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MINIREVIEW

Lipoteichoic Acids, Phosphate-Containing Polymers in the Envelope of Gram-Positive Bacteria

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Lipoteichoic acids (LTA) are polymers of alternating units of a polyhydroxy alkane, including glycerol and ribitol, and phosphoric acid, joined to form phosphodiester units that are found in the envelope of Gram-positive bacteria. Here we review four different types of LTA that can be distinguished on the basis of their chemical structure and describe recent advances in the biosynthesis pathway for type I LTA, D-alanylated polyglycerol-phosphate linked to di-glucosyl-diacylglycerol. The physiological functions of type I LTA are discussed in the context of inhibitors that block their synthesis and of mutants with discrete synthesis defects. Research on LTA structure and function represents a large frontier that has been investigated in only few Gram-positive bacteria.

Aiming to reconstitute coenzyme A biosynthesis from extracts of *Lactobacillus arabinocus*, James Baddiley detected large amounts of soluble nucleotides that were identified as CDP-glycerol and CDP-ribitol (1, 2). Earlier work had revealed that nucleotide-linked sugars, for example, UDP-glucose and UDP-galactose, contribute to metabolism and are polymerized into polysaccharide (3–5). Baddiley proposed that CDP-glycerol and CDP-ribitol contribute to the synthesis of polyglycerol-phosphate [poly(Gro-P)] and polyribitol-phosphate. Large amounts of these polymers could indeed be isolated from the cell walls of Gram-positive bacteria but not from Gram-negative microbes (6). These phosphate-containing polymers harbored glycosyl and D-alanine ester substituents and were eventually designated teichoic acids (7).

Due to their supramolecular structure, the murein sacculi (cell wall peptidoglycan) in extracts of mechanically broken Gram-positive bacteria sediment and teichoic acids associated with such preparations were named wall teichoic acid (WTA) (8). Poly-(Gro-P) in the supernatant, when centrifuged, associates with membranes, which explains the designations intracellular teichoic acid (9), membrane teichoic acid (10, 11), and, once the linkage of poly(Gro-P) to glycolipid was revealed, lipoteichoic acid (LTA) (12, 13). The characterization of the structure of teichoic acids and their nucleotide precursors represents a significant achievement at a time when sophisticated technologies such as mass spectrometry or nuclear magnetic resonance (NMR) were not available.

Although initially not appreciated as WTA and LTA, these molecules were identified independently in extracts of *Streptococcus pneumoniae* as immune-stimulatory compounds. Immunochemical studies had distinguished the somatic antigen fraction C (pneumococcal C-polysaccharide) (14) and a species-specific lipocarbohydrate, also designated heterophile antigen or pneumococcal F-antigen because of its Forssman antigenicity and fatty acid content (15). In contrast to highly variable capsular polysaccharides, the C-polysaccharide and lipocarbohydrate are conserved in strains of *S. pneumoniae* (15–17). Chemical studies eventually characterized C-polysaccharide as the WTA (15, 18–20) and lipocarbohydrate as the LTA (21) of pneumococci. Unlike other bacterial species, *S. pneumoniae* incorporates polymeric repeats with identical chemical compositions in both WTA and LTA (17, 22). While a biosynthesis pathway for pneumococcal WTA has been proposed, the synthesis of pneumococcal LTA has not yet been elucidated (see reference 23 for a review).

STRUCTURAL BASIS OF LTA TYPES

Werner Fischer used the generic term "amphiphile" to define polymeric chains associated with bacterial membranes and described five structural types (I to V) (24). The basic structure of LTA consists of a soluble polymer that is tethered to a membrane anchor and faces the outer leaflet of the plasma membrane. The polymer is formed of alternating units of a polyhydroxy alkane, including glycerol and ribitol, joined via phosphodiester linkages. The repeating units are further modified, providing chemical diversity between various bacterial species. It should be noted that methods for the extraction of intact LTA and for the elucidation of its structure have evolved since the early discovery of LTA (12, 13, 25, 26). These advances revised the structural models for some LTA molecules (27–30).

Type I LTA, the most frequently encountered polymer, displays the simple structure poly(Gro-P). Chemical diversity in type I LTA is based on (i) the chemical nature of substituents decorating Gro-P subunits, (ii) the length of the polymer, and (iii) the nature of the glycolipid anchor in the membrane. Type I LTA of *Firmicutes* (including *Staphylococcus aureus*, *Bacillus subtilis*, *Enterococcus faecalis*, *Listeria monocytogenes*, *Streptococcus agalactiae*, and *Streptococcus pyogenes*) is composed of 1,3-polyglycerolphosphate [poly(Gro-P)]. In *S. aureus* and *B. subitlis*, it is linked to the C-6 of the nonreducing glycosyl within the glycolipid anchor gentiobiosyldiacylglycerol Glc(β1–6)Glc(β 1–3)-diacylglycerol [Glc_2-DAG] (Fig. 1A) (27, 31, 32). The poly(Gro-P) chain encompasses between 15 and 50 Gro-P units (26). When staphylococci are grown in laboratory media, roughly 70% and 15% of the Gro-P moieties are substituted at position 2 with D-alanine esters and *N*-acetylglucosamine (GlcNAc), respectively (Fig. 1A) (27, 33, 34). *Streptococcus* sp. strain DSM 8747, although closely related to

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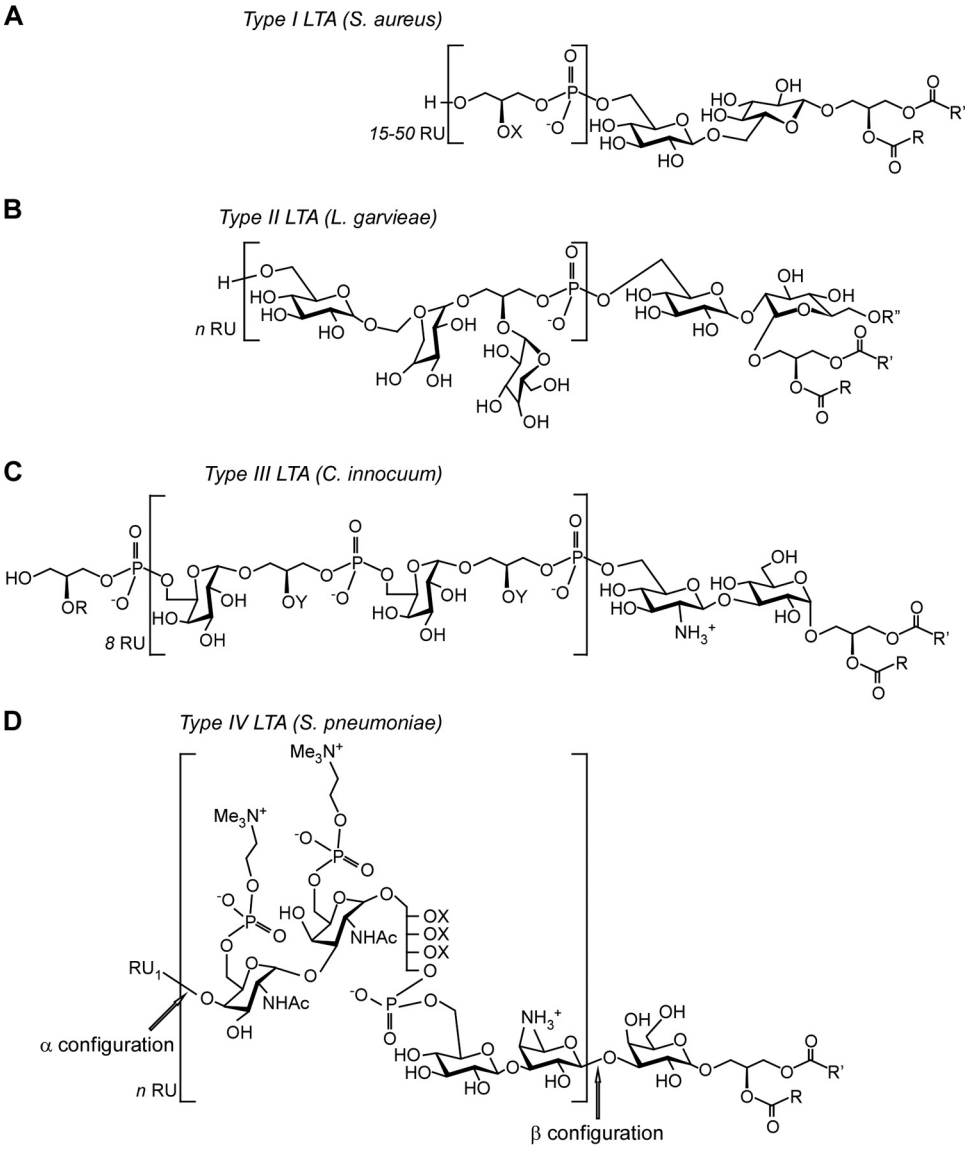

FIG 1 Structure of LTA molecules from various bacteria. The repeating unit, RU, of each LTA type is indicated within the brackets; it is always linked to a glycolipid. Where known, *n* (the number of RUs) is indicated. (A) Structure of type I LTA from *Staphylococcus aureus*. The 1,3-polyglycerol-phosphate RUs are substituted at the C2 position (X) with hydrogen proton (~15%), D-alanyl ester (~70%), or *N*-acetylglucosamine (~15%). (B) Structure of type II LTA from *Lactococcus garvieae*. (C) Structure of type III LTA from *Clostridium innocuum*. Gro-P in the RU can be substituted at the C2 position (Y) with hydrogen proton (~25%), glucosamine (~50%), or *N*-acetylglucosamine (~50%). (D) Structure of type IV LTA of *Streptococcus pneumoniae*. The RU may be substituted with hydrogen, D-alanyl, or *N*-acetylglucosamine (X). Substituents R, R′, and R″ in the glycolipids may be alkyl or branched alkyl chains.

S. pneumoniae (type IV LTA), produces type I LTA with the rare glycolipid anchor 3-O-(β-D-galactofuranosyl)-1,2-DAG (35). In group B *Streptococcus* type III strains, the polyglycerophosphate chain of LTA is substituted only with D-alanine and a kojibiose links the chain to the membrane anchor, resulting in a Glc-α-1,2Glc-α-1-3-DAG (36). The dihexosyl-DAG (Hex_2-DAG) appears to be the most common glycolipid anchor of type I LTA, although here too variations exist. For example, *Lactobacillus gasseri* JCM 1131 carries a tetrahexosylglycerol with either two or three fatty acid chains (37). Some species, including lactobacilli, streptococci, and listeria, may produce two variants of LTA where the glycolipid anchor may be substituted with acyl or phosphatidyl (25, 29, 38). For example, in lactobacilli the structure of the glycolipid anchor may be Glc(β1–6)Gal(α1–2)Glc(β1–3) DAG or Glc(β1–6)Gal(α1–2)6-O-acyl-6Glc(α1–3) DAG (25). The type I LTA of bacilli has been divided into two groups based on their side-chain substituents, α-GlcNAc in group A and α-Gal in group B (39). The fine structure of both type I LTA molecules of *L. monocytogenes* has recently been resolved by NMR and gas chromatography mass spectrometry, revealing variations in the specific fatty acid distributions between the two LTA molecules (29). Changes in temperature during bacterial growth presumably affect the production of one LTA molecule more than the other (29).

Type II and III LTA molecules contain repeat units of glycosylalditol-phosphate. This definition includes compounds where

the repeating units encompass glycosyl residues (24). For example, *Lactococcus garvieae* and *Clostridium innocuum* elaborate type II and III LTA molecules with the repeating units (Gal-Gal-Gro-P)$_n$ and (Gal-Gro-P)$_n$, respectively (Fig. 1B and C) (24, 40). In *L. garvieae*, the repeating unit is added to the disaccharide kojibiose linked to DAG. A third fatty acid chain modifies the 6-hydroxy group of the kojibiose. The glycerol moiety in the repeating unit is modified with Gal, and D-alanylation is not observed (40).

Type IV LTA refers to *S. pneumoniae* WTA and LTA which are substituted with choline (Cho). The structure of pneumococcal teichoic acid has been studied extensively (22, 41) and was recently revised (30). Its repeating unit consists of the pseudopentasaccharide 2-acetamido-4-amino-2,4,6-trideoxygalactose (AATGal), glucose (Glc), and ribitol-phosphate (Rib-P) followed by two *N*-acetylgalactosamine (GalNAc) moieties, both substituted in position O-6 with P-Cho (Fig. 1D). The terminal repeating unit can occur with or without 6-O-P-Cho substitution, and the hydroxyl groups of Rib-P can be substituted in nonstoichiometric amounts by D-Ala. The first repeating unit is β-linked to the sugar to which it is attached, in this case, the lipid anchor Glc(β 1–3)-diacylglycerol [Glc-DAG] (30). Additional repeating units in the polymer are α-linked to the precedent unit (30). The presence of the nonreducing terminal GalNAc-GalNAc and the common monoglucosyl-DAG provides the molecular basis for the Forssman antigenicity and the identity of the lipid anchor (41).

Type V LTA includes macroamphophiles such as lipoglycans, Gro-P-lipoglucogalactofuranan, and succinyl lipomannan from *Bifidobacterium bifidum* and *Micrococcus luteus* (42, 43). According to Fischer's definition for LTA structure, these compounds are not composed of repeating phosphodiester-linked units (42, 43). Type V lipoglycans therefore represent polysaccharides attached to lipid (glycolipid or phosphatidylinositol). Fischer excluded the lipopolysaccharide of Gram-negative bacteria from his classification (24). Some *Actinobacteria* and *Tenericutes* contain type V lipoglycans as well as type I LTA (44, 45). Nevertheless, the majority of *Firmicutes* are thought to synthesize only a single type of LTA (24, 45). Lipoglycans are found abundantly in the envelope of acid-fast bacteria, for example, *Mycobacterium tuberculosis*, which otherwise lack canonical teichoic acids (46, 47).

EARLY INSIGHTS INTO THE SYNTHESIS OF TYPE I LTA

The relatively simple structure of type I LTA invited biochemical approaches to study its biosynthesis in several Gram-positive organisms. Steps involved in the synthesis of the two main building blocks, the glycolipid anchor and the poly(Gro-P) chain, could be distinguished, and modifications of the assembled polymer, i.e., D-alanylation, were attributed to a third group of catalysts. Early attempts at identifying genes involved in LTA synthesis proved confusing, as mutants did not distinguish between specific requirements for WTA and LTA synthesis.

Genetic approaches focused initially on characterizing temperature-sensitive *B. subtilis* mutants that were also resistant to phage infection or displayed alterations in bacterial shape (48–51). Young used the nomenclature *gta* and *rod* to describe mutations in genes regulating the *g*lycosylation of *t*eichoic *a*cid and *rod* shape (48). Inability to maintain the rod shape under nonpermissive conditions was correlated with cell wall assembly defects, in particular, a decrease in envelope phosphate content and loss of TA enzyme activity in "particulate enzyme preparations" (see reference 52 for a review). Eventually, the *tar* and *tag* (*t*eichoic *a*cid *r*ibose and *g*lucose) genes, which are involved in WTA synthesis, were discovered (53). WTA synthesis is initiated on the murein linkage unit tethered to undecaprenol-phosphate, which is also the lipid carrier for peptidoglycan synthesis (54, 55). Nonetheless, several phage resistance alleles in *gtaA*, *gtaB*, and *gtaC* could not completely account for the loss of WTA glycosylation (48, 49, 56). Several decades later, it was discovered that *gtaA* encodes the enzyme TagE, which is responsible for transferring α-glucose from UDP-glucose to the C-2 position along the poly(Gro-P) polymer backbone of *B. subtilis* (57, 58). In contrast, *pgcA* (originally named *gtaC* or *gtaE*) and *gtaB* were shown to be involved in the synthesis of UDP-glucose (56, 59). UDP-glucose was shown to serve as a precursor for the synthesis of WTA in *B. licheniformis* (60) and as a precursor for the glycolipid anchor of *B. subtilis* LTA (61). Genetic studies in *B. subtilis* led Karamata and colleagues to propose that mutations in *gtaB* and *gtaC* (*pgcA*) affect the synthesis of both WTA and LTA (62) (Fig. 2); this model was later confirmed in *S. aureus* (63).

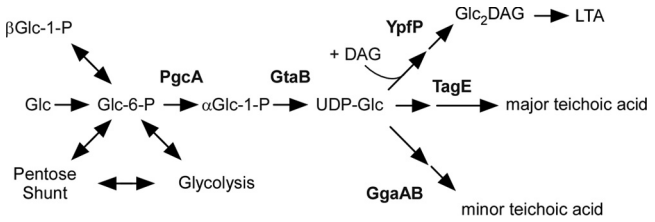

FIG 2 UDP-glucose contributes to wall teichoic acid and lipoteichoic acid synthesis. The diagram shows the flow of the UDP-glucose (UDP-Glc) pool in *Bacillus subtilis* and the key enzymes involved in synthesis of lipoteichoic acid (LTA) as well as minor and major wall teichoic acids (WTA) (adapted from reference 62). The major WTA from *B. subtilis* 168 is D-alanyl-[α-D-glycosylated poly(Gro-P)]. It is replaced with a phosphorus-free polysaccharide containing uronic acid residues (minor WTA) when *B. subtilis* is grown in phosphate-limited medium (89, 137). See the text for details.

The enzyme responsible for the synthesis of poly(Gro-P) was discovered only recently (64) even though its biochemical activity, substrate properties, and catalytic mechanism had been proposed for decades (65). It was initially assumed that type I LTA synthesis involves CDP-glycerol; however, *in vivo* pulse-labeling experiments revealed phosphatidylglycerol (PG) as a precursor for poly(Gro-P) synthesis (66–68). Careful characterization of lipid extracts from enterococci suggested that *sn*-glycerol 1-phosphate (Gro-P)-containing phosphoglycolipids contribute to LTA synthesis (69). Using membrane extracts from enterococci, Ganfield and Pieringer demonstrated that phosphatidylkojibiosyl DAG, not CDP-glycerol, functions as the acceptor of Gro-P moieties from PG (65). This study also demonstrated that the responsible enzymatic activity is membrane associated and that *in vitro*-synthesized poly(Gro-P) is not substituted with a glycolipid anchor (65). Elongation of the poly(Gro-P) moiety was examined in crude membrane preparations containing lipoteichoic acid-synthesizing activity and differential radioisotope labeling (70, 71). These experiments revealed that [^{14}C]acetate appears successively in glucosyl-diacylglycerol (Glc-DAG), Glc$_2$-DAG, and LTA, whereas [2-^{3}H]glycerol is first incorporated into PG and then into glycolipid-anchored LTA (70–72). Stepwise degradation of pulse-labeled LTA with phosphodiesterase and phosphomonoesterase from the glycerol terminus suggested that the polymer grows in a manner distal to the lipid anchor (70, 71). These observations

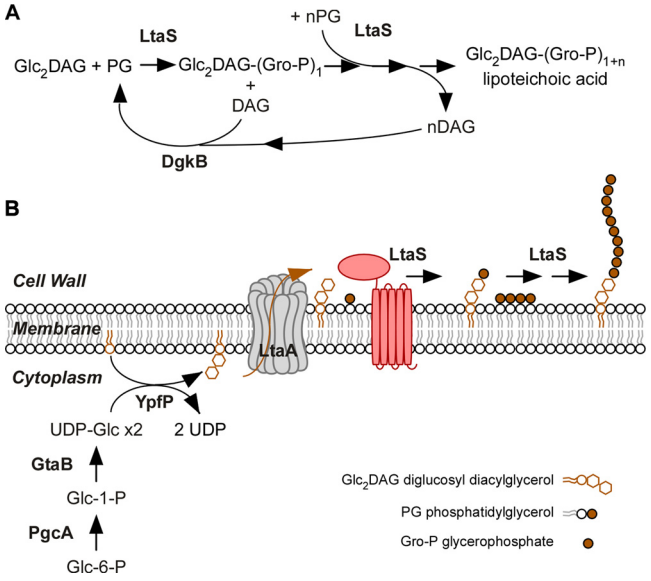

FIG 3 Synthesis of lipoteichoic acid in *Staphylococcus aureus*. (A) Diagram showing the reaction catalyzed by LtaS. The first glycerophosphate (Gro-P) subunit is cleaved from phosphatidyl glycerol (PG) and attached to the glycolipid anchor diglycosyl-diacylglycerol (Glc_2DAG). This reaction leads to the release of DAG. Polymerization of Gro-P by LtaS occurs at the distal end of $Glc_2DAG\text{-}(Gro\text{-}P)_1$, utilizing additional PG molecules ($n = \sim 50$). For some bacterial species, the first reaction is thought to require a specific primase (see the text for details). The DgkB enzyme is responsible for the recycling of DAG (see the text for details). (B) Diagram showing the biosynthesis of LTA in *S. aureus*. The enzymes PgcA, GtaB, and YpfP synthesize the Glc_2DAG glycolipid anchor. LtaA, a 12-transmembrane domain protein (in gray), flips Glc_2DAG across the plasma membrane. LtaS (red) spans the plasma membrane five times, and its C-terminal domain polymerizes the poly(Gro-P) chain on Glc_2DAG on the *trans* side of the membrane.

were incorporated into a unifying model whereby transfer of *sn*-glycerol 1-phosphate from PG to Glc_2-DAG and subsequent stepwise addition of Gro-P at the distal end of polymerizing chains lead to LTA assembly (Fig. 3A). This model correctly predicted that LTA synthesis would require enzymes for poly(Gro-P) synthesis and for the transfer of Gro-P from PG to Glc_2-DAG (73).

LTA SYNTHESIS IN *S. AUREUS*

It is now appreciated that the synthesis of the type I LTA glycolipid anchor, Glc_2-DAG, occurs via three enzymatic steps. First, α-phosphoglucomutase (PgcA) converts glucose-6-phosphate to glucose-1-phosphate (60, 62). Next, UTP:α-glucose-1-phosphate uridyl transferase (GtaB) converts glucose-1-phosphate to UDP glucose (UDP-Glc) (56, 59). Finally, the processive glycosyl-transferase YpfP strings two UDP-Glc molecules onto DAG to generate Glc_2-DAG (Fig. 2 and 3) (61, 74, 75). Analysis of *pgcA*, *gtaB*, and *ypfP* mutations in *S. aureus* corroborated this model (63). Importantly, *pgcA*, *gtaB*, and *ypfP* mutants continue to synthesize poly-(Gro-P); however, LTA is tethered to the membrane via a terminal DAG, instead of Glc_2-DAG (63, 75, 76). In staphylococci, *ypfP* is located in an operon with a second gene, *ltaA*, which is conserved in several *Firmicutes* species (63). Mutants lacking *ltaA* synthesize (Gro-P) chains attached to DAG instead of Glc_2-DAG, although Glc_2-DAG continues to be synthesized and accumulates in the membrane (63). Bioinformatic predictions identify LtaA as a member of the major facilitator superfamily (77); this polytopic membrane protein has been proposed to translocate Glc_2-DAG (63) (Fig. 3).

The enzyme responsible for the synthesis of poly(Gro-P) was discovered based on the model whereby PG is used as a substrate for stepwise addition of *sn*-glycerol 1-phosphate into poly(Gro-P)-polymerizing chains and transfer onto Glc_2-DAG (Fig. 3A) (73). As the membranes of *Escherichia coli* harbor PG and Glc_2-DAG but lack poly(Gro-P), it seemed plausible that expression of the gene for LTA synthesis in this organism could lead to the formation of poly(Gro-P). A plasmid library of staphylococcal genomic DNA fragments was introduced into *E. coli*, and several hundred clones were screened by immunoblotting of bacterial extracts with LTA [poly(Gro-P)]-specific monoclonal antibody (64). This approach identified *ltaS* (64), which encodes a polytopic membrane protein with a large C-terminal domain on the outer surface of the bacterial membrane, annotated in the Pfam database as a sulfatase domain (www.sanger.ac.uk/Software/Pfam; pfam00884) (64) (Fig. 3B). The protein was named LTA synthase (LtaS); mutations in the corresponding *S. aureus ltaS* gene cause severe cell division defects and loss of viability (64, 78).

LtaS proteins contain hydrophobic domains predicted to span the plasma membrane five times with short intervening loops (64) (Fig. 3B). The fifth hydrophobic segment is followed by a predicted signal peptidase I recognition motif that has been experimentally validated for *S. aureus* LtaS (79). The three-dimensional structures of the extracellular catalytic domain of *S. aureus* and *B. subtilis* LtaS (eLtaS) have been determined (80, 81). Overall, eLtaS assumes a sulfatase-like fold but with discrete changes in the active site (80). In *S. aureus* eLtaS, Thr^{300} (T300), together with E255, D475, and H476, coordinates a manganese ion and forms the binding pocket for Gro-P (80). Catalysis has been proposed to involve PG docking in the active site of eLtaS to enable the nucleophilic attack from the deprotonated hydroxyl of T300, thereby generating the Gro-P-threonine intermediate and releasing DAG. The Gro-P-threonine intermediate may subsequently be resolved by the nucleophilic attack of the terminal OH group of PG to extend the LTA chain (80).

LTA SYNTHESIS IN OTHER GRAM-POSITIVE BACTERIA

The chemical structure of type I LTA differs for various bacterial species, and the enzymes responsible for their synthesis and translocation and for polymerization of both the glycolipid anchor and the hydrophilic polymer must therefore also differ. In *S. aureus*, synthesis of Glc_2-DAG-linked LTA requires PgcA, GtaB, LtaA, and YpfP (32, 82); however, *ltaA* is not found in the genome of *B. subtilis* (61, 83). In *L. monocytogenes*, YpfP is replaced with two glycosyltransferases, LafA and LafB (LTA anchor formation protein), for the synthesis of the more complex GalGlc-DAG anchor and the transmembrane LafC protein is required to flip this glycolipid across the membrane (84). Pathways similar to the glycolipid anchor synthesis pathway in *L. monocytogenes* are thought to exist in *E. faecalis*, *S. agalactiae*, and *S. pneumoniae* (83). The function of LtaS homologues has been characterized in several Gram-positive bacteria, including *B. subtilis*, *L. monocytogenes*, and *B. anthracis*. These investigations confirmed that proteins with a predicted sulfatase domain (pfam00884 family) are indeed responsible for the polymerization of poly(Gro-P) and the anchoring of LTA to the membrane (81, 84–86). *B. subtilis* and *B. anthracis* encode four LtaS homologues, and genetic deletion of the corresponding genes suggested an overlap in activity or even redun-

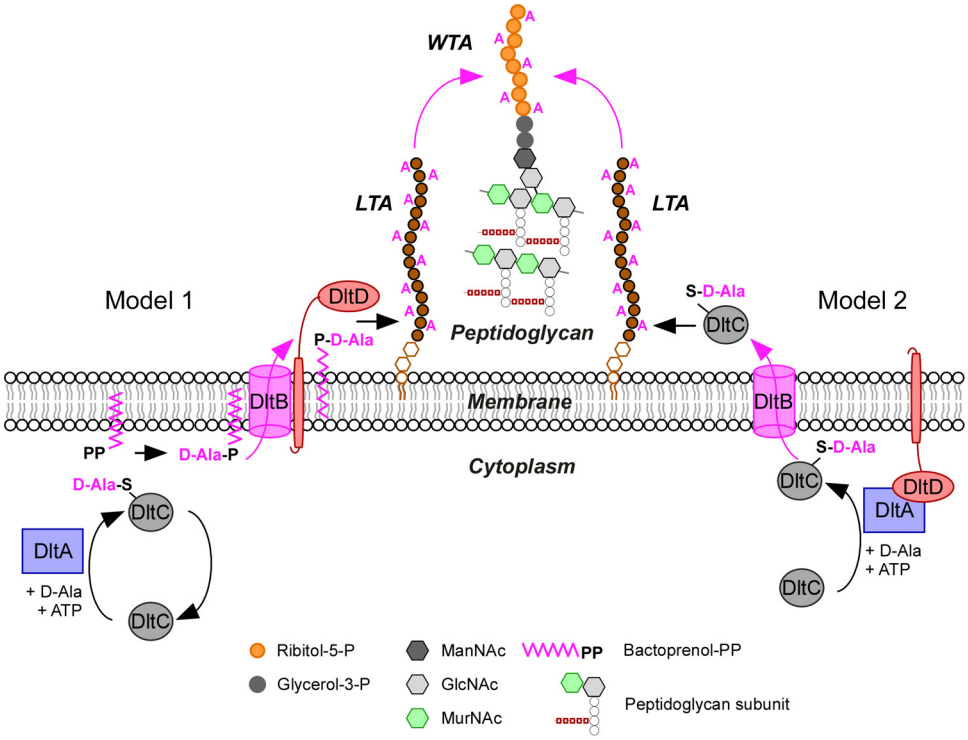

FIG 4 Models for D-alanylation of teichoic acids. DltA (Dcl), DltB, DltC (Dcp), and DltD are required for the D-alanylation of LTA, and two models have been proposed to explain the molecular basis. Model 1 proposes that the combined activity of DltA and DltC in the cytoplasm results in the transfer of D-Ala onto the lipid carrier bactoprenol pyrophosphate (PP) (95). Next, DltB flips D-Ala-bactoprenol-PP across the plasma membrane. It is unclear whether DltD assists the DltB flippase or contributes to D-Ala transfer onto LTA (96). In model 2, DltD facing the cytoplasm assists DltA for the loading of D-Ala onto the carrier protein DltC. Next, DltC-S-D-Ala is translocated across the membrane by DltB and transfers D-Ala onto LTA (89). D-Alanylation of WTA is not thought to require catalysis. Presumably, D-Ala moieties of LTA are transferred onto WTA. MurNAc, *N*-acetylmuramic acid; GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine.

dancy (see below) (64, 81, 85, 86). *L. monocytogenes* was found to encode two LtaS enzymes, and it was proposed that one of the enzymes, designated LTA primase, adds a single Gro-P moiety to the glycolipid anchor to initiate chain polymerization (84). It has been suggested that *B. subtilis* YvgJ may catalyze a similar reaction (85). Nevertheless, LTA primase is not required for the synthesis of glycolipid-linked LTA in either *B. subtilis* or *L. monocytogenes* (84, 85) and its molecular function may not yet be fully appreciated. These data can be incorporated into a predictive model whereby microbes expressing an *ltaS*-like gene are likely to synthesize a polyglycerol-containing LTA. *S. pneumoniae* with its more complex polymer lacks an *ltaS* orthologue. Here, synthesis of WTA and type IV LTA has been proposed to involve cytoplasmic CDP-ribitol polymerized on a lipid carrier that is subsequently flipped across the membrane for delivery to a peptidoglycan ligase or glycolipid transferase (41, 87, 88).

LTA D-ALANYLATION

Type I LTA is typically substituted with D-alanine esters, which confer positive charges onto an otherwise negatively charged polymer (89). Studying extracts of enterococci, Baddiley and Neuhaus detected an enzyme activity that activated D-alanine in the presence of ATP (90). In addition to this 56-kDa activating enzyme, a 6-kDa heat-stable protein, membranes, ATP, and D-alanine were required for the D-alanylation of LTA (89). D-Alanine activating ligase (Dcl) is a member of the family of proteins that activates amino acids or fatty acids and which functions here to ligate activated D-alanine to the 4′-phosphopantetheine prosthetic group of Dcp, the 6-kDa D-alanyl carrier protein (Fig. 4) (91, 92). Francis Neuhaus and colleagues identified the genetic determinants for Dcl and Dcp, which were designated *dltA* and *dltC*, respectively. Both genes are located in an operon along with two other determinants, *dltB* and *dltD* (93). In *S. aureus*, this operon consists of five genes, *dltXABCD* (94). Although also found in other species, the function of *dltX* is still unknown. In *B. subtilis*, each gene of the *dltABCD* operon is required for the D-alanylation of LTA (94, 95). Two models have been proposed to account for the contributions of DltB and DltD. DltB spans the plasma membrane several times, and DltD appears to be anchored to the membrane via an N-terminal hydrophobic sequence. In the first model, DltB transfers D-alanine from DltC to undecaprenol-phosphate and flips the lipid-linked intermediate across the membrane, whereas DltD, acting on the *trans* side of the membrane, transfers D-alanine to LTA (Fig. 4; model 1) (95). In the second model, DltD promotes transfer of D-alanine between DltA and DltC in the cytoplasm. Alanylated DltC is translocated across the membrane by DltB and may then transfer D-alanine directly onto LTA (Fig. 4; model 2) (89). An analysis of DltD membrane topology suggests that the protein may reside outside the cytoplasm, on the *trans* side of the membrane (96), as predicted by the first model (Fig. 4; model 1). Nevertheless, experimental support for this model lacks a demonstration that undecaprenol-phosphate or its synthetic intermediates

indeed contribute to LTA D-alanylation and transport of D-alanyl across the membrane. Of note, the Dlt system appears to D-alanylate LTA but not WTA. Both *in vitro* and *in vivo* pulse-chase experiments suggest that D-alanyl esters are transferred from LTA to WTA (97, 98). If so, one wonders whether D-alanylation of WTA requires a dedicated catalyst or whether catalysis is provided by D-alanylated LTA (Fig. 4).

TEICHOIC ACIDS AND BACTERIAL GROWTH

It had long been presumed that WTA and LTA synthesis is essential for bacterial growth (89, 99, 100). However, genes for enzymes that catalyze the first two steps in the WTA synthesis, *tagO* and *tagA*, can be deleted in *B. subtilis* or *S. aureus* without abolishing growth (101–103). In contrast, genes for enzymes that function downstream in the WTA synthesis cannot be deleted unless *tagA* or *tagO* is also mutated (102). A model has been developed to explain this synthetic viability phenotype: the accumulation of WTA synthesis intermediates limits the availability of undecaprenyl-phosphate for peptidoglycan synthesis, which is essential for bacterial growth (101). Thus, strictly speaking, WTA is not required for bacterial growth albeit *S. aureus* cells lacking WTA are enlarged and do not divide in the same manner as wild-type cells (see below). The genetic requirements for LTA are best discussed for each of its building blocks. For example, glycolipid synthesis enzymes such as those encoded by *pgcA*, *gtaB*, and *ypfP* are dispensable for *S. aureus* growth even though the mutants display morphological alterations such as increased size and aberrant cell shape (63, 75). These pleiotropic phenotypes could be attributed to the possibility that UDP-glucose may be perceived as a metabolic signal, coupling cell division with increased cell mass (104, 105). In *B. subtilis*, UgtP (YpfP) has been shown to inhibit FtsZ assembly (106). *ypfP* is dispensable for LTA synthesis, as *S. aureus ypfP* mutants anchor the polymer onto DAG, instead of Glc_2-DAG; nevertheless, LTA production is greatly reduced in *ltaS* mutants (63, 75, 76).

Reduced *ltaS* expression or chemical inhibition of the *ltaS* gene product results in severe cell division defects, a phenotype that is exacerbated when staphylococci are grown under conditions above 30°C (64, 78, 107). Some organisms carry more than one *ltaS*-like gene. For example, *B. subtilis* and *B. anthracis* carry four *ltaS* genes. The *B. subtilis yflE* mutant grows very slowly, which earned YflE the designation of a housekeeping LTA synthase (81, 85). Two housekeeping LtaS enzymes were identified in *B. anthracis*, and deletion of their corresponding genes, *ltaS1* and *ltaS2*, resulted in a three-log reduction in plating efficiency and in an inability to sporulate (86). Deletion of all four *ltaS* genes in *B. anthracis* could not be achieved (86). A mutant lacking all four paralogues of *ltaS* in *B. subtilis* remained viable albeit with severe morphological defects in cell structure and filament formation (81, 85). Further, *B. subtilis* does not tolerate the simultaneous deletion of *tagO* (WTA synthesis) and *ltaS* (LTA synthesis) (81). Deletion of the two *ltaS* genes in *L. monocytogenes* reduced bacterial plating efficiency by more than 6-log (81, 84–86). By screening a library of 167,405 compounds for inhibition of *S. aureus* growth at 42°C, compound 1771 was identified as an inhibitor of LTA synthesis (107). The spectrum of antibiotic activity for compound 1771 was analyzed against several Gram-positive bacteria harboring poly(Gro-P) LTA as well as LtaS homologues. Compound 1771 inhibited the growth of antibiotic-resistant methicillin-resistant *S. aureus* (MRSA), including USA300 LAC, and VRE, i.e., vancomycin-resistant *Enterococcus faecalis* and *E. faecium*, whose genomes harbor two *ltaS* homologues. Gram-positive bacteria with three *ltaS* homologues (*Clostridium perfringens*) or four *ltaS* homologues (*B. cereus* and *B. anthracis*) were also susceptible to compound 1771-mediated growth inhibition (107). Scanning and thin-section transmission electron microscopy of bacterial cultures treated with subinhibitory concentrations of 1771 revealed dispersal of bacterial cluster or chain formation, increases in cell size, deformation of cell surface and shape, thickening, and structural disorganization of the cell wall envelope (107). Thus, LTA synthesis, but not WTA, may be essential for growth in several different Gram-positive bacteria and chemical inhibition may be used to assess the presence of LtaS enzymes and type I LTA in less-well-characterized organisms.

Genetic suppression analyses also suggest a role of LTA and its building blocks in metabolism. For example, the *B. subtilis* housekeeping *ltaS* gene *yflE* was identified in a screen for suppressors of the Mg^{2+}-dependent growth defect of *mbl* mutants, a gene otherwise required for elongation of rod-shaped bacteria (81). Another investigation revealed that disruption of *B. subtilis yflE* or *yfnI* suppresses the lethality caused by *dgkB* repression (108). DAG is phosphorylated by diacylglycerol (diglyceride) kinase (DgkB) and thereby shunted into phosphatidylglycerol synthesis (Fig. 3A) (71). LtaS enzymes hydrolyze PG, a process that results in polymerization of poly(Gro-P) and formation of the byproduct DAG. The loss of viability of *dgkB* mutants has been attributed to accumulation of DAG, and it seems plausible that mutations in *ltaS* can alleviate this phenotype (108). In *S. aureus*, the growth defect caused by loss of LTA in an *ltaS* mutant could be rescued by inactivation of *gdpP* (109), a phosphodiesterase that hydrolyzes the essential signaling molecule cyclic-di-AMP (110). Increased cyclic-di-AMP in the *gdpP* mutant was shown to be associated with increased peptidoglycan cross-linking, which could somehow compensate for the lack of LTA (109). A receptor(s) for cyclic-di-AMP, i.e., the potassium transporters Kdp and Ktr, was identified, which suggests a role for the second messenger in ion transport, maintenance of cytoplasmic pH, and osmotic homeostasis in *S. aureus* (111, 112).

OTHER FUNCTIONS OF TEICHOIC ACIDS

Teichoic acids are zwitterionic molecules, and LTA D-alanylation is known to modulate ion hemostasis (89). D-Alanylation of LTA has been proposed to promote Mg^{2+} ion scavenging and to target autolysins to discrete locations in the bacterial envelope, which is essential for the separation of peptidoglycan in dividing cells (113, 114). D-Alanylated LTA may restrict the activity of autolysins and cell wall active antibiotics, thereby maintaining the integrity of the bacterial envelope (115, 116) (also reviewed in references 54 and 89). Modifications of TA polymers with D-alanyl esters or *N*-acetylglucosamine are also important for escape from innate immune defenses such as host antimicrobial peptides (117). In *B. subtilis*, D-alanylation-deficient mutants restore the protein secretion defect associated with mutations in *prsA*, which encodes a secretion chaperone (118). Presumably, an increased anionic charge through the increased binding of Ca^{2+} and Mg^{2+} to teichoic acids can stabilize and fold proteins in the absence of PrsA (118).

Other LTA types are not thought to contain D-alanine modifications. Instead, type IV LTA of *S. pneumoniae* is extensively modified with choline, a highly unusual component for bacteria (119).

LTA choline substituents are essential for the deposition of LytB glucosaminidase and LytA autolysin at the septum of dividing pneumococci, indicating that *S. pneumoniae* teichoic acids contribute to cell separation and that choline may play a role similar to that of D-alanylation (120).

LTA has been shown to bind the GW modules of internalin B of *L. monocytogenes* (121). Each GW module is approximately 80 amino acids long, with a highly conserved glycine-tryptophan dipeptide (121). The GW module is also present in staphylococcal autolysins AtlA and AtlE from *S. aureus* and *S. epidermidis* (122, 123). A structural model for their interaction with LTA was recently proposed (124). Because autolysins contribute to cell division and biofilm formation (123), it is not surprising that bacteria with reduced amounts of LTA display pleiotropic phenotypes (76).

LTA may play an important role in bacterial infectious diseases, as the polymer has been proposed to activate the immune system of infected hosts and promote inflammation (125–127). It was first proposed that LTA triggers innate immune responses by activating CD14 and Toll-like receptor (TLR) ligands (reviewed in references 128, 129, and 130). However, this model has been recently challenged with the argument that LTA extracted from bacteria may be contaminated with lipoproteins and peptidoglycan, compounds that are well known for their immune-modulatory activities (131, 132). The total chemical synthesis of various LTA types has provided valuable information for assessing their immunostimulatory attributes, as this technology can eliminate the concern of contamination with other cell wall compounds. Further, chemical synthesis of LTA building blocks affords the opportunity of assessing individual contributions of the glycolipid anchor, polymer repeat, and polymer substituents for immune system stimulation (36, 40, 132). These approaches have confirmed that LTA from several different bacterial species may be endowed with inflammatory activity, albeit Toll-like receptors (TLRs), in particular, TLR2, may not be involved in LTA recognition (40). Nevertheless, it seems unlikely that type I LTA displays an immunestimulatory function that is as potent as that observed for lipoproteins (131, 133) or lipopolysaccharides (132).

CONCLUSIONS AND PERSPECTIVES

In their landmark review, Neuhaus and Baddiley detailed the discovery, synthesis, and functions of teichoic acids, emphasizing the importance of LTA as a polyelectrolyte that forms a "continuum of anionic charges" between the plasma membrane and the extracellular milieu (89). To date, the molecular mechanisms whereby teichoic acids can contribute to the integrity of the bacterial cell wall envelope or to bacterial growth have remained poorly understood. Nonetheless, we have learned that type I LTA is positioned at the crossroads of several biosynthesis pathways that are perturbed by the accumulation or depletion of metabolites such as DAG, lipids, UDP-Glc, and cyclic-di-AMP (105, 108, 109). Only few of the enzymes that are involved in the synthesis of type I LTA synthesis have been examined in detail, and this gap needs to be addressed. Further, while we begin to appreciate the function of LtaS, it is not clear why some microbes synthesize LTA with only one type of synthesis enzyme whereas others employ multiple different LtaS enzymes. Both type I LTA and WTA contribute positional information for murein hydrolases that must act on peptidoglycan during bacterial growth and cell division (116, 124, 134). This cell biological function, i.e., the markings of new and old peptidoglycan or of discrete subcellular sites, may explain the essential contributions of teichoic acids to bacterial growth. Technological advances such as NMR and mass spectrometry have provided the means of identifying and characterizing the structures of new amphiphiles from Gram-positive bacteria (44, 135). Yet we remain unable to build models for the synthesis of many different types of LTA molecules. To quote Neuhaus and Baddiley, it seems that indeed "there may be no clear beginning and certainly there is no clear end to the cell surface. . ." of Gram-positive bacteria (89, 136).

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