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Genes Required for Glycolipid Synthesis and Lipoteichoic Acid Anchoring in *Staphylococcus aureus*▽

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Staphylococcus aureus **lipoteichoic acid (LTA) is composed of a linear 1,3-linked polyglycerolphosphate chain and is tethered to the bacterial membrane by a glycolipid (diglucosyl-diacylglycerol [Glc_2-DAG]). Glc_2-DAG is synthesized in the bacterial cytoplasm by YpfP, a processive enzyme that transfers glucose to diacylglycerol (DAG), using UDP-glucose as its substrate. Here we present evidence that the *S. aureus* α-phosphoglucomutase (PgcA) and UTP:α-glucose 1-phosphate uridyltransferase (GtaB) homologs are required for the synthesis of Glc_2-DAG. LtaA (*l*ipo*t*eichoic *a*cid protein *A*), a predicted membrane permease whose structural gene is located in an operon with *ypfP*, is not involved in Glc_2-DAG synthesis but is required for synthesis of glycolipid-anchored LTA. Our data suggest a model in which LtaA facilitates the transport of Glc_2-DAG from the inner (cytoplasmic) leaflet to the outer leaflet of the plasma membrane, delivering Glc_2-DAG as a substrate for LTA synthesis, thereby generating glycolipid-anchored LTA. Glycolipid anchoring of LTA appears to play an important role during infection, as *S. aureus* variants lacking *ltaA* display defects in the pathogenesis of animal infections.**

Lipoteichoic acid (LTA) is an abundant secondary wall polymer in the cell wall envelope of gram-positive bacteria (11, 13, 27). LTA plays an important role during host infection, as it is thought to be perceived by receptors on immune cells that trigger innate responses in an effort to defend host tissues from invading microbes (34). Other proposed functions of LTA include Mg^{2+} ion scavenging and proper targeting of autolysins to the bacterial envelope. Physiological functions of autolysins in degradation of cell wall envelopes or separation of dividing cells are essential for bacterial growth (6, 16, 31).

The chemical structure of LTA has been determined for several different gram-positive bacteria (13). *Staphylococcus aureus* LTA is composed of 1,3-linked polyglycerolphosphate chains linked to β-gentiobiosyldiacylglycerol (diglucosyl-diacylglycerol [Glc_2-DAG]), which anchors LTA in the bacterial membrane (8). Under laboratory growth conditions, 75 to 80% of LTA glycerolphosphate moieties have D-alanine ester substitutions at position 2 (15), and some staphylococcal strains have *N*-acetylglucosamine substitutions at the same position (43) (Fig. 1). The polyglycerolphosphate structure of LTA is found in many other gram-positive bacteria, including *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus cereus*, *Bacillus anthracis*, *Enterococcus faecalis*, *Listeria* spp., and group A and group B streptococci (11, 14).

YpfP, a processive glycosyltransferase, is required for glycolipid synthesis in *B. subtilis* and *S. aureus* (24, 25, 27). Expression of *B. subtilis ypfP* in *Escherichia coli*, an organism that does not synthesize glycolipids, leads to accumulation of several glycolipids and phosphoglycolipids (24, 25). Inactivation of *ypfP* in *S. aureus* abrogates all glycolipid synthesis and leads to morphological alterations, including an increase in cell size and aberrant cell shapes (27). Interestingly, LTA synthesis is not abolished in a *ypfP* mutant strain, and LTA with wild-type chain length is produced (27). However, LTA is anchored to the membrane via diacylglycerol (DAG) in the mutant, and increased amounts of LTA are found in the culture supernatant (27). Similarly, a defect in glycolipid synthesis in group B *Streptococcus* leads to shedding of LTA into the medium, and mutant bacteria have defects in invasion of eukaryotic cells and virulence (7).

YpfP successively transfers glucose onto DAG using UDP-glucose as its substrate (24, 25). In *B. subtilis*, UDP-glucose is synthesized by conversion of glucose-6-phosphate to α-glucose-1-phosphate, a reaction catalyzed by α-phosphoglucomutase (PgcA; formerly known as GtaC and GtaE) (32). GtaB (UTP:α-glucose-1-phosphate uridyltransferase) synthesizes UDP-glucose from α-glucose-1-phosphate and UTP (41, 46) (Fig. 2A). Similar to *ypfP* mutants, *B. subtilis* or *B. licheniformis* mutants unable to produce UDP-glucose are defective in the synthesis of glycolipids and glycolipid-anchored LTA (4, 32).

Using bioinformatic tools, we identified *S. aureus* homologs of *B. subtilis pgcA* and *gtaB*. Inactivation of *pgcA* or *gtaB* eliminated the synthesis of membrane glycolipids in *S. aureus*. Similar to an *S. aureus ypfP* mutant, variants lacking *pgcA* or *gtaB* had the ability to generate LTA. A new gene, *ltaA* (*l*ipo*t*eichoic *a*cid gene *A*) that is located in the same operon and is immediately adjacent to *ypfP* was identified. While *ltaA* is dispensable for the synthesis of membrane glycolipids, it is required for the efficient synthesis of LTA with a glycolipid anchor.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* strains were grown in Luria-Bertani medium, and *S. aureus* strains were grown in tryptic soy broth (TSB) at 37°C. All bacterial strains used in this study are listed in Table 1. *S. aureus* strain SEJ1 is a derivative of strain RN4220 (29) with an in-frame deletion in *spa* (encoding protein A). *S. aureus* strains harboring mariner transposon insertions in SAV1016 (*ltaA*), SAV1017 (*ypfP*), SAV2491 (*pgcA*), and SAV2500 (*gtaB*) were

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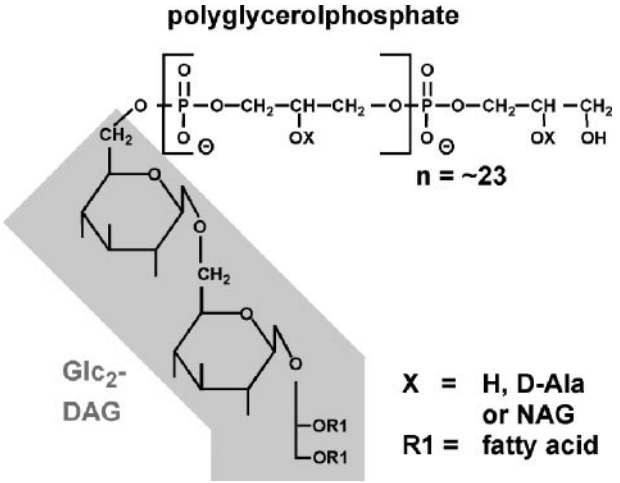

FIG. 1. Structure of *S. aureus* lipoteichoic acid. *S. aureus* LTA is composed of linear polyglycerolphosphate chains (average length, 23 repeating units), which are linked to a membrane anchor composed of Glc_2-DAG (shaded box). Position 2 (indicated by X) of the repeating glycerolphosphate subunits can be unsubstituted (hydrogen), esterified with D-alanyl, or linked to *N*-acetylglucosamine (NAG). The diagram was adapted from previously published schemes (12, 35).

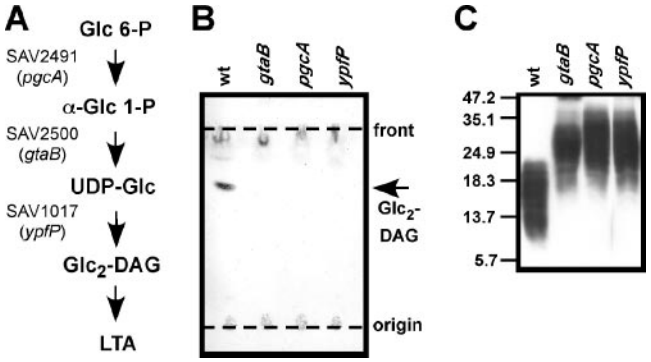

FIG. 2. (A) LTA glycolipid anchor synthesis in *S. aureus*. Glucose-6-phosphate (Glc 6-P) is converted by α-phosphoglucomutase (PgcA, encoded by SAV2941) to α-glucose-1-phospate (α-Glc 1-P), which is then activated by UTP:α-glucose-1-phosphate uridyltransferase (GtaB, encoded by SAV2500) to generate UDP-glucose (UDP-Glc). UDP-Glc and diacylglycerol (DAG) serve as substrates for YpfP, a processive glycosyltransferase that generates diglucosyl-diacylglycerol (Glc_2-DAG), which, after transfer of linear polyglycerolphosphate, functions as the membrane anchor for LTA. (B) TLC of glycolipids from *S. aureus*. Samples (300 μg) of membrane lipids isolated from *S. aureus* strains ANG361 (wild type) (wt), ANG372 (*gtaB*), ANG371 (*pgcA*), and ANG370 (*ypfP*) were separated by TLC, and glycolipids were visualized with α-naphthol/sulfuric acid. The positions of the origin and TLC solvent front are indicated by dashed lines. (C) Immunoblot analysis of LTA extracted from *S. aureus*. LTA was extracted from *S. aureus*, separated by 15% SDS—PAGE, and detected with a polyglycerolphosphate-specific antibody. The positions of protein molecular mass markers (in kDa) are indicated on the left.

grown in TSB supplemented with 10 μg/ml erythromycin. Ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) were used for selection of plasmid pCL55 (33) and its derivatives in *E. coli* and *S. aureus*, respectively. Gene expression from the tetracycline-inducible promoter was induced by addition of 200 ng/ml anhydrotetracycline.

Transposon insertion and phage transduction. Transposon insertion sites were determined by inverse PCR and DNA sequencing, as previously described (2). Strains ANG370 (transposon insertion in SAV1017 [*ypfP*]), ANG371 (insertion in SAV2941 [*pgcA*]), and ANG372 (insertion in SAV2500 [*gtaB*]) were generated by transducing transposons from bursa aurealis library mutants ΦNΞ171-39, ΦNΞ177-2, and ΦNΞ1952 (2) into strain SEJ1. In a separate study, mariner transposon mutagenesis was performed with *S. aureus* SEJ1. ANG361 (control strain with transposon insertion at an irrelevant site) and ANG359 (transposon insertion in SAV1016 [*ltaA*]) were obtained by moving transposon insertions from two of these library strains into a fresh SEJ1 background strain. Strain ANG460 was obtained by moving a transposon from strain ANG359 (SAV1016 [*ltaA*]) into *S. aureus* strain Newman. Strain ANG460 and parental strain Newman were used for virulence studies.

Lipoteichoic acid immunoblotting. For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis of cell-associated LTA, 1 ml of a staphylococcal overnight culture was mixed with 0.5 ml of 0.1-mm glass beads, and the bacteria were lysed by 45 min of vortexing in the cold. The glass beads and cell debris were sedimented by centrifugation at 200 × *g* for 1 min, and 0.5 ml of supernatant was transferred to a new tube. Staphylococcal membranes and LTA were sedimented by centrifugation at 16,000 × *g* for 10 to 15 min and suspended in 100 μl of sample buffer containing 2% SDS. Where indicated below, samples were normalized based on the optical density at 600 nm (OD_{600}); that is, samples from a culture with an OD_{600} of 6 were suspended in 90 μl sample buffer. To determine the amount of LTA shed into the culture medium, samples were prepared as follows. Bacteria from a 500-μl culture were pelleted by centrifugation at 16,000 × *g* for 5 min. One hundred microliters of culture supernatant was removed and mixed 1:1 with sample buffer. Samples were boiled for 20 to 30 min, insoluble material removed by centrifugation at 16,000 × *g* for 5 min, and samples were subjected to 15% SDS–PAGE followed by electrotransfer to polyvinylidene difluoride membranes. Supernatant LTA samples were normalized based on OD_{600}; that is, 10 μl of a culture with an OD_{600} of 5 was loaded. LTA (polyglycerolphosphate)-specific primary antibody (clone 55; HyCult Biotechnology) and horseradish peroxidase-linked anti-mouse secondary antibody (Cell Signaling) were used at dilutions of 1:2,500 and 1:5,000. Immunoreactive LTA species were detected by enhanced chemiluminescence.

Plasmid and strain construction. *S. aureus* RN4220 (29) chromosomal DNA was used as a template for PCRs. For expression of SAV1017 (*ypfP*) and SAV1017/SAV1016 (*ypfP*/*ltaA*) from the native promoter, plasmids pCL55-*ypfP* and pCL55-*ypfP*/*ltaA* were constructed. Primers 5-BamHI-P-SAV1017 (CGG GATCCGCTCCTTTTTCTACAATATGTTTATTATACACG) and 3-KpnI-SAV1017 (GGGGTACCTTATTTAACGAAGAATCTTGCATATAAAGGA ACC) and primers 5-BamHI-P-SAV1017 and 3-KpnI-SAV1016 (GGGGTACC TTACTTAGCTTTTTCTCTATTTGCTATAAAGTAGC) were used to amplify SAV1017 (*ypfP*) and SAV1017/SAV1016 (*ypfP*/*ltaA*) DNA, respectively. PCR products were cut with BamHI/KpnI and ligated with chromosomal integration vector pCL55 (33) cut with the same enzymes. For tetracycline-inducible gene expression, p*itet* was constructed. Primer 5-KpnI-tet (GGGGTACCTTGGTTA CCGTGAAGTTACCATCACGG) and the 5′-phosphorylated and restriction site-containing primer P-revSacIIPmeIBglIIAvrII-tet P-(CCGCGGGTTTAAA CAGATCTCCTAGGTCATTTGATATGCCTCCGAATTCG) were used to amplify the inducible tetracycline region from plasmid pYJ335 (23). The resulting PCR product was cut with KpnI and ligated with pCL55 that had been digested with KpnI/SmaI to generate p*itet*. p*itet*-*ltaA* was constructed for tetracycline-inducible expression of SAV1016 (*ltaA*). Primers 5-AvrII-SAV1016 (CC GCCTAGGCATCACAACCACAAGAGATTTATGGAAAGGTTCC) and 3-SacII-SAV1016 (TCCCCGCGGTTACTTAGCTTTTTCTCTATTTGCTATA AAGTAGC) were used to amplify SAV1016. The PCR product was digested with AvrII/SacII and ligated with p*itet* that had been cut with the same enzymes. All plasmids were cloned in *E. coli* strain XL1-Blue, and the DNA sequences of inserts were verified by fluorescent automated sequencing at the DNA sequencing facility of the University of Chicago Cancer Research Center. Plasmids were then integrated into the *geh* locus (lipase gene) in the *S. aureus* chromosome.

Membrane lipid isolation. Lipids from *S. aureus* and *E. coli* strains were extracted by using a modified Bligh-Dyer method (26). For isolation of *S. aureus* membrane lipids, 0.1 or 1 liter of TSB was inoculated with 1 or 10 ml of an overnight culture, and the resulting culture was grown at 37°C until the OD_{600} was ~3.5. Cultures were chilled on ice for 30 to 60 min, and staphylococci were collected by centrifugation. Bacteria were washed with 0.1 M sodium acetate (pH 4.7) or 0.1 M sodium citrate (pH 4.7) and lysed by shearing with 0.1-mm glass beads. For 0.1-liter cultures, bacterial suspensions were placed in 2-ml Fast Prep tubes containing 0.1-mm glass beads (~0.5 ml) and lysed with a Fast-Prep machine (Q-BIOgene) by shaking the tubes three times for 45 s at setting 6. The glass beads were sedimented by centrifugation at 200 × *g* for 1 min, and the supernatant containing bacterial lysate was transferred to new tubes. Lysed bacteria were sedimented by centrifugation at 16,000 × *g* for 5 min, and lipids

TABLE 1. Bacterial strains used in this study

Strain	Relevant features	Source or reference
Escherichia coli strains		
XL1-Blue		Stratagene
XL1-Blue/pYJ335	*E. coli*/*S. aureus* shuttle vector containing a tetracycline-inducible promoter	23
ANG243	pCL55 in XL1-Blue; *S. aureus* single-site integration vector	33
ANG284	p*itet* in XL1-Blue; pCL55 containing a tetracycline-inducible promoter	This study
ANG373	pCL55-*ypfP* in XL1-Blue; expression of YpfP (SAV1017) under native promoter control	This study
ANG374	pCL55-*ypfP*/*ltaA* in XL1-Blue; expression of YpfP (SAV1017) and LtaA (SAV1016) under native promoter control	This study
ANG375	p*itet-ltaA* in XL1-Blue; expression of LtaA (SAV1016) under tetracycline-inducible promoter control	This study
Staphylococcus aureus strains		
Newman	Human clinical isolate	9
ΦNΞ171-39	Newman with transposon insertion in SAV1017 (*ypfP*)	2
ΦNΞ177-2	Newman with transposon insertion in SAV2491 (*pgcA*)	2
ΦNΞ1952	Newman with transposon insertion in SAV2500 (*gtaB*)	2
ANG460	Newman with transposon insertion in SAV1016 (*ltaA*); transduced from strain ANG359	This study
RN4220	Transformable laboratory strain	29
SEJ1	RN4220Δspa; in-frame deletion in *spa* coding for protein A	Taeok Bae
ANG359	SEJ1 with transposon insertion in SAV1016 (*ltaA*); transduced	This study
ANG361	SEJ1 with transposon insertion in COL2559; transduced; wild type for LTA expression used as control strain	This study
ANG370	SEJ1 with transposon insertion in SAV1017 (*ypfP*); transduced from strain ΦNΞ171-39	This study
ANG371	SEJ1 with transposon insertion in SAV2491 (*pgcA*); transduced from strain ΦNΞ177-2	This study
ANG372	SEJ1 with transposon insertion in SAV2500 (*gtaB*); transduced from strain ΦNΞ1952	This study
ANG391	p*itet-ltaA* in strain ANG359 (*ltaA* [SAV1016] mutant)	This study
ANG393	p*itet* in strain ANG359 (*ltaA* [SAV1016] mutant)	This study
ANG395	p*itet* in strain ANG361 (wild type for LTA)	This study

were extracted. For large cultures, 40 ml of a washed bacterial suspension was mixed with an equal volume of glass beads; the bacteria were lysed with a bead beater (Biospec Products, Inc.) by shaking samples three times for 2 min at 4°C. Bacteria were chilled between each run for 5 min on ice. The glass beads were sedimented by centrifugation for 1 min at 200 × *g*. The membranes were removed with the supernatant, and the bacterial debris was sedimented by centrifugation at 12,000 × *g* for 20 min. The pellets were washed with 40 ml of 0.1 M sodium acetate (pH 4.7) or 0.1 M sodium citrate (pH 4.7), the wet weight was determined, and samples were stored frozen at −20°C. For lipid extraction, frozen pellets were suspended (0.4 g/ml) in 0.1 M sodium acetate (pH 4.7) or 0.1 M sodium citrate (pH 4.7). Chloroform and methanol were added to obtain a final methanol/chloroform/buffer ratio of 2:1:0.8. Lipids were extracted for 2 h at room temperature with vortexing. Insoluble material was removed by centrifugation at 2,600 × *g* for 20 min, and the extracted lipids were transferred with the supernatant to new tubes. The lipids were extracted again as described above; chloroform and buffer were added to combined extracts to obtain a methanol/chloroform/buffer ratio of 1:1:0.9. Following vigorous vortexing, samples were centrifuged at 2,600 × *g* for 20 min, and the chloroform phase containing lipids was transferred to a new tube. The lipids were dried under a stream of nitrogen, and the dry weight was determined. The lipids were then suspended in methanol-chloroform (1:1) at a concentration of 25 to 50 mg/ml and stored at −20°C. Membrane lipids from *E. coli* expressing YpfP (ANG373), from *E. coli* expressing YpfP and LtaA (ANG374), or from a control strain (ANG243) were extracted from 1-liter cultures. One liter of Luria-Bertani medium containing 100 μg/ml ampicillin was inoculated with 20 ml of an overnight culture, and the resulting culture was grown for 5 h at 37°C. Bacteria were collected by centrifugation and washed with 50 ml of 0.1 M sodium citrate (pH 4.7), and bacterial pellets were suspended in 8 ml of the same buffer. Bacteria were lysed by sonicating the preparations four times for 30 s at setting 7 using a Branson Sonifier 185 cell disruptor equipped with a microtip. Bacterial debris was collected by centrifugation at 100,000 × *g* for 30 min, and lipids were extracted as described above. Dried *E. coli* lipids were suspended in 1:1 in methanol-chloroform (1:1) at a concentration of 100 mg/ml, and 10-μl samples (1 mg lipid) were analyzed by thin-layer chromatography (TLC) and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry.

Lipid analysis by TLC. Lipids were separated by TLC using Whatman Silica Gel A60 plates. Prior to separation, TLC plates were dried for 2 h at 100°C. Samples (300 to 1,000 μg lipid in 10 to 20 μl) were spotted on plates and separated using chloroform-methanol-H_2O (70:30:4 or 65:25:4), as previously described (25–27). For detection of glycolipids, TLC plates were sprayed with 0.5% α-naphthol in 50% methanol and then with 95% H_2SO_4. The plates were subsequently incubated for 15 to 20 min at 100 to 120°C, which revealed glycolipids as pink spots (25–27). Digalactosyl-diacylglycerol (Sigma) was used as a chromatographic standard in some experiments. For structural analysis by MALDI-TOF mass spectrometry, glycolipids were purified following TLC by scraping silica gel from plates and extracting lipids twice with 3 ml of chloroform-methanol (1:1). Appropriate areas for lipid extraction were determined by developing one lane run in parallel with α-naphthol and H_2SO_4. Silica gel was sedimented by centrifugation at 2,600 × *g* for 10 min, and lipids in the methanol-chloroform extracts were transferred to new tubes. Lipids were recovered from the lower chloroform phase after the addition of H_2O to obtain a chloroform-methanol-H_2O (1:1:0.9) mixture, vortexing, and centrifugation for 10 min at 2,600 × *g*. Lipids were dried under a stream of nitrogen and prepared for MALDI-TOF analysis as described below. LTA glycolipid anchor structures were analyzed by TLC and were visualized with α-naphthol as described above. For nonglycolipid LTA anchor structures, lipids were separated on Silica Gel A60 plates using a heptane–isopropyl ether–acetic acid (60:40:4) solvent system; lipids were visualized by staining with a 0.2% amido black solution in 1 M NaCl, as previously described (40). 1,2-Dipalmitoyl-*sn*-glycerol (Avanti Polar Lipids, Inc.) was used for TLC calibration.

Lipid analysis by the MALDI-TOF method. Dried lipids were suspended in 10 μl of a 0.125 M 2,2′:5′,2″-terthiophene (Sigma Aldrich) MALDI matrix dissolved in chloroform-methanol (1:1). Tenfold dilutions of the samples were prepared using the same MALDI matrix, and, where indicated below, $NaHCO_3$ was added to a final concentration of 5 mM. Two 0.3-μl samples were spotted, and MALDI-TOF spectra were obtained with a Reflection time of flight instrument (ABI Biosystems) in the positive reflection mode.

LTA purification and lipid anchor isolation. LTA from *S. aureus* was purified by hydrophobic interaction chromatography using a 5-ml HiTrap Octyl FF column (Amersham Biosciences). Four liters of TSB was inoculated with 40 ml of

a wild-type or mutant *S. aureus* culture, and cultures were grown at 37°C to an OD_{600} of ~3.5. Cultures were chilled on ice for 45 min, and bacteria were sedimented by centrifugation at 6,000 × *g* for 10 min and washed with 360 ml of 0.1 M sodium citrate (pH 4.7). Then the bacteria were sedimented by centrifugation at 8,000 ×*g* for 10 min, suspended in 40 ml of 0.1 M sodium citrate (pH 4.7), and disrupted with a bead beater (Biospec Products, Inc.) by shearing them five times for 2 min at 4°C with 0.1-mm glass beads (40 ml). The glass beads were sedimented by centrifugation for 1 min at 200 × *g*. Cell debris was collected by centrifugation and washed with 0.1 M sodium citrate (pH 4.7), and samples were stored at −20°C. Pellets were suspended in 0.1 M sodium citrate (pH 4.7) (0.4 g/ml) and stirred at room temperature for 30 min with an equal volume of 1-butanol. Insoluble material was sedimented by centrifugation at 13,000 × *g* for 20 min, extracts were transferred to new tubes, and phases were separated by centrifugation at 13,000 × *g* for 20 min. The aqueous (lower) phase containing LTA was retrieved. Pellets were reextracted with 1-butanol as described above, and LTA-containing phases were combined and extracted again with 1-butanol. Following phase separation, the volume of the LTA-containing phase was reduced to 6 to 7 ml using a Speed Vac, and the preparation was dialyzed against three changes of 1 liter of 20 mM sodium citrate (pH 4.7) using Spectra/Por 6 dialysis membranes (1,000-Da cutoff; Spectrum Laboratories, Inc.). Following dialysis, samples were manipulated so that each 10-ml sample contained 15% 1-propanol in 0.1 M sodium citrate (pH 4.7). Samples (3 ml) were loaded onto 5-ml HiTrap Octyl FF columns equilibrated with 0.1 M sodium citrate (pH 4.7)–15% 1-propanol. The columns were washed with 90 ml of 50 mM sodium citrate (pH 4.7)–15% 1-propanol buffer, and LTA was eluted with a linear 15 to 65% 1-propanol gradient in 50 mM sodium citrate (pH 4.7) in 90 ml. The flow rate was set at 1.5 ml/min, and 2-ml fractions were collected. Fractions from two runs were combined, aliquots were analyzed by immunoblotting for the presence of LTA, and positive fractions were pooled. For lipid anchor analysis, purified LTA was dialyzed extensively against H_2O at 4°C and freeze-dried, and the dry weight was determined. Forty milligrams of LTA was hydrolyzed in 1 ml of 48% hydrofluoric acid (HF) for 44 to 48 h at 4°C. The reaction mixtures were neutralized with saturated $NaHCO_3$, and lipids were extracted for 1 h at room temperature by addition of chloroform-methanol to obtain a final chloroform/methanol/aqueous phase ratio of 1:1:0.9. Samples were centrifuged for 10 min at 2,600 × *g*, and the chloroform phase, containing the LTA lipid anchor, was transferred to a new tube. The lipids were dried under a stream of nitrogen and suspended in a small volume of chloroform-methanol (1:1), and the lipid anchor from 10 mg LTA was analyzed by TLC or MALDI-TOF mass spectrometry.

Bacterial growth curves and murine infection and abscess formation. For growth curves, three independent cultures of *S. aureus* strains Newman and ANG460 (*ltaA* mutant) were diluted 100-fold into 30 ml TSB and incubated at 37°C with shaking. At intervals culture aliquots were removed, and OD_{600} values were determined. The maximal doubling times during exponential growth (1.25 h to 3.75 h after inoculation) were calculated for wild-type and mutant strains. For virulence studies, strains Newman and ANG460 (*ltaA* mutant) were grown overnight in TSB at 37°C, diluted 100-fold into 25 ml fresh medium, and incubated at 37°C for 3 h. Staphylococci were collected by centrifugation, washed, and suspended in sterile phosphate-buffered saline. Bacterial suspensions were diluted to obtain an OD_{600} of 0.4, and 100-μl portions of the bacterial suspensions were administered intravenously via retroorbital injection to 45-day-old BALB/c mice (10 to 12 mice per group). Four days after injection, the mice were killed by CO_2 asphyxiation, and the kidneys and livers were removed. The organs were homogenized in 1 ml of phosphate-buffered saline–1% Triton X-100, and dilutions of the homogenates were plated on tryptic soy agar plates to determine the number of CFU/ml. The detection limit was 33 CFU/ml, and the value for organs of animals with levels below the detection limit was defined as 32 CFU/ml for statistical analysis using a one-tailed Student's *t* test.

RESULTS

Genes required for glycolipid synthesis in *S. aureus*. Membranes of *S. aureus* contain approximately 9% glycolipid (28); the predominant glycolipid, Glc_2-DAG, is also used as a membrane anchor for LTA (8). Previous studies have shown that *ypfP*, which encodes a processive glycosyltransferase, is absolutely required for Glc_2-DAG synthesis in *S. aureus* (27). YpfP employs UDP-glucose as a substrate for glycolipid synthesis (24, 27). In *B. subtilis*, two enzymes, encoded by *pgcA* and *gtaB*, are involved in UDP-glucose synthesis, using a pathway shown in Fig. 2A. BLAST searches with *B. subtilis* PgcA and GtaB as queries identified the *S. aureus* Mu50 genome-encoded SAV2491 (E-value, 2e-97) and SAV2500 (E-value, e-110) products as PgcA and GtaB homologs, respectively (Fig. 2A). Transposon insertions in the genes were isolated in a nonredundant collection of bursa aurealis mutants (2). Transposon insertions from original library strains ΦNΞ177-2 (*pgcA*), ΦNΞ1952 (*gtaB*), and ΦNΞ171-39 (*ypfP*) were transduced into the protein A-deficient RN4220-derived laboratory strain SEJ1. Transposon insertion sites in the transductants, strains ANG372 (*gtaB*, SAV2941), ANG371 (*pgcA*, SAV2941), and ANG370 (*ypfP*, SAV1017), were confirmed by inverse PCR and DNA sequencing. To determine the requirement for *S. aureus* PgcA and GtaB for glycolipid synthesis, total membrane lipids were extracted from mutant and wild-type strains and analyzed by TLC, followed by α-naphthol/sulfuric acid staining of glycolipids. As expected from previous work (27, 28), ANG361 (wild-type parent), which has a transposon insertion in a gene irrelevant for glycolipid production, contained one prominent α-naphthol-reactive species that comigrated with the calibration standard, Gal_2-DAG (Fig. 2B). The results of MALDI-TOF analysis of this compound were in agreement with predicted masses for Glc_2-DAG (see below). Furthermore, Glc_2-DAG was not detected in membrane extracts from *ypfP* mutant staphylococci. TLC analysis also revealed that Glc_2-DAG was not present in membrane extracts obtained from *pgcA* or *gtaB* mutant staphylococci (Fig. 2B). In *ypfP* mutants, LTA with a wild-type chain length is anchored in the membrane via DAG (27). This structural change resulted in altered (slower) mobility of LTA on SDS-PAGE, as visualized by immunoblotting with polyglycerolphosphate-specific antibody (20). Similar changes in LTA motility were observed for LTA isolated from *pgcA* and *gtaB* mutant *S. aureus* strains (Fig. 2C). Together, these results show that *pgcA* and *gtaB* are essential for glycolipid synthesis in *S. aureus* and that in the absence of these genes LTA with an altered structure is produced. The mobility of LTA extracted from *pgcA* and *gtaB* mutant strains is identical to the mobility of LTA isolated from a *ypfP* mutant, suggesting that all of these LTAs have similar lipid anchor structures.

Mutations in *ltaA* cause structural changes in staphylococcal LTA. In *S. aureus* strains, *ypfP* is located in an operon with another gene, annotated in the MU50 genome by locus tag SAV1016 (designated *ltaA* here), whose predicted start site overlaps the 3′ end of the *ypfP* coding sequence (Fig. 3A). LtaA homologs (E-value, >5e-158) are found in all *S. aureus* strains sequenced and in closely related species (*Staphylococcus epidermidis*, *Staphylococcus haemolyticus*, *Staphylococcus saprophyticus*) and are always preceded by *ypfP*. While YpfP is a cytoplasmic protein, LtaA is predicted to encode a 12-transmembrane-domain protein with an unknown function (Fig. 3B). LtaA encoded by the MU50 genome (Uniprot ID Q99V76) is annotated as a multidrug resistance-related protein, and multiple-sequence alignment using Pfam identified an MFS-1 domain in LtaA, which otherwise is found in members of the major facilitator superfamily clan (10). Genes that are located in an operon often encode proteins that function in the same biochemical pathway. To investigate a possible function of *ltaA* in LTA or glycolipid biosynthesis, we transduced a mutant allele (transposon insertion in *ltaA*) with phage Φ85

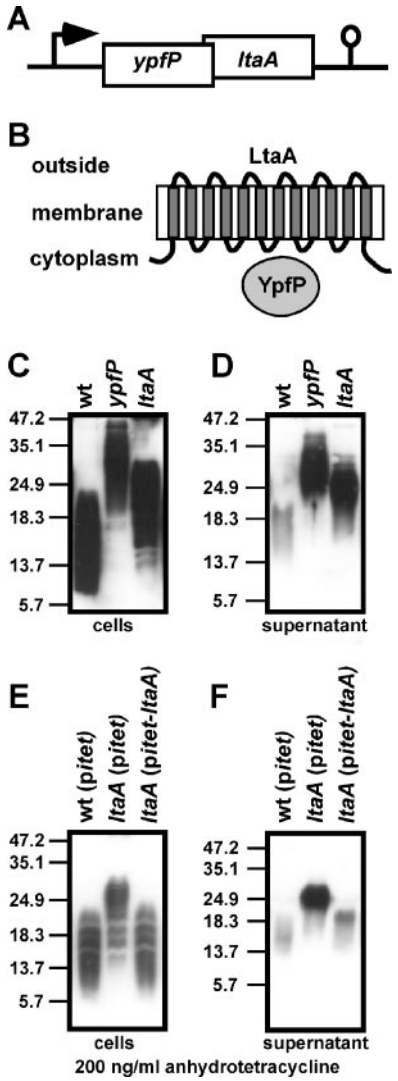

FIG. 3. (A) *S. aureus ypfP* operon. *ypfP* is located in an operon with *ltaA* (locus tag, SAV1016), which encodes a 12-transmembrane-segment protein predicted to function as a permease of the major facilitator superfamily. The diagram also shows a predicted transcriptional terminator downstream of *ltaA*. (B) Predicted subcellular locations of YpfP and LtaA. YpfP is a cytoplasmic protein, whereas LtaA is thought to reside in the cytoplasmic membrane. (C) Cell-associated LTA from *S. aureus* strains ANG361 (wild-type) (wt), ANG370 (*ypfP*), and ANG359 (*ltaA*) was extracted and normalized based on culture OD_{600} values, and the preparations were analyzed by immunoblotting. (D) Immunoblot analysis of LTA shed into the culture supernatant using the strains used for panel C. (E and F) For complementation analysis, a single-site chromosomal integration vector carrying *ltaA* under control of the tetracycline-inducible P_{itet} promoter was used (p*itet-ltaA*). *S. aureus* strains ANG395 (wild-type with empty vector p*itet*) (wt), ANG393 (*ltaA* mutant with empty vector p*itet*), and ANG391 (*ltaA* mutant with integrated p*itet-ltaA*) were grown in the presence of 200 ng/ml anhydrotetracycline. Cell-associated LTA (E) and LTA shed into the culture supernatant (F) were analyzed by immunoblotting. Samples were normalized based on culture OD_{600} values. The positions of protein molecular mass markers (in kDa) are indicated on the left.

into strain SEJ1 to generate *S. aureus* strain ANG359. Cell-associated LTA extracted from *S. aureus* ANG359 migrated more slowly on an SDS-PAGE gel than LTA isolated from the wild-type strain but faster than LTA from a *ypfP* mutant (Fig. 3C). Similar to the observation for a *ypfP* mutant, an increase in the amount of LTA released into the culture supernatant was observed for the *ltaA* mutant (Fig. 3D) (27). To examine a possible polar effect of the transposon insertion on *ypfP* expression in strain ANG359, we performed a complementation analysis. DNA including *ltaA* was cloned into the single-site integration vector p*itet* containing an inducible tetracycline promoter (P_{itet}) inserted into pCL55. The resulting plasmid, p*itet-ltaA*, was integrated into the *geh* lipase gene of strain ANG359 to generate *S. aureus* ANG391. When this strain was grown in the presence of the P_{itet} inducer anhydrotetracycline, cell-associated LTA from ANG391 migrated with the same mobility on an SDS-PAGE gel as wild-type LTA (Fig. 3E). Furthermore, similar to the observation for a wild-type strain, only small amounts of LTA were released into the culture supernatant by the complemented strain (Fig. 3F). Thus, inactivation of *ltaA* causes structural changes in LTA that can be detected by SDS-PAGE, which prompted the designation *ltaA* (for *l*ipo*t*eichoic *a*cid gene *A*).

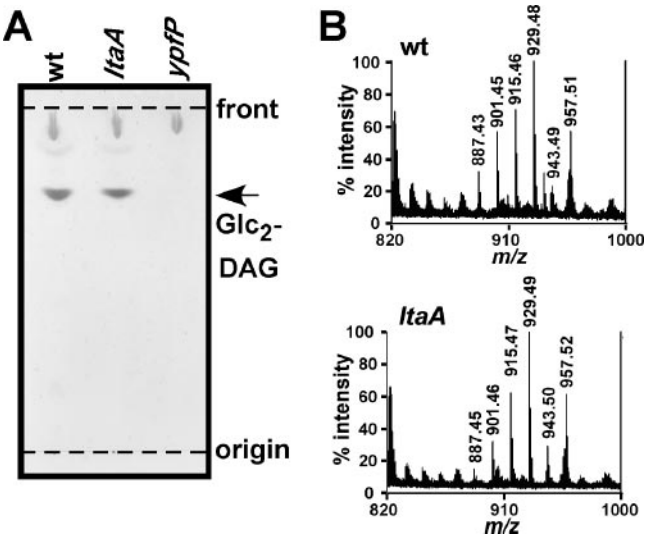

FIG. 4. (A) TLC analysis of *S. aureus* glycolipids. Membrane lipids were extracted from *S. aureus* strains ANG361 (wild-type) (wt), ANG359 (*ltaA*), and ANG370 (*ypfP*) and separated on TLC plates. Glycolipids were visualized with α-naphthol/sulfuric acid. The positions of the origin and solvent front are indicated by dashed lines. (B) MALDI-TOF mass spectrometry of *S. aureus* glycolipids. Glycolipids were isolated by TLC, extracted, dried, and suspended in 0.125 M 2,2′:5′,2″-terthiophene dissolved in chloroform-methanol (1:1). Spotted samples were dried and ionized by MALDI, and mass spectra were collected with a Reflection time of flight instrument in the positive ion mode.

Staphylococcal *ltaA* is not required for glycolipid synthesis. Glycolipids are not detectable in the membranes of *ypfP*, *pgcA*, or *gtaB* mutant *S. aureus* strains (see above). To investigate whether glycolipid production is affected in an *ltaA* mutant strain, total membrane lipids were extracted and separated by TLC, and glycolipids were visualized by α-naphthol/sulfuric acid staining. A major α-naphthol-reactive species was detected in membranes of *ltaA* mutant staphylococci, and this molecule migrated with the same mobility on TLC as Glc_2-DAG from membranes of wild-type staphylococci (Fig. 4A). To verify the identity of the α-naphthol-reactive species, lipids

TABLE 2. Masses of diglucosyl-diacylglycerol lipids isolated from membranes of wild-type and mutant *S. aureus* strains

Possible fatty acid chain length	Chemical formula	Predicted molecular mass	Observed molecular mass for:		
			Wild type	*ltaA* mutant	*ypfP* mutant
C_{15}/C_{15}	$C_{45}H_{84}NaO_{15}$	887.57	887.43	887.45	Absent
C_{15}/C_{16}	$C_{46}H_{86}NaO_{15}$	901.59	901.45	901.46	Absent
C_{15}/C_{17}	$C_{47}H_{88}NaO_{15}$	915.60	915.46	915.47	Absent
C_{15}/C_{18}	$C_{48}H_{90}NaO_{15}$	929.62	929.48	929.49	Absent
C_{16}/C_{18}	$C_{49}H_{92}NaO_{15}$	943.63	943.49	943.50	Absent
C_{17}/C_{18}	$C_{50}H_{94}NaO_{15}$	957.65	957.51	957.52	Absent

were extracted from TLC plates and analyzed by MALDI-TOF mass spectrometry, and spectra were recorded in the reflector positive ion mode. The mass-to-charge (*m*/*z*) ratios of all major ion signals of the α-naphthol-reactive species were in agreement with the predicted mass of Glc_2-DAG sodium adducts harboring fatty acids with chain lengths ranging from C_{15} to C_{18} (Fig. 4B) (i.e., observed *m*/*z* 929.5 and predicted *m*/*z* 929.6 for the sodium ion of Glc_2-DAG harboring C_{18} and C_{15} acyl chains). The differences of 14 mass units in the spectra in Fig. 4 correspond to addition or omission of methylene (CH_2) groups in Glc_2-DAG. A complete list of observed and predicted compound masses, chemical structures, and possible acyl chain lengths for glycolipids in wild-type and *ltaA* mutant strains is provided in Table 2. When analyzing membranes of *ypfP* mutant staphylococci, we could not detect ion signals in the range from *m*/*z* 880 to *m*/*z* 960 (Table 2). In summary, our results reveal that inactivation of LtaA does not affect glycolipid synthesis in *S. aureus*.

LtaA modulates YpfP-dependent glycolipid biosynthesis. Previous work showed that expression of *B. subtilis ypfP* in *E. coli* leads to the production of glycolipids (25). Moreover, expression of *S. aureus ypfP* in *E. coli* not only generates Glc_2-DAG but also leads to the formation of three other glycolipids, monoglycosyl-phosphatidylglycerol (Glc-PG) (also designated PL1), diglucosyl-diacyl-phosphatidylglycerol (Glc_2-DAPG) (also designated PL2), and triglucosyl-diacylglycerol (Glc_3-DAG) (24). In agreement with previous reports, we observed that expression of *ypfP* in *E. coli* ANG373(pCL55-*ypfP*) led to the production of four glycolipid species that could be detected by α-naphthol staining of TLC plates (compounds I to IV) (Fig. 5). As a control, glycolipids were not detected in membrane extracts of *E. coli* ANG243 harboring the empty vector pCL55 (Fig. 5). *E. coli* strain ANG374(pCL55-*ypfP*/*ltaA*) expressed both *ypfP* and *ltaA*. Only two glycolipid species, compounds II and IV, were detected in membranes of *E. coli* ANG374 (Fig. 5). To identify these compounds and to characterize the functions of LtaA in modulating YpfP-dependent glycolipid biosynthesis, lipids were purified from TLC plates and analyzed by MALDI-TOF mass spectrometry, and spectra were recorded in the positive reflector ion mode. The mass spectra collected for the four glycolipids (Glc_2-DAG, Glc-PG, Glc_2-DAPG, and Glc_3-DAG) produced by *E. coli* strain ANG373(pCL55-*ypfP*) were in agreement with previously reported masses and proposed structures (24) (Fig. 6). Membranes of *E. coli* ANG374(pCL55-*ypfP*/*ltaA*), expressing both *ltaA* and *ypfP*, harbored only Glc_2-DAG (compound II) and Glc-PG (compound IV) and not Glc_2-DAPG (compound I) or Glc_3-DAG (compound III) (Fig. 6). A complete list of the observed and predicted masses and chemical structures of glycolipids is provided in Table 3 and Fig. 6. Taken together, these experiments demonstrated that LtaA restricts the spectrum of YpfP-derived glycolipids to two compounds, Glc_2-DAG and Glc-PG, and abrogates the formation of the aberrant products Glc_2-DAPG and Glc_3-DAG, which are also not found in membranes of *S. aureus*.

The molecular details of YpfP-dependent biosynthesis of Glc_2-DAPG (PL2) are currently not understood. Presumably, YpfP adds two glucose molecules to phosphatidylglycerol, whereas an *E. coli* acyltransferase may subsequently transfer additional fatty acids to generate Glc_2-DAPG (24). Glc_3-DAG is likely synthesized by YpfP via successive addition of thee glucose molecules to DAG, presumably without the aid of *E. coli* enzymes. Addition of the third glucose residue appears to be the rate-limiting step, as the amount of Glc_2-DAG that accumulates in *E. coli* membranes is greater than the amount of Glc_3-DAG that accumulates. We presume that LtaA prevents formation of Glc_3-DAG by binding or sequestration of Glc_2-DAG from YpfP. For example, LtaA may promote Glc_2-DAG transport from the inner leaflet of the plasma membrane to the outer leaflet. If this is so, Glc_2-DAG may not be accessible for YpfP, which is located in the cytoplasm. The transmembrane topology and predicted permease function of LtaA are certainly in agreement with this model.

Diacylglycerol anchor structures of LTA in *ltaA* mutant staphylococci. Glycolipid synthesis occurs on membranes in the bacterial cytoplasm, whereas LTA biosynthesis is thought to be completed on membrane surfaces outside the cytoplasm

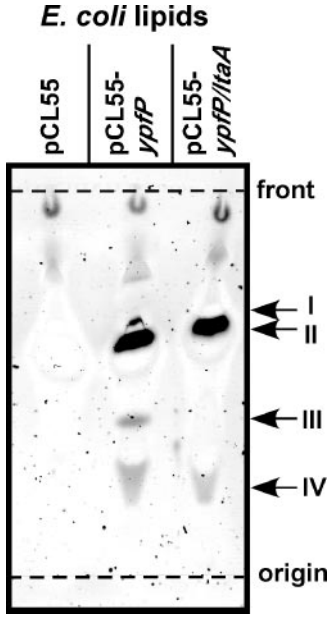

FIG. 5. TLC analysis of glycolipids generated by YpfP in *E. coli*. Membrane lipids isolated from *E. coli* strains ANG243 (vector control; pCL55), ANG373 (plasmid expressing YpfP; pCL55-*ypfP*), and ANG374 (plasmid expressing YpfP and LtaA; pCL55-*ypfP*/*ltaA*) were separated by TLC, and glycolipids were detected by α-naphthol/sulfuric acid staining. Glycolipid species were designated compounds I to IV.

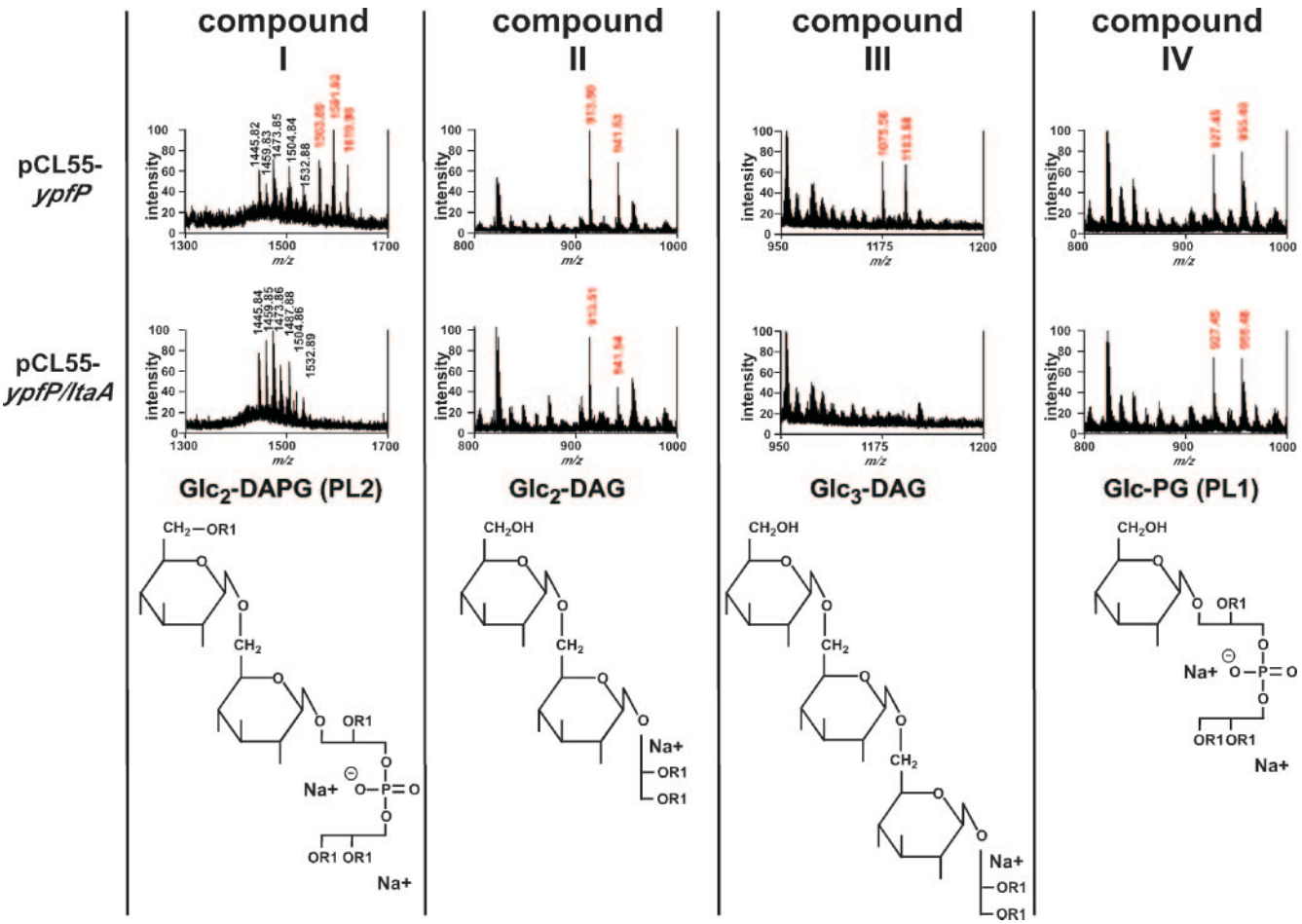

FIG. 6. MALDI-TOF mass analysis of YpfP-generated glycolipids in *E. coli*. Lipids from *E. coli* were separated by TLC, and compounds I to IV were isolated. Dried lipids were suspended in 0.125 M 2,2,5′,2″-terthiophene–5 mM $NaHCO_3$ and analyzed by using MALDI-TOF mass spectrometry, and mass spectra were collected in the positive ion mode. Mass-to-charge (*m*/*z*) ratios derived from glycolipid ion signals are indicated by red, and the corresponding structures (24) are shown at the bottom.

(12). Thus, even though Glc_2-DAG synthesis is not abolished in *ltaA* mutant staphylococci, mutations in *ltaA* could still affect the incorporation of Glc_2-DAG as an LTA anchor. One could assume that LtaA functions as a permease for glycolipid transport across the cytoplasmic membrane. To test this hypothesis, we purified LTA molecules extracted from membranes of wild-type (ANG361), *ypfP* (ANG370), and *ltaA* (ANG359) staphylococcal strains and analyzed their membrane anchor structures. Purified LTA was treated with 48% HF to hydrolyze the phosphodiester bonds of the glycerolphosphate subunits. LTA

TABLE 3. Masses of glucose-containing lipids isolated from *E. coli* membranes expressing YpfP or YpfP/LtaA

Compound	Glycolipid[a]	Chemical formula	Possible fatty acid chain length	Predicted molecular mass	Observed molecular mass for:	
					YpfP	YpfP and LtaA
I	PL2 (Glc_2-DAPG)[a]	$C_{82}H_{150}Na_2O_{22}P_1$	$2\times C_{16}/2\times C_{16:1}$	1,564.02	1,563.89	Absent
		$C_{84}H_{154}Na_2O_{22}P$	$C_{16}/2\times C_{16:1}/C_{18}$	1,592.05	1,591.92	Absent
		$C_{86}H_{158}Na_2O_{22}P$	$C_{16}/C_{16:1}/C_{18}/C_{18:1}$	1,620.08	1,619.96	Absent
II	Glc_2-DAG	$C_{47}H_{86}NaO_{15}$	$C_{16}/C_{16:1}$	913.59	913.50	913.51
		$C_{49}H_{90}NaO_{15}$	$C_{16}/C_{18:1}$	941.61	941.53	941.54
III	Glc_3-DAG	$C_{53}H_{96}NaO_{20}$	$C_{16}/C_{16:1}$	1,075.64	1,075.56	Absent
		$C_{55}H_{100}NaO_{20}$	$C_{16}/C_{18:1}$	1,103.67	1,103.58	Absent
IV	PL1 (Glc-PG)[a]	$C_{44}H_{82}Na_2O_{15}P$	$C_{16}/C_{16:1}$	927.52	927.45	927.45
		$C_{46}H_{86}Na_2O_{15}P$	$C_{16}/C_{18:1}$	955.55	955.48	955.48

[a] See reference 24.

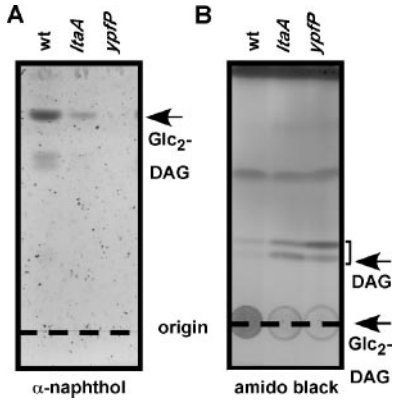

FIG. 7. LTA membrane anchor structures. LTA from *S. aureus* strains ANG361 (wild-type) (wt), ANG359 (*ltaA*), and ANG370 (*ypfP*) was purified, and polyglycerolphosphate was hydrolyzed with hydrofluoric acid. LTA anchor structures, which resist hydrofluoric acid treatment, were isolated by organic solvent extraction and analyzed by TLC. (A) LTA anchor structures were separated by TLC using chloroform-methanol-H_2O (70:30:4), and glycolipids visualized with α-naphthol/sulfuric acid. (B) Lipids were separated using heptane–isopropyl ether–acetic acid (60:40:4) and were visualized by staining with a 0.2% amido black solution. The chromatographic mobilities of diacylglycerol (1,2-dipalmitoyl-*sn*-glycerol) and Glc_2-DAG are indicated on the right.

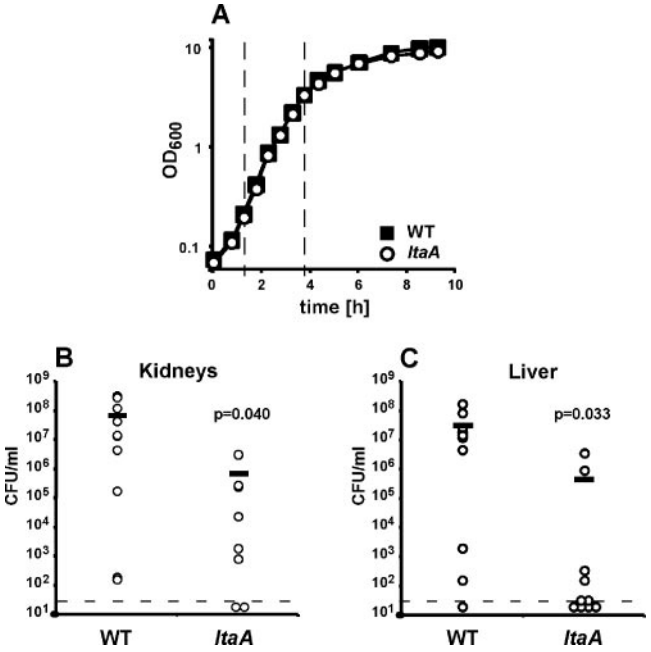

FIG. 8. *S. aureus* mutants lacking *ltaA* display a virulence defect in the murine abscess model. (A) Growth curves for *S. aureus* strain Newman and an isogenic *ltaA* mutant. Overnight cultures of *S. aureus* wild-type strain Newman (WT) and *ltaA* mutant strain ANG460 were diluted into fresh TSB, and the cultures were incubated at 37°C. At intervals samples were withdrawn, and OD_{600} values were determined. Average values for three independently grown cultures are shown. During logarithmic growth (1.25 to 3.75 h after inoculation, indicated by dashed lines) the wild-type and *ltaA* mutant strains had doubling times of 38 and 37 min, respectively. (B and C) Virulence test with *S. aureus* strain Newman and an isogenic *ltaA* mutant. BALB/c mice were challenged by retroorbital infection with *S. aureus* strain Newman or ANG460 (*ltaA*). Four days after infection, the animals were killed, and the kidneys and livers were removed. Infected organ tissues were homogenized and spread on agar plates, and the staphylococcal burden was quantified by using colony formation and enumeration. The dashed lines indicate the limits of detection (33 CFU/ml), and the horizontal bars indicate observation averages. The statistical significance of the reduction in the staphylococcal burden was assessed with the one-tailed Student *t* test, and *P* values were determined.

membrane anchor lipids, which resist HF treatment, were subsequently extracted with chloroform-methanol and analyzed by TLC. As previously reported, Glc_2-DAG is the predominant membrane anchor lipid of LTA in wild-type *S. aureus* strains (Fig. 7A). Small amounts of a lipid compound that comigrated with DAG were observed in HF-treated LTA samples from wild-type staphylococci (Fig. 7B). α-Naphthol-reactive glycolipids were not detected following HF treatment of LTA that had been purified from membranes of *ypfP* mutant staphylococci. Moreover, LTA anchor structures in *ypfP* mutant staphylococci were comprised of DAG. These findings are consistent with a model indicating that DAG or phosphatidylglycerol moieties retain biosynthetic LTA intermediates (polyglycerolphosphate polymerized via successive addition of glycerolphosphate derived from phosphatidylglycerol) in the membrane until transfer to Glc_2-DAG completes the synthesis pathway (5). However alternative models for LTA synthesis have been proposed, in which glycerolphosphate is directly polymerized on glycolipids (11, 19) and DAG-linked polyglycerolphosphate LTA may therefore be synthesized only in the absence of glycolipids. HF treatment of LTA purified from membranes of *ltaA* mutant strains released small amounts of glycolipids and also additional anchor lipids (Fig. 7). Compared to the LTA from the wild-type parent, the LTA from the *ltaA* mutant contained decreased amounts of Glc_2-DAG and increased amounts of DAG (Fig. 7). A mixture of glycolipid and nonglycolipid anchor structures is in agreement with our initial observation that there were discrete differences in mobility on SDS-PAGE gels between LTA isolated from the *S. aureus ltaA* mutant strain and LTA isolated from wild-type (Glc_2-DAG anchor) and *ypfP* mutant (DAG anchor) strains. Together, these results indicate that even though LtaA is not required for glycolipid synthesis, the predicted membrane permease is required for LTA attachment to Glc_2-DAG anchor structures.

***S. aureus* mutants lacking *ltaA* display a virulence defect in a murine abscess model.** To assess whether glycolipid anchoring of LTA is required for the pathogenesis of staphylococcal infections, an *ltaA* mutant was constructed using the *S. aureus* human clinical isolate Newman (9). LTA isolated from the Newman-derived *ltaA* mutant ANG460 displayed an electrophoretic mobility shift similar to the shift displayed by LTA isolated from the RN4220-derived mutant, indicating that strain ANG460 is also defective in glycolipid anchoring of LTA (data not shown). Wild-type strain Newman and the isogenic *ltaA* mutant replicated with similar doubling times (38 and 37 min, respectively) during logarithmic growth in TSB (Fig. 8A). The *ltaA* mutant had a slightly lower density during the postexponential growth phase (Fig. 8A), which may have coincided with changes in membrane lipid composition that are known to occur during later growth stages (28, 45). For virulence studies, 45-day-old BALB/c mice were infected with *S. aureus* strain Newman or an isogenic variant lacking *ltaA*. Infected animals were killed after 4 days, the livers and kidneys

were removed, and homogenized tissues were plated on agar plates to determine staphylococcal replication in organ abscesses. Compared to the replication of the wild-type parent, the replication of the staphylococcal variant lacking *ltaA* was significantly impaired in kidney and liver tissues (Fig. 8B and C). On balance, we observed a 2-log reduction in the number of CFU recovered, suggesting that glycolipid anchoring of LTA plays an important role in the functional assembly of staphylococcal cell wall envelopes during infection.

DISCUSSION

Despite rigorous biochemical analysis of LTA, the genetic determinants required for LTA synthesis have still not been defined. The *dlt* operon, encoding factors that catalyze D-alanyl esterification of glycerolphosphate and ribitolphosphate teichoic acids, has been characterized for several different gram-positive bacteria (22, 36, 37). D-Alanyl esterification of teichoic acids plays an important role during host-pathogen interactions of *S. aureus*, group A streptococci, *Listeria monocytogenes*, and *B. anthracis* (1, 17, 30, 39). Only one additional gene has been reported to be required for LTA biosynthesis in *S. aureus*. *ypfP*, which encodes a processive glycosyltransferase, is required for glycolipid and LTA anchor structure synthesis (27). Here we describe identification of three additional *S. aureus* genes, *pgcA*, *gtaB*, and *ltaA*, which are involved in the synthesis of LTA.

B. subtilis pgcA and *gtaB* encode enzymes required for the synthesis of UDP-glucose, which is used by YpfP as a substrate for glycolipid synthesis (32, 41, 46). Using bioinformatic analysis, we identified *S. aureus* PgcA and GtaB homologs, and here we present evidence that these proteins are also required for glycolipid biosynthesis in *S. aureus* (Fig. 2). In *B. subtilis*, UDP-glucose has a central role as a metabolite for the synthesis of cell wall envelope polymers. UDP-glucose is required not only for glycolipid synthesis but also for glycosylation of the major wall teichoic acid and for the synthesis of the minor wall teichoic acid (32). In addition, UDP-glucose plays a central role in *B. subtilis* biofilm formation through a pathway that is distinct from the requirement for UDP-glucose for glycolipid or wall teichoic acid synthesis (3, 32). Additional work is needed to investigate the requirement for *pgcA* and *gtaB* in *S. aureus* cell wall envelope assembly, cell shape determination, or biofilm formation.

Previous studies have shown that *pgcA*, *gtaB*, and *ypfP* mutant *Bacillus* strains, as well as *ypfP* mutant *S. aureus* strains, have aberrant cell shapes (18, 27, 32, 42). It is not yet clear whether such morphological alterations are caused by a lack of membrane glycolipids or by changes in LTA structure or function (for example, by increased release of LTA into the culture media of *ypfP* mutant staphylococci) (27). Experimental distinction between these possibilities may now be possible if workers investigate *gtaB*, *pgcA*, *ypfP*, and *ltaA* mutant staphylococci. To our knowledge, the *S. aureus ltaA* mutant described in this report is the first mutant capable of segregating glycolipid and LTA biosynthesis. Membrane glycolipids are synthesized in an *ltaA* mutant strain in a fashion similar to that in wild-type staphylococci; however, only a small amount of LTA is anchored by Glc_2-DAG, whereas a large portion is tethered to DAG, similar to LTA anchor structures in *ypfP* mutant membranes. These findings place LtaA activity downstream of the PgcA, GtaB, and YpfP activities in the LTA synthesis pathway outlined in Fig. 2A.

Glycolipid synthesis occurs in the bacterial cytoplasm, whereas LTA synthesis is thought to localize in the outer leaflet of the plasma membrane (12). We assume that spontaneous distribution of glycolipids from inner membrane leaflets to outer membrane leaflets occurs at a low rate. Transport of glycolipids by a permease may be required to achieve the necessary distribution and availability of glycolipids for LTA biosynthesis. *ltaA* codes for a hydrophobic protein with 12 predicted transmembrane domains. On the basis of Pfam amino acid sequence analysis, LtaA seems to be a member of the major facilitator superfamily clan (10). All members of the major facilitator superfamily possess either 12 or 14 transmembrane helices, and these proteins are membrane transporters that are ubiquitous in bacteria, archaea, and eukarya and function primarily in uniport, symport, or antiport of small solutes (38, 44). More recently, *E. coli* LplT, another member of the major facilitator superfamily, has been reported to catalyze the transmembrane movement of lysophospholipids from the membrane surface into the cytoplasm, where the lipid is acylated by a second protein encoded in the same operon (21). In the absence of LplT, lysophospholipids can still be incorporated into cells, albeit at a much lower rate, which presumably represents spontaneous flipping of lysophospholipid across membranes (21). These observations resemble our observations for LTA biosynthesis. In *ltaA* mutant strains, only small amounts of glycolipids may flip to the outside, as very little Glc_2-DAG is found in LTA anchor structures (Fig. 7). If one also assumes that addition of Glc_2-DAG to LTA continuously removes glycolipids from the outer leaflet of the plasma membrane, the slow diffusion of glycolipids across membranes may not fulfill the concentration requirements for glycolipid substrates in LTA biosynthesis. LtaA may therefore promote transfer of glycolipids across membranes following a concentration gradient.

A model in which LtaA functions as glycolipid flippase can also explain the role of LTA in modulating YpfP-dependent glycolipid synthesis in *E. coli*. In the absence of LtaA, but not in its presence, YpfP synthesizes Glc_3-DAG in *E. coli* membranes. The addition of the third glucose molecule to Glc_2-DAG is slow and rate limiting. If one assumes that LtaA mediates transport of Glc_2-DAG across the membrane, this mechanism may prevent YpfP-mediated synthesis of Glc_3-DAG in the bacterial cytoplasm. Nevertheless, our model cannot explain why YpfP-synthesized PL2 glycolipid with four acyl chains accumulates in the absence of LtaA but not in the presence of LtaA.

Even though in our study we could identify three *S. aureus* genes involved in glycolipid synthesis and LTA anchoring, other genes that are involved in LTA biosynthesis still remain to be discovered. Knowledge of the entire biosynthetic pathway is essential to increase our understanding of cell wall envelope assembly in gram-positive bacteria.

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