JOURNAL OF BACTERIOLOGY, Apr. 2006, p. 2463–2472 Vol. 188, No. 7
0021-9193/06/$08.00+0 doi:10.1128/JB.188.7.2463–2472.2006

Cross-Linked Peptidoglycan Mediates Lysostaphin Binding to the Cell Wall Envelope of *Staphylococcus aureus*†

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Received 29 November 2005/Accepted 19 January 2006

Staphylococcus simulans **bv. staphylolyticus secretes lysostaphin, a bacteriocin that cleaves pentaglycine cross bridges in the cell wall of *Staphylococcus aureus*. The C-terminal cell wall-targeting domain (CWT) of lysostaphin is required for selective binding of this bacteriocin to *S. aureus* cells; however, the molecular target for this was unknown. We used purified green fluorescent protein fused to CWT (GFP-CWT) to reveal species-specific association of the reporter with staphylococci. GFP-CWT bound *S. aureus* cells as well as purified peptidoglycan sacculi. The addition of cross-linked murein, disaccharides linked to interconnected wall peptides, blocked GFP-CWT binding to staphylococci, whereas murein monomers or lysostaphin-solubilized cell wall fragments did not. *S. aureus* strain Newman variants lacking the capacity for synthesizing polysaccharide capsule (*capFO*), poly-*N*-acetylglucosamine (*icaAC*), lipoprotein (*lgt*), cell wall-anchored proteins (*srtA*), or the glycolipid anchor of lipoteichoic acid (*ypfP*) bound GFP-CWT similar to wild-type staphylococci. A *tagO* mutant strain, defective in the synthesis of polyribitol wall teichoic acid attached to the cell wall envelope, displayed increased GFP-CWT binding. In contrast, a *femAB* mutation, reducing both the amount and the length of peptidoglycan cross-linking (monoglycine cross bridges), showed a dramatic reduction in GFP-CWT binding. Thus, the CWT domain of lysostaphin directs the bacteriocin to cross-linked peptidoglycan, which also serves as the substrate for its glycyl-glycine endopeptidase domain.**

Bacteria secrete ribosomally synthesized bacteriocins with the intent of selectively killing microbes that compete for limited resources (34). Research on the inhibitory mechanisms of bacteriocins has led to the discovery of fundamental bacterial processes such as DNA replication (17), membrane transport (50), and cell wall biosynthesis (64). Moreover, research on bacteriocins can also provide tangible results and supply therapeutics for human infections caused by bacterial pathogens. *Staphylococcus simulans* bv. staphylolyticus secretes lysostaphin, a bacteriocin that cleaves pentaglycine cross bridges in the cell wall of *Staphylococcus aureus* (58). The gram-positive bacterium *S. aureus* is a human pathogen and colonizes the human skin and nares (1, 39). Over the past three decades, treatment of staphylococcal infections has become increasingly difficult because of colonization with strains that are resistant to virtually all antimicrobial agents (6). Lysostaphin has been used for the elimination of staphylococci from skin and nares of individuals with increased risk for *S. aureus* infections (33).

The cell wall envelope of *S. aureus* and other gram-positive bacteria is a complex surface organelle, assembled from peptidoglycan precursor molecules and decorated with proteins and polysaccharides. *S. aureus* peptidoglycan (murein) comprises glycan strands with the repeating disaccharide *N*-acetylmuramic acid (β1–4)-*N*-acetylglucosamine (MurNac-GlcNac) units of various lengths (18, 19). Short wall peptides, composed of L-Ala-D-iGln-L-Lys-D-Ala, are linked via an amide bond to the D-lactyl moiety of MurNac (20, 44, 70). Neighboring wall peptides are cross-linked between the ε-amino group of L-Lys in one peptide and the carboxyl group of D-Ala in another via pentaglycine cross bridges. Together, the glycan strands and cross-linked wall peptides generate the three-dimensional exoskeletal network of peptidoglycan (68, 69). Other major constituents of the cell wall envelope are polyanionic wall teichoic acids (WTAs) and lipoteichoic acids (LTAs), as well as proteins (45, 47). *S. aureus* strains secrete poly-*N*-acetylglucosamine exopolysaccharide and are encapsulated with one or more types of cell wall-attached polysaccharides (11, 37). In addition to proteins that are covalently attached to peptidoglycan, non-covalently associated proteins fulfill important functions within the cell wall envelope (21, 45).

Lysostaphin is synthesized by *S. simulans* as a preproenzyme and exported from the bacterial cytoplasm by an N-terminal signal peptide (25, 55). Following membrane translocation and signal peptide cleavage, prolysostaphin is released and 14 tandem repeats of a 13-residue peptide at the N-terminal end are removed by extracellular proteases to generate mature, enzymatically active bacteriocin (25, 55). Lysostaphin binds to *S. aureus* cells and cleaves pentaglycine cross bridges within peptidoglycan, thereby removing the cell wall envelope and precipitating osmotic rupture of staphylococci (8, 58, 60). The mature form of lysostaphin encompasses two domains, the glycyl-glycine endopeptidase, which cleaves oligoglycine peptides (30), and a C-terminal cell wall-targeting domain (CWT) (2). The C-terminal 92 amino acid residues of lysostaphin are dispensable for enzymatic activity but necessary and sufficient for directing lysostaphin or fused reporter proteins to the cell wall envelope of *S. aureus* (2). Previous work left unresolved the molecular nature of the CWT receptor within the *S. aureus* cell wall envelope.

Here we have generated green fluorescent protein fused to CWT (GFP-CWT) to reveal species-specific association of this purified reporter protein with staphylococci. GFP-CWT bound

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† Supplemental material for this article may be found at http://jb.asm.org/.

S. aureus cells as well as purified peptidoglycan sacculi. The addition of cross-linked murein, i.e., disaccharides linked to interconnected wall peptides, blocked GFP-CWT binding to staphylococci, whereas murein monomers or lysostaphin-solubilized cell wall fragments did not. *S. aureus* mutants lacking polysaccharide capsule, poly-*N*-acetylglucosamine, lipoproteins, cell wall-anchored proteins, cell wall teichoic acids, or the glycolipid anchor of lipoteichoic acid bound GFP-CWT similar to wild-type bacteria. In contrast, a *femAB* mutation, reducing both the amount and the length of peptidoglycan cross-linking (monoglycine cross bridges), caused a dramatic reduction in GFP-CWT binding. These observations support a model whereby the CWT domain of lysostaphin directs the bacteriocin to cross-linked peptidoglycan, which also serves as substrate for its glycyl-glycine endopeptidase domain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C. All other strains were grown in tryptic soy broth (TSB) at the indicated temperature. *S. aureus* strains Newman (14), SA113 (ATCC 35556), and RN4220 (36), *S. simulans* TNK1 (2), *Enterococcus faecalis* FA2-2 (10), *Listeria monocytogenes* EGDe (obtained from M. Loessner, Institute of Food Science and Nutrition, Zürich), and *Bacillus subtilis* 168 (9) were used in this study. *S. aureus* Newman transposon mutants from the Phoenix library (3) with defined transposon insertion sites were transduced into wild-type Newman background using phage 85. Insertion sites were confirmed by DNA sequencing of inverse PCR fragments (3). The *S. aureus ypfP* mutant (29) was used in this study, and an isogenic chloramphenicol-sensitive wild-type strain was generated by passing the mutant at 30°C in the absence of antibiotic selection. The *femAB* mutant *S. aureus* strain AS145, isogenic wild-type strain BB270, and *femAB* complementation plasmid pBBB64 were obtained from B. Berger-Bächi (63). Antibiotics were used at the following concentrations: 100 μg/ml ampicillin for the selection of pQE30 (QIAGEN), pQE30-GFP, pQE30-GFP-CWT, pGEX2TK (Amersham Biosciences), and pGST-K-CWT (2) as well as plasmids pTS1 (48), pTS1-ΔtagO, pKOR1 (4), and pKOR1-ΔtagO in *E. coli*; and 7.5 or 10 μg/ml chloramphenicol was used for the selection of plasmid pKOR1-ΔtagO in *S. aureus*. *S. aureus* strains containing transposon insertions were grown in TSB medium with 10 μg/ml erythromycin. A list of all strains used in this study can be found in Table S1 in the supplemental material.

Strain and plasmid construction. *S. aureus* strain Newman Δ*tagO* and RN4220 Δ*tagO* variants were constructed by allelic replacement using plasmid pKOR1-ΔtagO. Primer pair 5-1kb-EcoRI-SAV0747 (CG*GAATTC*GATATCGATTAATAATAAAGCGATACTTTG) and 5-int-KpnI-SAV0747 (GG*GGTACC*TGTTACTGCAACTAGTAATAATGTAACCAT) was used to PCR amplify 1 kb of upstream DNA, including the first 30 nucleotides of *tagO* coding sequence and primer pair 3-int-KpnI-SAV0747 (GG*GGTACC*AGTCGGAAATCGTCACACAAAGAAGATTAG) and 3-1kb-BamHI-SAV0747 (CG*GGATCC*GTCCTGCGACAGATAACTTGTAGAATCG) was used to PCR amplify 1 kb downstream DNA including the last 27 nucleotides of *tagO* using Newman chromosomal DNA as template. PCR products were cut with the restriction enzymes EcoRI/KpnI and KpnI/BamHI, respectively, and ligated with vector pTS1 cut with EcoRI and BamHI, resulting in plasmid pTS1-ΔtagO. Next, primer pair attB1-SAV0747-F (GGGACAAGTTTGTACAAAAAAGCAGGCTGATATCGATTAATAATAAAGCGATAC) and attB2-SAV0747-R (GGGGACCACTTTGTACAAGAAAGCTGGGTGTCCTGCGACAGATAACTTGTAG) was used to amplify the *tagO* deletion fragment from plasmid pTS1-ΔtagO, and the fragment was moved to plasmid pKOR1 (4) by using Gateway technology (Invitrogen), resulting in plasmid pKOR1-ΔtagO. pKOR1-ΔtagO was introduced into *S. aureus* strains RN4220 and Newman by electroporation. Allelic replacement was performed as previously described (4), and chromosomal Δ*tagO* deletion was confirmed by PCR using the primer pair 5-O-outside check (TCACCTGATTTGTGTGAGTAGGTATCATTG) and 3-O-outside check (ATCAAAAGTCACACTTAATGGCGCTATTTG).

The GFP expression vector pQE30-GFP was constructed as described by Loessner et al. (38). For expression of the GFP lysostaphin cell wall-targeting domain fusion protein, primer pair 5′-SacI-CWT (TCTA*GAGCTC*ACGCCCAATACAGGTTGGAAAACAAAC) and 3′-PstI-CWTwithSTOP (AA*CTGCAG*TCACTTTATAGTTCCCCAAAGAACACC) was used to amplify the lysostaphin CWT domain by using *S. simulans* TNK1 chromosomal DNA as the template. The PCR product was cut with enzymes SacI and PstI and ligated with vector pQE30-GFP that had been cut with the same enzymes, thereby generating pQE30-GFP-CWT. DNA sequences of plasmid insertions were confirmed by fluorescence-automated sequencing at the Cancer Sequencing facility (The University of Chicago). Italicized nucleotides in primer sequences indicate restriction sites.

Protein purification. GFP and GFP-CWT were purified from cleared lysates of *E. coli* XL1-Blue by chromatography over 1.5 ml (column volume) nickel nitrilotriacetic (QIAGEN) (49), equilibrated with 50 mM Tris-HCl (pH 7.5), 150 mM NaCl (column buffer). The column was washed with 20 volumes of column buffer, 20 volumes of column buffer with 10% glycerol and again with 20 volumes of column buffer. Proteins were eluted with 500 mM imidazole in column buffer. *E. coli* BL21 (DE3) (65) (pGST-K-CWT) or *E. coli* CA8000 (23) (pGEX2TK) was used for glutathione *S*-transferase (GST)-CWT and GST overexpression, and proteins were purified by affinity chromatography from cleared lysates on 1.5 ml glutathione-Sepharose 4B resin (Bioworld) and eluted with 20 mM glutathione in column buffer. Proteins were dialyzed twice against 1 liter of 50 mM Tris-HCl (pH 7.5)–150 mM NaCl–30% glycerol buffer and stored at −20°C. Concentrations were determined using the bicinchoninic acid protein assay (Pierce).

Microscopy. Bacteria from 500 μl TSB culture were sedimented by centrifugation at 9,300 × *g* for 3 min, washed with 1 ml 50 mM Tris-HCl (pH 8.0) buffer, and suspended in 500 μl 50 mM Tris-HCl (pH 8.0). Optical densities at 600 nm (OD_{600}) of washed cultures were determined and adjusted to an OD_{600} of 1. For binding assays, 100 μl of 250 nM GFP or GFP-CWT protein solutions in 10 mg/ml bovine serum albumin (BSA)–50 mM Tris-HCl (pH 8.0) was mixed with 400 μl washed staphylococci to yield a final GFP or GFP-CWT concentration of 50 nM. Reaction mixtures were incubated for 10 min at room temperature. Bacteria and bound protein were sedimented by centrifugation at 16,000 × *g* for 3 min and suspended in 400 μl 50 mM Tris-HCl (pH 8.0). A drop of bacterial suspensions was placed on polylysine-coated glass coverslips and immediately analyzed by fluorescence microscopy.

Fluorescence plate assay. *ypfP* mutant and isogenic wild-type staphylococcus strains were grown overnight in TSB medium at 43°C with shaking. All other strains were grown overnight at 37°C. Bacteria of 3- to 4-ml culture were collected by centrifugation at 8,000 × *g* for 10 min, washed with 3 to 5 ml 50 mM Tris-HCl (pH 8.0) buffer, and suspended in 1.5 to 2 ml 50 mM Tris-HCl (pH 8.0). The cultures were adjusted to an OD_{600} of 10 and twofold dilutions thereof. For binding assays, 250 nM GFP or GFP-CWT solutions in 10 mg/ml BSA–50 mM Tris-HCl (pH 8.0) were prepared. Four hundred microliters of washed cells with an OD_{600} of 10 and twofold dilutions thereof were mixed with 100 μl 250 nM GFP or GFP-CWT protein solution and incubated for 10 min at room temperature. Bacteria and bound protein were removed by centrifugation at 16,000 × *g* for 10 min and 200 μl of the supernatants containing unbound GFP or GFP-CWT protein were removed and dispensed into black 96-well microtiter plates. Fluorescence readings for these supernatants were determined using the Synergy HT fluorescence plate reader (Bio-Tex) equipped with a 485 ± 20 nm excitation and 528 ± 20 nm emission filter set. Fluorescence values were autoscaled to a "low well" containing 200 μl 2 mg/ml BSA–50 mM Tris-HCl (pH 8.0). Fluorescence readings were plotted on the *y* axes, and bacterial OD_{600} values were plotted on the *x* axes. For competition assays, a 10-fold molar excess of GST or 10-fold, 5-fold, and 2.5-fold excesses of GST-CWT were added to bacterial suspensions before GFP-CWT binding assays were performed as described above.

Detection of lipoteichoic acid by immunoblot. *ypfP* mutant and isogenic wild-type *S. aureus* strains were grown overnight at 43°C in 4 ml TSB medium. One-milliliter culture was mixed with a 0.1-mm glass beads (~0.5 ml in volume) and cells were lysed by vortexing in the cold for 45 min. Glass beads were settled by centrifugation at 2,000 × *g* for 1 min and 0.5 ml of the supernatant was placed in a fresh tube. Cell membranes were collected by centrifugation at 16,000 × *g* for 10 min and suspended in 70 μl sample buffer with 2% sodium dodecyl sulfate (SDS). *S. aureus* strains Newman, RN4220, and SA113 were grown overnight in 4 ml TSB medium at 37°C with shaking. OD_{600} values were determined, and 1.5-ml-culture aliquots were placed in 2-ml fast prep tubes containing 0.1-mm glass beads (~0.5 ml in volume). Bacteria were lysed in a Fast-Prep machine (Q-BIOgene) by shaking three times for 45 s at setting 6. Tubes were chilled for 2 min on ice between runs. Glass beads were settled by centrifugation at 2,000 × *g* for 15 s, and 1 ml of the supernatant was placed in a fresh tube. Membranes were sedimented by centrifugation at 16,000 × *g* for 15 min and suspended in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer containing 2% SDS. Samples were normalized for their optical density, i.e., cultures with an initial OD_{600} of 6 were suspended in 100 μl. Samples were boiled for 30 min to solubilize lipoteichoic acid and insoluble material was removed by centrifugation

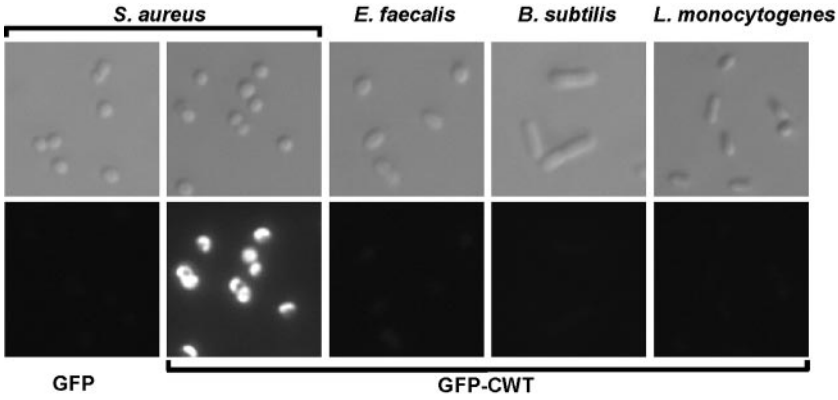

FIG. 1. GFP-CWT binds to the surface of *S. aureus* cells. Bacteria were incubated with purified 50 nM GFP or GFP-CWT, harboring a C-terminal fusion to the lysostaphin CWT. Bacteria with or without bound protein were sedimented by centrifugation and GFP or GFP-CWT binding was visualized by fluorescence microscopy. The top panel shows images captured via charge-coupled-device camera by phase-contrast microscopy; the bottom panel displays images captured via fluorescence microscopy.

at 16,000 × *g* for 5 min. Eight microliters of each sample was separated on 15% SDS-PAGE. Western blotting was performed using the polyglycerolphosphate specific lipoteichoic acid antibody Clone 55 (HyCult Biotechnology) at a 1:1,000 or 1:2,000 dilution as primary antibody and the horseradish peroxidase-linked anti-mouse antibody (Cell signaling) at a 1:5,000 dilution as secondary antibody. Immunoblotting was performed in triplicate and reactive signals developed with chemiluminescence.

Purification of peptidoglycan. *S. aureus* peptidoglycan sacculi from 1-liter overnight cultures were purified as previously described (13). Purified peptidoglycan sacculi were lyophilized and dried samples were treated with 48% hydrofluoric acid for 48 h at 4°C (<6.5 mg peptidoglycan per 1 ml hydrofluoric acid). Acid-extracted, purified peptidoglycan was washed with H_2O and stored at 4°C for future experiments. For fluorescence binding assays, 3.125 μl (and twofold dilutions) of purified peptidoglycan with an OD_{600} of 10 was suspended in a final volume of 400 μl 50 mM Tris-HCl (pH 8.0) buffer. Next, 100 μl 250 nM GFP or GFP-CWT protein solution in 10 mg/ml BSA–50 mM Tris-HCl (pH 8.0) and binding assays performed as described above. Peptidoglycan purification and assays were performed in duplicate.

Peptidoglycan digestion and HPLC separation. One milliliter of peptidoglycan (OD_{600}, 10) was digested with 5,000 units of mutanolysin (Sigma) in 100 mM sodium phosphate buffer (pH 5.9) with 1 mM phenylmethylsulfonyl fluoride for ~16 h at 37°C with shaking and enzyme subsequently heat inactivated by boiling for 10 min. Insoluble material was removed by centrifugation at 16,000 × *g* for 15 min. The amount of reducing amino sugars within these samples was determined by a modified Morgan-Elson reaction using 0 to 250 μM *N*-acetylglucosamine (NAG) solutions as standards (46). Mutanolysin-digested peptidoglycan samples were adjusted to NAG concentration of 400 μM and twofold serial dilutions thereof. The ability of these fractions to inhibit GFP-CWT binding to *S. aureus* Newman cells was assayed using the fluorescence plate assay. Reactions were set up as follows: 100 μl washed Newman culture of an OD_{600} of 10 was mixed with 100 μl 400 μM mutanolysin-digested peptidoglycan material (or twofold dilutions) and volume adjusted to 400 μl with 50 mM Tris-HCl (pH 8.0). Next, 100 μl 250 nM GFP or GFP-CWT solutions in 10 mg/ml BSA–50 mM Tris-HCl (pH 8.0) were added at a final concentration of 80 μM soluble peptidoglycan and a 50 nM GFP or GFP-CWT concentration in 2 mg/ml BSA–50 mM Tris-HCl (pH 8.0). As controls, a no-inhibition binding curve was established by incubating bacteria and GFP-CWT protein in the absence of solubilized peptidoglycan fragments and a maximal-inhibition curve was generated by incubating GFP-CWT and solubilized peptidoglycan fractions in the absence of bacteria. For high-performance liquid chromatography (HPLC) purification, 2 ml purified peptidoglycan adjusted to an OD_{600} of 20 was digested with 10,000 U mutanolysin in 50 mM sodium phosphate buffer (pH 5.9) containing 1 mM phenylmethylsulfonyl fluoride and incubation for 15 h at 37°C with shaking. Samples were boiled for 10 min, and insoluble material was removed by centrifugation at 16,000 × *g* for 15 min. Mutanolysin-solubilized peptidoglycan was either directly reduced and separated by HPLC or further digested with lysostaphin (AMBI Products, LLC). For lysostaphin digestion, 100 μl of a 2 mg/ml lysostaphin solution in 20 mM sodium acetate buffer (pH 4.6) was added to 1 ml mutanolysin-digested peptidoglycan adjusted to pH 7.0 by the addition of 50 μl 1 M Tris-HCl (pH 8.6). This reaction mixture was incubated for 16 h at 37°C with shaking. Reaction mixtures were boiled for 10 min and insoluble material was removed by centrifugation at 16,000 × *g* for 15 min. Digested peptidoglycan material was reduced in sodium borate buffer, pH 9.0, with solid sodium borohydrate as described by Navarre et al. (46). The pH of reduced samples was adjusted to 3.0 with 20% phosphoric acid and 500 μl material was injected for each HPLC run. An HPLC Gold system (Beckman Coulter) and a 250- by 4.6-mm C_{18} ODS hypersile 3-μm-particle-size guard column (Thermos) were used to separate peptidoglycan with H_2O–0.1% trifluoroacetic acid (TFA) (buffer A) and acetonitrile–0.1% TFA (buffer B) gradients as follows: 5 min, 0% buffer B; 10 min, linear gradient 0 to 7.5% buffer B; 110 min, linear gradient 7.5 to 15% buffer B; 10 min, linear gradient 15 to 30% buffer B; 10 min, linear gradient 30 to 50% buffer B; and finally, 50 min, 100% buffer B (at a flow rate of 0.5 ml per minute and collection of 0.50-ml fractions). Pooled fractions were taken to dryness and suspended in 300 μl H_2O, and OD_{206} values determined. Fractions were analyzed in a fluorescence plate assay for their ability to inhibit binding of GFP-CWT to *S. aureus* cells. One hundred microliters of each fraction set to a 206-nm reading of 3, and twofold dilutions thereof were used, resulting in a maximal 206-nm reading of 0.6 in a 500-μl binding reaction mixture.

RESULTS

GFP-CWT is targeted to the cell wall envelope of *S. aureus*. We sought to quantify lysostaphin binding to bacterial cell surfaces and constructed a fusion between the GFP and the lysostaphin cell wall targeting domain (GFP-CWT). Purified GFP or GFP-CWT was added to *S. aureus* cells and binding was viewed by fluorescence microscopy. GFP alone did not bind to *S. aureus*; however, GFP-CWT bound to *S. aureus* strain Newman, causing fluorescence of cell surfaces (Fig. 1). GFP-CWT did not bind to the surfaces of other gram-positive bacteria, including *Enterococcus faecalis* FA2-2, *Listeria monocytogenes* EGDe, and *Bacillus subtilis* 168 (Fig. 1). These results indicate that the CWT targets lysostaphin or hybrid reporter proteins specifically to the envelope of *S. aureus* cells.

To quantify GFP-CWT binding to the envelope of *S. aureus* cells, bacteria were sedimented by centrifugation and GFP-CWT depletion from the supernatant was measured with a fluorescence plate reader. Increasing numbers of staphylococcal cells sedimented increased amounts of GFP-CWT (Fig. 2A). As a control, GFP alone did not bind to staphylococci (Fig. 2A). GFP-CWT binding to staphylococci appears to be species specific, as suspensions of *E. faecalis* failed to sediment reporter protein from the supernatant (Fig. 2B). GST-CWT is a hybrid

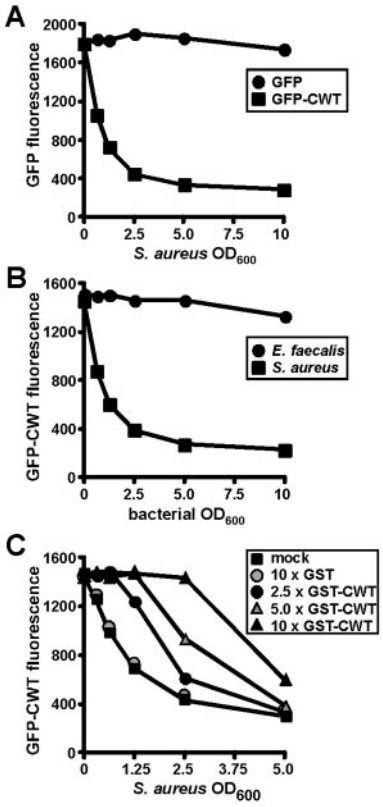

FIG. 2. GFP-CWT binding to receptors on the surface of *S. aureus*. A fluorescence plate assay was developed to quantify GFP or GFP-CWT binding to bacterial surfaces. (A) *S. aureus* Newman cells were washed and adjusted to an OD_{600} of 10, and twofold dilutions of staphylococci were mixed with GFP or GFP-CWT. Bacteria were sedimented by centrifugation and fluorescence of supernatant measured. (B) GFP-CWT binding to *S. aureus* Newman or *Enterococcus faecalis* strain FA2-2. (C) GFP-CWT binding to *S. aureus* Newman without (mock) or with the addition of a 2.5-fold, 5-fold, or 10-fold excess of purified GST-CWT protein or a 10-fold excess of GST.

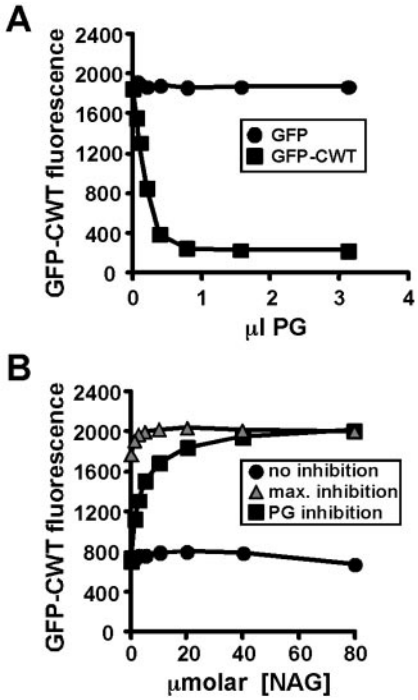

FIG. 3. GFP-CWT binding to purified peptidoglycan. (A) Highly purified peptidoglycan (PG) sacculi (carbohydrate, lipid, protein, and teichoic acid removed) from *S. aureus* Newman were adjusted to an OD_{600} of 10, and twofold dilutions of sacculi were mixed with GFP or GFP-CWT. Peptidoglycan sacculi were sedimented by centrifugation and supernatants analyzed for fluorescence. (B) Purified peptidoglycan was cleaved with mutanolysin and concentration of soluble amino sugars (NAG) determined with the Morgan-Elson reaction. *S. aureus* cells were mixed with solubilized peptidoglycan (or twofold serial dilutions) and GFP-CWT. Inhibition of GFP-CWT binding with solubilized peptidoglycan (PG) and mock inhibition, as well as maximal inhibition (GFP-CWT incubated with solubilized peptidoglycan in the absence of sedimentable bacteria), were analyzed.

between glutathione *S*-transferase and the CWT of lysostaphin. Purified GST-CWT binds to the surface of *S. aureus* cells in a manner that resembled binding of GFP-CWT (2). We wondered whether incubation of staphylococci with GST-CWT could occupy staphylococcal receptor sites for GFP-CWT, a phenomenon that would be expected for receptor-ligand interactions. Binding of GFP-CWT to the surface of *S. aureus* cells could indeed be inhibited by the addition of 2.5-fold, 5-fold, or 10-fold molar excess of purified GST-CWT in a dose-dependent manner (Fig. 2C). As a control, a 10-fold molar excess of GST without the CWT did not compete with the binding of GFP-CWT to the surface of *S. aureus* (Fig. 2C). Thus, GFP-CWT fluorescence and bacterial cosedimentation assays can be exploited to measure binding of the lysostaphin CWT to its receptor in the cell wall envelope.

GFP-CWT binds to purified peptidoglycan. To test whether peptidoglycan sacculi retained the ability of binding GFP-CWT, we purified sacculi from *S. aureus* strain Newman following established procedures (13). GFP-CWT bound to purified peptidoglycan as indicated by the rapid decrease in fluorescence of supernatants obtained after sedimentation of peptidoglycan sacculi (Fig. 3A). GFP-CWT cosedimentation was mediated by CWT binding to peptidoglycan, as GFP alone displayed no affinity for cell wall sacculi (Fig. 3A). Glycan strands within purified peptidoglycan were cleaved with mutanolysin, a muramidase that hydrolyzes the β1–4 bond in MurNAc-GlcNAc. The products of this reaction, amino sugar [NAG]-reducing equivalents, were measured with the modified Morgan-Elson reaction. To test whether muramidase-solubilized peptidoglycan occupies receptor sites on CWT, GFP-CWT binding to the surface of *S. aureus* was examined in the presence or absence of increasing amounts of peptidoglycan fragments. The addition of cell wall fragments blocked cosedimentation of GFP-CWT with intact staphylococci (Fig. 3B), indicating that muramidase-solubilized peptidoglycan fragments indeed occupy receptor sites on lysostaphin CWT.

Cross-linked peptidoglycan, but not murein monomers, inhibits GFP-CWT binding to staphylococci. To define the molecular properties of cell wall fragments capable of blocking GFP-CWT binding to staphylococci, mutanolysin-cleaved peptidoglycan fragments were separated by HPLC, and concentrations were measured as absorbances at 206 nm (A_{206}) (13) (Fig. 4A). Five to ten adjacent eluate fractions were pooled,

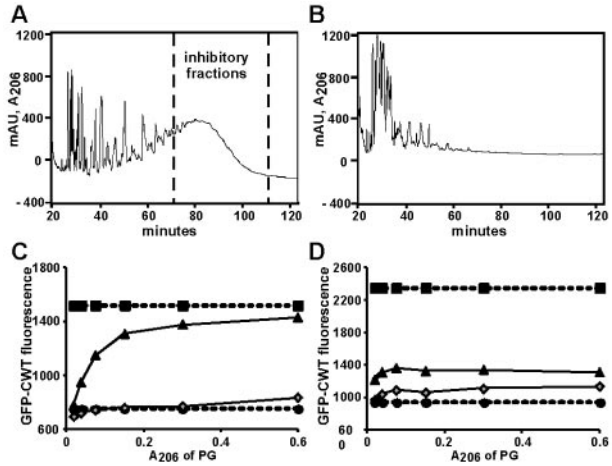

FIG. 4. Peptidoglycan fragments inhibit GFP-CWT binding to *S. aureus*. (A) HPLC chromatogram of mutanolysin-digested *S. aureus* peptidoglycan. AU, absorbance units. (B) HPLC chromatogram of mutanolysin/lysostaphin-digested *S. aureus* peptidoglycan. AU, absorbance units. (C) *S. aureus* cells were mixed with soluble, reduced peptidoglycan fragments (mutanolysin digested) at an A_{206} of 3 (or twofold serial dilutions thereof) and GFP-CWT. Following sedimentation of bacteria, GFP-CWT fluorescence was measured in supernatants. Low-molecular-weight peptidoglycan fragments (murein monomers and dimers in fractions 56 to 60; gray diamonds) did not inhibit binding of GFP-CWT to *S. aureus*. High-molecular-weight peptidoglycan (cross-linked fragments in fractions 91 through 95; black triangles) displayed strong inhibitory activity. As controls, no-inhibition-fluorescence values (filled circles) were determined by incubating bacteria and GFP-CWT in the absence of solubilized peptidoglycan fragments. Maximal-inhibition values (black squares) were determined by incubating GFP-CWT in the absence of bacteria and HPLC fractions. (D) *S. aureus* cells were mixed with soluble, reduced peptidoglycan fragments (mutanolysin/lysostaphin digested) at an A_{206} of 3 (or twofold serial dilutions thereof) and GFP-CWT. No inhibitory activity was found as shown for fractions 29 and 30 (gray diamonds) or 51 through 60 (black triangles). Filled circles and filled squares represent no-inhibition and maximal-inhibition curves, respectively.

taken to dryness, and adjusted to an A_{206} of 3 (see Table S2 in the supplemental material) and inhibition of GFP-CWT binding to *S. aureus* was examined by fluorescence assay. Fractions containing small peptidoglycan fragments (compounds eluted in fractions 21 to 70 of the HPLC chromatograms in Fig. 4A and C) did not interfere with the binding of GFP-CWT to *S. aureus*. A representative binding curve is shown for pooled fractions 56 to 60 in Fig. 4C. GFP-CWT inhibitory binding activity was found for fractions 71 to 80 and higher, which contain cross-linked peptidoglycan fragments with multiple murein subunits (13). Maximal inhibitory activity was observed in fractions at 91 to 95 min (Fig. 4C).

Mutanolysin-solubilized peptidoglycan was cleaved further with the glycyl-glycine endopeptidase lysostaphin, thereby separating all cross bridges and generating small, un-cross-linked murein cell wall fragments (Fig. 4B). HPLC fractions of mutanolysin/lysostaphin-released murein fragments were pooled and taken to dryness, and the concentrations were adjusted to an A_{206} of 3 (see Table S2 in the supplemental material). In contrast to mutanolysin-released cell wall, mutanolysin/lysostaphin-cleaved murein fragments were unable to inhibit GFP-CWT binding to *S. aureus* cells (Fig. 4D). Thus, cross-linked peptidoglycan, harboring murein disaccharides, wall peptides, and cross bridges, appears to serve as a receptor for CWT. Chemical or biochemical removal of protein, carbohydrate, lipid, and teichoic acid involves established procedures; however, the possibility of contamination of peptidoglycan fragments in our experiments is difficult to rule out. We therefore sought to test our hypothesis with molecular genetic experiments.

Binding of GFP-CWT to *S. aureus* mutants with defects in cell wall envelope assembly. To investigate the requirement of specific cell envelope structures for lysostaphin targeting, GFP-CWT binding was examined with *S. aureus* mutants defective in the synthesis of specific cell wall structures. Previous work established that *capF* and *capO* are required for capsular biosynthesis (31, 54), whereas *icaA* and *icaC* are involved in the synthesis of poly-*N*-acetylglucosamine exopolysaccharide (11, 24). The *oatA* gene product functions as an acetyltransferase in the cell wall envelope and is essential for all MurNAc O-acetylation (5). PBP4 is a low-molecular-weight penicillin binding protein with carboxypeptidase and transpeptidase activity involved in peptidoglycan cross-linking (26, 35, 72). Sortase A anchors surface proteins to pentaglycine cross bridges in the cell wall envelope, and *srtA* mutants are defective in the anchoring of all surface proteins with LPXTG motif type sorting signals (43). Lgt transfers diacylglycerol to cysteine side chain sulfhydryls of lipoprotein precursors, and *lgt* mutants are defective in the biosynthesis of all lipoproteins (62). *S. aureus* mutants with bursa aurealis transposon insertions (3) in the aforementioned genes were subjected to binding assays with GFP-CWT. The results showed that the physiological functions of *capFO* (Fig. 5A), *icaAC* (Fig. 5B), *oatA*, and *pbp4* (Fig. 5C), *srtA* (Fig. 5D), and *lgt* (Fig. 5E) are dispensable for lysostaphin CWT binding to staphylococcal cell surfaces.

D-Alanine modifications of secondary wall polymers impact GFP-CWT binding to staphylococci. Esterification of polyglycerol-phosphate LTA or polyribitol-phosphate WTA with D-Ala occurs on the extracellular surface of the staphylococcal membrane, i.e., after teichoic acids have been translocated across the membrane (47). Four gene products (DltABCD) are required for this process, during which D-Ala is first linked to a carrier protein and then translocated across the cytoplasmic membrane (47, 52). Translocated D-Ala residues are transferred to membrane-linked LTA and subsequently to WTA (47). Mutations in *dltB* abrogate this process (51, 52), and mutants with such defect displayed increased binding to GFP-CWT (Fig. 5E). This observation suggested that teichoic acids may actually interfere with bacteriocin binding, which seemed at first a surprising result. Cell wall binding proteins of other bacteria that display a similar domain structure as lysostaphin are known to bind lipoteichoic acids, and these include *Streptococcus pneumoniae* autolysin (LytA) (57) and *Listeria monocytogenes* internalin B (InlB) (27). One would expect that mutations which interfere with the synthesis of teichoic acids display reduced binding to various murein hydrolases and cell wall binding proteins, such as lysostaphin.

Lipoteichoic acid biosynthesis and GFP-CWT binding to staphylococci. We sought to analyze the role of LTA in the binding of lysostaphin to the cell wall envelope of *S. aureus* in greater detail. Biosynthetic components for LTA synthesis are thought to be located in the outer leaflet of the cytoplasmic membrane, the presumed site of lipoteichoic acid synthesis. A

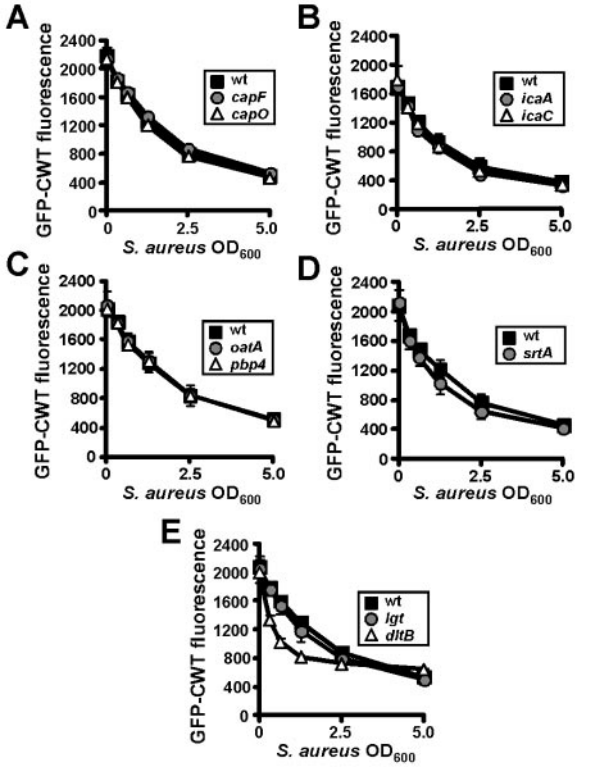

FIG. 5. GFP-CWT binding to *S. aureus* mutants with altered cell wall envelope properties. Wild-type (wt) *S. aureus* Newman and Phoenix library transposon mutants with insertions in defined genes were subjected to GFP-CWT binding assays. (A) Capsular polysaccharide mutants (Newman wt, ΦHE5448 [locus tag SAV0154, *capF*], and ΦHE5283 [locus tag SAV0163, *capO*]). (B) Poly-*N*-acetylglucosamine mutants (Newman wt, ΦHE4722 [locus tag SAV2666, *icaA*], and ΦHE14557 [locus tag SAV2669, *icaC*]). (C) Peptidoglycan mutants (Newman wt, ΦHE8500 [locus tag SAV2567, *oatA*], and ΦHE11552 [locus tag SAV0642, *pbp4*]). (D) Sortase mutants (Newman wt and ΦHE3486 [locus tag SAV2528, *srtA*]). (E) Lipoprotein and D-alanine modification of secondary wall polymer mutants (Newman wt, ΦHE106 [locus tag SAV0761, *lgt*], and ΦHE12076 [locus tag SAV0933, *dltB*]).

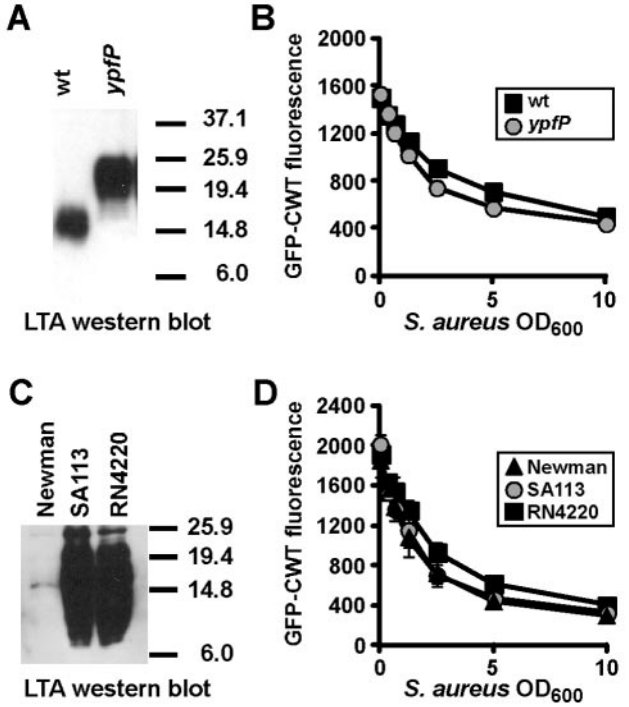

FIG. 6. GFP-CWT binding to *S. aureus* mutants with altered LTA properties. (A and C) Detection of LTA by immunoblotting. *S. aureus* cells were disintegrated with glass beads and bacterial lysates boiled in SDS buffer to solubilize LTA. Samples were separated by SDS-PAGE and LTA detected by immunoblotting with a polyglycerol phosphate-specific LTA antibody. Bars and numbers at the right of the panel indicate the positions and sizes of protein standards in kDa. wt, wild type. (B and D) GFP-CWT binding assays and measurement of fluorescence in supernatants. (B) GFP-CWT binding to isogenic wild-type (wt) and *ypfP* mutant *S. aureus* strains. (D) GFP-CWT binding to *S. aureus* Newman, SA113, and RN4220.

glycolipid anchor, Glc-(β1–6)-Glc-(β1–3)-diacylglycerol is extended by the transfer of glycerolphosphate units from phosphatidylglycerol with the formation of elongated LTA and diacylglycerol (16, 32). *S. aureus* mutants completely lacking lipoteichoic acid have not been described and the genes required for glycerolphosphate polymerization are not yet known. Nevertheless, YpfP, a diglucosyldiacylglycerol synthase, is required for synthesis of the LTA membrane anchor and *ypfP* mutant staphylococci synthesize LTA that is anchored by diacylglycerol (29). We prepared crude membrane extract from a wild-type and *ypfP* mutant strains and analyzed LTA production by immunoblotting. In agreement with previously published results (29), we observed that LTA synthesis is not abolished in the *ypfP* mutant strain (Fig. 6A). Altered electrophoretic mobility of the immunoreactive material is consistent with the possibility that LTA diacylglycerol assumes a unique mobility on SDS-PAGE or that the altered anchor structure imposes compositional differences in assembled teichoic acid. Importantly, no significant difference in the binding of the GFP-CWT fusion protein to a *ypfP* mutant strain was observed (Fig. 6B), indicating that the glycolipid anchor of LTA is not required for lysostaphin binding.

Different *S. aureus* isolates produce different amounts of LTA, as *S. aureus* strains SA113 and RN4220 contain significantly larger amounts of LTA than strain Newman (Fig. 6C). Even though these three strains contain different amounts of LTA, all three *S. aureus* strains bound similar amounts of GFP-CWT (Fig. 6D). Thus, it seems highly unlikely that GFP-CWT binding to the cell wall envelope of *S. aureus* involves LTA.

Wall teichoic acid affects binding of GFP-CWT to staphylococci. *S. aureus* mutants lacking wall teichoic acid can be constructed by inactivation of the *tagO* gene (71). Absence of wall teichoic acid in a Δ*tagO* variant of *S. aureus* strain Newman was confirmed by subjecting teichoic acid extracts to gel electrophoresis and alcian blue silver staining (data not shown). Binding of GFP-CWT to the surface of the Δ*tagO* variant was strongly increased (Fig. 7), and the reporter seemed to bind more uniformly to the surface of the Δ*tagO* mutant than to wild-type cells. Other *tagO* mutant *S. aureus* strains, including the previously described SA113 Δ*tagO* variant (71) and a RN4220 Δ*tagO* variant, displayed a similar increase in GFP-CWT binding. Thus, increased binding of GFP-CWT in the absence of wall teichoic acid is not restricted to specific strains

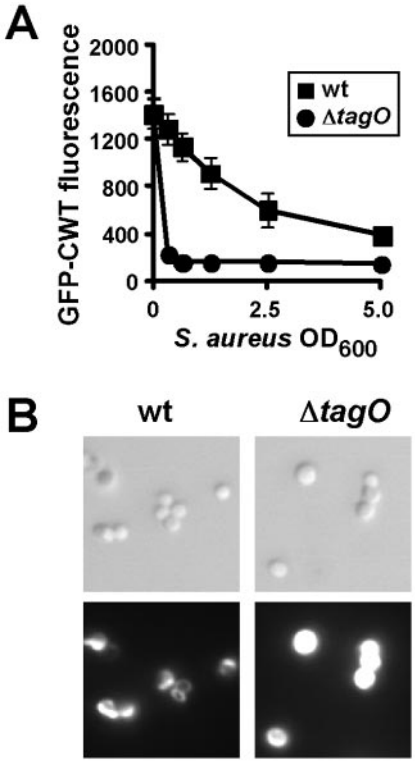

FIG. 7. GFP-CWT binding to *S. aureus* mutants with altered WTA properties. (A) GFP-CWT binding assays to the surface of *S. aureus* Newman wild-type (wt) and *tagO* mutant strains was measured. (B) GFP-CWT binding to the surface of *S. aureus* Newman wt and *tagO* mutant strains was viewed by fluorescence microscopy. Increased GFP-CWT binding to the surface of an *S. aureus* strains lacking WTA is reflected in a steeper binding curve (A) as well as brighter fluorescence of images captured with identical exposure times (B).

and is consistent with the phenotype of *dltB* mutants, which cannot catalyze esterification of teichoic acids.

Truncation of pentaglycine cross bridges in the cell wall of *femAB* mutants interferes with GFP-CWT binding. *S. aureus* mutants with altered peptidoglycan structure were examined for GFP-CWT binding. Pentaglycine cross bridges occur in the cell wall envelope of staphylococci but not in other bacterial species. Three catalysts, FemA, FemB, and FemX (*f*actors *e*ssential for *m*ethicillin resistance), are required for synthesis of the pentaglycine cross bridge. Charged glycine tRNAs and cell wall peptides are substrates for FemX synthesis of monoglycine [D-Ala-D-iGln-L-Lys(NH_2-Gly)-D-Ala-D-Ala], FemA synthesis of di- and triglycine [D-Ala-D-iGln-L-Lys(NH_2-Gly_{2-3})-D-Ala-D-Ala] and FemB synthesis of tetra- and pentaglycine cross bridges [D-Ala-D-iGln-L-Lys(NH_2-Gly_{4-5})-D-Ala-D-Ala] (15, 41, 56, 59). The *femAB* genes are deleted in *S. aureus* strain AS145, resulting in replacement of the pentaglycine cross bridges with monoglycine bridges and drastically reduced cross-linking between peptidoglycan strands (63). This strain is highly lysostaphin resistant, due to the lack of pentaglycine substrate for the glycyl-glycine endopeptidase lysostaphin (63). Here we examined the *femAB* mutant strain for its ability to bind the GFP-CWT fusion protein. A dramatic decrease in binding of GFP-CWT to the cell wall envelope of *femAB* mutant staphylococci was observed compared with the isogenic wild-type strain BB270 (Fig. 8). Previous work showed that introduction of plasmid pBBB64, containing an intact copy of the *femAB* operon, restored the lysostaphin sensitivity of strain AS145 (63). Similarly, GFP-CWT binding was restored by transforming strain AS145 with the complementing plasmid pBBB64 (Fig. 8). Thus, peptidoglycan structures that are unique to staphylococci, such as the pentaglycine cross bridge (with its involvement in peptidoglycan cross-linking), play an essential role in the binding of lysostaphin to its peptidoglycan receptor within the cell wall envelope of *S. aureus*.

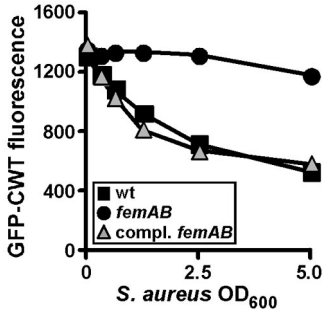

FIG. 8. GFP-CWT binding to a *S. aureus* mutant with altered peptidoglycan cross bridge structure. GFP-CWT binding to the surface of *S. aureus* BB270 wild type (wt) and isogenic *femAB* mutant strain AS145 without or with complementing (compl.) plasmid pBBB64 was measured.

DISCUSSION

Commensals of the human skin engage in microbial warfare to ensure their growth and survival among bacterial competitors. In order to compete with commensal *S. aureus*, *S. simulans* cells secrete lysostaphin, an endopeptidase that cleaves pentaglycine cross bridges in the cell wall peptidoglycan of all staphylococcal species (12, 55). Because gram-positive bacteria secrete polypeptides into the extracellular milieu, secreted bacteriocins or murein hydrolases are confronted with two obstacles that must be properly resolved for function. First, host cells that synthesize and secrete bacteriocins should not be killed by these molecules, and second, secreted murein hydrolases must be specifically targeted to *S. aureus* cells to avoid unproductive interactions with other bacterial species (only staphylococci harbor pentaglycine cross bridges).

S. simulans has solved the first problem by expressing immunity genes, *epr* (endopeptidase resistance) and $tRNA^{Ser}$ (12). Epr is homologous to Fem factors and, when presented with charged seryl-tRNA, catalyzes the incorporation of serine into cross bridges of peptidoglycan precursors (15). Products of the Epr reaction are then assembled within the cell wall envelope and peptidoglycan with glycine/serine cross bridges displays intrinsic resistance to lysostaphin cleavage (12, 15).

The second obstacle is solved by appending targeting domains to bacteriocins. For example, bioinformatic analysis of gram-positive murein hydrolases can distinguish between catalytically active domains, which are shared between many enzymes, even in gram-negative microbes, and targeting domains that are typically only found in few molecules and often clustered within a single bacterial species. Removal of the target-

ing domain generally does not interfere with murein hydrolase activity, a prediction that has been confirmed for lysostaphin (2). What are the receptors for the targeting domains of murein hydrolases in the cell wall of gram-positive bacteria? Seminal work on *S. pneumoniae* autolysin (LytA) demonstrated that choline binding domains of LytA promote binding to choline-containing teichoic acids (57). Choline binding domains can be found in several other proteins of *S. pneumoniae* (67). *L. monocytogenes* InlB, a surface protein that mediates bacterial invasion into epithelial cells, is tethered to the cell wall envelope via a C-terminal targeting domain that binds to LTA (7, 27). Finally, the C-terminal targeting domains of bacteriophage murein hydrolases Ply500 and Ply118 bind to carbohydrate polymers on the surface of *Listeria monocytogenes* (36). On the basis of these observations, murein hydrolases have been thought to bind cell wall-associated polymers that display chemically discrete receptor properties within the bacterial envelope. If so, biochemical and genetic studies aimed at identifying the receptors of murein hydrolases should uncover molecular interactions that govern the targeting of bacteriocins and of enzymes that perform physiological degradation of cell wall envelopes.

Lysostaphin CWT has been classified as an SH3b domain, a prokaryotic homologue of the eukaryotic src homology 3 (SH3) domain (40, 53). SH3 domains in eukaryotic proteins are involved in signal transduction and bind proline-rich protein sequences (22, 28, 53). Prokaryotic SH3b domains share only limited sequence identity with their eukaryotic counterpart, albeit that they display very similar secondary structures. The three-dimensional structure of two bacterial SH3b domains have been determined (40, 42). *Listeria monocytogenes* internalin B requires a C-terminal SH3b targeting domain for attachment to the bacterial LTA (27). LTA is tethered to a lipid anchor and inserted in bacterial membranes. In agreement with this model, internalin B fractionates with bacterial membranes but not with murein sacculi (27). In contrast, lysostaphin fractionates with cell wall sacculi (2).

While our studies were in progress, the structure of the CWT of ALE-1 has been solved (40). Closely related to lysostaphin, ALE-1 also functions as a glycyl-glycine endopeptidase that is secreted by *Staphylococcus capitis* EPK1 (66). Further, the CWT of ALE-1, which is highly similar to the lysostaphin CWT, was shown to bind purified peptidoglycan in a manner that required structural integrity of pentaglycine cross bridges (40). Because the CWT domains of lysostaphin and ALE-1 share a high degree of homology, it seems safe to assume that both enzymes likely bind to the same receptor in the cell wall envelope of *S. aureus*. In agreement with this study, we show here that lysostaphin CWT binds to purified peptidoglycan and that intact pentaglycine cross bridges are essential for binding. However, binding of GFP-CWT to the *S. aureus* cell wall was not inhibited by the addition of excess amounts of pentaglycine (data not shown). Further, muramidase-solubilized, HPLC-purified murein monomer or dimer (the latter harboring pentaglycine cross bridges) did not inhibit binding of the GFP-CWT reporter to cell wall sacculi; such inhibition could only be achieved with highly cross-linked peptidoglycan species. Together, these results suggest that CWT binding to peptidoglycan requires not only pentaglycine cross bridges but also other structural features of cross-linked peptidoglycan.

The LysM domain of the major autolysin AcmA of *Lactococcus lactis* functions as a targeting signal for this murein hydrolase (61). The AcmA LysM domain binds to peptidoglycan, and consistent with results reported here, this interaction can be inhibited by lactococcal LTA (61). Our results suggest that WTAs interfere with lysostaphin CWT binding, though *ypfP* deletion leading to alterations in the membrane anchor of staphylococcal LTA did not perturb CWT binding. Targeting of lysostaphin or other murein hydrolases with homologous CWT domains must be viewed as interactions of polypeptides with the three-dimensional peptidoglycan scaffold, comprised of glycan strands, wall peptides, and cross bridges. Such interaction is likely complex and can therefore be perturbed by secondary polymers (such as teichoic acids, carbohydrate, or even protein) that enter into the scaffold. The identification of cross-linked peptidoglycan as CWT receptor offers some interesting views on cell wall assembly. For example, fusion of the CWT domain upstream of LPXTG motif type sorting signals abolishes the physiological function of the latter, namely, cleavage of surface protein by sortase A and amide bond formation with the pentaglycine cross bridge of lipid II precursor (2). In view of the findings presented here, we think it is likely that the CWT prematurely sequesters polypeptides in the *S. aureus* cell wall envelope, which may then be inaccessible for sortase A. In keeping with this view, *S. aureus* expressing lysostaphin or reporter proteins with appended CWT direct these molecules to the cell wall envelope but not into the extracellular medium. In contrast, *S. simulans* secretes lysostaphin into the extracellular medium without deposition in the cell wall. Incorporation of serine residues into cross bridges of *S. simulans* cells expressing immunity factors may be responsible for this phenomenon; however, other cell wall envelope features, for example, teichoic acids, may also contribute to CWT discrimination of *S. aureus* peptidoglycan receptor and *S. simulans* host peptidoglycan.

ACKNOWLEDGMENTS

We thank Dominique Missiakas for *S. aureus* mutants, Brigitte Berger-Bächi for the *femAB* mutant strain and plasmid pBBB64, Ted Bae and Chris Cain for help with *tagO* plasmid construction, Mohammed Yousef for assistance with fluorescence plate reader assays, Luciano Marraffini and Christiaan van Ooij for critical reading of the manuscript and members of our laboratory for helpful discussions.

This work was supported in part by a grant from Biosynexus Inc. The study of lysostaphin provides important insight into assembly of surface proteins into the staphylococcal cell wall, work which is supported by United States Public Health Service Grants AI38897 and AI52474 from the National Institute of Allergy and Infectious Diseases, Division of Microbiology and Infectious Diseases to O.S.

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