). Binding to elastin is a common mechanism for pathogen–host interactions. Elastin imparts tissue with an increased ability to stretch or contract and is a prevalent ECM component in lung and skin. The mature cross-linked elastin mesh is derived from tropoelastin (TE), a 72 kDa protein composed of over 30 small exon modules (Wise and Weiss 2009). The sequences of the four TE module classes are dominated by conformationally flexible amino acids paired with varying patterns of hydrophobic residues and cross-linkable lysines. The modules control the multiple steps of TE interaction governing elastogenesis, the process that generates the order matrix of elastin molecules. Ag85 interacts with the KA cross-linking modules which are short alanine-based α-helices with an edge of (i + 3/i + 4) lysines (Kuo *et al.* 2013). The lysines form the Ag85-binding site along with a neighboring hydrophobic residue. Human TE contains 10 KA cross-linking modules that could act as binding sites.

The complementary binding site on Ag85 is located along the surface between the expected sites for Fn binding and mycomembrane docking. TE Exon 27 (Ex27) is the KA cross-linking module with the highest measured Ag85B affinity. Relative to the likely position of Ag85 on the mycomembrane, TE Ex27 docks along the side of Ag85 (Fig. 20.1). On Ag85B, a loop containing glutamic acid (E258) forms a grove that cradles TE Ex27 and creates a favorable electrostatic interaction for the positively charged lysines of TE Ex27 to bind (Kuo *et al*. 2013). The negative charge is conserved in MTB Ag85A and Ag85C as an aspartic acid. The docked Ag85B-TE Ex27 complex is strengthened by additional van der Waals contacts between the two proteins. The structure of the binding site pocket overlays closely in all of the native Ag85 structures (Ronning *et al*. 2000, 2004; Anderson *et al*. 2001); however, several Ag85C structures with inhibitors and mutations show a dramatic shift in the TE Ex27 binding-loop (Ronning *et al*. 2000; Favrot *et al*. 2013, 2014). The conformational dynamics of the loop and neighboring active site are linked to the enzyme's catalytic function. Elastin binding could inhibit flexibility in the loop, leading to allosteric inhibition of the mycolyl transferase activity. The possibility that allosteric regulation by ECM components might lock the enzyme onto its substrate is an intriguing mechanism by which the adhesion capability of Ag85 could be enhanced through a limitation of its geographical freedom.

20.4.4 Implication in Disease

Ag85 affords mycobacteria the ability to insulate against many of the host chemical defenses through the mycomembrane. Fn-binding and elastin-binding ability enhance the importance of Ag85 in the initial stage of infection that involves adhesion and dissemination. Disruption of either the Fn or elastin binding interaction with Ag85 can reduce the pathogen's ability to interact with an ECM-coated surface or host cell (Kuo *et al*. 2012, 2013). Adhesion blockers may have some potential in limiting infectivity. While *M. tuberculosis* commonly initiates infections through the lungs of humans and other mammals, other mycobacteria, including *M. avium* subsp. *paratuberculosis*, can invade the intestinal mucosa to cause disease (Bannantine and Bermudez 2013). Tissues susceptible to mycobacterial infection could be targeted by non-invasive drug administration methods. Additionally, Fn-binding provides a mechanism by which mycobacteria can control macrophage internalization. After the initial infection, mycobacteria invade and replicate within the endosomes of macrophages. When mycobacteria are invaginated, Ag85 proteins are downregulated during the adaptation to the intracellular environment (Rohde *et al*. 2007; Mustafa *et al*. 2014). In fact, mycobacteria have been shown to lose acid-fastness yet thickening of the cell wall, suggesting an adaptive change in the mycomembrane after cell invasion (Deb *et al*. 2009). Inhibitors targeting the Ag85 mycolyl transferase activity are being designed to disrupt mycomembrane production and allow a combination drug to enter the mycobacterial cell. Unfortunately, this approach will be more effective at fighting infections before the latency period. Corresponding to the expression profile of Ag85, a multistage tuberculosis vaccine that combines the use of early-stage (including Ag85B) and late-stage antigens, shows efficient protection against both initial exposure and latent infection (Aagaard *et al*. 2011).

20.5 Conclusion

Because Ag85 proteins are involved in numerous biological processes critical to infection, these proteins offer several opportunities for combating the dynamic and evolving threat of tuberculosis. As mycolyl transferases, Ag85 proteins help to strengthen the protective mycomembrane, while the secondary adhesin function of Ag85 proteins adds to the promiscuous host-binding sites on the mycobacterial surface. As two ECM-binding partners have been determined, the potential exists for additional unidentified host targets to interact with Ag85 proteins. Although Ag85 has long been recognized for its antigenic potential in vaccines, the importance of Ag85's multiple active roles in pathogenesis are now being realized and exploited for their even greater potential in fighting tuberculosis.

Acknowledgement

This work was supported by the Agriculture and Food Research Initiative (AFRI) of the USDA Cooperative State Research, Education and Extension Service (2008-55620-18710; JDIP), the National Research Initiative (NRI) of the USDA Cooperative State Research, Education and Extension Service (2008-35204-04626), the Agriculture and Food Research Initiative (AFRI) of the USDA Cooperative State Research, Education and Extension Service (2009-65119-05993), and the Animal Formula Fund (NY-478455 and NY- 478437).

References

Aagaard, C; Hoang, T; Dietrich, J; Cardona, PJ; Izzo, A; Dolganov, G; Schoolnik, GK; Cassidy, JP; Billeskov, R; Andersen, P (2011) A multistage tuberculosis vaccine that confers efficient protection before and after exposure. *Nature Medicine* 17: 189–224.

Abouzeid, C; Ratliff, TL; Wiker, HG; Harboe, M; Bennedsen, J; Rook, GaW (1988) Characterization of fibronectin-binding antigens released by *Mycobacterium tubeculosis* and *Mycobacterium bovis* BCG. *Infection and Immunity* 56: 3046–3051.

Anderson, DH; Harth, G; Horwitz, MA; Eisenberg, D (2001) An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the Mycobacterium tuberculosis 30 kDa major secretory protein (antigen 85B), a mycolyl transferase. *Journal of Molecular Biology* 307: 671–681.

Backus, KM; Dolan, MA; Barry, CS; Joe, M; Mcphie, P; Boshoff, HIM; Lowary, TL; Davis, BG; Barry, CE (2014) The three *Mycobacterium tuberculosis* antigen 85 isoforms have unique substrates and activities determined by non-active site regions. *Journal of Biological Chemistry* 289: 25041–25053.

Bannantine, JP; Bermudez, LE (2013) No holes barred: invasion of the intestinal mucosa by *Mycobacterium avium* subsp *paratuberculosis*. *Infection and Immunity* 81: 3960–3965.

Belisle, JT; Vissa, VD; Sievert, T; Takayama, K; Brennan, PJ; Besra, GS (1997) Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* 276: 1420–1422.

Bhatt, A; Fujiwara, N; Bhatt, K; Gurcha, SS; Kremer, L; Chen, B; Chan, J; Porcelli, SA; Kobayashi, K; Besra, GS; Jacobs, WR (2007) Deletion of kasB in *Mycobacterium tuberculosis* causes loss of acid-fastness and subclinical latent tuberculosis in immunocompetent mice. *Proceedings of the National Academy of Science, USA* 104: 5157–5162.

Borremans, M; Dewit, L; Volckaert, G; Ooms, J; Debruyn, J; Huygen, K; Vanvooren, JP; Stelandre, M; Verhofstadt, R; Content, J (1989) Cloning, sequence determination, and expression of a 32-kilodalton-protein gene of *Mycobacterium tuberculosis*. *Infection and Immunity* 57: 3123–3130.

Chandra, S; Faisal, SM; Chen, JW; Chen, TT; Mcdonough, SP; Liu, S; Moreira, MA; Akey, BL; Chang, CF; Chang, YF (2012) Immune response and protective efficacy of live attenuated *Salmonella* vaccine expressing antigens of *Mycobacterium avium* subsp. *paratuberculosis* against challenge in mice. *Vaccine* 31: 242–251.

Changeux, JP (2012) Allostery and the Monod-Wyman-Changeux model after 50 years. *Annual Review of Biophysics* 41: 103–133.

Deb, C; Lee, C-M; Dubey, VS; Daniel, J; Abomoelak, B; Sirakova, TD; Pawar, S; Rogers, L; Kolattukudy, PE (2009) A novel in vitro multiple-stress dormancy model for *Mycobacterium tuberculosis* generates a lipid-loaded, drug-tolerant, dormant pathogen. *PloS One* 4: 0006077.

Dheenadhayalan, V; Shin, KS; Chang, CF; Chang, CD; Wang, SJ; Mcdonough, S; Mcdonough, P; Stehman, S; Shin, S; Torres, A; Chang, YF (2002) Cloning and characterization of the genes coding for antigen 85A, 85B and 85C of *Mycobacterium avium* subsp. *paratuberculosis*. *DNA Sequencing* 13: 287–294.

Dye, C; Williams, BG (2010) The population dynamics and control of tuberculosis. *Science.* 328: 856–861.

Favrot, L; Grzegorzewicz, AE; Lajiness, DH; Marvin, RK; Boucau, J; Isailovic, D; Jackson, M; Ronning, DR (2013) Mechanism of inhibition of *Mycobacterium tuberculosis* antigen 85 by ebselen. *Nature Communications* 4: 10.1038/ncomms3748.

Favrot, L; Lajiness, DH; Ronning, DR (2014) Inactivation of the Mycobacterium tuberculosis Antigen 85 complex by covalent, allosteric inhibitors. *Journal of Biological Chemistry* 289: 25031–25040.

Fukui, Y; Hirai, T; Uchida, T; Yoneda, M (1965) Extracellular proteins of tubercule bacili. 4. Alpha and beta antigens as major extracellular protein products and as cellular components of a strain (H37Rv) of *Mycobacterium tuberculosis*. *Biken Journal* 8: 189–199.

Gao, BL; Gupta, RS (2012) Phylogenetic framework and molecular signatures for the main clades of the phylum *Actinobacteria*. *Microbiology and Molecular Biology Reviews* 76: 66–112.

George, KL; Falkinham, JO, 3rd (1989) Identification of cytoplasmic membrane protein antigens of *Mycobacterium avium*, *M. intracellulare*, and *M. scrofulaceum*. *Canadian Journal of Microbiology* 35: 529–534.

Greenstein, RJ (2003) Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infectious Diseases* 3: 507–514.

Henderson, B; Nair, S; Pallas, J; Williams, MA (2011) Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. *FEMS Microbiology Reviews* 35: 147–200.

Huc, E; De Sousa-D'auria, C; De La Sierra-Gallay, IL; Salmeron, C; Van Tilbeurgh, H; Bayan, N; Houssin, C; Daffe, M; Tropis, M (2013) Identification of a mycoloyl transferase selectively involved in O-acylation of polypeptides in corynebacteriales. *Journal of Bacteriology* 195: 4121–4128.

Huygen, K (2014) The immunodominant T-cell epitopes of the mycolyl-transferases of the antigen 85 complex of M. tuberculosis. *Frontiers in Immunology* 5: 321.

Jackson, M; Raynaud, C; Laneelle, MA; Guilhot, C; Laurent-Winter, C; Ensergueix, D; Gicquel, B; Daffe, M (1999) Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Molecular Microbiology* 31: 1573–1587.

Kathaperumal, K; Park, SU; Mcdonough, S; Stehman, S; Akey, B; Huntley, J; Wong, S; Chang, CF; Chang, YF (2008) Vaccination with recombinant Mycobacterium avium subsp. paratuberculosis proteins induces differential immune responses and protects calves against infection by oral challenge. *Vaccine* 26: 1652–1663.

Kathaperumal, K; Kumanan, V; Mcdonough, S; Chen, LH; Park, SU; Moreira, MA; Akey, B; Huntley, J; Chang, CF; Chang, YF (2009) Evaluation of immune responses and protective efficacy in a goat model following immunization with a coctail of recombinant antigens and a polyprotein of Mycobacterium avium subsp. paratuberculosis. *Vaccine* 27: 123–135.

Kuo, CJ; Bell, H; Hsieh, CL; Ptak, CP; Chang, YF (2012) Novel *Mycobacteria* Antigen 85 Complex Binding Motif on Fibronectin. *Journal of Biological Chemistry* 287: 1892–1902.

Kuo, CJ; Ptak, CP; Hsieh, CL; Akey, BL; Chang, YF (2013) Elastin, a Novel Extracellular Matrix Protein Adhering to Mycobacterial Antigen 85 Complex. *Journal of Biological Chemistry* 288: 3886–3896.

Lappalainen, I; Hurley, MG; Clarke, J (2008) Plasticity within the obligatory folding nucleus of an immunoglobulin-like domain. *Journal of Molecular Biology* 375: 547–559.

Lenfant, N; Hotelier, T; Bourne, Y; Marchot, P; Chatonnet, A (2013) Proteins with an alpha/beta hydrolase fold: Relationships between subfamilies in an ever-growing superfamily. *Chemico-Biological Interactions* 203: 266–268.

Madison, B (2001) Application of stains in clinical microbiology. *Biotechnic and Histochemistry* 76: 119–125.

Marrakchi, H; Laneelle, MA; Daffe, M (2014) Mycolic Acids: Structures, Biosynthesis, and Beyond. *Chemical Biology* 21: 67–85.

Matsuo, K; Yamaguchi, R; Yamazaki, A; Tasaka, H; Yamada, T (1988) Cloning and expression of the Mycobacteirum Bovis BCG gene for extracellular alpha-antigen. *Journal of Bacteriology* 170: 3847–3854.

Mustafa, T; Leversen, NA; Sviland, L; Wiker, HG (2014) Differential in vivo expression of mycobacterial antigens in Mycobacterium tuberculosis infected lungs and lymph node tissues. *BMC Infectious Diseases* 14: 535.

Naito, M; Ohara, N; Matsumoto, S; Yamada, T (1998) The novel fibronectin-binding motif and key residues of mycobacteria. *Journal of Biological Chemistry* 273: 2905–2909.

Naito, M; Fukuda, T; Sekiguchi, K; Yamada, T (2000) The domains of human fibronectin mediating the binding of alpha antigen, the most immunopotent antigen of mycobacteria that induces protective immunity against mycobacterial infection. *Biochemical Journal* 347: 725–731.

Park, SU; Kathaperumal, K; Mcdonough, S; Akey, B; Huntley, J; Bannantine, JP; Chang, YF (2008) Immunization with a DNA vaccine cocktail induces a Th1 response and protects mice against *Mycobacterium avium* subsp. *paratuberculosis* challenge. *Vaccine* 26: 4329–4337.

Patti, JM; Allen, BL; Mcgavin, MJ; Hook, M (1994) MSCRAMM-Mediated adherence of microorganisms to host tissues *Annual Review of Microbiology* 48: 585–617.

Portevin, D; Sousa-D'auria, CC; Houssin, C; Grimaldi, C; Chami, M; Daffe, M; Guilhot, C (2004) A polyketide synthase catalyzes the last condensation step of mycolic acid biosynthesis in mycobacteria and related organisms. *Proceedings of the National Academy of Science, USA* 101: 314–319.

Potts, JR; Campbell, ID (1994) Fibronectin structure and assembly *Current Opinion in Cell Biology* 6: 648–655.

Principi, N; Esposito, S (2015) The present and future of tuberculosis vaccinations. *Tuberculosis* 95: 6–13.

Rohde, KH; Abramovitch, RB; Russell, DG (2007) *Mycobacterium tuberculosis* invasion of macrophages: Linking bacterial gene expression to environmental cues. *Cell Host and Microbe* 2: 352–364.

Ronning, DR; Klabunde, T; Besra, GS; Vissa, VD; Belisle, JT; Sacchettini, JC (2000) Crystal structure of the secreted form of antigen 85C reveals potential targets for mycobacterial drugs and vaccines. *Nature Structural Biology* 7: 141–146.

Ronning, DR; Vissa, V; Besra, GS; Belisle, JT; Sacchettini, JC (2004) *Mycobacterium tuberculosis* antigen 85A and 85C structures confirm binding orientation and conserved substrate specificity. *Journal of Biological Chemistry* 279: 36771–36777.

Sharma, A; Askari, JA; Humphries, MJ; Jones, EY; Stuart, DI (1999) Crystal structure of a heparin- and integrin-binding segment of human fibronectin. *EMBO Journal* 18: 1468–1479.

Vakonakis, I; Campbell, ID (2007) Extracellular matrix: from atomic resolution to ultrastructure. *Current Opinion in Cell Biology* 19: 578–583.

Wiker, HG; Harboe, M (1992) The antigen 85 complex: a major secretion product of *Mycobacterium tuberculosis*. *Microbiology Reviews* 56: 648–661.

Wiker, HG; Sletten, K; Nagai, S; Harboe, M (1990) Evidence for three separate genes encoding the proteins of the mycobacterial antigen 85 complex. *Infection and Immunity* 58: 272–274.

Wiker, HG; Harboe, M; Nagai, S (1991) A localization index for distinction between extracellular and intracellular antigens of *Mycobacterium tuberculosis*. *Journal of General Microbiology* 137: 875–884.

Wilson, RA; Maughan, WN; Kremer, L; Besra, GS; Futterer, K (2004) The structure of *Mycobacterium tuberculosis* MPT51 (FbpC1) defines a new family of non-catalytic alpha/beta hydrolases. *Journal of Molecular Biology* 335: 519–530.

Wise, SG; Weiss, AS (2009) Tropoelastin. *International Journal of Biochemistry and Cell Biology* 41: 494–497.

Wolfe, LM; Mahaffey, SB; Kruh, NA; Dobos, KM (2010) Proteomic Definition of the Cell Wall of Mycobacterium tuberculosis. *Journal of Proteome Research* 9: 5816–5826.

Young, DB; Garbe, TR (1991) Lipoprotein antigens of *Mycobacterium tuberculosis*. *Research in Microbiology* 142: 55–65.

3.8

Bacterial Moonlighting Proteins that Function as Cytokine Binders/Receptors

21

Miscellaneous IL-1β-Binding Proteins of *Aggregatibacter actinomycetemcomitans*

Riikka Ihalin

Department of Biochemistry, University of Turku, Turku, Finland

21.1 Introduction

The Gram-negative capnophilic bacterium *Aggregatibacter actinomycetemcomitans* forms robust biofilms on a variety of surfaces by producing long fimbriae that are encoded by its *tad*-locus (Schreiner *et al.* 2003; Tomich *et al.* 2007). *A. actinomycetemcomitans*' main ecological niche is the oral cavity where it thrives in gingival pockets, but it can also be found in the epithelium, where it has the potential to invade epithelial cells (Meyer *et al.* 1991). In subgingival biofilm, the opportunistic pathogen is involved in the etiology of periodontitis, especially the localized aggressive form of the disease (Zambon *et al.* 1985; Haffajee and Socransky 1994; Åberg *et al.* 2015) in which the host response destroys the tooth-supporting tissues. Because *A. actinomycetemcomitans* can be found in the oral microbiota of healthy individuals, host–microbe interactions as well as microbe–microbe interactions (Fine *et al.* 2013) are likely to play an important role in the progression of the disease.

Although the species primarily colonizes the oral cavity, *A. actinomycetemcomitans* may cause health problems in distant sites from its main ecological niche. *A. actinomycetemcomitans* may promote the development of cardiovascular diseases (Kozarov *et al.* 2005; Hyvärinen *et al.* 2012) and, as a member of the HACEK group of Gram-negative bacteria, it can cause endocarditis (Das *et al.* 1997). Moreover, it has been found in abscesses in the brain (Rahamat-Langendoen *et al.* 2011), demonstrating its potential to spread from the oral cavity.

A. actinomycetemcomitans uses various virulence mechanisms when colonizing and causing disease in humans. The most well-known virulence factors of the species are the repeat-in-toxin (RTX) leukotoxin and cytolethal distending toxin (CDT). Leukotoxin is coded by the *ltxCABD* operon in which *ltxA* codes for the toxin, *ltxC* is needed for post-translational modifications, and the products of *ltxB* and *ltxD* take part in the transport of the toxin (Johansson 2011). Modifications in the promoter region of *ltxCABD* have been shown to change

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

the expression levels of leukotoxin in some clinical isolates of *A. actinomycetemcomitans* (Brogan *et al.* 1994; He *et al.* 1999). In the highly leukotoxic strain JP2, the *ltxCABD* promoter has a 530 bp deletion (Haubek 2010). Leukotoxin can kill various human cells, although its primary targets are polymorphonuclear leukocytes (Johansson *et al.* 2000) and monocytes/macrophages (Tsai *et al.* 1979). Lymphocytes (Simpson *et al.* 1988; Rabie *et al.* 1988; Mangan *et al.* 1991; Shenker *et al.* 1994) and erythrocytes (Balashova *et al.* 2006) are also affected by leukotoxin, making it a versatile factor for manipulating the immune response. Monocytes are especially sensitive to the cytotoxic effect of leukotoxin, which lyses the cells in a caspase-1-dependent manner (Kelk *et al.* 2003). This result is in accordance with the finding that LtxA-treated macrophages rapidly secrete bioactive interleukin (IL)-1β, which requires caspase-1 activation for maturation (Kelk *et al.* 2005). The main toxic function of CDT is to prevent eukaryotic cells from entering mitosis, thus leading to the eventual apoptosis of the host cell (Shenker *et al.* 1999). In addition to preventing cell proliferation, *A. actinomycetemcomitans* CDT disturbs cell–cell contacts in the epithelium (Damek-Poprawa *et al.* 2013), facilitating the entrance of bacteria into the deeper layers of gingival tissue.

21.2 *A. actinomycetemcomitans* Biofilms Sequester IL-1β

It has been known since the early 1990s that bacteria are able to bind certain proinflammatory cytokines (Porat *et al.* 1991; Luo *et al.* 1993; Zav'yalov *et al.* 1995; Kanangat *et al.* 2001; Wu *et al.* 2005; McLaughlin and Hoogewerf 2006). Although cytokine binding is generally considered a property of opportunistic pathogens, opportunistic periodontal pathogens other than *A. actinomycetemcomitans* have not been shown to bind cytokines (Fletcher *et al.* 1997). Whether these negative results are partly explained by the selected experimental conditions, such as the chosen culture media, is still unclear.

Activated macrophages and the proinflammatory cytokine IL-1β advance the progression of periodontitis *in vivo*. Significantly more IL-1β-producing macrophages have been found in chronic periodontal lesions than in healthy sites (Gemmell and Seymour 1998), and IL-1 antagonism reduces the inflammation progression and periodontal tissue destruction in a nonhuman primate model (Delima *et al.* 2002). In addition, IL-1β induces the synthesis of RANKL, which promotes the differentiation of osteoclasts into bone-degrading cells (Darveau 2010). *A. actinomycetemcomitans* biofilm cells release an array of small molecules *in vitro* (Zijnge *et al.* 2012), and the released molecules (<0.02 μm) enhance the production of proinflammatory cytokines in human whole blood (Oscarsson *et al.* 2008). Moreover, the leukotoxin of *A. actinomycetemcomitans* stimulates human macrophages to produce IL-1β (Kelk *et al.* 2005, 2008).

Clinical isolate strains of *A. actinomycetemcomitans* that form tough biofilms by producing fimbriae, as well as different single-gene *tad*-locus mutant strains devoid of fimbriae, are able to bind IL-1β (Paino *et al.* 2011). There seems to be no difference between the IL-1β-binding capacity of planktonic and biofilm cells of *A. actinomycetemcomitans* (Paino *et al.* 2011), contrary to earlier findings that

S. aureus biofilm cells can bind IL-1β more efficiently than planktonic cells (McLaughlin and Hoogewerf 2006). In the oral cavity, the majority of the *A. actinomycetemcomitans* cell population is located in biofilms; whether the biofilm as an intact community is able to sequester the central proinflammatory cytokine is therefore a very relevant and significant question. To answer that question, a considerable amount of recombinant IL-1β or a system capable of producing biologically active IL-1β, preferably in contact with the biofilm, would be needed. We therefore utilized a technique where a separately grown *A. actinomycetemcomitans* biofilm was combined with an organotypic gingival mucosa tissue culture model (Paino *et al*. 2012). The biofilm was co-cultured with the gingival mucosa model in the absence/presence of penicillin and streptomycin, which have been shown to decrease the viability of the biofilm. The amount of IL-1β in the growth medium and histological sections of the co-cultures was evaluated. When the co-culture was assembled in the absence of antibiotics, the viable *A. actinomycetemcomitans* biofilm bound substantial amounts of IL-1β (Paino *et al*. 2012). However, when antibiotics were used in the system, IL-1β leaked to the growth medium and there were no signs of IL-1β in the biofilm (Paino *et al*. 2012).

21.3 *A. actinomycetemcomitans* Cells Take in IL-1β

Bacteria use several mechanisms to sense environmental signals. Perhaps the most common strategy is to use an extracellular receptor which binds the signal molecule and transmits the message through a special pathway. Excellent examples of systems that function in this manner are the two-component signal transduction systems. Another option is to internalize the signal, or part of it, inside the cell where the signal could directly interact with other molecules. Although *S. aureus* has been hypothesized to internalize peptides originating from IL-1β digestion (Kanangat *et al*. 2001), there was no direct evidence that bacteria could actively take in IL-1β prior to our studies.

21.3.1 Novel Outer Membrane Lipoprotein of *A. actinomycetemcomitans* Binds IL-1β

The identification of the front-line receptor or binder of IL-1β in the outer membrane of *A. actinomycetemcomitans* has been our primary aim since we discovered that *A. actinomycetemcomitans* is able to bind this central proinflammatory cytokine. However, this proved to be a very challenging task in which we have only recently succeeded (Paino *et al*. 2013). Unlike the two other intracellular bacterial IL-1β binders described below, this novel outer membrane protein had no previously described function. From the sequence analysis, we could conclude that the protein was a lipoprotein. In addition, it could be expressed as a soluble recombinant protein without its signal sequence and purified from the expression host *E. coli* (Paino *et al*. 2013). The microplate binding assays confirmed that the protein moiety of the novel lipoprotein bound IL-1β *in vitro*, although it also seemed to bind the similarly sized control protein, soy trypsin inhibitor (Paino *et al*. 2013).

In addition to the conserved lipidation site, the sequence analysis of BilRI revealed interesting properties about the protein. After the first 50 amino acids, including the 19 amino acid long signal sequence, there was a 40 amino acid long sequence which repeated almost unchangeable three times. An outer membrane signal sequence is underlined and the lipoprotein lipidation site is highlighted with * (Fig. 21.1).

A similarity search with BLAST detected similar proteins only in the *Pasteurellaceae* family members; the most alike proteins could be found in various strains of *A. actinomycetemcomitans* and *Aggregatibacter aphrophilus* (Paino *et al*. 2013).

The only similar protein with a known function is from *Haemophilus ducreyi* which has a lipoprotein, fibrinogen binder A (FgbA), that binds host fibrinogen and thus plays a role in virulence and helps the pathogen to invade the host (Bauer *et al*. 2009). The sequence alignment performed using Clustal O indicated that the 156 amino acid long FgbA of *H. ducreyi*, which has been verified to bind fibrinogen (Bauer *et al*. 2009), had only a slightly truncated three-unit long repeating sequence (Fig. 21.2). Other strains of *H. ducreyi* produce at least two shorter variants (105 and 98 amino acids) of FgbA that only possess the middle part of the repeating sequences (Fig. 21.2). Our recent study showed that BilRI does not bind either to fibrinogen or collagen (Ahlstrand *et al*. 2016). However, BilRI interacted with various host cytokines and the structural studies indicated that BilRI was intrinsically disordered, that is, it did not have any stable folds

MKKSVLAALV LGVTLSVTGC* DDSKTSPQAE QAKTSVSEAK DAVVNAANDV
KDATVEAAKD AQNMAADKMV EVKDAISEKM DAMTTQASEM
KDAAVEAAKD AKDAAADKMA EVKDAISEKM DAMATQVNEM
KDTAAEAVKD AKDAAADKMT EVKDAVSEKM GATATQTNEM
KDAVKSETESK

Figure 21.1 Amino acid sequence of a novel bacterial interleukin receptor I (BilRI), which is an outer membrane (signal sequence underlined) lipoprotein (lipidation site marked with *). The protein contained a triplicate repetitive 40 amino acid long sequence after the first 50 amino acids (including the signal sequence).

```
CLUSTAL O(1.2.1) multiple sequence alignment

A. actinomycetemcomitans BilRI  MKKSVLAALVLGVTLSVTGCDDSKTSPQAEQAKTSVSEAKDAVVNAANDVKDATVEAAKDAQN 62
H. ducreyi FgbA; 156 aa         MKKSILA-LVLGTTFTLAACDKPQVEEMKQTVTDTAANTKAIVVEKAGDMKESASE------- 55
H. ducreyi FgbA; 105 aa         MKKSILA-LVLGTTFTLAACDKPQVEEMKQTVTDTAANTKAIVVEKAGDMK----------- 50
H. ducreyi FgbA; 98 aa          MKKSILA-LVLGTTFTLAACDKPQVEEMKQTVTDTAANTKAIVVEKA---------------- 46


A. actinomycetemcomitans BilRI  MAADKMVEVKDAISEKMDAMTTQASEMKDAAVEAAKDAKDAAADK---MAEVKDAISEKM-- 120
H. ducreyi FgbA; 156 aa         --------MKDAAKAKLEDMKESAAEAKESLAEKANEMKDAAKAK---LEGMKESAAEAK-- 104
H. ducreyi FgbA; 105 aa         ----------------------ESANEMKDAAKAKLEDMKEAAADKKAEMAEKKDEMKEKMDT 91
H. ducreyi FgbA; 98 aa          ----------------GDMKESASEMKDAAKAKLEDMKEAAADKKAEMAEKMDSAA----- 86


A. actinomycetemcomitans BilRI  --DAMATQVNEMKDTAAEAVKDAKDAAADKMTEVKDAVSEKMGATATQTNEMKDAVKSETESK 181
H. ducreyi FgbA; 156 aa         --ESLAEKANEMKDAAKAKLEDMKEAAADKKAEMAEKMDSAADAMKEKVDEMKK--------- 156
H. ducreyi FgbA; 105 aa         AADAMKEKVDEMKK---------------------------------------------------- 105
H. ducreyi FgbA; 98 aa          --DAMKEKVDEMKK---------------------------------------------------- 98
```

Figure 21.2 Clustal O sequence alignment of the *A. actinomycetemcomitans* BilRI amino acid sequence with three different variants of *H. ducreyi* fibrinogen binder A (FgbA).

(Ahlstrand *et al*. 2016). BilRI therefore has two characteristics typical of a certain group of moonlighting proteins: it is capable of interacting with several ligands; and there is no stable fold without a binding ligand (Tompa *et al*. 2005).

21.3.2 IL-1β Localizes to the Cytosolic Face of the Inner Membrane and in the Nucleoids of *A. actinomycetemcomitans*

In our experimental set up, IL-1β could be detected inside viable *A. actinomycetemcomitans* biofilm cells grown in close contact with the organotypic gingival mucosa (Paino *et al*. 2012). In most cases, gold-particle-labeled anti-IL1β antibodies were shown by immuno-electron microscopy to localize to either the cytosolic side of the inner membrane or within the nucleoids of *A. actinomycetemcomitans* (Fig. 21.3a). The uptake of IL-1β could also be detected in single cells of bacteria that were located in close vicinity to the epithelium (Fig. 21.3b). Only viable *A. actinomycetemcomitans* cells could take up IL-1β; cells treated with antibiotics were smaller and showed no signs of IL-1β uptake (Paino *et al*. 2012).

21.3.3 Inner Membrane Protein ATP Synthase Subunit β Binds IL-1β

Because IL-1β localized inside the bacterial cells, we became interested in the possible intracellular proteins that might interact with IL-1β. The intracellular proteins were first separated by native-polyacrylamide gel electrophoresis (PAGE), and blotted onto a membrane that was incubated with biotinylated IL-1β and detected with avidin-HRP. A similar native-PAGE gel with intracellular proteins was also silver-stained. The protein bands that interacted with IL-1β were extracted from the silver-stained gel and identified by mass spectrometry.

One of the intracellular proteins that interacted with IL-1β was F_1F_0 ATP synthase subunit β. F_1F_0 ATP synthase is a well-conserved multi-subunit protein

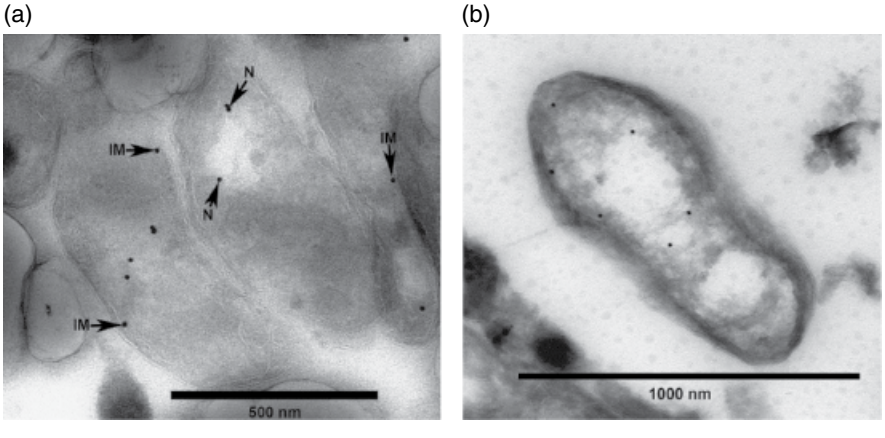

Figure 21.3 Viable *A. actinomycetemcomitans* cells internalize IL-1β both (a) in biofilm and (b) as planktonic cells. Pre-grown *A. actinomycetemcomitans* biofilms were co-cultured with an organotypic gingival mucosa, and the localization of IL-1β was investigated using immuno-electron microscopy. IL-1β was detected both attached to the inner membrane (IM) and in the outer edges of nucleoids (N).

complex that can be found in all living organisms from bacteria, mitochondria, and chloroplasts to the surfaces of several eukaryotic cell types (Hong and Pedersen 2008). We showed that IL-1β bound specifically to the trimeric form of subunit β, which was produced as a recombinant protein (Paino *et al*. 2011). The binding of IL-1β to the recombinant protein was detected with an electrophoretic mobility shift assay (EMSA) combined with immunodetection using anti-IL-1β antibodies. The catalytic domain of the F_1 part of the ATP synthase consists of three β subunits (Nakamoto *et al*. 2008), suggesting that IL-1β targets the complete catalytic domain of the enzyme. Different antimicrobial peptides with α-helical structures have been shown to inhibit the F_1F_0 ATP synthase of *E. coli* by specifically binding to its β subunit (Laughlin and Ahmad 2010). The positively charged α-helix in antimicrobial peptides interacts with the negatively charged DELSEED sequence in the β-subunit of *E. coli* (Laughlin and Ahmad 2010). In IL-1β, the potential interacting α-helical portion is the extension loop with a QGQDMEQQ amino acid sequence.

F-type ATP synthase has served as a target for antimicrobial drug development against tuberculosis. The novel drug R207910 binds the *c* subunit, inhibiting ATP synthesis, which eventually eliminates even drug-resistant *Mycobacterium tuberculosis* infection (Andries *et al*. 2005). However, *in vitro* studies show that *M. tuberculosis* may also develop resistance to R207910 (Petrella *et al*. 2006). Of the more conventional antimicrobial agents, iodine deactivates the F_1 part of ATP synthase (Petrone *et al*. 1987) by covalently modifying the β subunit.

A. actinomycetemcomitans is known to be especially resistant to host defenses and antimicrobials due to its ability to invade epithelial cells (Meyer *et al*. 1991) and vascular endothelial cells (Schenkein *et al*. 2000). *Salmonella enterica*, an intracellular pathogen, exploits the specific virulence protein MtgC to inhibit its own F_1F_0 ATP synthase; this improves persistence within macrophages by balancing ATP levels in an environment with an elevated pH (Lee *et al*. 2013). Whether the binding of IL-1β to the F_1F_0 ATP synthase subunit β of *A. actinomycetemcomitans* is antimicrobial or enhances the virulence and robustness of the biofilm or intracellular planktonic cells needs to be confirmed.

21.3.4 DNA-Binding Histone-Like Protein HU Interacts with IL-1β

Because IL-1β is located along the outer edges of *A. actinomycetemcomitans*, it is not surprising that intracellular histone-like protein HU interacted with IL-1β (Paino *et al*. 2012). The interaction was originally detected in a similar way as the interaction with F_1F_0 ATP synthase subunit β, and was confirmed with recombinant HU in a microplate binding assay and by EMSA (Paino *et al*. 2012). Our results also suggested that the interaction with IL-1β changed the capacity of HU to bind to DNA, which might result in changes in gene expression. This hypothesis is supported by the finding that IL-1β changes the gene expression pattern of *S. aureus* (Kanangat *et al*. 2007) and the role of the highly conserved HU in the regulation of a large set of genes in various bacteria. For instance, in *E. coli*, HU regulates the expression of 8% of the total genes (Oberto *et al*. 2009), many of which are associated with stress response and metabolism (Kar *et al*. 2005; Oberto *et al*. 2009).

In *Porphyromonas gingivalis*, an opportunistic Gram-negative periodontal pathogen, HU is required for the proficient synthesis of the K-antigen polysaccharide capsule (Alberti-Segui *et al*. 2010) as well as the expression of genes involved in cell division and the uptake of iron (Priyadarshini *et al*. 2013). HU, and probably other nucleoid-associated proteins (NAPs), might therefore play an important role in the process by which commensal *P. gingivalis* transforms into a periodontal pathogen (Priyadarshini *et al*. 2013). It is tempting to suggest that this might also be true in other periodontal pathogens and that at least some of them could use environmental cues, such as IL-1β, to change the properties of a central NAP such as HU.

Certain bacterial species have endogenous proteins that interact with the DNA-binding protein HU, indicating that HU is regulated by direct protein–protein interactions. Some *Neisseria* species produce conserved protein DMP12, which belongs to a group of DNA mimic proteins. This novel small 12 kDa protein interacts as a monomer with the dimeric form of the *Neisseria* HU protein (Wang *et al*. 2013). Because DMP12 had approximately four times lower affinity to HU than to double-stranded DNA, the authors concluded that *Neisseria* DMP12 most likely functions as a regulator of HU and not as a competitive inhibitor (Wang *et al*. 2013). When expressed as a recombinant protein in *E. coli*, DMP12 increased the growth rate of the expression host (Wang *et al*. 2013). *Haemophilus influenzae*, which belongs to the Pasteurellaceae family of bacteria along with *A. actinomycetemcomitans*, produces the dsDNA mimic protein HI1450 (Parsons *et al*. 2004), which interacts with the α-form of the HU of *H. influenzae* with the same affinity as dsDNA (Parsons *et al*. 2005). A BLAST search for a similar protein in *A. actinomycetemcomitans* revealed an almost identical protein with 80% identity (unpublished data), suggesting that *A. actinomycetemcomitans* has intrinsic means of regulating HU binding to dsDNA. Intracellular IL-1β might bring a new dimension to this HU regulation.

21.4 The Potential Effects of IL-1β on *A. actinomycetemcomitans*

By sequestering the central proinflammatory cytokine IL-1β, *A. actinomycetemcomitans* might slow down the launching of the inflammatory reaction that is vital for the efficient eradication of a harmful pathogen. However, binding IL-1β may be doubly advantageous for the bacteria because it has been shown that the binding of IL-1β to bacterial cells increases the growth of virulent bacteria (Porat *et al*. 1991; Meduri *et al*. 1999). Furthermore, binding of IL-1β by *Staphylococcus aureus* biofilms (McLaughlin and Hoogewerf 2006) changes the virulence gene expression of *S. aureus* (Kanangat *et al*. 2007). Opportunistic pathogens could therefore dampen the inflammatory reaction and change their own behavior in tandem. We have shown that *A. actinomycetemcomitans* acts in this manner.

21.4.1 Biofilm Amount Increases and Metabolic Activity Decreases

When exposed to 10 ng mL^{-1} IL-1β for six hours, *A. actinomycetemcomitans* from two different clinical isolate strains increased their biofilm formation

(Paino *et al*. 2011). Whether the detected increase in biofilm mass was due to an increase in the total number of biofilm cells or enhanced production of extracellular polymeric substance (EPS) is unknown. *A. actinomycetemcomitans* biofilms were pre-exposed to IL-1β in RPMI medium, which is a nutrient-poor medium. Nutrient limitation can enhance EPS production by *A. actinomycetemcomitans* biofilms (Amarasinghe *et al*. 2009). Similar types of regulation of EPS production by starvation have been detected in *Pseudomonas* (Wrangstadh *et al*. 1986) and *Vibrio cholera* (Wai *et al*. 1998).

When IL-1β binds to the catalytic domain of the trimeric form of the β subunit of the F-type ATP synthase, it might significantly decrease the ATP production of the cell. F-type ATP synthase generates a substantial amount of cellular ATP by oxidative phosphorylation, and its inhibition may result in a significant drop in the cellular energy level if not substituted by other means. We have not yet measured the effects of IL-1β on the ATP levels of *A. actinomycetemcomitans*. However, we found indirect evidence of the effects on the overall metabolic activity using alamarBlue™ (resazurin), which forms fluorescent products when reduced by agents such as $FADH_2$, NADH, and NADPH in metabolically active bacterial cells. We discovered that, in the presence of a physiologically relevant amount of IL-1β (i.e., $10\,ng\,mL^{-1}$), the metabolic activity of the *A. actinomycetemcomitans* biofilm decreased for approximately three hours. In other words, IL-1β diminished the amount of reducing agents that are used in oxidative phosphorylation to produce ATP. Our current knowledge of the molecular mechanisms of how IL-1β inhibits the metabolism of *A. actinomycetemcomitans* does not provide a direct mechanism for the decreased reduction potential of the biofilm cells. However, the net effect of IL-1β is to decrease the energy availability of the cell in the form of ATP, which in turn may increase energy production via pathways other than oxidative phosphorylation, such as glycolysis (Koebmann *et al*. 2002; Holm *et al*. 2010). In *E. coli*, ATP has been shown to have a crucial role in regulating the proteolysis of a group 2 σ^{70} factor RpoS (Peterson *et al*. 2012) that primarily regulates gene expression in the stationary phase of growth and allows the cells to survive stressful conditions in a variety of demanding environments (McCann *et al*. 1991). If bacteria other than *E. coli* regulate their transcription initiation factors by sensing ATP, the decreased ATP synthesis may function as a link between IL-1β sensing and modulation of gene expression because F-type ATP synthase is highly conserved among bacterial species.

21.4.2 Potential Changes in Gene Expression

The central proinflammatory cytokine IL-1β has been shown to change the gene expression pattern of *S. aureus*, that is, to decrease the expression of leukotoxin genes and increase the expression of microbial surface proteins that recognize host adhesive matrix molecules such as fibronectin, fibrinogen, and collagen (Kanangat *et al*. 2007). The fact that IL-1β localizes in the outer edges of nucleoids and that the DNA-binding protein HU interacts with IL-1β (Paino *et al*. 2012) suggests possible mechanisms that might allow IL-1β to modulate gene expression in *A. actinomycetemcomitans*. Moreover, if other bacterial species also take up IL-1β, IL-1β might have the potential to modulate gene expression in

a variety of bacterial species because HU is a well-conserved DNA-binding protein in both Gram-negative and Gram-positive species.

As discussed above, the inhibition of ATP synthesis via F-type ATP synthase, which inevitably leads to decreased ATP levels in the cell, may also modulate gene expression. No detailed knowledge is available regarding *A. actinomycetemcomitans*' sigma factors and their regulation, and our hypotheses have been based on the roles of alternative sigma factors in *E. coli* and their usage in the stress response. However, nature tends to repeat the same formula, so it is tempting to suggest that ATP levels, especially decreases in ATP, might affect the gene expression of *A. actinomycetemcomitans*. This hypothesis is supported by the finding that nutrient limitation increases the production of EPS by *A. actinomycetemcomitans* biofilms. This is an interesting field that warrants further investigation, and molecular methods such as RNA sequencing and high-throughput proteomics allow for the study of how proinflammatory mediators may help the opportunistic pathogen to adapt to the inflammatory milieu.

21.5 Conclusions

It has been known for decades that some pathogenic bacteria are able to sequester various cytokines (Porat *et al.* 1991; Luo *et al.* 1993; Zav'yalov *et al.* 1995; Kanangat *et al.* 2001; Wu *et al.* 2005; McLaughlin and Hoogewerf 2006) and that this binding affects bacterial virulence properties by increasing growth (Porat *et al.* 1991; Meduri *et al.* 1999) and biofilm formation (McLaughlin and Hoogewerf 2006), downregulating the expression of toxin genes (Kanangat *et al.* 2007), and increasing the expression of adhesion molecules that interact with host matrix proteins (Kanangat *et al.* 2007). Part of these responses might originate from the interaction of cytokines with moonlighting bacterial proteins that have other primary functions. When bacteria enter the host, the interaction between the various bacterial components with the host non-immune and immune cells leads to the production of an inflammatory milieu with increased amounts of proinflammatory cytokines. In the case of *A. actinomycetemcomitans*, we have shown that the gatekeeper cytokine in inflammation, IL-1β, is sequestered and taken up by the viable biofilm cells (Paino *et al.* 2012). Inside the bacterium, IL-1β may interact with two moonlighting proteins, F-type ATP synthase subunit β and DNA-binding protein HU, which could result in decreased ATP levels and changes in gene expression patterns. A subset of the cells within a biofilm are always hibernating (Lewis 2007), and this increases the resistance of biofilms to host defenses and antibiotics. Sequestering IL-1β might therefore be an additional means to decrease metabolic activity and increase the resistance of *A. actinomycetemcomitans* biofilms. The bacterium might augment its adaptation to the changing inflammatory milieu by regulating appropriate sets of genes that would be needed for optimal interplay with the host.

Because cytokines are produced to orchestrate the function of eukaryotic host cells, bacterial sequestration of these molecules undoubtedly alters the fine balance of cytokine signaling. We have determined how the binding of central host signal molecules to bacteria might affect the host defense, epithelial cells, and

immune cells, of which cell trafficking, maturation, and proper function depend on proper cytokine and chemokine signaling. *A. actinomycetemcomitans* may possess specific IL-1β binders that disturb the inflammatory reaction. However, part of the impact is most likely mediated by moonlighting proteins, such as F-type ATP synthase subunit β and DNA-binding protein HU, that have other primary functions in the bacteria. This novel virulence mechanism may facilitate the ability of the bacterium to evade the host immune system and to colonize the host.

References

Åberg CH, Kelk P, Johansson A (2015) *Aggregatibacter actinomycetemcomitans*: virulence of its leukotoxin and association with aggressive periodontitis. *Virulence* 6(3): 188–195.

Ahlstrand T, Tuominen H, Beklen A, Torittu A, Oscarsson J, Sormunen R, Pöllänen MT, Permi P, Ihalin R (2016) A novel intrinsically disordered outer membrane lipoprotein of *Aggregatibacter actinomycetemcomitans* binds various cytokines and plays a role in biofilm response to interleukin-1β and interleukin-8. *Virulence*, published online 26 July, doi: 10.1080/21505594.2016.1216294.

Alberti-Segui C, Arndt A, Cugini C, Priyadarshini R, Davey ME (2010) HU protein affects transcription of surface polysaccharide synthesis genes in *Porphyromonas gingivalis*. *Journal of Bacteriology* 192: 6217–6229.

Amarasinghe JJ, Scannapieco FA, Haase EM (2009) Transcriptional and Translational Analysis of Biofilm Determinants of *Aggregatibacter actinomycetemcomitans* in Response to Environmental Perturbation. *Infection and Immunity* 77: 2896–2907.

Andries K, Verhasselt P, Guillemont J, Gohlmann HW, Neefs JM, Winkler H, Van Gestel J, Timmerman P, Zhu M, Lee E, *et al.* (2005) A diarylquinoline drug active on the ATP synthase of *Mycobacterium tuberculosis*. *Science* 307: 223–227.

Balashova NV, Crosby JA, Al Ghofaily L, Kachlany SC (2006) Leukotoxin confers beta-hemolytic activity to *Actinobacillus actinomycetemcomitans*. *Infection and Immunity* 74: 2015–2021.

Bauer ME, Townsend CA, Doster RS, Fortney KR, Zwickl BW, Katz BP, Spinola SM, Janowicz DM (2009) A fibrinogen-binding lipoprotein contributes to the virulence of *Haemophilus ducreyi* in humans. *Journal of Infectious Diseases* 199: 684–692.

Brogan JM, Lally ET, Poulsen K, Kilian M, Demuth DR (1994) Regulation of *Actinobacillus actinomycetemcomitans* leukotoxin expression: analysis of the promoter regions of leukotoxic and minimally leukotoxic strains. *Infection and Immunity* 62: 501–508.

Damek-Poprawa M, Korostoff J, Gill R, DiRienzo JM (2013) Cell junction remodeling in gingival tissue exposed to a microbial toxin. *Journal of Dental Research* 92: 518–523.

Darveau RP (2010) Periodontitis: a polymicrobial disruption of host homeostasis. *Nature Reviews Microbiology* 8: 481–490.

Das M, Badley AD, Cockerill FR, Steckelberg JM, Wilson WR (1997) Infective endocarditis caused by HACEK microorganisms. *Annual Review of Medicine* 48: 25–33.

Delima AJ, Karatzas S, Amar S, Graves DT (2002) Inflammation and tissue loss caused by periodontal pathogens is reduced by interleukin-1 antagonists. *Journal of Infectious Diseases* 186(4): 511–516.

Fine DH, Markowitz K, Fairlie K, Tischio-Bereski D, Ferrendiz J, Furgang D, Paster BJ, Dewhirst FE (2013) A consortium of *Aggregatibacter actinomycetemcomitans*, *Streptococcus parasanguinis*, and *Filifactor alocis* is present in sites prior to bone loss in a longitudinal study of localized aggressive periodontitis. *Journal of Clinical Microbiology* 51: 2850–2861.

Fletcher J, Reddi K, Poole S, Nair S, Henderson B, Tabona P, Wilson M (1997) Interactions between periodontopathogenic bacteria and cytokines. *Journal of Periodontal Research* 32(1 Pt 2): 200–205.

Gemmell E, Seymour GJ (1998) Cytokine profiles of cells extracted from humans with periodontal diseases. *Journal of Dental Research* 77(1): 16–26.

Haffajee AD, Socransky SS (1994) Microbial etiological agents of destructive periodontal diseases. *Periodontology* 2000 5: 78–111.

Haubek D (2010) The highly leukotoxic JP2 clone of *Aggregatibacter actinomycetemcomitans*: evolutionary aspects, epidemiology and etiological role in aggressive periodontitis. *APMIS Suppl* (130): 1–53.

He T, Nishihara T, Demuth DR, Ishikawa I (1999) A novel insertion sequence increases the expression of leukotoxicity in *Actinobacillus actinomycetemcomitans* clinical isolates. *Journal of Periodontology* 70: 1261–1268.

Holm AK, Blank LM, Oldiges M, Schmid A, Solem C, Jensen PR, Vemuri GN (2010) Metabolic and transcriptional response to cofactor perturbations in *Escherichia coli*. *Journal of Biological Chemistry* 285: 17498–17506.

Hong S, Pedersen PL (2008) ATP synthase and the actions of inhibitors utilized to study its roles in human health, disease, and other scientific areas. *Microbiology and Molecular Biology Reviews* 72: 590–641.

Hyvärinen K, Mäntylä P, Buhlin K, Paju S, Nieminen MS, Sinisalo J, Pussinen PJ (2012) A common periodontal pathogen has an adverse association with both acute and stable coronary artery disease. *Atherosclerosis* 223: 478–484.

Johansson A (2011) *Aggregatibacter actinomycetemcomitans* leukotoxin: a powerful tool with capacity to cause imbalance in the host inflammatory response. *Toxins (Basel)* 3: 242–259.

Johansson A, Claesson R, Hänström L, Sandström G, Kalfas S (2000) Polymorphonuclear leukocyte degranulation induced by leukotoxin from *Actinobacillus actinomycetemcomitans*. *Journal of Periodontal Research* 35: 85–92.

Kanangat S, Bronze MS, Meduri GU, Postlethwaite A, Stentz F, Tolley E, Schaberg D (2001) Enhanced extracellular growth of *Staphylococcus aureus* in the presence of selected linear peptide fragments of human interleukin (IL)-1beta and IL-1 receptor antagonist. *Journal of Infectious Diseases* 183: 65–69.

Kanangat S, Postlethwaite A, Cholera S, Williams L, Schaberg D (2007) Modulation of virulence gene expression in *Staphylococcus aureus* by interleukin-1beta: novel implications in bacterial pathogenesis. *Microbes and Infection* 9: 408–415.

Kar S, Edgar R, Adhya S (2005) Nucleoid remodeling by an altered HU protein: reorganization of the transcription program. *Proceedings of the National Academy of Science, USA* 102: 16397–16402.

Kelk P, Johansson A, Claesson R, Hänström L, Kalfas S (2003) Caspase 1 involvement in human monocyte lysis induced by *Actinobacillus actinomycetemcomitans* leukotoxin. *Infection and Immunity* 71: 4448–4455.

Kelk P, Claesson R, Hänström L, Lerner UH, Kalfas S, Johansson A (2005) Abundant secretion of bioactive interleukin-1beta by human macrophages induced by *Actinobacillus actinomycetemcomitans* leukotoxin. *Infection and Immunity* 73: 453–458.

Kelk P, Claesson R, Chen C, Sjöstedt A, Johansson A (2008) IL-1beta secretion induced by *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans* is mainly caused by the leukotoxin. *International Journal of Medical Microbiology* 298: 529–541.

Koebmann BJ, Westerhoff HV, Snoep JL, Nilsson D, Jensen PR (2002) The glycolytic flux in *Escherichia coli* is controlled by the demand for ATP. *Journal of Bacteriology* 184: 3909–3916.

Kozarov EV, Dorn BR, Shelburne CE, Dunn WA,Jr, Progulske-Fox A (2005) Human atherosclerotic plaque contains viable invasive *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*. *Arteriosclerosis, Thrombosis and Vascular Biology* 25: e17-8.

Laughlin TF, Ahmad Z (2010) Inhibition of *Escherichia coli* ATP synthase by amphibian antimicrobial peptides. *International Journal of Biological Macromolecules* 46: 367–374.

Lee EJ, Pontes MH, Groisman EA (2013) A bacterial virulence protein promotes pathogenicity by inhibiting the bacterium's own F1Fo ATP synthase. *Cell* 154: 146–156.

Lewis K (2007) Persister cells, dormancy and infectious disease. *Nature Reviews Microbiology* 5: 48–56.

Luo G, Niesel DW, Shaban RA, Grimm EA, Klimpel GR (1993) Tumor necrosis factor alpha binding to bacteria: evidence for a high-affinity receptor and alteration of bacterial virulence properties. *Infection and Immunity* 61: 830–835.

Mangan DF, Taichman NS, Lally ET, Wahl SM (1991) Lethal effects of *Actinobacillus actinomycetemcomitans* leukotoxin on human T lymphocytes. *Infection and Immunity* 59: 3267–3272.

McCann MP, Kidwell JP, Matin A (1991) The putative sigma factor KatF has a central role in development of starvation-mediated general resistance in *Escherichia coli*. *Journal of Bacteriology* 173: 4188–4194.

McLaughlin RA, Hoogewerf AJ (2006) Interleukin-1beta-induced growth enhancement of *Staphylococcus aureus* occurs in biofilm but not planktonic cultures. *Microbes and Pathogens* 41: 67–79.

Meduri GU, Kanangat S, Stefan J, Tolley E, Schaberg D (1999) Cytokines IL-1beta, IL-6, and TNF-alpha enhance in vitro growth of bacteria. *American Journal of Respiratory and Critical Care Medicine* 160: 961–967.

Meyer DH, Sreenivasan PK, Fives-Taylor PM (1991) Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. *Infection and Immunity* 59: 2719–2726.

Nakamoto RK, Baylis Scanlon JA, Al-Shawi MK (2008) The rotary mechanism of the ATP synthase. *Archives of Biochemistry and Biophysics* 476: 43–50.

Oberto J, Nabti S, Jooste V, Mignot H, Rouviere-Yaniv J (2009) The HU regulon is composed of genes responding to anaerobiosis, acid stress, high osmolarity and SOS induction. *PLoS One* 4(2): e4367.

Oscarsson J, Karched M, Thay B, Chen C, Asikainen S (2008) Proinflammatory effect in whole blood by free soluble bacterial components released from planktonic and biofilm cells. *BMC Microbiology* 8: 206.

Paino A, Tuominen H, Jääskeläinen M, Alanko J, Nuutila J, Asikainen SE, Pelliniemi LJ, Pöllänen MT, Chen C, Ihalin R (2011) Trimeric form of intracellular ATP synthase subunit beta of *Aggregatibacter actinomycetemcomitans* binds human interleukin-1beta. *PLoS One* 6: e18929.

Paino A, Lohermaa E, Sormunen R, Tuominen H, Korhonen J, Pöllänen MT, Ihalin R (2012) Interleukin-1beta is internalised by viable *Aggregatibacter actinomycetemcomitans* biofilm and locates to the outer edges of nucleoids. *Cytokine* 60: 565–574.

Paino A, Ahlstrand T, Nuutila J, Navickaite I, Lahti M, Tuominen H, Välimaa H, Lamminmäki U, Pöllänen MT, Ihalin R (2013) Identification of a novel bacterial outer membrane interleukin-1Beta-binding protein from *Aggregatibacter actinomycetemcomitans*. *PLoS One* 8: e70509.

Parsons LM, Yeh DC, Orban J (2004) Solution structure of the highly acidic protein HI1450 from *Haemophilus influenzae*, a putative double-stranded DNA mimic. *Proteins* 54: 375–383.

Parsons LM, Liu F, Orban J (2005) HU-alpha binds to the putative double-stranded DNA mimic HI1450 from *Haemophilus influenzae*. *Protein Science* 14: 1684–1687.

Peterson CN, Levchenko I, Rabinowitz JD, Baker TA, Silhavy TJ (2012) RpoS proteolysis is controlled directly by ATP levels in *Escherichia coli*. *Genes and Development* 26: 548–553.

Petrella S, Cambau E, Chauffour A, Andries K, Jarlier V, Sougakoff W (2006) Genetic basis for natural and acquired resistance to the diarylquinoline R207910 in mycobacteria. *Antimicrobial Agents and Chemotherapy* 50: 2853–2856.

Petrone G, Garboczi DN, Pedersen PL (1987) Mitochondrial ATP synthase complex: interaction of its F1 adenosinetriphosphatase moiety with the heavy atom iodine. *Biochemistry* 26: 4016–4021.

Porat R, Clark BD, Wolff SM, Dinarello CA (1991) Enhancement of growth of virulent strains of *Escherichia coli* by interleukin-1. *Science* 254: 430–432.

Priyadarshini R, Cugini C, Arndt A, Chen T, Tjokro NO, Goodman SD, Davey ME (2013) The nucleoid-associated protein HUbeta affects global gene expression in *Porphyromonas gingivalis*. *Microbiology* 159: 219–229.

Rabie G, Lally ET, Shenker BJ (1988) Immunosuppressive properties of *Actinobacillus actinomycetemcomitans* leukotoxin. *Infection and Immunity* 56: 122–127.

Rahamat-Langendoen JC, van Vonderen MG, Engstrom LJ, Manson WL, van Winkelhoff AJ, Mooi-Kokenberg EA (2011) Brain abscess associated with *Aggregatibacter actinomycetemcomitans*: case report and review of literature. *Journal of Clinical Periodontology* 38: 702–706.

Schenkein HA, Barbour SE, Berry CR, Kipps B, Tew JG (2000) Invasion of human vascular endothelial cells by *Actinobacillus actinomycetemcomitans* via the receptor for platelet-activating factor. *Infection and Immunity* 68: 5416–5419.

Schreiner HC, Sinatra K, Kaplan JB, Furgang D, Kachlany SC, Planet PJ, Perez BA, Figurski DH, Fine DH (2003) Tight-adherence genes of *Actinobacillus actinomycetemcomitans* are required for virulence in a rat model. *Proceedings of the National Academy of Science, USA* 100: 7295–7300.

Shenker BJ, Vitale LA, Keiba I, Harrison G, Berthold P, Golub E, Lally ET (1994) Flow cytometric analysis of the cytotoxic effects of *Actinobacillus actinomycetemcomitans* leukotoxin on human natural killer cells. *Journal of Leukocyte Biology* 55: 153–160.

Shenker BJ, McKay T, Datar S, Miller M, Chowhan R, 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. *Journal of Immunology* 162: 4773–4780.

Simpson DL, Berthold P, Taichman NS (1988) Killing\ of human myelomonocytic leukemia and lymphocytic cell lines by *Actinobacillus actinomycetemcomitans* leukotoxin. *Infection and Immunity* 56: 1162–1166.

Tomich M, Planet PJ, Figurski DH (2007) The *tad* locus: postcards from the widespread colonization island. *Nature Reviews Microbiology* 5: 363–375.

Tompa P, Szasz C, Buday L (2005) Structural disorder throws new light on moonlighting. *Trends in Biochemical Sciences* 30(9): 484–489.

Tsai CC, McArthur WP, Baehni PC, Hammond BF, Taichman NS (1979) Extraction and partial characterization of a leukotoxin from a plaque-derived Gram-negative microorganism. *Infection and Immunity* 25: 427–439.

Wai SN, Mizunoe Y, Takade A, Kawabata SI, Yoshida SI (1998) *Vibrio cholerae* O1 strain TSI-4 produces the exopolysaccharide materials that determine colony morphology, stress resistance, and biofilm formation. *Applied and Environmental Microbiology* 64: 3648–3655.

Wang HC, Wu ML, Ko TP, Wang AH (2013) Neisseria conserved hypothetical protein DMP12 is a DNA mimic that binds to histone-like HU protein. *Nucleic Acids Research* 41: 5127–5138.

Wrangstadh M, Conway PL, Kjelleberg S (1986) The production and release of an extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion. *Archives of Microbiology* 145: 220–227.

Wu L, Estrada O, Zaborina O, Bains M, Shen L, Kohler JE, Patel N, Musch MW, Chang EB, Fu YX, Jacobs MA, Nishimura MI, Hancock RE, Turner JR, Alverdy JC (2005) Recognition of host immune activation by *Pseudomonas aeruginosa*. *Science* 309: 774–777.

Zambon JJ (1985) *Actinobacillus actinomycetemcomitans* in human periodontal disease. *Journal of Clinical Periodontology* 12: 1–20.

Zav'yalov VP, Chernovskaya TV, Navolotskaya EV, Karlyshev AV, MacIntyre S, Vasiliev AM, Abramov VM (1995) Specific high affinity binding of human interleukin 1 beta by Caf1A usher protein of *Yersinia pestis*. *FEBS Letters* 371: 65–68.

Zijnge V, Kieselbach T, Oscarsson J (2012) Proteomics of Protein Secretion by *Aggregatibacter actinomycetemcomitans*. *PLoS One* 7: e41662.

3.9

Moonlighting Outside of the Box

22

Bacteriophage Moonlighting Proteins in the Control of Bacterial Pathogenicity

Janine Z. Bowring[1], *Alberto Marina*[2,3], *José R. Penadés*[1], *and Nuria Quiles-Puchalt*[1]

[1] *Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK*
[2] *Instituto de Biomedicina de Valencia, IBV-CSIC, Valencia, Spain*
[3] *CIBER de Enfermedades Raras (CIBERER-ISCIII)*

22.1 Introduction

Bacteriophages (phages) are viruses that infect and replicate within bacterium following injection of their genome into the bacterial cell. Phages are basically composed of a proteic particle that encapsulates the DNA or RNA genome. They are found with enormous diversity in genome composition, virion structures, and lifestyles, being widely distributed in a great variety of habitats. With a global estimated population of 10^{30}, phages are considered the most abundant organisms in the biosphere (Brüssow and Hendrix 2002).

Phages play critical roles in bacterial virulence, ecology and diversity. Thus, bacteriophages can alter the genome architecture of bacteria as they serve as points for genomic rearrangement due to their mosaic nature (Brüssow *et al*. 2004). Integrated temperate phages can influence the global regulators of the host, allowing them to adapt to a specific niche (Clokie *et al*. 2011), they confer protection to possible lytic infections by other phages (Berngruber *et al*. 2010), and can be involved in different processes of ecological competition between different bacterial species racing for the same niche (Selva *et al*. 2009). Bacteriophages can also contribute greatly to the pathogenicity of bacteria as they encode for different virulence factors and for many of the toxins that cause specific toxin-mediated diseases (toxinoses). The best known of these toxins are responsible for diseases such as diphtheria, cholera, dysentery, botulism, food poisoning, scalded skin syndrome, necrotizing pneumonia, or scarlet fever (Boyd *et al*. 2012). As well as their own transfer, phages can mobilize host genomic segments and other mobile genetic elements (MGEs) by classical generalized transduction. This widespread mechanism, common to most bacteria, allows the transfer of any

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

gene from one bacterium to another and greatly contributes to bacterial evolution (Penadés *et al*. 2015).

Despite the wide diversity in size and genomic content of bacteriophages, a common characteristic to all of them is that they encode the essential genes needed to complete their life cycle and all the factors required to successfully take advantage of the host cellular machineries. The constraints imposed by the limited amount of genomic content that the phage capsids can package renders the question of which genes bacteriophages should encode a matter of vital importance. It is therefore assumed that the genes encoded by a bacteriophage should have a vital function for the phage so that the genetic content is optimized to obtain the best outcome. This restriction in genome size could hinder the acquisition of new and adaptive functions, so the acquisition of a novel function by an already encoded protein could be highly beneficial for the bacteriophage. On that account, it could be expected that within the different bacteriophage genomes studied so far, plenty of proteins with more than one function would be found. Unfortunately, and probably due to the difficulty in predicting a moonlighting function for a protein, few examples of bacteriophage moonlighting proteins have been extensively studied regarding their secondary function and their impact on the pathogenicity of bacteria.

The most extensively studied bacteriophage moonlighting protein is the capsid decorator protein Psu of the P4 bacteriophage. This protein has a structural role in the assembly of the viral particle by helping to stabilize the capsid. Additionally, Psu also acts as a Rho-dependent transcriptional antiterminator, interacting with the Rho factor and hence with the bacterial RNA transcription machinery. Psu therefore prevents the termination of different transcripts and controls the expression of bacterial genes that could have an impact on overall bacterial pathogenesis (Ranjan *et al*. 2013, 2014). Not all bacteriophage proteins that exhibit a moonlighting function in the control of bacterial pathogenicity interact directly with bacteria factors, as in the case of Psu. Given the impact of phage on bacterial growth and in horizontal gene transfer, some moonlighting functions affect the phage while indirectly impacting on bacterial pathogenicity. An example of this is found in some structural bacteriophage proteins with a lytic role, essential for the viavility of the phage (Boulanger *et al*. 2008; Rodríguez-Rubio *et al*. 2013). This is also the case for the T4 I-TevI homing endonuclease of the T4 bacteriophage that, additionally to its role in promoting intron mobility, acts as an auto-repressor of its own transcription and so controls the persistance in its host (Edgell *et al*. 2004).

One of the most important contributions of the bacteriophages to bacterial pathogenicity is the mobilization of other mobile genetics elements and the virulence factors that they encode. In this sense, the study of the mobilization of the *Staphylococcus aureus* pathogenicity islands by their helper bacteriophages has led to the identification of different bacteriophage moonlighting proteins that, apart from their role in the biology of the phage, interact with these elements and activate their transfer (Tormo-Más *et al*. 2010). All of the examples mentioned previously refer to bacteriophage-encoded proteins with a dual role, interacting either with the phage, the bacteria, or with other mobile genetic elements, but there are also examples of bacteria-encoded proteins that have a moonlighting

role in phage biology. The *Escherichia coli* thioredoxin protein that has an antioxidant function for the bacteria also moonlights as an enhancer of the replication of the T7 bacteriophage (Bedford *et al*. 1997; Ghosh *et al*. 2008). In this chapter the dual role of these proteins is described in more detail, along with their impact on the bacteriophage biology and the repercussion in bacterial pathogenicity.

22.2 Bacteriophage T4 I-TevI Homing Endonuclease Functions as a Transcriptional Autorepressor

The genome of the *E. coli*-infecting bacteriophage, T4 contains a thymidylate synthase group 1A intron encoding the homing endonuclease I-TevI. The homing endonuclease I-TevI functions as a selfish genetic element that spreads between related genomes by a "homing" mechanism (Chu *et al*. 1984; Bell-Pedersen *et al*. 1989; Quirk *et al*. 1989). Homing occurs when the translated endonuclease protein binds to a DNA recognition site and cleaves a double-strand break (DSB) (Clyman & Belfort 1992; Mueller *et al*. 1996). The intron and associated homing endonuclease are then incorporated through DSB-repair, which uses the intron-containing allele as a template (Burt & Koufopanou 2004; Belfort & Bonocora 2014; Stoddard 2014). The generalized mechanism of group 1A intron-encoded homing can be seen in Figure 22.1. For a more comprehensive review on the homing endonucleases, readers are directed to the recent reviews by Stoddard (2014) and Belfort & Bonocora (2014). This mechanism allows the homing endonuclease and the encoding intron to exist in mutual association, with the intron providing the endonuclease with a phenotypically neutral location to reside and the endonuclease promoting the intron-carrying genes to spread between genomes (Belfort 1989).

Group I intron-encoded homing endonucleases contain a lengthy recognition sequence for their target DNA, to prevent random cleavage of the host genome (Jurica & Stoddard 1999). The intron-containing allele is also protected from cleavage by its homing endonuclease due to the intron itself interrupting the recognition sequence. Although these recognition sequences limit the homing endonucleases to site-specific cleavage, this is in a sequence-tolerant fashion. As such they can cleave variant homing sites, sometimes with near-wild-type efficacy, to maximize spread to related genomes (Bryk *et al*. 1993, 1995; Jurica & Stoddard 1999; Chevalier & Stoddard 2001). Indeed these intron-encoded mobile elements are encoded across organellar genomes, microbial genomes, and phage, with highly efficient transfer and transmission.

Enterobacteria phage T4, one of the T-even phage, has been found to encode 15 homing endonucleases. Of these 12 are "free-standing" endonucleases that are encoded in the intergenic regions, while the other 3 are group 1 intron-encoded endonucleases (Edgell *et al*. 2010). These 3 introns were found to be inserted into the genes for thymidylate synthase (*td*) and the genes for aerobic (*nrd*B) and anaerobic (*nrd*D) ribonucleotide reductase (Chu *et al*. 1984; Gott *et al*. 1986; Young *et al*. 1994). The homing endonucleases encoded by the introns of *td* and *nrd*D were named I-TevI and I-TevII, respectively, while the T4 *nrd*B

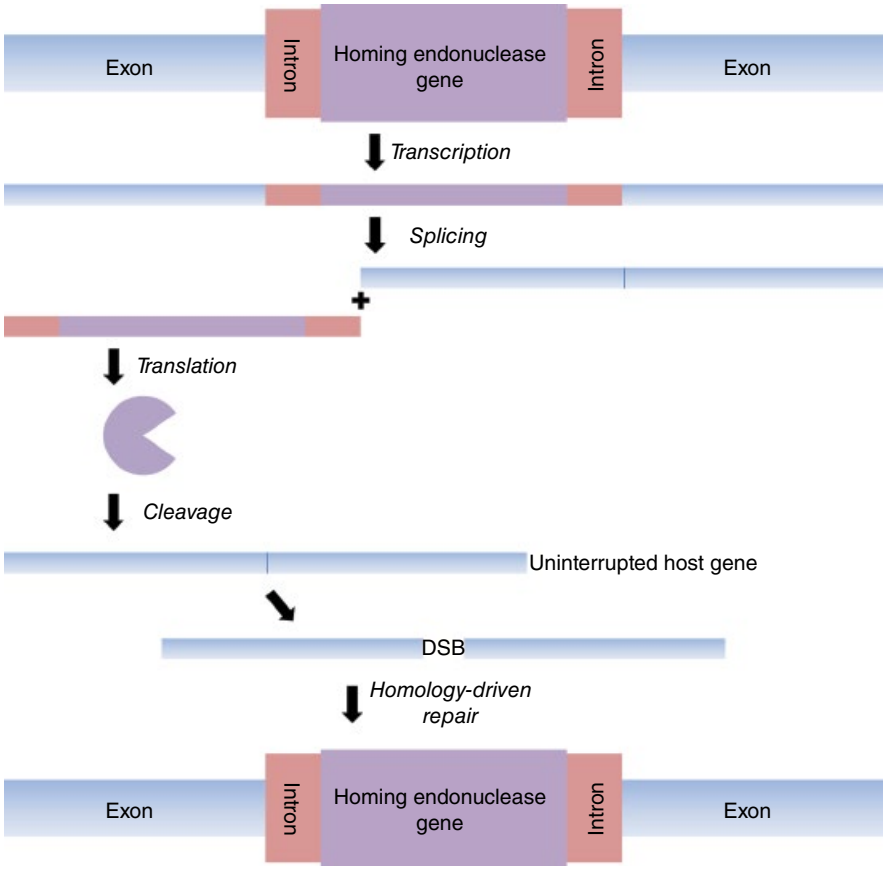

Figure 22.1 The homing mechanism for mobile group I introns. The endonuclease and flanking introns are transcribed, splicing occurs, and the homing endonuclease is translated as a free-standing protein. The endonuclease activity will cleave an uninterrupted host gene at the corresponding cleavage site, forming a double-strand break (DSB) close to the intron insertion site. Repair by homology-driven strand invasion, recombination, and replication using the intron-containing allele leads to incorporation of the intron and associated endonuclease into the gene. Source: Stoddard (2014).

intron contained a mutation rendering the intron immobilized and the encoded endonuclease non-functional (Quirk *et al*. 1989). This endonuclease (I-TevIII) is found in non-mutated, active form in the related phage RB3 (Eddy & Gold 1991). These intron-encoded homing endonucleases are each subject to translational control, with T4 middle and/or late promotors and Shine-Dalgarno sequences preceding the coding sequences of the open reading frames of each intron (Gott *et al*. 1988). This transcriptional regulation could be important for the expression of the genes within which the introns reside (Semrad & Schroeder 1998). In the case of I-TevI, further transcriptional regulation is provided by the homing endonuclease itself (Edgell *et al*. 2004). This endonuclease moonlights as an autorepressor of its own transcription in order to ensure persistence in the phage population (Edgell *et al*. 2004).

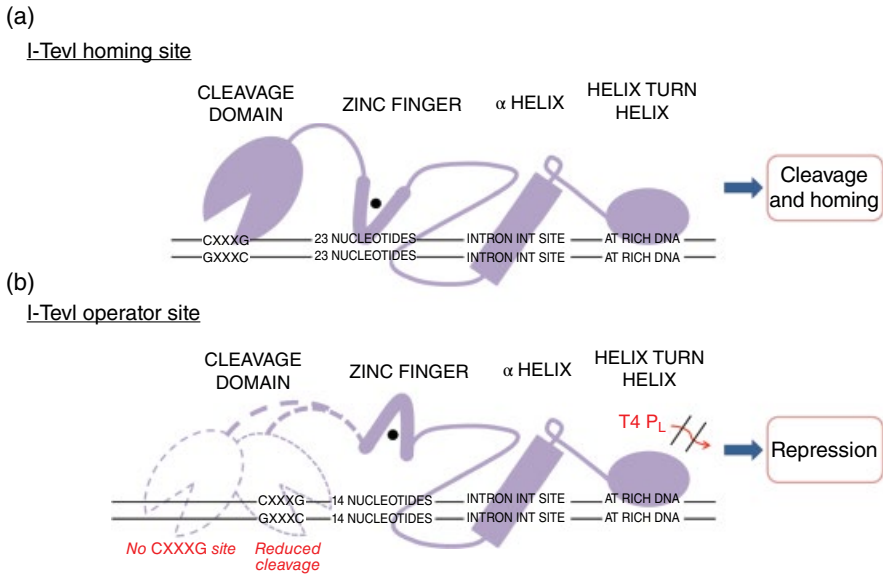

Figure 22.2 A cartoon depicting the two separate functions of homing endonuclease I-TevI. (a) This section shows the homing function of I-TevI in which the helix-turn-helix interacts with the AT-rich section of a homing site, leading to the zinc finger directing the catalytic domain 23–25 nucleotides upstream of the intron integration site (Intron Int Site). The catalytic domain cleaves as the CXXXG site and this cleavage forms the DSB necessary for intron homing and incorporation. (b) This section depicts the autorepressor function of the I-TevI. In this case the helix-turn-helix interacts with an AT-rich site displaying 15 bp identity to the homing AT-rich site, but upstream of an operator site. This operator AT-rich sequence may overlap with the T4 late promotor. Binding of the helix-turn-helix to the operator site directs the catalytic domain to interact with a CXXXG site 9 nt closer to the DNA-binding site. Cleavage at this site is reduced *c*. 100-fold compared to at the homing site, and this reduction in catalytic activity silences transcription.

I-TevI is therefore able to act as a homing endonuclease while moonlighting as a transcriptional autoregulator due to its distinct functional domains. The homing site of I-TevI consists of 38 bp of the *td* coding sequence, but only 20 bp are crucial for interaction with the appropriate intron-less gene (Derbyshire *et al.* 1997; Van Roey *et al.* 2001). Examination of the *td* intron immediately upstream of the I-TevI indicated a 15 bp identity between the I-TevI homing site and the I-TevI potential operator sites, which overlap with the T4 middle and late promoters (Edgell *et al.* 2004). This 15 bp includes an A-T-rich section critical for the interaction of the endonuclease helix-turn-helix (HTH) module with the substrate. This module directs the zinc finger to position the catalytic domain to cleave at the optimal distance on the homing substrate.

As Figure 22.2 shows, at a normal homing site of the I-TevI the zinc finger directs the catalytic domain 23–25 nucleotides (nt) upstream of the intron integration site, at a CXXXG sequence, leading to cleavage and homing of the intron into the gene. At operator site DNA the helix-turn-helix module interacts with the A-T-rich section displaying identity to the homing site and which can overlap with the T4 late promoter. As the substrate lacks the CXXXG sequence at the

preferred distance of 23–25 nt, the catalytic domain scans the substrate for the sequence elsewhere. In the operator site this sequence is located 9 nt closer to the DNA binding domain and cleavage at this site is reduced by a factor of 100 compared to at the homing site (Edgell *et al.* 2004). Cleavage therefore occurs at a much lower frequency; this reduction in catalytic activity silences transcription and in this way I-TevI acts as an autorepressor. Through these mechanisms the separate functional domains of I-TevI allow for two separate functions.

This bacteriophage homing endonuclease that moonlights as a repressor of its own transcription facilitates both dissemination throughout intronless *td* alleles (*td* homing sites which form further copies of the gene following recombination of I-TevI), whilst also minimizing host genome cleavage to ensure persistence in the phage population (Bryk *et al.* 1995; Edgell *et al.* 2004). Given the importance of horizontal transfer of phage genes to bacterial pathogenicity, the horizontal transfer of the I-TevI within a lysogenic phage population may have impact on phage life cycle and bacterial pathogenicity. Indeed, a study on the related T4 homing endonuclease, SegF, indicated that T4 contains many more homing endonuclease genes than its nearest relatives (Belle *et al.* 2002). It appears T4 is more able to withstand the DSBs entailed in encoding homing endonucleases and this allows phage T4 to integrate many more into its genome. The greater incorporation of homing endonucleases gives T4 a general advantage over its relatives; research indicates that phage T2 is partially excluded from mixed infection progeny in the presence of T4 and that T4 genes are preferentially incorporated into progeny with genetic crosses from mixed populations (Belle *et al.* Shub 2002). This indicates that homing endonucleases can confer a selective advantage in phage T4, the inference being that homing endonucleases can play a role in phage evolution. The impact of phage evolution on the evolution of the bacteria they infect can lead to changes in bacterial pathogenicity, either through horizontal gene transfer or selective pressures. Furthermore, there is a possibility of homing endonuclease horizontal transfer between phage and eukarya indicating such transfer could have a wider impact (Michel & Dujon 1986; Shub *et al.* 1988).

22.3 Capsid Psu Protein of Bacteriophage P4 Functions as a Rho Transcription Antiterminator

The Enterobacteria phage-plasmid P4 encodes another example of a phage protein that moonlights to control elements of bacterial pathogenicity. The bacteriophage-plasmid P4 is an 11.6 kb linear, double-stranded DNA phage-plasmid, originally isolated from *E. coli* as phage-forming plaques on P2 lysogenic strains (Six and Klug 1973; Briani *et al.* 2001). The P4 phage-plasmid can be found either as a multicopy plasmid or integrated into the host genome in an immune-integrated condition similar to the lysogenic state; however, P4 requires the presence of an unrelated helper phage, such as P2, to undergo the lytic cycle (Briani *et al.* 2001; Six & Klug 1973). The P2 helper phage encoded tail proteins, capsid proteins, and late-stage lytic functions are utilized by the P4, which encodes only three of the proteins necessary for the P4 lytic cycle (Six 1975; Souza *et al.* 1978). The δ gene encoded by the P4 initiates transactivation of the P2 prophage late

stage lysis genes (Souza *et al.* 1977). The capsid size determining scaffold protein Sid is encoded by the P4 *sid* gene. This protein forms a hexamer responsible for the assembly of P2 produced capsid proteins into a 45 nm P4 capsid, rather than the normal 60 nm P2 capsid (Shore *et al.* 1978). The third protein encoded by the P4 phage-plasmid is the capsid-decorating protein Psu, which acts to stabilize the smaller P4 capsid (Dokland *et al.* 1993; Isaksen *et al.* 1993). This 21 kD protein also moonlights as a transcription antiterminator of bacterial Rho-dependent termination (Pani *et al.* 2006; Ranjan *et al.* 2014).

Rho-dependent transcriptional termination is the mechanism by which the homohexameric RNA helicase Rho disassociates an elongation complex (EC) containing bacterial RNA polymerase from template DNA and nascent RNA (Peters *et al.* 2011). Along with intrinsic termination which does not require the participation of Rho, this process is essential for the proper expression of bacterial genes. Although Rho-dependent termination has been the subject of study for many years, the exact molecular mechanism by which Rho binds nascent RNA and disassociates the EC from template DNA and transcript RNA remains unclear. A number of reviews and studies summarize the current knowledge on Rho-dependent transcriptional termination and should be referred to for further information (Banerjee *et al.* 2006; Epshtein *et al.* 2010; Mooney *et al.* 2009; Peters *et al.* 2011). The main steps in the pathway of Rho termination are: the Rho protein binding to nascent RNA transcript; activation of the Rho ATP-powered translocase activity; translocation of the RNA 5'→3' through the Rho central channel; and finally Rho dissociation of an EC halted at a pause site from the template DNA and nascent RNA.

Psu was identified as an antiterminator of *E. coli* Rho-dependent transcription over two decades ago, making it the first moonlighting bacteriophage protein to be identified (Lagos *et al.* 1986; Linderoth & Calendar 1991). The antitermination function of Psu does not require a specific mRNA sequence, and the activity is not dependent on any other phage gene (Lagos *et al.* 1986; Linderoth & Calendar 1991). As shown in Figure 22.3a the resolved structure of the Psu monomer consists of seven α-helices forming three distinct subsections (Banerjee *et al.* 2012). The tight coiled-coil structure formed by N-terminal α1 and α2 helices is named the CC-stem and is encircled by the C-terminal α5–α7 helices, known as the CT-belt. The final subsection of the Psu protein is the central region comprising α3 and α4 helices (Banerjee *et al.* 2012). Studies indicate that the C-terminal 20 amino acids are important for antitermination of Rho-dependent termination (Ranjan *et al.* 2014).

Previous Psu studies suggested that the protein exists as a dimer in solution and the solved crystal structure confirmed the protein formed a symmetric "V"-shaped homodimer (Pani *et al.* 2006; Banerjee *et al.* 2012; Ranjan *et al.* 2013, 2014). Figure 22.3 indicates how the central region of the Psu monomer folds into a self-assembled knotted structure that forms the dimerization interface. At this interface two Psu monomers "knot" together, leading to the "V"-shaped homodimer (Banerjee *et al.* 2012). Self-tying knots formed by folds in the polypeptide backbone have been previously described, though it is interesting that the Psu has been shown to form this knotted conformation spontaneously during *in vitro* folding assays (King *et al.* 2010; Ranjan *et al.* 2014). A combination of the knotted

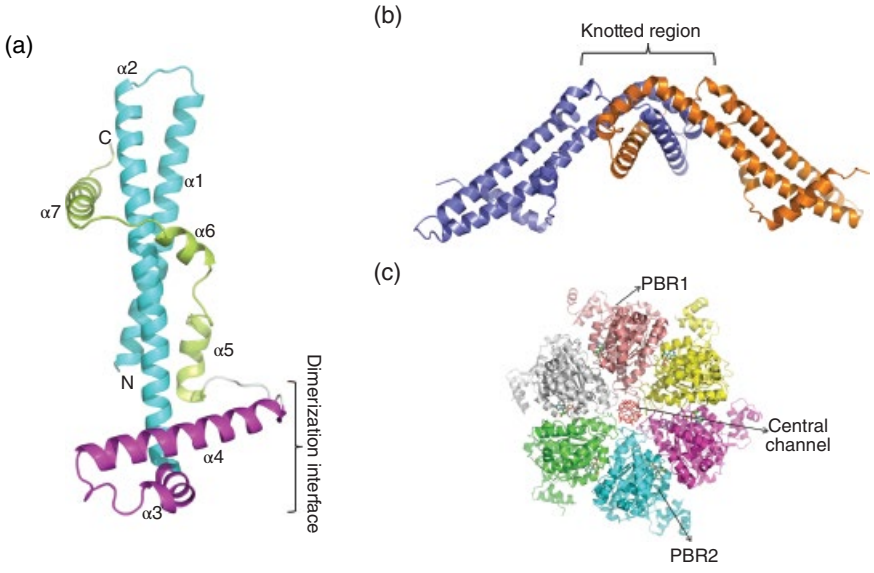

Figure 22.3 (a) The crystal structure of the Psu monomer with seven α-helices and dimerization interface. The N-terminal, α1, and α2 helices form a tight coiled-coil structure termed the "CC-stem," surrounded by the three C-terminal helices (α5–α7) known as the CT-belt. The central region, shown here as the dimerization interface and consisting of helices α3 and α4, forms a knotted structure with another monomer. CC-stem in cyan, CT-belt in green and central region in magenta. (b) The dimeric structure shown here is formed when the two Psu monomers "knot," and this knotted region (as indicated) acts as a hinge allowing the other regions to swing inward and outward. This dimer formation allows Psu to interact with and bind the Rho hexamer. (PDB code: 3RX6). The figure shows the dimer with domain colour differences. (c) A top view of the closed, hexameric structure of Rho bound to RNA (PDB code: 3ICE). The two Psu-binding regions (PBR1 and PBR2) are indicated by arrows along with the central region containing the RNA. Source: Banerjee *et al*. (2012) and Ranjan *et al*. (2014). (*See color plate section for the color representation of this figure*.)

dimer interface and the CC-stem form a stable architecture for the CT-belt to form around. The knotted region of the dimer also acts like a hinge, allowing some movement of the CC-stem and CT-belt. This allows the C-terminal of the two Psu monomers to interact with the Psu-binding regions (PBR) of the Rho hexamer. These binding regions are indicated in Figure 22.3c, which shows a top view of the Rho homohexameric protein. Rho amino acids 139–153 make up PBR1, the primary interaction site for Psu binding. The existence of a secondary site (PBR2) at residues 347–355 has also been identified. It is predicted that the major interaction of Rho: Psu occurs at PBR1, with PBR2 playing a lesser role (Ranjan *et al*. 2014).

The interaction of Psu with PBR1 and PBR2 is predicted to lead to the docked structure modeled in Figure 22.4, with a Psu dimer bound to diagonally opposite intersubunit niches on the Rho hexamer (Ranjan *et al*. 2014). The Psu interaction occurs near to both the ATP- and secondary RNA-binding sites of the Rho, inhibiting ATP binding and ATPase activity (Pani *et al*. 2006). The positioning of the dimer over the central channel of the Rho is comparable to the positioning of Psu over P4 hexameric capsid proteins (Dokland *et al*. 1993). Psu bound to P4 capsid

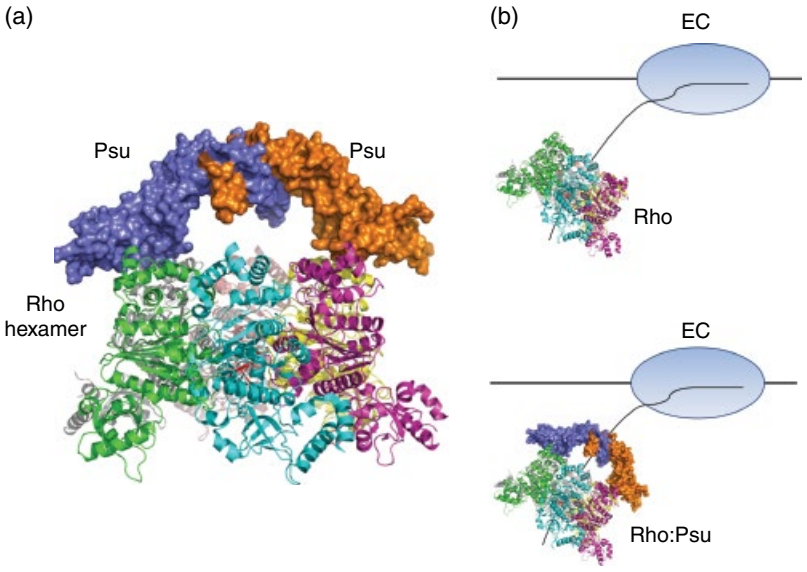

Figure 22.4 (a) A cartoon representation of a Psu dimer (PDB: 3RX6) interacting with a Rho hexamer (PDB:3ICE). The Psu dimer occupies diagonally opposite intersubunit niches of the hexamer, near Rho ATP-binding sites. This interaction positions the Psu dimer over the central channel of the Rho hexamer, which is predicted to mechanically impede the translocase activity of the Rho. (b) A cartoon showing the action of Psu as a transcription antiterminator. The initial figure shows the Rho hexamer on the nascent RNA of the elongation complex (EC), which will lead to the termination of transcription once Rho disassociates the EC from the RNA and template DNA. The second image represents the antitermination mechanism, in which Psu dimer blocks the central channel of the Rho, preventing the translocation of nascent RNA through the Rho and thus preventing the disassociation of the EC from the RNA and template DNA. The Psu dimers here are depicted by surface representation. Source: Banerjee *et al*. (2012) and Ranjan *et al*. (2014).

proteins has been shown to block the hexamer "hole" and the structure of the Psu dimer indicates it binds RNA bound Rho with a preference for the "closed" state of the Rho central channel (Ranjan *et al*. 2014). As such, it is predicted that Psu interaction with Rho blocks the central channel in a similar manner to the capsid proteins. By covering the central channel of the Rho hexamer the Psu dimer mechanically impedes the exit path of nascent RNA, blocking the translocase activity of the Rho (Ranjan *et al*. 2014). Furthermore, the ATPase activity of the Rho has been directly linked to the translocation rate of the Rho along the RNA, so the bound Psu also blocks Rho translocation by inhibiting ATP binding and hydrolysis (Chalissery *et al*. 2007). Unable to translocate the nascent RNA the Rho would not dissociate the EC from the template DNA and nascent RNA transcript, thus preventing the termination of transcription.

The moonlighting function of the P4 Psu protein as a Rho-termination antiterminator has implications for bacterial pathogenicity. Transcription and Rho-dependent transcription termination are essential for bacterial gene expression, which controls pathogenicity. Indeed there are indications that Rho-dependent termination has a role in "silencing" horizontally transferred DNA, such as

prophage DNA (Cardinale *et al*. 2008). Important pathogenicity genes such as toxins and antibiotic resistance genes could be lost through this silencing, and the antiterminator function of Psu could prevent the loss of such genes. Furthermore, Rho is highly conserved throughout a number of different bacterial pathogens. Studies have found this conservation extends to the PBR1 site, indicating Psu could have an inhibiting effect on the Rho proteins of other pathogens (Ranjan *et al*. 2014).

22.4 Bacteriophage Lytic Enzymes Moonlight as Structural Proteins

There are other examples of morphogenetic proteins that have a secondary role other than their structural function. Bacteriophage T5, a member of the *Siphoviridae* family that infects *E. coli*, encodes a tape measure protein, Pb2, with the dual role of acting as a structural protein involved in the formation of the tail and also as a pore-forming protein with a peptidoglycan hydrolase activity (Feucht *et al*. 1990; Boulanger *et al*. 2008). This lytic activity is related to the formation of the channel through which the phage genome crosses the cell membrane. Another example of a morphogenetic protein with a moonlighting activity is the peptidoglycan hydrolase encoded by the *S. aureus* bacteriophage φ11. The gene product 49 of the bacteriophage φ11 has a virion-associated peptidoglycan hydrolase domain (VAPGH) in the C terminal region of the protein with a muralytic activity that involves this protein in the lysis of the bacteria after infection of the phage (Rodríguez *et al*. 2011). Nonetheless, phage mutants in this gene have been reported to lose their DNA, suggesting that this protein might be located in the baseplate of the viral particle where it could have a structural role preventing the exit of the phage DNA and stabilizing the capsid particle (Rodríguez-Rubio *et al*. 2013). This structural role seems more a consequence of the position of this protein in the forming capsid rather than be related to the main lytic activity of the protein, and it is crucial for the correct formation of the viral particle.

Although the way these proteins are involved in bacterial pathogenesis is not clearly defined, they have an essential role in the formation of the viral particle and the correct development of the phage life cycle; they are therefore implicated in the transfer of the virulent factors the phage encode or in the horizontal gene transfer mediated by bacteriophages. In this sense, while these proteins are not directly linked to bacterial pathogenicity, they are involved in the viability of the phage and represent an example of the potentially novel phage proteins with an essential secondary role.

22.5 Moonlighting Bacteriophage Proteins De-Repressing Phage-Inducible Chromosomal Islands

Recently, an unexpected moonlighting role for some staphylococcus bacteriophage proteins has been described related to their activity as de-repressors of the *S. aureus* pathogenicity islands (SaPIs). The highly mobile *S. aureus* pathogenicity

islands are chromosomal islands with a conserved genetic organization that generally encode genes involved in virulence, like the staphylococcal enterotoxin B (EntB), the toxic shock syndrome toxin (TSST-1), and other superantigens and factors involved in the host adaptation process (Lindsay *et al*. 1998; Viana *et al*. 2010). These elements are generally distributed in *S. aureus*; in fact, sequencing of a large number of *S. aureus* strains revealed that all sequenced strains possess one or more islands in their genome, and it is now assumed that almost all clinical strains contains at least one of these elements integrated in their genomes (Novick *et al*. 2010).

The *S. aureus* pathogenicity islands are closely related to certain *S. aureus* temperate (helper) bacteriophages, whose life cycles they parasitize. These mobile genetic elements do not encode for the morphogenetic genes needed for the assembly of the viral particle, instead hijacking the machinery from their helper phages for their transduction (Tallent *et al*. 2007; Tormo *et al*. 2008). This is not the only vital process where the presence of a helper bacteriophage is needed; the mobilization of the SaPIs requires the induction of a resident helper prophage or superinfection by a helper phage. One of the main characteristics of SaPIs is that they encode for the master repressor Stl which, like classic prophage repressors, binds to a region between two divergent promoters that controls transcription of the two major transcripts. Stl inhibits the expression of most of the island genes and promotes the maintenance into the host genome of the SaPI (Ubeda *et al*. 2008) (Fig. 22.5). Unlike the classical bacteriophage repressors which sense the activation of the SOS response followed by entry to the lytic cycle of the phage, the Stl repressor is insensitive to the activation of the SOS response. So, how does the SaPI sense the stressful situations that could lead to the bacterial death and escape before that happens? In other words, how is the SaPI activated? The answer to this question is a stunning example of how a protein can acquire a novel function to be used by a different element than that which encodes it.

The de-repression of the SaPIs is initiated by the interaction of a specific phage protein that, by a protein–protein interaction with the Stl repressor, disrupts the Stl-DNA complex allowing the initiation of the excision-replication-packaging cycle of the island (Tormo-Más *et al*. 2010) (Fig. 22.5). There is high sequence diversity among the different repressors encoded by the different islands and, as the interaction is highly specific, each different repressor interacts with a different bacteriophage protein. Accordingly, there is a difference in the ability of each Staphylococcal phage to induce the different islands, depending on whether they encode for the specific phage-encoded antirepressor. For example, bacteriophage 80α can induce almost all of the islands tested so far, including SaPI1, SaPI2, SaPIn1, SaPIbov1, SaPIbov2, and SaPIbov5, while bacteriophage φ11 only induces SaPIbov1 and SaPIbov5.

The study of the activation of the different islands has led to the identification of three de-repressor proteins encoded by the *S. aureus* bacteriophages (Tormo-Más *et al*. 2010). The de-repressor protein that interacts with the repressor encoded by the *S. aureus* pathogenicity islands SaPIbov1 and SaPIbov5 (as they encode for the same repressor) is the phage-encoded trimeric dUTPase. The dUTPases (Duts) are ubiquitous enzymes responsible for the regulation of

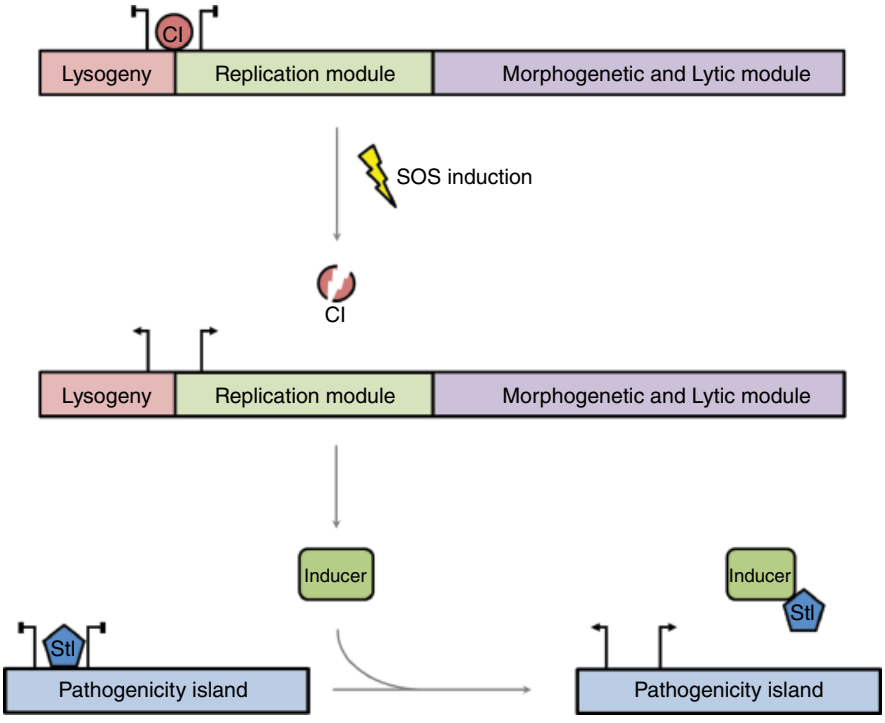

Figure 22.5 SaPI induction is mediated by a helper phage-encoded protein. The helper phage and the SaPI reside stably integrated into the bacteria chromosome due to the activity of their respective encoded master repressors, CI for the phage and Stl for the SaPI. When the SOS response is activated the repressor of the phage CI is degraded, allowing the expression of the lytic genes of the phage and initiating its replication. The interaction of a phage-encoded protein (Inducer) with the Stl repressor of the SaPI allows the de-repression of the main SaPI promoters and the activation of the island. (*See color plate section for the color representation of this figure.*)

cellular dUTP levels in the bacteria to prevent the misincorporation of the uracil into DNA, which could lead to chromosome fragmentation and cell death (Vértessy and Tóth 2009; Kouzminova and Kouzminov 2004). The role of the Duts in the activation of the island SaPIbov1 has been extensively studied and will be further examined in Section 22.6. The phage-encoded antirepressor for SaPI1 is the ORF22 of phage 80α, also known as Sri. Sri is a small protein of 52 residues that also plays a role as an inhibitor of bacterial replication. Sri has been shown to bind to the *S. aureus* homolog of *Bacillus subtilis* DnaI, a protein that is essential during the initation of the DNA replication process of the bacteria where it is required for the assembly of the primosome complex (Liu *et al*. 2004). Finally, the SaPIbov2 inducer is encoded by the ORF15 of the phage 80α, a protein with unknown function in the biology of the phage.

Closely related elements with the same genetic organization of the *S. aureus* pathogenicity islands have been found in other Gram-positive bacteria such as *Enterococcus faecalis*, *Lactococcus lactis*, and *Streptococcus* spp. and the "phage-related chromosomal islands" (PRCIs) term was proposed to group these genetic elements (Novick *et al*. 2010). These elements, of which the SaPIs are the best

characterized, have lately been re-designated as PICIs (phage-inducible chromosomal islands) and represent a new family of highly mobile genetic elements (Penadés *et al*. 2015). So far, the best-characterized PICI element studied in a non-staphylococcal species is the one present in the *E. faecalis* V583 strain (EfsCIV583). Although it was first identified as a defective prophage that interacted with the *E. faecalis* V583 prophage φV1 to form a composite functional phage (Duerkop *et al*. 2012), a subsequent study identified it as a PICI element (Matos *et al*. 2013). In this study, it was shown that prophage φV1 would act as the helper phage, and the PICI will be packaged in small capsids, a common feature with SaPIs. Nonetheless, it was also proposed that activation of the element is dependent on a SOS-sensitive repressor with activity controlled by a helper phage-independent mechanism (Matos *et al*. 2013). The highly conserved structural homology between these elements renders the question whether the mechanism by which SaPIs are activated through the employment of a moonlighting-phage-encoded protein is used by any of the other PICI elements. As such, further studies of these elements are needed to determine the process involved in the activation of these elements.

The use of bacteriophage-encoded proteins as de-repressor proteins is an elegant strategy that allows the SaPI to be induced only when the helper phage has entered the lytic cycle. This couples the lifecycles of both elements and assures that the SaPI will be activated when the phage is producing the proteins that the element is going to parasitize, resulting in high-frequency horizontal transfer of the SaPI and the virulence genes it encodes, intra- and inter-generically (Maiques *et al*. 2007; Chen and Novick 2009; Chen *et al*. 2015). In addition to any function they may have in the biology of the phage itself, these bacteriophage-encoded proteins have an important role as promoters of the dissemination of other mobile genetic elements, such as SaPIs, and the virulence factors encoded by these elements, playing a crucial role in the bacterial pathogenicity.

22.6 dUTPase, a Metabolic Enzyme with a Moonlighting Signalling Role

The dUTPases (dUTP pyrophosphatase; Dut; EC 3.6.1.23) are metabolic enzymes encoded by all free-living organisms and many viruses, with a fundamental role in the prevention of the misincorporation of the uracil into the nascent DNA that could result in chromosomal fragmentation and cell death (Vértessy and Tóth 2009). Most DNA polymerases cannot distinguish between thymine and uracil, and so the different levels of the respective nucleotides incorporated into the DNA will depend on the relative levels of dUTP and dTTP present in the cell. The noncanonical nucleoside dUTP is constantly produced in the pyrimidine biosynthesis pathway either by phosphorilation of dUDP or by deamination of dCTP. The dUTPase enzymes which catalyze the hydrolysis of the deoxyuridine triphosphate (dUTP) to deoxyuridine monophosphate (dUMP) and inorganic pyrophosphate are responsible for keeping the levels of available dUTP low, and therefore strictly control the ratio of dUTP/dTTP. Additionally, this key enzyme in the nucleotide metabolism is also responsible for the production of the nucleotide dUMP, the precursor for dTTP.

The dUTPases are divided into three families of proteins according to oligomerization state: monomeric; homodimeric; and homotrimeric (Penadés *et al*. 2013). The homotrimeric Duts, the largest family of Duts, are found in most eukaryotes, prokaryotes, and some viruses, like some staphylococcal bacteriophages and retroviruses infecting non-primate cells. *E. coli* and human Duts are considered the prototype model of this family of Duts (Cedergren-Zeppezauer *et al*. 1992; Mol *et al*. 1996). The different subunits fold into β-pleated sheets with five conserved motifs distributed over the entire sequence, with each trimer subunit involved in the formation of the every active site present in the trimer (Vértessy and Tóth 2009). The homodimeric Duts are found in the trypanosomatides *Leishmania major* and *Trypanosoma cruzi*, in some bacterial species such as *Campylobacter jejuni*, and in some bacteriophages (Moroz *et al*. 2004; Hemsworth *et al*. 2011). Homodimeric Duts are all α-helices proteins, the nucleotide-binding catalytic core of which consists of four conserved α-helices that is decorated by additional helices variable in length and sequence. Although dimeric Duts are not related either in sequence or structurally to the homotrimeric enzymes, the active center is also formed by five motifs. Finally, the monomeric Duts are present in mammalian and avian herpesviruses (Tarbouriech *et al*. 2005). They seem to have emerged from a process of gene duplication, fusion, and reorganization from a trimeric dUTPase. The folding of these proteins resembles the active center of the trimeric Duts, and the characteristic five motifs forming the active site are also present (McGeehan *et al*. 2001).

As mentioned in the previous section (Section 22.5), in addition to their role in controlling the pool of dUTP, the dUTPases encoded by the staphylococcal bacteriophages moonlight as de-repressor proteins of the *S. aureus* islands SaPIbov1 and SaPIbov5. Although it was initially described how the enzymatic activity was unrelated to this secondary function of the protein (Tormo-Más *et al*. 2010), more recent studies suggest that this activity could be crucial in this role (Tormo-Más *et al*. 2013; Szabó *et al*. 2014). As with other members of the trimeric Dut family, the staphylococcal phage-encoded Duts proteins present the five conserved motifs involved in the enzymatic activity: motifs I, II, and IV coordinate the metal ion and the phosphate chain of the nucleotide, while a strictly conserved catalytic aspartate residue in motif III localizes and polarizes a water molecule for an in-line nucleophilic attack on the αP during the dUTP hydrolysis (Barabás *et al*. 2004; Chan *et al*. 2004). The conserved C-terminal motif V undergoes a conformational change during the enzymatic activity, flipping over the active site and creating a microenvironment that facilitates the nucleophilic attack (Vértessy *et al*. 1998). Surprisingly, the staphylococcal bacteriophage-encoded dUTPases possess an additional extra region, named domain VI, involved in SaPI induction. This domain that is not involved in the enzymatic activity is highly variable between the different bacteriophage-encoded dUTPases and has been shown to be responsible for the specifity in the interaction with the Stl repressor of the SaPI (Tormo-Más *et al*. 2010, 2013). The extra VI domain, although necessary, is not sufficient for SaPI induction; it has been shown that the conformational changes suffered by the motif V are also involved in this process. Binding of the nucleotide dUTP orders the C-terminal P-loop-like motif V and this conformational change is needed for the inductor activity of the dUTPase. The hydrolysis

of the dUTP disorders the C-terminal motif V, generating a Dut protein unable to induce the SaPI (Tormo-Más *et al*. 2013). Uncontrolled replication of the SaPIs results in diminished bacterial viability (Ubeda *et al*. 2008) and, as SaPIs need some cellular components for their transfer, this on/off mechanism for SaPI induction is a remarkable evolutionary adaptation allowing the prevention of uncontrolled replication. The proposed mechanism for activation of the island by this switching between the active (dUTP-bond) and inactive (apo state) conformations resembles that described for the proto-oncogenic G-proteins. G-proteins are involved in a number of different signaling pathways, including modulation of cell behaviors such as proliferation, differentiation, motility, and death (Etienne-Manneville *et al*. 2002).

In contrast to this proposed mechanism, other studies suggest that the Stl repressor of the SaPI could act as a competitor of dUTP for binding to the dUTPase protein and that the proposed extra motif VI is not needed for induction of the island. It has been proposed that high levels of dUTP inhibit the formation of the dUTPase:Stl complex and, conversely, the formation of this complex strongly inhibits the enzymatic activity of the dUTPase (Szabó *et al*. 2014; Hirmondó *et al*. 2015; Nyíri *et al*. 2015). In this scenario, only after the dNTP pool is cleared from dUTP will the dUTPase bind to the Stl repressor of the SaPI, activating the transfer of the island. The latest studies on this subject depicting these apparently contradictory mechanisms only highlight that this is a thrilling topic that is at the forefront of their study.

Recent studies support the hypothesis that virus-encoded Duts could be moonlighting proteins with different regulatory functions. For example, the Epstein–Barr virus (EBV) Dut activates NF-κB expression and produces immune dysregulation in the host by binding to the cellular Toll-like receptor 2 (TLR2) (Ariza *et al*. 2009). In the case of MHV-68, expression of the encoded viral Dut blocks the expression of the type I interferon signaling pathway (Leang *et al*. 2011). Finally, it has been reported that Kaposi's sarcoma Dut downregulates the immune response by targeting several cytokine receptors (Madrid and Ganem 2012). Taking into account these studies, and since it is assumed that bacteriophage-encoded proteins must have a role in the phage biology, it is tempting to hypothesize that the *S. aureus* bacteriophage-encoded Duts could have a role for the biology of the phage, while the SaPIs may be taking advantage of this mechanism for the activation of its own cycle. What could be the hypothetical function of the Dut in the phage biology? If we consider that other virus-encoded Duts have regulatory functions and the on/off switch mechanism that the *S. aureus* Duts have to activate the SaPIs, we could hypothesize that this would be a relevant regulatory process for the biology of the phage that has to be strictly regulated; this would certainly have relevance in the biology of the phage and an impact on bacterial pathogenicity. In this hypothetical scenario the *S. aureus* bacteriophage Duts would have different roles: one as a metabolic enzyme; one as an activator of the SaPI; and lastly one for the biology of the phage.

More studies are needed to clarify the mechanism by which the SaPIs are induced by the dUTPase and whether or not this enzyme is involved in an uncharacterized mechanism related to the phage biology. However, it is clear that the moonlighting role described for the dUTPase, a well-characterized

metabolic enzyme, as the activator for the mobilization of SaPIs and their encoded pathogenicity virulence factors is a remarkable feature implicating this protein in the regulation of horizontal gene transfer.

22.7 *Escherichia coli* Thioredoxin Protein Moonlights with T7 DNA Polymerase for Enhanced T7 DNA Replication

We have included this final instance of bacteriophage-related control of bacterial pathogenicity as an example of bacterial protein moonlighting in relation to phage. Bacteriophage T7 is an obligate lytic phage of *E. coli* that has been extensively studied (Lee & Richardson 2011). Although the phage encodes its own replicative DNA polymerase, encoded by gene 5, this polymerase is nonprocessive, dissociating from the template after polymerizing only 1–20 nucleotides (Huber *et al*. 1987). On infection of *E. coli* the polymerase forms a complex with the bacterial trx^2 and this interaction increases the polymerase processivity to *c*. 800 nucleotides per binding event (Huber *et al*. 1987; Tabor *et al*. 1987). The *E. coli* thioredoxin, including trx^2, has an antioxidant function in the host *E. coli*, a role completely independent of its role in T7 replication. An extensive review of this antioxidant process can be found in the following references (Arnér & Holmgren 2000; Huber *et al*. 1986; Lu & Holmgren 2014).

In its role as a processivity factor for the T7 phage the bacterial thioredoxin binds to a 71 amino acid loop on the T7 polymerase, designated the thioredoxin-binding domain (TBD). This forms a sliding clamp, allowing the polymerase to bind the template DNA more strongly, as well as remodeling to allow the interaction of other replication proteins (Bedford *et al*. 1997; Ghosh *et al*. 2008). Although the thioredoxin is part of one of the major antioxidant systems for *E. coli*, this moonlighting function is an example of the phage exploiting a bacterial protein for enhanced replication, with seemingly no positive effect for the host bacteria. The T7 bacteriophage is lytic and therefore DNA replication of the phage will lead to lysis of the host cell. In this situation, the bacteriophage-related moonlighting protein has a detrimental effect on the bacteria, impacting bacterial pathogenicity.

22.8 Discussion

In this chapter we have examined the dual role of various well-known and well-characterized bacteriophage moonlighting proteins and their impact on bacterial pathogenicity. The first example of moonlighting proteins studied was the homing endonuclease T4 I-TevI encoded by the T4 bacteriophage. This homing endonuclease, in addition to its main cleavage activity, has a role as a transcriptional regulator controlling its own transfer. There has to be a tightly regulated expression of the endonucleases since unregulated synthesis or non-temporally controlled expression could led to the generation of undesirable double-strand breaks.

This could affect the phage viability and even affect the host bacterial genome (Edgell *et al*. 2004), which would have repercussions on the overall pathogenicity of the bacterial population.

Interestingly, in some of the phage-encoded moonlighting proteins characterized so far it seems that, in addition to the primary function within the phage, the other moonlighting function is related to the biology of the bacteria (Ranjan *et al*. 2014) or to other mobile genetic elements (Tormo-Más *et al*. 2010, 2013). It should be noted that bacteriophages need the bacterial machinery to complete their biological processes, so employing an already-encoded phage protein for interaction with the host and modification of its metabolism for their own benefit could be an adaptive response. This is the case for Psu, which moonlights as a decorator protein of the capsid and a transcriptional antiterminator modifying bacterial gene expression by overcoming Rho terminator factor activity. In contrast, the use of bacteriophage-encoded proteins as activators by other mobile genetic elements, as described for the SaPIs, could be detrimental for the phage. SaPIs interact with their helper phages, hijacking the phage proteins for their own transfer and encoding a variety of mechanisms to cause interference with the phage (Christie and Dokland 2012; Ram *et al*. 2012, 2014). This raises the question as to why these phage proteins do not lose a secondary role that could be detrimental. A possible explanation for this would be the fact that SaPI repressors display a high variability in their gene sequence, suggesting that they are under a selective pressure to find new de-repressor bacteriophage-encoded proteins (Tormo-Más *et al*. 2010; Frigols *et al*. 2015). This co-evolution would lead to the acquisition of this de-repressor function in other bacteriophage-encoded proteins.

Not so surprisingly, bacteriophages can exploit bacterially encoded proteins, using them for a different function than that originally carried out in the bacteria. An example of this is bacteriophage T7 which uses the thioredoxin protein, an antioxidant protein produced by the bacteria *E. coli*, as an enhancer of bacteriophage replication (Bedford *et al*. 1997; Ghosh *et al*. 2008). Thioredoxin associates with the DNA polymerase, increasing the processivity of the replication process for the phage, leading to an increment of bacteriophage progeny and impacting on bacterial pathogenicity.

Despite the importance of bacteriophages in bacterial virulence and the key role moonlighting proteins have in this process, just a few examples of moonlighting proteins have been identified in bacteriophages. This could be explained either because bacteriophages do not encode for proteins with additional functions, or because of the difficulty in predicting a secondary role for these proteins, making it difficult to recognize them and indicating more bacteriophage moonlighting proteins are yet to be discovered. This last hypothesis seems to be correct, as recent studies have identified new phage-encoded proteins with a dual role (Tormo-Más *et al*. 2010). Particularly in the case of phages, the moonlighting phenomenon could be of more importance than previously thought. Bacteriophages encode in their genome the genes needed for replication, encapsidation, and infection of their bacteria host. New acquisition of genes, and accordingly new advantageous functions, is strictly restricted by the genome size that the phage can package into its capsid. In this sense the possibility that an

already existing protein could acquire a novel and moonlighting function is highly beneficial for the biology of the bacteriophage.

The difficulty in identifying a moonlighting protein is not unique to bacteriophages, as the same problem is encountered in the study of moonlighting proteins in prokaryotes and eukaryotes alike. In the likely scenario that numerous bacteriophage moonlighting proteins are yet to be discovered, this opens a new and challenging situation where a more complex network of interactions between bacteriophages and bacteria or other mobile genetic elements would be expected, which would certainly have an impact on bacterial virulence.

Understanding the biology of bacteriophages is of great importance due to their crucial role in bacterial pathogenicity, as well as for the study of the different proteins and functions that they have for their own biology. In this sense, the study of new proteins with moonlighting activities will lead to increase in the knowledge of these mobile genetic elements and their impact upon bacterial pathogenicity.

References

Ariza ME, Glaser R, Kaumaya PTP, Jones C, Williams MV (2009) The EBV-encoded dUTPase activates NF-kappa B through the TLR2 and MyD88-dependent signaling pathway. *The Journal of Immunology*, 182: 851–859.

Arnér ES, Holmgren A (2000) Physiological functions of thioredoxin and thioredoxin reductase. *European Journal of Biochemistry/FEBS*, 267(20): 6102–6109.

Banerjee S, Chalissery J, Bandey I, Sen R (2006) Rho-dependent transcription termination: more questions than answers. *Journal of Microbiology*, 44(1): 11–22.

Banerjee R, Nath S, Ranjan A, Khamrui S, Pani B, Sen R, Sen U (2012) The first structure of polarity suppression protein, Psu from enterobacteria phage P4, reveals a novel fold and a knotted dimer. *The Journal of Biological Chemistry*, 287(53): 44667–44675.

Barabás O, Pongrácz V, Kovári J, Wilmanns M, Vértessy BG (2004) Structural insights into the catalytic mechanism of phosphate ester hydrolysis by dUTPase. *The Journal of Biological Chemistry*, 279: 42907–42915.

Bedford E, Tabor S, Richardson CC (1997) The thioredoxin binding domain of bacteriophage T7 DNA polymerase confers processivity on *Escherichia coli* DNA polymerase I. *Proceedings of the National Academy of Sciences of the United States of America*, 94(2): 479–484.

Belfort M (1989) Bacteriophage introns: parasites within parasites? *Trends in Genetics*, 5(7): 209–213.

Belfort M, Bonocora RP (2014) Homing endonucleases: from genetic anomalies to programmable genomic clippers. *Methods in Molecular Biology, (Clifton, N.J.)*, 1123: 1–26.

Belle A, Landthaler M, Shub DA (2002) Intronless homing: site-specific endonuclease SegF of bacteriophage T4 mediates localized marker exclusion analogous to homing endonucleases of group I introns. *Genes and Development*, 16(3): 351–362.

Bell-Pedersen D, Quirk SM, Aubrey M, Belfort M (1989) A site-specific endonuclease and co-conversion of flanking exons associated with the mobile td intron of phage T4. *Gene*, 82(1): 119–126.

Berngruber TW, Weissing FJ, Gandon S (2010) Inhibition of superinfection and the evolution of viral latency. *Journal of Virology*, 84: 10200–10208.

Boulanger P, Jacquot P, Plancon L, Chami M, Engel A, Parquet C, Herbeuval C, Letellier L (2008) Phage T5 straight tail fiber is a multifunctional protein acting as a tape measure and carrying fusogenic and muralytic activities. *The Journal of Biological Chemistry*, 283: 13556–13564.

Boyd EF (2012) Bacteriophage-encoded bacterial virulence factors and phage-pathogenicity island interactions. *Advances in Virus Research*, 82: 91–118.

Briani F, Dehò G, Forti F, Ghisotti D (2001) The plasmid status of satellite bacteriophage P4. *Plasmid*, 45(1): 1–17.

Brüssow H, Hendrix RW (2002) Phage genomics: small is beautiful. *Cell*, 108: 13–16.

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

Bryk M, Quirk SM, Mueller JE, Loizos N, Lawrence C, Belfort M (1993) The td intron endonuclease I-TevI makes extensive sequence-tolerant contacts across the minor groove of its DNA target. *The EMBO Journal*, 12(5): 2141–2149.

Bryk M, Belisle M, Mueller JE, Belfort M (1995) Selection of a remote cleavage site by I-tevI, the td intron-encoded endonuclease. *Journal of Molecular Biology*, 247(2): 197–210.

Burt A, Koufopanou V (2004) Homing endonuclease genes: the rise and fall and rise again of a selfish element. *Current Opinion in Genetics & Development*, 14(6): 609–615.

Cardinale CJ, Washburn RS, Tadigotla VR, Brown LM, Gottesman ME, Nudler E (2008) Termination factor Rho and its cofactors NusA and NusG silence foreign DNA in *E. coli*. *Science (New York, NY)*, 320(5878): 935–938.

Cedergren-Zeppezauer ES, Larsson G, Nyman PO, Dauter Z, Wilson KS (1992) Crystal structure of a dUTPase. *Nature*, 355: 740–743.

Chalissery J, Banerjee S, Bandey I, Sen R (2007) Transcription termination defective mutants of Rho: Role of different functions of Rho in releasing RNA from the elongation complex. *Journal of Molecular Biology* 371(4): 855–872.

Chan S, Segelke B, Lekin T, Krupka H, Cho US, Kim MY, So M, Kim CY, Naranjo CM, Rogers YC, Park MS, Waldo GS, Pashkov I, Cascio D, Perry JL, Sawaya MR (2004) Crystal structure of the *Mycobacterium tuberculosis* dUTPase: insights into the catalytic mechanism. *Journal of Molecular Biology*, 341: 503–517.

Chen J, Novick RP (2009) Phage-mediated intergeneric transfer of toxin genes. *Science*, 323: 139–141.

Chen J, Carpena N, Quiles-Puchalt N, Ram G, Novick RP, Penadés JR (2015) Intra- and inter-generic transfer of pathogenicity island-encoded virulence genes by cos phages. *The ISME Journal*, 9(5): 1260–1263.

Chevalier BS, Stoddard BL (2001) Homing endonucleases: structural and functional insight into the catalysts of intron/intein mobility. *Nucleic Acids Research*, 29(18): 3757–3774.

Christie GE, Dokland T (2012) Pirates of the Caudovirales. *Virology*, 434: 210–221.
Chu FK, Maley GF, Maley F, Belfort M (1984) Intervening sequence in the thymidylate synthase gene of bacteriophage T4. *Proceedings of the National Academy of Sciences of the United States of America*, 81(10): 3049–3053.
Clokie MR, Millard AD, Letarov AV, Heaphy S (2011) Phages in nature. *Bacteriophage*, 1: 31–45.
Clyman J, Belfort M (1992) Trans and cis requirements for intron mobility in a prokaryotic system. *Genes & Development*, 6(7): 1269–1279.
Derbyshire V, Kowalski JC, Dansereau JT, Hauer CR, Belfort M (1997) Two-domain structure of the td intron-encoded endonuclease I-TevI correlates with the two-domain configuration of the homing site. *Journal of Molecular Biology*, 265(5): 494–506.
Dokland T, Isaksen ML, Fuller SD, Lindqvist BH (1993) Capsid localization of the bacteriophage P4 Psu protein. *Virology* 194(2): 682–687.
Duerkop BA, Clements CV, Rollins D, Rodrigues JLM, Hooper LV (2012) A composite bacteriophage alters colonization by an intestinal commensal bacterium. *Proceedings of the National Academy of Sciences of the United States of America*, 109: 17621–17626.
Eddy SR, Gold L (1991) The phage T4 nrdB intron: a deletion mutant of a version found in the wild. *Genes & Development*, 5(6): 1032–1041.
Edgell DR, Derbyshire V, Van Roey P, LaBonne S, Stanger MJ, Li Z, Boyd TM, Shub DA, Belfort M (2004) Intron-encoded homing endonuclease I-TevI also functions as a transcriptional autorepressor. *Nature Structural & Molecular Biology*, 11(10): 936–944.
Edgell DR, Gibb EA, Belfort M (2010) Mobile DNA elements in T4 and related phages. *Virology Journal*, 7: 290.
Epshtein V, Dutta D, Wade J, Nudler E (2010) An allosteric mechanism of Rho-dependent transcription termination. *Nature*, 463(7278): 245–249.
Etienne-Manneville S, Hall A (2002) Rho GTPases in cell biology. *Nature*, 420: 629–635.
Feucht A, Schmid A, Benz R, Schwarz H, Heller KJ (1990). Pore formation associated with the tail-tip protein pb2 of bacteriophage T5. *The Journal of Biological Chemistry*, 265: 18561–18567.
Frigols B, Quiles-Puchalt N, Mir-Sanchis I, Donderis J, Elena SF, Buckling A, Novick RP, Marina A, Penadés JR (2015) Virus satellites drive viral evolution and ecology. *PLOS Genetics*, 11(10): e1005609.
Ghosh S, Hamdan SM, Cook TE, Richardson CC (2008) Interactions of *Escherichia coli* thioredoxin, the processivity factor, with bacteriophage T7 DNA polymerase and helicase. *The Journal of Biological Chemistry*, 283(46): 32077–32084.
Gott JM, Shub DA, Belfort M (1986) Multiple self-splicing introns in bacteriophage T4: evidence from autocatalytic GTP labeling of RNA in vitro. *Cell*, 47(1): 81–87.
Gott JM, Zeeh A, Bell-Pedersen D, Ehrenman K, Belfort M, Shub DA (1988) Genes within genes: independent expression of phage T4 intron open reading frames and the genes in which they reside. *Genes & Development*, 2(12B): 1791–1799.
Hemsworth GR, Moroz OV, Fogg MJ, Scott B, Bosch-Navarrete C, González-Pacanowska D, Wilson KS (2011) The crystal structure of the *Leishmania major*

deoxyuridine triphosphate nucleotidohydrolase in complex with nucleotide analogues, dUMP, and deoxyuridine. *The Journal of Biological Chemistry*, 286: 16470–16481.

Hirmondó R, Szabó JE, Nyíri K, Tarjányi S, Dobrotka P, Tóth J, Vértessy BG (2015) *DNA Repair*, 30: 21–27.

Huber HE, Russel M, Model P, Richardson CC (1986) Interaction of mutant thioredoxins of *Escherichia coli* with the gene 5 protein of phage T7. The redox capacity of thioredoxin is not required for stimulation of DNA polymerase activity. *The Journal of Biological Chemistry*, 261(32): 15006–15012.

Huber HE, Tabor S, Richardson CC (1987) *Escherichia coli* thioredoxin stabilizes complexes of bacteriophage T7 DNA polymerase and primed templates. *The Journal of Biological Chemistry*, 262(33): 16224–16232.

Isaksen ML, Dokland T, Lindqvist BH (1993) Characterization of the capsid associating activity of bacteriophage P4's Psu protein. *Virology* 194(2): 674–681.

Jurica MS, Stoddard BL (1999) Homing endonucleases: structure, function and evolution. *Cellular and Molecular Life Sciences*, 55(10): 1304–1326.

King NP, Jacobitza A, Sawayab MR, Goldschmidta L, Yeates TO (2010) Structure and folding of a designed knotted protein. *Proceedings of the National Academy of Sciences of the United States of America*, 107(48): 20732–20737.

Kouzminova EA, Kuzminov A (2004) Chromosomal fragmentation in dUTPase-deficient mutants of *Escherichia coli* and its recombinational repair. *Molecular Microbiology*, 51: 1279–1295.

Lagos R, Jiang RZ, Kim S, Goldstein R (1986) Rho-dependent transcription termination of a bacterial operon is antagonized by an extrachromosomal gene product. *Proceedings of the National Academy of Sciences of the United States of America*, 83(24): 9561–9565.

Leang RS, Wu TT, Hwang S, Liang LT, Tong L, Truong JT, Sun R (2011) The anti-interferon activity of conserved viral dUTPase ORF54 is essential for an effective MHV-68 infection. *PLOS Pathogens*, 7(10): e1002292.

Lee SJ, Richardson CC (2011) Choreography of bacteriophage T7 DNA replication. *Current Opinion in Chemical Biology*, 15(5): 580–586.

Linderoth NA, Calendar RL (1991) The Psu protein of bacteriophage P4 is an antitermination factor for rho-dependent transcription termination. *Journal of Bacteriology*, 173(21): 6722–6731.

Lindsay J A, Ruzin A, Ross HF, Kurepina N, Novick RP (1998) The gene for toxic shock toxin is carried by a family of mobile pathogenicity islands in *Staphylococcus aureus*. *Molecular Microbiology* 29: 527–543.

Liu J, Dehbi M, Moeck G, Arhin F, Bauda, Bergeron D, Callejo M, Ferretti V, Ha N, Kwan T, McCarty J, Srikumar R, Williams D, Wu JJ, Gros P, Pelletier J, DuBow M (2004) Antimicrobial drug discovery through bacteriophage genomics. *Nature Biotechnology*, 22: 185–191.

Lu J, Holmgren A (2014) The thioredoxin antioxidant system. *Free Radical Biology & Medicine*, 66: 75–87.

Madrid AS, Ganem D (2012) Kaposi's sarcoma-associated herpes-virus ORF54/dUTPase downregulates a ligand for the NK activating receptor NKp44. *Journal of Virology*, 86: 8693–8704.

Maiques E, Ubeda C, Tormo MA, Ferrer MD, Lasa I, Novick RP, Penadés JR (2007) Role of staphylococcal phage and SaPI integrase in intra- and interspecies SaPI transfer. *Journal of Bacteriology*, 189: 5608–5616.

Matos RC, Lapaque N, Rigottier-Gois L, Debarbieux L, Meylheuc T, Gonzalez-Zorn B, Repoila F, Lopes Mde F, Serror P (2013) *Enterococcus faecalis* prophage dynamics and contributions to pathogenic traits. *PLOS Genetics*, 9: e1003539.

McGeehan JE, Depledge NW, McGeoch DJ (2001) Evolution of the dUTPase gene of mammalian and avian herpesviruses. *Current Protein & Peptide Science*, 2(4): 325–333.

Michel F, Dujon B (1986) Genetic exchanges between bacteriophage T4 and filamentous fungi? *Cell*, 46(3): 323.

Mol CD, Harris JM, McIntosh EM, Tainer JA (1996) Human dUTP pyrophosphatase: uracil recognition by a beta hairpin and active sites formed by three separate subunits. *Structure*, 4: 1077–1092.

Mooney RA, Davis SE, Peters JM, Rowland JL, Ansari AZ, Landick R (2009) Regulator trafficking on bacterial transcription units in vivo. *Molecular Cell*, 33(1): 97–108.

Moroz OV, Harkiolaki M, Galperin MY, Vagin AA, González- Pacanowska D, Wilson KS (2004) The crystal structure of a complex of *Campylobacter jejuni* dUTPase with substrate analogue sheds light on the mechanism and suggests the "basic module" for dimeric d(C/U)TPases. *Journal of Molecular Biology*, 342: 1583–1597.

Mueller JE, Clyman J, Huang YJ, Parker MM, Belfort M (1996) Intron mobility in phage T4 occurs in the context of recombination-dependent DNA replication by way of multiple pathways. *Genes & Development*, 10(3): 351–364.

Novick RP, Christie GE, Penadés JR (2010) The phage-related chromosomal islands of Gram-positive bacteria. *Nature Reviews Microbiology*, 8: 541–551.

Nyíri K, Papp-Kádár V, Szabó JE, Németh V, Vértessy BG (2015) Exploring the role of the phage-specific insert of bacteriophage ϕ11 dUTPase. *Structural Chemistry*, 26: 1425–1432.

Pani B, Banerjee S, Chalissery J, Muralimohan A, Loganathan RM, Suganthan RB, Sen R (2006) Mechanism of inhibition of Rho-dependent transcription termination by bacteriophage P4 protein Psu. *The Journal of Biological Chemistry*, 281(36): 26491–26500.

Penadés JR, Donderis J, García-Caballer M, Tormo-Más MÁ, Marina A (2013) dUTPases, the unexplored family of signalling molecules. *Current Opinion in Microbiology*, 16(2): 163–170.

Penadés JR, Chen J, Quiles-Puchalt N, Carpena N, Novick RP (2015) Bacteriophage-mediated spread of bacterial virulence genes. *Current Opinion in Microbiology*, 23: 171–178.

Peters JM, Vangeloff AD, Landick R (2011) Bacterial transcription terminators: the RNA 3'-end chronicles. *Journal of Molecular Biology*, 412(5): 793–813.

Quirk SM, Bell-Pedersen D, Belfort M (1989) Intron mobility in the T-even phages: high frequency inheritance of group I introns promoted by intron open reading frames. *Cell*, 56(3): 455–465.

Ram G, Chen J, Kumar K, Ross HF, Ubeda C, Damle PK, Lane KD, Penadés JR, Christie GE, Novick RP (2012) Staphylococcal pathogenicity island interference

with helper phage reproduction is a paradigm of molecular parasitism. *Proceedings of the National Academy of Sciences of the United States of America*, 109(40): 16300–16305.

Ram G, Chen J, Ross HF, Novick RP (2014) Precisely modulated pathogenicity island interference with late phage gene transcription. *Proceedings of the National Academy of Sciences of the United States of America*, 111(40): 14536–14541.

Ranjan A, Sharma S, Banerjee R, Sen U, Sen R (2013) Structural and mechanistic basis of anti-termination of Rho-dependent transcription termination by bacteriophage P4 capsid protein Psu. *Nucleic Acids Research*, 41(14): 6839–6856.

Ranjan A, Banerjee R, Pani B, Sen U, Sen R (2014) The moonlighting function of bacteriophage P4 capsid protein, Psu, as a transcription antiterminator. *Bacteriophage*, 3(2): e25657.

Rodríguez L, Martínez B, Zhou Y, Donovan DM, Rodríguez A, García P (2011) Lytic activity of the virion-associated peptidoglycan hydrolase HydH5 of *Staphylococcus aureus* bacteriophage vB_SauS-phiIPLA88. *BMC Microbiology*, 11: 138.

Rodríguez-Rubio L, Quiles-Puchalt N, Martínez B, Rodríguez A, Penadés JR, García P (2013) The peptidoglycan hydrolase of the *Staphylococcus aureus* bacteriophage ϕ11 plays a structural role in the viral particle. *Applied and Environmental Microbiology*, 79: 6187–6190.

Selva L, Viana D, Regev-Yochay G, Trzcinski K, Corpa JM, Lasa I, Novick RP, Penadés JR (2009) Killing niche competitors by remote-control bacteriophage induction. *Proceedings of the National Academy of Sciences of the United States of America*, 106: 1234–1238.

Semrad K, Schroeder R (1998) A ribosomal function is necessary for efficient splicing of the T4 phage thymidylate synthase intron in vivo. *Genes & Development*, 12(9): 1327–1337.

Shore D, Dehò G, Tsipis J, Goldstein R (1978) Determination of capsid size by satellite bacteriophage P4. *Proceedings of the National Academy of Sciences of the United States of America*, 75(1): 400–404.

Shub DA, Gott JM, Xu MQ, Lang BF, Michel F, Tomaschewski J, Pedersen-Lane J, Belfort M (1988) Structural conservation among three homologous introns of bacteriophage T4 and the group I introns of eukaryotes. *Proceedings of the National Academy of Sciences of the United States of America*, 85(4): 1151–1155.

Six EW (1975) The helper dependence of satellite bacteriophage P4: Which gene functions of bacteriophage P2 are needed by P4? *Virology*, 67(1): 249–263.

Six EW, Klug CA (1973) Bacteriophage P4: a satellite virus depending on a helper such as prophage P2. *Virology*, 51(2): 327–344.

Souza L, Calendar R, Six EW, Lindqvist BH (1977) A transactivation mutant of satellite phage P4. *Virology*, 81(1): 81–90.

Souza L, Geisselsoder J, Hopkins A, Calender R (1978) Physical mapping of the satellite phage P4 genome. *Virology*, 85(2): 335–342.

Stoddard BL (2014) Homing endonucleases from mobile group I introns: discovery to genome engineering. *Mobile DNA*, 5(1): 7.

Szabó JE, Németh V, Papp-Kádár V, Nyíri K, Leveles I, Bendes AÁ, Zagyva I, Róna G, Pálinkás HL, Besztercei B, Ozohanics O, Vékey K, Liliom K, Tóth J, Vértessy BG (2014) Highly potent dUTPase inhibition by a bacterial repressor protein

reveals a novel mechanism for gene expression control. *Nucleic Acids Research*, 42(19): 11912–11920.

Tabor S, Huber HE, Richardson CC (1987) *Escherichia coli* thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. *The Journal of Biological Chemistry*, 262(33): 16212–16223.

Tallent SM, Langston TB, Moran RG, Christie GE (2007) Transducing particles of *Staphylococcus aureus* pathogenicity island SaPI1 are comprised of helper phage-encoded proteins. *Journal of Bacteriology*, 189: 7520–7524.

Tarbouriech N, Buisson M, Seigneurin J-M, Cusack S, Burmeister WP (2005) The monomeric dUTPase from Epstein–Barr virus mimics trimeric dUTPases. *Structure*, 13: 1299–1310.

Tormo MA, Ferrer MD, Maiques E, Ubeda C, Selva L, Lasa I, Calvete JJ, Novick RP, Penadés JR (2008) *Staphylococcus aureus* pathogenicity island DNA is packaged in particles composed of phage proteins. *Journal of Bacteriology*, 190: 2434–2440.

Tormo-Más MA, Mir I, Shrestha A, Tallent SM, Campoy S, Lasa I, Barbé J, Novick RP, Christie GE, Penadés JR (2010) Moonlighting bacteriophage proteins derepress staphylococcal pathogenicity islands. *Nature*, 465: 779–782.

Tormo-Más MA, Donderis J, García-Caballer M, Aaron A, Mir-Sanchis I, Marina A, Penadés JR (2013) Phage dUTPases control transfer of virulence genes by a proto-oncogenic G protein- like mechanism. *Molecular Cell*, 49: 947–958.

Ubeda C, Barry P, Novick RP, Penadés JR (2008) Characterization of mutations defining SaPI functions and enabling autonomous replication in the absence of helper phage. *Molecular Microbiology*, 67: 493–503.

Van Roey P, Waddling CA, Fox KM, Belfort M, Derbyshire V (2001) Intertwined structure of the DNA-binding domain of intron endonuclease I-TevI with its substrate. *The EMBO Journal* 20(14): 3631–3637.

Vértessy BG, Tóth J (2009) Keeping uracil out of DNA: physiological role, structure and catalytic mechanism of dUTPases. *Accounts of Chemical Research*, 42: 97–106.

Vértessy BG, Larsson G, Persson T, Bergman AC, Persson R, Nyman PO (1998) The complete triphosphate moiety of non-hydrolyzable substrate analogues is required for a conformational shift of the flexible C-terminus in *E. coli* dUTP pyrophosphatase. *FEBS Letters*, 421: 83–88.

Viana D, Blanco J, Tormo-Más MA, Selva L, Guinane CM, Baselga R, Corpa J, Lasa I, Novick RP, Fitzgerald JR, Penadés JR (2010) Adaptation of *Staphylococcus aureus* to ruminant and equine hosts involves SaPI-carried variants of von Willebrand factor-binding protein. *Molecular Microbiology*, 77: 1583–1594.

Young P, Ohman M, Xu MQ, Shub DA, Sjöberg BM (1994) Intron-containing T4 bacteriophage gene sunY encodes an anaerobic ribonucleotide reductase. *The Journal of Biological Chemistry*, 269(32): 20229–20232.

23

Viral Entry Glycoproteins and Viral Immune Evasion

Jonathan D. Cook and Jeffrey E. Lee

Department of Laboratory Medicine and Pathobiology, Faculty of Medicine, University of Toronto, Toronto, Canada

23.1 Introduction

Viruses are obligate intracellular pathogens. During viral egress, some are encapsidated by a highly symmetrical proteinaceous coat while others are enveloped in a host-derived lipid membrane, protecting a similarly ordered tegument and/or nucleocapsid. In both cases virtually all viruses are geometrically limited in size, and therefore limited in the amount of genetic information that they can carry with them (with some notable exceptions found within several DNA viruses). This limitation precludes many common strategies for adaptation that are employed by prokaryotes and eukaryotes alike. The typical prokaryotic example that is highlighted in every introductory microbiology course involves the acquisition of genetic information from the environment. Classically, genes that are acquired horizontally provide the bacterium with a quantum genetic leap to thrive in a new environmental niche (Wiedenbeck and Cohan 2011). These genes are often encoded within circular plasmid DNA, which are routinely larger than 1 kilobase pair (kbp) and can replicate autonomously within the prokaryotic cell. Additional size-limiting means of adaptation in both eukaryotes and prokaryotes arise from gene-duplication events followed by divergence (Bergthorsson *et al*. 2007), thereby expanding the size of an organism's genome (sometimes dramatically) and allowing for the mutation of essential genes to generate novel functions.

As mentioned above, viral adaptations to environmental pressures are often restricted by the amount of genetic material that a virus can transport, thereby leaving genetic shift and genetic drift as the standard mechanisms by which smaller viruses obtain adaptive diversity. Genome size has an inverse correlation to random point-mutation rate (Holmes 2011), lending a higher propensity for single-stranded (ss) RNA and ssDNA viruses to obtain point mutations. Additionally, the error-prone nature of viral encoded RNA-dependent RNA polymerases can drive the higher mutation rates observed for RNA viruses

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

(Lauring *et al*. 2013). However, this is offset by the restraints of survival. For instance, a nonsense mutation resulting in the loss of a single gene-product in the HIV-1 viral genome could result in a virus that is incapable of a productive infection (Sheehy *et al*. 2002). Despite the apparent risk associated with highly mutable genomes, viruses represent a class of organism undergoing rapid evolution under strong selective pressures embodied by a host with an adaptive immune system. Viruses have therefore evolved moonlighting activities within essential proteins, likely as a means to work around the constraints of small genome size and constant challenge. In this chapter, we describe the moonlighting functions of viral envelope and entry proteins, the first viral moonlighting proteins that the host encounters during the viral challenge.

23.2 Enveloped Viral Entry

Enveloped viruses such as influenza A virus (IAV), human immunodeficiency virus (HIV), herpes versus simplex virus (HSV), hepatitis C virus (HCV), Dengue virus (DENV), and Ebola virus (EBOV) are the causative agents of some of the world's most pervasive and lethal infectious diseases; they are therefore among the most relevant viruses to modern human health. Like all viruses, enveloped viruses must deposit their genetic material within the host to ensure their propagation and survival. In the case of enveloped viruses, this is achieved through the fusion of the viral envelope with the host membrane, allowing the virus access to the cell. Although enveloped viruses vary in morphology and pathological manifestation, the strategies they employ for the initial penetration of the host membrane display striking convergence.

Mechanistically, upon encountering the host, the enveloped viral particle must first recognize its target through either a cell-surface receptor or attachment cofactor. This interaction is mediated via a surface viral glycoprotein (GP) complex. Viral surface GPs can be organized into three main categories based upon structural similarities in their membrane-fusion machinery. Filoviruses, retroviruses, orthomyxoviruses, paramyxoviruses, and coronaviruses, among others, contain envelope GPs that fall into the class I family (White *et al*. 2008). The class I GPs exist as metastable trimers on the virion and undergo an irreversible conformational change to a hallmark post-fusion six-helix bundle. On the other hand, class II GPs found in flaviviruses and alphaviruses are predominantly composed of β-sheets and are displayed as antiparallel dimers on the viral surface. During fusion, they undergo a reversible shift to a stable trimeric post-fusion conformation (Chao *et al*. 2014). In contrast class III viral GPs, such as those from herpesviruses and Epstein–Barr virus (EBV), are hybrids between the class I and class II GP families. Class III GPs are trimeric in nature, but undergo a reversible conformational change during fusion. Moreover, while class I and II GPs are composed of a single fusogenic glycoprotein, host cell-viral membrane fusion by class III GPs may require the formation of a larger viral entry complex. For example, it has been suggested that HSV-1 entry requires the formation of a core fusion complex composed of gB, gH/gL, gD, and a gD receptor (Turner *et al*. 1998; Rey 2006; Heldwein and Krummenacher 2008).

Nevertheless, once the receptor-binding GP or subunit interacts with its cognate receptor, the virus then undergoes a triggering event whereby the fusion GP is rendered fusion-competent. In the case of influenza viruses, this triggering event is multi-staged, comprising an initial proteolytic cleavage event at the plasma membrane followed by receptor-driven endocytosis and a pH-induced fusion subunit rearrangement (Harrison 2008; Li *et al*. 2010; Ivanovic *et al*. 2013). In the case of EBOV, the precise triggering event is currently unknown but is dependent upon GP interaction with the cholesterol transporter Niemann-Pick C1 (NPC1) (Carette *et al*. 2011) and multiple rounds of receptor-binding subunit proteolysis rather than being directly pH driven (Lee *et al*. 2008). In the canonical HIV-1 fusion event, triggering is achieved at the plasma membrane through a series of binding events between the virus and the host receptor CD4 and co-receptor(s) CCR5/CXCR4, and culminating in fusion subunit rearrangement (Blumenthal *et al*. 2012). Regardless of the method by which the enveloped virus becomes fusion-competent, the membrane fusion event itself is thought to occur by equivalent mechanisms (Li *et al*. 2010; Connolly *et al*. 2011). The metastable fusion-competent GP extends an N-terminal fusion peptide or fusion loop into the host cell membrane, thereby creating a pre-hairpin intermediate that spans both membranes. This intermediate inverts, drawing the outer leaflets of each membrane together into a state of hemi-fusion. In this manner, the energetic barrier to fusion is overcome by a dramatic conformational rearrangement of the fusogenic complex to an extremely stable conformation (Aydin *et al*. 2014). The hemi-fusion intermediate resolves into a fusion pore, which allows the virus access to the host cytoplasm. Fusion events using class I machinery are irreversible, resulting in a six-helix bundle comprising a trimer of hairpins formed by two heptad-repeats. Heptad-repeat 1 (HR1) is N-terminal to heptad-repeat 2 (HR2) and forms the core three-helix bundle that the HR2 interfaces with. A schematic of the general viral-host cell fusion process is depicted in Figure 23.1.

Extensive electron micrograph reconstructions have been used to determine the many conformations of the various classes of viral entry GPs. Pre-fusion viral GPs have been described at atomic level resolution through X-ray crystallographic studies and corroborate the interpretations of the electron microscopic data. These proteinaceous protrusions from the viral envelope are poised to interact with host cellular proteins, but are also prime targets for humoral immunity.

23.3 Moonlighting Activities of Viral Entry Glycoproteins

Viral entry proteins regularly exhibit moonlighting activities regardless of the structural classification of the fusion machinery. The conformationally labile nature of viral entry GPs ensure that at least two conformations can be present on the surface of free virus and on the cellular membrane of an infected cell, allowing for interactions with host proteins at multiple stages during infection. Viral envelope and entry GPs primarily function as viral adhesins and viral invasins with moonlighting roles relegated to other tasks. In keeping with the

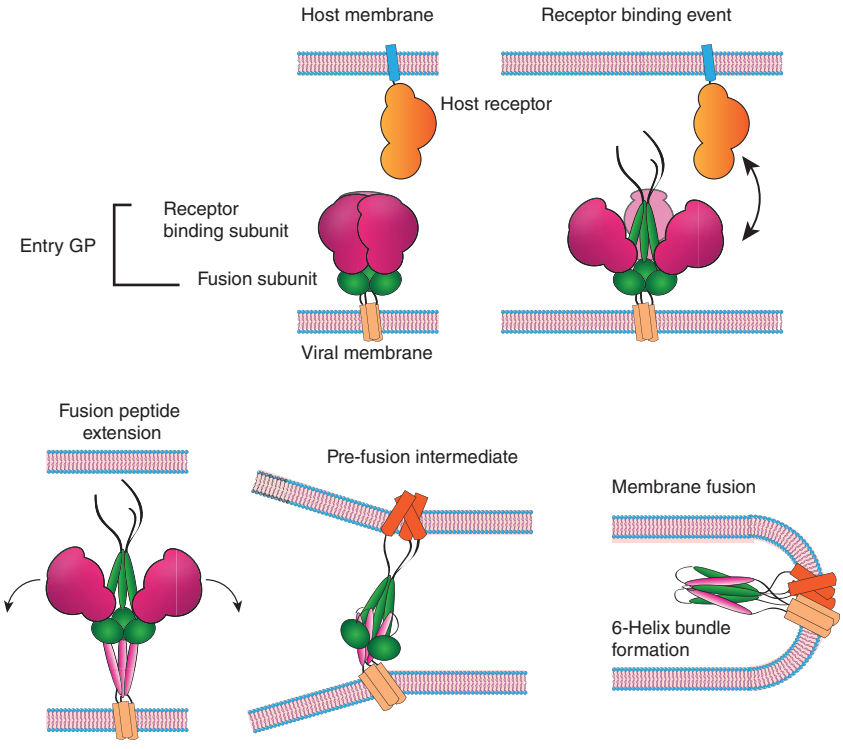

Figure 23.1 Illustration of a typical viral fusion event catalyzed by viral envelope and entry glycoproteins with moonlighting activities.

language of this edition, the moonlighting activities of viral entry proteins can be categorized into only two main groups: (1) entry proteins that directly facilitate the evasion of the host immune response; and (2) entry proteins that sabotage cellular processes to aid in the establishment of a productive infection.

23.3.1 Viral Entry Glycoproteins Moonlighting as Evasins

Humoral immunity towards viral infection encompasses both innate and adaptive arms of the immune system. Cross-talk between innate and adaptive immunity is crucial to the successful clearance of pathogens from the host and dysfunction in either will leave the host at great disadvantage. Viral GPs effectively evade neutralization by host humoral and antibody responses through several modes. Antigenic drift within GPs, most apparent in the seasonal varieties of influenza A virus, can effectively thwart the humoral immune response to challenge by enveloped viruses (Das *et al*. 2010). Central to this chapter, viral evasins have evolved to undermine the purpose of host immunity by presenting targets for innate and adaptive humoral immunity that are at best irrelevant and sometimes damaging to the host response. These strategies are distinct from the genetic drift and shift responsible for our need for an annual flu vaccination, although the moonlighting activities of viral evasins likely arose from some combination of selective pressure and mutation over evolutionary time.

23.3.2 Evading the Complement System

The complement system is a large collection of soluble serum proteins and cellular receptors that presents a major obstacle to a productive viral infection (Carroll and Isenman 2012). There are at least three complement pathways that lead to the enhancement of humoral immunity, virion lysis, opsonization, and the enhancement of T-cell-mediated immunity. Accordingly, viral envelope GPs have evolved moonlighting activities to evade and interrupt complement-mediated neutralization. This is accomplished through several different mechanisms by various viruses, and a list of known viral moonlighting proteins and their interactions with the complement system is provided in Table 23.1.

Viral envelope and entry proteins that have viral evasin moonlighting activity against the complement system include the HSV-1 and HSV-2 gC glycoproteins. HSV-1 gC is responsible for the binding of HSV-1 to heparan sulfate (Laquerre *et al*. 1998), thereby allowing the virus to tether to dendritic cells expressing CD209 and initiate a productive infection (de Jong *et al*. 2008). The complement component C3b is also bound by HSV-1 gC, interrupting the complement cascade through the steric occlusion of properdin and C5 from C3b (Kostavasili *et al*. 1997). This occlusion effectively shuts down the alternative pathway of the complement system and inhibits C5b, the first member of the membrane attack complex (MAC), from forming.

The classical pathway of the complement system is another target of viral evasins. The first step of the classical pathway is binding of C1q to immune complexes formed by native IgM or IgG antibodies and invading pathogen. Interruption of this binding event is achieved by the Influenza A matrix protein M1, via M1 binding to C1q (Zhang *et al*. 2009). Other viral envelope proteins such as HIV-1 gp41 have been shown to bind to C1q *in vitro* through the HIV-1 gp41 immunodominant region 1 (Kojouharova *et al*. 2003), however the contribution to HIV-1 pathogenesis has not been experimentally determined. Interestingly, a peptide corresponding to a region of HIV-1 gp41 known as the 3S region has been shown to ligate the C1q receptor gC1qR and cause an increase in natural killer (NK)-mediated CD4+ T-cell lysis (Fausther-Bovendo *et al*. 2010). It is tempting to speculate that C1q:HIV-1 gp41 complexes are targeted to T-cells bearing gC1qR on their surface, thereby facilitating ligation of the gC1qR receptor by HIV-1 gp41 and immune suppression.

Herpesviruses and poxviruses also frequently regulate the complement system; however, many of the proteins that they use to accomplish this regulation have not been demonstrated as classical moonlighting proteins. For instance, the EBV gp350/220 glycoprotein binds to the complement receptor CR2 to tether to B-cells and enhance fusion (Janz *et al*. 2000); however, the EBV gp350/220 glycoprotein is not absolutely necessary for a productive infection in these cells. Ligation of the B-cell receptor in the presence of C3b cleavage products can sensitize B-cells towards activation (Hebell *et al*. 1991); however, it has not been shown that ligation by gp350/220 can interfere with this process during a productive infection. On the other hand, it is known that expression of viral entry glycoproteins in infected cells can often downregulate their cognate receptors by co-complexing prior to delivery to the cell surface (Cao *et al*. 1996), and exosomes derived from EBV infected cell lines can present virus-free gp350/220 to B-cells

Table 23.1 Viral moonlighting proteins and their interactions with the complement system.

Virus or genus	Moonlighting protein	Complement system interaction	Reference
Baculovirus	gp64	CD55/DAF recruitment	Kaname *et al.* 2010
Epstein-Barr virus	gp350/220	CR2	Janz *et al.* 2000
Flavivirus	NS1	factor H	Chung *et al.* 2006
		C1s	Avirutnan *et al.* 2006
		C4	Avirutnan *et al.* 2010
		clusterin	Kurosu *et al.* 2007
Hepatitis C virus	CP	gC1qR	Kittlesen *et al.* 2000
Herpes simplex virus-1/2	gC	C3	Kostavasili *et al.* 1997
Human astrovirus-1	CP	MBL	Hair *et al.* 2010
		C1q	Bonaparte *et al.* 2008
Human cytomegalovirus	unknown	CD59 recruitment	Spear *et al.* 1995
		CD55/DAF recruitment	
Human immunodeficiency virus-1	gp41	C1q	Kojouharova *et al.* 2003
		gC1qR	Fausther-Bovendo *et al.* 2010
	unknown	CD59 recruitment	Saifuddin *et al.* 1997
		CD55/DAF recruitment	
		CD46/MCP recruitment	
Human T-lymphotropic virus-1	unknown	CD59 recruitment	Spear *et al.* 1995
		CD55/DAF recruitment	
Influenza A virus	M1	C1q	Zhang *et al.* 2009
Mumps virus	unknown	CD46/MCP recruitment	Johnson *et al.* 2012
		CD55/DAF recruitment	
Simian virus 5	unknown	CD46/MCP recruitment	Johnson *et al.* 2009

in culture (Vallhov *et al.* 2011). Other such ambiguities arise throughout the literature, as host complement control proteins can be recruited to the nascent virions of retroviruses, lentiviruses, paramyxoviruses, and flaviviruses to facilitate complement evasion (Stoermer and Morrison 2011). The mechanism of this recruitment remains elusive, but recruitment of host factors into budding viruses

is often controlled through direct viral protein interactions (Douaisi *et al*. 2004; Jorgenson *et al*. 2009; Bleck *et al*. 2014). The direct interaction of complement control factors with viral entry GPs has been previously described; baculovirus recruitment of the complement control protein CD55/decay-accelerating factor (DAF) is mediated by the baculovirus entry glycoprotein gp64 as shown through co-immunoprecipitation assays (Kaname *et al*. 2010). The complexity of the complement system and its interactions with both viral pathogens and the adaptive arm of the immune system is still an active area of research and clearly a prime target for viral moonlighting proteins.

23.3.3 Evading Antibody Surveillance

Viral GPs adopt multiple mechanisms to evade antibody surveillance; these include utilizing a glycan shied, releasing shed/soluble GP and producing multiple viral GP decoys or distractions. A summary of various viral GPs in antibody evasion is presented in Table 23.2.

23.3.3.1 The Viral Glycan Shield

The surfaces of viral GPs, as their name suggests, are decorated with carbohydrates. Carbohydrates are often attached to asparagine residues found in the context of an Asn-X-Thr/Ser sequon (N-linked) or through an O-glycosidic linkage to serine or threonine (O-linked). These glycans are conformationally flexible and can extend a significant distance (30–40 Å) from the protein surface. Glycosylation promotes correct protein folding, proper trafficking, and, in some cases, mediates host receptor binding.

Carbohydrates positioned on GPs have been well characterized in their role to mask antigenic sites from immune surveillance. For example, the HIV-1 envelope glycoprotein (gp160) is composed of a gp120 attachment ectodomain and an α-helical gp41 fusion subunit. The gp120 subunit contains *c*. 25 N-linked oligomannose-type carbohydrates clustered in an outer domain that account for *c*. 50% of the molecular weight of gp160. Structural and biochemical studies reveal a constantly evolving oligomannose glycan shield that sterically hinders neutralizing antibody binding, but does not reduce viral fitness (Wei *et al*. 2003). Neutralizing antibodies are typically generated against the V1/V2/V3 loops; however, these antibodies are not broadly neutralizing since the primary amino acid sequence within these loops is not conserved. HIV-1 glycans effectively direct the immune system to produce non-effective antibodies against highly variable regions on the viral surface, allowing the virus to persist in the host. The use of a glycan shield or glycocalyx to evade antibody responses is also a common strategy for other RNA and DNA viruses. The surface of the EBOV GP is covered with N- and O-linked glycans. Survivors of EBOV infection have low to insignificant titers of neutralizing antibodies, and glycosylation is thought to protect the virus from antibody surveillance. In HCV, the ectodomains of the E glycoprotein are highly glycosylated: E1 contains four conserved N-linked glycosylation sites and nine are conserved across all genotypes in E2. These glycans likely allow HCV to evade the humoral immune response and, as a result, 80% of HCV-infected individuals progress from an acute to a chronic infection (Helle *et al*.

Table 23.2 Role of various viral GPs in antibody evasion.

Family	Virus	Protein	Function/mechanism	Reference
Arenaviridae	Lassa virus	Soluble GP1	Attachment subunit of Lassa virus GP can be shed to act as an antigen decoy and distraction	Branco *et al*. 2010
Filoviridae	Ebola virus	GP	Virion-attached GP is covered with a thick layer of N- and O-linked glycans that can act as a glycan shield	Lennemann *et al*. 2014
		sGP	Primary gene product that is secreted in abundance; acts as an antibody decoy and/or misdirects the humoral immune system	Sanchez *et al*. 1996; Mohan *et al*. 2012
		ssGP	Minor gene product that is secreted like sGP; its role is poorly defined however, could act as a potential decoy and distraction	Mehedi *et al*. 2011
Flaviviridae	Hepatitis C virus	E1/E2	The E1/E2 glycoprotein is covered with N-linked glycans that allows escape from neutralizing antibodies	Falkowska *et al*. 2007; Helle *et al*. 2007, 2010; Di Lorenzo *et al*. 2011
Herpesviridae	Epstein–Barr virus	gp350	The gp350 surface is covered with N-linked glycans that protect it from antibody surveillance	Szakonyi *et al*. 2006
	Bovine herpes virus	gp180	O-linked glycans shield the virus from elicited antibodies; these O-linked glycans are conserved in all other gammaherpesvirus gp350 glycoproteins	Machiels *et al*. 2011
Orthomyxoviridae	Influenza A virus	HA	N-linked glycans on the virion-attached HA protect diverse influenza A strains from host immune responses	Wei *et al*. 2010; Das *et al*. 2010
Paramyxoviridae	Nipah virus	F	N-linked glycans protect the Nipah virus fusion glycoprotein from host antibodies	Aguilar *et al*. 2006
	Respiratory syncytial virus	Soluble G	Attachment protein for RSV can be shed; may act as an antibody decoy and distraction	Bukreyev *et al*. 2008
Retroviridae	Human immuno-deficiency virus-1	gp160	N-linked glycans cover the surface of gp160 and shields the virus from immune surveillance	Wei *et al*. 2003
		Soluble gp120	Shed gp120 can act as an antibody decoy and distraction	Moore and Sodroski 1996

2011; Kong *et al*. 2012; Drummer 2014). For IAV, there are four conserved glycans in the stalk of H1N1 hemagglutinin (HA). During IAV adaptation to humans, there has been a step-wise accumulation of several N-linked glycosylation sites near antigenic regions on the globular head of HA that are predicted to interfere with antibody-mediated neutralization (Wei *et al*. 2010). In the case of Nipah virus, glycosylation on the envelope glycoprotein has a dual role. The N-linked glycans provide enhanced resistance to neutralizing antibodies, but also appear to attenuate fusion and entry (Aguilar *et al*. 2006). In EBV, a DNA herpesvirus, structural studies revealed that the gp350 surface is completely covered with N-linked glycans, with the exception of a patch of residues that are involved in binding to the cell-surface receptor CR2 (Szakonyi *et al*. 2006). In addition, a related gammaherpesvirus is highly O-linked glycosylated and provides a glycan shield for an otherwise accessible viral surface (Machiels *et al*. 2011). It is clear that N- and O-linked glycans are important in directing the accessibility of antibodies.

23.3.3.2 Shed Viral Glycoproteins: An Antibody Decoy

The shedding of soluble viral GPs exemplifies another viral strategy of humoral misdirection. Many enveloped viruses generate free GPs that moonlight to act as either "antibody sinks" or "antibody decoys." In HIV-1, the gp120 attachment subunit is non-covalently linked to gp41 and can be dissociated from the surface of the virus. The loss of gp120 occurs as the virions age and correlates with a decrease in infectivity (Gelderblom *et al*. 1985, 1987; McKeating *et al*. 1991). In EBOV, the shed GP is generated through a proteolytic cleavage on the virion-attached GP at its membrane-proximal external region by the tumor necrosis factor-α converting enzyme (TACE). The released trimeric shed GP is immunologically similar to the virion-attached EBOV GP. In a guinea pig model of EBOV infection, shed GP inhibits the neutralizing activity of EBOV antibodies (Dolnik *et al*. 2004). Soluble or shed GP have also been detected from Lassa virus (Branco and Garry 2009; Branco *et al*. 2010), VSV (Kang and Prevec 1971; Little and Huang 1977), rabies (Dietzschold *et al*. 1983), and HSV infections (Chen *et al*. 1978). The shed GPs may either distract the immune system to make non-neutralizing antibodies or act as a sponge to soak up any elicited neutralizing antibodies. However, it is important to note that the role of many of these shed GPs in pathogenesis remains speculative.

23.3.3.3 Antigenic Variations in Viral Glycoproteins

In addition to shed GP, some viruses, in particular Ebola viruses, are able to encode multiple soluble "antigenic distractions." The EBOV genome contains seven genes (*NP*, *VP35*, *VP40*, *GP*, *VP30*, *VP24*, and *L*) but, unique to EBOV, more than three different proteins are produced from the *GP* gene due to co-transcriptional editing and post-translational cleavages (Fig. 23.2). Each protein produced plays a different role to facilitate a productive Ebola virus infection.

The primary *GP* open reading frame (*c*. 70% of transcripts) encodes for a non-structural 364-amino acid protein, termed (pre-sGP), that is secreted in abundance during infection. Post-translational furin cleavage of pre-sGP produces a mature sGP and a small heavily O-glycosylated peptide (Δ-peptide). The mature sGP forms covalently linked, parallel homodimers. A co-transcriptional stuttering

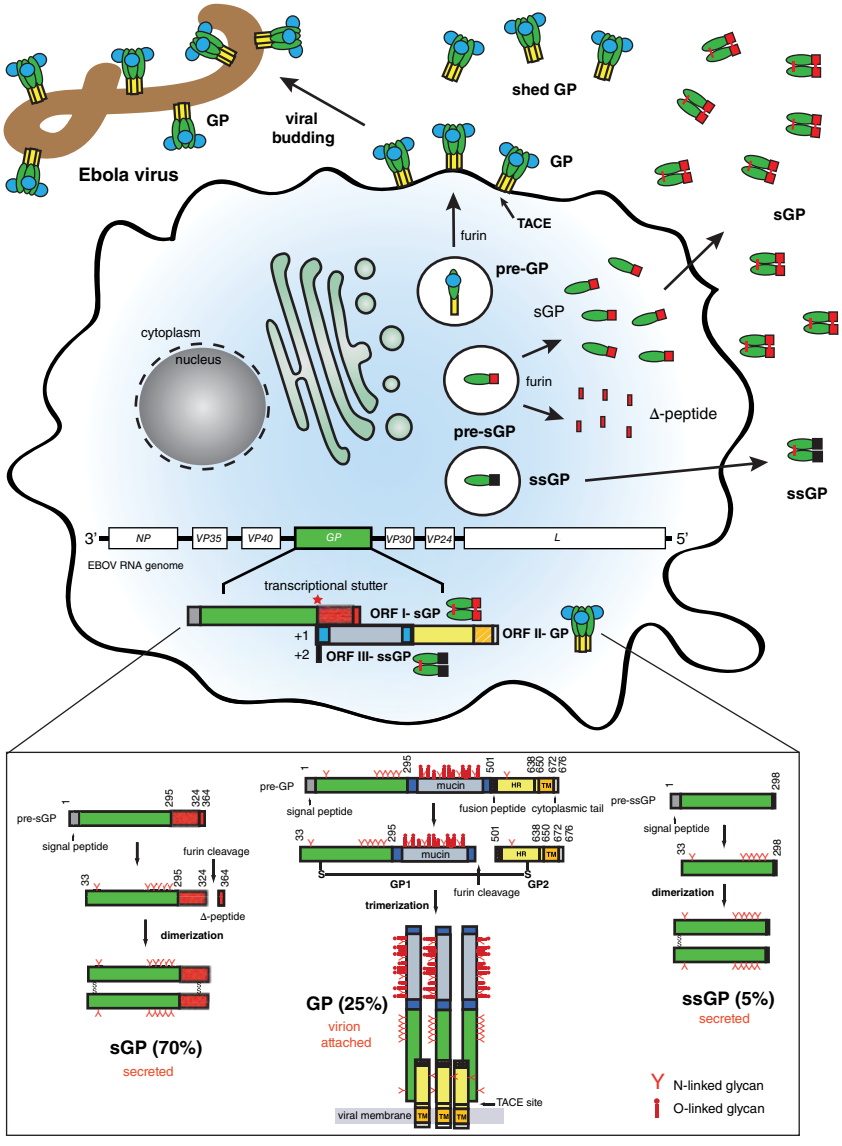

Figure 23.2 Shed, soluble, and virion attached Ebola virus glycoproteins. (*See color plate section for the color representation of this figure.*)

event allows the insertion of an extra adenosine in *c.* 25% of the transcripts. This insertion eliminates the stop codon in sGP and produces a second open reading frame to encode the larger 676-amino acid, trimeric virion-attached GP (pre-GP). The pre-GP is cleaved by furin to result in two disulfide-linked subunits: GP1 is involved in host cell attachment and GP2 in viral-host membrane fusion. Three GP1-GP2 heterodimers assemble to form the mature, trimeric, pre-fusion GP peplomer on the viral surface. While the oligomeric states and disulfide bond linkages of sGP and GP are different, the first 295-amino acids are the same. In Ebola virus-infected patients, significant quantities of sGP have been detected in

the blood (Sanchez *et al*. 1996) and sGP is able to inhibit the neutralization potential of EBOV GP antisera (Ito *et al*. 2001). Antibodies identified from EBOV survivor sera or mice vaccinated with a Venezuelan equine encephalitis replicon system preferentially recognize sGP over pre-fusion GP (Maruyama *et al*. 1999; Wilson *et al*. 2000). It has therefore been suggested that sGP can control the host adaptive immune response by distracting the immune system to make antibodies against an unrelated target or acting as an "antibody sink" to absorb elicited antibodies. Indeed, sGP has also been shown to "subvert" the humoral immune system by inducing antibodies that focus on epitopes that are shared between sGP and GP, allowing sGP to absorb any anti-GP neutralizing antibodies (Mohan *et al*. 2012). A third open reading frame is produced by the insertion of two adenosine nucleotide (+2 shift) in 5% of the transcripts to encode a small, secreted 298-residue GP termed ssGP (Mehedi *et al*. 2011). ssGP is secreted as a disulfide-linked homodimer, similar to the structural organization of sGP. The role of ssGP is poorly defined, but may also act as an antibody decoy or distraction as for sGP.

23.3.3.4 Shed Viral Glycoproteins and Immune Signal Modulation

Shed/soluble GPs do not always act as an antibody decoy, but can also modulate immune signaling pathways. Here, we describe two examples from respiratory syncytial virus (RSV) and EBV. In RSV infection, soluble forms of its G glycoprotein have been detected. Soluble RSV G lacks the first 65 amino acids, which contains the viral transmembrane anchor. RSV is unique in that it can infect and re-infect its host regardless of the presence of maternally derived antibodies or previous immune clearance. Soluble RSV glycoprotein G is able to serve as a decoy, allowing infection and re-infection (Bukreyev *et al*. 2008), but also plays another role in modifying CXCL1-mediated responses. The RSV G protein has a conserved cysteine region encompassing a CXC3 fractalkine motif that is able to bind to a CSCL1-specific receptor, CX3CR1, and compete with CX3CL1 binding to CX3CR1 (Tripp *et al*. 2001). Mimicry by the RSV G CXC3 motif prevents the trafficking of $CX3CR1^{+}$ T-cells to the lung, thereby limiting cellular immune responses to RSV infection (Harcourt *et al*. 2006). In EBV infection, gp42 is a co-receptor for viral entry into B-cells. The membrane-attached EBV gp42 can be proteolytically cleaved in the endoplasmic reticulum to remove a type II transmembrane anchor to generate a soluble gp42 (Ressing *et al*. 2005). Soluble EBV gp42 inhibits the presentation of MHC class II-restricted antigens to $CD4^{+}$ T-cells (Ressing *et al*. 2003). EBV gp42 binds the MHC class II molecule HLA-DR1 using an interaction surface that is distant from the receptor binding sites (Mullen *et al*. 2002).

23.3.4 Evading Host Restriction Factors

By now it should be clear that moonlighting activities of viral proteins are necessary adaptations to a hostile host. Viral restriction strategies outside of the classical definition of innate or adaptive immunity are now respected as powerful tools in the fight against host invasion.

One such protein known as the restriction factor BST-2 (also known as tetherin or CD317) is a common target of viral GP moonlighting activity. BST-2

restricts viral budding by tethering nascent virions to the cell following scission (Neil *et al*. 2008; Lehmann *et al*. 2011). Although HIV-1 makes use of the non-structural protein Vpu to downregulate BST-2, EBOV and HIV-2 both achieve this same goal through moonlighting activities of their respective viral GPs (Kaletsky *et al*. 2009). Effective BST-2 antagonism requires the presence of a clathrin assembly protein (AP) -binding motif on the long cytoplasmic tail of HIV-2 gp41 (Lau *et al*. 2011). Interestingly, EBOV GP lacks a significant cytoplasmic tail and unlike the HIV-2 viral GP has no identifiable Yxxϕ clathrin-AP-binding motif. These facts and others suggest that BST-2 antagonism by EBOV GP is mediated through a different mechanism than that described for HIV-2.

A conserved group of proteins known as interferon-induced transmembrane proteins (IFITMs) have recently been identified as host restriction factors (Brass *et al*. 2009). These proteins have two transmembrane domains and are expressed in response to interferon signaling. There are several different proteins that belong to the IFITM family, each with differing activities against pseudotyped murine leukemia viruses (MLVs) expressing IAV, SARS-CoV, and filovirus GPs (Feeley *et al*. 2011; Huang *et al*. 2011). Although the mechanism of this particular host restriction strategy remains unknown, the differential activity demonstrated by the IFITMs is suggestive of host-viral GP interactions. Possibly, it represents a novel mechanism of reinforcement of the endocytic pathway against viral pathogens, and is a likely target of yet-undiscovered viral moonlighting activities by viral glycoproteins.

23.3.5 Modulation of Other Immune Pathways

It has been known for decades that the HIV-1 gp41 glycoprotein has immunosuppressive activity. This protein, whose main function is to catalyze the merger of the host and viral membranes, has been implicated in numerous moonlighting functions (summarized in Table 23.3). In addition to its principal role in HIV-1 pathology, HIV-1 gp41 also contains two unique immunodominant regions and two independent immunomodulatory regions. Unfortunately much of the research into the functions of these regions has been stymied by the lack of a tenable recombinant molecule for use in investigation; consequently, small peptides encompassing 15–25 amino acid stretches of HIV-1 gp41 are routinely employed in biochemical and virological research. Figure 23.3 illustrates the numerous HIV-1 gp41 moonlighting motifs in the context of the viral envelope.

Most of the HIV-1 envelope is littered with prematurely triggered HIV-1 gp41 in the post-fusion conformation. The various amino acid motifs associated with immune dominance and suppression are inaccessible in the pre-fusion conformation. Some of the moonlighting functions of these motifs include extensive cytokine and chemokine modulation, interruption of the T-cell receptor complex (as described in Section 23.4.2), ligation of the macrophage migration inhibitory factor receptor CD74 (Zhou *et al*. 2011), and driving the generation of non-neutralizing antibodies against immunodominant epitopes found within the GP (Robinson *et al*. 1991).

In 1985, George Cianciolo and his colleagues described a synthetic peptide derived from retroviral fusion glycoproteins with putative immunomodulatory

Table 23.3 Moonlighting activities of the HIV-1 gp41 viral entry glycoprotein.

Primary structure boundaries*	Host factor(s)	Region	Function	Reference
Amino acids: 512–528	TCRα	FP	Inhibition of T-cell proliferation and secretion of proinflammatory cytokines	Cohen *et al.* 2008
Amino acids: 574–592	unknown	ISD	Canonical immunosuppressive region responsible for cytokine modulation	Denner *et al.* 2013
Amino acids: 596–610	CD74	–	Increase MAP/ERK phosphorylation to putatively support HIV-1 infectivity	Zhou *et al.* 2011
Amino acids: 596–606	Immunoglobulin	ID1	Generation of non-neutralizing and infectivity enhancing antibodies	Robinson *et al.* 1991
Amino acids: 601–613	C1q	–	Unknown	Kojouharova *et al.* 2003
Amino acids: 609–623	gC1qR	–	NK cell inhibition	Fausther-Bovendo *et al.* 2010
Amino acids: 615–632	TCRα	ISLAD	Activation blockade in T-cells and cytokine modulation at lymph nodes	Ashkenazi *et al.* 2013
Amino acids: 644–663	Immunoglobulin	ID2	Generation of non-neutralizing and infectivity enhancing antibodies	Robinson *et al.* 1991
Amino acids: 683–711	CD3	TM	Interruption of T-cell activation and proliferation	Cohen *et al.* 2010

* Sequence corresponds to HIV-1 gp41 strain HXB2

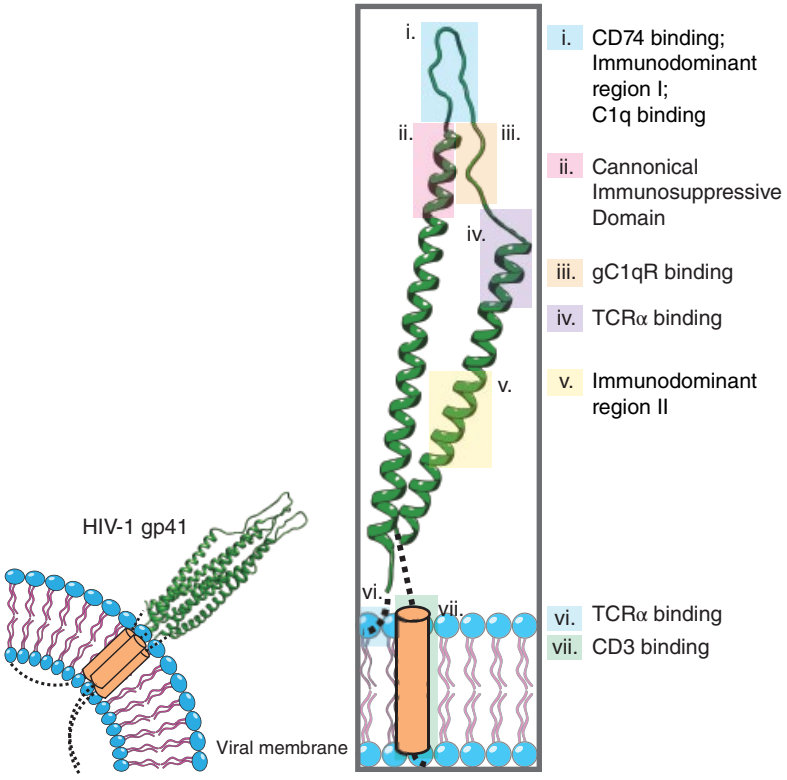

Figure 23.3 A schematic diagram placing the moonlighting activities of the HIV-1 gp41 viral entry glycoprotein into a structural context.

activity (Cianciolo *et al.* 1985). In this seminal paper, the 17-amino acid peptide, termed CKS-17, was capable of curbing the proliferation of murine lymphocytes in culture. Mechanistic studies performed in the laboratory of Robert Good and others clearly show that retrovirally derived immunosuppressive peptide causes upregulation of cyclic-AMP, PI3-K activation, PKCμ activation, and modulate cytokine expression (Haraguchi *et al.* 1997; Luangwedchakarn *et al.* 2003; Fan *et al.* 2005). Despite years of research into the effects of this immunomodulatory peptide, identification of the receptor(s) responsible for CKS-17 signal transduction remains elusive. This region is structurally conserved across retroviral fusion proteins and filoviruses (Cook and Lee 2013). Indeed, inactivated Zaire EBOV or peptides derived from the conserved immunosuppressive region of various filoviruses inhibit cytokine expression in cultured human peripheral blood mononuclear cells (PBMC). These synthetic peptides also have a profound effect on the surface distribution of CD4 and CD8 on these cells, supporting the putative immunomodulatory effects of this conserved motif (Yaddanapudi *et al.* 2006). The CKS-17 peptide maps to the chain reversal region of retroviral and filoviral fusion proteins and a region of gp41 that comprises the terminal end of the HR1 three-helix bundle (Cook and Lee 2013); however, crystallographic evidence indicates that subtle amino acid differences between the lentiviral and retroviral

immunosuppressive motif produce significantly different conformations between the two viral genera. Regardless of the structural differences, peptides corresponding to the HIV-1 immunosuppressive motif have been shown to modulate cytokine expression in PBMC culture (Denner *et al.* 2013). Furthermore, recombinant HIV-1 gp41 with a single point mutation in the immunosuppressive region illustrated that the immunosuppressive region could suppress antibody responses in rats (Morozov *et al.* 2012). This is reminiscent of results reported for antibody responses in mice immunized with the retroviral ectodomains of the Friend murine leukaemia virus, human T-lymphotropic virus-1, xenotropic murine leukaemia virus-related virus, and mutated syncytin-1 (Schlecht-Louf *et al.* 2010).

Retroviruses, filoviruses and human endogenous retroviruses (HERV) that contain a CX_6CC motif within their fusion GPs have been touted as immunosuppressive by numerous researchers. The Heidmann group has shown that retroviral fusion domains stably expressed in various tumor lines could facilitate xenograph immune-evasion in a murine model (Mangeney and Heidmann 1998). This immunosuppressive activity was then separated from the fusogenic activity of several retroviral envelope proteins through the introduction of point mutations in the viral envelope proteins (Schlecht-Louf *et al.* 2010). By separating the two activities of these proteins in this manner, Heidmann and colleagues have provided compelling proof of the moonlighting activities of these proteins. Of note, the HERV-derived syncytin-2 may have been co-opted for its moonlighting activity rather than its primary fusogenic role as it has been implicated in maternal–fetal tolerance rather than cell–cell fusion during placental development (Dupressoir *et al.* 2012).

23.4 Viral Entry Proteins Moonlighting as Saboteurs of Cellular Pathways

A productive viral infection requires the manipulation of host proteins to avoid adaptive immune detection, apoptosis, viral lysis, and innate immune restriction. Viral GPs moonlighting as viral saboteurs are fast becoming recognized as modulators of cell-surface proteins that are involved in both signal transduction and host antiviral defense.

23.4.1 Sabotaging Signal Transduction Cascades

Considering that many of the cognate receptors of viral entry proteins are surface receptors important in signal transduction, it is not unexpected that these proteins can initiate signaling events in both target and bystander host cells. One of the more recognized viral attachment subunits that accomplish this to the benefit of the virus is HIV-1 gp120. It has long been accepted that soluble HIV-1 gp120 can cause apoptosis in bystander cells through the ligation of HIV-1 co-receptors (Cicala *et al.* 2000). It is also understood that HIV-1 gp120 can modulate dendritic cells via the induction of a cytokine feed forward loop centering on the IL-6/STAT3 axis (Del Cornò *et al.* 2014). Both of these moonlighting activities of HIV-1 gp120 alter the cellular milieu during a productive infection.

Like HIV-1 gp120, hepatitis C virus can also use its attachment glycoprotein E2 to modulate host immune cells by binding and utilizing CD81 as a viral receptor. However, ligation of CD81 on NK cells by HCV E2 can either block NK cell activation, and all of the concomitant cytokine production, or deliver a co-stimulatory signal in the case of NK-like T-cell clones (Crotta *et al*. 2002). Additionally, HCV E2 mimics activation sites on host proteins eIF2α and the interferon inducible protein kinase PKR, effectively encouraging protein synthesis and growth in infected cells (Taylor *et al*. 1999). Both HIV-1 and HCV utilize their viral attachment proteins to bind their cognate host receptor with advantageous outcomes. It is interesting to speculate that these moonlighting activities would have been systematically selected for, as not all receptor-binding modes would necessitate a signal transduction event. Interruption of signaling events via receptor ligation is at least as probable as the alternative and, after all, the result of the moonlighting activity is what drives the adaptation.

23.4.2 Host Surface Protein Sabotage

Originally, the EBOV GP was thought to cause the downregulation of the major histocompatibility complex (MHC) class I, β7 integrins and the viral GPs themselves (Simmons *et al*. 2002). However, interestingly EBOV GP does not facilitate the loss of expression or removal of MHC class I and β7 integrins from the cell surface. Instead, the flexible and heavily glycosylated mucin-like domain of EBOV GP acts as a "glycan umbrella" to sterically shield the smaller MHC class I and β7 integrins from performing their native functions on the cell surface. In 2010, Francica *et al*. described a particularly salient example of the utility of this mechanism in viral pathogenesis. Primary cytotoxic T-lymphocytes (CTLs) specific for cells that had been co-transduced with EBOV GP were incapable of cytolytic response due to the occlusion of the MHC class I protein by the viral glycoprotein (Francica *et al*. 2010). This strategy is particularly relevant as the immune system relies heavily on signal transduction for its activation and, correspondingly, is commonly targeted by viral saboteurs encoded by numerous viral pathogens. For instance, the EBV fusion-trigger, gp42, forms a stable complex with the MHC class II receptor HLA-DR1 (Mullen *et al*. 2002). Although this interaction does not downregulate the MHC class II receptor or interfere with its maturation, it was shown through *in vitro* proliferation assays to hinder the activation T-cells specific to presented antigen (Ressing *et al*. 2003). Moreover, literature supports the observation that EBV generates a soluble version of EBV gp42 that is cleaved from the infected cell surface via signal-sequence cleavage. This soluble form of EBV gp42 is sufficient to dampen CD4+ T-cell response in an antigen-specific *in vitro* assay (Ressing *et al*. 2005).

In the canonical immune activation paradigm, MHC molecules on an antigen-presenting cell (APC) arrange a peptide antigen for recognition by the T-cell receptor (TCR) complex. In two separate reports, the transmembrane domain and the fusion peptide of HIV-1 gp160 both interfere with TCRα/CD3 interactions, thereby inhibiting antigen-dependent activation of primary T-cells (Cohen *et al*. 2008, 2010). Fluorescence resonance energy transfer analysis between the TCRα transmembrane domain and the HIV-1 gp41 FP domain showed a high degree of specific interactions between these host and viral proteins. In support

of this finding, it has also been illustrated by confocal fluorescence microscopy that TCRα and HIV-1 gp160 migrate to identical cap-like structures following T-cell activation (Cohen *et al*. 2010). Interestingly, another conserved sequence of HIV-1 gp41 termed the immunosuppressive-loop-associated determinant (ISLAD) has been implicated in the amelioration of pathogenesis in a murine model of multiple sclerosis through direct interactions with TCRα, establishing an activation blockade in T-cells and cytokine modulation at the lymph nodes (Ashkenazi *et al*. 2013). As such, the TCR appears to be a hotspot of immunomodulatory activities of viral moonlighting GPs.

23.5 Conclusions

Scientific research promises that we can better equip ourselves to handle challenges from old and new infectious diseases alike by understanding the strengths of the viral pathogen and the selective pressures that have shaped both its primary and moonlighting activities. Novel pharmaceutical interventions and efficacious vaccination strategies rely on a fine understanding of the host–pathogen interaction. Moonlighting strategies, although stumbled upon stochastically throughout evolutionary time, are no longer happenstance, and have been selected for by the intricate demands placed on pathogen persistence. We would be remiss to ignore the evolutionary clues that moonlighting proteins provide about our own biological armaments. We have discussed the roles of shed filoviral and lentiviral envelope proteins in antibody evasion, suggesting that appropriately directed antibody therapies will be effective. Indeed, the explosion in the recent literature reporting the discovery of broadly neutralizing antibodies against HIV-1 and EBOV are evidence of this (Lee and Saphire 2009; Burton *et al*. 2012). Excitingly, this chapter only highlights the first of many lessons to come on how moonlighting proteins can direct our endeavors to design superior treatments and prophylactics to combat some of the world's most pervasive and deadly infectious diseases.

References

Aguilar, H.C., Matreyek, K.A., Filone, C.M., Hashimi, S.T., Levroney, E.L., Negrete, O.A., Bertolotti-Ciarlet, A., Choi, D.Y., McHardy, I., Fulcher, J.A., *et al*. (2006). N-glycans on Nipah virus fusion protein protect against neutralization but reduce membrane fusion and viral entry. *Journal of Virology* 80, 4878–4889.

Ashkenazi, A., Faingold, O., Kaushansky, N., Ben-Nun, A., and Shai, Y. (2013). A highly conserved sequence associated with the HIV gp41 loop region is an immunomodulator of antigen-specific T cells in mice. *Blood* 121, 2244–2252.

Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., Jairungsri, A., Kanlaya, R., Tangthawornchaikul, N., Puttikhunt, C., *et al*. (2006). Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. *Journal of Infectious Diseases* 193, 1078–1088.

Avirutnan, P., Fuchs, A., Hauhart, R.E., Somnuke, P., Youn, S., Diamond, M.S., and Atkinson, J.P. (2010). Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. *Journal of Experimental Medicine* 207, 793–806.

Aydin, H., Cook, J.D., and Lee, J.E. (2014). Crystal structures of beta- and gammaretrovirus fusion proteins reveal a role for electrostatic stapling in viral entry. *Journal of Virology* 88, 143–153.

Bergthorsson, U., Andersson, D.I., and Roth, J.R. (2007). Ohno's dilemma: evolution of new genes under continuous selection. *Proceedings of the National Academy of Science, USA* 104, 17004–17009.

Bleck, M., Itano, M.S., Johnson, D.S., Thomas, V.K., North, A.J., Bieniasz, P.D., and Simon, S.M. (2014). Temporal and spatial organization of ESCRT protein recruitment during HIV-1 budding. *Proceedings of the National Academy of Science, USA* 111, 12211–12216.

Blumenthal, R., Durell, S., and Viard, M. (2012). HIV entry and envelope glycoprotein-mediated fusion. *Journal of Biological Chemistry* 287, 40841–40849.

Bonaparte, R.S., Hair, P.S., Banthia, D., Marshall, D.M., Cunnion, K.M., and Krishna, N.K. (2008). Human astrovirus coat protein inhibits serum complement activation via C1, the first component of the classical pathway. *Journal of Virology* 82, 817–827.

Branco, L.M. and Garry, R.F. (2009). Characterization of the Lassa virus GP1 ectodomain shedding: implications for improved diagnostic platforms. *Virology Journal* 6, 147.

Branco, L.M., Grove, J.N., Moses, L.M., Goba, A., Fullah, M., Momoh, M., Schoepp, R.J., Bausch, D.G., and Garry, R.F. (2010). Shedding of soluble glycoprotein 1 detected during acute Lassa virus infection in human subjects. *Virology Journal* 7, 306.

Brass, A.L., Huang, I.C., Benita, Y., John, S.P., Krishnan, M.N., Feeley, E.M., Ryan, B.J., Weyer, J.L., van der Weyden, L., Fikrig, E., *et al.* (2009). The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 139, 1243–1254.

Bukreyev, A., Yang, L., Fricke, J., Cheng, L., Ward, J.M., Murphy, B.R., and Collins, P.L. (2008). The secreted form of respiratory syncytial virus G glycoprotein helps the virus evade antibody-mediated restriction of replication by acting as an antigen decoy and through effects on Fc receptor-bearing leukocytes. *Journal of Virology* 82, 12191–12204.

Burton, D.R., Poignard, P., Stanfield, R.L., and Wilson, I.A. (2012). Broadly neutralizing antibodies present new prospects to counter highly antigenically diverse viruses. *Science* 337, 183–186.

Cao, J., Park, I.W., Cooper, A., and Sodroski, J. (1996). Molecular determinants of acute single-cell lysis by human immunodeficiency virus type 1. *Journal of Virology* 70, 1340–1354.

Carette, J.E., Raaben, M., Wong, A.C., Herbert, A.S., Obernosterer, G., Mulherkar, N., Kuehne, A.I., Kranzusch, P.J., Griffin, A.M., Ruthel, G., *et al.* (2011). Ebola virus entry requires the cholesterol transporter Niemann-Pick C1. *Nature* 477, 340–343.

Carroll, M.C., and Isenman, D.E. (2012). Regulation of humoral immunity by complement. *Immunity* 37, 199–207.

Chao, L.H., Klein, D.E., Schmidt, A.G., Peña, J.M., and Harrison, S.C. (2014). Sequential conformational rearrangements in flavivirus membrane fusion. *Elife* 3, e04389.

Chen, A.B., Ben-Porat, T., Whitley, R.J., and Kaplan, A.S. (1978). Purification and characterization of proteins excreted by cells infected with herpes simplex virus and their use in diagnosis. *Virology* 91, 234–242.

Chung, K.M., Liszewski, M.K., Nybakken, G., Davis, A.E., Townsend, R.R., Fremont, D.H., Atkinson, J.P., and Diamond, M.S. (2006). West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proceedings of the National Academy of Science, USA* 103, 19111–19116.

Cianciolo, G.J., Copeland, T.D., Oroszlan, S., and Snyderman, R. (1985). Inhibition of lymphocyte proliferation by a synthetic peptide homologous to retroviral envelope proteins. *Science* 230, 453–455.

Cicala, C., Arthos, J., Rubbert, A., Selig, S., Wildt, K., Cohen, O.J., and Fauci, A.S. (2000). HIV-1 envelope induces activation of caspase-3 and cleavage of focal adhesion kinase in primary human CD4(+) T cells. *Proceedings of the National Academy of Science, USA* 97, 1178–1183.

Cohen, T., Pevsner-Fischer, M., Cohen, N., Cohen, I.R., and Shai, Y. (2008). Characterization of the interacting domain of the HIV-1 fusion peptide with the transmembrane domain of the T-cell receptor. *Biochemistry* 47, 4826–4833.

Cohen, T., Cohen, S.J., Antonovsky, N., Cohen, I.R., and Shai, Y. (2010). HIV-1 gp41 and TCRalpha trans-membrane domains share a motif exploited by the HIV virus to modulate T-cell proliferation. *PLoS Pathogens* 6, e1001085.

Connolly, S.A., Jackson, J.O., Jardetzky, T.S., and Longnecker, R. (2011). Fusing structure and function: a structural view of the herpesvirus entry machinery. *Nature Reviews Microbiology* 9, 369–381.

Cook, J.D. and Lee, J.E. (2013). The secret life of viral entry glycoproteins: moonlighting in immune evasion. *PLoS Pathogens* 9, e1003258.

Crotta, S., Stilla, A., Wack, A., D'Andrea, A., Nuti, S., D'Oro, U., Mosca, M., Filliponi, F., Brunetto, R.M., Bonino, F., *et al.* (2002). Inhibition of natural killer cells through engagement of CD81 by the major hepatitis C virus envelope protein. *Journal of Experimental Medicine* 195, 35–41.

Das, S.R., Puigbò, P., Hensley, S.E., Hurt, D.E., Bennink, J.R., and Yewdell, J.W. (2010). Glycosylation focuses sequence variation in the influenza A virus H1 hemagglutinin globular domain. *PLoS Pathogens* 6, e1001211.

de Jong, M.A., de Witte, L., Bolmstedt, A., van Kooyk, Y., and Geijtenbeek, T.B. (2008). Dendritic cells mediate herpes simplex virus infection and transmission through the C-type lectin DC-SIGN. *Journal of General Virology* 89, 2398–2409.

Del Cornò, M., Donninelli, G., Varano, B., Da Sacco, L., Masotti, A., and Gessani, S. (2014). HIV-1 gp120 activates the STAT3/interleukin-6 axis in primary human monocyte-derived dendritic cells. *Journal of Virology* 88, 11045–11055.

Denner, J., Eschricht, M., Lauck, M., Semaan, M., Schlaermann, P., Ryu, H., and Akyüz, L. (2013). Modulation of cytokine release and gene expression by the immunosuppressive domain of gp41 of HIV-1. *PLoS One* 8, e55199.

Di Lorenzo, C., Angus, A.G., and Patel, A.H. (2011). Hepatitis C virus evasion mechanisms from neutralizing antibodies. *Viruses* 3, 2280–2300.

Dietzschold, B., Wiktor, T.J., Wunner, W.H., and Varrichio, A. (1983). Chemical and immunological analysis of the rabies soluble glycoprotein. *Virology* 124, 330–337.

Dolnik, O., Volchkova, V., Garten, W., Carbonnelle, C., Becker, S., Kahnt, J., Ströher, U., Klenk, H.D., and Volchkov, V. (2004). Ectodomain shedding of the glycoprotein GP of Ebola virus. *EMBO Journal* 23, 2175–2184.

Douaisi, M., Dussart, S., Courcoul, M., Bessou, G., Vigne, R., and Decroly, E. (2004). HIV-1 and MLV Gag proteins are sufficient to recruit APOBEC3G into virus-like particles. *Biochemical and Biophysical Research Communications* 321, 566–573.

Drummer, H.E. (2014). Challenges to the development of vaccines to hepatitis C virus that elicit neutralizing antibodies. *Frontiers in Microbiology* 5, 329.

Dupressoir, A., Lavialle, C., and Heidmann, T. (2012). From ancestral infectious retroviruses to bona fide cellular genes: role of the captured syncytins in placentation. *Placenta* 33, 663–671.

Falkowska, E., Kajumo, F., Garcia, E., Reinus, J., and Dragic, T. (2007). Hepatitis C virus envelope glycoprotein E2 glycans modulate entry, CD81 binding, and neutralization. *Journal of Virology* 81, 8072–8079.

Fan, T., Day, N.K., Luangwedchakarn, V., Chang, Y., Ikehara, S., Lerner, D.L., and Haraguchi, S. (2005). The phosphorylation of phospholipase C-gamma1, Raf-1, MEK, and ERK1/2 induced by a conserved retroviral peptide. *Peptides* 26, 2165–2174.

Fausther-Bovendo, H., Vieillard, V., Sagan, S., Bismuth, G., and Debré, P. (2010). HIV gp41 engages gC1qR on CD4+ T cells to induce the expression of an NK ligand through the PIP3/H2O2 pathway. *PLoS Pathogens* 6, e1000975.

Feeley, E.M., Sims, J.S., John, S.P., Chin, C.R., Pertel, T., Chen, L.M., Gaiha, G.D., Ryan, B.J., Donis, R.O., Elledge, S.J., *et al*. (2011). IFITM3 inhibits influenza A virus infection by preventing cytosolic entry. *PLoS Pathogens* 7, e1002337.

Francica, J.R., Varela-Rohena, A., Medvec, A., Plesa, G., Riley, J.L., and Bates, P. (2010). Steric shielding of surface epitopes and impaired immune recognition induced by the ebola virus glycoprotein. *PLoS Pathogens* 6, e1001098.

Gelderblom, H.R., Reupke, H., and Pauli, G. (1985). Loss of envelope antigens of HTLV-III/LAV, a factor in AIDS pathogenesis? *Lancet* 2, 1016–1017.

Gelderblom, H.R., Hausmann, E.H., Ozel, M., Pauli, G., and Koch, M.A. (1987). Fine structure of human immunodeficiency virus (HIV) and immunolocalization of structural proteins. *Virology* 156, 171–176.

Hair, P.S., Gronemus, J.Q., Crawford, K.B., Salvi, V.P., Cunnion, K.M., Thielens, N.M., Arlaud, G.J., Rawal, N., and Krishna, N.K. (2010). Human astrovirus coat protein binds C1q and MBL and inhibits the classical and lectin pathways of complement activation. *Molecular Immunology* 47, 792–798.

Haraguchi, S., Good, R.A., Cianciolo, G.J., Engelman, R.W., and Day, N.K. (1997). Immunosuppressive retroviral peptides: immunopathological implications for immunosuppressive influences of retroviral infections. *Journal of Leukocyte Biology* 61, 654–666.

Harcourt, J., Alvarez, R., Jones, L.P., Henderson, C., Anderson, L.J., and Tripp, R.A. (2006). Respiratory syncytial virus G protein and G protein CX3C motif adversely affect CX3CR1+ T cell responses. *Journal of Immunology* 176, 1600–1608.

Harrison, S.C. (2008). Viral membrane fusion. *Nature Structural and Molecular Biology* 15, 690–698.

Hebell, T., Ahearn, J.M., and Fearon, D.T. (1991). Suppression of the immune response by a soluble complement receptor of B lymphocytes. *Science* 254, 102–105.

Heldwein, E.E. and Krummenacher, C. (2008). Entry of herpesviruses into mammalian cells. *Cellular and Molecular Life Sciences* 65, 1653–1668.

Helle, F., Goffard, A., Morel, V., Duverlie, G., McKeating, J., Keck, Z.Y., Foung, S., Penin, F., Dubuisson, J., and Voisset, C. (2007). The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. *Journal of Virology* 81, 8101–8111.

Helle, F., Vieyres, G., Elkrief, L., Popescu, C.I., Wychowski, C., Descamps, V., Castelain, S., Roingeard, P., Duverlie, G., and Dubuisson, J. (2010). Role of N-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. *Journal of Virology* 84, 11905–11915.

Helle, F., Duverlie, G., and Dubuisson, J. (2011). The hepatitis C virus glycan shield and evasion of the humoral immune response. *Viruses* 3 1909–1932.

Holmes, E.C. (2011). What does virus evolution tell us about virus origins? *Journal of Virology* 85, 5247–5251.

Huang, I.C., Bailey, C.C., Weyer, J.L., Radoshitzky, S.R., Becker, M.M., Chiang, J.J., Brass, A.L., Ahmed, A.A., Chi, X., Dong, L., *et al.* (2011). Distinct patterns of IFITM-mediated restriction of filoviruses, SARS coronavirus, and influenza A virus. *PLoS Pathogens* 7, e1001258.

Ito, H., Watanabe, S., Takada, A., and Kawaoka, Y. (2001). Ebola virus glycoprotein: proteolytic processing, acylation, cell tropism, and detection of neutralizing antibodies. *Journal of Virology* 75, 1576–1580.

Ivanovic, T., Choi, J.L., Whelan, S.P., van Oijen, A.M., and Harrison, S.C. (2013). Influenza-virus membrane fusion by cooperative fold-back of stochastically induced hemagglutinin intermediates. *Elife* 2, e00333.

Janz, A., Oezel, M., Kurzeder, C., Mautner, J., Pich, D., Kost, M., Hammerschmidt, W., and Delecluse, H.J. (2000). Infectious Epstein-Barr virus lacking major glycoprotein BLLF1 (gp350/220) demonstrates the existence of additional viral ligands. *Journal of Virology* 74, 10142–10152.

Johnson, J.B., Grant, K., and Parks, G.D. (2009). The paramyxoviruses simian virus 5 and mumps virus recruit host cell CD46 to evade complement-mediated neutralization. *Journal of Virology* 83, 7602–7611.

Johnson, J.B., Lyles, D.S., Alexander-Miller, M.A., and Parks, G.D. (2012). Virion-associated complement regulator CD55 is more potent than CD46 in mediating resistance of mumps virus and vesicular stomatitis virus to neutralization. *Journal of Virology* 86, 9929–9940.

Jorgenson, R.L., Vogt, V.M., and Johnson, M.C. (2009). Foreign glycoproteins can be actively recruited to virus assembly sites during pseudotyping. *Journal of Virology* 83, 4060–4067.

Kaletsky, R.L., Francica, J.R., Agrawal-Gamse, C., and Bates, P. (2009). Tetherin-mediated restriction of filovirus budding is antagonized by the Ebola glycoprotein. *Proceedings of the National Academy of Science, USA* 106, 2886–2891.

Kaname, Y., Tani, H., Kataoka, C., Shiokawa, M., Taguwa, S., Abe, T., Moriishi, K., Kinoshita, T., and Matsuura, Y. (2010). Acquisition of complement resistance

through incorporation of CD55/decay-accelerating factor into viral particles bearing baculovirus GP64. *Journal of Virology* 84, 3210–3219.

Kang, C.Y. and Prevec, L. (1971). Proteins of vesicular stomatitis virus. 3. Intracellular synthesis and extracellular appearance of virus-specific proteins. Virology 46, 678–690.

Kittlesen, D.J., Chianese-Bullock, K.A., Yao, Z.Q., Braciale, T.J., and Hahn, Y.S. (2000). Interaction between complement receptor gC1qR and hepatitis C virus core protein inhibits T-lymphocyte proliferation. *Journal of Clinical Investigations* 106, 1239–1249.

Kojouharova, M.S., Tsacheva, I.G., Tchorbadjieva, M.I., Reid, K.B., and Kishore, U. (2003). Localization of ligand-binding sites on human C1q globular head region using recombinant globular head fragments and single-chain antibodies. *Biochimica et Biophysica Acta* 1652, 64–74.

Kong, L., Giang, E., Robbins, J.B., Stanfield, R.L., Burton, D.R., Wilson, I.A., and Law, M. (2012). Structural basis of hepatitis C virus neutralization by broadly neutralizing antibody HCV1. *Proceedings of the National Academy of Science, USA* 109, 9499–9504.

Kostavasili, I., Sahu, A., Friedman, H.M., Eisenberg, R.J., Cohen, G.H., and Lambris, J.D. (1997). Mechanism of complement inactivation by glycoprotein C of herpes simplex virus. *Journal of Immunology* 158, 1763–1771.

Kurosu, T., Chaichana, P., Yamate, M., Anantapreecha, S., and Ikuta, K. (2007). Secreted complement regulatory protein clusterin interacts with dengue virus nonstructural protein 1. *Biochemical and Biophysical Research Communications* 362, 1051–1056.

Laquerre, S., Argnani, R., Anderson, D.B., Zucchini, S., Manservigi, R., and Glorioso, J.C. (1998). Heparan sulfate proteoglycan binding by herpes simplex virus type 1 glycoproteins B and C, which differ in their contributions to virus attachment, penetration, and cell-to-cell spread. *Journal of Virology* 72, 6119–6130.

Lau, D., Kwan, W., and Guatelli, J. (2011). Role of the endocytic pathway in the counteraction of BST-2 by human lentiviral pathogens. *Journal of Virology* 85, 9834–9846.

Lauring, A.S., Frydman, J., and Andino, R. (2013). The role of mutational robustness in RNA virus evolution. *Nature Reviews Microbiology* 11, 327–336.

Lee, J.E. and Saphire, E.O. (2009). Neutralizing ebolavirus: structural insights into the envelope glycoprotein and antibodies targeted against it. *Current Opinion in Structural Biology* 19, 408–417.

Lee, J.E., Fusco, M.L., Hessell, A.J., Oswald, W.B., Burton, D.R., and Saphire, E.O. (2008). Structure of the Ebola virus glycoprotein bound to an antibody from a human survivor. *Nature* 454, 177–182.

Lehmann, M., Rocha, S., Mangeat, B., Blanchet, F., Uji-I, H., Hofkens, J., and Piguet, V. (2011). Quantitative multicolor super-resolution microscopy reveals tetherin HIV-1 interaction. *PLoS Pathogens* 7, e1002456.

Lennemann, N.J., Rhein, B.A., Ndungo, E., Chandran, K., Qiu, X., and Maury, W. (2014). Comprehensive functional analysis of N-linked glycans on Ebola virus GP1. *MBio* 5, e00862–00813.

Li, L., Jose, J., Xiang, Y., Kuhn, R.J., and Rossmann, M.G. (2010). Structural changes of envelope proteins during alphavirus fusion. *Nature* 468, 705–708.

Little, S.P. and Huang, A.S. (1977). Synthesis and distribution of vesicular stomatitis virus-specific polypeptides in the absence of progeny production. *Virology* 81, 37–47.

Luangwedchakarn, V., Day, N.K., Hitchcock, R., Brown, P.G., Lerner, D.L., Rucker, R.P., Cianciolo, G.J., Good, R.A., and Haraguchi, S. (2003). A retroviral-derived peptide phosphorylates protein kinase D/protein kinase Cmu involving phospholipase C and protein kinase C. *Peptides* 24, 631–637.

Machiels, B., Lété, C., Guillaume, A., Mast, J., Stevenson, P.G., Vanderplasschen, A., and Gillet, L. (2011). Antibody evasion by a gammaherpesvirus O-glycan shield. *PLoS Pathogens* 7, e1002387.

Mangeney, M. and Heidmann, T. (1998). Tumor cells expressing a retroviral envelope escape immune rejection in vivo. *Proceedings of the National Academy of Science, USA* 95, 14920–14925.

Maruyama, T., Parren, P.W., Sanchez, A., Rensink, I., Rodriguez, L.L., Khan, A.S., Peters, C.J., and Burton, D.R. (1999). Recombinant human monoclonal antibodies to Ebola virus. *Journal of Infectious Diseases* 179 Suppl 1, S235–239.

McKeating, J.A., McKnight, A., and Moore, J.P. (1991). Differential loss of envelope glycoprotein gp120 from virions of human immunodeficiency virus type 1 isolates: effects on infectivity and neutralization. *Journal of Virology* 65, 852–860.

Mehedi, M., Falzarano, D., Seebach, J., Hu, X., Carpenter, M.S., Schnittler, H.J., and Feldmann, H. (2011). A new Ebola virus nonstructural glycoprotein expressed through RNA editing. *Journal of Virology* 85, 5406–5414.

Mohan, G.S., Li, W., Ye, L., Compans, R.W., and Yang, C. (2012). Antigenic subversion: a novel mechanism of host immune evasion by Ebola virus. *PLoS Pathogens* 8, e1003065.

Moore, J.P. and Sodroski, J. (1996). Antibody cross-competition analysis of the human immunodeficiency virus type 1 gp120 exterior envelope glycoprotein. *Journal of Virology* 70, 1863–1872.

Morozov, V.A., Morozov, A.V., Semaan, M., and Denner, J. (2012). Single mutations in the transmembrane envelope protein abrogate the immunosuppressive property of HIV-1. *Retrovirology* 9, 67.

Mullen, M.M., Haan, K.M., Longnecker, R., and Jardetzky, T.S. (2002). Structure of the Epstein-Barr virus gp42 protein bound to the MHC class II receptor HLA-DR1. *Molecular Cell* 9, 375–385.

Neil, S.J., Zang, T., and Bieniasz, P.D. (2008). Tetherin inhibits retrovirus release and is antagonized by HIV-1 Vpu. *Nature* 451, 425–430.

Ressing, M.E., van Leeuwen, D., Verreck, F.A., Gomez, R., Heemskerk, B., Toebes, M., Mullen, M.M., Jardetzky, T.S., Longnecker, R., Schilham, M.W., *et al.* (2003). Interference with T cell receptor-HLA-DR interactions by Epstein-Barr virus gp42 results in reduced T helper cell recognition. *Proceedings of the National Academy of Science, USA* 100, 11583–11588.

Ressing, M.E., van Leeuwen, D., Verreck, F.A., Keating, S., Gomez, R., Franken, K.L., Ottenhoff, T.H., Spriggs, M., Schumacher, T.N., Hutt-Fletcher, L.M., *et al.* (2005). Epstein-Barr virus gp42 is posttranslationally modified to produce soluble gp42 that mediates HLA class II immune evasion. *Journal of Virology* 79, 841–852.

Rey, F.A. (2006). Molecular gymnastics at the herpesvirus surface. *EMBO Report* 7, 1000–1005.

Robinson, W.E., Gorny, M.K., Xu, J.Y., Mitchell, W.M., and Zolla-Pazner, S. (1991). Two immunodominant domains of gp41 bind antibodies which enhance human immunodeficiency virus type 1 infection in vitro. *Journal of Virology* 65, 4169–4176.

Saifuddin, M., Hedayati, T., Atkinson, J.P., Holguin, M.H., Parker, C.J., and Spear, G.T. (1997). Human immunodeficiency virus type 1 incorporates both glycosyl phosphatidylinositol-anchored CD55 and CD59 and integral membrane CD46 at levels that protect from complement-mediated destruction. *Journal of General Virology* 78 (Pt 8) 1907–1911.

Sanchez, A., Trappier, S.G., Mahy, B.W., Peters, C.J., and Nichol, S.T. (1996). The virion glycoproteins of Ebola viruses are encoded in two reading frames and are expressed through transcriptional editing. *Proceedings of the National Academy of Science, USA* 93, 3602–3607.

Schlecht-Louf, G., Renard, M., Mangeney, M., Letzelter, C., Richaud, A., Ducos, B., Bouallaga, I., and Heidmann, T. (2010). Retroviral infection in vivo requires an immune escape virulence factor encrypted in the envelope protein of oncoretroviruses. *Proceedings of the National Academy of Science, USA* 107, 3782–3787.

Sheehy, A.M., Gaddis, N.C., Choi, J.D., and Malim, M.H. (2002). Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* 418, 646–650.

Simmons, G., Wool-Lewis, R.J., Baribaud, F., Netter, R.C., and Bates, P. (2002). Ebola virus glycoproteins induce global surface protein down-modulation and loss of cell adherence. *Journal of Virology* 76, 2518–2528.

Spear, G.T., Lurain, N.S., Parker, C.J., Ghassemi, M., Payne, G.H., and Saifuddin, M. (1995). Host cell-derived complement control proteins CD55 and CD59 are incorporated into the virions of two unrelated enveloped viruses. Human T cell leukemia/lymphoma virus type I (HTLV-I) and human cytomegalovirus (HCMV). *Journal of Immunology* 155, 4376–4381.

Stoermer, K.A., and Morrison, T.E. (2011). Complement and viral pathogenesis. *Virology* 411, 362–373.

Szakonyi, G., Klein, M.G., Hannan, J.P., Young, K.A., Ma, R.Z., Asokan, R., Holers, V.M., and Chen, X.S. (2006). Structure of the Epstein-Barr virus major envelope glycoprotein. *Nature Structural and Molecular Biology* 13, 996–1001.

Taylor, D.R., Shi, S.T., Romano, P.R., Barber, G.N., and Lai, M.M. (1999). Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. *Science* 285, 107–110.

Tripp, R.A., Jones, L.P., Haynes, L.M., Zheng, H., Murphy, P.M., and Anderson, L.J. (2001). CX3C chemokine mimicry by respiratory syncytial virus G glycoprotein. *Nature Immunology* 2, 732–738.

Turner, A., Bruun, B., Minson, T., and Browne, H. (1998). Glycoproteins gB, gD, and gHgL of herpes simplex virus type 1 are necessary and sufficient to mediate membrane fusion in a Cos cell transfection system. *Journal of Virology* 72, 873–875.

Vallhov, H., Gutzeit, C., Johansson, S.M., Nagy, N., Paul, M., Li, Q., Friend, S., George, T.C., Klein, E., Scheynius, A., *et al.* (2011). Exosomes containing glycoprotein 350 released by EBV-transformed B cells selectively target B cells through CD21 and block EBV infection in vitro. *Journal of Immunology* 186, 73–82.

Wei, X., Decker, J.M., Wang, S., Hui, H., Kappes, J.C., Wu, X., Salazar-Gonzalez, J.F., Salazar, M.G., Kilby, J.M., Saag, M.S., *et al.* (2003). Antibody neutralization and escape by HIV-1. *Nature* 422, 307–312.

Wei, C.J., Boyington, J.C., Dai, K., Houser, K.V., Pearce, M.B., Kong, W.P., Yang, Z.Y., Tumpey, T.M., and Nabel, G.J. (2010). Cross-neutralization of 1918 and 2009 influenza viruses: role of glycans in viral evolution and vaccine design. *Science Translational Medicine* 2, 24ra21.

White, J.M., Delos, S.E., Brecher, M., and Schornberg, K. (2008). Structures and mechanisms of viral membrane fusion proteins: multiple variations on a common theme. *Critical Reviews in Biochemistry and Molecular Biology* 43, 189–219.

Wiedenbeck, J. and Cohan, F.M. (2011). Origins of bacterial diversity through horizontal genetic transfer and adaptation to new ecological niches. *FEMS Microbiology Reviews* 35, 957–976.

Wilson, J.A., Hevey, M., Bakken, R., Guest, S., Bray, M., Schmaljohn, A.L., and Hart, M.K. (2000). Epitopes involved in antibody-mediated protection from Ebola virus. *Science* 287, 1664–1666.

Yaddanapudi, K., Palacios, G., Towner, J.S., Chen, I., Sariol, C.A., Nichol, S.T., and Lipkin, W.I. (2006). Implication of a retrovirus-like glycoprotein peptide in the immunopathogenesis of Ebola and Marburg viruses. *FASEB Journal* 20, 2519–2530.

Zhang, J., Li, G., Liu, X., Wang, Z., Liu, W., and Ye, X. (2009). Influenza A virus M1 blocks the classical complement pathway through interacting with C1qA. *Journal of General Virology* 90, 2751–2758.

Zhou, C., Lu, L., Tan, S., Jiang, S., and Chen, Y.H. (2011). HIV-1 glycoprotein 41 ectodomain induces activation of the CD74 protein-mediated extracellular signal-regulated kinase/mitogen-activated protein kinase pathway to enhance viral infection. *Journal of Biological Chemistry* 286, 44869–44877.

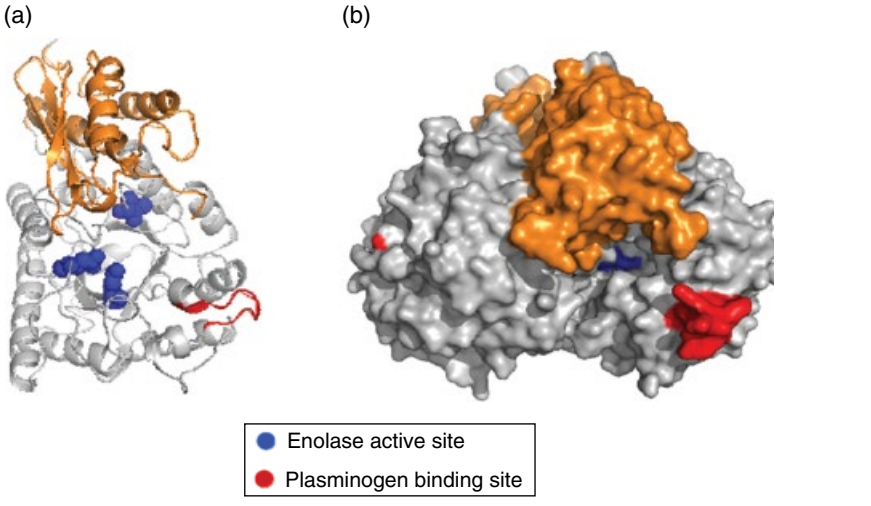

Figure 2.3 α-Enolase. (a) Single chain of Enolase showing the enzyme active site in blue and the plasminogen-binding site in red. (b) Enolase monomer displayed as surface. Different domains are colored in gray and orange (PDB:1W6T).

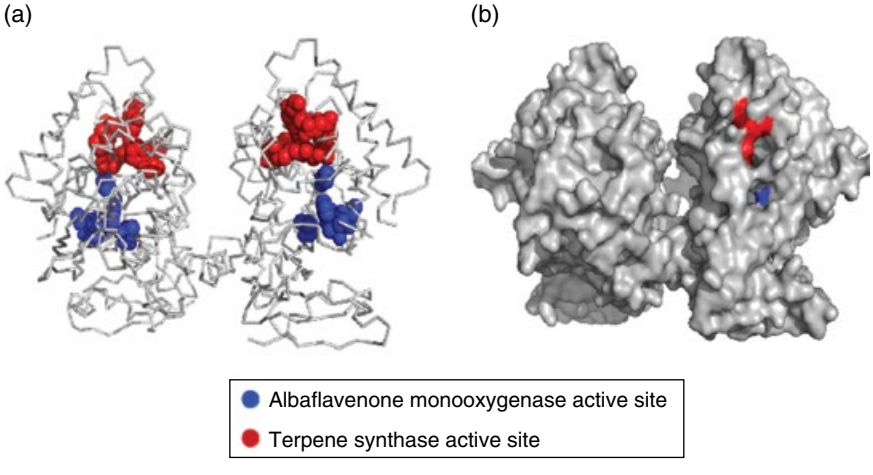

Figure 2.4 Albaflavenone monooxygenase. The monooxygenase and terpene synthase active sites are shown in blue and red respectively in the (a) cartoon and (b) surface representation of Albaflavenone monooxygenase (PDB: 3EL3).

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

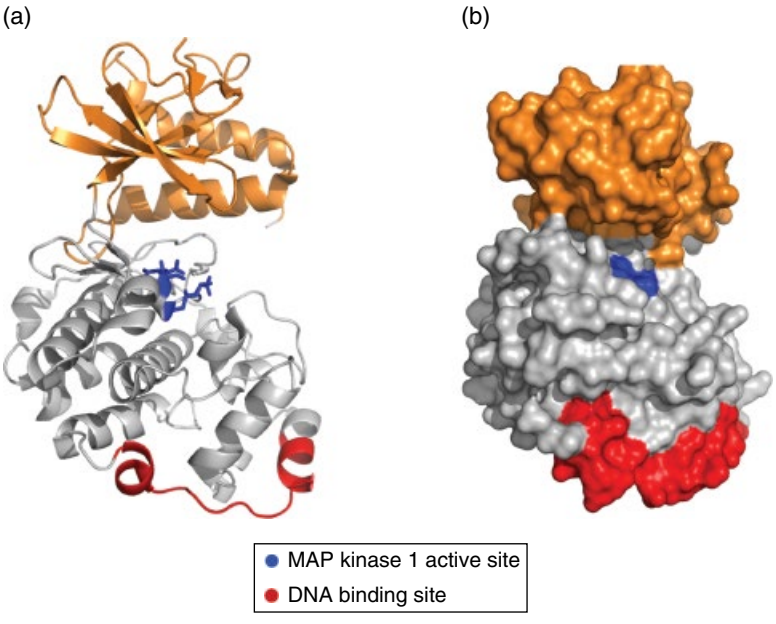

Figure 2.5 Human MAPK1/ERK2. The MAPK1 active site is shown in blue and the DNA-binding motif is highlighted in red. Different domains are shown in gray and orange (PDB:4G6N).

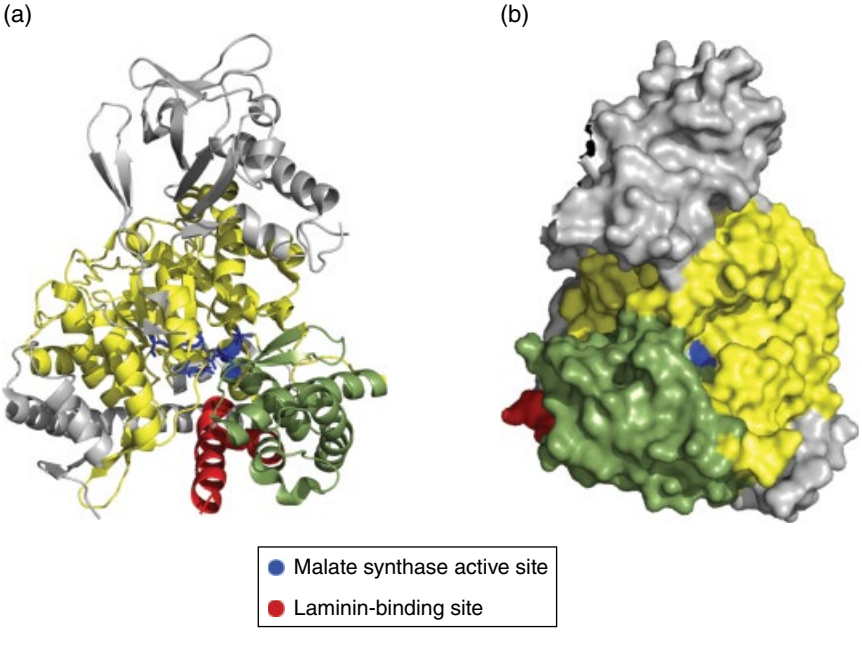

Figure 2.6 Malate synthase. The enzyme active site is shown in blue and the laminin- binding site is shown in red. Different domains are shown in different colors (PDB:2GQ3).

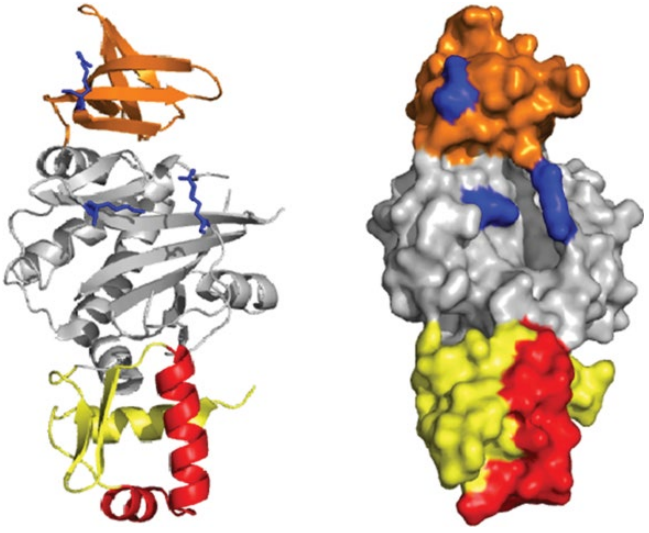

Figure 2.7 BirA. The catalytic site residues are shown in blue while the H-T-H motif involved in binding DNA (moonlighting function) is shown in red. Different domains are shown in different colors (PDB:1BIB).

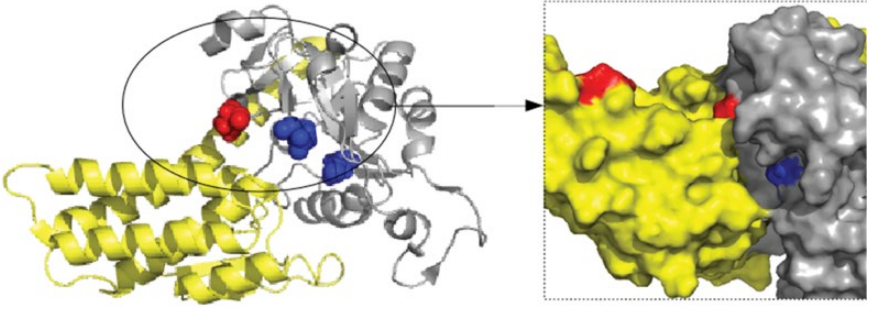

Figure 2.8 Human MRDI. The active site residues are shown in blue while the residues implicated in controlling invasion (moonlighting function) is shown in red. Different domains are shown in different colors (PDB:4LDQ).

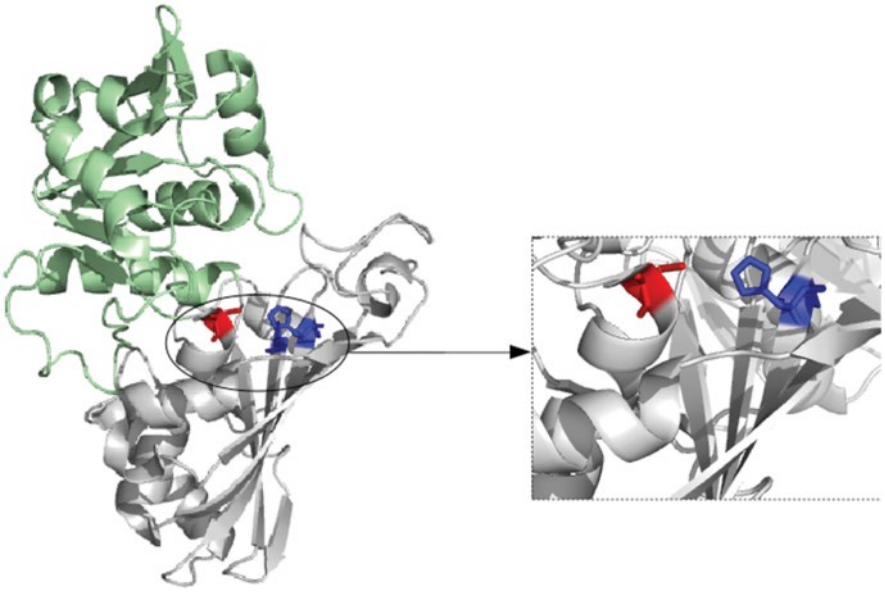

Figure 2.9 GAPDH. The catalytic site residue Cys149 (shown in red) is the residue known to be involved for both the canonical and moonlighting functions of *E. coli* GAPDH. The other catalytic residue His179 is shown in blue (PDB:1DC5).

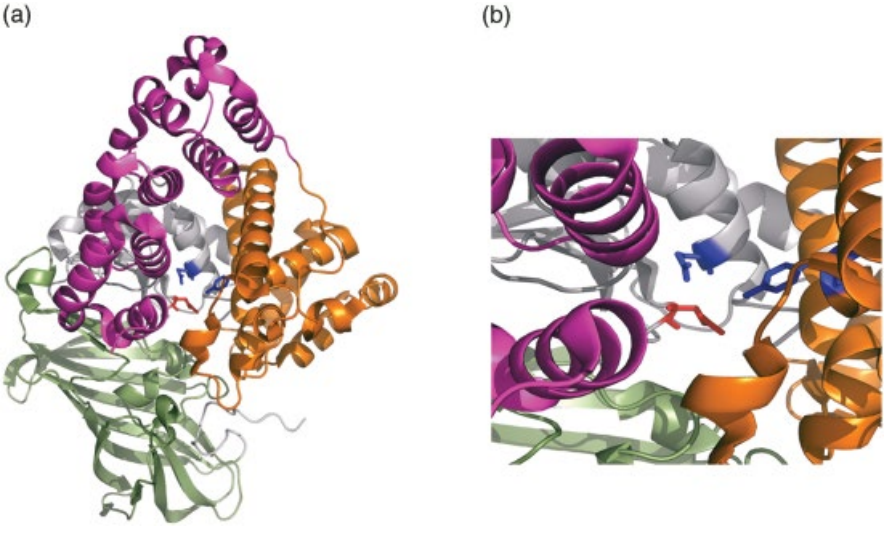

Figure 2.10 Leukotriene A4 hydrolase. The LTA4 catalytic site residues Glu296 and Tyr383 are shown in blue. The catalytic site residue Glu271, involved in two separate functions in two different catalytic reactions is shown in red (PDB:2R59).

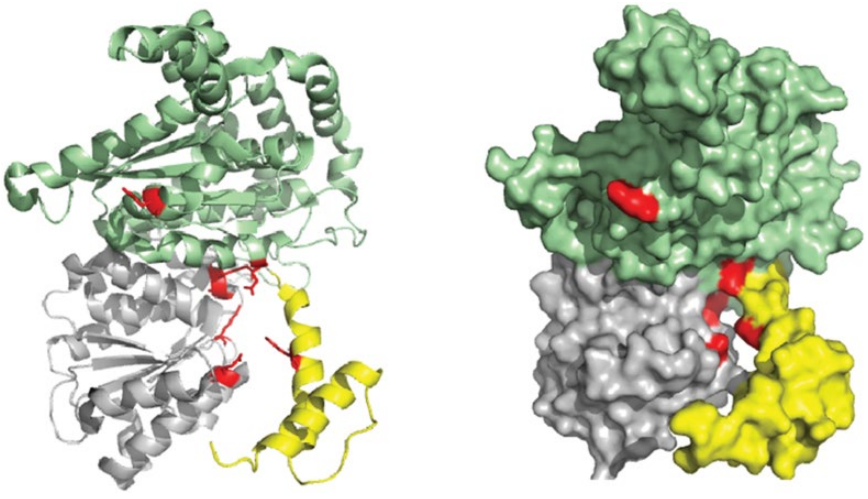

Figure 2.11 Phosphoglucose isomerase (PGI). Catalytic residues are shown as red sticks. Inhibition of enzymatic and AMF functions of PGI by the PGI inhibitor and mutational analysis of the catalytic residues have indicated overlapping regions of both functions in the human PGI (PDB:1IAT).

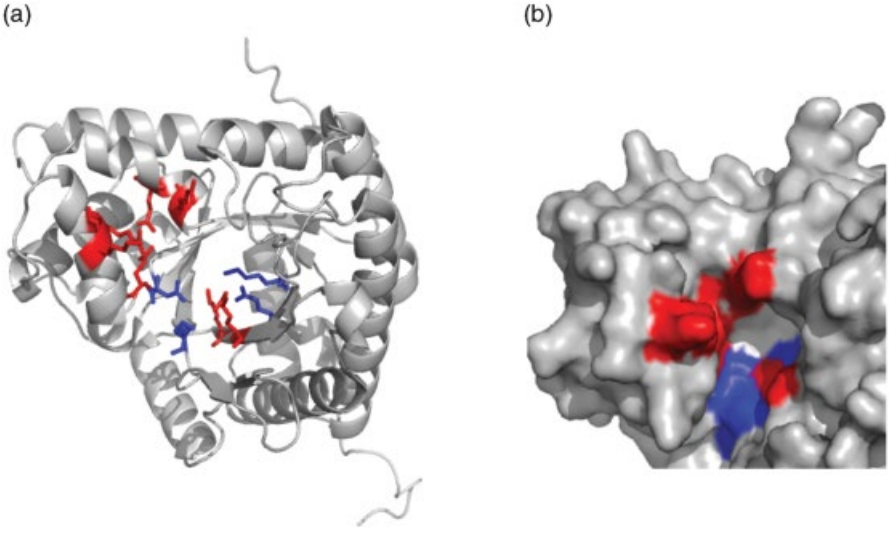

Figure 2.12 Aldolase. The enzyme active site is shown in blue and the actin-binding site is shown in red (PDB:2PC4).

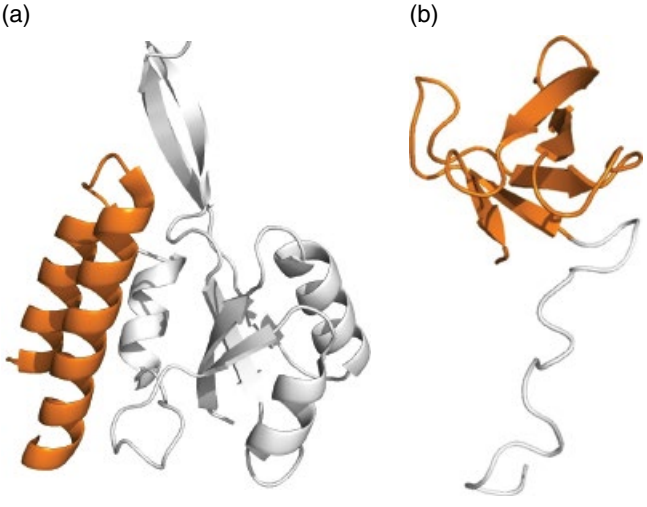

Figure 2.13 RfaH The Rfah CTD is colored in orange. In the closed form of RfaH (a), the CTD (α-helix form) and NTD tightly interacts and works as a transcription factor (PDB:2OUG). The subsequent (or simultaneous) refolding of the CTD into a (b) β-barrel transforms RfaH into a translation factor (PDB:2LCL).

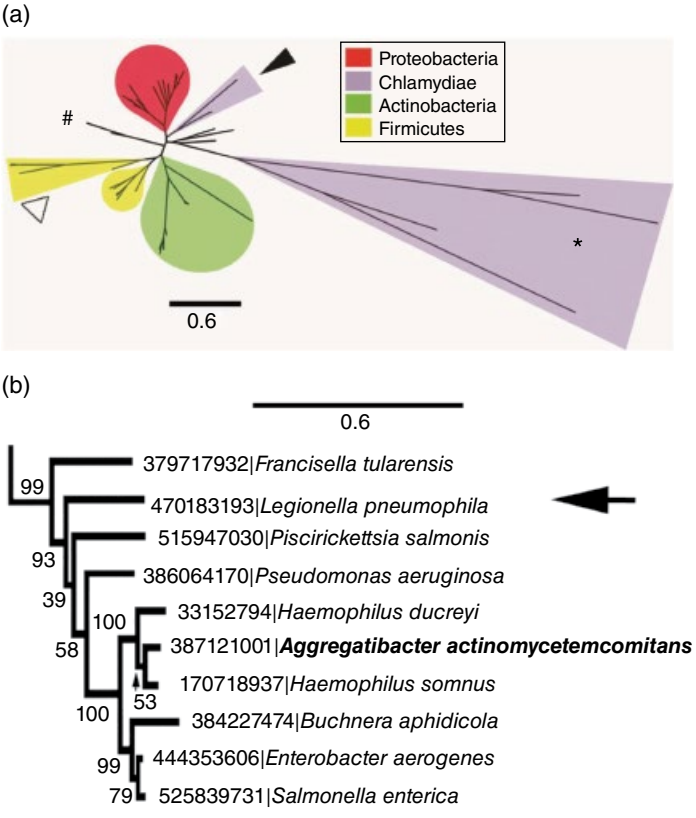

Figure 6.7 Phylogenetic relationships between 48 chaperonins (belonging to 35 bacterial species) known to have moonlighting functions and (or) extra-cytoplasmic locations. (a) Diagram of an unrooted maximum likelihood phylogenetic tree of the 48 chaperonins. Colors indicate phylum as per legend. Asterisk marks the highly divergent Cpn60.2 and Cpn60.3 of *Chlamydia trachomatis* and *Chlamydophila pneumoniae*, whereas the black arrowhead shows the positions of the Cpn60.1 of these two chlamydiales, which are more closely related to the Cpn60 of *Borrelia burgdorferi*, *Porphyromonas gingivalis*, and *Leptospira interrogans* (black branches between the two purple triangles). The open triangle marks the group formed by the three Cpn60s of *Mycoplasma gallisepticum*, *Mycoplasma genitalium*, and *Mycoplasma pneumoniae*, whereas the pound sign marks the position of the *Mycoplasma penetrans* Cpn60, which closely groups with the *Helicobacter pylori*'s Cpn60. Branch lengths are proportional to the number of amino acid substitutions per site (scale shown at the bottom of the diagram). (b) Diagram of part of the rooted tree of the same 48 chaperonins shown in (a). The part shown would be equivalent to the central part of the Proteobacteria branch (red bubble) depicted in (a), and includes HtpB (marked with the black arrow) and its closest moonlighting Cpn60s from the intracellular pathogens *Piscirickettsia salmonis* and *Francisella tularensis*. The tip labels include the amino acid sequence identification number (GIs for the Pfam database) followed by the bacterial taxonomic name. Branch lengths are proportional to the number of substitutions per site (scale given at the top of the panel). Bootstrap values (%) based on 100 replications are given for every branch.

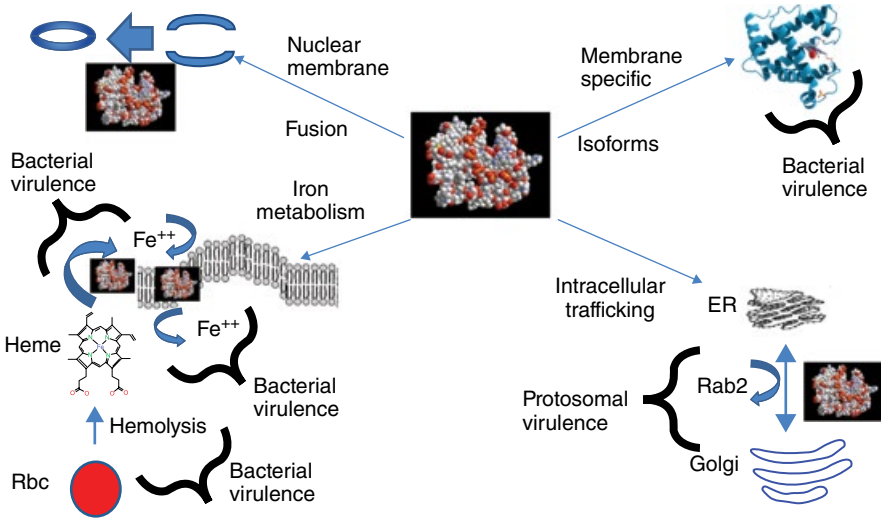

Figure 8.2 Membrane-associated GAPDH: structure and function.

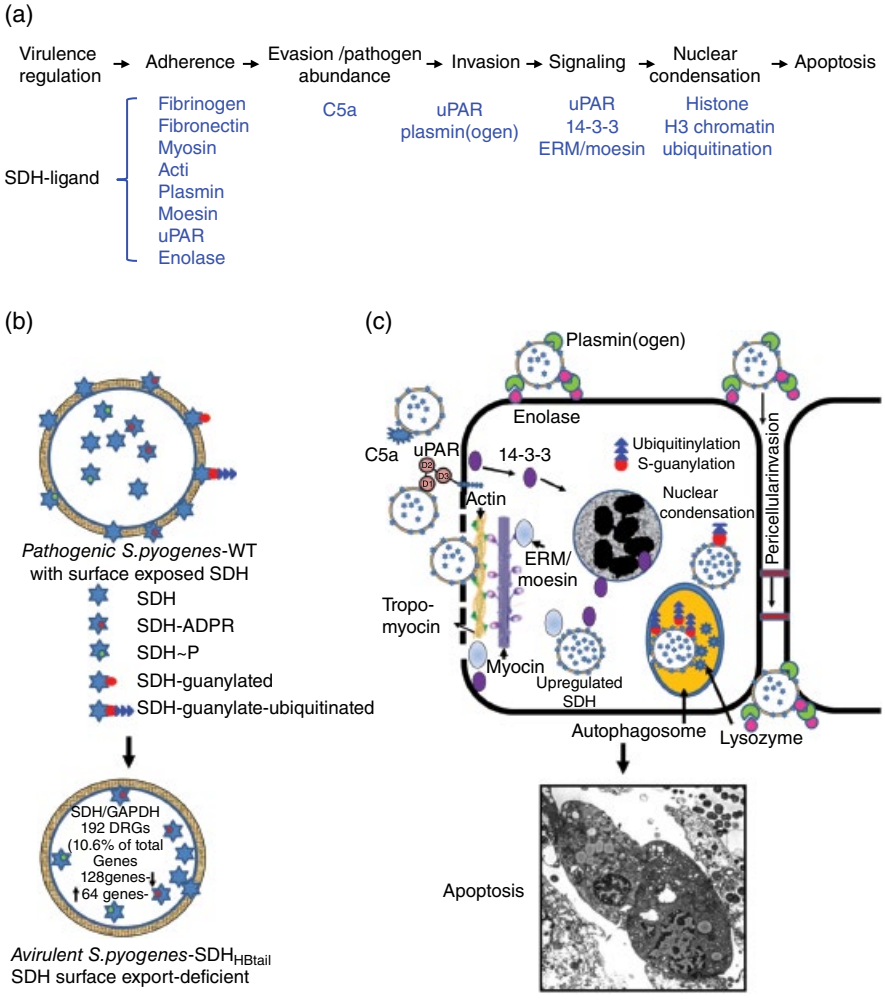

Figure 9.1 Schematic diagram showing the role of SDH in the *S. pyogenes* pathogenesis. (a) Various stages of *S. pyogenes* infection. (b) During infection, as a successful pathogen *S. pyogenes* adheres to and invades host cells. Subsequently, it hides and proliferates within the host tissues by evading host innate immune responses and maintains its abundance in a local microenvironment. *S. pyogenes* also causes apoptosis of host cells. In all these different stages of the infection, SDH participates in almost all stages by binding through different receptor ligands as illustrated. (c) The SDH surface export is also important to maintain GAS virulence as, by retaining SDH within cytoplasm, 128 genes, including 25 major virulence genes, are downregulated. SDH is therefore a quintessential important virulence regulator. The surface export of the cytoplasmic SDH and its role in the *S. pyogenes* virulence regulation may be mediated via several post-translational modifications including reported phosphorylation, ADP-ribosylation, S-guanylation, and ubiquitination. The mechanism underlying this possible and predicted regulation is however unknown.

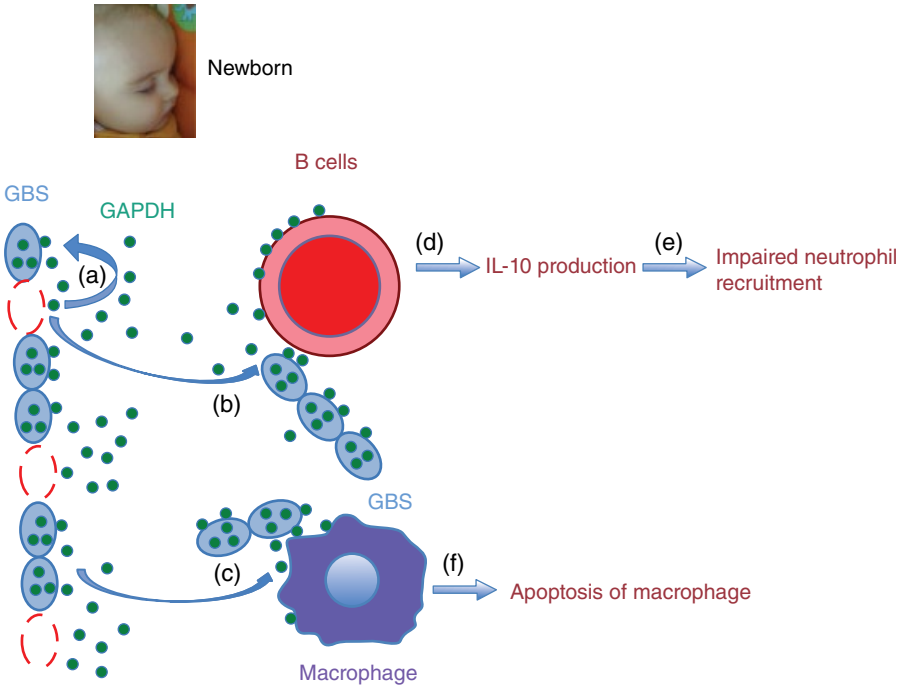

Figure 10.1 Group B *Streptococcus* GAPDH modulates the neonatal inflammatory response. (a) GBS cocci growing in chains may spontaneously lyse (dashed red cocci) and the released cytosolic GAPDH (green dot) can then re-associate with living bacteria. Free or GBS-bound GAPDH can interact with (b) B cells or with (c) macrophages. Upon interaction with B cells, GAPDH (d) triggers IL-10 production and (e) impairs neutrophil recruitment. (f) GAPDH can also interact with macrophages to induce their apoptosis.

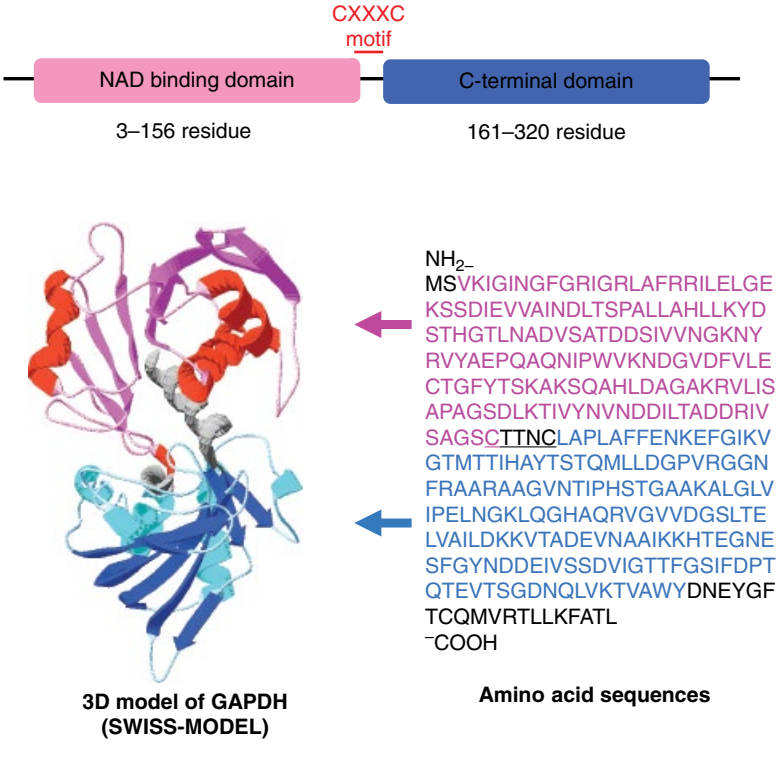

Figure 12.2 The three-dimensional structure and amino acid sequences of GAPDH of *L. plantarum* LA 318. The underline indicates CXXXC motif.

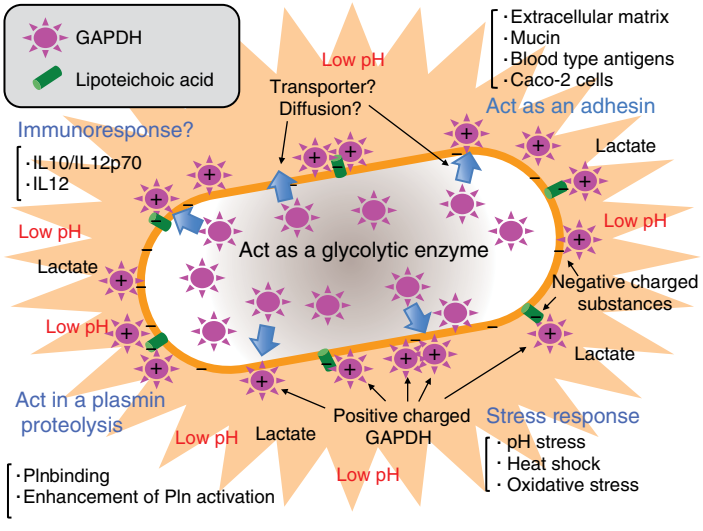

Figure 12.3 Overview diagram of GAPDH in probiotics.

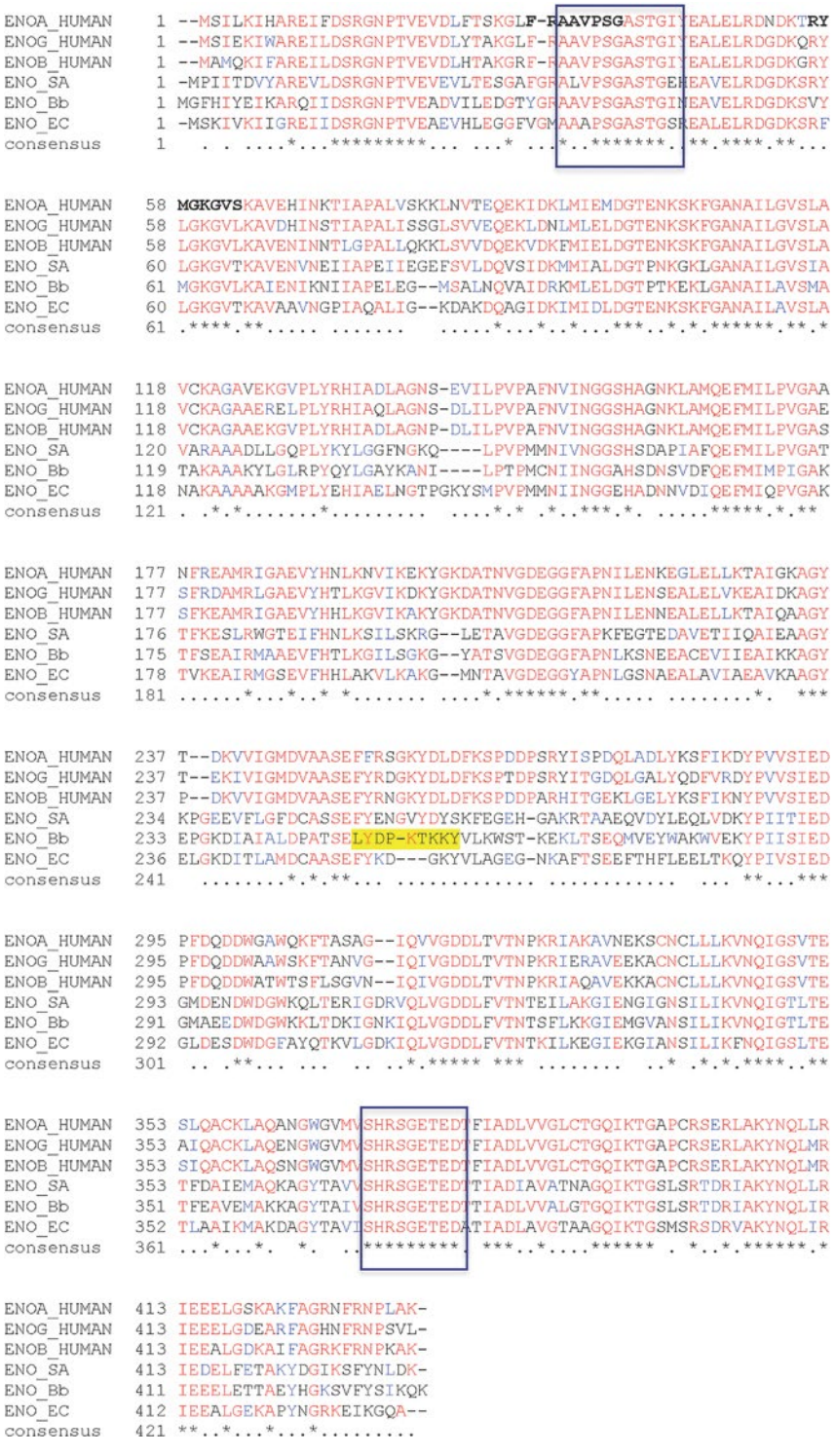

Figure 15.1 Alignment of enolase protein sequences from human (three isoforms), *Staphylococcus aureus*, *Escherichia coli*, and *B. burgdorferi*. Identical amino acids are in red; similar amino acids are in blue font. A consensus line underneath the alignment indicates identity (*) or similarity (.). The box at the top of the alignment indicates a hydrophobic domain thought to play a role in membrane association (Pancholi 2001). The box at the bottom of the alignment indicates the catalytic site of the enzyme. Yellow highlighted text indicates an internal plasminogen-binding motif (Noguiera *et al*. 2012). Bold black text indicates cross-reactive epitopes associated with autoimmunity in cancer (Adamus *et al*. 1998).

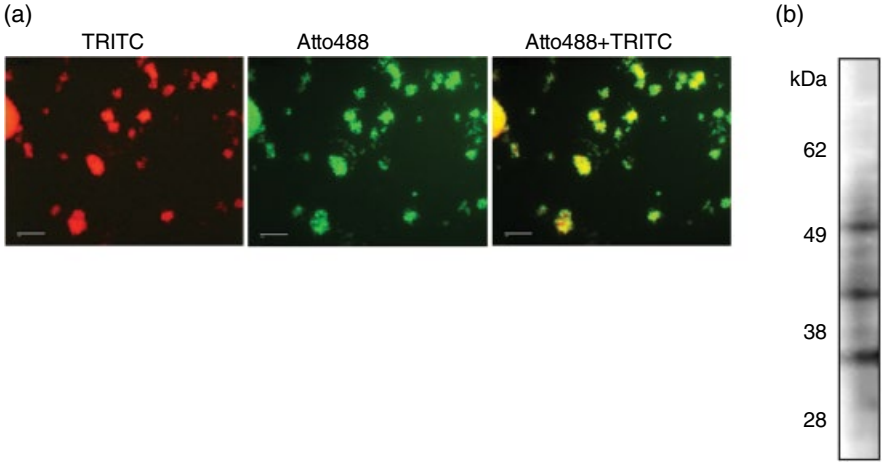

Figure 18.1 Binding of human plasminogen to *M. pneumoniae*. (a) Immunofluorescence of fixed *M. pneumoniae* cells after incubation with Atto488-labeled plasminogen (50 µg mL^{-1}). Antiserum to Triton X-insoluble proteins detected with TRITC-labeled secondary antibody is used as control. Bar: 10 µm. (b) Ligand immunoblotting assay using SDS-PAGE-separated total proteins of *M. pneumoniae* incubated with plasminogen (15 µg mL^{-1}) detected with rabbit anti-plasminogen.

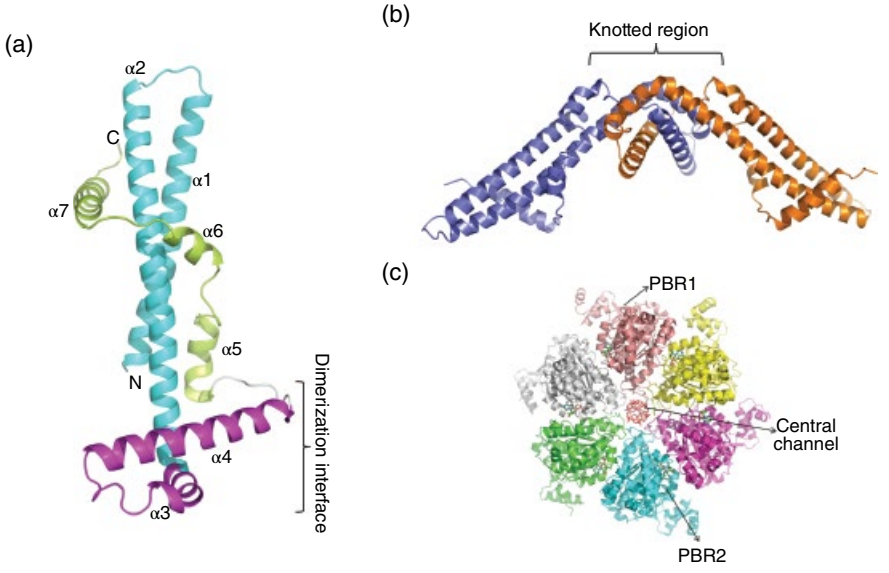

Figure 22.3 (a) The crystal structure of the Psu monomer with seven α-helices and dimerization interface. The N-terminal, α1, and α2 helices form a tight coiled-coil structure termed the "CC-stem," surrounded by the three C-terminal helices (α5–α7) known as the CT-belt. The central region, shown here as the dimerization interface and consisting of helices α3 and α4, forms a knotted structure with another monomer. CC-stem in cyan, CT-belt in green and central region in magenta. (b) The dimeric structure shown here is formed when the two Psu monomers "knot," and this knotted region (as indicated) acts as a hinge allowing the other regions to swing inward and outward. This dimer formation allows Psu to interact with and bind the Rho hexamer. (PDB code: 3RX6). The figure shows the dimer with domain colour differences. (c) A top view of the closed, hexameric structure of Rho bound to RNA (PDB code: 3ICE). The two Psu-binding regions (PBR1 and PBR2) are indicated by arrows along with the central region containing the RNA. Source: Banerjee *et al*. (2012) and Ranjan *et al*. (2014).

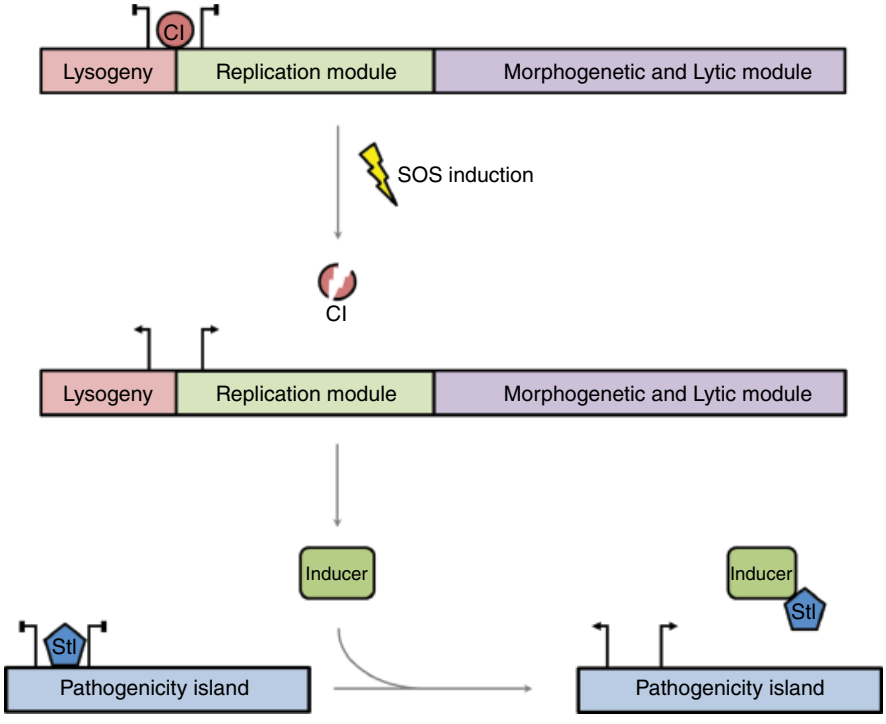

Figure 22.5 SaPI induction is mediated by a helper phage-encoded protein. The helper phage and the SaPI reside stably integrated into the bacteria chromosome due to the activity of their respective encoded master repressors, CI for the phage and Stl for the SaPI. When the SOS response is activated the repressor of the phage CI is degraded, allowing the expression of the lytic genes of the phage and initiating its replication. The interaction of a phage-encoded protein (Inducer) with the Stl repressor of the SaPI allows the de-repression of the main SaPI promoters and the activation of the island.

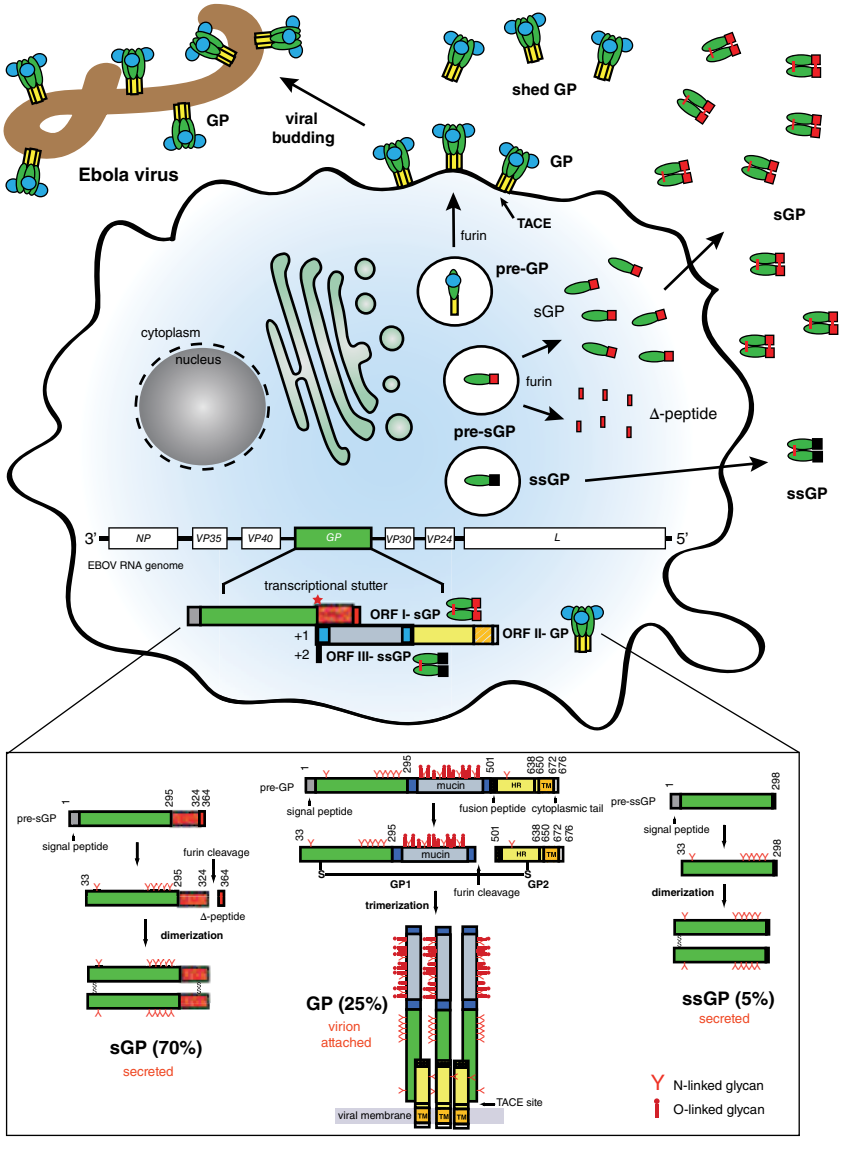

Figure 23.2 Shed, soluble, and virion attached Ebola virus glycoproteins.

Index

Moonlighting Proteins: Novel Virulence Factors in Bacterial Infections, First Edition.
Edited by Brian Henderson.

b

f

g

h

m

p

t