Wall Teichoic Acids of Gram-Positive Bacteria

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Abstract

The peptidoglycan layers of many gram-positive bacteria are densely functionalized with anionic glycopolymers called wall teichoic acids (WTAs). These polymers play crucial roles in cell shape determination, regulation of cell division, and other fundamental aspects of gram-positive bacterial physiology. Additionally, WTAs are important in pathogenesis and play key roles in antibiotic resistance. We provide an overview of WTA structure and biosynthesis, review recent studies on the biological roles of these polymers, and highlight remaining questions. We also discuss prospects for exploiting WTA biosynthesis as a target for new therapies to overcome resistant infections.

Introduction

Bacteria are surrounded by a complex cell envelope that performs a variety of functions (114). Cell envelopes are varied in structure, but all contain layers of peptidoglycan (PG), a crosslinked matrix of linear carbohydrate (glycan) chains linked to one another via covalent bonds between attached peptides (130). This PG matrix is essential for survival, and in gram-positive organisms it is densely functionalized with other polymers. Wall teichoic acids (WTAs) are the most abundant PG-linked polymers in many gram-positive organisms (91). They are intimately involved in many aspects of cell division and are essential for maintaining cell shape in rod-shaped organisms (120). WTAs are required for β-lactam resistance in methicillin-resistant *S. aureus* (MRSA), and they modulate susceptibility to cationic antibiotics in several organisms (23; 25; 144). Due to their importance in pathogenesis, WTAs are possible targets for new therapeutics to overcome resistant bacterial infections. Here we review recent work on WTAs and discuss studies implicating the pathway as a therapeutic target.

Teichoic Acid Definition

Teichoic acids were discovered in 1958 by Armstrong and co-authors while trying to determine the function of CDP-glycerol and CDP-ribitol in *Lactobacillus arabinosus, Bacillus subtilis* and several other bacteria (6). The term teichoic acid encompasses a diverse family of cell surface glycopolymers containing phosphodiester-linked polyol repeat units (133). Teichoic acids include both lipoteichoic acids (LTAs), which are anchored in the bacterial membrane via a glycolipid, and WTAs, which are covalently attached to

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peptidoglycan (Figure 1) (91; 133). This review focuses primarily on WTAs. For information on LTAs the reader is directed to a recent review by Reichmann and Gründling (106).

Wall Teichoic Acid Backbone Structure

Wall teichoic acids are highly abundant modifications of gram-positive cell walls (90). In *B. subtilis* and *S. aureus*, it has been estimated that every ninth PG MurNAc residue contains an attached WTA polymer containing 40 to 60 polyol repeats (12; 71). The total mass of WTAs in these and other organisms comprises up to 60% of the cell wall (44; 124). Consistent with their estimated length, cryo-EM images suggest that *S. aureus* WTAs extend well beyond the PG layer (81; 82; 106).

The wall teichoic acid polymer can be divided into two components, a disaccharide linkage unit and a main chain polymer composed of phosphodiester-linked polyol repeat units (Figure 2) (91). The disaccharide linkage unit, which is highly conserved across bacterial species, is comprised of N-acetylmannosamine (β1→4) N-acetylglucosamine-1-phosphate (ManNAc (β1→4) GlcNAc-1P) with one to two glycerol-3-phosphate (GroP) units attached to the C4 oxygen of ManNAc (5; 71; 91). The anomeric phosphate of the linkage unit is covalently attached to peptidoglycan (PG) via a phosphodiester bond to the C6 hydroxyl of N-acetylmuramic acid (MurNAc). The phosphodiester-linked polyol repeats extend from the GroP end of the linkage unit (91; 133).

The best-characterized WTA structures contain repeat units of 1,5-D-ribitol-phosphate (RboP) or 1,3-L-α-glycerol-phosphate (GroP), but WTA monomer structures can be highly diverse and many published structures are more unusual (45; 46; 90). Other WTA repeat units include variations of glycosyl-polyol phosphate or glycosyl-phosphate polyol-phosphate (Figure 2)(90; 91). WTA structural diversity can exist within the same species, as in *B. subtilis*, where strains 168 and W23 contain GroP and RboP repeat units, respectively. *Lactobacillus plantarum* has the ability to switch backbone composition (21). Furthermore, single strains of *S. aureus* and *B. coagulans* have been found to contain polymers with distinct repeats expressed simultaneously, but at different levels (102; 128). WTA structural variations may represent adaptations to different environments. Relatedly, bacteria are known to modulate transcription of WTA biosynthetic genes in response to stress conditions (87). Despite their diversity, all WTAs contain a negatively charged anionic backbone and share common functions.

Tailoring Modifications on the Wall Teichoic Acid Polymer

Additional WTA structural diversity arises from the presence or absence of substituents attached to the repeating monomers (Figure 3). The repeat unit hydroxyls can be tailored with cationic D-alanine esters or a variety of mono- or oligosaccharides, commonly glucose or GlcNAc (91). In RboP polymers, D-alanyl residues are installed at position 2 of ribitol while sugars are commonly found at position 4 (91; 128). D-Alanine ester content is variable and depends on several factors, including pH, salt concentration, and temperature (64; 91). In contrast, the WTA sugar substituents do not appear to fluctuate with changes in the cellular environment (32). These tailoring modifications are typically highly abundant. For example, nearly all of the RboP repeats in *S. aureus* contain O-GlcNAc substituents (64). Depending on the bacterial strain, the anomeric configuration of the glycosidic linkage to the repeat unit can be exclusively α, exclusively β, or a mixture of the two anomers (29; 45; 46; 88). Some *S. aureus* strains have been found to contain, in the same cell wall, two different poly(RboP) WTAs, one fully α-glycosylated and the other fully β-glycosylated

(125). In *B. subtilis* W23 it was observed that some WTA strands were fully glycosylated, whereas other strands contained no sugar substituents (29).

Wall Teichoic Acid Biosynthesis

Overview

Much of the early work to understand WTA biosynthesis utilized particulate enzyme preparations. Though this work enabled characterization of the enzymatic steps in WTA polymer formation, identification of the enzymes responsible for these steps remained elusive (133). Later, genetic analyses of temperature-sensitive and phage-resistant mutants led to the identification of the poly(GroP) WTA gene cluster in *B. subtilis* 168 and the poly(RboP) WTA gene cluster in *B. subtilis* W23 (65; 148). Sequence analysis led to proposals for the enzymatic activity encoded by each gene (75; 91; 133). However, it proved challenging to characterize the pathway fully using genetics because many of the biosynthetic genes are essential (14; 15; 34; 36; 69). Efforts to characterize WTA enzymes biochemically were hampered because the substrates contain 55-carbon lipid chains that make them difficult to handle. Moreover, these lipid-linked substrates are present in very low abundance and cannot be isolated in useful quantities from bacterial cells. The development of chemoenzymatic methods to make WTA substrates has not only allowed elucidation of biosynthetic pathways, but also has permitted mechanistic studies on purified teichoic acid enzymes in the absence of membranes (22; 24; 54; 110; 113; 150).

Linkage Unit Synthesis

In 1978 Archibald and colleagues used electron microscopy to show that WTA synthesis begins in the cytoplasm at the wall-membrane interface and that the WTA polymer subsequently appears on the outer surface of the cell (3; 133). TagO catalyzes the first synthetic step: the transfer of GlcNAc-1-phosphate from UDP-GlcNAc to a membrane-anchored undecaprenyl-phosphate carrier lipid, an intermediate shared with peptidoglycan biosynthesis (107; 116). The TagO product is used in the synthesis of several *B. subtilis* 168 cell wall polymers, including the major wall teichoic acid (poly(GroP)), the minor teichoic acid (poly(glucosyl N-acetylgalactosamine 1-phosphate)) and teichuronic acid (poly(glucuronyl N-acetylgalactosamine)) (116). Consequently, the first committed step in WTA biosynthesis is the transfer of ManNAc from UDP-ManNAc to the C4 hydroxyl of GlcNAc to form the ManNAc-β1,4-GlcNAc disaccharide, catalyzed by TarA (33; 54; 150). The UDP-ManNAc donor sugar is derived from UDP-GlcNAc via MnaA (*yvyH*), an epimerase that catalyzes stereochemical inversion at the C2 position (117). TagB, a glycerophosphotransferase, catalyzes the transfer of a glycerol-phosphate unit from CDP-glycerol to the C4 position of ManNAc (16; 54). These first three steps to complete the synthesis of the WTA linkage unit are highly conserved across all thus far characterized strains (Figure 3). After these steps, WTA pathways diverge.

Poly(glycerol-phosphate) Wall Teichoic Acid Synthesis

The genes encoding proteins involved in poly(GroP) WTA synthesis are annotated as tag genes (teichoic acid glycerol) (19). *B. subtilis* 168 is the most studied of the WTA poly(GroP)-producing organisms (Figure 3). After the action of TagO, TagA, and TagB to complete synthesis of the linkage unit, TagF adds 45 – 60 glycerol-phosphate units to the TagB product to assemble the polymer (78; 98; 109; 110; 113). The GroP moiety originates from the activated precursor CDP-glycerol, which is synthesized by TagD, a cytidylyltransferase that catalyzes the transfer of L-α-glycerol-3-phosphate to CTP, releasing pyrophosphate (94). While the Tag enzymes from *B. subtilis* 168 remain the most characterized of the poly(GroP) WTA biosynthetic enzymes, homologs have been identified

and loosely characterized in other bacteria known to contain poly(GroP), including *Enterococcus faecalis, Lactobacillus plantarum, Staphylococcus epidermidis*, and several *Streptomycetes* (37; 39; 50; 86; 89).

In 2010, Strynadka and co-authors published the first and so far only crystal structure of a teichoic acid enzyme involved in polymer synthesis (78). The structure showed that *S. epidermidis* TagF possesses a GT-B glycosyltransferase fold and an extended open active site that appears capable of accommodating the rebinding of CDP-glycerol without product release. While this observation suggests a processive mechanism, kinetic studies found that polymer length depended on the ratio of CDP-glycerol to lipid acceptor, supporting a distributive mechanism (98; 110; 113). The crystal structure of TagF should allow for further mechanistic studies on this class of phosphotransferases. TagB and TagF share 50% similarity in their catalytic domains, but features governing primase versus polymerase activity await identification.

Poly(ribitol-phosphate) Wall Teichoic Acid Synthesis

Enzymes making poly(RboP) WTAs are designated Tar for teichoic acid ribitol (19), but it should be noted that TarO, TarA, TarB, and TarD have the same biochemical functions as TagO, TagA, TagB, and TagD. In *S. aureus*, following formation of the lipid-diphospho-ManNAc-GlcNAc-GroP product by TarO, TarA and TarB, poly(RboP) synthesis continues with the TarF-mediated transfer of a (predominantly) single unit of glycerol-phosphate from CDP-glycerol, synthesized by TarD (9; 22; 24; 85; 135). As described above, the synthesis of poly(GroP) WTAs requires a GroP primase (TagB) as well as a GroP polymerase (TagF), and it was proposed that poly(RboP) WTA synthesis in *S. aureus* requires both a RboP primase and a RboP polymerase to make the main chain polymer (75; 105). Recent work has shown, however, that *S. aureus* contains a single enzyme, TarL, that primes the linkage unit and then attaches more than 40 ribitol-phosphates to complete the polymer (Figure 3) (24; 85; 97). The CDP-ribitol substrate utilized by TarL is made by the combined action of TarI, a cytidylyl transferase, and TarJ, an alcohol dehydrogenase (96; 151). All *S. aureus* strains contain two sets of *tarIJL* genes (the second set is designated *tarI'J'K*) (105). The significance of these duplications is still unclear (85; 97; 105; 120; 144).

Whereas poly(GroP) WTA biosynthesis has only been fully characterized in one organism, *B. subtilis* 168, poly(RboP) WTA biosynthesis has been characterized in both *B. subtilis* W23 and *S. aureus* (Figure 3) (22; 24). These two organisms produce very similar RboP-WTAs, which differ only in the number of GroP repeats in the linkage unit (one versus two) and in the sugar tailoring modifications (the addition of Glc versus GlcNAc) (91; 147). Both organisms appeared to contain the same *tar* genes (75; 105), but their WTA biosynthetic pathways were found to differ. Although *S. aureus* utilizes a single enzyme to prime the linkage unit and build the RboP polymer chain, *B. subtilis* W23 requires a RboP primase, TarK (22; 85). Furthermore, TarK acts directly on the TagB product. Thus, *B. subtilis* W23 does not require the enzymatic activity of TarF even though it contains a *tarF* gene. In *S. aureus tarF* is essential, but genetic analysis has established that *B. subtilis tarF* is neither essential nor transcribed under laboratory culture conditions (22). The WTA polymer in *B. subtilis* W23 is completed by the RboP polymerase TarL, which can attach upwards of 40 RboP units (147). Thus, *B. subtilis* W23 TarL is not a bifunctional primase-polymerase like its *S. aureus* homolog, but can only utilize WTA lipid-linked substrates that are primed with RboP by TarK (22; 85).

The biochemical and genetic studies of wall teichoic acid biosynthesis described above have substantially expanded our understanding of how WTA polymers are made, but also highlight challenges in predicting enzymatic pathways from genome sequences. Other

pathways will most likely begin with the same initial steps, but may then diverge (Figure 2). In principle, the presence of *tagD* and *tarIJ* genes, required to make CDP-glycerol (*tagD*) and CDP-ribitol (*tarIJ*) substrates, should facilitate prediction of WTA polymer composition (124) (11; 39). It should be noted, however, that strains containing both types of genes may produce only one type of polymer. For example, some strains of *L. plantarum* that make only poly(GroP) polymers contain both *tarIJKL* and *tagDF* homologs, but the *tarIJ* gene homologs are not transcribed (21; 124). More perplexing is a recent report of an *E. faecalis* strain making a WTA ribitol-phosphate polymer that does not have a *tarJ* homolog (122).

Attachment of sugars to the polyol chain

In the past few years several WTA glycosyltransferases have been identified. In *B. subtilis* 168, TagE modifies WTAs with α-glucose using UDP-glucose as a donor substrate (1; 10). In *B. subtilis* W23, TarQ attaches β-glucose units to poly(RboP)-WTAs (23). In *S. aureus*, RboP-WTA α-O-GlcNAc modifications are installed by TarM (145) and β-O-GlcNAc modifications are installed by TarS (23). As mentioned above, WTAs extracted from cells appear to be heavily modified with sugars of the same stereochemistry or not modified at all (29; 45; 46; 125), suggesting that the glycosyltransferases are processive enzymes that fully glycosylate WTAs. Processivity may ensure that individual polymers are densely modified and, in organisms that contain more than one glycosyltransferase, may also prevent the attachment of sugars of different stereochemistries to the same polymer. The presence or absence of sugars and the glycosidic linkage conformation affects polymer structure and likely influences interactions with other components in the cell envelope or at the cell interface (13). Strains lacking modifications have altered phenotypes with regard to antibiotic susceptibility and virulence (23; 144). Although progress has been made with regard to the roles of the WTA sugar substituents, numerous questions remain.

Polymer Export

The final steps in the WTA biosynthetic pathway, those following polymer formation and sugar tailoring, are not as clearly defined as earlier steps. For both poly(GroP) and poly(RboP) WTA polymers, the polymer is translocated through the membrane by a two-component ABC (<u>A</u>TP-<u>b</u>inding <u>c</u>assette) transporter, TagGH (or TarGH) (76). TagH, containing an ATPase domain, provides energy to drive a conformational change in the transmembrane component, TagG, which somehow facilitates translocation across the membrane (76; 111). The WTA transporter exports polymers containing main chains that can be more than ten times longer than the width of the lipid bilayer. In principle, there are two models for how export may occur: one involves a threading mechanism in which the polymer is fed through the transporter from the non-reducing end, eventually pulling the linkage unit across; the other involves a flipping mechanism in which the lipid-pp-GlcNAc-ManNAc linkage unit is somehow flipped across the membrane, with the polymer chain following (Figure 4). While the former model is conceptually simpler, experimental studies support the latter model, as they show that the linkage unit, and not the main chain, is recognized by TagGH (21; 111). It is not known whether translocation occurs concomitantly with or following polymerization. Reconstitution of the translocation process in proteoliposomes would provide valuable mechanistic insight and may be possible now that all previous steps have been characterized and enzymes to make the precursors are available.

Wall Teichoic Acid Attachment to PG

Once the polymer is outside the cell, the final stage of wall teichoic acid biosynthesis is formation of a phosphodiester linkage between the WTA polymer and the C6 hydroxyl of the PG MurNAc unit. Based on genetic evidence, a set of redundant enzymes, TagTUV, was

recently proposed to be responsible for catalyzing this coupling reaction in *B. subtilis* 168 (66), and similar evidence supports a related function for three homologous enzymes in *S. aureus* (Figure 4) (40; 62; 92). TagT was found to have geranyl pyrophosphatase activity (42; 66). This activity was presumed to be a surrogate for attack of the WTA phosphodiester bond by the C6 MurNAc hydroxyl of PG, but reconstitution of an authentic coupling reaction must be done to confirm the proposed WTA ligase activity.

When and where WTAs are synthesized and attached to peptidoglycan is still debated. Decades ago, some studies suggested the WTA polymer is attached to nascent (new) peptidoglycan, while others indicated that it may be attached to mature (old) PG. Still others suggested it can be attached to concurrently synthesized as well as pre-existing PG (20; 83; 123; 133; 141). Recently, the timing and location of WTA synthesis has been revisited. In cryo-EM images, Matias and Beveridge identified densely-staining material at the septa of *B. subtilis* and *S. aureus* as WTA, and proposed that WTAs are attached to new PG during cell division (80–82). Consistent with this hypothesis, this densely-staining material disappears in a time-dependent fashion when the first step in WTA synthesis is inhibited (25). Furthermore, studies using fluorescent protein fusions show that WTA biosynthetic proteins localize predominantly to the septum in *B. subtilis* and *S. aureus* (7; 16; 51), suggesting that WTAs are attached to nascent peptidoglycan during cell division (septum formation). However, other studies suggest that WTAs are attached to older PG (4; 112). Fluorescently-labeled concanavalin A (ConA), a lectin reported to bind to α-GlcNAcylated WTAs, was used to probe the location of WTAs in *S. aureus*. Fluorescent ConA bound to the half of the cell containing old PG, but not to the half containing PG produced during cross wall formation (cell division) (4; 112). Additional studies using alternative detection methods are required to resolve the location of WTA attachment.

ᴅ-Alanylation of the Wall Teichoic Acid repeat unit

Attachment of ᴅ-alanine esters to WTAs is an important mechanism by which bacteria modulate surface charge (32). Unlike glycosylation, which occurs inside the cell, ᴅ-alanylation occurs following export of WTAs to the cell surface. Four enzymes, encoded by the *dltABCD* operon, attach ᴅ-alanines to both LTAs and WTAs (Figure 5) (72; 84; 95). DltA resembles the adenylation domains of nonribosomal peptide synthetases. It is responsible for activating ᴅ-alanine as an AMP ester (58), and then transferring the aminoacyl adenylate to DltC. DltC possesses a pantothenate cofactor that forms a thioester with ᴅ-alanine through nucleophilic attack of the sulfhydryl on the mixed anhydride (59; 129). What happens after DltC is charged with ᴅ-alanine is unclear, but involves both DltB and DltD (Figure 5). DltB, which contains several predicted membrane-spanning helices, is an uncharacterized membrane protein of the membrane-bound O-acetyltransferase (MBOAT) family. DltD is a membrane-anchored protein predicted to have a short intracellular N-terminus and a large extracellular C-terminal domain with predicted esterase/thioesterase activity. DltD was shown to hydrolyze ᴅ-alanine from acyl carrier protein (ACP), which is normally involved in fatty acid biosynthesis; on this basis DltD was suggested to have an editing function and to remove ᴅ-alanine from mischarged ACP (38). It was also shown to modestly accelerate the rate of transfer of ᴅ-alanine to DltC, suggesting an accessory role in the transfer reaction (38). These proposals for DltD require it to function in the cytoplasm, which is inconsistent with its predicted topology. At this point, the specific function of DltD is unclear.

It has been proposed based on pulse-chase experiments that ᴅ-alanines are transferred to LTAs by DltC and then to WTAs in an isoenthalpic transesterification reaction (56). Supporting such a mechanism, ᴅ-alanyl esters could be transferred between LTAs of differing lengths in nonnative micelles without an enzyme catalyst, though the reaction was

slow (28). Alternatively, it has been suggested that D-alanyl esters are transferred back from LTAs to DltC and then to WTAs (68). Since this transfer reaction required bacterial membranes, other factors may also play a role in the process. DltB is also required for D-alanylation, and it has been suggested to facilitate membrane translocation of D-alanine-charged DltC so that it can serve as the direct donor to LTA (91). DltC has not been identified in any proteomic analyses of secreted proteins, and the evidence that it serves as the direct D-alanylation donor is limited (41; 68; 91). While it is clear that D-alanylation of LTAs and WTAs requires the same biosynthetic machinery, many questions remain regarding the enzymology of the process.

Nonessential & Conditionally Essential Wall Teichoic Acid genes

It is possible to delete *tagO* or *tagA*, the first two genes in the WTA biosynthetic pathway, and produce bacteria lacking WTA polymers. These bacteria display a range of defects, but are viable *in vitro*. With the exception of the tailoring enzymes, however, most of the downstream WTA genes are essential and their deletion results in a lethal phenotype *unless* flux into the WTA pathway is blocked at an early stage (34; 36). The lethality of a late stage block may be due to accumulation of toxic intermediates in the cell or to depletion of cellular pools of undecaprenyl phosphate, which is also required for the synthesis of PG (34–36; 120; 144). Conditionally essential genes are found in pathways for other cell wall polysaccharides that are synthesized on the undecaprenyl phosphate carrier lipid. For example, it was found that deletion of late-stage genes involved in synthesis of the capsular polysaccharide of *S. pneumoniae* generated bacteria with suppressor mutations in the initiating enzyme that turned off capsule production (63; 142).

Roles of Wall Teichoic Acids

Wall teichoic acid polymers play numerous, varied roles in the cell wall owing to their location, abundance, and polyanionic nature. The molecular mechanisms underlying their many critical functions are not well understood. Genetic and small molecule tools are enabling more detailed studies to elucidate these mechanisms.

Regulation of Cell Morphology and Division

Bacteria lacking WTAs grow slower than wildtype and clump in solution. These strains exhibit numerous morphological abnormalities, including a non-uniform thickening of the peptidoglycan cell wall, increased cell size, and defects in septal positioning and number (4; 25; 31; 34; 93; 112). Furthermore, rod-shaped bacteria such as *B. subtilis* and *L. monocytogenes* lose their shape, becoming spherical (14; 19; 43; 47; 116; 117). These phenotypes imply that wall teichoic acids are necessary for proper localization, assembly, and/or activation of cell wall elongation and division machinery. Consistent with this idea, many WTA and PG biosynthetic enzymes have been shown to colocalize and physically interact in *B. subtilis* (51; 66).

Evidence for functional interactions between WTAs and PG machinery comes from *S. aureus*, where it was observed that the putative PG cross-linking enzymes PBP4 and FmtA lose septal localization in the absence of WTAs(7; 104). This mislocalization correlates with decreased PG cross-linking found in Δ*tarO* mutants (112). In addition, Campbell *et al.* showed that TarO inhibitors potentiate β-lactams in MRSA strains, indicating a functional connection between the PG and WTA biosynthetic pathways (25). Farha *et al.* used a panel of β-lactams to argue that this synthetic lethality was specifically due to combined disruption of the PG cross-linking enzymes PBP2 (via β-lactams) and PBP4 (via WTA biosynthesis inhibition) (48). Although many details remain to be clarified, there is considerable evidence

that WTAs regulate the function of PG biosynthetic machinery, and one mechanism by which they do so is through the recruitment of cell wall assembly proteins.

Regulation of autolytic activity

The failure of mutants to effectively septate and separate during cell division implicates WTAs in coordinating the localization, stability, and/or activity of PG lytic proteins. Strains lacking WTAs have an altered endogenous autolysin expression profile and increased autolysis rates (18; 79; 112; 134). *In vitro* studies performed with purified LTAs, enzymes, and cell wall substrates explain these observations using a recruitment model, in which polyanionic phosphate backbones bind and sequester positively-charged autolysins (17; 49; 91). Positively-charged D-alanyl esters help mask this charge and free autolysins to degrade PG. This model requires WTAs to be either absent from the septum, or present in a highly D-alanylated state, as autolytic activity must be regulated to ensure separation specifically at the cross wall during cell division (146). Recent studies support an exclusion model (52; 112). Finally, others have proposed that WTAs could indirectly modulate enzyme activity through ion chelation (8; 18; 60). Whether directly or indirectly, or by recruitment or exclusion, it is clear WTAs play a role in the localization of autolytic enzymes (52; 104; 112).

Regulation of Ion Homeostasis

It is thought that WTAs can bind extracellular metal cations by extending beyond the PG layer (67; 140). WTAs have a high affinity for metals and their biosynthesis is upregulated under metal limitation (8; 45). Wall teichoic acids also bind protons, and cells lacking WTAs having a 23% decrease in their proton binding capacity (18). While WTAs do not influence the pH gradient across the cell wall, it has been suggested that they can create localized changes in pH, indirectly modulating the function of some enzymes, (e.g. autolysins) (18). WTA ion binding is also thought to minimize repulsion between nearby phosphate groups, which can affect polymer structure and therefore cell wall integrity (67; 140). D-alanylation masks negatively charged sites on the polymer, reducing WTAs' binding capacity for cations (60). Some have also speculated ion binding to WTAs could help prevent fluctuations in osmotic pressure between the inside and the outside of the cell (4; 60; 120).

Protection from Host Defenses and Antibiotics

Wall teichoic acids and their attached substituents contribute to bacterial cell surface charge and hydrophobicity, which in turn affects binding of extracellular molecules. This plays a role in protecting bacteria from various threats and adverse conditions. In the absence of WTAs, bacteria are sensitive to high temperatures and unable to grow in high salt media, indicating WTAs are involved in temperature tolerance and osmotic stress (7; 61; 117; 127). WTA deficient cells are more susceptible to human antibacterial fatty acids (AFAs), presumably because hydrophobic AFAs penetrate the less hydrophilic mutant cell wall more easily, and more efficiently bind to the cell membrane where they can cause damage (70).

Preventing D-alanylation through gene deletion removes D-alanine esters from both LTAs and WTAs, consequently, researchers have not yet been able to identify roles specific to each polymer. Nevertheless, it has been established that TA D-alanylation protects against host-defense mechanisms (73; 74). *S. aureus* lacking D-alanine have an increased susceptibility to phagocytes, to neutrophil killing (32), and to lysostaphin and lysozyme (100). A reduction in the D-alanyl content of the cell wall results in increased susceptibility to glycopeptide antibiotics and to certain cationic antimicrobials peptides (CAMPs) (32; 99; 100; 126; 131). It was reasoned that the absence of D-alanyl esters increases the overall negative cell surface

charge (126), attracting cationic molecules and thus increasing susceptibility to CAMPs. Accordingly, *B. subtilis* and *S. aureus* lacking TA D-alanine modifications bind more positively-charged cytochrome c as compared to wildtype (99; 134). In Group B *Streptococci*, however, D-alanylation showed little influence on the surface-association of fluorescently-labeled CAMPs (108). These data suggest that charge effects are not solely responsible for the observed changes in CAMP susceptibility. Saar-Dover *et al.* suggest the increased cell wall density observed in strains with D-alanylation prevents penetration of CAMPs, thereby conferring protection (108).

Blocking WTA synthesis renders *B. subtilis* and MRSA sensitive to β-lactam antibiotics (23; 25; 48; 79; 99; 119; 134). Recent data has replicated this sensitivity by the sole removal of WTA β-GlcNAc modifications (23). It has been speculated that the WTA β-GlcNAc moieties scaffold proteins involved in resistance. Accordingly, recent work has shown that *S. aureus* WTAs specifically interact with PBP2A (104), the resistant transpeptidase found in MRSA. Since wall teichoic acid glycosyl substituents are also receptors for phage (10; 143), there is precedence for WTA sugar residues mediating protein binding. It is possible that glycosylated WTAs function as ligands for cell surface proteins with carbohydrate recognition sites.

Effects on Adhesion and Colonization

The presence of WTAs and their D-alanyl esters influences bacterial interactions with various surfaces (91). WTA deficient cells have a reduced capability to form biofilms (55; 61; 127; 131). Removal of D-alanine esters also decreases biofilm formation and bacterial adhesion to plastic and glass surfaces, an effect potentially linked to the resultant increase in negative cell surface charge creating increased repulsive forces between bacteria and a stratum (55; 61).

In 1980, Aly *et al.* showed a 71% reduction of *S. aureus* binding to nasal epithelial cells that were preincubated with teichoic acid (2; 73; 131; 135; 136; 139). Since that time, several animal studies have established that bacteria lacking TA d-alanine esters or WTAs are attenuated in host colonization and infection (32; 48; 118; 119; 132; 136). *Lactobacilli* lacking TA D-alanylation colonized mice gastrointestinal tracts at 1% of wildtype levels (131). Similarly, WTA-free *S. aureus* cells colonized rat nares at levels 90% lower than wildtype and in rabbits these mutant cells were unable to proliferate to adjacent organs (135; 139). TA D-alanine esters are considered virulence factors, since their deletion attenuates pathogenicity but does not cause major cellular defects *in vitro* (4; 14; 25; 27; 31). WTAs have also been categorized as virulence factors, although they exhibit such profound defects *in vitro* that they may be better described as "*in vivo* essential" (30; 138). The decreased ability of WTA null mutants to survive in challenging host environments is due to the combined effects of an inability to adhere to host tissue, an increased susceptibility to host defenses, and cell division defects (74; 91; 131; 137).

The wall teichoic acid pathway as an antibiotic target

The vital roles of WTAs in physiology and pathogenesis promote this pathway as a target for antibacterial drugs and vaccines (120; 137). The WTA pathway contains three distinct target categories: antivirulence targets, β-lactam potentiator targets, and essential targets (Figure 6).

Antivirulence Targets

Proponents of virulence factors as antibacterial targets argue that resistance is less likely to emerge to virulence factor inhibitors since there is no selection pressure for survival (30).

Despite arguments against these inhibitors, such as their inability to cure established infections or unforeseen *in vivo* selection pressures, it is worth investigating virulence factor targets, particularly for resistant pathogens. Enzymes of the *dlt* operon are targets for antivirulence agents since strains lacking TA ᴅ-alanine esters are strongly attenuated *in vivo* but show minimal growth defects *in vitro* (137). A DltA inhibitor was reported in 2005, but was not further optimized and is likely not specific (84). The enzymes involved in initiating WTA polymer synthesis, TarO and TarA, have also been described as virulence factor targets but, as noted above, these deletion strains are more than attenuated *in vivo*; they are non-viable (135). Hence, it is possible that inhibitors of these initiating enzymes would behave more like traditional antibiotics *in vivo*, although this remains to be established. The natural product tunicamycin inhibits TarO at very low concentrations (25; 57; 101), but at higher concentrations it also blocks MraY, an essential UDP-MurNAc-pentapeptide transferase involved in peptidoglycan biosynthesis (103). Tunicamycin has been used to probe the *in vitro* effects of inhibiting WTA biosynthesis, unfortunately however, it cannot be used in animals because it inhibits GPT, an essential phosphotransferase involved in eukaryotic N-linked glycan biosynthesis (103). Hence, there is still a need for specific nontoxic inhibitors that target TarO, TarA, and Dlt enzymes for use *in vivo*.

β-lactam potentiator targets

β-lactams are one of the safest and most widely used classes of antibiotics (53), and there is considerable interest in compounds that restore β-lactam sensitivity to resistant microorganisms. β-lactamase inhibitors, for example, have been highly successful as components of compound combinations to treat many β-lactam resistant infections (53). β-lactam resistance in MRSA is not mediated by β-lactamases, but by the resistant transpeptidase PBP2A (149). Since the activity of this transpeptidase depends on the function of various host factors, compound combinations are possible as therapeutic modalities (149). TarO, TarA and TarS are β-lactam potentiator targets. Unlike Δ*tarO* and Δ*tarA* strains, Δ*tarS* mutants divide normally and do not show appreciable growth defects (23). Furthermore, they are not strongly attenuated in virulence. Hence, TarS inhibitors should act exclusively as potentiators, whereas, inhibitors of TarO and TarA would serve as both β-lactam potentiators and antivirulence agents. A cell-based high-throughput β-lactam potentiation screen recently identified the clinically used antiplatelet drug ticlopidine as a TarO inhibitor, although target selectivity was not demonstrated (48). It should be possible to also identify TarS inhibitors from β-lactam potentiation screens, as they can be readily discriminated from TarO or TarA inhibitors based on their cellular effects (23).

Traditional Antibiotic Targets

Inhibitors of the essential late stage WTA biosynthetic enzymes TarB through TarH should have lethal effects on bacterial cells, and thus would be akin to traditional antibiotics. Late stage WTA pathway inhibitors (*e.g.* targocil) were discovered by screening a Δ*tarO* mutant and a wildtype strain against the same library to identify compounds that selectively killed the wildtype strain. All identified compounds were found to inhibit TarG (77; 121; 132). It has been suggested that the identification of several different inhibitors against TarG indicates that this is a druggable target. It is likely that WTA precursor export is the rate-limiting step in the pathway, and that TarG may also be more accessible to inhibitors than most other WTA targets since it spans the membrane. All identified TarG inhibitors suffer from a relatively high frequency of resistance. Consequently, the TarG inhibitors were shown to have efficacy in an MRSA infection model only in combination with β-lactams (26; 132). It would be desirable to identify compounds with a lower frequency of resistance to validate late stage enzymes as targets independent of β-lactams.

Conclusion

Wall teichoic acids comprise a huge percentage of the gram-positive cell wall and are thus extremely important for cell wall integrity. They are important for pathogenesis and play fundamental roles in bacterial physiology. Furthermore, their tailoring modifications modulate their structures and interactions with other molecules and cells in complex ways. The variety of enzyme classes involved in WTA biosynthesis (glycosyltransferases, polymerases, ABC transporters) and the diverse functions of WTAs make the ongoing study of this pathway challenging. Small molecule inhibitors of WTA enzymes can be used to probe enzyme mechanism and WTA function in cells, and to explore the potential of the different target types.

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Acronym List

WTA	wall teichoic acid
PG	peptidoglycan
TA	teichoic acid
LTA	lipoteichoic acid
poly(GroP)	poly(glycerol-3-phosphate)
poly(RboP)	poly(ribitol-5-phosphate)
GlcNAc	N-acetyl-D-glucosamine
MRSA	methicillin resistant *Staphylococcus aureus*
tag (**Tag**)	<u>t</u>eichoic <u>a</u>cid <u>g</u>lycerol
tar (**Tar**)	<u>t</u>eichoic <u>a</u>cid <u>r</u>ibitol

Mini-Glossary

polyol	a compound having multiple hydroxyl functional groups
transesterification	the intra- or intermolecular nucleophilic attack of a TA D-alanyl ester by a free TA backbone hydroxyl
PBP	<u>p</u>enicillin <u>b</u>inding <u>p</u>rotein, which synthesizes peptidoglycan by elongating and crosslinking the glycan chains
synthetic lethality	synthetic lethality occurs when the simultaneous disruption of two nonessential genes or processes results in a loss of viability

autolysins	bacterial enzymes that hydrolyze peptidoglycan during cell growth and division; they enable incorporation of new material into the cell wall and promote cell separation (115)
virulence factor	a factor essential for bacterial colonization and pathogenesis, but non-essential for survival *in vitro*
biofilm	multilayered communities of bacterial cells embedded in a self-produced matrix (127)
traditional antibiotic target	a bacterial protein or pathway that is essential for survival so that small molecule inhibition is lethal
potentiator	a small molecule that increases the potency of an antibiotic when used in combination

Summary Points List

1. WTAs are covalently attached to PG and comprise up to 60% of the cell wall mass in gram-positive bacteria.

2. The first three steps in WTA biosynthesis, catalyzed by TagO, TagA, and TagB, lead to the formation of a lipid-anchored disaccharide-glycerol-phosphate and are conserved across gram-positive bacteria.

3. Cells lacking WTAs have gross defects in cell division and cell morphology. This and other evidence indicate that the WTA and PG biosynthetic pathways are connected.

4. How WTAs function to regulate normal cell physiology is still unclear. They are thought to scaffold some cell wall assembly proteins, exclude other such proteins, and influence protein activity through ion chelation.

5. WTAs are required for survival in a host.

6. WTA tailoring modifications are involved in biofilm formation, virulence, and antimicrobial resistance.

7. The WTA pathway is a possible target for β-lactam potentiators, antivirulence agents, and novel antibiotics.

Future Issues List

1. While most of the steps in WTA biosynthesis have been elucidated, major questions remain regarding the mechanisms of polymer export and attachment to PG.

2. The pathway for D-alanylation of TAs is poorly understood. In particular, the enzymatic functions of DltB and DltD are unknown.

3. There is a long standing controversy regarding whether WTA precursors are attached to old or new PG. This issue has a direct bearing on how WTAs may regulate PG biosynthesis and cell division.

4. The roles of distinct tailoring modifications in modulating WTA function remain unclear. Glycosylated WTAs are required to maintain β-lactam resistance in MRSA, but a molecular mechanism to explain this observation is lacking.

5. While small molecules that inhibit WTA synthesis have been identified and some have been shown to have efficacy in animal models, the WTA pathway as a therapeutic target has yet to be validated.
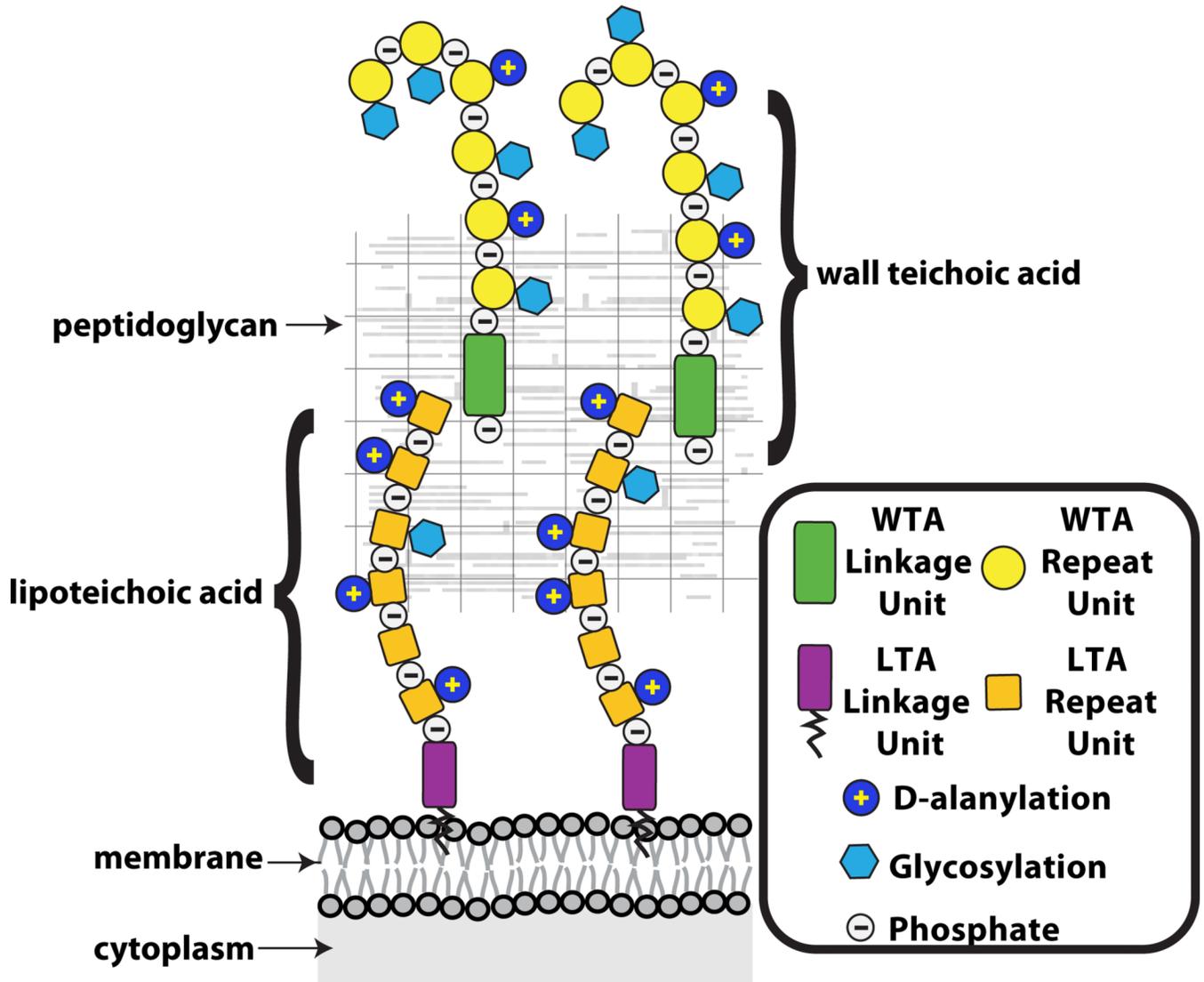
Figure 1. Teichoic acid polymers are located within the gram-positive cell wall
Schematic of the gram-positive cell wall showing that wall teichoic acids are covalently anchored to peptidoglycan and lipoteichoic acids are tethered to the membrane. The WTAs extend beyond the PG layer, whereas fully extended LTAs may not be able to reach pass the PG layer.
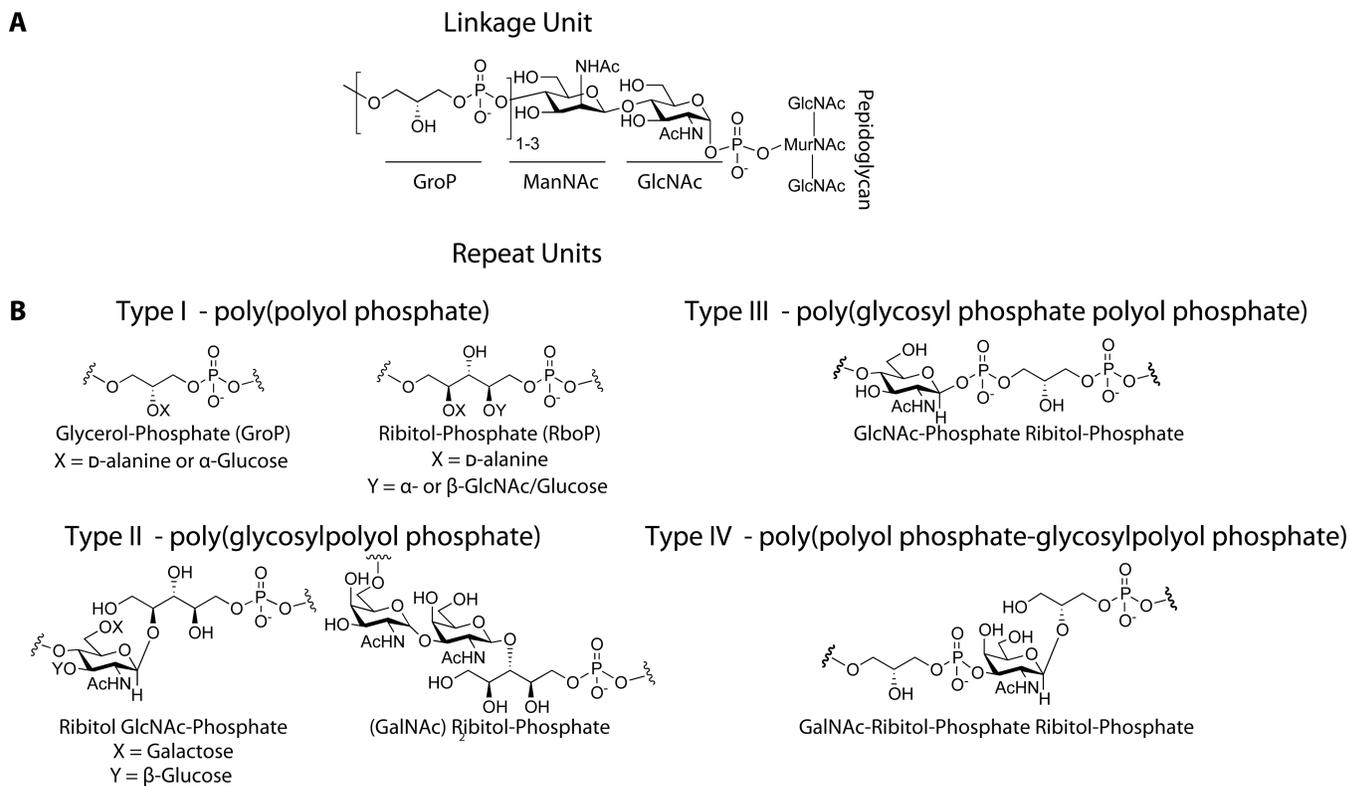
A   Linkage Unit

| GroP | ManNAc | GlcNAc |

Repeat Units

B   Type I - poly(polyol phosphate)   Type III - poly(glycosyl phosphate polyol phosphate)

Glycerol-Phosphate (GroP)   Ribitol-Phosphate (RboP)   GlcNAc-Phosphate Ribitol-Phosphate
X = D-alanine or α-Glucose   X = D-alanine
                                  Y = α- or β-GlcNAc/Glucose

Type II - poly(glycosylpolyol phosphate)   Type IV - poly(polyol phosphate-glycosylpolyol phosphate)

Ribitol GlcNAc-Phosphate   (GalNAc)₂ Ribitol-Phosphate   GalNAc-Ribitol-Phosphate Ribitol-Phosphate
   X = Galactose
   Y = β-Glucose

Figure 2. Wall teichoic acid polymers share a common linkage unit, but exhibit structural diversity in their repeat units

A. The commonly characterized WTA linkage unit consists of a GroP-ManNAc-GlcNAc-Phosphate that is covalently attached to peptidoglycan. While the stereochemistry of the glycosidic linkage has not been clearly established, it is assumed to be retained as α based on predicted enzymatic catalysis.

B. Four different classes of WTA repeat units found in gram-positive bacteria (90).
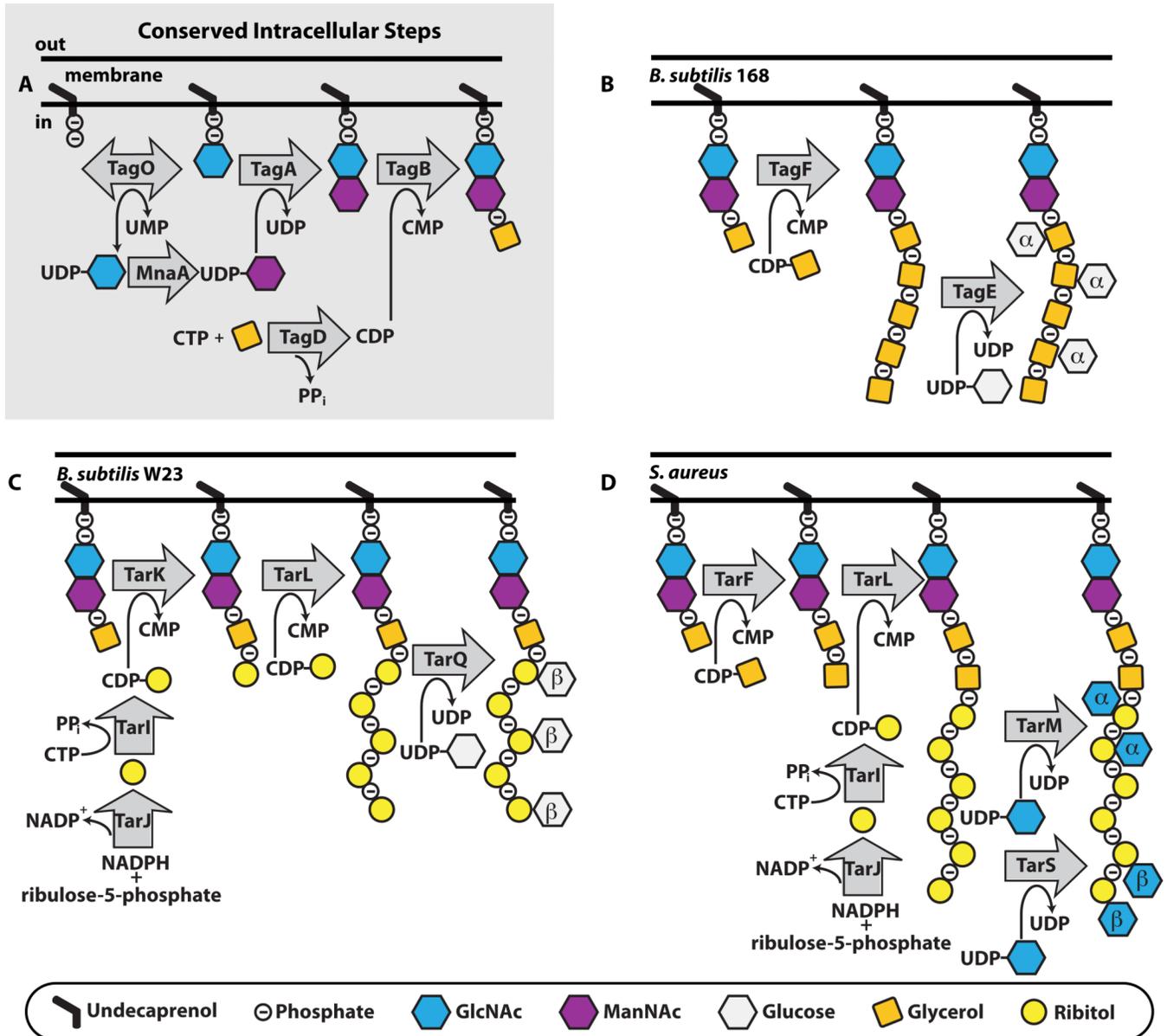
Figure 3. In different organisms wall teichoic acid biosynthesis begins with the same initial steps, but then the pathways diverge

A. The initial steps to WTA polymer formation that has been found in all thus far characterized pathways. B,C,D. The end stage intracellular steps to form the WTA polymer in *B. subtilis* 168 (B), *B. subtilis* W23 (C) and S. aureus (D). TarIJ reactions to form CDP-ribitol exist in both *B. subtilis* W23 and *S. aureus*. The α- and β- within the sugar denotes the stereochemistry of attachment. We have drawn α- and β-GlcNAc attachment to *S. aureus* occurring on the same strand, but it is possible that the TarS and TarM enzymes do not attach GlcNAc to the same polymer.
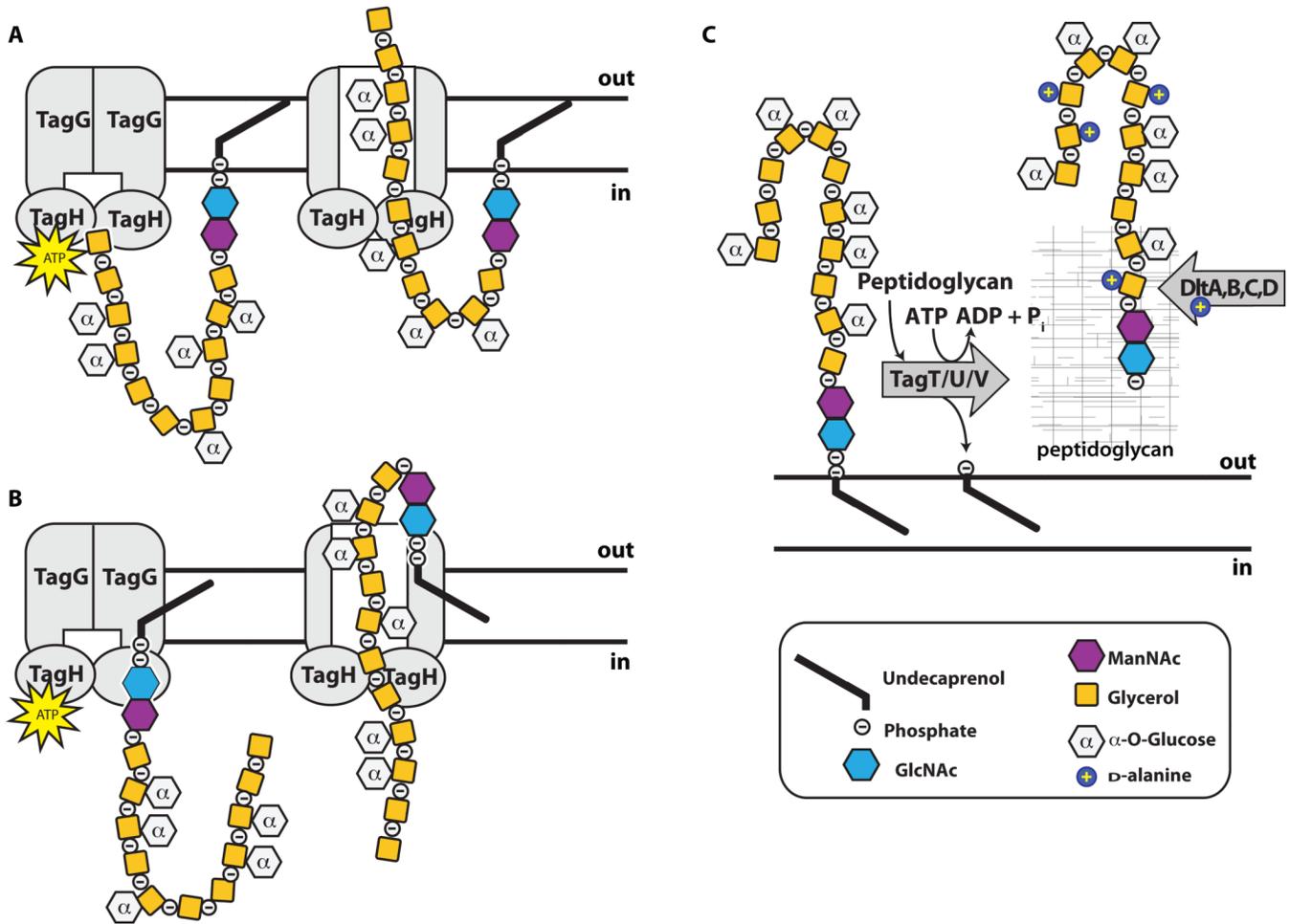
Figure 4. Wall teichoic acid polymer flipping and attachment to peptidoglycan in *B. subtilis* 168 proceeds through a series of enzymes

Graphical representation of the poly(GroP) WTA polymer in *B. subtilis* 168 transported through the cell membrane by TagGH. Homology suggests energy generated by TagH ATP hydrolysis drives a conformational change that allows for polymer transport. TagGH can presumably either thread the polymer through the membrane by the non-reducing end (A) or recognize the disaccharide linkage unit and flip the polymer across (B). Following transport (C), the polymer is covalently attached to PG by TagTUV. It is unknown whether the TagTUV enzymes work independently or together to ligate WTA to PG. The presumed energy source for this ligation reaction, which we designated as ATP hydrolysis, is undetermined. It remains unknown whether extracellular D-alanylation by DltABCD occurs before or after WTA is covalently attached to PG.
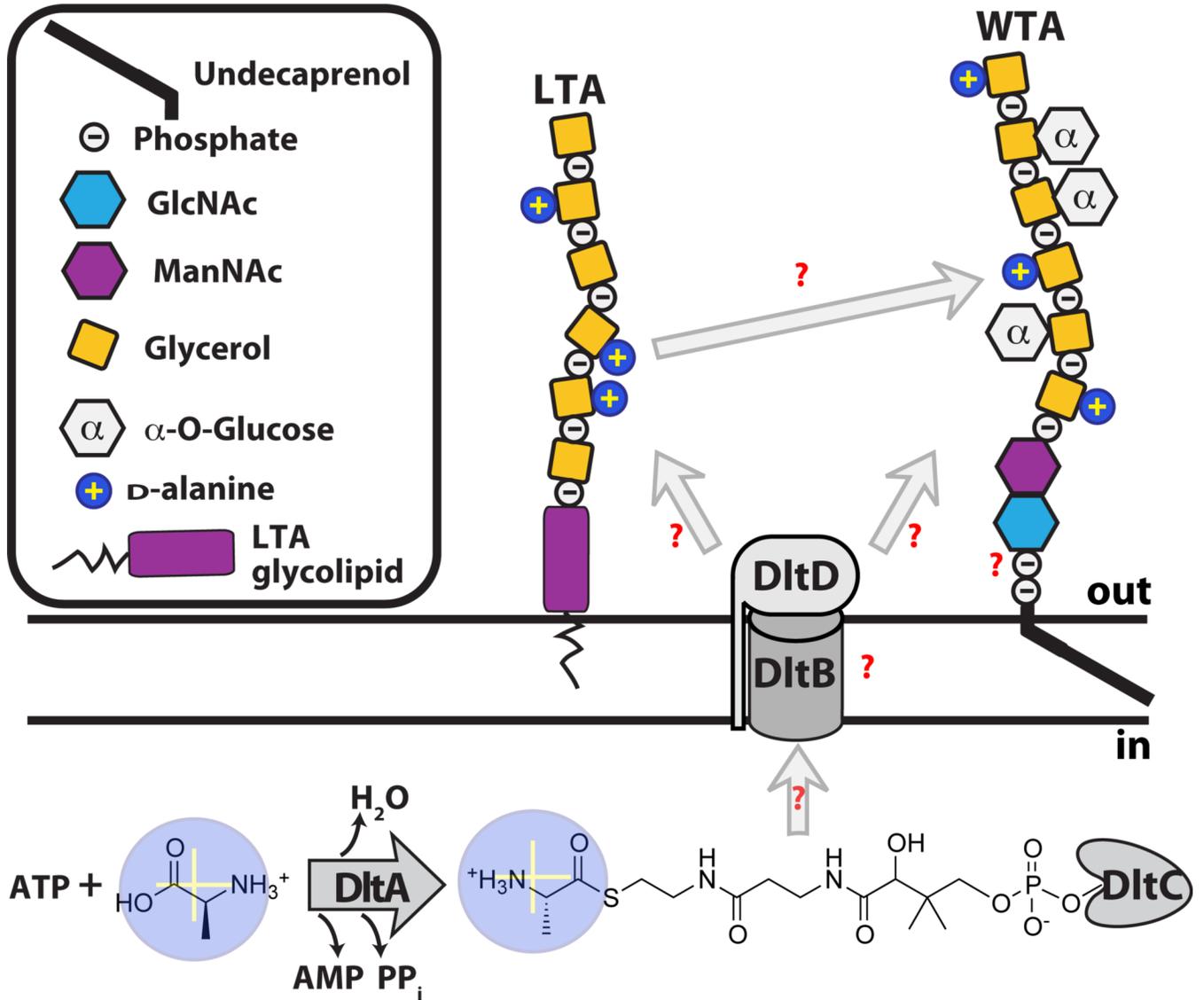
Figure 5.
D-alanylation of teichoic acids. The DltABCD machinery is responsible for the activation and attachment of D-alanines to extracellular TAs. Many questions remain regarding the roles of DltB and DltD, the nature of their substrates, and whether D-alanine transesterification is enzymatically catalyzed.
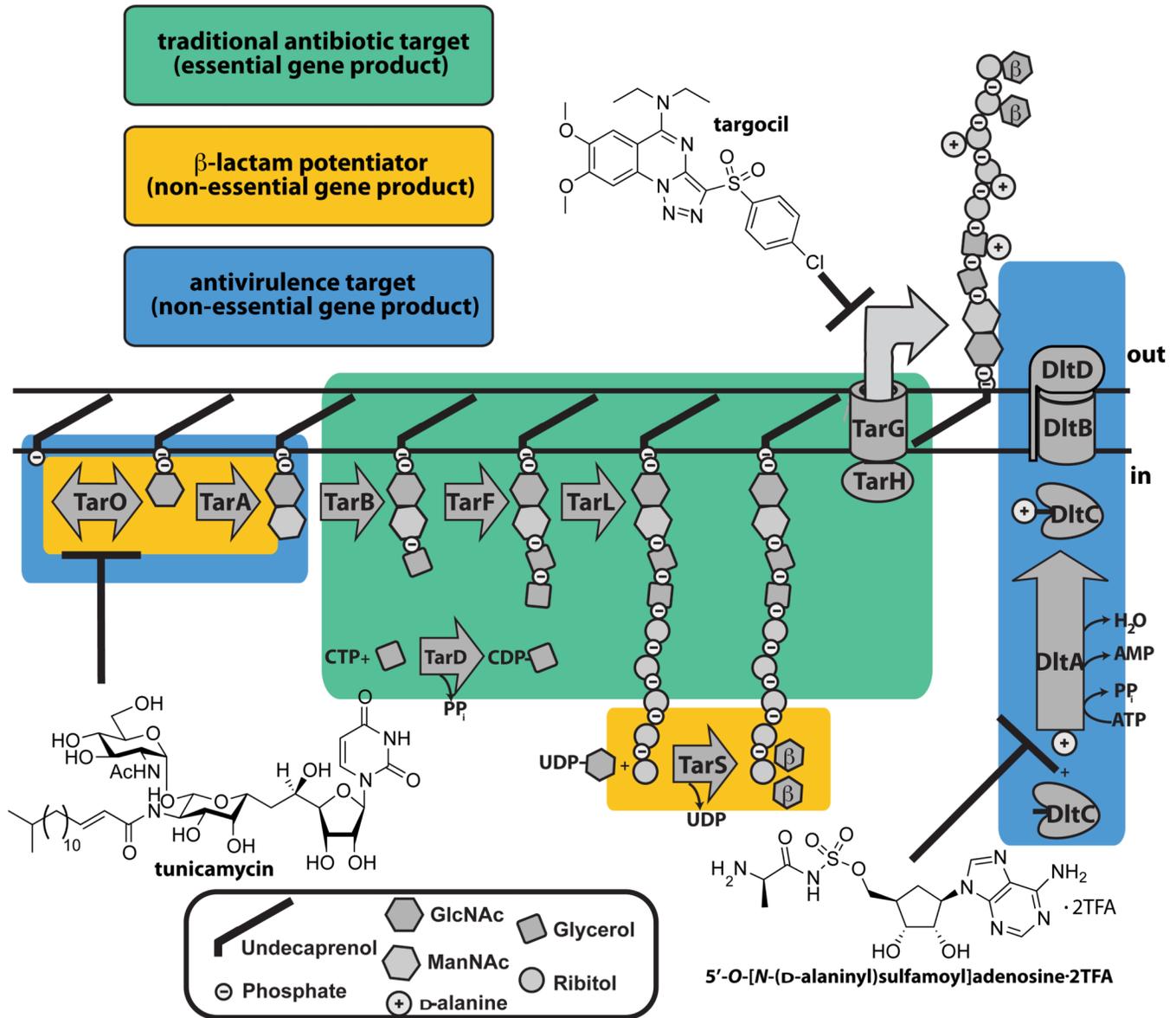
Figure 6. Wall teichoic acid biosynthetic enzymes are potential antibiotic targets
Schematic showing the three types of antibacterial targets present in the *S. aureus* WTA pathway. Enzymes that are traditional, β-lactam potentiators, or antivirulence antimicrobial targets are boxed. The three chemical structures illustrated are small molecules that are known to inhibit the WTA enzymes, TarO, TarG, and DltA.