The fnta gene is a member of the Staphylococcus aureus core cell wall stimulon. TheFmtA protein interacts with β-lactams through formation of covalent species. Here, we show thatFmtA has weak d-Ala-d-Ala-carboxypeptidase activity and is capable of covalently incorporating C14-Gly into cell walls. The fluorescence microscopy study showed that the protein is localized to the cell division septum. Furthermore, we show that wteichoic acids interact specifically withFmtA and mediate recruitment ofFmtA to the S. aureus cell wall. Subjection ofS. aureus toFmtA concentrations of 0.1 μM or less induces autolysis and biofilm production. This effect requires the presence of wteichoic acids. AtFmtA concentrations greater than 0.2 μM, autolysis and biofilm formation inS. aureus are repressed and growth is enhanced. Our findings indicate dual roles ofFmtA inS. aureus growth, whereby at low concentrations,FmtA may modulate the activity of the major autolysin (AtlA) ofS. aureus and, at high concentrations, may participate in synthesis of cell wall peptidoglycan. These two roles ofFmtA may reflect dual functions ofFmtA in the absence and presence of cell wall stress, respectively.

T he fnta gene was identified as a methicillin resistance factor inStaphylococcus aureus by Komatsuzawa et al. (23). Inactivation of fmtA leads to increased sensitivity of methicillin-resistantS. aureus strains (MRSA) to Triton X-100 and β-lactams and decreases the level of highly cross-linked peptidoglycan (PG) (22, 23). Cell wall inhibitors such as oxacillin, methicillin, cefoxitin, phosfomycin, and bacitracin cause upregulation of the trascripton levels offmtA (22).

Several studies on the genome-wideS. aureus response to cell wall inhibitors have demonstrated that their biological activity causes upregulation offmtA (3, 26, 27, 41, 46). In addition, inactivation of genes involved with cell wall biosynthesis, such asmurF, leads to higher levels offmtA (41). Hence, fmtA is considered part of the core cell wall stimulon (26).

The function offmtA is not known. The fmtA gene has been linked to biofilm formation and autolysis, two interconnected processes in bacteria whereby the ability ofS. aureus to produce biofilms is linked to its ability to undergo a controlled autolysis process (2, 25). Inactivation offmtA diminishes the ability ofS. aureus to form biofilms (4, 44) and enhances autolysis (4, 23). Boles et al. showed the cell wall of theS. aureus fmtA mutant to lack wteichoic acids (WTAs) (4). Wteichoic acids are phosphate-rich glycopolymers referred to as WTAs (Fig. 1) when connected to the peptidoglycan (PG) or as lipoteichoic acids (LTAs) when connected to the cytoplasmic membrane (51, 54). They make up as much as 60% of the total cell mass in Gram-positive bacteria and have been implicated in a variety of processes, including biofilm production and autolysis (13, 21, 33, 39, 49). Recent reports have implicated WTAs in spatial and temporal regulation of theS. aureus major autolysin (AtlA) and penicillin-binding protein 4 (PBP4) (1, 40).

The primary structure ofFmtA harbors two of the three conserved motifs ofPBPs, SXXK andS(Y)XN (where S indicates any amino acid) while lacking the KTG motif. The protein forms covalent species with β-lactam compounds; however, this interaction is weak, suggesting thatFmtA is intrinsically resistant to β-lactam inactivation (10). The other four native PBPs ofS. aureus are sensitive to β-lactams. The methicillin-resistantS. aureus strains have acquired an additional PBP, PBP2a, which also interacts poorly with β-lactams (36).

S. aureus remains a major concern for public health due to resistance to a wide range of antibiotics. Understanding how it copes with cell wall stress would provide insights toward new treatment strategies. Further, genome-wide screening studies have shown that inhibition of nonessential genes could be a successful strategy in sensitizingS. aureus to antibiotics that otherwise have failed in clinical settings due to resistance (5, 20, 28, 43).

Here we investigate the relationship offmtA to cell wall biosynthesis. Our new findings shed light on the role offmtA inS. aureus methicillin resistance, autolysis, and biofilm formation.

MATERIALS AND METHODS

Materials and chemical reagents. Growth media were purchased from EMDBioscience. Enzymes and chemicals were purchased from Sigma and New England BioLabs. Lipoteichoic acids were purchased from InvivoGen. Ampex Red (AR) was purchased from Molecular Probes, Inc. 14C-Gly was purchased from Perkin Elmer. Monoclonal anti-FmtA serum was purchased from GeneScript Corporation. Sterile 96-well tissue culture plates were purchased from Corning Incorporated. Safranin was purchased from bioMérieux Canada.

Construction ofFmtA S127A mutant. FmtA S127 refers to the mature protein sequence. The signal peptide sequence (27 amino acids at the N terminus) was not included in the cloning process offmtA (10). This facilitated the isolation of the protein from the cytoplasm. The mature protein lacks any cysteine residues in the primary structure. Hence, isolation of this otherwise periplamomic protein from the cytoplasm should not have any effect on the folding of the protein (10).

Mutation of Ser127 to Ala was carried out using a QuikChange site-
directed mutagenesis kit (Stratagene). The pET24a (+):fmtA<sup>27</sup> vector (10) was amplified using *Pfu* Turbo DNA polymerase and a pair of mutagenic primers, DirS127A (5′-CGATGTITTTTAAGGCTGCTCAA AATTTTC-3′) and RevS127A (5′-GAAAATTTTGGACCTGACCTAT TAAAACATCG-3′) (the mutated nucleotides are italicized). The PCR product was digested with the DpnI restriction endonuclease and used to transform *Escherichia coli* BL21(DE3). The nucleotide sequence of the mutant variants was verified by DNA sequencing. The purifications of FmtA<sup>27</sup> and the S12A mutant were carried as described by Fan et al. (10).

**Transpeptidase activity assays.** Transpeptidase activity of FmtA<sup>27</sup> was investigated using radioactive <sup>14</sup>C-labeled glycine (<sup>14</sup>C-Gly) as the acceptor. Cross-linking experiments were carried out using cell wall isolated from *S. aureus* RN2420. A typical 100-μl reaction mixture consisted of 50 μM of cell wall, FmtA<sup>27</sup> at different concentrations (0 to 30 μM), and 1 mM <sup>14</sup>C-Gly, all in 0.1 M Tris (pH 8.5) buffer. In a control reaction, albumin (15 μM) or ovalbumin (15 μM) was incubated with cell wall (50 μg) and <sup>14</sup>C-Gly (1 mM).

Reaction mixtures were incubated for 2 h at 37°C. Following incubation, samples were spun down at 25,000 × *g* for 30 min at 4°C. Pellets containing cell walls were washed twice with 0.1 M Tris (pH 8.5) buffer to remove any nonspecifically bound <sup>14</sup>C-Gly. The incorporated radioactivity was recorded by a Tri-carb liquid scintillation analyzer (Packard). The same experiments were carried out with FmtA<sup>27</sup>S127A at 30 μM.

**Preparation of S. aureus cell walls with and without wall teichoic acid.** Cell walls from *S. aureus* RN2420 were isolated as previously reported (7). Removal of WTAs from cell walls was carried out using DNA sequencing. The purifications of FmtA<sup>27</sup> and the S12A mutant were carried as described by Fan et al. (10). A typical 100-μl reaction mixture consisted of 50 μM of cell wall, FmtA<sup>27</sup> at different concentrations (0 to 30 μM), and 1 mM <sup>14</sup>C-Gly, all in 0.1 M Tris (pH 8.5) buffer. In a control reaction, albumin (15 μM) or ovalbumin (15 μM) was incubated with cell wall (50 μg) and <sup>14</sup>C-Gly (1 mM).

Reaction mixtures were incubated for 2 h at 37°C. Following incubation, samples were spun down at 25,000 × *g* for 30 min at 4°C. Pellets containing cell walls were washed twice with 0.1 M Tris (pH 8.5) buffer to remove any nonspecifically bound <sup>14</sup>C-Gly. The incorporated radioactivity was recorded by a Tri-carb liquid scintillation analyzer (Packard). The same experiments were carried out with FmtA<sup>27</sup>S127A at 30 μM.

**Investigation of FmtA<sup>27</sup> hydrolase activity.** FmtA<sup>27</sup> (2.5 μM) was incubated with PG (1 mg/ml) in 50 mM sodium phosphate (pH 7.0) buffer (1 ml) at 37°C for different times (0.5, 1, 2, 3, 4, and 5 h). The change in absorbance was monitored at 450 nm. Mutanolysin from *Streptomyces globisporus* was used as a positive control.

In another experiment, PG (1 mg/ml)–50 mM phosphate (pH 7.0) buffer was incubated with 10 μM FmtA<sup>27</sup> in a 20-μl reaction mixture. In control experiments, PG was digested with mutanolysin and undigested PG. The reaction mixture was loaded into a C<sub>18</sub> reverse-phase high-performance liquid chromatography (HPLC) column (Persuit; Varian) (5 μl; 250 by 4.6 mm). A linear gradient of 0% to 30% methanol in a 100 mM phosphate (pH 2.8) buffer was used to elute any peptidoglycan fragments.

**Investigation of FmtA<sup>27</sup> interaction with WTA by the use of acidic nPAGE/H<sup>+</sup>.** Interaction of FmtA<sup>27</sup> with WTAs was investigated with native polyacrylamide gel electrophoresis (nPAGE)/H<sup>+</sup>. A typical reaction mixture (15 μl) consisted of 10 μg of FmtA<sup>27</sup> (15 μM final concentration) and 5 μg of WTA in the presence of NaCl at different concentrations (100, 300, or 500 mM), all in 50 mM sodium phosphate (pH 7.0) buffers. Another reaction mixture containing 8 μg of PBP2a (15 μM final concentration) was also prepared using a method similar to that described above. Bovine serum albumin (BSA), ovalbumin, RNaseA, and carbonic anhydrase (10 μg each) were used as negative controls. The PAGE gels were stained with Coomassie blue.

**Investigation of FmtA<sup>27</sup> interaction with WTA by trypsin digestion.** FmtA<sup>27</sup> (40 μM) was incubated either with or without WTAs (1.25 μg in 12.5 mM sodium phosphate [pH 5.5] buffer and 0.2% sodium azide; 80-μl reaction volume) for 1 h at 37°C. The mixture was subjected to trypsin digestion based on a protocol from a ProteoExtract kit (Cal Biochem) (trypsin assay concentration = 0.2 μg/μl) and further incubated at 37°C. Aliquots of 10 μl were removed at defined time intervals, and the digestion was terminated with 10 μl of sodium dodecyl sulfate (SDS) loading dye, followed by heating at 60°C for 5 min. Samples were analyzed by 15% Tris-Tricine SDS-PAGE. Each experiment was repeated three times.

In the case of limited trypsin digestion, the samples were subjected to trypsin only for 15 min and the protein samples were analyzed by matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectroscopy (MS) at the Advanced Protein Technology Center, Hospital for Sick Children, Toronto, Canada.

**CD spectroscopy studies of FmtA<sup>27</sup> in the presence of WTAs.** Far-UV (200 to 260 nm) circular dichroism (CD) spectra of FmtA<sup>27</sup> (14 μM in 50 mM sodium phosphate [pH 7.0] buffer), in the presence of WTAs, were recorded at 22°C using a Jasco J-810 instrument and a cuvette with a 1.0-mm path length. The average of three scans was recorded and corrected for the signals from the buffer, PG fragment, and WTAs.

**Autolysis assays.** *S. aureus* RN4220 cells were grown in tryptic soy broth (TSB) overnight at 37°C. Cells were diluted 200-fold in fresh TSB medium and grown with shaking at 37°C to an optical density at 620 nm (OD<sub>620</sub>) of 0.8. Cells were harvested by centrifugation (6,300 × *g*, 10 min) and washed twice with ice-cold water. The pellet was resuspended in 1× phosphate-buffered saline (PBS) (pH 7.0) to an OD<sub>620</sub> of 1.0 and dispensed into a 96-well plate (200 μl per well). Cells were incubated at 37°C in the presence of various concentrations of FmtA<sup>27</sup>, with agitation at 200 rpm. Autolysis was measured as a decrease in OD<sub>620</sub> over time. In the case of the *S. aureus* ΔtarO mutant and its parental strain (EBII16), Mueller-Hinton broth (MHB) medium was used and FmtA<sup>27</sup> was kept at 0.2 μM.

**Biofilm assay.** Cultures of *S. aureus* RN4220 in TSB, or of the ΔtarO mutant and EBII16 strain in MHB, were grown overnight and normalized by optical density. The cell cultures were next diluted 50-fold into fresh TSB supplemented with 0.2% glucose. Wells of a sterile, 96-well flat-bottomed tissue culture plate (Corning Incorporated) were filled with 200-μl aliquots of the diluted culture. FmtA<sup>27</sup> was added to the assay at various final concentrations (0 to 6 μM). Cultures were incubated for 24 h at 37°C in the closed plate and assayed for biofilm formation according to the method of Christensen et al. (6). Adherent cells were fixed with Bouin fixative (Sigma) and stained with 0.1% Safranin (bioMérieux, Canada) for 10 min. Wells were then washed with running tap water. Images of the inverted plates were taken using a digital camera. Biofilms were dissolved using 30% glacial acetic acid for 15 min, and relative biofilm formation was also assayed by reading absorbance at 562 nm using a plate reader.
Construction of gfp-fmtA<sup>277</sup> fusion. The fmtA<sup>277</sup> ampiclon was ligated into a pDNA3.1/TNT-GFP-TOPO vector (Invitrogen). To facilitate subcloning of gfp-fmtA chimeras into the pET24a vector, a silent mutation was introduced by QuikChange site-directed mutagenesis (Stratagene) to remove the Ndel restriction site in the gfp gene. The following mutagenic primers were used: Dir (5'-GGCTTATCCGGATCATAGAAGCCGAC GAC-3') and Rev (5'-GTCATGCGGTTTCATGTGATCGGATAAGG-3'). Introduction of the mutation was confirmed by DNA sequencing analysis. Another set of primers was designed to amplify the gfp-fmtA<sup>277</sup> fusion gene for the final cloning to the pET24a vector: Dir (5'-AGGCTAT ATGCGCCGAAGGAGAAGACGAC-3') and Rev (5'-AGCGAAATTCTT ATTATGGACAATACACCTCG-3') (Ndel and EcoRI restriction sites are italicized). The successful cloning of the fusion gene into pET24a was also confirmed by DNA sequencing analysis.

The final construct, gfp-fmtA<sup>277</sup>-pET24a, was used to transform E. coli BL21 (DE3) cells. Expression and purification of green fluorescent protein-FmtA<sup>277</sup> (GFP-FmtA<sup>277</sup>) was carried out as described before (10). In addition, the activity of the chimeric FmtA<sup>277</sup> was investigated using fluorescent penicillin (Bocillin) and binding to cell walls isolated from S. aureus EBII16 and ΔtarO mutant, as previously described (10).

Investigation of GFP-FmtA<sup>277</sup> localization to S. aureus by fluorescence microscopy. S. aureus EBII16 and ΔtarO mutant strains were grown overnight in MHB growth media. Cells were diluted 200-fold in fresh MHB and grown to an OD<sub>600</sub> of ~0.6. Cells were harvested by centrifugation at 9,300 x g for 10 min. The cells were washed with 1X phosphate-buffered saline (PBS) and resuspended in 1X PBS buffer to give a final OD<sub>600</sub> of 1.2.

In a typical binding assay, 100 μl of 5 μM GFP-FmtA<sup>277</sup> protein solution was mixed with 400 μl of washed S. aureus cells to yield a final concentration of 1 μM GFP-FmtA<sup>277</sup>. Reaction mixtures were incubated for 10 min at room temperature. Staphylococcal cells with bound protein were sedimented by centrifugation at 16,000 x g for 3 min. The cells were washed with PBS and resuspended in PBS. A drop of bacterial suspension was placed on a polylysine-coated glass slide and immediately analyzed using epifluorescence microscopy performed with a laser-scanning confocal microscope (Fluoview FV300). Similar experiments were carried out at GFP-FmtA<sup>277</sup> concentrations of 2, 5, and 10 μM. Prior to analysis by microscopy, mixtures were incubated with 1 μM protein for 0.5, 1, and 2 h to measure binding time of GFP-FmtA<sup>277</sup> to S. aureus cells.

To assess the effect of oxacillin and vancomycin on the localization of FmtA, S. aureus EBII16 and ΔtarO mutant strains were exponentially grown in MHB and grown to an OD<sub>600</sub> of ~0.6. Cells were harvested by centrifugation and washed with PBS and resuspended in PBS. A drop of bacterial suspension was placed on a polylysine-coated glass slide and immediately analyzed using epifluorescence microscopy performed with a laser-scanning confocal microscope (Fluoview FV300).

In this study, we worked with the mature FmtA protein, FmtA<sup>277</sup>, which lacks the signal peptide sequence (10). FmtA<sup>277</sup> catalyzed the removal of the d-Ala from the diacetyl tripeptide substrate (a surrogate for the PG donor strand). The rate of d-Ala removal increased in the presence of Gly-Gly, which is a surrogate for the PG acceptor strand. Different ratios of donor to acceptor were investigated, and the optimum ratio was 1:10. Under these conditions, the catalytic efficiency of d-carboxypeptidase activity of FmtA was measured to be 0.745 x 10<sup>-6</sup> M<sup>-1</sup> s<sup>-1</sup>. The FmtA<sup>Δ717S127A</sup> mutant was completely devoid of this activity.

Potential transpeptidase activity of FmtA<sup>277</sup> was investigated by monitoring the catalytic incorporation of <sup>14</sup>C-Gly into S. aureus cell walls. Albumin and ovalbumin, proteins unrelated to PG biosynthesis, served as negative controls in these experiments (Fig. 2A). FmtA<sup>277</sup> covalently incorporated <sup>14</sup>C-Gly into the cell walls, but albumin and ovalbumin did not. The <sup>14</sup>C labeling of cell walls by FmtA<sup>277</sup> depended on the concentration of the protein (Fig. 2A). The level of <sup>14</sup>C-Gly incorporation into the cell walls by the FmtA<sup>277</sup>S127A mutant was the same as the background levels (in the absence of FmtA<sup>277</sup>) (Fig. 2B).

FmtA<sup>277</sup> interacts with S. aureus WTAs. FmtA<sup>277</sup> binds to cell walls (10). To identify the cell wall component essential for FmtA<sup>277</sup> recruitment, we investigated the binding of FmtA<sup>277</sup> to cell walls digested with either lysostaphin or mutanolysin. Lysostaphin cleaves the pentaglycine bridge in PG and decreases the degree of PG cross-linking (glycan strands are less cross-linked). Mutanolysin cleaves the glycan strands after N-acetylmuramic acid and produces different oligomers of muropeptides.

Digestion of cell walls with mutanolysin resulted in as much as an 80% decrease of FmtA<sup>277</sup> binding to cell walls (Fig. 3), whereas digestion of cell walls with lysostaphin resulted in a 40% decrease of FmtA<sup>277</sup> binding (Fig. 3). The persistence of FmtA<sup>277</sup> binding to cell walls digested by lysostaphin was investigated further. We looked at the components of cell wall present in our cell wall preparations and took note that WTAs were not removed from the cell wall preparations. WTAs were removed from the cell wall preparations by HF. The newly prepared cell walls are referred to as peptidoglycan (PG) here.

FmtA<sup>277</sup> failed to bind to PG (Fig. 4). This experiment was repeated with cell walls isolated from the ΔtarO mutant. The tarO gene is involved in the first step of WTA biosynthesis, and, as such, S. aureus tarO mutant cell walls lack WTAs (8). The in vitro bind-
ing assays with cell walls isolated from the ΔtarO mutant showed that these cell walls could not recruitFmtAΔ27; the protein remained predominately in the unbound fraction (Fig. 4).

The WTA-binding properties ofFmtAΔ27 were further studied using WTAs isolated from Staphylococcus aureus RN4220. Incubation ofFmtAΔ27 with WTAs resulted in a lack of electrophoretic resolution of the protein by the use of an nPAGE/H+ gel (Fig. 5). We concluded thatFmtAΔ27 could not enter the gel in the presence of WTAs; however, addition of NaCl to the reaction mixture facilitated resolution ofFmtAΔ27 by the nPAGE/H+ procedure. At 500 mM NaCl, the protein was resolved completely (Fig. 5A). The presence of salt alone had no effect on the electrophoretic resolution ofFmtAΔ27 by nPAGE/H+ (Fig. 5B). These results strongly indicate thatFmtAΔ27 interacts with WTAs. Similar experiments were carried out with PB2p2a, a penicillin-binding protein that has transpeptidase activity. PB2p2a displayed an affinity for the WTAs and behaved in the same way asFmtA (Fig. 5C). Proteins unrelated to cell wall metabolism, including RNase A, and carbonic anhydrase, did not show any binding to WTAs, while BSA showed a weak affinity for teichoic acids (Fig. 6).

WTAs induce a conformational change inFmtAΔ27 structure. The CD experiments showed thatFmtAΔ27 undergoes a dose-dependent conformational change in the presence of teichoic acids (Fig. 7). The conformational change is associated with a reduction inFmtAΔ27 α-helix content, as interpreted from a decrease in the CD signal at 222 nm. The reduction in α-helix content was calculated to be 6% in the presence of WTAs at 6.25 μg/ml, 14% in the presence of WTAs at 12.5 μg/ml, and 21% in the presence of WTAs at 25 μg/ml (Fig. 7).

The interpretation of the effect of WTAs onFmtAΔ27 secondary structure was supported by trypsin digestion experiments (Fig. 8). The digestion profiles ofFmtAΔ27 differed in the presence and absence of WTAs. Three major fragments were released upon incubation ofFmtAΔ27 with trypsin; however, when WTAs (6.25 μg/ml) were present, six fragments were produced (Fig. 8). These observations provide further evidence that WTAs interact withFmtAΔ27.

To identify the specific region in the protein involved with binding to WTAs,FmtAΔ27 was subjected to a 15-min trypsin digestion in the absence and presence of WTAs. Analysis of the released peptides by MALDI-TOF MS (see Fig. S1 in the supplemental material) showed a notable difference: a peptide with a mass of 1,369 Da was not detected when the digestion was carried out in the presence of WTA. Instead, a peptide with a mass of 853 Da exhibited more abundance. The peptide with a mass of 1,369 Da mapped to amino acids 312 to 323 in the C-terminal portion ofFmtAΔ27, and the peptide with a mass of 853 Da mapped to amino acids 155 to 163 ofFmtAΔ27.

FmtAΔ27 induces autolysis ofStaphylococcus aureus. Binding ofFmtAΔ27 to WTA suggests that it could affect processes regulated by WTA. One of the important processes controlled by WTA and LTA is autolysis. The activity ofAtlA, the major autolysin ofStaphylococcus aureus, has been shown to be controlled by teichoic acids (14, 40, 50, 51). We investigated the potential effect ofFmtAΔ27 on autolysis of Staphylococcus aureus RN4220 by incubating cells with different concentrations ofFmtAΔ27. We discovered thatFmtAΔ27 exogenously added toStaphylococcus aureus cells affected autolysis of cells in a dual mode. At concentrations of up to 0.1 μM,FmtAΔ27induced autolysis over 1 h, whereas at concentrations higher than 0.2 μM, an enhanced growth of bacteria was observed instead (Fig. 9A). A similar observation was also made for the ΔtarO mutant, which lacks WTAs (8) (Fig. 9B).

FmtAΔ27 induces biofilm formation inStaphylococcus aureus. Controlled autolysis is essential for formation of biofilms (15, 19); therefore,FmtAΔ27-induced autolysis suggests thatFmtAΔ27 might be involved in biofilm production inStaphylococcus aureus. Indeed, recent reports show that inactivation ofFmtA leads to cell biofilm formation deficiency (4, 44). Our study showed that biofilm production in the Staphylococcus aureus RN4220 strain increases with theFmtAΔ27 concentration up to 0.1 μM and that it decreases atFmtA concentrations above 0.2 μM (Fig. 10), much like the dual effect observed forFmtA-induced autolysis.

We investigated the WTA effect on the ability ofFmtAΔ27 to induce biofilm formation by comparing biofilm formation of the ΔtarO mutant with that of the wild-type strain in the presence or absence of 0.2 μMFmtAΔ27. These studies showed that, although
FmtA\textsuperscript{Δ27} induced maximum biofilm formation in the *S. aureus* wild-type strain, it was entirely unable to induce this process in the \(\Delta\text{tarO}\) mutant strain despite similar conditions (Fig. 10C).

FmtA\textsuperscript{Δ27} localizes in the division septum. To investigate localization of FmtA to *S. aureus*, we fused FmtA\textsuperscript{Δ27} to the C terminus of GFP. GFP-FmtA\textsuperscript{Δ27} expressed well in *E. coli* (25 mg/liter), and the protein was purified using the protocol for FmtA\textsuperscript{Δ27} purification (10). The GFP tag showed no effect on the interaction of FmtA with \(\beta\)-lactams or cell walls (see Fig. S2S in the supplemental material). We therefore concluded that GFP-fused FmtA\textsuperscript{Δ27} is indistinguishable from wild-type FmtA\textsuperscript{Δ27}.

Gründling and Schneewind reported that the GFP protein alone does not bind to *S. aureus* cells (16). Our fluorescence microscopy studies showed that GFP-FmtA\textsuperscript{Δ27} bound at the division septum in the dividing *S. aureus* RN4220 cells, where cell wall synthesis is reported to occur in *S. aureus* (33). In nondividing cells, GFP-FmtA\textsuperscript{Δ27} was localized as two dots corresponding to the ring of the future division plane (Fig. 11). A localization pattern similar to that of GFP-FmtA\textsuperscript{Δ27} has been reported for PBP4 of *S. aureus* (1). In the \(\Delta\text{tarO}\) mutant, GFP-FmtA\textsuperscript{Δ27} was observed all over the cell wall but with no specific accumulation at the division septum (Fig. 11).

Exposure of *S. aureus* to either oxacillin or vancomycin (each of which inhibits the cross-linking step in peptidoglycan biosynthesis by binding, respectively, to the transpeptidase domain of PBPs or the \(\beta\)-Ala-\(\beta\)-Ala portion of the peptidoglycan) did not have any effect on the localization of GFP-FmtA\textsuperscript{Δ27}. A similar observation was made for PBP4 (1). In the case of PBP2, however, the presence of these antibiotics caused delocalization of the PBP2 from the division site, suggesting that PBP2 requires substrate binding for its correct localization (35).

**DISCUSSION**

The *fmtA* gene was identified as a methicillin resistance factor (23), and it is present in all *S. aureus* strains. It is part of the core cell wall stimulon (26). Its expression level is elevated by the presence of cell wall inhibitors and knockout of genes that are directly involved with peptidoglycan biosynthesis (3, 23, 27, 42, 46). Furthermore, inactivation of *fmtA* results in a lack of WTAs on *S. aureus* cell walls and deficiency in biofilm production (4, 44).

The function of FmtA is not known. In an earlier study, we showed that FmtA is capable of forming covalent species with \(\beta\)-lactams involving the serine-active site residue of the SXXK conserved motif (10). The \(\beta\)-lactam binding activity of FmtA suggests that it is a PBP. Of the four well-known PBPs of *S. aureus*, PBP1 is a monofunctional transpeptidase that is essential for \(\beta\)-lactam resistance. PBP2 is a bifunctional enzyme with both transglycosylase and transpeptidase activity, and it is essential for peptidoglycan biosynthesis.
viability of *S. aureus* (37). PBP3 and PBP4 are believed to be monofunctional and dispensable transpeptidases. Furthermore, PBP2 and PBP4 were shown to work together to synthesize a highly cross-linked peptidoglycan (24).

In this report, we show that FmtA has DD-carboxypeptidase activity; it catalyzes the removal of the last D-Ala residue from the Nε,Nε-diacetyl-L-Lys-D-Ala-D-Ala peptide (a surrogate for the PG donor strand). However, it shows low enzymatic activity. In the presence of Gly-Gly (a surrogate for the PG acceptor strand), the catalytic efficiency of FmtA was determined to be 0.745 M⁻¹ s⁻¹. In comparison, the catalytic efficiency of the DD-carboxypeptidase activity of PBP4 of *S. aureus* was determined to be 15.7 M⁻¹ s⁻¹ (30). The activity of other well-studied PBPs has also been reported to be higher, ranging from 10 M⁻¹ s⁻¹ for PBP5 of *E. coli* to 2.9 × 10⁴ M⁻¹ s⁻¹ for PBP3 of *N. gonorrhoeae*, according to a study using the same substrate as used in our study (42).

The low enzymatic activity of FmtA could be due to three main reasons: (i) it lacks the third conserved motif, the KTG motif (this motif has been shown to be involved in substrate binding and catalysis) (9, 29, 52); (ii) the L-Lys-D-Ala-D-Ala is not the FmtA native substrate; (iii) the DD-carboxypeptidase activity is reminiscent of that of a PBP from which FmtA had evolved; and (iv) the enzyme may require interaction with a ligand in the periplasmic space to become active. Several proteins involved with either peptidoglycan biosynthesis or its remodeling have been shown to become active in the presence of either the native substrate or a particular ligand. For example, PBP2a of *S. aureus* becomes active upon binding to PG (12), *E. coli* PBP1b and PBP1a (two lipoproteins) are activated through interactions with membrane-bound proteins LpoA and LpoB (32, 45) and PBP5 of *E. coli* requires binding to the membrane to become active (38).

In spite of the poor activity of the enzyme observed using PG surrogates, we discovered that FmtA is capable of incorporating ¹⁴C-Gly into isolated *S. aureus* cell walls. This observation suggests that FmtA may have transpeptidase activity, which agrees with previous reports that inactivation of *fmtA* is associated with a decrease in the level of the highly cross-linked peptidoglycan and an increase in the sensitivity of *S. aureus* to oxacillin (22, 23).

The reported link between FmtA and the level of cross-linked peptidoglycan is consistent with previous findings that inactivation of *fmtA* leads to a decrease in the level of the highly cross-linked peptidoglycan and an increase in the sensitivity of *S. aureus* to oxacillin (22, 23).
PG should be noted, as the cross-linked sites in PG are reported to be targets of teichoic acid attachments in *Streptococcus pneumoniae* and *Bacillus licheniformis* (11, 53). Interestingly, inactivation of fmtA leads to a lack of teichoic acid attachment in *S. aureus* (4). In light of these reports, it is possible that the native function ofFmtA might be involved with teichoic acid attachment through mediation of cross-linked PG synthesis or marking the teichoic acid attachment sites in PG. In this study, we demonstrated thatFmtA interacts specifically with WTAs. Proteins unrelated to cell wall biosynthesis (BSA, ovalbumin, RNaseA, and carbonic anhydrase) showed no interactions with WTAs, suggesting that the negative charge on WTAs does not enable indiscriminate protein binding.

WTAs are proposed to be temporal and spatial regulators of peptidoglycan cross-linking that operate by mediating localization of proteins associated with PG biosynthesis and remodeling, such as PBP4 and AtlA (1, 40). Although a direct interaction between these two proteins and WTAs has not been demonstrated, our study shows that interaction of WTAs with PG-associated proteins is not limited to FmtA. PBP2a, the major source of resistance to β-lactams in *S. aureus*, interacts with WTAs in the same way as FmtA. This finding suggests that interaction of PG-associated proteins, such as PBPs, with WTAs could be more common than previously thought.

The interaction ofFmtA with teichoic acids led us to investigate two processes in *S. aureus* that are closely linked to teichoic acids, autolysis and biofilm production. We observed that exogenous FmtA<sup>27</sup> induced autolysis and biofilm production in *S. aureus* at concentrations lower than 0.1 μM. However, at FmtA<sup>27</sup> concentrations higher than 0.2 μM, *S. aureus* exhibited enhanced growth and deficiency in biofilm production. These observations corroborate recent reports that *S. aureus* ΔfmtA mutants have lost their ability to precisely control the autolysis activity of autolysins (4) and their ability to effectively form biofilms (4, 44). The ability of *S. aureus* to form biofilms is intimately linked to its ability to undergo controlled autolysis (2, 25). We therefore propose that the effect FmtA has on biofilm formation is linked to its ability to induce controlled autolysis.

TheFmtA role in autolysis and, hence, biofilm formation does not result from a direct role ofFmtA in autolysis, as FmtA does not have autolysis activity (see Fig. S3S in the supplemental material). It is possible that binding ofFmtA to WTAs may activate *S. aureus* autolysins. The major autolysin of *S. aureus* is AtlA (30), which is involved in the separation of daughter cells after cell division. Studies have shown that WTAs control the activity of AtlA (14, 40, 50, 51) and that modulation of the WTA negative charge affects AtlA activity (33, 39). Binding ofFmtA to WTAs might affect the activity of AtlA by modulating the negative charge of WTAs; however, we cannot exclude the possibility of interactions between FmtA and AtlA.

The dual mode ofFmtA activity for *S. aureus*, induction and inhibition of autolysis, suggests that FmtA might serve two different roles in *S. aureus*, both dependent of the FmtA concentration in the cell; at low concentrations (<0.1 μM), FmtA may be involved in positively controlling the autolysis process, and at higher protein concentrations (>0.2 μM), FmtA may repress the activity of autolysins and may be involved in PG biosynthesis.

In this study, we discovered that FmtA localizes at the cell division septum, most likely by binding to the cross wall (Fig. 11). PBP4 and AtlA also localize in the cell division septum (1, 40). The localization data clearly show that FmtA is initially localized at the division poles and then moves toward the center of the bacterium as the cross-wall is synthesized (Fig. 11B). The presence of cell wall inhibitors did not cause delocalization of the protein, demonstrating that PG does not mediate the FmtA recruitment to the division septum. Our finding that FmtA interacts with WTAs suggests that WTAs might recruit FmtA to the cell wall. However, no mature WTAs have been shown to be present in the cross-wall region (40). It is plausible that FmtA can be recruited either by a precursor of WTAs in the cross-wall region or by another target. Based on the effect that FmtA has on autolysis, we cannot exclude the possibility that FmtA interacts with AtlA; AtlA also localizes in the cross-wall region (40). Incidentally, the structural genes of atlA and fmtA are close to each other in *S. aureus* genomes and are separated by three structural gene loci, SA0906, SA0907, and SA0908 (gene numbering per the *S. aureus* N315 strain), with no known functions.

On a final note, it is intriguing that one autolysin and two PBPs (FmtA and PBP4) colocalize in the same region of the cell wall during the process of cell division. It is proposed that PG-synthesizing enzymes and autolysins form a complex, which would be
significantly more efficient in PG biosynthesis (18). Our localization studies, and those performed with AtLA and PBP4, suggest that AtLA, FmtA, and/or PBP4 may work cooperatively to mediate PG remodeling during normal cell growth conditions. In the presence of cell wall inhibitors, FmtA could repress autolysis and mediate growth, probably by enhancing PG biosynthesis.

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