Current Topics in Microbiology and Immunology

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Protein and Sugar Export and Assembly in Gram-positive Bacteria

Springer

Current Topics in Microbiology and Immunology

Volume 404

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ISSN 0070-217X ISSN 2196-9965 (electronic)
Current Topics in Microbiology and Immunology
ISBN 978-3-319-56012-0 ISBN 978-3-319-56014-4 (eBook)
DOI 10.1007/978-3-319-56014-4

Library of Congress Control Number: 2017934881

Printed on acid-free paper

This Springer imprint is published by Springer Nature
The registered company is Springer International Publishing AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Declaration of Interest

Fabio Bagnoli and Rino Rappuoli are employees of GSK Vaccines and own GSK stocks and patents on vaccines against Gram positive pathogens. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Authorship

Fabio Bagnoli and Rino Rappuoli were involved in the conception and design of the book and approved its content before publication.

Preface

This book provides an overview of the current knowledge on the envelope structures of Gram-positive bacteria, their biosynthesis and assembly, their functions as well as their role as antibacterial targets and in biotechnology applications. This is a concise volume containing eleven chapters, from renowned experts in the field, reviewing recent findings and knowledge on very diverse arguments and at the same time linked to each other. That is the uniqueness behind a book like this and the added value towards a search in literature databases.

The cell envelope of these bacteria includes surface proteins, capsular polysaccharides, peptidoglycan, teichoic acids, and phospholipids. These components play key roles in cell viability, virulence and evasion of host defences. Many virulence factors of pathogenic species reside on the bacterial surface. Surface proteins have very diverse functions (e.g., adhesion, invasion, signalling, conjugation, interaction with the environment and immune-evasion). On the other hand, polysaccharides often play a mechanical protective role for the bacterium and the remarkable structural diversity in capsular polysaccharides favours immune evasion. Peptidoglycan is a well-established target for antibiotics and can undergo modification to decrease susceptibility to the drugs.

Both surface proteins and sugars, being the most exterior components, are also accessible to antibodies and represent important vaccine targets. Certain proteins assemble into complexes forming secretion apparatuses, such as the type VII secretion system, pili (or fimbrae) and flagella. These macromolecular structures have very diverse functions, which include secretion, conjugation, adhesion, biofilm formation and motility. Obviously, different species have different envelope structures and the knowledge on most important species (e.g., Actinomyces spp., Bacillus spp., Clostridium spp., Enterococcus spp., Streptococcus spp., and Staphylococcus spp.) is rapidly increasing.

Given the complexity and breath of the literature behind this argument we decided to write this book in the attempt to give an overview of the current knowledge on the envelope structures of Gram-positive bacteria, their biosynthesis, and functions. Secretion systems, spatial organization of cell wall-anchored proteins and bioinformatic algorithms for predicting subcellular localization of proteins are

explained in a simple but detailed fashion. Assembly mechanisms of structures such as pili and sugar polymers are described along with the recently discovered Type VII secretion system. The latter one has been described in low-GC Gram-positive bacteria and they can show a very complex organization with up to five chromosomal-encoded systems (ESX-1 to ESX-5) in mycobacteria to a much simpler organization in Firmicutes.

Finally, relevant examples of applied science which exploit knowledge on Gram-positive bacteria are also included. Possible targets for new antimicrobials are noted. We highlighted the development of the Twin-arginine protein translocation system (Tat) for the biotechnological secretion of fully folded and co-factor-containing proteins and its potential use as an anti-microbial drug target. The use of these bacteria in biotechnology for the production of heterologous proteins and methodologies for analyzing surface and secreted proteins with a particular emphasis to vaccine antigen discovery are also discussed.

In conclusion, this book is useful to any researcher, clinician or technician who is involved with basic or applied science projects on Gram-positive bacteria.

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Envelope Structures of Gram-Positive Bacteria

Mithila Rajagopal and Suzanne Walker

Abstract Gram-positive organisms, including the pathogens *Staphylococcus aureus, Streptococcus pneumoniae,* and *Enterococcus faecalis,* have dynamic cell envelopes that mediate interactions with the environment and serve as the first line of defense against toxic molecules. Major components of the cell envelope include peptidoglycan (PG), which is a well-established target for antibiotics, teichoic acids (TAs), capsular polysaccharides (CPS), surface proteins, and phospholipids. These components can undergo modification to promote pathogenesis, decrease susceptibility to antibiotics and host immune defenses, and enhance survival in hostile environments. This chapter will cover the structure, biosynthesis, and important functions of major cell envelope components in gram-positive bacteria. Possible targets for new antimicrobials will be noted.

Abbreviations

PG	Peptidoglycan
MRSA	Methcillin-resistant *Staphylococcus aureus*
GlcNAc	*N*-acetylglucosamine
GalNAc	*N*-acetylgalactosamine
MurNAc	*N*-acetlymuramic acid
PBP	Penicillin-binding protein
PGT	Peptidoglycan glycosyltransferase
Und-P	Undecaprenyl phosphate
TA	Teichoic acid

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Current Topics in Microbiology and Immunology (2017) 404:1–44
DOI 10.1007/82_2015_5021

Published Online: 16 February 16

WTA	Wall teichoic acid
LTA	Lipoteichoic acid
CPS	Capsular polysaccharides
PIA	Polysaccharide intercellular adhesin

Contents

1 Introduction

The cell envelope is a complex, dynamic, multilayered structure that serves to protect bacteria from their unpredictable and often hostile surroundings. The cell envelopes of most bacteria fall into one of two major groups. Gram-negative bacteria have an inner, cytoplasmic membrane surrounded by a thin layer of peptidoglycan (PG) and an outer membrane containing lipopolysaccharide. The outer membrane functions as a permeability barrier to control the influx and egress of ions, nutrients, and environmental toxins, and it also contributes to osmoprotection. Gram-positive bacteria lack a protective outer membrane but the PG layers are many times thicker than those in gram-negative organisms (Silhavy et al. 2010; Vollmer et al. 2008). Embedded in the inner membrane and attached to the PG layers are long anionic polymers called teichoic acids (TAs), which play multiple roles in cell envelope physiology as well as pathogenesis (Brown et al. 2013; Percy and Gründling 2014; Schneewind and Missiakas 2014). Membrane-embedded and wall-associated

proteins serve as environmental sensors, regulate passage of nutrients and ions across the cytoplasmic membrane, facilitate efflux of toxins and other molecules, modulate surface adhesion, and participate in enzymatic synthesis, degradation, and remodeling of the cell envelope during growth and division, and in response to environmental stress (Buist et al. 2008; Kovacs-Simon et al. 2011; Navarre and Schneewind 1999; Stock et al. 2000; Zhen et al. 2009). Other important cell envelope components in gram-positive organisms include capsular polysaccharides (CPS), which are covalently attached to PG, and extracellular polysaccharides, which form an amorphous outer layer (Arciola et al. 2015; Yother 2011).

The importance of the cell envelope for bacterial survival makes it a target for antibiotics, and several classes of clinically used antibiotics inhibit biosynthesis of PG, resulting in osmotic rupture. Other antibiotics damage the membrane barrier (Walsh 2003). Because resistance to clinically used antibiotics has become widespread, there is a push to better understand cell envelope biogenesis and regulation, and to identify new cell envelope targets that can be exploited in the development of next-generation antibiotics. In this chapter, we will focus on important cell envelope components of gram-positive pathogens using *Staphylococcus aureus* as a focal point, except where other gram-positive pathogens are better studied. Attention will also be given to the nonpathogenic *Bacillus subtilis* because its genetic tractability and other biological characteristics have led to its adoption as the principal gram-positive model organism.

2 Cell Membrane

Gram-positive organisms are surrounded by bilayer membranes that can vary substantially in composition but typically include large amounts of phosphatidylglycerol and cardiolipin. In *Bacillus* species, phosphatidylethanolamine is abundant as well (Clejan et al. 1986; Haque and Russell 2004; Minnikin and Abdolraimzadeh 1974). Many gram-positive species express at least one type of aminoacylated phosphatidylglycerol (Epand et al. 2007; Parsons and Rock 2014). For example, in *S. aureus*, lysyl-phosphatidylglycerol is found in significant amounts, particularly during logarithmic growth (Ernst et al. 2009). This phospholipid is synthesized by a polytopic membrane protein, MprF, which catalyzes the transfer of lysine from lysyl-tRNA to phosphatidylglycerol on the inner leaflet of the membrane and then translocates this species to the outer leaflet of the membrane (Ernst et al. 2009; Kristian et al. 2003). Lysyl-phosphatidylglycerol reduces susceptibility to antimicrobial peptides produced during host infection (Peschel et al. 2001) and also provides protection against aminoglycosides, bacitracin, daptomycin, and some β-lactams (Nishi et al. 2004; Komatsuzawa et al. 2001). Daptomycin-resistant *S. aureus* clinical isolates frequently contain mutations that increase MprF expression or translocase activity (Friedman et al. 2006; Julian et al. 2007; Jones et al. 2008; Yang et al. 2009b). Other species of gram-positive bacteria have MprF homologs that have been implicated in similar functions (Ernst and Peschel 2011). It is thought that the positive

charges of lysyl-phosphatidylglycerol serve to repel positively charged antibiotics or antibiotic-metal complexes (Ernst and Peschel 2011; Nishi et al. 2004).

The composition of both the head groups and the fatty acyl chains in membrane phospholipids can change rapidly in response to environmental conditions, such as low pH, osmotic stress, or temperature extremes (Zhang and Rock 2008). For example, branched chain fatty acid content in membranes can vary substantially depending on growth conditions. Membrane lipid composition affects membrane viscosity, which modulates membrane permeability and can influence both solute transport and protein interactions. Membrane lipid homeostasis is thus a crucial process and interfering with it can compromise viability (de Mendoza 2014; Zhang and Rock 2008).

In addition to the lipid components, the cell membrane contains the lipid anchor component of lipoteichoic acid (LTA) and includes numerous transmembrane and

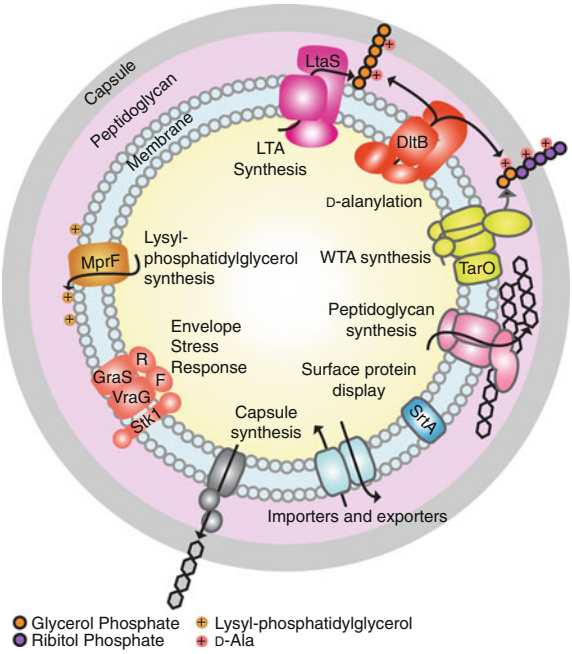

Fig. 1 The gram-positive cell envelope. The complex gram-positive cell envelope is the first line of defense for the organism. Here, the *S. aureus* envelope is shown as an example. Major pathways involved in the synthesis of the cell envelope include capsule, PG, and TA syntheses. TAs can be modified by D-alanylation. D-alanylation and lysyl-phosphatidylglycerol synthesis are known factors for antibiotic resistance. Envelope stress response regulators modulate the organism's response to toxic molecules or conditions that perturb the cell envelope. Importers and exporters, ubiquitously present among bacteria, serve the necessary role of channeling in nutrients and pumping out the toxic molecules. Finally, surface protein display systems function to tether proteins to the cell membrane or cell wall, which perform important roles in adhesion and interaction with the environment

lipoproteins with functions in cell envelope synthesis, transport of cell envelope precursors and nutrients, and export of toxic compounds (Fig. 1). Among these, transmembrane proteins are the sensory components of several two-component sensing systems that regulate the cell's response to external stimuli, including cell density and presence of damaging toxins. For instance, the amount of lysyl-phosphatidylglycerol in *S. aureus* is regulated by a complex of proteins that includes a two-component signaling system, GraRS, and a two-component ABC-transporter-like system, VraFG. This complex, which senses and responds to a variety of stimuli, including the presence of antimicrobial peptides, also regulates D-alanylation of TAs (Falord et al. 2011; Li et al. 2007a, b; Yang et al. 2012). Modulating the negative charge density of the cell envelope through lysinylation of phosphatidylglycerol and D-alanylation of TAs decreases susceptibility of *S. aureus* to antimicrobial peptides produced during host infection and increases resistance to cationic antibiotics administered to treat infection (Ernst and Peschel 2011; Brown et al. 2013; Revilla-Guarinos et al. 2014; Bayer et al. 2013).

3 Peptidoglycan

Gram-positive bacteria are surrounded by many layers of peptidoglycan (PG), which form a protective shell that is 30–100 nm thick (Silhavy et al. 2010). The PG layers are covalently modified with carbohydrate polymers including wall teichoic acids (WTAs) or functionally related anionic glycopolymers as well as CPS. The PG layers also scaffold numerous proteins, some of which are bound non-covalently through interactions with PG-binding modules such as LysM domains (Buist et al. 2008) while others are covalently attached by sortases (Schneewind and Missiakas 2012). Some wall-associated proteins play important roles in cell envelope remodeling during growth and division, whereas others scavenge nutrients and metals from the environment or serve as adhesins that promote surface binding and colonization (Navarre and Schneewind 1999). PG has numerous important functions but perhaps the most important is that it stabilizes the cell membrane, enabling it to withstand high internal osmotic pressures. This function is critical for cell survival because the turgor pressure pushing against the cell membrane can reach 20 atmospheres in some gram-positive bacteria (Mitchell and Moyle 1956; Norris and Sweeney 1993). Since PG is essential for viability and the biosynthetic pathway is highly conserved in gram-positive and gram-negative organisms, PG biosynthesis is a target for many clinically used antibiotics, including β-lactams, which are the most successful class of antibiotics in history, and vancomycin, which is still widely used to treat serious gram-positive infections, including methicillin-resistant *S. aureus* (MRSA) infections.