Published in final edited form as:

Nat Chem Biol. 2017 April ; 13(4): 396–401. doi:10.1038/nchembio.2302.

***In vitro* reconstitution demonstrates the cell wall ligase activity of LCP proteins**

Kaitlin Schaefer^{1,2}, Leigh M. Matano², Yuan Qiao^{1,2}, Daniel Kahne^{1,*}, and Suzanne Walker^{2,*}

¹Department of Chemistry and Chemical Biology, Harvard University, Cambridge, Massachusetts, 02138, United States

²Department of Microbiology and Immunology, Harvard Medical School, Boston, Massachusetts, 02115, United States

Abstract

Sacculus is a peptidoglycan matrix that protects bacteria from osmotic lysis. In Gram-positive organisms, the sacculus is densely functionalized with glycopolymers important for survival, but how assembly occurs is not known. In *Staphylococcus aureus*, three LCP family members have been implicated in attaching the major glycopolymer wall teichoic acid (WTA) to peptidoglycan, but ligase activity has not been demonstrated for these or any other LCP proteins. Using WTA and peptidoglycan substrates produced chemoenzymatically, we show that all three proteins can transfer WTA precursors to nascent peptidoglycan, establishing that LCP proteins are peptidoglycan-glycopolymer ligases. Although all *S. aureus* LCP proteins have the capacity to attach WTA to PG, we show that their cellular functions are not redundant. Strains lacking *lcpA* have phenotypes similar to WTA null strains, indicating that this is the most important WTA ligase. This work provides a foundation for studying how LCP enzymes participate in cell wall assembly.

Introduction

The bacterial sacculus, also known as the cell wall, is a complex macromolecular structure that is required for survival^{1,2}. In Gram-positive organisms, peptidoglycan (PG), the biosynthesis of which is targeted by many clinically used antibiotics, makes up only about fifty percent of the sacculus by mass³. The other fifty percent of the mass largely comprises glycopolymers that are covalently bound to peptidoglycan⁴. In *Staphylococcus aureus* (*S. aureus*), WTA is the most abundant glycopolymer attached to peptidoglycan and it plays crucial roles in the physiology and pathogenesis of this organism⁵. Strains lacking WTA

*Correspondence to: kahne@chemistry.harvard.edu; suzanne_walker@hms.harvard.edu.

Contributions

S.W., D.K., K.S., and L.M.M. designed experiments. K.S. prepared wall teichoic acid substrates, and peptidoglycan substrates were prepared with help from Y.Q.; L.M.M. performed the Tn-seq experiments; K.S. and L.M.M. prepared the spot dilution assays and the MIC experiments; L.M.M. performed the Tn-seq experiments; K.S. and L.M.M. made strains used in this study except those received from others as noted (Supplementary Table 5); K.S. cloned, expressed, and purified all LCP proteins and performed reconstitution experiments; S.W. designed and supervised the project; S.W., D.K., and K.S. wrote the manuscript with input from all authors.

Competing financial interests

The authors declare no competing financial interests.

have major cell division defects, key autolysins are dysregulated, and peptidoglycan synthesis machinery is mislocalized^{6,7}. These strains are unable to colonize nasal epithelial cells and are highly attenuated *in vivo*^{8,9}. In addition, methicillin-resistant *S. aureus* (MRSA) strains become sensitive to beta lactam antibiotics when WTA synthesis is prevented^{7,10,11}. In order to complete cell wall assembly, the pathways for peptidoglycan and WTA biosynthesis must converge (Fig. 1). How this is accomplished has largely remained a mystery because key questions about the process can only be answered through reconstitution, and there are major challenges involved in reconstituting the coupling of two non-protein polymers.

Peptidoglycan and WTA precursors are assembled intracellularly on a carrier lipid, undecaprenyl phosphate (Und-P), and are then exported through dedicated transporters (Fig. 1)^{9,12–14}. The final steps of cell wall assembly, which occur on the extracytoplasmic face of the membrane, involve forming nascent (uncrosslinked) peptidoglycan polymers, crosslinking these polymers to one another, and coupling WTA to peptidoglycan (Fig. 1). While it has long been known that WTA is coupled to peptidoglycan via a phosphodiester bond to the C6 hydroxyl of intermittent N-acetyl muramic acid (MurNAc) units in the PG backbone^{15,16}, it was not known whether coupling occurred to Lipid II or peptidoglycan polymer^{17,18}, and until recently there were not even any proposed candidates for the enzymes that catalyze the reaction (Fig. 1).

In 2011, a study in *Bacillus subtilis* (*B. subtilis*) implicated three similar genes in the attachment of WTA to peptidoglycan¹⁸. These genes, encoding proteins belonging to the LCP (LytR-CpsA-Psr) family, were identified by their co-purification with a tagged variant of MreB. Their close proximity to known WTA biosynthetic genes in the chromosome prompted studies to assess their roles in WTA biosynthesis, and it was found that deletion of *lcp* genes resulted in loss of WTA from the sacculus¹⁸. It was proposed that LCPs are enzymes with ligase activity, but it was not possible to demonstrate that LCPs transfer glycopolymers to PG due to the challenges involved in reconstitution¹⁸. It was reported, however, that one of the *B. subtilis* LCP proteins hydrolyzed a polyprenyl pyrophosphate, and this activity provided the only biochemical support that LCP proteins are directly involved in attaching glycopolymers to peptidoglycan. Since then, LCP genes have been linked to glycopolymer biosynthesis in other organisms, and while some suggestive biochemical results have been reported, conclusive evidence that LCP proteins have ligase activity is still lacking^{19–23}. *Staphylococcus aureus*, like *B. subtilis*, contains three *lcp* genes, now called *lcpA*, *lcpB*, and *lcpC*^{20,24}. Deleting *lcpA* or *lcpB* was shown to reduce WTA levels, but complete loss of WTA only occurred when all three *lcp* genes were deleted, and it has thus been suggested that these genes play redundant roles in WTA biosynthesis^{19,20}.

Here we use WTA and peptidoglycan substrates prepared chemoenzymatically to show that all three *S. aureus* LCP proteins act as phosphosugar transferases that can attach WTA precursors to nascent peptidoglycan. In addition to establishing LCPs as enzymes that directly ligate cell wall glycopolymers to peptidoglycan, these studies provide insights into the structural features required in both substrates for transfer to occur. We have also addressed the cellular functions of the *S. aureus* LCPs. By comparing phenotypes of *lcp* knockouts to WTA null strains that lack a functional copy of *tarO*, the first gene in the WTA

pathway, we have established that the three LCP proteins do not have redundant cellular functions even though they all have the capacity to attach WTAs to peptidoglycan. LcpA is the most important glycopolymer ligase for WTA-related functions and LcpC is the least important. Our studies lay the groundwork for characterizing LCP enzymes as antibiotic targets and for investigating how cells use these related enzymes for different cellular functions.

Results

LCP proteins attach radiolabeled WTA to peptidoglycan

To establish whether LCP proteins play a direct enzymatic role in attaching glycopolymers to peptidoglycan, we sought to reconstitute WTA ligation to peptidoglycan. WTA precursors comprise a disaccharide linked at the reducing end to undecaprenyl pyrophosphate (Und-PP) and at the non-reducing end to two glycerol phosphate units and up to forty ribitol phosphate units (Fig. 1)⁹. We previously developed methods to make the full WTA precursor on a model carrier lipid^{11,25,26}, but it did not seem likely that the full polymer would be required to reconstitute ligation because the reaction takes place on the reducing end of the WTA molecule. Therefore, we prepared two short WTA precursors, LII_A^{WTA} and LII_B^{WTA}, attached to a hexaprenyl lipid chain (Fig. 2a). The hexaprenyl chain length was chosen to balance substrate solubility with the likelihood that the lipid chain forms extensive interactions with the protein, as suggested by crystal structures¹⁸. LI^{WTA} was synthesized in six chemical steps and we converted this monosaccharide precursor enzymatically to the corresponding disaccharide, LII_A^{WTA}, using the UDP-ManNAc transferase TagA, UDP-GlcNAc, and the UDP-GlcNAc 2-epimerase, MnaA (Fig. 2a)^{27,28}. A radiolabel was optionally incorporated using UDP-[¹⁴C]-GlcNAc. To obtain LII_B^{WTA}, which contains a glycerol phosphate at the C4 position of ManNAc, purified LII_A^{WTA} was incubated with CDP-glycerol and TagB (Fig. 2a)^{26,29}. A radiolabel was optionally incorporated into this substrate using CDP-[¹⁴C]-glycerol³⁰.

We purified a soluble *S. aureus* LcpA construct lacking the predicted transmembrane helix and tested whether it would transfer radiolabeled LII_A^{WTA} or LII_B^{WTA} to a chemically synthesized Lipid II precursor (Lipid II, Fig. 2b)^{31,32}. We did not detect a shifted band via polyacrylamide gel electrophoresis (PAGE), as would have been expected for a ligation product. There could have been many explanations for this outcome. For example, others have suggested that LCP proteins might require some form of energy, additional proteins, or a membrane interface in order to function¹⁸. We decided to first explore the possibility that LcpA might only transfer WTA substrates to larger peptidoglycan substrates^{17,18}. Therefore, we tested whether LcpA would incorporate the short radiolabeled WTA substrates into *S. aureus* sacculi isolated from a WTA null strain (*tarO*), but did not detect the radiolabel in sacculi after incubation with LcpA.

We decided that in order to properly evaluate ligase activity, we would need peptidoglycan oligomers of short, defined lengths because only these would permit unambiguous product characterization. To obtain short peptidoglycan oligomers, we incubated synthetic Lipid II with a mutant form of *S. aureus* SgtB, a monofunctional peptidoglycan glycosyltransferase that polymerizes Lipid II. The mutant, SgtB*, contains a Y181D amino acid substitution in

the active site cleft where the elongating polymer binds³³, and as a result products are released prematurely (Fig. 2b). Using radiolabeled Lipid II, we developed conditions to obtain products containing 1 to 7 disaccharide repeats, which can be visualized as a discrete ladder after gel electrophoresis (Fig. 2c). A similar distribution of non-radiolabeled peptidoglycan oligomers was prepared from cold Lipid II for the LCP reactions, and SgtB* was removed by precipitation. The cold peptidoglycan oligomers and radiolabeled LII_B^{WTA} were then incubated in the presence and absence of LcpA, and reactions were analyzed by PAGE autoradiography (Fig. 2c). In the presence of LcpA, a ladder of radiolabeled products was visible. Similar results were obtained using the shorter WTA substrate, radiolabeled LII_A^{WTA} (Fig. 2c). These ladders were only observed when both LcpA and peptidoglycan oligomers were included in the reaction, consistent with transfer of radiolabeled WTA precursors to the cold peptidoglycan oligomers (Fig. 2c; Supplementary Results, Supplementary Fig. 1). As the peptidoglycan oligomers used in this reconstitution lack the carboxamide modification and pentaglycine branch found in the stem pentapeptide of native *S. aureus* peptidoglycan³⁴, we have concluded that ligase activity does not depend on these features.

With conditions developed to reconstitute LcpA *in vitro*, we tested the other two *S. aureus* LCP proteins, LcpB and LcpC, for WTA ligase activity. For both proteins, we detected a ladder of radiolabeled products in reactions containing peptidoglycan oligomers, radiolabeled WTA substrate, and active enzyme (Supplementary Fig. 2). These results establish that all three LCP proteins have the capacity to act directly as WTA ligases. Furthermore, they can transfer WTA precursors as small as a disaccharide to peptidoglycan oligomers. Because the peptidoglycan precursor Lipid II is also a disaccharide attached to a polyprenyl chain through a pyrophosphate linkage, it closely resembles the WTA substrates used in our reconstitution. This structural similarity prompted us to test whether LcpA would transfer radiolabeled Lipid II to cold PG oligomers, but we did not observe a ladder of radiolabeled products. Therefore, LcpA can use a WTA disaccharide as the electrophile in the ligation reaction, but it cannot use Lipid II even though large portions of the molecules are identical. It is possible that the lactoyl-pentapeptide on the C3 position of Lipid II impedes productive binding.

Mass spectrometry provides chemical proof of ligation

While PAGE analysis provided convincing evidence that radiolabeled WTA substrates were transferred to peptidoglycan, it did not provide any information about the chemical structure of the products. To confirm the formation of phosphodiester-linked products, we used high-resolution mass spectrometry. MS analysis of lipid-linked peptidoglycan oligomers is challenging because the molecules ionize poorly; however, we previously found that peptidoglycan oligomers prepared from synthetic Lipid II containing a short lipid chain (tetraprenyl, *i.e.*, C20) ionize well enough to enable LC/MS analysis³⁵. Therefore, we prepared peptidoglycan oligomers from C20-Lipid II, verified by autoradiography that LcpA could transfer radiolabeled LII_A^{WTA} to these C20-linked oligomers (Supplementary Fig. 3), and then repeated the reaction using non-radiolabeled substrates exclusively (Fig. 3). In the absence of LcpA, we detected masses for C20-Lipid II, peptidoglycan oligomers containing up to four disaccharides, and LII_A^{WTA} (Supplementary Table 1). In the presence of LcpA,

we detected these as well as new species with exact masses corresponding to ligation products consisting of a peptidoglycan oligomer coupled via a phosphodiester bond to a single WTA disaccharide. WTA ligation products were observed for Lipid IV, Lipid VI, and Lipid VIII (Fig. 3; see also Supplementary Table 2 and Supplementary Fig. 4), but not Lipid II even though the latter species was a major component of the starting material (Supplementary Fig. 3). Because C20-Lipid II ionizes better than corresponding peptidoglycan oligomers, and we would expect the C20-Lipid II ligation product to ionize better than the larger peptidoglycan ligation products that were actually observed, we have concluded that Lipid II is not a substrate for ligation. Transfer takes place only to peptidoglycan oligomers.

We also assessed whether LCP-dependent hydrolysis of $\text{LII}_A^{\text{WTA}}$ occurred under the reaction conditions, but in the absence of peptidoglycan acceptor. We did not detect any enzymatic hydrolysis, despite reports from other labs that some LCP proteins will hydrolyze pyrophosphoryl lipid substrates^{18,36}. Notably, the conditions we developed for WTA transfer to peptidoglycan oligomers are milder than those that have been used to achieve hydrolysis of pyrophosphoryl lipids.

***S. aureus* LCP proteins play non-redundant cellular roles**

We have shown that all three *S. aureus* LCP proteins have the capacity to attach WTA to peptidoglycan oligomers *in vitro*. Others have shown that overexpression of any *S. aureus* *lcp* gene from a high copy number plasmid in a strain lacking all three genes partially restores WTA levels. The overexpression results have been taken as evidence that LCP proteins have redundant cellular functions, but other evidence suggests that they play different roles in glycopolymer attachment^{21,24}. We decided to employ chemical probes that inhibit the growth of WTA-deficient strains to compare the roles of LcpA, LcpB, and LcpC in WTA biosynthesis. One compound, amsacrine, inhibits DltB³⁷, a core component of the teichoic acid D-alanylation machinery. *S. aureus* contains two types of teichoic acids, wall teichoic acids and membrane-anchored lipoteichoic acids (LTA; Supplementary Fig. 5), and both are modified with positively charged D-alanyl ester groups, installed by the Dlt pathway^{4,38}. Neither D-alanylation nor the presence of WTA is essential for viability *in vitro*³⁹, but removal of both WTA and the D-alanyl ester modifications on LTA is lethal (Supplementary Fig. 5)⁴⁰. Therefore, strains lacking *tarO*, the first gene in the WTA biosynthetic pathway, cannot grow on the DltB inhibitor amsacrine³⁷. By probing a *S. aureus* transposon mutant library with amsacrine to identify genes that are synthetically lethal with depletion of teichoic acid D-alanyl esters, we observed that insertions mapping to *lcpA* were dramatically depleted (>10-fold; Fig. 4a and Supplementary Fig. 6). In contrast, there was no statistically significant difference in the reads mapping to *lcpB* and *lcpC* in inhibitor-treated and untreated samples. The transposon sequencing results indicated that *lcpA*, like *tarO*, becomes essential when D-alanylation is inhibited, while *lcpB* and *lcpC* are unaffected.

We confirmed the transposon results by plating single and double *lcp* deletion strains on amsacrine (Fig. 4b). As expected, the wildtype strain grew at all dilutions whereas the *tarO* strain did not grow at any³⁷. The *lcpB* and *lcpC* strains grew similarly to the wildtype

strain whereas the *lcpA* strain behaved like the *tarO* strain. Even strains lacking both *lcpB* and *lcpC* were fully viable on amsacrine (Fig. 4b). To confirm the plating phenotypes, we measured the minimum inhibitory concentration (MIC) of amsacrine and a related DltB inhibitor, O-amsa³⁷, against wildtype, *tarO*, *lcpA*, *lcpB*, *lcpC*, and all double mutant combinations (Supplementary Table 3), and found that only strains lacking *lcpA* were sensitive to DltB inhibition. Finally, we addressed whether the sensitivity of *lcpA* mutants to amsacrine was a strain-dependent phenomenon. We constructed *lcpA* mutants in several additional *S. aureus* strains and found that all mutants were sensitive to amsacrine (Supplementary Fig. 7). Complementation of a *lcpA* strain with a single copy of *lcpA* that integrates into an ectopic locus in the chromosome restored growth on amsacrine, confirming that the lethal phenotype was due solely to the absence of *lcpA* (Supplementary Fig. 8).

Other small molecules, including the azo dye Congo red and beta lactams such as oxacillin, have also been shown to inhibit growth of *tarO* strains^{7,10,11,41}. Although the target of Congo red has not been established, *tarO* strains are 1000-fold more susceptible than wildtype strains to this compound⁴¹. A *lcpA* strain (known then as *msrR*) was also reported to be susceptible to Congo red⁴², but it was not compared to *tarO* or to other *lcp* mutants. We compared the Congo red sensitivity of all *lcp* single and double knockouts to the corresponding *tarO* and wildtype strains using a spot dilution assay. The *lcpA* strain was sensitive to Congo red, although slightly less so than the *tarO* strain (Fig. 4b), whereas the other *lcp* single mutants grew comparably to wildtype on Congo red. The *lcpAB* double mutant, however, was just as sensitive to Congo red as the *tarO* strain. Thus, the growth phenotypes of the double mutants confirmed that *lcpA* is critical for preventing lethality on Congo red, and also revealed a role for *lcpB* in Congo red resistance. As deletion of *lcpC* did not affect growth phenotypes on either Congo red or amsacrine, we have concluded that it is less important than *lcpA* or *lcpB* with respect to WTA biosynthesis. Notably, LcpC has been identified as the key LCP protein for capsular polysaccharide biosynthesis in *S. aureus*²¹.

We next tested the importance of *lcpA* and *lcpB* for oxacillin resistance in MRSA. MRSA strains have acquired a gene, *mecA*, encoding an intrinsically resistant penicillin binding protein (PBP), PBP2a, which can crosslink peptidoglycan when other PBPs are inhibited by beta lactams^{43,44}. While *mecA* is necessary for beta lactam resistance, it is not sufficient: other genes are also involved in the resistant phenotype, including *tarO*⁷. While the role of wall teichoic acids in protecting MRSA strains from beta lactams is not fully understood, it has been proposed that these cell wall glycopolymers scaffold proteins involved in peptidoglycan biosynthesis^{10,11}. Consistent with this, WTA deletion results in mislocalization of PBP4⁶. We deleted *lcpA* and *lcpB* from two different MRSA strains and plated them on oxacillin. The *lcpA* strains were highly sensitive to oxacillin like the *tarO* null strains, but the *lcpB* mutants grew at all dilutions like the wildtype strain (Fig. 4b; Supplementary Fig. 9). As the plating experiments were done at a single beta lactam concentration, we also measured the MICs of oxacillin as well as another beta lactam, cefaclor, against the mutant strains. The MICs confirmed that deleting *lcpA* has a greater impact on beta lactam susceptibility than deleting *lcpB*, although the fold-change in MICs depend on both the strain background and the beta lactam (Supplementary Table 4).

Discussion

Here we have reported the first conclusive demonstration that LCP proteins catalyze glycopolymer attachment to peptidoglycan. Through an *in vitro* reconstitution, we have shown that all three LCP proteins in *Staphylococcus aureus* can catalyze the transfer of WTA precursors to peptidoglycan. Notably LCP enzymes recognize these two large cell wall polymers without requiring the presence of other proteins as previously suggested¹⁸. However, we have also shown that these proteins are non-redundant in cells, with one of them, LcpA, being more important than the others for WTA-related functions.

The reconstitution results, in addition to demonstrating ligase activity, have provided insight into the structural features that make a competent WTA or peptidoglycan substrate. The WTA substrate need only contain two saccharides, and the native undecaprenyl lipid can be truncated to a hexaprenyl chain, which is approximately the length found to fit within the hydrophobic channel observed in a LCP crystal structure^{18,36}. It is possible that even smaller WTA analogs would be transferred, but the substrates identified here are small enough to be tractable and may enable a wide range of biochemical and biophysical studies to better understand LCP function.

Another key finding was that WTA precursors were only transferred to peptidoglycan polymers. Lipid II was not a substrate under the reconstitution conditions even when present in large amounts relative to oligomeric species that were good substrates. How the LCP proteins discriminate against Lipid II is unclear as there is no information yet about where in these enzymes the PG polymer binds. However, there are two possibilities, which are not mutually exclusive. First, the pyrophosphoryl lipid on the anomeric position of Lipid II could act as a steric barrier to productive binding of the MurNAc sugar near the pyrophosphate of the WTA precursor. Second, the binding site may require that the MurNAc sugar be located between flanking GlcNAcs. Although our results were obtained using soluble LCP constructs and substrates that have shorter lipids than the native substrates, it seems likely that similar discrimination against Lipid II occurs in cells. We draw this conclusion based on structures of peptidoglycan glycosyltransferases, including one with a Lipid II substrate bound in the active site cleft.⁴⁵ The C6 hydroxyl of the N-acetyl muramic acid sugar of Lipid II points directly into the active site and the presence of a large glycopolymer at this position would prevent substrate binding and thus block formation of peptidoglycan polymer (Supplementary Fig. 10). For a similar reason, we suspect that Lipid IV, which is a competent peptidoglycan substrate for WTA transfer *in vitro*, is not a physiologically relevant substrate: the active site clefts of peptidoglycan glycosyltransferases are long and a MurNAc C6 hydroxyl would not be exposed until at least Lipid VI has formed. Whether WTA precursors can only be transferred to uncrosslinked peptidoglycan oligomers, *i.e.*, at an early stage of cell wall synthesis, or are also transferred to crosslinked cell wall is unclear at this point. We cannot exclude crosslinked peptidoglycan as a substrate based on the negative results obtained for isolated sacculi, but we note that methods to make crosslinked peptidoglycan of defined composition should allow this issue to be resolved.

In this paper we have also addressed whether the three LCP proteins in *S. aureus* play equivalent roles in WTA biosynthesis because previous studies have made conflicting

claims. Some concluded that these three proteins have redundant functions; the evidence for redundancy was that all three proteins must be deleted to achieve complete loss of WTA from sacculi and that each of the three proteins, when overexpressed from a high copy number plasmid, substantially restored WTA levels. Although overexpression-based complementation studies can reveal that proteins share a similar functional capability, they do not demonstrate equivalent cellular functions. Therefore, to probe the functional roles of the LCP proteins, we compared the chemical sensitivities of *lcp* knockout strains to the corresponding *tarO* knockout strains. Several compounds with different mechanisms had previously been found to inhibit the growth of strains lacking *tarO*, and we used three distinct compound classes in this study. The results showed that *lcpA* knockouts most closely phenocopy strains lacking *tarO*. Other studies have found that *lcpA* strains, but not *lcpB* or *lcpC* strains, have misplaced and thickened septa, display reduced colony spreading compared with wildtype, and have a cell clumping phenotype^{24,42}. Notably, these characteristics are also observed in *tarO* strains^{7,46}. We also found evidence that *lcpB* contributes to WTA-related functions, but found no role for *lcpC*. In this regard, deleting *lcpC* was previously shown to have no effect on wall teichoic acid levels in the cell wall, whereas deleting either *lcpA* or *lcpB* reduced these levels^{19,20}. We speculate that factors such as differential localization and regulation may explain the greater importance of LcpA compared with LcpB in WTA synthesis¹⁹. Although further studies are required to elucidate the different functions of LCP proteins in cells, the reconstitution reported here lays the groundwork for discovering and characterizing LCP inhibitors. As knockouts of *lcpA* are sensitive to several families of chemical inhibitors, including beta lactams, LcpA is an appealing target for new therapeutics.

Online Methods

Reagents and general methods

Strains are listed in Supplementary Table 5. *S. aureus* was grown in tryptic soy broth (TSB) or on TSB with 1.5% agar at 30 and 37 °C. Phage 80alpha was used for transductions and the methods for transduction were described previously⁴⁰. All constructed LCP-pET28-plasmids were transformed into NovaBlue(DE3) *E. Coli*, and LCP proteins were overexpressed in BL21(DE3) *E. Coli* grown in lysis broth (LB). Antibiotics were used at the following concentrations: 10 µg ml⁻¹ chloramphenicol, 10 µg ml⁻¹ erythromycin, 50 µg ml⁻¹ kanamycin, 50 µg ml⁻¹ neomycin, 50 µg ml⁻¹ carbenicillin. L-[¹⁴C]-Glycerol-3-phosphate was purchased from Person-Elmer. UDP-[¹⁴C]-GlcNAc was purchased from American Radiolabeled Chemicals, Inc (ARC). Amsacrine was purchased from Abcam (ab142742).

Cloning pET28(b)_*lcpA*, pET28(b)_*lcpB*, pET28(b)_*lcpC*, *plcpA*, pKFC_*lcpB*

Soluble LCP constructs (LcpA (58–327 aa), LcpB (31–405 aa), and LcpC (30–307)) with a N-terminal His₈ tag were prepared by cloning *lcp* genes into pET28(b)(–). Using *S. aureus* NCTC8325 DNA, *lcp* genes (*lcpA* (SAOUHSC_01361), *lcpB* (SAOUHSC_00997), *lcpC* (SAOUHSC_02583)) were PCR amplified using primer sets (F'*lcp*(A,B, or C) and R'*lcp*(A,B, or C)). The plasmid backbone was amplified using pET28(b)(–) (Novagen) as template DNA and primer sets (F'pET28_*lcp*(A,B, or C) and R'pET28_*lcp*(A,B, or C)). The complementary plasmid *plcpA* was constructed for integrating a single copy of *lcpA* into an

ectopic locus of the *S. aureus* chromosome. The *lcpA* gene was amplified using *S. aureus* NCTC8325 DNA and primer sets (F'pTP63_lcpA and R'pTP63_lcpA), and the pTP63 plasmid was amplified using the primer sets (F'pTP63 and R'pTP63) amplified from pTP63 template. A kanamycin-marked deletion of *lcpB* was constructed using the *E. coli*-*S. aureus* shuttle vector pKFC⁴⁷. Nucleotides upstream and downstream *lcpB*, and a kanamycin resistance gene (*aphA-3*) were amplified using primer sets (F'+1000_lcpB, R'+1000_lcpB, F'-1000_lcpB, R'-1000_lcpB, F'kan_lcpB, R'kan_lcpB). Using the pKFC plasmid as a template, pKFC was amplified with primer sets (F'pKFC and R'pKFC). All primer sets are listed in Supplementary Table 6. Plasmids were assembled using the Gibson assembly kit (NEB).

Protein overexpression and purification

Proteins used for the chemoenzymatic preparation of WTA precursors (TagA, TagB, TagD, MnaA)^{26,27,29,30} were described previously. The mutant SgtB* used to prepared peptidoglycan oligomers, was also overexpressed using the previously reported purification³³. For overexpression of TM-LCP proteins, 1.5 liters of LB media with BL21(DE3) *E. coli* containing the plasmid pET28b(-)_lcpA, pET28b(-)_lcpB, or pET28b(-)_lcpC were grown at 37 °C until OD₆₀₀=0.6, and then protein expression was induced with 1 mM IPTG. The temperature of the culture was reduced to 16 °C and grown for 18 hours before cells were pelleted by centrifugation at 4,200 x g at 4 °C. Using a cell disruptor, cells were lysed in 30 ml of lysis buffer (100 mM Tris-HCl (pH 8), 500 mM NaCl, 0.6% CHAPS, 0.5% Triton X-100 reduced, lysozyme, and PMSF). Clarified lysate was purified by nickel affinity chromatography. The equilibrated nickel resin was washed with 20 mM imidazole in buffer B (100 mM Tris-HCl (pH 8), 500 mM NaCl, 10% glycerol), 40 mM imidazole in buffer B, and then eluted with 250 mM imidazole in buffer B. Elution fractions were purified further using size exclusion chromatography on the Superdex 75 10/300 GL (Äkta Pure), pre-equilibrated with buffer C (50 mM Tris (pH 8), 500 mM NaCl, 20% glycerol). TM- LcpA, LcpB, and LcpC were collected and the concentrations were calculated by a Bradford assay using bovine serum albumin (BSA) as the standard and absorption at 280 nm as measured by the Nanodrop 2000. SDS-PAGE confirmed the purity of TM- LcpA, LcpB, and LcpC.

Preparation of WTA and PG substrates

LI^{WTA}, hexaprenyl-pyrophospho-GlcNAc, was prepared previously from published chemical routes^{28,29}. In the preparation of cold LII_A^{WTA}, LI^{WTA} (100 μM) was incubated with TagA (15 μM), UDP-GlcNAc (300 μM), and MnaA (3 μM) in previously described conditions²⁷. For PAGE autoradiography experiments, radiolabeled LII_A^{WTA} was prepared with UDP-[¹⁴C]-GlcNAc (specific activity= 300 nCi nmol⁻¹). These Lipid II_A^{WTA} products were purified using a BakerbondTM spe Octadecyl (C18) disposable column (Catalog number # 7020-01). The C18 column was equilibrated with 2× 750 μl MeOH/0.1% NH₄OH and 2× 750 μl H₂O/0.1% NH₄OH. The column was washed 4× with 500 μl H₂O/0.1% NH₄OH and eluted with 3× 500 μl MeOH/0.1% NH₄OH. Product yield was obtained by measuring [¹⁴C] counts using a liquid scintillation counter, and using a standard curve of the UDP-[¹⁴C]-GlcNAc to assess product conversion. Side-by-side cold LII_A^{WTA} and radiolabeled LII_A^{WTA} were prepared to approximate similar yield for cold LII_A^{WTA}. Cold

reactions were analyzed by LC/MS to confirm the formation of product as described previously²⁶. To prepare LII_B^{WTA}, cold UDP-GlcNAc was used under the same conditions for LII_A^{WTA} preparation. Radiolabeled LII_B^{WTA} was prepared using CDP-[¹⁴C]-glycerol (500 μM; prepared from TarD and [¹⁴C]-glycerol-3-phosphate, specific activity= 150 nCi nmol⁻¹, as previously reported³⁰) and TagB in previously described conditions²⁶. These products were purified on a Bakerbond™ spe octadecyl (C18) disposable column under similar wash and elution conditions used to prepare LII_A^{WTA}. Product yield was obtained by measuring [¹⁴C] counts using a liquid scintillation counter, and a standard curve of [¹⁴C]-glycerol-3-phosphate was made to assess product conversion. LC/MS was used to confirm cold LII_B^{WTA} product formation²⁶.

To prepare peptidoglycan oligomers either synthetic tetraprenyl (C20)- or heptaprenyl (C35)-Lipid II was used as indicated^{31,48,49}. Radiolabeled C20- and C35- Lipid II was prepared following previously published routes^{31,50}. C35-linked peptidoglycan oligomers were prepared from incubating SgtB* (800 nM), 20% DMSO, 32 μM C35-Lipid II, reaction buffer (12.5 mM HEPES (pH 8), 2 mM MnCl₂, 0.25 mM Tween-80) at room temperature for 20 minutes. Reaction mixtures were quenched with equal volume (10 μl) of methanol, and SgtB* was removed by collecting the supernatant which was dried *in vacuo*. C20-peptidoglycan oligomers were prepared by incubating SgtB* (5 μM), 20% DMSO, 32 μM C20-Lipid II, reaction buffer (12.5 mM HEPES (pH 8), 2 mM MnCl₂, 0.25 mM Tween-80) for 2 hours at room temperature, quenched with equal volume of methanol, and the supernatant was collected and dried *in vacuo*.

Assays for LcpA, LcpB, and LcpC activity

For gel electrophoresis analysis, peptidoglycan oligomers (dried following precipitation of SgtB*; prepared from 32 μM or C35- or C20-Lipid II) were resuspended in H₂O and sonicated for approximately five minutes. LcpA reaction buffer (12.5 mM HEPES (pH 8), 2 mM MnCl₂, 0.25 mM Tween-80), [¹⁴C]-LII_A^{WTA} or [¹⁴C]-LII_B^{WTA} (8 μM; DMSO), and active LCP protein (LcpA, LcpB, or LcpC; 2 μM) was added and the reaction proceeded for 6 hours at room temperature. Similar reactions lacking LCP protein were similarly set up. Reactions were quenched with 10 μl MeOH prior to drying *in vacuo*. For LC/MS analysis, peptidoglycan oligomers (dried; prepared from 32 μM C20-Lipid II) were resuspended in H₂O and sonicated for approximately five minutes. LcpA reaction buffer, LII_A^{WTA} (8 μM; DMSO), and LcpA (2 μM) were added and reactions proceeded at room temperature for 6 hours. Reactions were quenched with equal reaction volume of MeOH (10 μl).

PAGE analysis of the LcpA, LcpB, and LcpC

The acrylamide gels used were similar to those described previously⁵¹. Gels (20 cm × 16 cm (H × W); 1.0 mm thickness) were constructed with 10% acrylamide/Tris⁵¹. LcpA, LcpB, and LcpC reactions were quenched directly with 10 μl MeOH and dried down *in vacuo*. Reactions were resuspended in 10 μL of 2× SDS loading buffer and loaded onto the gel. The gels were electrophoresed with anode and cathode buffer as previously described^{51,52}. The anode buffer consisted of 100 mM Tris (pH 8.8), and the cathode buffer consisted of 100 mM Tris (pH 8.25), 100 mM Tricine, and 0.1% SDS. At a 30 mA voltage, gels ran for approximately 5 hours. The gels were dried with a gel dryer (Labconco, Catalog #4330100).

Dried gels were exposed to a general purpose storage phosphor screen for approximately 12–24 hours. An autoradiograph was imaged using a Typhoon 9400 imager and analyzed using the software ImageQuant TL.

Mass spectrometry methods for analyzing LCP reactions

C20-PG oligomer mixtures, reaction mixtures lacking LcpA, and LcpA reaction mixtures were resuspended in 1:1 H₂O/MeOH. High-resolution mass spectrometry (Q-ExactiveTM Hybrid Quadrupole-Orbitrap Mass Spectrometer) was used to separate peptidoglycan oligomers. With a 10 µl injection volume, samples were separated on a C18 (50×20 mm; Gemini-NX 5 µm 110 Å, Phenomenex). Products were eluted with 100% Solvent A (95:5 Water:Methanol/0.1% NH₄OH) over 9.5 minutes with a flow rate of 0.4 ml min⁻¹. Mass spectra were analyzed using the Exactive series 2.5, XCalibur 3.0 (Thermo Scientific).

Transposon Sequencing

Transposon sequencing was performed as described previously⁵³. Briefly, a pooled transposon library was grown in the presence and absence of 10 µg ml⁻¹ of amsacrine at 30°C until an OD₆₀₀=1.0. Genomic DNA was prepared and sent for Illumina sequencing. Data was processed using the Tufts University Genomics Core Facility Galaxy server^{54–56} and mapped to *S. aureus* NCTC8325 using Bowtie⁵⁷. The Mann-Whitney U test was used to identify genes with depleted reads, and genes that had more than a tenfold change in the number of reads between the untreated control and treated were considered depleted. The threshold for significance was set to P < 0.05 after correcting for false discovery with the Benjamini-Hochberg procedure. Six genes, one of which is *lcpA*, contained depleted reads between all samples.

Spot dilutions of *S. aureus* strains on amsacrine, Congo red, and oxacillin plates

S. aureus cultures were grown overnight to stationary phase and diluted 1:100 in fresh TSB, shaking at 37°C. Cultures were re-grown for approximately four hours, and normalized to OD₆₀₀=1.0. Each culture was diluted in a series of tenfold dilutions and plated on TSA containing amsacrine (10 µg ml⁻¹), Congo red (100 µg ml⁻¹), or oxacillin (0.625 or 0.125 µg ml⁻¹). Plates were incubated at 30 or 37 °C overnight.

MICs against *S. aureus* strains

S. aureus strains were grown overnight in TSB, and diluted 1:100 in TSB the following morning. At OD₆₀₀=1.0, strains were diluted 1/1000 in 150 µl of TSB with 1.5 µl of compound (amsa, O-amsa, oxacillin, cefaclor) in 96-well plates and incubated at 30 °C. OD₆₀₀ values were measured to determine MIC values after 16–18 hours.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dr. Vanina Haunreiter for her generous gift of the strains J126, JH100, RH53, RH72, PS60, PS109, and PS47^{19,42}, Dr. Sunia Trauger and the Bauer Core Facilities for assistance with mass spectrometry, and

the Dr. Ting Pang and the Bernhardt lab for their gift of the plasmid pTP63. This research was supported by the NIH (P01AI083214 and R01 AI099144 to S.W.; R01 GM076710 to D.K. & S.W.).

Main Text References

1. Silhavy TJ, Kahne D, Walker S. The bacterial cell envelope. *Cold Spring Harb Perspect Biol.* 2010; 2:a000414. [PubMed: 20452953]
2. Vollmer W, Blanot D, de Pedro MA. Peptidoglycan structure and architecture. *FEMS Microbiol Rev.* 2008; 32:149–67. [PubMed: 18194336]
3. Rajagopal M, Walker S. Envelope Structures of Gram-Positive Bacteria. *Curr Top Microbiol Immunol.* 2016
4. Neuhaus FC, Baddiley J. A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in gram-positive bacteria. *Microbiol Mol Biol Rev.* 2003; 67:686–723. [PubMed: 14665680]
5. Weidenmaier C, Peschel A. Teichoic acids and related cell-wall glycopolymers in Gram-positive physiology and host interactions. *Nat Rev Microbiol.* 2008; 6:276–87. [PubMed: 18327271]
6. Atilano ML, et al. Teichoic acids are temporal and spatial regulators of peptidoglycan cross-linking in *Staphylococcus aureus*. *Proc Natl Acad Sci USA.* 2010; 107:18991–6. [PubMed: 20944066]
7. Campbell J, et al. Synthetic lethal compound combinations reveal a fundamental connection between wall teichoic acid and peptidoglycan biosyntheses in *Staphylococcus aureus*. *ACS Chem Biol.* 2011; 6:106–16. [PubMed: 20961110]
8. Weidenmaier C, et al. Role of teichoic acids in *Staphylococcus aureus* nasal colonization, a major risk factor in nosocomial infections. *Nat Med.* 2004; 10:243–5. [PubMed: 14758355]
9. Brown S, Santa Maria JP Jr, Walker S. Wall teichoic acids of gram-positive bacteria. *Annu Rev Microbiol.* 2013; 67:313–36. [PubMed: 24024634]
10. Farha MA, et al. Inhibition of WTA Synthesis Blocks the Cooperative Action of PBPs and Sensitizes MRSA to beta-Lactams. *ACS Chem Biol.* 2013; 8:226–233. [PubMed: 23062620]
11. Brown S, et al. Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids. *Proc Natl Acad Sci USA.* 2012; 109:18909–14. [PubMed: 23027967]
12. Sham LT, et al. Bacterial cell wall. MurJ is the flippase of lipid-linked precursors for peptidoglycan biogenesis. *Science.* 2014; 345:220–2.
13. Ruiz N. Bioinformatics identification of MurJ (MviN) as the peptidoglycan lipid II flippase in *Escherichia coli*. *Proc Natl Acad Sci USA.* 2008; 105:15553–7. [PubMed: 18832143]
14. Mohammadi T, et al. Identification of FtsW as a transporter of lipid-linked cell wall precursors across the membrane. *EMBO J.* 2011; 30:1425–32. [PubMed: 21386816]
15. Yokoyama K, Miyashita T, Araki Y, Ito E. Structure and functions of linkage unit intermediates in the biosynthesis of ribitol teichoic acids in *Staphylococcus aureus* H and *Bacillus subtilis* W23. *Eur J Biochem.* 1986; 161:479–89. [PubMed: 3096735]
16. Bera A, et al. Influence of wall teichoic acid on lysozyme resistance in *Staphylococcus aureus*. *J Bacteriol.* 2007; 189:280–3. [PubMed: 17085565]
17. Schlag M, et al. Role of staphylococcal wall teichoic acid in targeting the major autolysin Atl. *Mol Microbiol.* 2010; 75:864–73. [PubMed: 20105277]
18. Kawai Y, et al. A widespread family of bacterial cell wall assembly proteins. *EMBO J.* 2011; 30:4931–41. [PubMed: 21964069]
19. Dengler V, et al. Deletion of hypothetical wall teichoic acid ligases in *Staphylococcus aureus* activates the cell wall stress response. *FEMS Microbiol Lett.* 2012; 333:109–20. [PubMed: 22640011]
20. Chan YG, Frankel MB, Dengler V, Schneewind O, Missiakas D. *Staphylococcus aureus* mutants lacking the LytR-CpsA-Psr family of enzymes release cell wall teichoic acids into the extracellular medium. *J Bacteriol.* 2013; 195:4650–9. [PubMed: 23935043]
21. Chan YG, Kim HK, Schneewind O, Missiakas D. The capsular polysaccharide of *Staphylococcus aureus* is attached to peptidoglycan by the LytR-CpsA-Psr (LCP) family of enzymes. *J Biol Chem.* 2014; 289:15680–90. [PubMed: 24753256]

22. Liszewski Zilla M, Chan YG, Lunderberg JM, Schneewind O, Missiakas D. LytR-CpsA-Psr enzymes as determinants of *Bacillus anthracis* secondary cell wall polysaccharide assembly. *J Bacteriol.* 2015; 197:343–53. [PubMed: 25384480]
23. Harrison J, et al. Lcp1 Is a Phosphotransferase Responsible for Ligating Arabinogalactan to Peptidoglycan in *Mycobacterium tuberculosis*. *MBio.* 2016; 7:e00972–16. [PubMed: 27486192]
24. Over B, et al. LytR-CpsA-Psr proteins in *Staphylococcus aureus* display partial functional redundancy and the deletion of all three severely impairs septum placement and cell separation. *FEMS Microbiol Lett.* 2011; 320:142–51. [PubMed: 21554381]
25. Brown S, Meredith T, Swoboda J, Walker S. *Staphylococcus aureus* and *Bacillus subtilis* W23 make polyribitol wall teichoic acids using different enzymatic pathways. *Chem Biol.* 2010; 17:1101–10. [PubMed: 21035733]
26. Brown S, Zhang YH, Walker S. A revised pathway proposed for *Staphylococcus aureus* wall teichoic acid biosynthesis based on in vitro reconstitution of the intracellular steps. *Chem Biol.* 2008; 15:12–21. [PubMed: 18215769]
27. Gale RT, Sewell EW, Garrett TA, Brown ED. Reconstituting poly(glycerol phosphate) wall teichoic acid biosynthesis in vitro using authentic substrates. *Chemical Science.* 2014; 5:3823–3830.
28. Lee W, et al. The Mechanism of Action of Lysobactin. *J Am Chem Soc.* 2015; 138:100–3. [PubMed: 26683668]
29. Ginsberg C, Zhang YH, Yuan Y, Walker S. In vitro reconstitution of two essential steps in wall teichoic acid biosynthesis. *ACS Chem Biol.* 2006; 1:25–8. [PubMed: 17163636]
30. Badurina DS, Zolli-Juran M, Brown ED. CTP:glycerol 3-phosphate cytidyltransferase (TarD) from *Staphylococcus aureus* catalyzes the cytidyl transfer via an ordered Bi-Bi reaction mechanism with micromolar $K(m)$ values. *Biochim Biophys Acta.* 2003; 1646:196–206. [PubMed: 12637027]
31. Ye XY, et al. Better substrates for bacterial transglycosylases. *J Am Chem Soc.* 2001; 123:3155–6. [PubMed: 11457035]
32. Lebar MD, et al. Forming cross-linked peptidoglycan from synthetic gram-negative Lipid II. *J Am Chem Soc.* 2013; 135:4632–5. [PubMed: 23480167]
33. Rebets Y, et al. Moenomycin resistance mutations in *Staphylococcus aureus* reduce peptidoglycan chain length and cause aberrant cell division. *ACS Chem Biol.* 2014; 9:459–67. [PubMed: 24255971]
34. Tipper DJ, Strominger JL, Ensign JC. Structure of the cell wall of *Staphylococcus aureus*, strain Copenhagen. VII Mode of action of the bacteriolytic peptidase from *Myxobacter* and the isolation of intact cell wall polysaccharides *Biochemistry.* 1967; 6:906–20. [PubMed: 4290597]
35. Perlstein DL, Zhang Y, Wang TS, Kahne DE, Walker S. The direction of glycan chain elongation by peptidoglycan glycosyltransferases. *J Am Chem Soc.* 2007; 129:12674–5. [PubMed: 17914829]
36. Eberhardt A, et al. Attachment of capsular polysaccharide to the cell wall in *Streptococcus pneumoniae*. *Microb Drug Resist.* 2012; 18:240–55. [PubMed: 22432711]
37. Pasquina L, et al. A synthetic lethal approach for compound and target identification in *Staphylococcus aureus*. *Nat Chem Biol.* 2016; 12:40–5. [PubMed: 26619249]
38. Percy MG, Grundling A. Lipoteichoic acid synthesis and function in gram-positive bacteria. *Annu Rev Microbiol.* 2014; 68:81–100. [PubMed: 24819367]
39. Peschel A, Vuong C, Otto M, Gotz F. The D-alanine residues of *Staphylococcus aureus* teichoic acids alter the susceptibility to vancomycin and the activity of autolytic enzymes. *Antimicrob Agents Chemother.* 2000; 44:2845–7. [PubMed: 10991869]
40. Santa Maria JP Jr, et al. Compound-gene interaction mapping reveals distinct roles for *Staphylococcus aureus* teichoic acids. *Proc Natl Acad Sci USA.* 2014; 111:12510–5. [PubMed: 25104751]
41. Suzuki T, et al. Wall teichoic acid protects *Staphylococcus aureus* from inhibition by Congo red and other dyes. *J Antimicrob Chemother.* 2012; 67:2143–51. [PubMed: 22615298]
42. Hubscher J, et al. MsrR contributes to cell surface characteristics and virulence in *Staphylococcus aureus*. *FEMS Microbiol Lett.* 2009; 295:251–60. [PubMed: 19459977]

43. Hartman BJ, Tomasz A. Low-affinity penicillin-binding protein associated with beta-lactam resistance in *Staphylococcus aureus*. *J Bacteriol.* 1984; 158:513–6. [PubMed: 6563036]
44. Matsuhashi M, et al. Molecular cloning of the gene of a penicillin-binding protein supposed to cause high resistance to beta-lactam antibiotics in *Staphylococcus aureus*. *J Bacteriol.* 1986; 167:975–80. [PubMed: 3638304]
45. Huang CY, et al. Crystal structure of *Staphylococcus aureus* transglycosylase in complex with a lipid II analog and elucidation of peptidoglycan synthesis mechanism. *Proc Natl Acad Sci USA.* 2012; 109:6496–501. [PubMed: 22493270]
46. Kaito C, Sekimizu K. Colony spreading in *Staphylococcus aureus*. *J Bacteriol.* 2007; 189:2553–7. [PubMed: 17194792]

Online Methods References

47. Kato F, Sugai M. A simple method of markerless gene deletion in *Staphylococcus aureus*. *J Microbiol Methods.* 2011; 87:76–81. [PubMed: 21801759]
48. Chen L, et al. Intrinsic Lipid Preferences and Kinetic Mechanism of *Escherichia coli* MurG⁺. *Biochemistry.* 2002; 41:6824–6833. [PubMed: 12022887]
49. Tsukamoto H, Kahne D. N-methylimidazolium chloride-catalyzed pyrophosphate formation: application to the synthesis of Lipid I and NDP-sugar donors. *Bioorg Med Chem Lett.* 2011; 21:5050–3. [PubMed: 21592792]
50. Perlstein DL, Wang TS, Doud EH, Kahne D, Walker S. The role of the substrate lipid in processive glycan polymerization by the peptidoglycan glycosyltransferases. *J Am Chem Soc.* 2010; 132:48–9. [PubMed: 20017480]
51. Barrett D, et al. Analysis of glycan polymers produced by peptidoglycan glycosyltransferases. *J Biol Chem.* 2007; 282:31964–71. [PubMed: 17704540]
52. Wang TS, et al. Primer preactivation of peptidoglycan polymerases. *J Am Chem Soc.* 2011; 133:8528–30. [PubMed: 21568328]
53. Santiago M, et al. A new platform for ultra-high density *Staphylococcus aureus* transposon libraries. *BMC Genomics.* 2015; 16:252. [PubMed: 25888466]
54. Goecks J, Nekrutenko A, Taylor J, Galaxy T. Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* 2010; 11:R86. [PubMed: 20738864]
55. Blankenberg D, et al. Galaxy: a web-based genome analysis tool for experimentalists. *Curr. Protoc. Mol. Biol.* Chapter 19. Unit. 2010; 19(10):1–21.
56. Giardine B, et al. Galaxy: a platform for interactive large-scale genome analysis. *Genome Res.* 2005; 15:1451–5. [PubMed: 16169926]
57. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 2009; 10:R25. [PubMed: 19261174]

Figure 1. Schematic depicting the two major biosynthetic pathways that converge to assemble the cell wall of *S. aureus*

Peptidoglycan (green arrows) and wall teichoic acid precursors (purple arrows) are biosynthesized intracellularly on a shared phospholipid carrier, Und-P, and then flipped outside the cell. How these two pathways come together has not been established.

Figure 2. Reconstitution using purified protein and defined cell wall substrates shows that LcpA is a WTA transferase

(a) Scheme for enzymatic synthesis of WTA precursors LII_A^{WTA} and LII_B^{WTA} from synthetic Lipid I^{WTA}. Radiolabels were optionally incorporated using UDP-[¹⁴C]-GlcNAc for LII_A^{WTA} and CDP-[¹⁴C]-glycerol for LII_B^{WTA}. (b) Scheme for enzymatic synthesis of peptidoglycan oligomers from synthetic Lipid II, which optionally contained [¹⁴C]-GlcNAc. SgtB* contains a Y181D substitution and produces short peptidoglycan oligomers (n=1–7 disaccharide repeats). (c) An autoradiograph showing that LcpA transfers [¹⁴C]-WTA precursors to cold peptidoglycan oligomers (lanes 2 and 4). Control lanes 5 and 6 contain [¹⁴C]-Lipid II and [¹⁴C]-peptidoglycan oligomers, respectively.

Figure 3. High-resolution mass spectrometry shows that LcpA couples WTA precursors to nascent peptidoglycan, but not to Lipid II

(a) LcpA was incubated with $\text{LII}_A^{\text{WTA}}$ and peptidoglycan oligomers prepared from C20-Lipid II to produce coupled products (see also Supplementary Fig. 3). (b) The control reaction (no LcpA) and the LcpA reaction were analyzed using a QTM-exact LC-MS (see also Supplementary Table 1 and 2). Extracted ion chromatograms for coupled products are shown. A single WTA-disaccharide was attached via a phosphodiester to a peptidoglycan tetrasaccharide (Lipid IV), hexasaccharide (Lipid VI), and octasaccharide (Lipid VIII), respectively. (c) A schematic depicting a WTA disaccharide attached to Lipid IV is shown. Because WTA is not transferred to Lipid II, we conclude that the WTA disaccharide on Lipid IV must be attached to the MurNAc flanked by GlcNAc sugars. (d) The experimental isotope pattern ($z=-2$) for WTA-Lipid IV is consistent with the theoretical isotope pattern. See Supplementary Figure 4 for additional product isotope patterns.

Figure 4. LcpA is the major LCP protein involved in *S. aureus* WTA biosynthesis

(a) Treating a HG003 transposon library with a D-alanylation inhibitor (amsacrine) resulted in depletion of *tn::lcpA* mutants. *Tn::lcpB* and *tn::lcpC* mutants were abundant in presence of amsacrine, as indicated by the large number of transposon insertions. (b) Spot dilutions show that *lcpA* strains are susceptible to three chemical probes (amsacrine, Congo red, oxacillin) known to inhibit the growth of *tarO* strains, which lack wall teichoic acids (see Fig. 1). Growth of wildtype, *lcpB*, and *lcpC* strains is unaffected by the probes.