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REVIEW ARTICLE

# Taking aim at wall teichoic acid synthesis: new biology and new leads for antibiotics

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**Wall teichoic acids are a major and integral component of the Gram-positive cell wall. These structures are present across all species of Gram-positive bacteria and constitute roughly half of the cell wall. Despite decades of careful investigation, a definitive physiological function for wall teichoic acids remains elusive. Advances in the genetics and biochemistry of wall teichoic acid synthesis have led to a new understanding of the complexity of cell wall synthesis in Gram-positive bacteria. Indeed, these innovations have provided new molecular tools available to probe the synthesis and function of these cell wall structures. Among recent discoveries are unexpected roles for wall teichoic acid in cell division, coordination of peptidoglycan synthesis and β-lactam resistance in methicillin-resistant *Staphylococcus aureus* (MRSA). Notably, wall teichoic acid biogenesis has emerged as a *bona fide* drug target in *S. aureus*, where remarkable synthetic-viable interactions among biosynthetic genes have been leveraged for the discovery and characterization of novel inhibitors of the pathway.**
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## INTRODUCTION

Wall teichoic acids are a major component of the Gram-positive cell wall, present in roughly equal proportions to peptidoglycan (Figure 1).[1] Initially thought to have an ancillary role in bacterial physiology, wall teichoic acids have emerged in recent years as a critical component of the cell wall of Gram-positive bacteria. Functional roles for wall teichoic acids include determination of cell shape in the model Gram-positive bacterium *Bacillus subtilis*, and in *Staphylococcus aureus*, these structures serve important roles in host colonization, coordination of peptidoglycan synthesis and resistance to β-lactam antibiotics.[2–8] Interestingly, wall teichoic acid biosynthetic proteins are encoded by genes that exhibit paradoxical gene dispensability patterns; deletions in early genes in the pathway result in viable but non-infectious organisms, and exhibit synthetic-viable interactions with otherwise essential late-acting genes.[2,7,9,10] Synthetic viability describes a genetic interaction in which the lethal phenotype associated with interruption of an essential gene is suppressed by simultaneous disruption of a second (non-essential) gene. The discovery of such synthetic-viable interactions in wall teichoic acid synthesis has enabled the rational discovery and characterization of novel antibacterial compounds that block wall teichoic acid biogenesis in *S. aureus*. Indeed, where methicillin-resistant *S. aureus* (MRSA) is a leading Gram-positive pathogen in an infectious disease crisis of global scale, wall teichoic acid appears to be an exciting new target for antibacterial chemical matter of unique chemical class and mechanism.[11–13]

Early foundational work on the chemistry and biology of wall teichoic acids, named after the Greek word τειχοζ (teichos; wall), was carried out during the 1950s and 1960s on strains of *Bacilli*, *Lactobacilli* and *Staphylocci*.[14] Nucleotide-activated ribitol-phosphate and glycerol-phosphate were first identified in lysates of *Lactobacillus arabinosus* and, based on their similarity to Park's nucleotide and precursors to other glycosyl-based macromolecules, were predicted to have a role in cell wall formation.[15,16] Follow-up inquiry into the biological function of activated glycosyl units led to the first identification of poly(ribitol-phosphate) in the wall of *L. arabinosus*.[17,18] Early examination of the structure of teichoic acids showed the common presence of polymers composed of either 1–3 linked units of glycerol-3-phosphate or 1–5 linked units of ribitol-5-phosphate decorated with hexose or *N*-acetylhexosamine residues, respectively, and D-alanine.[19,20] This work was followed by the demonstration that both nucleotide precursors and wall teichoic acids were present across a wide range of Gram-positive organisms.[14,21] As technologies in sample preparation and analysis advanced, a common linkage unit that anchored wall teichoic acid polymers to peptidoglycan was described, containing (glycerol-phosphate)-*N*-acetylmannosamine-β(1–4)-*N*-acetylglucosamine, and covalently linked to the 6′ hydroxyl of *N*-acetylmuramic acid residues of peptidoglycan (Figure 2).[22–24] Advancements in tools for molecular biology and biochemistry enabled elucidation of the genetic requirements for wall teichoic acid biosynthesis, and the indispensable phenotype of various wall teichoic acid biosynthetic genes suggested an essential role for these structures in *B. subtilis*.[25–27] Most recently, the development of soluble substrate analogs for teichoic acid biosynthetic intermediates has allowed for *in vitro*

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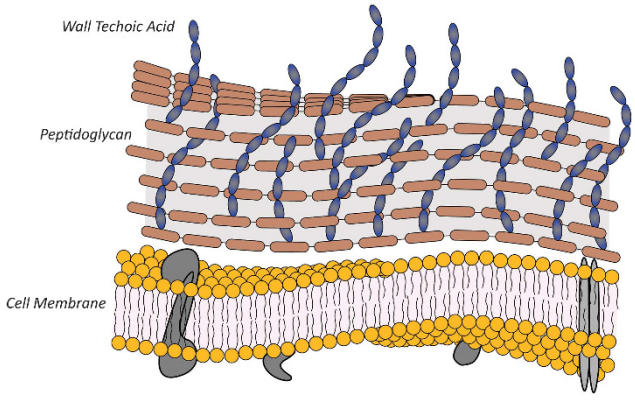



**Figure 1** The Gram-positive cell wall.

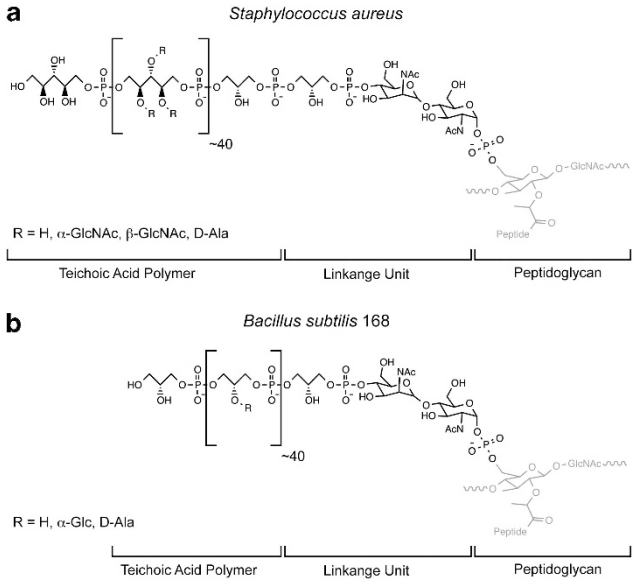



**Figure 2** Structures of common wall teichoic acids. (**a**) Poly(ribitol-phosphate) wall teichoic acid from *S. aureus*. (**b**) Poly(glycerol-phosphate) wall teichoic acid from *B. subtilis* 168. Ala, alanine; Glc, glucose; GlcNAc, *N*-acetylglucosamine.

biochemical characterization of recombinant wall teichoic acid biosynthetic proteins.[28–32] Cumulatively, this work led to the definition of both the structures and biosynthetic requirements of canonical poly(glycerol-phosphate) and poly(ribitol-phosphate) wall teichoic acids in *B. subtilis* 168 and *S. aureus*, respectively (Figure 2).

## SYNTHESIS OF WALL TEICHOIC ACID IN *B. SUBTILIS* 168 AND *S. AUREUS*

A number of Gram-positive bacteria are known to produce wall teichoic acid polymers having complex repeating units containing, for example, mannitol, erythritol, glucose or *N*-acetylglucosamine; however, a large proportion produce either poly(ribitol-phosphate) or poly(glycerol-phosphate) as the major polymer.[33–35] Much of our knowledge of the genetics and biochemistry of wall teichoic acid synthesis has been elaborated in recent years in the model Gram-positive bacterium *B. subtilis* 168 and the pathogen *S. aureus*, which, respectively, produce poly(glycerol-phosphate) and poly(ribitol-phosphate) wall teichoic acids. In these organisms, wall teichoic acid is synthesized by *tag* (teichoic acid glycerol) or *tar* (teichoic acid ribitol) gene products. Regardless of polymer structure, wall teichoic acid synthesis occurs intracellularly on an undecaprenyl-phosphate lipid carrier that anchors the nascent polymer to the cytoplasmic face of the cellular membrane (Figure 3). Synthesis of a common linkage unit is initiated by TagO/TarO catalyzed transfer of an *N*-acetylglucosamine-1-phosphate residue from UDP-*N*-acetylglucosamine to undecaprenyl-phosphate to form the teichoic acid intermediate lipid $\alpha$[36] (Table 1 describes a straightforward nomenclature for teichoic acid intermediates based on the cognate enzyme.[37] Lipid $\alpha$ is the substrate for TagA, lipid $\beta$ is the substrate for TagB, etc. For polymeric substrates, such as the TagF substrate lipid $\phi.n$, *n* denotes the number of repeating units in the polymer). Sequentially, TagA/TarA catalyzes the transfer of an *N*-acetylmannosamine residue to lipid $\alpha$ to generate lipid $\beta$.[28,29] Linkage unit synthesis is finalized by TagB/TarB with the addition of an *sn*-glycerol-3-phosphate unit yielding lipid $\phi.1$.[28,38] Following linkage unit synthesis, steps for the completion of poly(glycerol-phosphate) and poly(ribitol-phosphate) polymers are unique to the respective polymers. In poly(glycerol-phosphate)-containing organisms, roughly 40 glycerol phosphate units are polymerized directly on lipid $\phi.1$ via the TagF enzyme.[39–41] Lipid-linked $\phi.40$ is modified with multiple $\alpha$-glucose units via the TagE enzyme, and exported to the extracellular surface of the cytoplasmic membrane via the TagGH ABC transport system.[42–45] In contrast, in poly(ribitol-phosphate)-containing organisms, lipid $\phi.1$ is elaborated with an additional glycerol phosphate unit via the TarF enzyme to yield lipid $\phi.2$, before ribitol phosphate polymerization via TarL.[10,32,46] Intracellular decoration of lipid $\lambda.40$ in *S. aureus* includes modifications with both $\alpha$-linked and $\beta$-linked *N*-acetylglucosamine, catalyzed by TarM and TarS, respectively.[8,47] Similar to the export of poly(glycerol-phosphate), poly(ribitol-phosphate) is transported to the extracellular surface of the cytoplasmic membrane by the TarGH ABC transport system. Once exported, the lipid-linked teichoic acid polymer is thought to be D-alanylated by gene products from the *dltABCD* operon and transferred from the undecaprenol lipid carrier to peptidoglycan.[48] The final transfer of wall teichoic acid polymers from undecaprenol phosphate to muramic acid of peptidoglycan is thought to be carried out by a redundant set of three enzymes, namely LytR, Cps2a, Psr in *B. subtilis* 168 and Msr, SA0908 and SA2101 in *S. aureus*.[49,50] The latter biosynthetic assignments await *in vitro* biochemical confirmation.

## WALL TEICHOIC ACID IS A VIRULENCE FACTOR REQUIRED FOR HOST COLONIZATION

Wall teichoic acid is believed to have diverse functional roles including cation binding, osmotic tolerance, heat tolerance, regulation of autolysins, phage-binding and cell-shape determination.[2,51–54] Further, the more recent discovery of the essential role for wall teichoic acid in coordinating mechanisms required for host infection and drug resistance in *S. aureus* has bolstered a concerted effort to uncover mechanisms underlying this physiology, and to discover inhibitors of wall teichoic acid biosynthesis for development as antimicrobial leads.[5,6,55,56]

The importance of wall teichoic acid as a virulence factor was first elaborated by the Peschel group.[3] In this study, the authors used a Δ*tarO* strain of *S. aureus* in a cotton rat nasal colonization model. Remarkably, after a 7-day incubation period, there was a complete absence of bacterial colonization in rats inoculated with the Δ*tarO* strain, whereas all rats in the test group inoculated with the isogenic wild-type strain were colonized ($n = 15$ per group). Furthermore, the

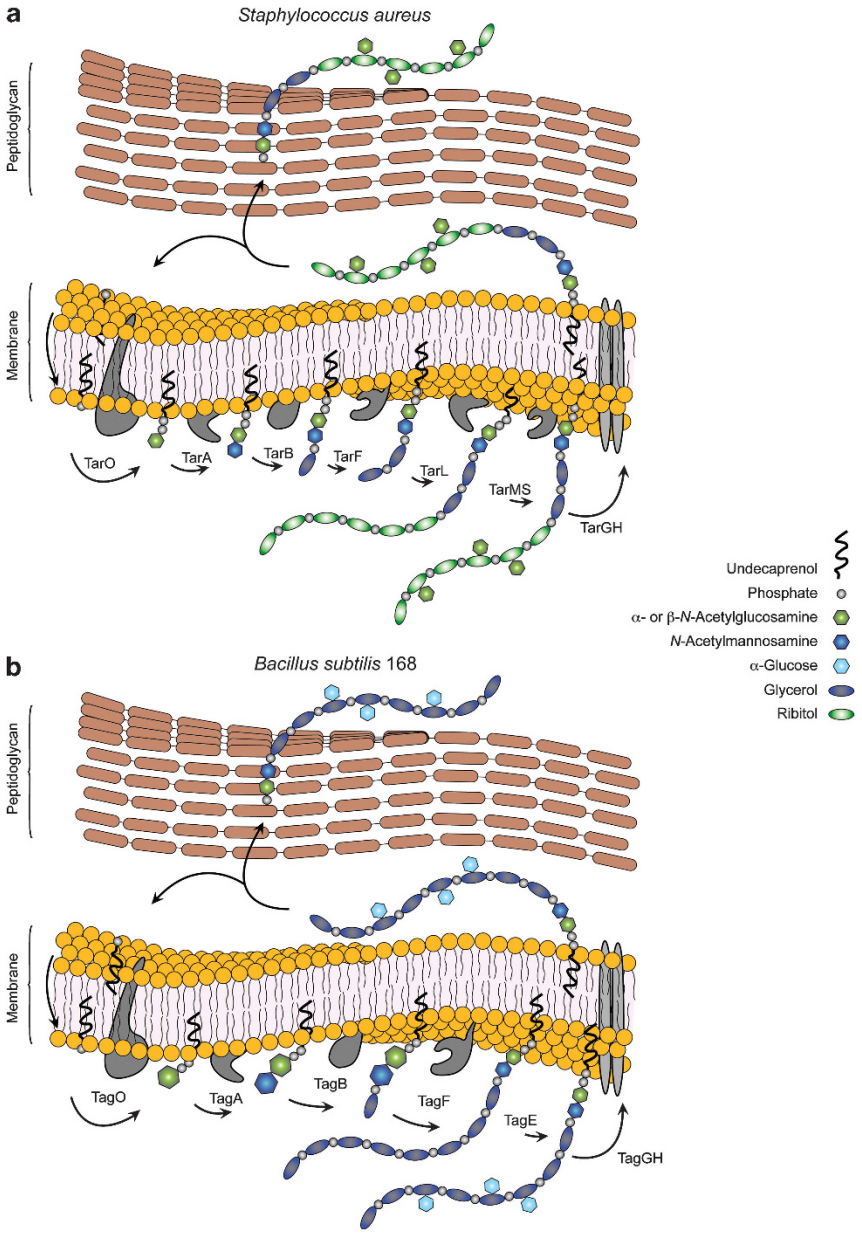



**Figure 3** Wall teichoic acid synthesis in (**a**) *S. aureus* and (**b**) *B. subtilis* 168.

Δ*tarO* mutation caused a decrease in the capacity of *S. aureus* to both establish and disseminate infection in a rabbit endocarditis model.[58] Careful investigation of the mechanistic underpinnings of the contribution of wall teichoic acid to these phenotypes was pursued. In an assessment of the effect of the Δ*tarO* genotype on the capacity of *S. aureus* to adhere to blood constituents, it was discovered that the mutation led to a reduction in bacterial adhesion to endothelial cells in *in vitro* models.[58] These data established a role for wall teichoic acids in colonization via host tissue adhesion, and indicated a connection between deficiencies in *in vivo* bacterial proliferation and wall teichoic acid. The association between defects in wall teichoic acid and bacterial proliferation *in vivo* has been substantiated by reports of additional Δ*tarO* phenotypes in cell division and in susceptibility to innate immune components, including antimicrobial peptides, defencins and antimicrobial fatty acids.[5,57,59,60] Collectively, this work established a strict requirement for wall teichoic acid in the virulence of *S. aureus*, and helped to substantiate wall teichoic acid biosynthesis as an antibacterial drug target.

## COMPLEX DISPENSABILITY PATTERNS GIVE RISE TO SYNTHETIC-VIABLE GENETIC INTERACTIONS AMONG WALL TEICHOIC ACID BIOSYNTHETIC GENES

Before the aforementioned reports investigating virulence of a Δ*tarO* *S. aureus* strain, wall teichoic acids were thought to be essential cellular structures. This conclusion was based on essential phenotypes evident for various wall teichoic acid biosynthetic genes.[25–27] The revelation by the Peschel group that the *tarO* gene was dispensable in *S. aureus* led the Brown laboratory to undertake a meticulous reassessment of the dispensability of wall teichoic acid biosynthetic genes in *B. subtilis* 168 and *S. aureus* strain RN4220, chosen for its genetic malleability.[2,9] These investigations confirmed and extended

**Table 1 Nomenclature for lipid-linked wall teichoic acid intermediates**

| *Enzyme* | *Lipid-linked substrate* | *Chemical composition* |
|---|---|---|
| TagA/ TarA | Lipid α | GlcNAc-P-P-und |
| TagB/ TarB | Lipid β | ManNAc-β(1–4)-GlcNAc-P-P-und |
| TarF | Lipid ϕ.*1* | GroP-ManNAc-β(1–4)-GlcNAc-P-P-und |
| TagF | Lipid ϕ.*n* | $(GroP)_n$-ManNAc-β(1–4)-GlcNAc-P-P-und |
| TarL | Lipid λ..*n* | $(RboP)_n$-$(GroP)_2$-ManNAc-β(1–4)-GlcNAc-P-P-und |

Abbreviations: GlcNAc, *N*-acetylglucosamine; GroP, *sn*-glycerol-3-phosphate; ManNAc, *N*-acetylmannosamine; P, phosphate; RboP, ribitol-5-phosphate; und, undecaprenol. Intermediates are named according to the cognate enzyme that utilizes the molecule as a substrate. For oligomeric and polymeric substrates, the number of repeating units is represented by *n*.

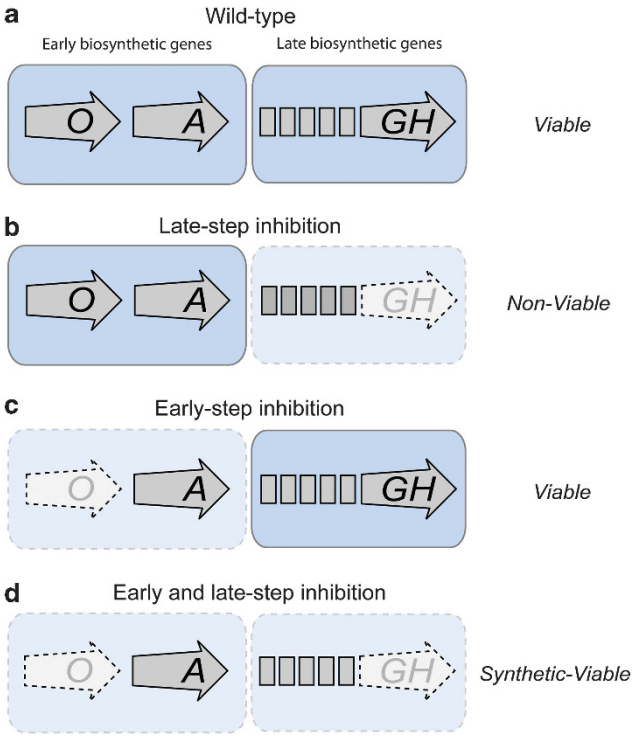



**Figure 4** Synthetic-viable interactions between early- and late-acting wall teichoic acid biosynthetic genes. Deletions in late steps in wall teichoic acid biosynthesis are non-viable, whereas deletions in early steps are viable and can rescue the lethal phenotype of a late-step interruption. Depicted is a series of examples using the early gene *tarO* and late genes *tarGH*. (**a**) Wild-type organisms with intact early- and late-acting wall teichoic acid biosynthetic genes synthesize wall teichoic acid and are viable. (**b**) Interruption of late-steps, Δ*tarGH* shown, blocks wall teichoic acid synthesis and is non-viable. (**c**) Interruption of early steps, Δ*tarO* shown, blocks wall teichoic acid synthesis is and viable. (**d**) Simultaneous interruption of early and late steps blocks wall teichioc acid synthesis and is viable. Additional synthetic-viable combinations include Δ*tarO*/Δ*tagO* in combination with any late-acting gene deletion (*tarBDFGHIJL/tagBDFGH*), or Δ*tarA*/Δ*tagA* in combination with any late-acting gene deletion (*tarBDFGHIJL/tagBDFGH*).

previous studies revealing essential phenotypes both in *B. subtilis* 168 and *S. aureus* for genes encoding wall teichoic acid priming (*tagB*, *tarB*, *tarF*), polymerization (*tagF*), nucleotide-activated precursor synthesis (*tagD*, *tarD*, *tarI*, *tarJ*) and export (*tarH*). Paradoxically, these investigations also confirmed that viable and stable deletions could be generated in the first step in wall teichoic acid biosynthesis carried out by the *tagO*/*tarO* gene product. This paradox was resolved by demonstrating synthetic-viable genetic interactions between early and late steps. Indeed, both in *B. subtilis* 168 and *S. aureus*, the lethal phenotypes of all of the so-called 'essential' wall teichoic acid genes could be suppressed by deletion of *tagO*/*tarO*. The *tagA*/*tarA* gene, encoding the second step in wall teichoic acid synthesis, was also found to be dispensable and deletions therein could similarly suppress lethal phenotypes associated with loss of the downstream genes.[7] The presence of two poly(ribitol-phosphate) polymerase genes in *S. aureus* has confounded definitive assignment of the dispensability of these genes, with indications that either one or both of the *tarK* and *tarL* polymerase genes are required to support growth in a wild-type background.[10,46] It is clear, however, that non-viable interruptions in polymerization step(s) can be rescued by deletion of *tarO* or *tarA*, similarly to remaining late-acting steps in wall teichoic acid synthesis. Through these genetic experiments, teichoic acid biosynthetic genes have been grouped into dispensable early-acting genes (*tagO*, *tagA*, *tarO*, *tarA*), or conditionally essential late-acting genes (*tagB*, *tagD*, *tagF*, *tarB*, *tarD*, *tarF*, *tarI*, *tarJ*, *tarK*, *tarL*). Furthermore, late-acting genes are essential in a wild-type background, but become dispensable in a Δ*tagO*/Δ*tarO* or Δ*tagA*/Δ*tarA* genetic background (Figure 4).

Interestingly, additional synthetic-viable interactions have been noted recently in the MRSA strain COL. The TarG-mediated wall teichoic acid export step in this strain is essential in a wild-type background, however, viable mutations in strains resistant to inhibitors of the TarG exporter have been uncovered in the late-acting genes *tarB* and *tarD*.[55] Although *tarB* and *tarD* have essential phenotypes in *S. aureus* strain RN4220,[9] characterization of the *S. aureus* COL *tarB* and *tarD* suppressor mutants suggested loss-of-function mutations, and teichoic acid was found to be absent from the cell walls of these mutants. Nevertheless, *tarB* and *tarD* mutations conferred sensitivity to osmotic, temperature and antibiotic stresses that could be rescued by inactivation of *tarO*.[55] Thus, although the observation of viable interruptions in late-acting genes in *S. aureus* COL is at odds with the binary essential/dispensable phenotypes documented in strain RN4220, a general relationship remains consistent: interruptions in late-acting genes give rise to compromised fitness, albeit of varying severity, that can be rescued by simultaneous inactivation of an early-acting gene. Genetic differences between *S. aureus* strains RN4220 and COL may explain the varying severity of phenotypes associated with deletions in late-acting steps within these strains. *S. aureus* strain RN4220 has been subjected to repeated mutagenesis to render it amenable to transformation with foreign DNA.[61] This strain harbors interruptions in $\sigma^B$ regulatory machinery, preventing the expression of associated stress response mechanisms.[62] Stress sensing and response machinery is a vital component of an organism's ability to survive cellular stressors. In the absence of this system, interruption of late-acting steps in wall teichoic acid synthesis may go unrecognized in strain RN4220 until a cell-death cascade is triggered. Conversely, in strain COL, a $\sigma^B$-mediated response could coordinate the required machinery to mitigate the deleterious effects of inhibition of wall teichoic acid biosynthesis. Ultimately, it is clear that genetic interactions among wall teichoic acid biosynthetic genes are complex and strain-dependent. Nevertheless, strong trends have emerged from a large body of work on this topic: the presence of teichoic acid polymers in the cell walls of *B. subtilis* 168 or *S. aureus* are not strictly essential, but deletion of a late-acting biosynthetic gene

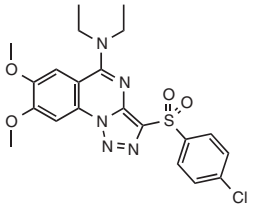
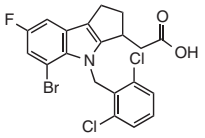
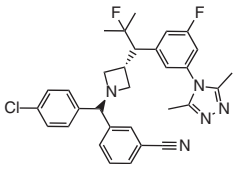
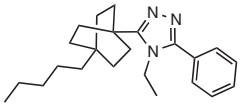
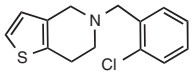
results in a fitness defect, the severity of which may range from sensitization to antibiotic stress, to death, and is dependent on both the bacterial strain and identity of the interrupted gene. Furthermore, late-acting genes display synthetic-viable interactions with early-acting genes, where the lethal phenotype caused by deletion of a late step can be suppressed by inactivation of an early step.

Insight into the mechanism underlying synthetic-viable interactions between early- and late-acting genes in wall teichoic acid synthesis was gained through an unbiased systems-scale investigation charting gene–gene and gene–chemical interactions in *B. subtilis* 168.[63] In this study, the promoter $P_{ywaC}$ was identified through microarray analysis as mounting an especially strong response following depletion of late enzymes in *B. subtilis* 168. Probing the $P_{ywaC}$ promoter with a well-characterized library of bioactive small molecules (bioactives) demonstrated a similar induction of the promoter by cell–wall bioactives, with a strong bias toward chemicals that inhibit undecaprenol-linked metabolism. Furthermore, bioinformatic analysis examining gene–gene connectivity through genomic context inferred strong connectivity among genes involved in the biosynthesis of wall teichoic acid, undecaprenol and peptidoglycan. Taken together, these data suggested a model for the synthetic viability observed among early and late wall teichoic acid genes: deletion of a late-acting gene interrupts wall teichoic acid biosynthesis, resulting in the accumulation of dead-end undecaprenol-linked intermediates. Accumulation of undecaprenol-linked wall teichoic acid intermediates may deplete a limited cellular store of undecaprenol, similarly required for the synthesis of peptidoglycan, and thus result in cell death. Indeed, in a *B. subtilis* 168 strain with the *tagF* polymerase gene under the control of an inducible promoter, depletion of TagF resulted in a decrease in incorporation of D-[$^{14}$C]glutamate into newly synthesized peptidoglycan.[63] Alternately, as the structural integrity of membrane bilayers is determined by the physicochemical properties of bilayer components, it is possible that accumulation of undecaprenol-linked wall teichoic acid intermediates on the inner leaflet of the cytoplasmic membrane has a direct destabilizing effect on the bilayer.[64] In either scenario, the deleterious accumulation of undecaprenol-linked wall teichoic acid intermediates can be avoided by preventing metabolite flux into the pathway with the deletion of the *tagO* or *tagA* genes.

## SYNTHETIC VIABILITY OFFERS A PLATFORM FOR PATHWAY-SPECIFIC, CELL-BASED SCREENING FOR WALL TEICHOIC ACID INHIBITORS

The discovery of unique synthetic-viable genetic interactions among wall teichoic acid genes underpinned the development of pathway-specific, cell-based screening for inhibitors of wall teichoic acid synthesis. The approach, which enables facile mechanistic follow-up on whole-cell-bioactive compounds while eliminating off-target nuisance compounds, has been successfully used in both academe and the pharmaceutical sector to discover inhibitors of the wall teichoic acid transport protein TarG.[55,56] First published by the Walker laboratory, compounds that are growth inhibitory to *S. aureus* are counter-screened against a *tarO* deletion strain.[56] Chemicals leading to growth inhibition in the wild-type, and not the Δ*tarO* strain, are predominantly late-step inhibitors of wall teichoic acid synthesis. Compound 1835F03 was discovered and its potency was optimized through a structure–activity relationship analysis to yield targocil (Table 2).[65] Interestingly, targocil has selective efficacy against *S. aureus* and no growth inhibitory effects on *B. subtilis* 168. *In vitro* enzymatic assays have been published for all intracellular steps in wall teichoic acid synthesis and these assays were subsequently used to rule out TarBDFIJL enzymes as potential targets. This pointed to either export or teichoic acid-peptidoglycan ligase enzymes as likely targets of targocil. Overexpression of *tarGH* was found to confer resistance, whereas expression of *tagGH* from the unsusceptible *B. subtilis* 168 in *S. aureus* conferred complete resistance. Mapping of spontaneous suppressor mutations conferring resistance to the action of targocil also identified TarG, the translocase component of the ABC export complex, was the antibiotic target.

**Table 2 Wall teichoic acid synthesis inhibitors**

| *Chemical name or series* | *Structure* | *Target* | *Reference* |
|---|---|---|---|
| Targocil |  | TarG | 56,65 |
| Tricyclic indole acid |  | TarG | 55 |
| *N*-aryl triazole |  | TarG | 55 |
| *C*-aryl triazole |  | TarG | 55 |
| Ticlopidine |  | TarO | 6 |

Similarly, Merck and Company used this methodology to uncover inhibitors of wall teichoic acid biosynthesis within its whole-cell-bioactive collection of 20 000 anti-*Staphylococcal* small molecules.[55] In this work, three diverse chemical classes of late-stage wall teichoic acid inhibitors were uncovered: tricyclic indole acids, *N*-aryl-triazoles and *C*-aryl-triazoles (Table 2). Mapping of suppressor mutations to the *tarG* gene once again implicated TarG as a target, and the inhibitors were found to be effective in reducing bacterial burden in a mouse thigh infection model. In both studies, the frequency of resistance to TarG inhibitors was high ($\sim 7 \times 10^6$ at 8 × MIC), as resistance could be generated not only from mutations in the target protein but additionally from loss-of-function mutations in *tarO* or *tarA*. However, as deletions in early steps in wall teichoic acid synthesis render *S. aureus* avirulent, TarG inhibitor-resistant mutants with

loss-of-function mutations in *tarO* or *tarA* were not viable in an *in vivo* model.

Taken together, efforts to search for late-step inhibitors at Merck and in the Walker laboratory, respectively, suggest that TarG is a highly druggable target that can be perturbed by chemicals of diverse structural class. The ease of discovery of TarG inhibitors is curious and due perhaps to its partial extracellular structure, or to its role as a wall teichoic acid translocase protein. Furthermore, the challenging biochemistry of this multitransmembrane domain protein remains an obstacle to rational *in vitro* optimization of bioactives targeting this structure, and efforts continue to discover leads that are active against more tractable late-step targets in the wall teichoic acid biosynthetic pathway.

## A ROLE FOR WALL TEICHOIC ACID IN β-LACTAM RESISTANCE IN MRSA

The β-lactams are the most successful class of antibiotics in clinical history.[66] Despite the clinical success of these molecules and the transformative role they have played in modern medicine, resistance to all β-lactam antibiotics has been documented, often shortly after introduction into the clinic. Resistance to β-lactams is commonly achieved via β-lactamase enzymes that inactivate the pharmacophore by hydrolyzing its β-lactam ring, rendering the antibiotic inert.[67] To salvage efficacy of these drugs, β-lactams are commonly coformulated with a β-lactamase inhibitor, (e.g. Augmentin; amoxicillin + clavulanic acid). Although this approach is broadly successful for the treatment of Gram-negative infections, clinically important Gram-positive pathogens such as MRSA achieve β-lactam-resistance via additional mechanisms. In MRSA, resistance is determined in part by the presence of a horizontally acquired penicillin-binding protein (PBP), PBP2A, which is unsusceptible to inactivation by β-lactam antibiotics.[68] While the presence of PBP2A was once thought to be the sole determinant for β-lactam resistance in *S. aureus*, it has become increasingly clear that this phenotype is supported by a complex network of resistance determinants, including PBPs (PBP2 and PBP4), and intriguingly, wall teichoic acid.

In 1994, the Murakami group[69] conducted transposon-mediated mutagenesis of a clinical MRSA isolate to identify methicillin resistance determinants. The authors demonstrated that selective disruption of the *llm* gene could reverse high-level methicillin resistance in clinical isolates of MRSA. The *llm* gene was identified as the wall teichoic acid biosynthetic gene *tarO* almost a decade later and a focused evaluation of the connectivity between wall teichoic acid biosynthesis and β-lactam resistance in MRSA ensued.[5,36] This work demonstrated that selective genetic inactivation of *tarO* caused a β-lactam sensitization phenotype in both methicillin-sensitive and methicillin-resistant strains of *S. aureus*. The β-lactam sensitization phenotype was observed only upon interruption of the early-acting enzyme TarO and not the late-acting enzyme TarG, although coadministration of imipenem or oxacillin with a TarG inhibitor was able to elicit a three-log decrease in the frequency of TarG inhibitor-resistant mutations.[5,55] Furthermore, the β-lactam sensitization phenotype was found to be heterogeneous across a spectrum of β-lactam antibiotics, suggesting a very specific interaction between wall teichoic acids and peptidoglycan biosynthetic machinery. Further investigation of the connection between wall teichoic acid and peptidoglycan synthesis by electron microscopy uncovered clear phenotypes for defects in wall teichoic acid synthesis in cell division in *S. aureus*; interruption of TarO was linked to defects in cell septation and cell separation.[5] Further analysis of the structural requirements for wall teichoic acid in β-lactam resistance linked this phenotype to the β-*O*-*N*-acetylglucosamine modification, rather than to the presence of the teichoic acid polymer itself.[8] This observation is fascinating in the context of *S. aureus* wall teichoic acid modifications including both α- and β-*O*-*N*-acetylglucosamine, with only the β-linked sugar having a role in β-lactam resistance.[8,47]

## TICLOPIDINE REVERSES β-LACTAM RESISTANCE IN MRSA: A NEW PROBE OF WALL TEICHOIC ACID SYNTHESIS AND A PROMISING DRUG COMBINATION

Given the requirement for wall teichoic acid in β-lactam resistance, there is strong therapeutic potential for a small-molecule inhibitor of an early step in teichoic acid synthesis. Toward this goal, the Brown laboratory developed a chemical combination screening strategy to exploit signature chemical–genetic interactions observed between *tarO* and β-lactam antibiotics.[6] A pairwise screen of 2080 previously approved drugs in combination with cefuroxime was conducted against MRSA strain USA300. Previously approved drugs represent a rich source of bioactivity with the potential to be repurposed for new therapeutic benefit.[70,71] Furthermore, downstream drug development efforts with repurposed bioactives are advantaged by the availability of extensive safety and pharmacological data. While a screen for β-lactam sensitization should enrich for compounds targeting early steps in wall teichoic acid synthesis, Tan *et al.*[72] recently demonstrated that inhibition of other cellular targets in *S. aureus*, including peptidoglycan biosynthesis and cell division, could likewise sensitize MRSA to β-lactams. Accordingly, molecules discovered to restore the efficacy of cefuroxime against MRSA were advanced to a counter screen to identify those that caused β-lactam sensitization via inhibition of an early step in wall teichoic acid biosynthesis. Bioactive molecules showing synergy in the primary screen were tested in a Δ*tarO* background for suppression of cefuroxime sensitization. Ticlopidine (Ticlid), an antihypertension drug that interrupts ADP-dependent platelet formation, was the only molecule to advance from this counter screen.[73] Ticlopidine also showed signature synthetic-viable interactions with late-acting wall teichoic acid biosynthetic genes, suppressing the lethal phenotype caused by genetic or chemical inactivation of TarG. While ticopidine did not display any growth-inhibition activity, the ticlopidine–cefuroxime combination displayed potent and synergistic *in vitro* efficacy against 10 clinical *S. aureus* isolates, including nine MRSA strains, and efficacy in an *in vivo* *S. aureus* infection model. Phenotypes for ticlopine-treated *S. aureus*, including sensitization to β-lactam antibiotics, absence of teichoic acid from the cell wall and resistance to teichoic acid-specific phage, were consistent with phenotypes for *tarO/tarA* gene deletions, and provided further support for a mechanism for ticlopidine in inhibition of an early-step in wall teichoic acid biosynthesis. *In vitro* enzyme assays confirmed TarO to be the target, and the ticlopidine–cefuroxime combination was found to be selective for *S. aureus*.

The availability of a small-molecule inhibitor for TarO provided an opportunity to probe the mechanism of synergy between early steps in wall teichoic acid biosynthesis and transpeptidation steps in peptidoglycan biosynthesis. Indeed, well-characterized bioactive small molecules have frequently proved highly useful in understanding complex biological processes.[74–76] Here, growth-inhibition phenotypes in MRSA were explored with a diverse collection β-lactam antibiotics in conjunction with TarO inhibition. Interestingly, blocking TarO function either with ticlopidine or by gene deletion sensitized MRSA only to β-lactams with a high affinity for PBP2, particularly the cephalosporins, and not to all β-lactams.[6] The selective interaction of ticlopidine with PBP2-specific β-lactams is

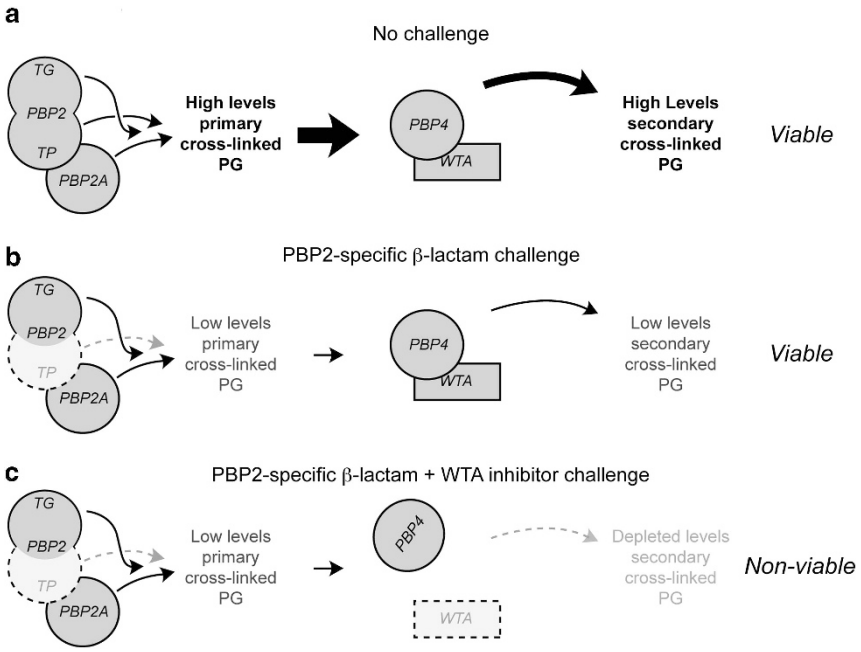



**Figure 5** A model for wall teichoic acid in β-lactam resistance in MRSA. (**a**) In the absence of β-lactam challenge, transglycosylase (TG) and transpeptidase (TP) domains in PBP2 work in concert with PBP2A to produce high levels of primary crosslinked peptidoglycan (PG). Wall teichoic acid (WTA) recruits PBP4 to the division septum where it catalyzes formation of highly crosslinked PG. (**b**) Under β-lactam challenge, the TP site of PBP2 is inhibited. TG activity of PBP2 and TP activity of PBP2A work in concert to produce reduced levels of primary crosslinked PG compared with the unchallenged condition. Primary crosslinked PG is further crosslinked by PBP4 in a WTA-dependent manner. (**c**) Under simultaneous challenge with a β-lactam and WTA inhibitor, PBP4 is mislocalized and does not synthesize highly crosslinked PG. The presence of low levels of primary crosslinked PG synthesized by PBP2 and PBP2A under β-lactam challenge are insufficient to support life.

fascinating in the context of an emerging literature on the unique functions of individual PBPs in MRSA. PBP2 is the only bifunctional PBP with transpeptidase and transglycosylase activities in *S. aureus* and is essential to support life.[77,78] Localization of PBP2 to the division septum is required for the essential transglycosylase activity of this enzyme, and is mediated by an interaction between the transpeptidase active site and its transpeptidation substrate.[79] Under β-lactam challenge, the transpeptidase active site on PBP2 is acylated, and autonomous localization to the division septum is inhibited. Under this stress, PBP2A, a horizontally acquired transpeptidase unsusceptible to inhibition by β-lactam antibiotics, carries out transpeptidation activities, and acts as a structural scaffold to recruit acylated PBP2 to the division septum.[77,79] In this manner, PBP2 remains properly localized under β-lactam challenge and can perform its essential transglycosylation function. This cooperative function of PBP2 and PBP2A yields peptidoglycan strands with low-level crosslinking, up to approximately five units in length.[4,80] Glycan strands with low-level crosslinking are substrates for PBP4, which functions as a secondary transpeptidase at the division septum to generate highly crosslinked peptidoglycan.[4,81] Thus, the activities of PBP2, PBP2A and PBP4 are required to synthesize the highly crosslinked cell wall in MRSA. Indeed, many MRSA strains are able to survive inhibition of transpeptidase activities on either PBP2 or PBP4, albeit with decreased amounts of highly crosslinked cell wall. However, simultaneous inhibition of both PBP2 and PBP4 transpeptidase activities is lethal.[82] Remarkably, Atilano and co-workers demonstrated that the localization of PBP4 at the division site is dependent on the presence of the wall teichoic acid polymer.[4] Taken together, these data allowed the Brown group to propose a model for the mechanistic basis for the synergy observed in MRSA between the TarO inhibitor ticlopidine and PBP2-specific antibiotics: PBP2-specific β-lactams inhibit the transpeptidation activity of PBP2, resulting in lowered levels of primary crosslinked cell wall, whereas ticlopidine inhibits the synthesis of wall teichoic acid, preventing proper localization and function of PBP4, and preventing secondary cell wall crosslinking. The low level of primary crosslinked cell wall synthesized under this challenge is insufficient to support life (Figure 5). Although untested at this time, it appears likely that β-lactam sensitivity reported to be associated with the loss of β-*O*-*N*-acetylglucosamine modifications to wall teichoic acid may also be due to a loss of PBP4 localization.[8] Indeed, it is an intriguing idea that the wall teichoic acid polymer may well be a specific scaffold for subtle functions in peptidoglycan synthesis and cell division that are nevertheless absolutely critical to the complex manifestation of β-lactam resistance in MRSA.

## CONCLUDING REMARKS

Wall teichoic acid biosynthesis has recently emerged as an exciting new target in antibiotic research. Long thought to be ancillary structures in the cell wall of Gram-positive organisms, new roles for wall teichoic acid in the coordination of cell division, peptidoglycan synthesis and resistance to β-lactam antibiotics have emerged. A detailed understanding of the biology underpinning these events and the description of unique chemical and genetic interactions within these processes have provided the opportunity to develop focused cell-based screening tools for the discovery of inhibitors of both wall teichoic acid biosynthesis and β-lactam resistance in MRSA. Indeed, mechanistic follow-up on bioactives from high-throughput screening efforts is a time- and resource-intensive endeavor with low rates of success. The pathway-specific, cell-based screening strategies

highlighted herein have exploited synthetic-viable interactions to enable focused discovery of pathway-specific bioactives, facilitating streamlined follow-up, elimination of nuisance compounds and ultimately, the discovery of inhibitors for TarO and TarG. Indeed, TarG appears to be a highly druggable target that is susceptible to a variety of chemical classes. Ticlopidine, a selective inhibitor of TarO, is strongly synergistic with PBP2-specific β-lactam antibiotics and restores the efficacy of these once-successful drugs for treatment of MRSA infections. Cumulatively, inhibitors of wall teichoic acid synthesis offer an exciting opportunity for the development of novel antibacterial leads with a new mechanism of action to treat drug-resistant *Staphylococcal* infections.

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