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Staphylococcus aureus Mutants Lacking the LytR-CpsA-Psr Family of Enzymes Release Cell Wall Teichoic Acids into the Extracellular Medium

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The LytR-CpsA-Psr (LCP) proteins are thought to transfer bactoprenol-linked biosynthetic intermediates of wall teichoic acid (WTA) to the peptidoglycan of Gram-positive bacteria. In *Bacillus subtilis*, mutants lacking all three LCP enzymes do not deposit WTA in the envelope, while *Staphylococcus aureus* Δ*lcp* mutants display impaired growth and reduced levels of envelope phosphate. We show here that the *S. aureus* Δ*lcp* mutant synthesized WTA yet released ribitol phosphate polymers into the extracellular medium. Further, Δ*lcp* mutant staphylococci no longer restricted the deposition of LysM-type murein hydrolases to cell division sites, which was associated with defects in cell shape and increased autolysis. Mutations in *S. aureus* WTA synthesis genes (*tagB*, *tarF*, or *tarJ2*) inhibit growth, which is attributed to the depletion of bactoprenol, an essential component of peptidoglycan synthesis (lipid II). The growth defect of *S. aureus tagB* and *tarFJ* mutants was alleviated by inhibition of WTA synthesis with tunicamycin, whereas the growth defect of the Δ*lcp* mutant was not relieved by tunicamycin treatment or by mutation of *tagO*, whose product catalyzes the first committed step of WTA synthesis. Further, sortase A-mediated anchoring of proteins to peptidoglycan, which also involves bactoprenol and lipid II, was not impaired in the Δ*lcp* mutant. We propose a model whereby the *S. aureus* Δ*lcp* mutant, defective in tethering WTA to the cell wall, cleaves WTA synthesis intermediates, releasing ribitol phosphate into the medium and recycling bactoprenol for peptidoglycan synthesis.

The peptidoglycan layer protects Gram-positive bacteria from osmotic lysis and serves as a barrier against compounds toxic to the membrane (1). Peptidoglycan also functions as a scaffold for the immobilization of capsular polysaccharides (2), wall teichoic acids (WTAs) (3), and proteins (4). Surface proteins are anchored by sortase A, a membrane-embedded transpeptidase that scans secreted polypeptides for the LPXTG motif of sorting signals (5). Sortase A cleaves the peptide bond between the threonine and the glycine of the LPXTG motif to form a thioester-linked intermediate between the carboxyl group of threonine at the C-terminal end of surface proteins and its active-site cysteine residue (5, 6). The sortase A acyl intermediate is resolved by the nucleophilic attack of the free amino group of the pentaglycine cross bridge within lipid II (7, 8), the substrate for peptidoglycan biosynthesis (9). Surface protein linked to lipid II is incorporated into the cell wall envelope via the transpeptidation and transglycosylation reactions of peptidoglycan synthesis (10–12). In *Staphylococcus aureus*, lipid II is comprised of C_{55}-PP–MurNAc–[D-Ala–D-iGln-(NH_2-Gly_5)–L-Lys–D-Ala–D-Ala]–GlcNAc, i.e., a murein disaccharide-pentapeptide subunit linked to the membrane carrier bactoprenol (C_{55}). In Gram-positive bacteria, bactoprenol/undecaprenyl is also used for the synthesis of extracellular polymers, such as WTA (13), teichuronic acid (14), and cell wall polysaccharides (15).

The *S. aureus* WTA is a polymer of 30 to 50 ribitol phosphate (Rbo-P) subunits connected via 1,5-phosphodiester bonds (16). Rbo-P_n is tethered to peptidoglycan via the murein linkage unit, GlcNAc-ManNAc-(glycerol phosphate [Gro-P])$_{2-3}$ (17). Synthesis of the murein linkage unit is initiated by TagO (also referred to as TarO), which links UDP-GlcNAc and undecaprenyl phosphate to generate C_{55}-PP–GlcNAc (14, 18, 19). The other WTA subunits are added to the undecaprenyl-linked intermediate via the enzymes TagA (ManNAc) (17, 20), TagBDF (Gro-P), and TarILJ (Rbo-P) (21–23). The product of this pathway, C_{55}-PP–GlcNAc–ManNAc–(Gro-P)$_{2-3}$–(Rbo-P)$_{30-50}$, is presumably flipped across the plasma membrane by the TagGH transporter (24). Attachment of the WTA polymer to the C-6 hydroxyl of *N*-acetylmuramic acid within peptidoglycan [MurNAc-P–GlcNAc–ManNAc–(Gro-P)$_{2-3}$–(Rbo-P)$_{30-50}$] occurs during cell wall assembly (25). The first two genes of the WTA pathway (*tagO*, *tagA*) can be deleted without abolishing staphylococcal growth (20, 26, 27). In contrast, *tagBDFGH* and *tarIJL* cannot be deleted unless staphylococci carry inactivating mutations in *tagA* or *tagO* (28). This synthetic viable phenotype is explained by the limited availability of bactoprenol and its undecaprenyl phosphate derivatives (C_{55}-PP and C_{55}-P) for peptidoglycan cell wall biosynthesis (28). WTA synthesis is blocked by tunicamycin, an antibiotic from *Streptomyces clavuligerus* that inhibits TagO (29).

Kawai et al. proposed that a family of genes encoding the LytR-CpsA-Psr (LCP) proteins catalyzes attachment of the murein linkage unit of WTA to the peptidoglycan of *Bacillus subtilis* (30). *B. subtilis* encodes three *lcp* gene homologues, designated *tagTUV*, which are positioned within gene clusters for WTA biosynthetic enzymes (30). Simultaneous deletion of all three genes (*tagTUV*) is not compatible with bacterial growth unless bacilli lack the *tagO*

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Genotype or insert	Description	Reference or source
Strains			
RN4220	Wild type	*S. aureus* laboratory strain, a restriction-deficient derivative of NCTC 8325-4	56
RN4220 Δ*tagO*	RN4220 *tagO*	RN4220 lacking *tagO*	57
RN4220 *tagB*::*erm*	RN4220 *tagB*	RN4220 with a *bursa aurealis* insertion in *tagB*	This work
RN4220 *tarF*::*erm*	RN4220 *tarF*	RN4220 with a *bursa aurealis* insertion in *tarF*	This work
RN4220 *tarJ2*::*erm*	RN4220 *tarJ2*	RN4220 with a *bursa aurealis* insertion in *tarJ2*	This work
MSSA 1112	Wild type	MSSA clinical isolate	58
MSSA 1112 Δ*lcpA*	MSSA 1112 *lcpA*::*erm*	MSSA 1112 with *ermB* replacing *lcpA* (*msrR*)	46
MSSA 1112 Δ*lcpB*	MSSA 1112 *lcpB*	MSSA 1112 lacking *lcpB* (*sa0908*)	33
MSSA 1112 Δ*lcpC*	MSSA 1112 *lcpC*	MSSA 1112 lacking *lcpC* (*sa2103*)	33
MSSA 1112 Δ*lcpAB*	MSSA 1112 *lcpA lcpB*	MSSA 1112 lacking *lcpA* and *lcpB*	33
MSSA 1112 Δ*lcpAC*	MSSA 1112 *lcpA lcpC*	MSSA 1112 lacking *lcpA* and *lcpC*	33
MSSA 1112 Δ*lcpBC*	MSSA 1112 *lcpB lcpC*	MSSA 1112 lacking *lcpB* and *lcpC*	33
MSSA 1112 Δ*lcp*	MSSA 1112 *lcp*	MSSA 1112 lacking all three *lcp* genes	33
MSSA 1112 Δ*lcp* Δ*tagO*	MSSA 1112 *lcp tagO*	MSSA 1112 lacking all three *lcp* genes and *tagO*	This work
Plasmids			
pΔ*tagO*	DNA segments flanking *tagO*	pKOR1 carrying a 1-kbp DNA segment upstream and downstream of *tagO*, used for allelic replacement	This work
pHTT4	SEB-MH_6-CWS	pOS1 vector encoding SEB with the CWS motif of protein A	10
pBursa	Modified mariner transposon	Plasmid carrying the mariner-based transposon with erythromycin resistance (thermosensitive replicon)	36
pFA545	Mariner transposase	Plasmid carrying the transposase (thermosensitive replicon)	36
p*lcpA*	*lcpA* (*msrR*)	Plasmid pGC2 (pT194 based) encoding *lcpA* for complementation studies	33
p*lcpB*	*lcpB* (*sa0908*)	Plasmid pGC2 (pT194 based) encoding *lcpB* for complementation studies	33
p*lcpC*	*lcpC* (*sa2103*)	Plasmid pGC2 (pT194 based) encoding *lcpC* for complementation studies	33

gene. Blocking the expression of *tagTUV* causes a concomitant decrease in the synthesis of WTA (30). Further, X-ray crystallography identified polyprenyl phosphate bound to recombinant *B. subtilis* TagT or its *Streptococcus pneumoniae* Cps2A homologue, both of which also exert *in vitro* phosphatase activity (30, 31). On the basis of these observations, Kawai et al. proposed that LCP enzymes recognize WTA or capsular polysaccharide synthesis intermediates as the substrates for the formation of phosphodiester linkages formed between the C6-hydroxyl of MurNAc in peptidoglycan and GlcNAc-ManNAc murein linkage units (30). In agreement with this model, mutants (with the Δ*lcp* mutation) lacking all of the three LCP homologues of *S. aureus*—*lcpA* (*mrsR*), *lcpB* (*sa0908*), and *lcpC* (*sa2103*)—do not harbor phosphate residues in the cell wall envelope (32) and cannot properly place cell division septa (33). Previous work left unresolved whether *S. aureus* Δ*lcp* mutants are defective in the synthesis and/or the cell wall anchoring of WTA and whether their associated cell division defect is due to the sequestration of lipid II within the WTA pathway (32, 33). The present study was performed to address these questions.

MATERIALS AND METHODS

Bacterial strains, bacterial growth, and reagents. *S. aureus* strains were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) supplemented with appropriate antibiotics. Erythromycin (Erm) and chloramphenicol (Cm) were used at a concentration of 10 μg/ml, and tunicamycin (Tun), unless otherwise specified, was used at a concentration of 1 μg/ml. To examine the effect of tunicamycin on bacterial growth, overnight cultures grown in the absence of tunicamycin were diluted (1:100) into 100 μl fresh TSB with or without tunicamycin and growth at 37°C was monitored every 15 min for 12 h in a Synergy HT plate reader (BioTek) by measuring the absorbance at 600 nm (A_{600}). To assess cell viability, overnight cultures of candidate strains were started by inoculating an isolated colony from a fresh plate into fresh medium. On the next day, a subculture was prepared by diluting an aliquot of the overnight culture in tubes containing fresh medium (1:100). The tubes were placed at 37°C with shaking. Sample aliquots were removed from the cultures after 3.5 h or 16 h, A_{600} values were recorded, and aliquots were serially diluted and plated on agar. The numbers of CFU were determined following incubation of the plates at 37°C overnight. The bacterial strains and plasmids utilized in this study are listed in Table 1. Mutations in the three LCP-encoding genes, *msrR*, *sa0908*, and *sa2103*, have been described earlier (32). For convenience, the genes are designated herein *lcpA*, *lcpB*, and *lcpC*, respectively. The nomenclature of the single, double, and triple mutants has been changed accordingly: each of the single or double mutants is referred to as the Δ*lcpA* (*msrR*), Δ*lcpB* (*sa0908*), Δ*lcpC* (*sa2103*), Δ*lcpAB* (*msrR* and *sa0908*), Δ*lcpAC* (*msrR* and *sa2103*), and Δ*lcpBC* (*sa0908* and *sa2103*) mutants. The Δ*lcp* mutant is the triple mutant lacking all three *lcp* genes (*msrR*, *sa0908*, *sa2103*). Deletion of *tagO* (Δ*tagO*) was achieved by allelic replacement using plasmid pKOR1 (34, 35). Transposon mutagenesis was performed using two plasmids, pFA545 and pBursa (36). Transposon mutants were isolated on agar plates containing tunicamycin, and candidate clones were rescreened for tunicamycin-dependent growth. To analyze the anchor structure of surface proteins, *S. aureus* strains were transformed with plasmid pHTT4 (10), which provides for the expression of the hybrid protein SEB-MH_6-CWS, where SEB and CWS represent secreted staphylococcal enterotoxin B and the cell wall-sorting signal of

protein A, respectively, separated by the engineered methionine–6-histidine (MH_6) affinity tag for purification.

Purification of SEB-MH_6-CWS and analysis of C-terminal anchor peptides. Methicillin-sensitive *S. aureus* (MSSA) 1112 strains (wild type and Δ*lcp* mutant) harboring pHTT4 (10) were grown to an A_{600} of 0.8. Cells were washed, suspended in 50 ml of 50 mM Tris-HCl buffer (pH 7.5) supplemented with 5 mM phenylmethylsulfonyl fluoride (PMSF), and broken in a BeadBeater instrument (Biospec Products Inc.). Crude lysates were centrifuged at 33,000 × *g* for 15 min, and sedimented material was suspended in 100 ml of 100 mM KH_2PO_4 (pH 7.5), containing 1% Triton X-100 and 1 mM PMSF. Samples were incubated for 3 h at 4°C with stirring to extract membrane lipids. Cell wall material containing SEB-MH_6-CWS was sedimented by centrifugation at 33,000 × *g* for 15 min, washed three times with 100 ml of 100 mM sodium phosphate buffer (pH 6.0), and suspended in 30 ml of 100 mM Tris-HCl buffer (pH 7.5) supplemented with 1 mM PMSF. SEB-MH_6-CWS was purified as described previously (10, 37). Briefly, the sample was incubated with 2 mg of lysostaphin for 16 h at 37°C and centrifuged at 33,000 × *g* for 15 min. The supernatant containing SEB-MH_6-CWS was applied to gravity-flow columns packed with Ni-nitrilotriacetic acid (NTA) beads. The column was washed with 30 column volumes of buffer A (100 mM Tris-HCl buffer [pH 7.5], 150 mM NaCl) containing 10 mM imidazole. SEB-MH_6-CWS was eluted in buffer A with 0.5 M imidazole. Protein in the eluate was precipitated with methanol-chloroform, dried under vacuum, and solubilized in 3 ml 70% formic acid prior to the addition of a cyanogen bromide crystal. Samples were incubated for 16 h at room temperature in the dark. Reaction products were washed twice with water, suspended in 1 ml of buffer B (10 mM Tris-HCl, 100 mM NaH_2PO_4, 6 M guanidine hydrochloride, pH 8.0), and applied to a gravity-flow column packed with Ni-NTA beads equilibrated in buffer B. The column was washed with 10 volumes each of buffer B, buffer C (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl) at pH 8.0, and buffer C at pH 6.3. Anchor peptides including H_6-CWS were eluted in buffer C containing 0.5 M imidazole at pH 8.0. For mass spectrometry analysis, peptides were desalted onto C_{18} matrix cartridges (Waters) that had been prewashed with 10 ml of acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (TFA) and 10 ml of 0.1% TFA in water. Peptides were eluted in 3 ml 60% CH_3CN, 0.1% TFA, dried under vacuum, and suspended in 50 μl 30% CH_3CN, 0.1% TFA. A 0.5-μl aliquot of this suspension was cospotted with 0.5 μl of matrix, α-cyano-4-hydroxycinnamic acid (10 mg/ml in 50% CH_3CN, 0.1% TFA). Samples were subjected to matrix-assisted laser desorption ionization (MALDI)–time of flight (TOF) mass spectrometry (MS) using an Autoflex Speed Bruker MALDI instrument. Ions were detected in linear positive mode.

WTA preparations. WTA was extracted from murein sacculi as described previously (38). Briefly, cells from a 30-ml culture of *S. aureus* grown in TSB at 37°C to an A_{600} of 1.0 were washed with 30 ml of buffer D [50 mM 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.5] and suspended in 30 ml of buffer D containing 4% sodium dodecyl sulfate (SDS), followed by boiling for 1 h in a water bath and centrifugation for 10 min at 23,000 × *g*. The sediment was washed twice in 1 ml buffer D containing 4% SDS, once in buffer D containing 2% NaCl, and once in buffer D. Following each wash, murein sacculi were sedimented by centrifugation at 23,000 × *g* for 10 min, and after the final wash, the peptidoglycan was suspended in 1 ml of 20 mM Tris-HCl buffer at pH 8.0 containing 0.5% SDS and 20 μg proteinase K and the mixture was incubated at 50°C for 4 h. Protease-digested murein sacculi were washed once in buffer D containing 2% NaCl and thrice in water and suspended in 1 ml of 0.1 M NaOH for a 16-h incubation at room temperature with rotation, to hydrolyze WTA. Following sedimentation of NaOH-extracted murein sacculi at 23,000 × *g* for 10 min, the supernatant containing the released WTA was transferred to a new tube and neutralized with 50 μl of 0.1 M acetic acid and 100 mM Tris-HCl, pH 8.5, for subsequent analysis by polyacrylamide gel electrophoresis (PAGE).

WTA was also precipitated from 100-ml culture supernatants of *S. aureus* grown in TSB at 37°C to an A_{600} of 0.5 by adding 3 volumes of 95% ethanol and incubating at 4°C for 30 min. Precipitated material was sedimented by centrifugation (16,000 × *g* for 15 min), washed with 70% ethanol, air dried, and suspended in 5 ml of 100 mM Tris-HCl (pH 7.5) containing 5 mM $CaCl_2$, 25 mM $MgCl_2$, DNase (10 μg/ml), and RNase (50 μg/ml). Samples were incubated for 3 h at 37°C and subjected to methanol-chloroform extraction. The teichoic acid in the aqueous layer was dried under vacuum and solubilized in 1 ml of 20 mM Tris-HCl (pH 8.0).

Quantifying the phosphate content of the staphylococcal envelope. Murein sacculi were prepared as described previously and stored in water (39). The phosphate content was determined by incubating 45 μl of a murein sacculus sample (A_{600}, 1.0) with 5 μl of trichloroacetic acid at 80°C for 16 h. The inorganic phosphate released by this treatment was quantified with a colorimetric assay where a mix composed of 6 N H_2SO_4, water, 2.5% ammonium molybdate, and 10% ascorbic acid (in a ratio of 1:2:1:1) was added at 1:1 (vol/vol) to trichloroacetic acid-treated preparations, and the mixture was incubated at 37°C for 90 min. Product formation corresponding to free phosphate was measured in a spectrophotometer at 820 nm (A_{820}), and the phosphate concentration in the samples was calculated from NaH_2PO_4 standards (concentration, 0 to 800 μM).

Gel electrophoresis and immunoblotting. WTA was separated on polyacrylamide gels using a Bio-Rad Protean II xi electrophoresis cell (20 cm by 16 cm by 1 mm) (40). Following separation of the extracts by electrophoresis, gels were incubated twice in 1 mg/ml alcian blue for 20 min, followed by two 30-min washes with deionized H_2O and staining with a Silver Stain Plus kit (Bio-Rad). For comparative analyses of proteins, aliquots of the cultures used for WTA analysis were removed, treated with lysostaphin (4 μg), and incubated at 37°C for 10 min. Proteins in these lysates were precipitated with 7% trichloroacetic acid, washed once with acetone, dried, solubilized in 100 μl of 0.5 M Tris-HCl, pH 8.0, containing 4% SDS, and heated at 90°C for 10 min. Proteins were separated on 12% SDS-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane (Millipore) for immunoblot analysis with appropriate polyclonal antibodies. Immunoreactive signals were revealed by using a secondary antibody coupled to IRDye 680 and visualized with a LI-COR Biosciences Odyssey imager.

Phenotypic characterization of mutants. The ability of the *S. aureus* mutants to bind purified LysM-mCherry fluorescent protein was assessed as described previously (34, 41). Briefly, cells of logarithmically growing cultures were sedimented by centrifugation, washed in phosphate-buffered saline (PBS), and incubated with purified LysM-mCherry for 10 min. mCherry was used as a control. Following incubation, cells were washed twice in PBS and fixed in 4% paraformaldehyde in PBS prior to analysis by flow cytometry and fluorescence microscopy. Incubation with propidium iodide (Invitrogen) was used to evaluate the membrane integrity of the mutants compared to that of the wild type. Briefly, overnight cultures were diluted 1:100 into TSB containing 0 or 1 μg/ml tunicamycin and cultured at 37°C for 3.5 h. Following centrifugation of 1 ml culture, cells were washed twice with PBS, fixed for 20 min with 4% paraformaldehyde, and then blocked for 1 h in PBS with 1% bovine serum albumin. Cells were incubated for 15 min with 10 nM SYTO 9 (Invitrogen) and 2 μg/ml propidium iodide in PBS, washed twice, and suspended in PBS for flow cytometry measurements.

Flow cytometry and microscopy. Flow cytometric analyses were performed using a BD LSR-II cytometer. mCherry fluorescence was quantified using the allophycocyanin (APC) parameter and gating on single cells using forward and side scatter. Staphylococci were gated using forward and side scatter, and SYTO 9-positive cells captured under the fluorescein isothiocyanate parameter were analyzed for propidium iodide staining in the peridinin chlorophyll protein A parameter. The parameters for negative propidium iodide staining were determined using an unstained control. For fluorescence microscopy, images were captured on a Leica TCS SP2 AOBS confocal laser scanning microscope with a ×100 objective using identical settings and exposure times between samples. For transmission electron microscopy, bacterial cells were washed twice with 50

mM Tris-HCl (pH 7.5), 150 mM NaCl, bathed in fixative (2% glutaraldehyde, 4% phosphonoformic acid, 0.1 M sodium cacodylate buffer) overnight at 4°C, and postfixed with 1% OsO_4 in 0.1 M sodium cacodylate buffer for 60 min. Fixed samples were stained in 1% uranyl acetate in maleate buffer for 60 min, serially dehydrated with increasing concentrations of ethanol, embedded in Spurr resin for 48 h at 60°C, thin sectioned (90 nm) using a Reichert-Jung Ultracut device, and poststained in uranyl acetate and lead citrate. The samples were imaged on an FEI Tecnai F30 transmission electron microscope with a Gatan charge-coupled-device (CCD) digital micrograph.

RESULTS

The *S. aureus* Δ*lcp* mutant releases WTA into the extracellular medium. A *S. aureus* mutant with all three *lcp* genes deleted harbors very little phosphate in its cell wall envelope, suggesting that the mutant does not synthesize WTA (32). We wondered whether *S. aureus lcp* mutants may also be defective in the cell wall anchoring of WTA (30). To examine these possibilities, murein sacculi were isolated from wild-type *S. aureus* MSSA 1112 and its isogenic mutants lacking one *lcp* gene (the Δ*lcpA*, Δ*lcpB*, and Δ*lcpC* mutants), two *lcp* genes (the Δ*lcpAB*, Δ*lcpAC*, and Δ*lcpBC* mutants), or all three *lcp* genes (the Δ*lcp* mutant). WTA was released from murein sacculi via alkaline hydrolysis; Rbo-P polymers of variable length were resolved by PAGE and visualized by staining with alcian blue-silver. As controls, the murein sacculi of wild-type *S. aureus* harbored WTA, whereas treatment of staphylococci with tunicamycin, an inhibitor of TagO, blocked the synthesis and incorporation of WTA into the cell wall (Fig. 1A, lanes 1 and 2). The MIC of tunicamycin required to block WTA synthesis in *S. aureus* MSSA 1112 was 1 μg/ml (see Fig. S1 in the supplemental material). WTA was detected in the murein sacculi of each single and double *lcp* mutant (Fig. 1A, lanes 3 to 8) but not in sacculi prepared from the Δ*lcp* strain (Fig. 1A, lane 9). This WTA synthesis defect of the Δ*lcp* mutant was restored by transformation with plasmids expressing any one of the three LCP homologues, *lcpA*, *lcpB*, or *lcpC* (Fig. 1B, lanes 11 to 13). We also determined the cell wall phosphate content of the wild-type versus mutant strains via colorimetric assay. Here, peptidoglycan was extracted and purified from exponentially growing bacteria, and the inorganic phosphate was liberated by incubation in 10% trichloroacetic acid. Staphylococci incubated with tunicamycin synthesized and incorporated very little phosphate into the cell wall envelope (Fig. 1C). As already noted by Dengler et al. (32), Rbo-P was not detected in cell wall extracts derived from the Δ*lcp* mutant. Furthermore, the Δ*lcpA* and Δ*lcpB* single mutants but not the Δ*lcpC* single mutant contained reduced levels of phosphate compared to wild type, as did each of the double mutants (Fig. 1C). Nevertheless, loss of two *lcp* genes did not cause clear synergistic effects in reducing the phosphate content of staphylococcal cell walls (Fig. 1C).

We next examined whether the Rbo-P polymer can be detected in the culture medium of Δ*lcp* staphylococci. Following growth in the presence or absence of tunicamycin, carbohydrates and teichoic acid polymers in the supernatant of centrifuged cultures from wild-type MSSA 1112 or its Δ*lcp* variant were precipitated with ethanol. Teichoic acids were sedimented by centrifugation, and contaminating nucleic acids were digested with DNase and RNase. Samples were extracted with methanol and chloroform to remove proteins. Teichoic acids in the aqueous layer were concentrated by lyophilization, suspended in water, subjected to PAGE, and revealed by alcian blue-silver staining. As controls, murein sacculi of wild-type MSSA 1112 harbored Rbo-P WTA, whose

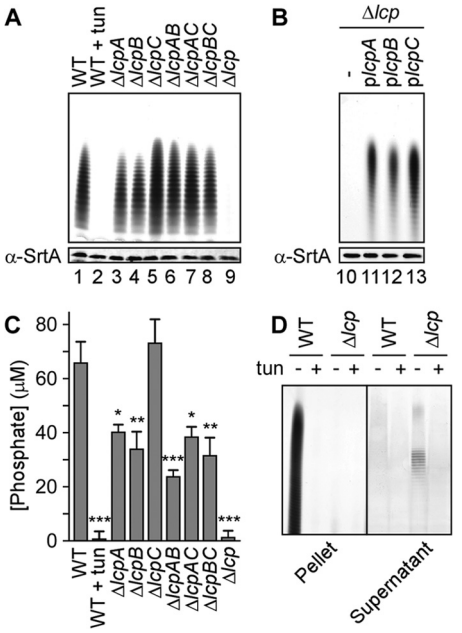

FIG 1 Δ*lcp* mutant cells lack cell-associated wall teichoic acid. (A and B) Alcian blue- and silver-stained acrylamide gels of cell wall-associated WTA. (A) WTA was extracted from the *S. aureus* MSSA 1112 wild type (WT) grown without (lane 1) or with (lane 2) tunicamycin and from single mutant strains (the Δ*lcpA*, Δ*lcpB*, and Δ*lcpC* mutants), double mutant strains (the Δ*lcpAB*, Δ*lcpAC*, and Δ*lcpBC* mutants), and a triple mutant strain (the Δ*lcp* mutant) (lanes 3 to 9, respectively). (B) WTA was extracted from the triple mutant strain (the Δ*lcp* mutant) carrying an empty vector (lane 10) or complementing plasmid p*lcpA*, p*lcpB*, or p*lcpC* (lanes 11 to 13, respectively). As a control for the number of staphylococci, cell extracts were immunoblotted with antibodies specific for sortase A. (C) Quantification of phosphate levels in purified peptidoglycan from the strains described for panel A. Average phosphate levels were derived from three independent experiments, and the standard errors of the means were calculated. Statistical significance between the wild type and each group was determined using one-way analysis of variance (ANOVA) with Bonferroni's multiple-comparison test. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (D) Identification of teichoic acid in the supernatant of Δ*lcp* cultures. Wild-type and Δ*lcp* strains were grown in the absence (−) or presence (+) of tunicamycin, and teichoic acids in cell pellets (left) or culture supernatants (right) were resolved by PAGE and visualized by staining with alcian blue and silver.

synthesis was blocked by tunicamycin treatment (Fig. 1D). Of note, the Rbo-P polymer was detected in the extracellular medium of cultures of the Δ*lcp* mutant but not those of wild-type *S. aureus* (Fig. 1D). Tunicamycin treatment of Δ*lcp* mutant cultures abolished the release of the Rbo-P polymer into the culture medium (Fig. 1D). To ascertain that Rbo-P polymer release did not result from cell lysis or aberrant peptidoglycan turnover, we asked whether the Δ*lcp* mutant also released cytoplasmic proteins or other cell wall polymers, such as sortase-anchored proteins, into the medium. We showed that a hybrid protein with the cell wall-sorting signal of protein A, SEB-MH_6-CWS, is properly tethered to the murein sacculus of the Δ*lcp* mutant. Immunoblotting analyses revealed that the hybrid is not aberrantly released into the extracellular milieu of Δ*lcp* mutant cultures compared to cultures of wild-type staphylococci (see Fig. S2 in the supplemental material). The cytoplasmic protein L6 was not detected in extracts of spent culture medium, ruling out bacterial lysis as a mechanism for surface protein release into the culture medium (see Fig. S2 in the supplemental material). Thus, we conclude that the Δ*lcp* mu-

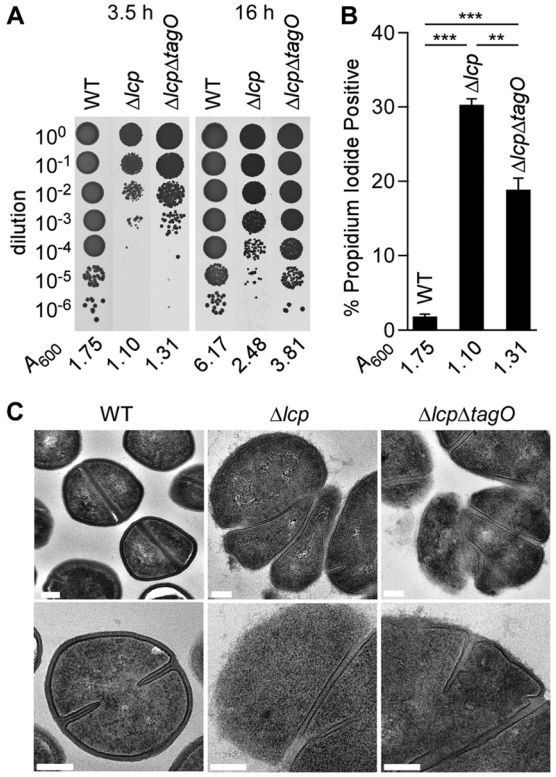

FIG 2 Deletion of *tagO* does not improve growth of the Δ*lcp* mutant. (A) Staphylococcal viability was examined by plating serial dilutions (0- to 6-fold) of culture aliquots of the wild type and the Δ*lcp* and Δ*lcp* Δ*tagO* mutants that had been grown for 3.5 and 16 h. Images of the agar plates are shown along with the A_{600} values of the cultures at the time of plating. (B) Membrane integrity of staphylococci was assessed with propidium iodide staining. Culture aliquots of the wild type and the Δ*lcp* and Δ*lcp* Δ*tagO* mutants that had been grown for 3.5 h were fixed with paraformaldehyde and stained with SYTO 9 (total cells) and propidium iodide. SYTO 9-positive cells were analyzed for propidium iodide staining using flow cytometry. Combined data from two independent experiments with triplicate analyses of 20,000 cells per sample are presented. Statistical significance was determined using the Student *t* test. **, $P < 0.01$; ***, $P < 0.001$. (C) Transmission electron micrographs of wild-type, Δ*lcp*, and Δ*lcp* Δ*tagO* bacteria. Cells were cultured for 3.5 h, as described for panel A. Bars, 200 nm.

tant fails to deposit WTA in the cell wall envelope and instead releases Rbo-P into the extracellular medium. Taken together, these data suggest that LcpA, LcpB, and LcpC display overlapping functions in depositing WTA in the cell wall envelope, presumably by forming a phosphodiester bond between the C-6 hydroxyl of MurNAc and the murein linkage unit tethered to Rbo-P_n.

Cell division and envelope defects of Δ*lcp* and Δ*lcp* Δ*tagO* mutant staphylococci. In *B. subtilis*, deletion of all three *lcp* genes is not compatible with bacterial growth, unless the mutant cells also harbor a deletion of the *tagO* gene (30). In contrast, the *S. aureus* Δ*lcp* mutant, which also lacks all three *lcp* genes, continues to grow (A_{600}), albeit at a lower rate than the wild type (see Fig. S3 in the supplemental material). To assess the viability of staphylococcal cells, culture aliquots were removed, serially diluted, and plated after 3.5 h to enumerate the number of viable CFU. We observed a 3-log-unit reduction in plating efficiency for the Δ*lcp* mutant compared to wild-type MSSA 1112 (Fig. 2A). Introduction of the Δ*tagO* allele into the Δ*lcp* mutant neither restored the plating efficiency to wild-type levels nor enhanced the growth of the mutant at the earlier 3.5-h time point (Fig. 2A), when the LCP enzymes are predominantly expressed (42). Further, when growth was measured via determination of the absorbance of liquid cultures (A_{600}), the Δ*lcp* Δ*tagO* mutant replicated at a rate similar to that for the Δ*lcp* mutant but not at the rate of its wild-type parent (see Fig. S3 in the supplemental material). To measure the number of nonviable staphylococci, culture aliquots of the three strains were stained with propidium iodide to identify lysed bacteria with membrane damage via flow cytometry (Fig. 2B). These experiments revealed that 30.2% of Δ*lcp* mutant cells and 18.7% of Δ*lcp* Δ*tagO* mutant cells were positive for propidium iodide, whereas only 1.68% of wild-type cells were positive for propidium iodide (Fig. 2B). Wild-type and mutant staphylococci were fixed, thin sectioned, and viewed by transmission electron microscopy. As expected, wild-type strain MSSA 1112 formed round cells with a thick cell wall envelope and cross-wall septa positioned at midcell, perpendicular to previous cell division planes (Fig. 2C). Uranyl acetate staining revealed the uniform deposition of WTA as electron-dense deposits in the envelope of wild-type staphylococci (Fig. 2C) (43). In contrast, the Δ*lcp* and Δ*lcp* Δ*tagO* variants generated deformed cells with thin, irregular envelopes lacking the WTA staining of wild-type staphylococci (Fig. 2C). Of note, the Δ*lcp* and Δ*lcp* Δ*tagO* variants generated cross-wall septa parallel to the previous division planes, indicating that the physiological mechanisms for the selection of cell division planes had been abolished in the Δ*lcp* and Δ*lcp* Δ*tagO* mutants (Fig. 2C). Interestingly, this phenotype was also observed for the isolated *tagO* and *lcpA* mutants, the latter of which still produces WTA (33, 43). These data suggest that both WTA synthesis and assembly are important for physiological cell division (43). In conclusion, the growth delay observed in the Δ*lcp* mutant is not a direct result of the continued synthesis of WTA and cannot be restored by preventing WTA synthesis.

Effect of tunicamycin on the generation of nonviable daughter cells by Δ*lcp* mutant staphylococci. Dengler et al. (32) reported that tunicamycin treatment could partially alleviate the growth defect of Δ*lcp* mutant staphylococci, a phenotype that was also observed in our experiments (Fig. 3A). We wondered whether tunicamycin treatment also suppressed the phenotype of the Δ*lcp* mutant of nonviable daughter cells. Bacterial cultures were grown without or with 1 μg/ml of tunicamycin for 3.5 h. Culture aliquots were serially diluted and plated on TSA for colony formation (Fig. 3A). As expected, the plating efficiency of wild-type MSSA 1112 was slightly reduced when the strain was grown in the presence of tunicamycin. In contrast, the addition of tunicamycin did not affect the plating efficiency of the Δ*lcp* strain (Fig. 3A), a phenotype that is in stark contrast to that of strains with mutational lesions in genes of the WTA biosynthetic pathway. For example, disruption of *S. aureus tagB*, *tarF*, or *tarJ2* via *bursa aurealis* transposon insertion was conditional for the presence of tunicamycin in agar medium, whereas the plating efficiency of the Δ*tagO* mutant was not (see Fig. S3 in the supplemental material). The plating efficiency of the Δ*lcp* strain and its sensitivity to tunicamycin were almost restored to wild-type levels upon transformation with plasmids that provide for the expression of any one of the three *lcp* genes (Fig. 3A). Of note, the reduction in plating efficiency was not always correlated with the bacterial growth measured for wild-type and Δ*lcp* cultures via increases in the A_{600} (Fig. 3A). As before, this difference can be explained by the generation of nonviable daughter cells, revealed by the increase of propidium iodide-pos-

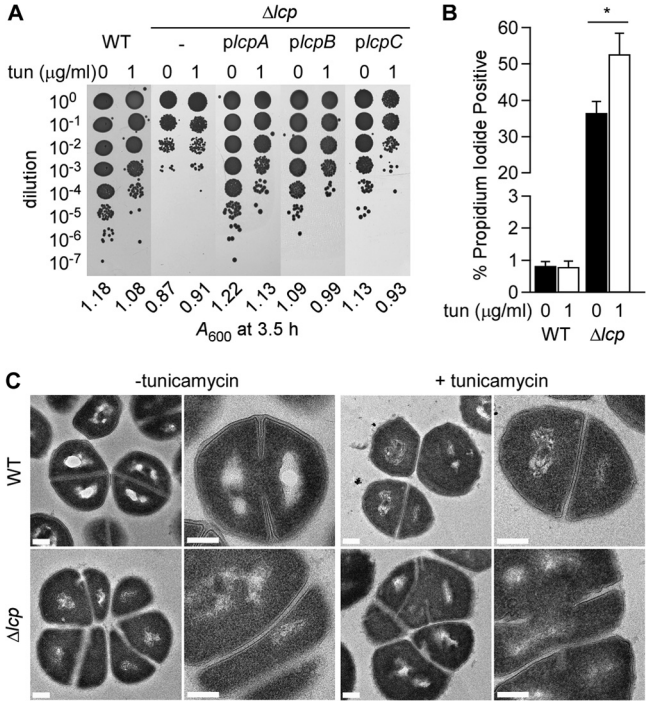

FIG 3 Inhibition of TagO with tunicamycin does not suppress the growth defects of the Δ*lcp* mutant. (A) Bacterial growth was examined by plating serial dilutions of cultures grown for 3.5 h without or with tunicamycin (0 or 1 μg/ml). Culture aliquots of the wild type and the Δ*lcp* mutant without or with p*lcpA*, p*lcpB*, or p*lcpC* were spotted on agar plates. A_{600} values were recorded at the time of plating and are reported under the images of the agar plates. (B) Membrane integrity of staphylococci assessed with propidium iodide staining. Culture aliquots of the wild type and the Δ*lcp* mutant grown for 3.5 h without or with tunicamycin (0 or 1 μg/ml) were fixed and stained as described in the legend to Fig. 2B. The data were analyzed as described in the legend to Fig. 2B. *, $P < 0.05$). (C) Transmission electron micrographs of wild-type and Δ*lcp* mutant cells from cultures grown for 3.5 h without or with tunicamycin (0 or 1 μg/ml). Bars, 200 nm.

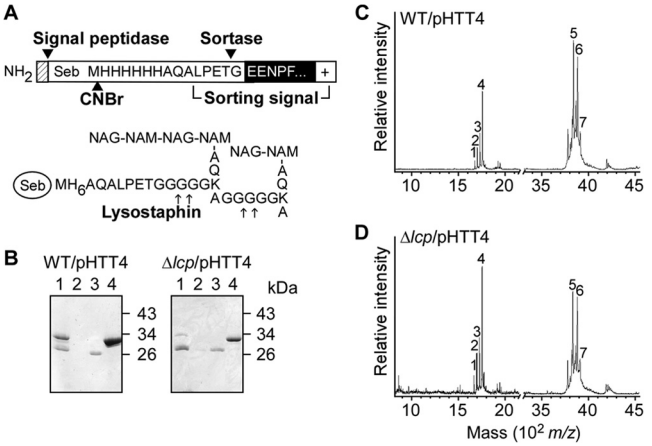

FIG 4 Sortase A anchoring of proteins to the cell wall is not affected in the Δ*lcp* mutant. (A) Diagram of recombinant SEB-MH_6-CWS encoded by the pHTT4 plasmid. Cleavage sites of the signal peptidase, sortase A, and CNBr are indicated. (B) Coomassie brilliant blue-stained SDS-polyacrylamide gel of SEB-MH_6-CWS released with lysostaphin from the cell wall of either wild-type (left) or Δ*lcp* mutant (right) staphylococci. Solubilized cell wall preparations (lanes 1) were subjected to affinity chromatography on Ni-NTA beads. Material not retained on the column is shown in lanes 2. The beads were washed (lanes 3), and SEB-MH_6-CWS was eluted with buffer containing 0.5 M imidazole (lanes 4). Molecular mass markers are indicated on the right side of the gel. (C and D) Mass spectrometry of CNBr-cleaved SEB-MH_6-CWS purified from the wild type (C) and the Δ*lcp* mutant (D). Proteins eluted in lanes 4 of panel B were digested with CNBr, and fragments were purified once more over Ni-NTA, eluted, desalted, and subjected to MALDI-TOF MS. The analysis of the ion signals labeled 1 to 7 and structural predictions are listed in Table 2.

itive cells in the Δ*lcp* mutant culture. While incubation of cultures with tunicamycin led to further increases in propidium iodide-positive Δ*lcp* cells, tunicamycin treatment did not affect the membrane integrity of wild-type staphylococci (Fig. 3B). Transmission electron microscopy revealed that tunicamycin treatment did not affect the overall morphology and cell division septum formation in wild-type cells; as expected, the outer electron-dense layer of WTA was abolished when staphylococci were grown in the presence of the antibiotic (Fig. 3C). Further, tunicamycin treatment of Δ*lcp* staphylococci did not change the aberrant gross morphology and septation defect of the mutant cells (Fig. 3C). These data show that treatment with tunicamycin, an inhibitor of WTA synthesis, suppresses neither the cell division defect of Δ*lcp* staphylococci nor its generation of nonviable daughter cells.

The *lcp* genes are not required for surface protein anchoring to the staphylococcal cell wall. Previous work identified lipid II to be the peptidoglycan substrate of the sortase A-catalyzed transpeptidation reaction of surface protein anchoring to the cell wall envelope (8). Consistent with this model, surface protein anchoring can be blocked by treating staphylococci with inhibitors of lipid II polymerization (vancomycin or moenomycin) or bactoprenol metabolism (nisin) (8). LCP proteins have been proposed to transfer WTA precursors [C_{55}-PP–GlcNAc–ManNAc–(Gro-P)$_{2-3}$–(Rbo-P)$_{30-50}$] onto the glycan strands of peptidoglycan (30). Since both reactions—surface protein and WTA anchoring—require bactoprenol-linked intermediates, we wondered whether sortase A-mediated protein anchoring is altered in *S. aureus* strains lacking the three LCP phosphotransferases (Δ*lcp* strain). To test this possibility, the wild-type parent strain MSSA 1112 and the Δ*lcp* mutant were transformed with plasmid pHTT4, which provides for the expression of SEB-MH_6-CWS, an engineered surface protein consisting of the N-terminal signal peptide and staphylococcal enterotoxin B (SEB) fused to the C-terminal cell wall-sorting signal (CWS) of protein A (Fig. 4A). Peptidoglycan preparations from both MSSA 1112 strains (wild type/pHTT4 and Δ*lcp* mutant/pHTT4) were treated with lysostaphin to solubilize anchored SEB-MH_6-CWS for purification via metal-chelating affinity chromatography (Fig. 4B). Purified proteins were subjected to cyanogen bromide (CNBr) cleavage at methionine residues. C-terminal anchor peptides with their N-terminal six-histidine (H_6) tag were again purified by affinity chromatography and analyzed by MALDI MS (Fig. 4CD). Ion signals labeled with *m/z* 1,665 (compound 1) and *m/z* 1,722 (compound 3) correspond to surface protein anchor peptides with two or three glycine residues [H_2N-H_6AQALPETGG(G)], which are released from the cell wall via lysostaphin cleavage (Fig. 4C and D; Table 2). Ion signals with *m/z* 1,693 (compound 2) and *m/z* 1,750 (compound 4) represent formylated peptides of compounds 1 and 3, respectively (Table 2). Compounds with *m/z* 3,826 (compound 5), *m/z* 3,854 (compound 6), and *m/z* 3,903 (compound 7) represent formylated or carbamylated anchor peptides with two or three glycine residues, also liberated via lysostaphin cleavage from the cell wall [NH_2-VDSKDVKIEVYLTTKKGTMH$_6$AQALPETG(G)]; these peptides

TABLE 2 Sortase A-catalyzed surface protein anchoring to the cell wall is not altered in the Δ*lcp* mutant[a]

Ion	Observed *m/z*		Calculated *m/z*	Proposed structure
	Wild type/pHTT4	Δ*lcp*/pHTT4		
1	1,665.941	1,665.811	1,665.729	NH_2-H_6AQALPET-Gly_2-CO_2H
2	1,693.893	1,693.830	1,693.739	HCO-NH-H_6AQALPET-Gly_2-CO_2H
3	1,722.888	1,722.827	1,722.781	NH_2-H_6AQALPET-Gly_3-CO_2H
4	1,750.956	1,750.867	1,750.791	HCO-NH-H_6AQALPET-Gly_3-CO_2H
5	3,826.572	3,826.436	3,826.215	NH_2-VDSKDVKIEVYLTTKKGTMH_6AQALPET-Gly_2-CO_2H, Na^+
6	3,854.116	3,854.111	3,854.225	HCO-NH-VDSKDVKIEVYLTTKKGTMH_6AQALPET-Gly_2-CO_2H, Na^+
7	3,901.934	3,902.469	3,903.301	H_2NCO-NH-VDSKDVKIEVYLTTKKGTMH_6AQALPET-Gly_3-CO_2H

[a] Cyanogen bromide cleavage products of cell wall-anchored SEB-MH_6-CWS liberated by lysostaphin treatment and purified by Ni-NTA chromatography (Fig. 4) were analyzed by MALDI-TOF MS in linear positive mode. The program CS ChemDraw was used for *m/z* calculations.

were generated via incomplete CNBr cleavage of SEB-MH_6-CWS at methionyl residues (Table 2). Thus, SEB-MH_6-CWS anchor peptides with similar structures and relative abundances were liberated via lysostaphin treatment from the cell wall envelope of wild-type and Δ*lcp* mutant staphylococci. These data indicate that the absence of LCP enzymes does not affect sortase A-mediated anchoring of surface proteins in *S. aureus*.

Deposition of LysM murein hydrolases in the envelope of Δ*lcp* mutant staphylococci. The decoration of the staphylococcal peptidoglycan with WTA restricts the binding of secreted murein hydrolases to the cell wall and limits the autolytic activity of these enzymes to the cross-wall compartment of dividing staphylococci (44). For example, the deposition of the LytN and Sle1 murein hydrolases is restricted to the cross wall (34, 41). Each of these enzymes harbors LysM domains at their N termini (34). LysM domains are both necessary and sufficient for the targeting of Sle1 to the cross wall (34). Inhibition of WTA synthesis, for example, by treatment of staphylococci with tunicamycin or deletion of *tagO*, abolishes the specificity of LysM domain targeting to the bacterial envelope (34). In a confocal fluorescence microscopy experiment, an abundance of the LysM domain of LytN ($LysM_{LytN}$)-mCherry and the LysM domain of Sle1 ($LysM_{Sle1}$)-mCherry was found to be deposited uniformly throughout the envelope of *tagO* mutant but not wild-type staphylococci (34) (Fig. 5A). As a control, mCherry alone did not bind to the staphylococcal envelope. We wondered whether the Δ*lcp* mutant, which fails to deposit WTA in the staphylococcal envelope, is also defective for the targeting of LysM-type murein hydrolases. Similar strong binding activities of $LysM_{LytN}$-mCherry and $LysM_{Sle1}$-mCherry were observed for the Δ*lcp* mutant strain but not for its MSSA 1112 parent (Fig. 5A).

The deposition of mCherry, $LysM_{LytN}$-mCherry, and $LysM_{Sle1}$-mCherry on the staphylococcal surface was quantified by measuring bacterial fluorescence in a flow cytometry experiment (Fig. 5B). Ten- and 100-fold increases in $LysM_{LytN}$-mCherry and $LysM_{Sle1}$-mCherry fluorescence, respectively, were measured with Δ*lcp* mutant staphylococci relative to that measured with wild-type strain MSSA 1112. These differences were comparable to

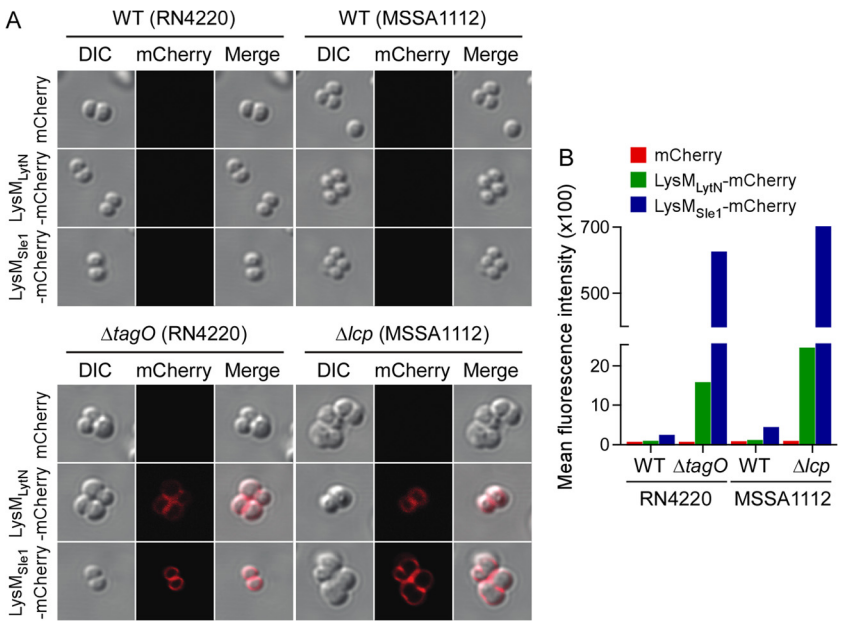

FIG 5 The LysM domains of LytN and Sle1 bind uniformly to the envelope of the Δ*lcp* mutant. Purified mCherry, $LysM_{LytN}$-mCherry, or $LysM_{Sle1}$-mCherry was incubated with wild-type parent (strains RN4220 and MSSA 1112), Δ*tagO* mutant, or Δ*lcp* mutant staphylococci. Binding of hybrid mCherry to the bacterial envelope was analyzed by fluorescence microscopy (A) and flow cytometry (B). (A) (Left) Differential inference contrast (DIC) images of staphylococcal cells analyzed by fluorescence microscopy (middle); (right) merged images derived from both data sets.

those observed between wild-type and Δ*tagO* mutant staphylococci (Fig. 5B). As a control, mCherry did not bind to the envelope of wild-type, Δ*tagO* mutant, or Δ*lcp* mutant staphylococci (Fig. 5A and B). These data suggest that the WTA deposition defect of the Δ*lcp* mutant causes the unrestricted deposition of murein hydrolases in the bacterial envelope, a phenotype that likely contributes to the decreased viability of Δ*lcp* daughter cells generated during cell division.

DISCUSSION

Recent reports implicated a family of genes encoding the LCP proteins in WTA synthesis and in the attachment of secondary cell wall polymers to bacterial peptidoglycan (30, 31). LCP-encoding genes are present in virtually all Gram-positive bacteria, and often, numerous homologues are present within a single genome (45). The LCP proteins share a similar predicted secondary structure, consisting of a short N-terminal cytoplasmic tail, a single transmembrane domain, and an extracellular C-terminal region encompassing a mixed α-helical/β-sheet LCP domain (30, 45). While the biochemical activity of these proteins is still not known, variants carrying defective alleles of *lcp* genes display pleiotropic phenotypes. For example, in both *S. aureus* and *S. pneumoniae*, loss of one or more of these genes resulted in aberrant septum formation (33, 46, 47), increased susceptibility to β-lactam antibiotics (33, 46), autolysis (33), aberrant biofilm formation (46), induction of cell wall envelope stress responses (32), and reduced cell wall phosphate (32). *B. subtilis* contains three homologues of the LCP genes, *tagTUV*, which cluster with genes of the WTA synthesis pathway (30). Deletion of all three genes is not compatible with growth of the mutant bacilli (30). Similar to late-stage WTA mutants, this phenotype can be rescued through the additional deletion of *tagO* (28); furthermore, *tagTUV* mutants cannot assemble WTA in the bacterial cell wall (30). In *S. pneumoniae*, mutants with deletions of one or two of the LCP homologues (Cps2A, LytR, and Psr) synthesize reduced amounts of capsular polysaccharide and release some of this material into the extracellular medium (31, 48, 49). The crystal structures of recombinant *B. subtilis* TagT and *S. pneumoniae* Cps2A revealed the presence of a polyprenyl phosphate lipid bound to the purified protein (30, 31). These findings have led to the hypothesis that LCP enzymes transfer secondary cell wall polymers from their undecaprenyl phosphate precursors and link them to the bacterial peptidoglycan (30). Specifically, LCP enzymes are proposed to tether the murein linkage unit of WTA or other secondary cell wall polysaccharides to the C-6 hydroxyl of MurNAc in the peptidoglycan of Gram-positive bacteria (30).

Although several layers of evidence support the hypothesis of Kawai and colleagues (30), there are also arguments against it. For example, *S. pneumoniae* is not known to express functional *tagO* and *tagA* homologues or synthesize murein linkage units (C_{55}-PP–GlcNAc–ManNAc); pneumococci are thought to anchor their teichoic acid polymers and capsular polysaccharides to the C-6 hydroxyl of MurNAc via phosphodiester bonds with other carbohydrate structures (50). Further, if *B. subtilis* TagTUV or *S. aureus* LcpABC were to tether the murein linkage units of WTA to the cell wall, one would expect that the corresponding mutant strains either harbor WTA precursors [C_{55}-PP–GlcNAc–ManNAc–Gro$_{2-3}$–(Rbo-P$_n$)] in the envelope or release unanchored products into the extracellular medium. Although this was tested, the *B. subtilis tagTUV* mutant did not release WTA into the medium and also did not deposit WTA in the envelope (30).

We are interested in sortase-mediated anchoring of surface proteins to the cell wall envelope (5), a biosynthetic pathway that also utilizes an undecaprenyl precursor, lipid II, to immobilize polypeptides in the bacterial envelope (7, 8, 51). We presumed that the *S. aureus* Δ*lcp* mutant may accumulate undecaprenyl-linked intermediates of the WTA pathway, thereby causing a block in surface protein anchoring. This, however, was not observed; instead, the *S. aureus* Δ*lcp* mutant synthesized WTA and released the Rbo-P polymers into the extracellular medium. We surmise that the observed release of WTA may be catalyzed by undecaprenyl pyrophosphate phosphatases (UppPs), which are otherwise involved in the recycling of translocated lipid II (52). If so, this could explain why undecaprenyl recycling occurs in the Δ*lcp* variant but not in *tagB*, *tarF*, or *tarJ2* mutants, which require mutations in *tagO* or *tagA* or inhibition of WTA synthesis with tunicamycin for growth. We entertain a model whereby the *S. aureus* Δ*lcp* mutant translocates its WTA precursors via TagGH for subsequent hydrolysis via UppPs, thereby enabling the return of bactoprenol into the cytoplasm. This model could also explain why other WTA biosynthetic intermediates, those accumulating in *tagB*, *tarF*, or *tarJ2* mutants (without substrate properties for TagGH), either deplete staphylococci of the lipid carrier or accumulate toxic intermediates. It seems plausible that UppPs do not have access to cytoplasmic intermediates of the WTA pathway and therefore cannot relieve the growth-inhibitory attributes of *tagB*, *tarF*, or *tarJ2* mutations. Our model offers insights into the development of new drugs that target key steps of WTA synthesis on the bacterial surface. At least for *S. aureus*, the causative agent of human infections with antibiotic-resistant strains (53), small-molecule inhibitors of LCPs would not be expected to cause bactericidal activity, whereas the combined inhibition of LCPs and UppPs may be associated with antibiotic activity. In contrast, the *B. subtilis tagTUV* mutant appears to sequester WTA synthesis intermediates, as these mutations inhibit the growth of the mutant, unless WTA synthesis is blocked via *tagO* mutation (30). Thus, *B. subtilis* may lack the relevant UppP activity and cannot recycle WTA synthesis intermediates. A combined literature and GenBank search suggests that *S. aureus* encodes three genes with undecaprenyl pyrophosphate phosphatase activity. The canonical *bacA-uppP* gene has been described and is dispensable for growth (54). *S. aureus* also encodes two predicted phosphatases with a PAP2 domain reminiscent of *ybjG* and *pgpB*, two genes that have been shown to encode redundant UppP activities *E. coli* (52). A similar search performed for *B. subtilis* suggests that this organism encodes only two undecaprenyl pyrophosphate phosphatases, *bacA* (formerly *yubB*) and *bcrC* (formerly *ywoA*) (55).

In sum, data reported here support the model of Kawai and colleagues, whereby LCP enzymes catalyze the transfer of WTA synthesis intermediates to the cell wall peptidoglycan (30). The observed pleiotropic phenotypes of the Δ*lcp* mutant are likely due to its WTA synthesis and WTA cell wall deposition defects. Without WTA, staphylococci display aberrant cell size, septum formation, autolysis, and susceptibility to antibiotics and defects in biofilm formation (32, 33, 45, 46). The molecular basis of the observed phenotypes is likely due to defects in the positioning and functional coordination of the peptidoglycan biosynthesis and cell wall separation machines, which involve a wide spectrum of different enzymes.

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