# Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions

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Abstract | Most Gram-positive bacteria incorporate membrane- or peptidoglycan-attached carbohydrate-based polymers into their cell envelopes. Such cell-wall glycopolymers (CWGs) often have highly variable structures and have crucial roles in protecting, connecting and controlling the major envelope constituents. Further important roles of CWGs in host-cell adhesion, inflammation and immune activation have also been described in recent years. Identifying and harnessing highly conserved or species-specific structural features of CWGs offers excellent opportunities for developing new antibiotics, vaccines and diagnostics for use in the fight against severe infectious diseases, such as sepsis, pneumonia, anthrax and tuberculosis.

Capsular polysaccharide
A glycopolymer, usually of variable composition and structure, that forms capsule-like protective layers around microbial cells.

S-layer protein
Forms a crystalline two-dimensional lattice on microbial-cell surfaces.

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The Gram-negative cell envelope comprises a thin peptidoglycan sacculus that is covered by an outer membrane. By contrast, Gram-positive bacteria have developed a profoundly different cell-envelope structure; they lack the normal outer membrane, and the cell wall is usually much thicker than that of Gram-negative species, with multiple peptidoglycan layers[1,2]. In addition, most Gram-positive bacteria possess other protective surface structures. These are highly variable and include capsular polysaccharides, S-layer proteins, mycolic acids that form an outer-membrane-like structure (in certain Actinobacteria) or combinations of these elements. One constant motif of Gram-positive cell envelopes, however, is the presence of additional glycopolymers, which form part of the fabric of the cell wall and are attached either to the peptidoglycan or to membrane lipids[3–11] (FIG. 1). These cell-wall glycopolymers (CWGs) include the teichoic acids, which are probably the best studied type of CWG[7,12]; the 'secondary cell-wall polysaccharides', which are found in many bacilli and their relatives[9]; and the branched mycobacterial arabinose-containing polymers[13].

Our knowledge of the diversity, structure and function of CWGs is still far from complete. Teichoic acids were discovered in the 1950s by James Baddiley and co-workers[14] and were assumed to have important roles in Gram-positive cell envelopes. However, only in recent years have mutants without or with altered CWGs become available, which has enabled a more detailed analysis of the physiological functions of CWGs[15–17]. Moreover, an increasing body of evidence indicates that certain CWGs have important roles in infections caused by major human pathogens, such as *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Bacillus anthracis* and *Mycobacterium tuberculosis*, and they are, therefore, important candidate targets for novel anti-infective drugs, vaccines and diagnostics (FIG. 1).

The structures of CWGs are highly diverse and often species- or even strain-specific. Most Gram-positive bacteria have two types of CWG, one peptidoglycan-attached and one lipid-attached[7,12], although some bacterial species have three or four different CWGs. *Bacillus subtilis*, for example, has one lipoteichoic acid (LTA), two distinct wall teichoic acids (WTAs) and one teichuronic acid, which is expressed only under low-phosphate conditions[18]. CWGs differ according to their type of sugar, net charge and decoration of the repeating units (FIG. 2). The backbone can contain sugars of various sizes that range from trioses to hexoses, which can be reduced (polyols) or oxidized (uronic acids) and can adopt pyranose or furanose configurations. In addition to CWGs, Gram-positive cell envelopes can contain many other glycosylated molecules that, together, form a major constituent of the cellular biomass (BOX 1).

This Review summarizes recent findings on the functions of CWGs and discusses common functional themes that have begun to emerge.

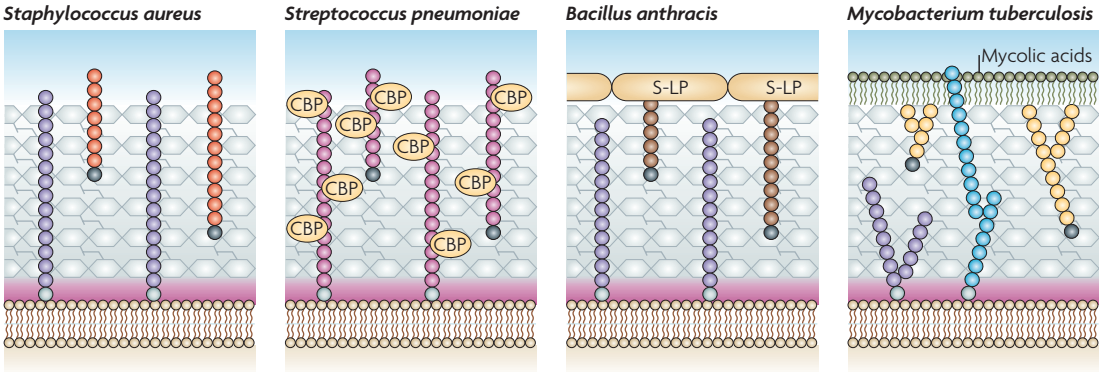



Figure 1 | **Cell-wall glycopolymers (CWGs) in the cell walls of major human pathogens.** CWG polymers are shown as chains of circles within the cell wall. Differences in the composition of CWG repeating units are indicated by different colours. Linkage units that connect CWGs with peptidoglycan or lipids are shown as dark- or light-grey circles, respectively. See FIG. 2 for compositional details. CWGs connect choline-binding proteins (CBPs) in *S. pneumoniae*, S-layer proteins (S-LPs) in *B. anthracis* and mycolic acids in *M. tuberculosis*. Bacilli and mycobacteria often contain more than the two types of CWG shown here.

Mycolic acid
Very-long-chain fatty acid that contains up to 60 carbon atoms and unusual modifications that form an outer-membrane-like layer on the surface of mycobacteria and their relatives. Mycolic acids are responsible for mycobacterial surface hydrophobicity and resistance to most conventional antibiotics.

Teichoic acid
A cell-envelope glycopolymer that is composed of many identical sugar-phosphate repeating units, which are usually modified with D-alanine and additional sugars.

Lipoteichoic acid (LTA)
A teichoic acid species that is connected to membrane glycolipids. The stereochemistry of LTAs and the biosynthetic origin of the glycerolphosphates are different from those of wall teichoic acids, which have glycerol-phosphate backbones.

Teichuronic acid
A teichoic acid-like polymer that lacks phosphate groups and possesses polyanionic properties because of the presence of uronic acid-containing repeating units.

Uronic acid
A sugar-derived acid, such as glucuronic acid or galacturonic acid.

Zwitterionic
The occurrence of both negatively and positively charged groups in a molecule.

## Different types of CWG

It is beyond the scope of this article to provide a comprehensive overview of the full structural diversity of CWGs (for more detailed reviews, see REFS 3–6,8,9,11). Peptidoglycan-anchored CWGs (P-CWGs) are connected to the cell wall by phosphodiester bonds between *N*-acetylmuramic acid and a special linkage unit that is often formed by an *N*-acetylglucosamine–*N*-acetylmannosamine disaccharide[19]. Membrane-connected CWGs (M-CWGs) are attached to glycolipids of varying size and composition[12] (FIG. 2). As the current nomenclature of CWGs is often ambiguous and confusing, in this Review, we suggest a classification system that is based on their electrostatic properties rather than their chemical composition.

***Zwitterionic CWGs.*** The presence of phosphate in CWG repeating units is the hallmark of the 'classical' teichoic acids. Most teichoic acids have zwitterionic properties because of the presence of negatively charged phosphate groups and modifications with free amino groups that are contained in residues such as D-alanine[7,20]. Peptidoglycan-attached WTAs are frequently formed by glycerol or ribitol groups that are connected by phosphodiester bonds (for example, in *S. aureus*), but tetroses, hexoses or complex sugar combinations have also been described (for example, in *S. pneumoniae*)[11,21–23].

The membrane-anchored LTAs are usually less diverse than WTAs, which is probably due to the peculiar LTA biosynthetic pathway (discussed below)[6,12]. LTA polymers are usually formed by glycerol-phosphate repeating units and are connected to glycolipids. However, more complicated structures have been described; for example, in pneumococci, the LTA repeating units of which are identical to pneumococcal WTA[23], in *Clostridium innocuum* and in *Lactococcus garvieae*[24]. Most teichoic acids are decorated with additional sugars and amino acids. WTAs and LTAs from low-G+C Gram-positive bacteria are usually modified with D-alanine[7], whereas acetyl, glutamyl and lysyl modifications have been described in WTAs from high-G+C Gram-positive bacteria[22,25].

***Anionic CWGs.*** Many Gram-positive bacteria produce polyanionic CWGs, which lack phosphate groups in their polymer backbone and do not, therefore, belong to the classical teichoic acids (FIG. 2). Teichuronic acid is produced instead of WTA by *B. subtilis*, other bacilli and micrococci under conditions of phosphate limitation. Their anionic properties result from the presence of uronic acids[4,26]. The secondary cell-wall polysaccharides of the P-CWG type from certain members of the Bacillaceae are polyanionic because of their modification with pyruvyl groups[9]. The structure of such a CWG from *B. anthracis* has recently been determined[27]. Pyruvylated CWGs seem to be integral cell-wall components in bacteria that possess S-layer proteins with an S-layer homology domain that binds CWGs in a pyruvylation-dependent manner[9,28]. *Micrococcus luteus*, *Mycobacterium bovis* and other bacteria of the high-G+C branch of Gram-positive bacteria produce lipoglycans that are esterified with anionic succinyl groups[24,29,30]. Some atypical teichoic acids, such as the minor WTA of *B. subtilis*, contain only negatively charged phosphate groups and positive charges are absent[31].

***Uncharged CWGs.*** Uncharged CWGs have been described in many actinomycetes, such as mycobacteria, corynebacteria and rhodococci[13,32,33]. Many of these polymers seem to be branched and contain mannose, arabinose and/or galactose. The peptidoglycan-anchored mycobacterial arabinogalactans are covalently connected to mycolic acids at the cell surface[8,34]. Lipoarabinomannans (LAMs) and similar lipoglycans are attached to the cytoplasmic membranes by inositol-phosphate-containing glycolipids[35,36].

Most CWGs have similar biosynthetic pathways that involve assembly of a polymer on the universal isoprenoid lipid carrier (bactoprenol) and resemble the

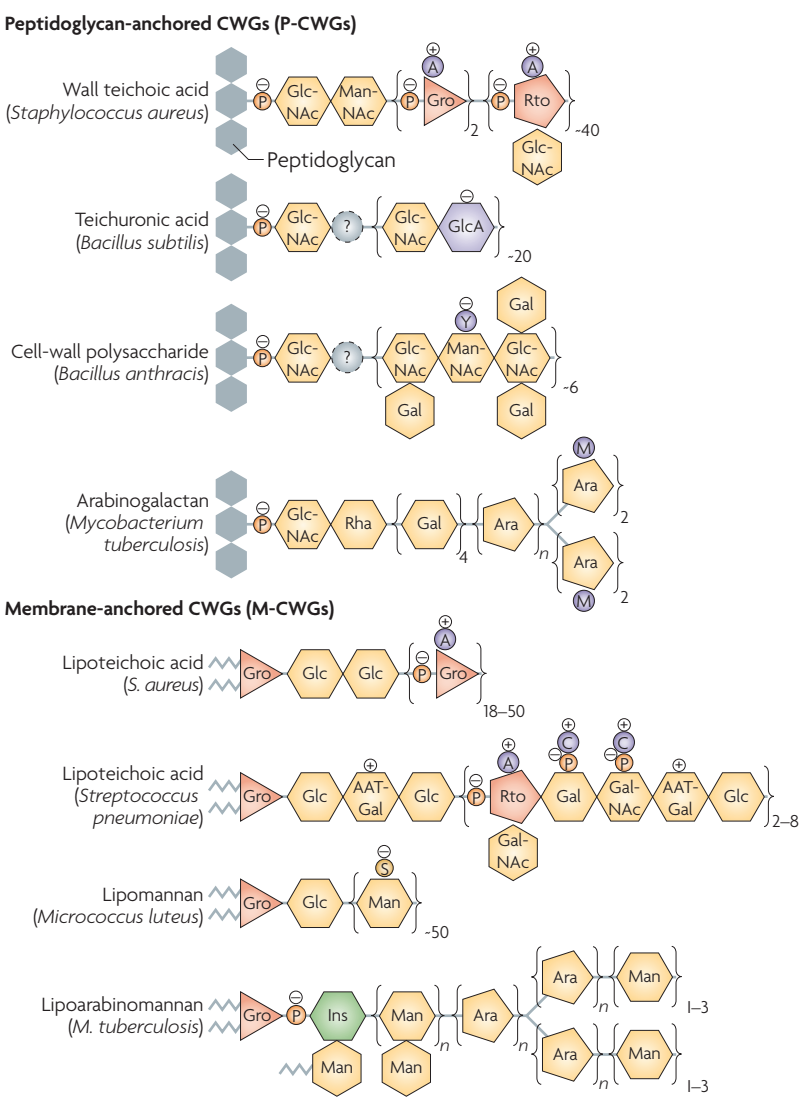



Figure 2 | **Schematic structures of selected cell-wall glycopolymers (CWGs).** Trioses, peptoses and hexoses are shown as triangles, pentagons and hexagons, respectively. Sugars, sugar-derived alcohols and sugar-derived acids are shown in yellow, orange and purple, respectively. Fatty acids are shown as zigzag lines. Many structural details, such as sugar-bonding patterns, are not included. *N*-acetylglucosamine (GlcNAc) at the first position of *B. subtilis* teichuronic acid is thought to be most likely, as the *tagO* gene, which is responsible for the incorporation of GlcNAc at this position, has been shown to be indispensable for teichuronic acid biosynthesis[153]. A GlcNAc residue at position one of *B. anthracis* P-CWG is also thought probable because of the presence of *tagO* in the *B. anthracis* genome, although no experimental evidence is available to confirm this. Unclear second positions in P-CWG linkage units are indicated by question marks. The exact position of pyruvylation in *B. anthracis* P-CWG is unknown. Mycobacterial CWGs are more extensively branched than indicated in this simple schematic. Non-glycosyl residues: A, D-alanine; C, choline; M, mycolic acid; P, phosphate; S, succinate; Y, pyruvate. Glycosyl residues: AAT-Gal, 2-acetamido-4-amino-2,4,6-trideoxy-D-galactose; Ara, arabinose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcA, glucuronic acid; Gro, glycerol; Ins, inositol; Man, mannose; ManNAc, *N*-acetylmannosamine; Rha, rhamnose; Rto, ribitol.

biosynthesis of peptidoglycan, capsular polysaccharides or, in Gram-negative bacteria, lipopolysaccharides. Precursor molecules are usually nucleotide-activated sugars, such as uridine diphosphate–glucose or cytosine diphosphate–ribitol, which are transferred to bactoprenol at the inner leaflet of the cytoplasmic membrane. Dedicated flippase proteins translocate the polymers to the outer leaflet[37,38], and the CWGs are then transferred to acceptor sites on the peptidoglycan or glycolipids[13,39]. Substituents, such as D-alanine, are eventually introduced into the mature polymers[7]. Many genes are involved in CWG biosynthesis, and these genes are often found in large gene clusters[26,40,41]. In terms of biosynthesis, the glycerol-phosphate LTA is unique; this LTA is assembled at the outer surface of the cytoplasmic membrane by a single protein, the LTA polymerase LtaS, using glycolipids as anchor structures and phosphoglyceryl units from phosphatidylglycerol as building blocks[6,42].

## Roles of CWGs in bacterial physiology

The universal presence of CWGs in Gram-positive bacteria has prompted scientists to propose important roles for these molecules in cell-wall function, maintenance and turnover (FIG. 3), and it is clear that bacterial cells commit considerable amounts of energy to their biosynthesis. However, it is only recently that defined mutants which lack certain CWGs[15,16], have reduced amounts of CWGs[17] or produce altered CWG structures[43,44] have become available, and these mutants have helped to elucidate CWG functions in greater detail.

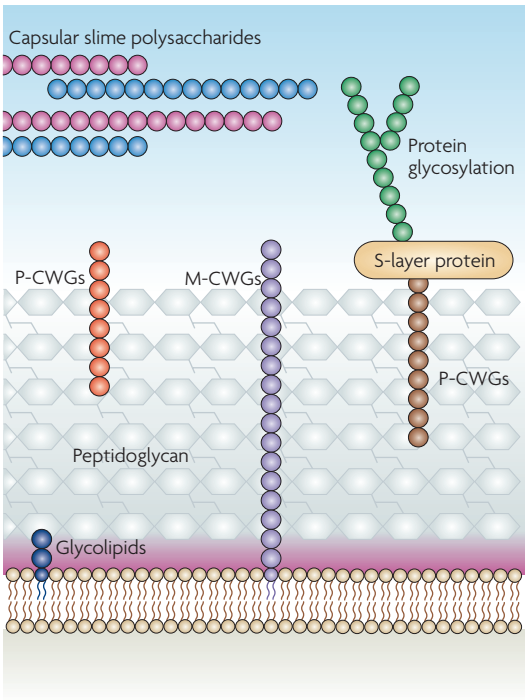
Contrary to previous proposals, WTA is dispensable for viability in *S. aureus* and *B. subtilis*, and the corresponding mutants seem to be more healthy than anticipated, at least under laboratory conditions[15,45]. By contrast, LTA is indispensable in *S. aureus*[42]. However, *S. aureus* mutants with approximately 90% reduced LTA content are viable, and only minor phenotypic changes have been observed during *in vitro* cultivation[17]. These results, and the fact that CWGs are extremely variable in structure, suggest that many CWG functions are non-essential and could involve indirect rather than highly specific interactions with other cell-wall components. There seems to be a tendency for the concomitant occurrence of LTAs with WTAs, and other M-CWG types (such as lipoglycans) often correlate with non-teichoic acid types of P-CWG. Accordingly, there could be two distinct physicochemical cell-envelope environments in Gram-positive bacteria — one that contains highly charged, zwitterionic CWGs and one that contains less charged, preferentially anionic CWGs.

A common theme that has emerged from many studies is that CWGs have an important role in protecting Gram-positive cell envelopes. These strong mechanical barriers are less efficient at preventing access of small harmful substances than Gram-negative cell envelopes because of their lack of an outer membrane. The protective role of CWGs can be indirect; for example, by attaching an S-layer protein in *B. anthracis* or mycolic acids in mycobacteria and their relatives. Alternatively, in the absence of additional protective layers, such as the capsule[46], the protective role can be direct; for example, by clogging the pores and cavities between peptidoglycan strands or modifying the physicochemical properties of the fabric of the cell wall to impede the passage of, for example, host-defence molecules,

Box 1 | **The Gram-positive 'glycobiome'**

The surfaces of bacteria are formed mainly by polysaccharides and glycosylated molecules, which account for a major portion of the bacterial biomass[1,2] (see the figure; differences in glycopolymer-repeating-unit composition are indicated by different colours). Although we understand the roles of surface-associated proteins to a certain degree, the roles of surface-associated polysaccharides are only beginning to be elucidated. Peptidoglycan is the largest component of the Gram-positive 'glycobiome' in terms of dimension and molecular weight[1]. Although the structure of peptidoglycan is largely invariable, most other surface glycopolymers are highly diverse and often have a species- or strain-specific composition. The diversity of cell-wall glycopolymers (CWGs) is exceeded by that of capsular polysaccharides; for example, pneumococci have at least 90 different types of capsular polysaccharide[72]. Glycosylation of bacterial surface proteins has been described in geobacilli, *Bacillus anthracis*, *Listeria monocytogenes* and *Streptococcus gordonii* and seems to be much more abundant than previously thought[161–164]. Glycolipids occur in the cytoplasmic membranes of most Gram-positive bacteria and in the mycolic acid layers of certain actinobacteria[165,166]. It remains difficult to study the three-dimensional structures of bacterial cell walls using the available methodology, and several controversial models on the positioning of cell-wall molecules are currently being investigated[167,168].

The roles of most of these glycosyl components are poorly understood, and our knowledge of their variability under different environmental conditions and their underlying regulation is also lacking. It can be assumed that many constituents of the glycobiome are equally important in mediating specific interactions as protein mediators. In-depth studies will help us to understand the roles of glycopolymer diversity in microbial physiology and in interactions with host cells, other bacteria and multiple environmental factors. The development of easy-to-use glycomics methodologies is a major challenge for the future[169,170]. M-CWG, membrane-connected CWG; P-CWG, peptidoglycan-anchored CWG.




bacteriocins, antibiotics, surfactants and phages. This could explain the limited impact of CWG deficiency under laboratory conditions, and is supported by previous findings which show that altering the structure or amounts of CWGs increases the susceptibility of bacteria to cationic antimicrobial peptides and antibiotics[43,47–49]. Moreover, WTA has been shown to contribute to lysozyme resistance in staphylococci, as they prevent lysozyme from binding to its peptidoglycan target[50].

The hydrophilic nature of surface-exposed CWGs has a strong impact on the physicochemical properties of bacterial cell surfaces (FIG. 3). Accordingly, *S. aureus* or *Enterococcus faecalis* mutants that contain altered CWGs show strongly reduced biofilm formation on biomaterials and attenuated virulence in animal models of artificial implant infections[17,51–53]. Charged CWGs have ion-exchanger-like properties that seem to have important roles in shaping the ionic milieu in the cell wall[54]. Accordingly, CWGs might contribute to maintenance of the proton gradient across the cytoplasmic membrane, which is a crucial component of bacterial energy metabolism[55]. Teichoic acids have a particular affinity for bivalent cations, and have been proposed to provide an ion-storage mechanism for magnesium[54].

Most of the antimicrobial molecules that bacteria encounter are positively charged, which ensures a high affinity for the negatively charged bacterial cytoplasmic membrane. This is also true for defensin-like antimicrobial host defence peptides, antimicrobial enzymes, such as human group IIA phospholipase A2 and lysozyme, and bacteriocins, such as nisin and epidermin[47,56]. The electrostatic properties of CWGs are, therefore, crucial for bacterial susceptibility to charged antimicrobial substances. It is assumed that bacteria modify teichoic acids with positively charged D-alanine to achieve partial resistance to cationic antimicrobial molecules, as D-alanine-deficient mutants are highly susceptible to cationic host defence molecules, bacteriocins and antibiotics[43,49,57,58]. The *dltABCD* operon, which is responsible for teichoic acid alanylation[59], is controlled by the three-component regulatory system ApsXRS (also named GraXRS) in *S. aureus* and *Staphylococcus epidermidis*[60,61]. The sensor kinase ApsS (also known as GraS) has recently been shown to recognize defensins and other antimicrobial peptides directly and to regulate alanylation in response to antimicrobial challenge[60,62]. In addition, several regulatory systems affect *dltABCD* expression in response to cell-envelope stress[63,64].

LTA seems to play a crucial part in cell division, which might explain why it is indispensable for viability. Accordingly, a conditionally LTA-deficient *S. aureus* mutant has revealed distorted cell shapes and division sites, which suggests that components of the cell-division machinery are positioned appropriately by their interactions with LTA[42]. Similar observations have been made in WTA-deficient *B. subtilis*[45].

Certain CWGs bind specifically to cell-wall proteins in a non-covalent manner. Choline-binding proteins, which are important virulence factors for *S. pneumoniae*, are tethered by choline residues in pneumococcal WTA and LTA[65]. The internalin B protein of *Listeria monocytogenes* binds to LTA[66]. The S-layer-protein-binding CWG of *B. anthracis* and its relatives is another example of a non-covalent CWG-binding protein[28]. In *S. aureus* and other Gram-positive bacteria, autolysins — enzymes that cleave peptidoglycan strands to

**Bacteriocin**
A bacterially produced, small, heat-stable peptide that is active against other bacteria and to which the producer has a specific immunity mechanism. Bacteriocins can have a narrow or broad target spectrum.

**Defensin**
A cationic peptide that is produced by the innate immune system, and kills bacteria, for example, by disrupting the phospholipid bilayer.

separate daughter cells upon cell division — also have high affinities for teichoic acids and have been proposed to be positioned in cell-wall septa by CWGs[67,68]. However, *S. aureus* mutants that lacked WTA or had a 90% reduction in LTA did not show major differences in their amounts of autolysin[17]. As the activity of the major autolysins depends on bivalent cations, it is possible that providing cations could be a more important role of teichoic acids in controlling autolysins than a direct interaction. CWGs are also important ligands for non-bacterial receptors. Many phages use CWGs as binding sites, and differences in CWG structure are important criteria in defining phage specificity[69–71]. A growing number of receptors from mammalian hosts bind specifically to CWGs, which permits important interactions during Gram-positive colonization and infection (discussed below).

CWGs share many features with capsular polysaccharides, including sugar building blocks, bactoprenol-dependent biosynthesis and structural variability[46,72]. In some cases, it can become difficult to discriminate between CWGs and capsular polysaccharides; for example, when some bacterial strains shed CWGs to incorporate them into polysaccharide capsules, slimes and biofilm matrices. Such observations have been made with WTAs from staphylococci[73,74] and enterococci[75], LTAs from streptococci[76] and arabinomannans from *Mycobacterium avium*[77]. The mechanisms of CWG shedding remain unknown.

## Roles of CWGs in attachment and colonization

Several recent studies indicate important roles for CWGs in the ability of Gram-positive bacteria to adhere to, and infect, host cells (FIG. 4; TABLE 1). These roles have been studied thoroughly for *S. aureus* WTA, which has been implicated in the binding of *S. aureus* to epithelial and endothelial cells. *S. aureus* mutants that lack WTA are profoundly inhibited in their ability to bind to nasal epithelial cells, and were unable to colonize the nares of cotton rats in an experimental nasal colonization model[15,78]. WTA seems to play a similar part in *S. aureus* adhesion to endothelial cells[79]. The impact of WTA deficiency is markedly increased under flow conditions in which the bacterial cells move over the host cell surface with sufficient velocity. *In vitro* and *in vivo* evidence indicate that WTA contributes to a similar extent to *S. aureus* adhesion to epithelial and endothelial cells as staphylococcal proteinaceous adhesins that bind, for example, to fibronectin or fibrinogen, and a lack of WTA does not seem to influence adhesin activities[78]. Altering the teichoic acid structure often has a similar impact on bacterial adherence as deleting WTA. Inactivation of the *dlt* operon leads to the absence of D-alanine from teichoic acids, which results in a significantly reduced ability of *S. aureus* to bind to epithelial and endothelial cells and cause infections[15,80]. Similar observations have been made upon deletion of the *dlt* operon in *L. monocytogenes*[81] and *Streptococcus pyogenes*[44]. LTA also plays a part in host cell binding; altering the structure of LTA influences the interaction of *Streptococcus agalactiae* with the blood–brain-barrier endothelium and abrogates the pathogenicity of *S. agalactiae* in experimental neonatal meningitis[82].

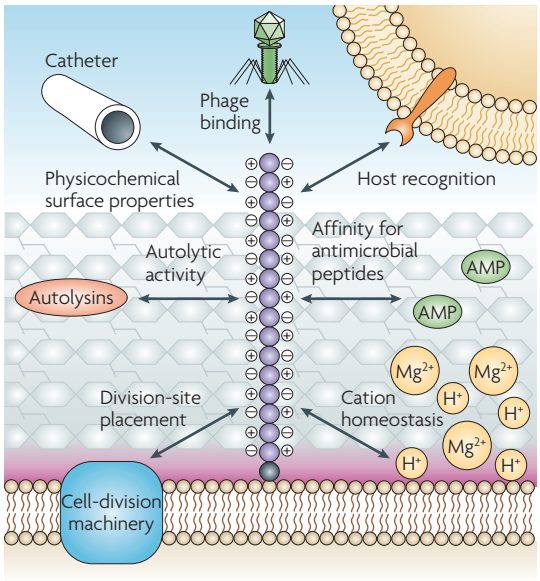



Figure 3 | **Roles of wall teichoic acid (WTA) and lipoteichoic acid (LTA) in the cell wall of *Staphylococcus aureus*.** The roles of WTA and LTA in directing the cell-division machinery; controlling autolysin activity; shaping physicochemical surface properties and biofilm formation (for example, on catheter surfaces); serving as phage receptors; mediating interactions with host receptors; controlling susceptibility and/or resistance to antimicrobial peptides (AMPs); and maintaining cation homeostasis are shown. *S. aureus* cell-wall glycopolymers (CWGs) are zwitterionic because of the presence of negatively and positively charged phosphate and amino groups in CWG repeating units, respectively.

The identification of receptors that mediate CWG-dependent bacterial adhesion to epithelial and endothelial cells is only in its infancy. Several lines of evidence point towards the specific, receptor-mediated binding of CWGs to host cells. Latex beads that are coated with WTA from wild-type *S. aureus* adhere specifically and in a dose-dependent manner to human epithelial and endothelial cells, whereas beads that are coated with WTA that lacks D-alanine do not adhere[15]. Pre-incubation of epithelial cells with purified WTA inhibits *S. aureus* adherence to these cells in a specific and dose-dependent manner[15]. Several host receptors, mainly from the C-type lectin or scavenger receptor (SR) families, bind to purified CWGs or CWG-displaying bacteria[24,83–85] (FIG. 4). In line with these findings, specific SR inhibitors, such as polyinosinic acid, block the binding of wild-type *S. aureus* to primary nasal epithelial cells, but not the residual binding of a WTA-deficient mutant[78].

Brown and Goldstein[86] defined SRs as membrane proteins that facilitate the binding and internalization of oxidized low-density lipoproteins (LDLs) or acetylated LDLs, but not native LDLs. Eight different SR classes (A–H) have been defined according to their multi-domain structures[85–87]. The prototypic class A SR (SRA) can bind *S. aureus* or purified LTA[88] . SRA is a trimeric type-I transmembrane glycoprotein that has distinct cytoplasmic, transmembrane, spacer, α-helical coiled-coil, collagenous

and C-terminal cysteine-rich domains. The specificity of binding depends on the net charge of the LTA, which is governed by the amount of D-alanylation[24]. In parallel, SRA$^{-/-}$ mice are more susceptible to *S. aureus* infections than wild-type mice, possibly owing to impaired non-opsonic phagocytosis of SR1$^{-/-}$ macrophages[89].

At least six other SRs, some of which are expressed on epithelial or endothelial cells, have been shown to bind bacteria such as *S. aureus*. Although the bacterial-binding partners are unknown, CWGs are among the most likely ligand candidates. MARCO (macrophage receptor with a collagenous structure) is another class A SR that seems to have a role in the *in vivo* clearance of *S. aureus*[90,91]. Murine SR5 (SCARA5) has a domain organization that is related to that of MARCO, binds *S. aureus* and is expressed, for example, on airway epithelial cells[92]. By contrast, SRCL I/II contains a C-type lectin domain instead of the carboxy-terminal cysteine-rich domain that is found in SRA[93]. LOX1 (lectin-like oxidized low-density lipoprotein receptor 1) is a member of the SRE family that is expressed on endothelial cells and binds *S. aureus*. This receptor has a carboxy-terminal lectin domain[94]. Adhesion of Gram-positive bacteria to the SRG-type SRPSOX can be blocked by several polyanionic inhibitors[95]. The SRH members FEEL1 and FEEL2 have also both been shown to bind Gram-positive bacteria[96]. In addition to mediating adherence, SRs are also involved in innate immune activation, as discussed in the next section.

Another well-investigated CWG–host-cell-receptor interaction is the binding of pneumococcal LTA to the G-protein-coupled platelet-activating factor (PAF) receptor[11]. The LTA of *S. pneumoniae* has phosphorylcholine modifications (FIG. 2) that are similar to that of the natural ligand PAF[97]. Accordingly, activation of PAF-receptor expression during inflammation leads to increased invasion of *S. pneumoniae* into epithelial and endothelial cells.

## Roles of CWGs in the innate immune system

Recognition of conserved microbial structures called pathogen-associated molecular patterns (PAMPs) is achieved by germ-line-encoded pattern-recognition receptors (PRRs), such as the SRs discussed above. The signalling potential of SRs is still unclear, whereas other PRRs, such as the Toll-like receptors (TLRs), have been shown to sense PAMPs and activate cells through complex signal-transduction events[98]. TLRs are type I transmembrane proteins that have an extracellular domain which contains leucine-rich repeats and a cytoplasmic region that has homology to the interleukin 1 receptor family[99].

Interestingly, most M-CWGs seem to be PAMPs that activate the human TLR2 receptor[11] (FIG. 4 ). The LTAs of *S. aureus, S. pneumoniae* and many other bacteria are thought to have immunostimulatory activity[100]. However, pneumococcal LTA seems to be generally less active compared with *S. aureus* LTA[101]. Conflicting data suggest important roles for either TLR1 or TLR6 heterodimerization in TLR2-dependent activation[101,102]. The co-receptors CD14, lipopolysaccharide-binding protein (LBP) and CD36 seem to contribute to TLR2 stimulation by LTA[103,104]. Experiments that used

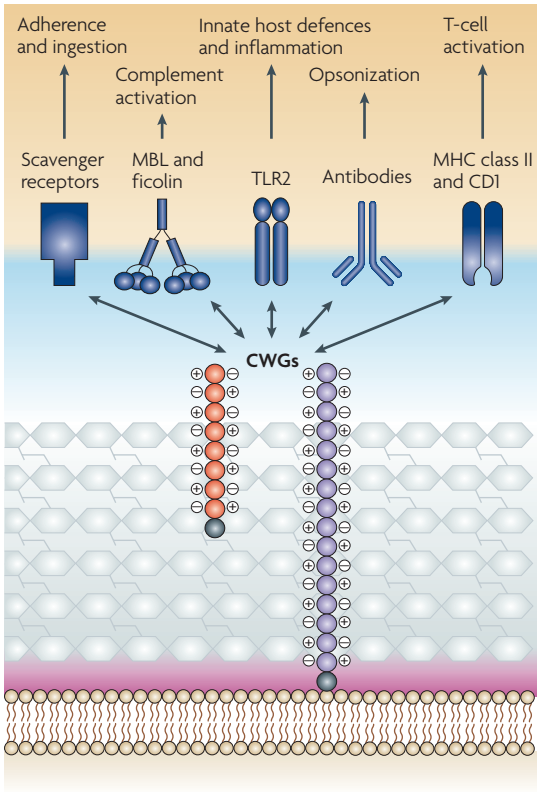



Figure 4 | **Interaction of cell-wall glycopolymers (CWGs) with host molecules.** The main consequences of CWG interactions with the indicated recognition molecules are shown. Scavenger receptors or mannose-binding lectin (MBL) and ficolins interact with various CWGs, which leads to binding and internalization by host cells or complement activation, respectively. Several M-CWGs seem to elicit proinflammatory responses through Toll-like receptor 2 (TLR2), although the relevance of these findings has recently been questioned. The adaptive immune response to Gram-positive bacteria involves the binding of antibodies to surface-exposed CWGs, and there is growing evidence that zwitterionic CWGs can be recognized by specific T cells upon presentation by major histocompatibility complex (MHC) class II molecules.

synthetic LTA derivatives have revealed that the membrane anchor and a few repeating units are the active components in the LTA-mediated TLR2 interaction[105]. The polyglycerol-phosphate backbone amplifies the response to the lipid anchor efficiently only if modified with D-alanine[106]. The nature of the fatty acids in the glycolipid does not have a major influence on LTA activity. Recent studies have questioned a dominant proinflammatory role for LTA from *S. aureus*, as a considerable amount of the activity in LTA preparations has been assigned to lipoprotein contamination, even in highly purified samples[107,108]. Accordingly, the inflammatory potential of LTA, compared with that of other PAMPs, remains to be clarified.

Mycobacterial LAM is another type of M-CWG that activates host cells via TLR2, probably through TLR2–TLR6 (REF. 109) or TLR1–TLR2 (REF. 110) heterodimers,

Pathogen-associated molecular pattern
A small molecular motif that is conserved across microbial species and engages innate immune receptors (for example, Toll-like receptors).

Pattern-recognition receptor
A host receptor (such as a Toll-like receptor) that can sense pathogen-associated molecular patterns and initiate signalling cascades that lead to an innate immune response.

Type I transmembrane protein
A protein which contains a single membrane-spanning domain that has its carboxyl terminus orientated towards the cytoplasm and its amino terminus orientated towards the lumen of membrane compartments or in an extracellular direction.

Table 1 | **Host receptors that bind cell-wall glycopolymers (CWGs)**

| CWG-binding receptor | Receptor family | Main cell or tissue of expression | Identified CWG ligands |
|---|---|---|---|
| Scavenger receptor A | Scavenger receptor | Macrophages | LTA (several species)[24,88] |
| CD36 | Scavenger receptor | Macrophages | LTA (*Staphylococcus aureus*)[104] |
| DC-SIGN | C-type lectin receptor | Dendritic cells and macrophages | LAM (mycobacteria)[116] |
| Mannose receptor | C-type lectin receptor | Macrophages and endothelial cells | LAM (mycobacteria)[115] |
| PAF receptor | G-protein-coupled receptor | Epithelial and endothelial cells, several leukocytes and platelets | LTA (*Streptococcus pneumoniae*)[97] |
| TLR2 | TLR | Several leukocytes, and epithelial and endothelial cells | LTA (several species)[103] and LAM (mycobacteria)[110] |
| CD14 | Glycosylphosphatidyl-inositol-anchored surface protein | Macrophages and monocytes | LTA (several species)[101,103] and LAM (mycobacteria)[111] |
| LBP | Soluble protein | Liver | LTA (several species)[103] and LAM (mycobacteria)[112] |
| Surfactant protein A | Soluble C-type lectin; collectin | Lung | LAM (mycobacteria)[125] |
| Surfactant protein D | Soluble C-type lectin; collectin | Lung | LAM (mycobacteria)[126] and LTA (*S. aureus*)[124] |
| MBL | Soluble C-type lectin; collectin | Liver | LTA (several species)[122], lipomannan (*Micrococcus luteus*)[122] and LAM (mycobacteria and rhodococci)[33,121] |
| L-ficolin | Soluble C-type lectin; collectin | Liver | LTA (several species)[123] |

LAM, lipoarabinomannan; LBP, lipopolysaccharide-binding protein; LTA, lipoteichoic acid; MBL, mannose-binding lectin; PAF, platelet-activating factor; TLR, Toll-like receptor.

with the assistance of CD14 (REF. 111) and LBP[11,112]. In different mycobacterial species, LAM can be either uncapped at its terminal arabinan domain (AraLAM) or capped with mannosyl (ManLAM) or phosphoinosite (PiLAM) residues[113]. AraLAM seems to signal through TLR2 (REF. 114). PiLAM is also able to activate different cell types through TLR2 (REF. 111), whereas ManLAM, which is found, for example, in *M. tuberculosis,* exhibits anti-inflammatory activity[115,116]. ManLAM modulates TLR-dependent signalling in dendritic cells (DCs) by an additional interaction with the C-type lectin receptor DC-SIGN (discussed below)[116,117]. The TLR2-dependent immune-stimulatory ligand in ManLAM-producing mycobacterial species seems to be the LAM precursor lipomannan. Therefore, the ratio of lipomannan to ManLAM determines the outcome of the innate immune response[113].

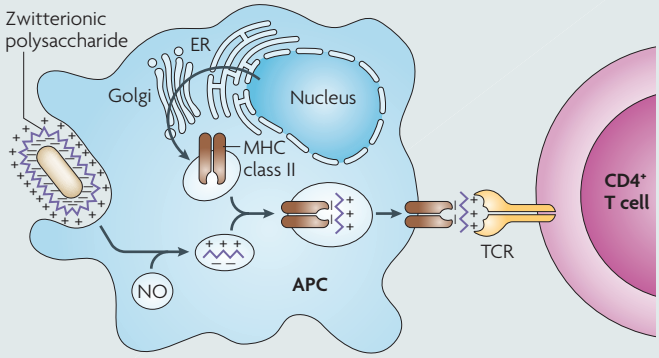
The soluble C-type lectins are another family of important innate immune PRR that recognize CWGs[118]. Classical C-type lectins, such as mannose-binding lectin (MBL) and L-ficolin, contain a hydrophobic fold that is termed the carbohydrate-recognition domain. All these proteins can activate the lectin pathway of complement activation upon binding to CWGs on the surface of a pathogen together with a cognate serine protease[119,120]. Activation of complement has several consequences, such as pathogen opsonization, generation of proinflammatory molecules and direct bacterial killing. One of the strongest bacterial ligands for MBL is *M. luteus* lipomannan, followed by enterococcal LTAs that contain monoglucosyl, diglucosyl and oligoglucosyl substituents[121]. MBL also binds mycobacterial LAMs, which contributes to the uptake of mycobacteria into human macrophages[122]. LTAs from *S. pyogenes* and *S. aureus* that lack terminal sugars, and LTAs from *Listeria* and *Lactococcus* spp. that contain galactosyl substituents, bind only poorly to MBL[121]. The major complement-activating receptor for *S. aureus* LTA is L-ficolin[123]. Other soluble lectins, such as lung surfactant proteins, also bind CWGs[124]. Surfactant proteins A (SPA) and D (SPD) have important roles in the innate immunity of the lung and bind to LAMs[125,126]; SPD can also bind to LTAs[124].

Cell-surface lectin receptors on DCs and macrophages play important parts in the innate immune response. The interaction between DC-SIGN and *M. tuberculosis* ManLAM leads to the suppression of DC function by the modulation of TLR expression[116,117]. In addition, DC-SIGN binds *S. pneumoniae*; however, the bacterial ligand is unknown[127]. The DC mannose receptor is another example of a C-type lectin that is involved in the modulation of DC function by ManLAM[115].

## Roles of CWGs in adaptive immunity

CWGs are recognized by the adaptive immune system and can be targeted effectively by antibodies[128–131] (FIG. 4). Antibodies that are directed against *S. aureus* peptidoglycan, teichoic acids and the capsular polysaccharides CP5 and CP8 have been detected in human sera. Antibody levels are elevated upon infection, depending on the infection severity or whether vaccination has occurred with defined CWGs (discussed below)[132,133]. CWGs might also be involved in the mechanisms that allow Gram-positive bacterial pathogens to evade host responses; for example, by antigen variation or as capsular components that prevent opsonization.

Box 2 | **Zwitterionic glycopolymers and MHC class II presentation**




Glycopolymers have traditionally been regarded as T-cell-independent antigens. However, recent studies demonstrated that certain zwitterionic polysaccharides are processed and presented by the major histocompatibility complex (MHC) class II pathway, which leads to activation of specific T cells[139,141,171] (see the figure). The prototypic zwitterionic polysaccharide capsular polysaccharide A (PSA) of the Gram-negative *Bacteroides fragilis* is taken up into endosomes and subsequently degraded by oxidative depolymerization, which requires nitric oxide generation by nitric oxide synthase 2 (NOS2)[171]. PSA fragments are then loaded onto the MHC class II molecule human leukocyte antigen (HLA)-DR after the MHC class II-associated invariant-chain peptide (CLIP) has been removed with the help of HLA-DM. This allows zwitterionic polysaccharide fragments to be presented on MHC class II molecules to $CD4^+$ T cells that carry αβ-T-cell receptors[141,171]. In addition, PSA upregulates the expression of MHC class II molecules and co-stimulatory molecules that are required for T-cell activation. Treatment of $CD4^+$ T cells with PSA leads to the expression of interleukin (IL)-10 in a subset of the $CD4^+$ T cells, which are termed $CD45RB^{low}$ (REF. 172). This might explain why the *B. fragilis* capsular polysaccharide complex (which contains PSA and PSB) can, on the one hand, induce abscesses but, on the other hand, protect from abscess formation following the subsequent injection of capsular polysaccharides, or whole *B. fragilis* cells if administered systemically[173]. IL-10 is an anti-inflammatory cytokine, and elevated levels of IL-10 might explain the anti-inflammatory potential of the *B. fragilis* zwitterionic polysaccharides. *Staphylococcus aureus* wall teichoic acids and capsular polysaccharide CP8 also have zwitterionic properties and behave similarly to PSA in inducing abscesses. The involvement of $CD4^+$ T cells has been confirmed using CP8 (REF. 142). Although the molecular mechanisms are still unclear, these preliminary findings suggest that zwitterionic cell-wall glycopolymers play important parts in activating adaptive immunity through the pathway described above. APC, antigen-presenting cell; ER, endoplasmic reticulum, TCR; T-cell receptor.

Most carbohydrates are T-cell-independent antigens[134,135] that are capable of eliciting the production of specific immunoglobulin (Ig) M by B cells but fail to induce Ig class switching to IgG isotypes and thus the creation of immunological memory. Protein-conjugated vaccines have therefore been developed to improve the efficacy of polysaccharide antigens[136,137]. Such glycoconjugates seem to elicit T-cell help by presenting the protein component to $CD4^+$ T cells[138]. This results in production of the carbohydrate-component-specific antibodies and creates immunological memory.

Recently, a structurally distinct group of zwitterionic carbohydrates has been shown to elicit immune responses by activating T cells directly without the help of conjugated proteins[139–141]. Zwitterionic polysaccharides, which include many CWGs, contain both a positive and a negative charge in each repeating subunit. The capsular polysaccharide from *Bacteroides fragilis* is the best-studied example of this group (BOX 2), but detailed studies of zwitterionic polymers from other bacterial species are still largely elusive. The zwitterionic *S. aureus* capsular polysaccharide CP8 induces the formation of $CD4^+$ T-cell-dependent intra-abdominal abscesses in animal models[142], but the underlying molecular basis of this CP8-dependent abscess formation is still under investigation. Interestingly, *S. aureus* WTA, which also contains zwitterionic repeating units, functions similarly to CP8 in the intra-abdominal abscess model. It is tempting to assume that similar mechanisms are responsible for the ability of WTA to induce abscesses. In another study that used *S. aureus* for abscess formation, hindpaw infection and surgical-wound infection models, significantly fewer bacteria were recovered from the tissues of mice that were deficient in αβ-T-cell receptors, and the inflammatory responses of these mice were considerably diminished compared with those of wild-type animals[143]. These interesting findings suggest that zwitterionic CWGs are important in T-cell-dependent immune stimulation, although more detailed investigations are required.

Certain M-CWGs, such as uncharged mycobacterial LAMs, can activate CD1b-restricted αβ-T cells through presentation on CD1b[144]. The CD1 family of molecules is able to present glycolipid antigens to CD1-restricted T cells[145]. These examples indicate that activation of adaptive immune responses by CWGs is of general importance and can include major histocompatibility complex (MHC) class II- and CD1-dependent antigen presentation.

## CWGs — diagnostics, vaccines and antibiotics

The easy accessibility of CWGs in the cell wall makes these polymers interesting candidate targets for novel anti-infective strategies. Most of the emerging highly antibiotic-resistant bacterial strains, such as meticillin-resistant *S. aureus*, vancomycin-resistant enterococci, penicillin-resistant *S. pneumoniae* and multi-resistant *M. tuberculosis* and *Clostridium difficile*, produce CWGs, which supports the notion that these surface components should be considered when seeking novel target structures. Targeting CWGs is particularly tempting for developing new diagnostics and vaccines. However, only certain CWG biosynthetic enzymes are conserved among most Gram-positive species and these

enzymes usually have intracellular locations, which must be taken into account when considering CWG biosynthesis as a potential drug target.

The variable, often species- or strain-specific structures of CWGs offer promising opportunities for diagnostics. In fact, some broadly used techniques for the detection of Gram-positive pathogens already rely on CWG recognition. For example, the differentiation of streptococcal species by specific antibodies, which was developed by Rebecca Lancefield[146–148] in the 1920s, is based on species-specific differences in CWGs. Different *S. aureus* lineages have been detected for many decades by phage typing, which, in part, takes advantage of strain-specific WTA structures[149]. The P-CWG from *B. anthracis* has recently been shown to have a species-specific structure[27,150], which makes it an interesting target candidate for new rapid-detection methods, an issue of particular urgency in times of bioterrorism.

Polysaccharides, such as *S. pneumoniae* capsular polysaccharide, have been used successfully for vaccination[136]. CWGs, which are usually less variable than capsular polysaccharides, could represent even more promising vaccination targets. LTA-specific monoclonal antibodies have yielded promising results as a passive vaccine for severe *S. aureus* infections[151]. In fact, the LTA structure is almost identical in many bacterial pathogens, which makes LTAs suitable vaccination targets. CWGs might also be suitable targets for active immunization if they can induce immunological memory directly or become protein-conjugated. Promising results have been obtained using enterococcal LTA in animal studies[131,132].

CWGs are usually essential for the viability and/or virulence of Gram-positive pathogens. Blocking the biosynthesis of these polymers with specific inhibitors is, therefore, another promising antimicrobial strategy. Accordingly, ethambutol, an inhibitor of mycobacterial arabinan biosynthesis, is already one of the most important anti-tuberculosis drugs[152]. Most P-CWGs have a conserved linkage unit in which *N*-acetylglucosamine is the first sugar to be linked to bactoprenol. The TagO enzyme that is responsible for this initial biosynthetic step has been analysed in *B. subtilis* and *S. aureus*[15,153]. *tagO*-related genes occur in the genomes of virtually all P-CWG-producing bacteria and many Gram-negative species (data not shown) that might use this type of enzyme for lipopolysaccharide biosynthesis. Therefore, TagO should be a promising target for novel antimicrobial compounds. Recently, some steps of WTA biosynthesis have been reconstituted *in vitro*, which could be used as the basis for high-throughput screening assays[154–156]. Another group of target candidates, the products of the *dltABCD* operon, are present in almost all Gram-positive pathogens that have D-alanylated WTAs and LTAs[7]. The D-alanyl residues are highly important in providing resistance to antimicrobial host defence factors, such as defensins[43], in adherence to and colonization of host tissues[79] and in biofilm formation on artificial medical devices[51]. Accordingly, *dlt* operon inactivation has led to profound virulence attenuation in many different bacterial species[44,57,81]. Notably, specific inhibitors of the DltA protein-mediated transfer of D-alanine to the carrier protein DltC have recently been described. Treatment of bacteria with such a compound leads to profoundly increased susceptibility to cationic antibiotics[157] and clearance of infection in animal models[158], which underscores the future potential of drugs that would not kill bacteria, but would severely compromise bacterial fitness and virulence. Finally, the use of phages for antimicrobial therapies has recently attracted increasing interest[159]. Such concepts further support the potential use of CWGs as target structures for antimicrobial therapies, as many Gram-positive phages are known to use CWGs for attachment and host recognition.

## Conclusions and perspectives

Previously, the different types of CWGs were not generally regarded as members of a common family. However, the obvious similarities in chemical nature, location, attachment to acceptor sites and biosynthetic pathways suggest that the CWGs discussed in this Review are variations on a common theme. Despite the enormous structural diversity, unifying concepts are emerging from recent bacteriological, biochemical and immunological studies. Nevertheless, different CWGs probably have only partial functional overlaps, which could be one reason for the presence of two or more different CWGs in one particular bacterial strain. Defining the most crucial roles and associating these roles with specific CWG composition and structure are major challenges for future studies. As defined CWG mutants are now more readily available, comprehensive functional studies can be anticipated in the near future.

In addition to the functions of CWGs in maintaining and protecting bacterial-cell envelopes, the role of these polymers in bacteria–host interactions deserves much more attention. The proinflammatory, endotoxin-like activities of M-CWGs need to be further clarified by, for example, using synthetic polymers or defined bacterial mutants that lack M-CWGs. Moreover, data on MHC class II presentation of CWGs are still indirect and require much more detailed investigation.

As elucidating CWG composition and structure is complicated and laborious, our knowledge of CWG diversity and variability is still limited and comes mainly from bacteria that were grown in culture broth in the laboratory. How CWG composition and amounts vary between different environmental conditions, for example, during colonization and infection, is almost completely unknown. Recent DNA microarray studies suggest that CWG biosynthesis is a regulated process that undergoes changes during infection[160]. Moreover, the example of D-alanine transfer into staphylococcal WTA and LTA, which is regulated in response to exposure to antimicrobial peptides, indicates that CWG structures could be much less static than previously thought[60].

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