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The *Staphylococcus aureus* Methicillin Resistance Factor FmtA Is a D-Amino Esterase That Acts on Teichoic Acids

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ABSTRACT The methicillin resistance factor encoded by *fmtA* is a core member of the *Staphylococcus aureus* cell wall stimulon, but its function has remained elusive for the past two decades. First identified as a factor that affects methicillin resistance in *S. aureus* strains, FmtA was later shown to interact with teichoic acids and to localize to the cell division septum. We have made a breakthrough in understanding FmtA function. We show that FmtA hydrolyzes the ester bond between D-Ala and the backbone of teichoic acids, which are polyglycerol-phosphate or polyribitol-phosphate polymers found in the *S. aureus* cell envelope. FmtA contains two conserved motifs found in serine active-site penicillin-binding proteins (PBPs) and β-lactamases. The conserved SXXK motif was found to be important for the D-amino esterase activity of FmtA. Moreover, we show that deletion of *fmtA* (Δ*fmtA*) led to higher levels of D-Ala in teichoic acids, and this effect was reversed by complementation of Δ*fmtA* with *fmtA*. The positive charge on D-Ala partially masks the negative charge of the polyol-phosphate backbone of teichoic acids; hence, a change in the D-Ala content will result in modulation of their charge. Cell division, biofilm formation, autolysis, and colonization are among the many processes in *S. aureus* affected by the D-Ala content and overall charge of the cell surface teichoic acids. The esterase activity of FmtA and the regulation of *fmtA* suggest that FmtA functions as a modulator of teichoic acid charge, thus FmtA may be involved in *S. aureus* cell division, biofilm formation, autolysis, and colonization.

IMPORTANCE Teichoic acids are involved in cell division, cell wall synthesis, biofilm formation, attachment of bacteria to artificial surfaces, and colonization. However, the function of teichoic acids is not fully understood. Modification by glycosylation and/or D-alanylation of the polyol-phosphate backbone of teichoic acids is important in the above cell processes. The intrinsic negative charge of teichoic acid backbone plays a role in the charge and/or pH of the bacterial surface, and D-alanylation represents a means through which bacteria modulate the charge or the pH of their surfaces. We discovered that FmtA removes D-Ala from teichoic acids. We propose FmtA may provide a temporal and spatial regulation of the bacterial cell surface charge in two ways, by removing the D-Ala from LTA to make it available to wall teichoic acid (WTA) in response to certain conditions and by removing it from WTA to allow the cell to reset its surface charge to a previous condition.

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*S*taphylococcus aureus is the leading cause of hospital- and community-acquired infections (1). *S. aureus* was once inherently susceptible to most antibiotics. However, it is now a pathogen of great concern due to its intrinsic virulence and its remarkable ability to rapidly adapt to different environmental conditions by mutation and DNA transfer (2–4). Multiple-drug-resistant *S. aureus* strains, such as methicillin-resistant *S. aureus* (MRSA), have become notoriously difficult to treat, with 20 to 40% of cases causing mortality (1). Emergence of MRSA strains resistant to vancomycin (5), an antibiotic reserved for the treatment of severe MRSA infections, has led to limited treatment options for *S. aureus* infections (6–9). To make matters worse, infections caused by multiple MRSA strains have reached epidemic proportions (8, 10).

The need for novel antibiotics for the treatment of *S. aureus*-related infections is as urgent today as it was in 1940, when penicillin was introduced to treat *S. aureus* infections (11, 12). Antibiotics that target cell wall biosynthesis (referred to as cell wall inhibitors), such as β-lactams and glycopeptides, are among the most efficient antibacterial agents for treating *S. aureus* infections; however, their biological activities have been compromised by the emergence of resistance mechanisms (11, 12). Recent reports have shown that MRSA can be resensitized to β-lactams and vancomycin by inhibiting nonessential genes involved in the biosynthesis of cell envelope components, such as peptidoglycan and teichoic acids, and these reports have rekindled interest in targeting the cell wall for drug discovery and provide evidence that antibiotic potency can be rescued (13–19).

Komatsuzawa et al. reported that *fmtA* was a factor in the methicillin resistance of MRSA strains (20) and that deletion of *fmtA* reduced the methicillin MIC for *S. aureus* Col (an MRSA strain) from 1,024 μg/ml to 128 μg/ml (20). In addition, deletion of *fmtA* was shown to disrupt the homogeneity of methicillin resistance (20). Further, *fmtA* was identified as a core member of the

cell wall stimulon; *fmtA* expression increased in the presence of cell wall inhibitors and when genes involved in cell wall biosynthesis were deleted (21–24). The primary structure of FmtA shares similarities with D,D-carboxypeptidase from *Streptomyces* R61 (D,D-carboxypeptidase R61) and class C β-lactamases (25). FmtA harbors two of the three conserved motifs, SXXK and SND, found in the serine active site of penicillin-binding proteins (PBPs) and β-lactamases. The third conserved motif, KTG, has not been identified in FmtA. Our previous studies on FmtA revealed that it interacts covalently with β-lactams via a serine residue located in the conserved SXXK motif. We also showed that FmtA has very weak D,D-carboxypeptidase activity and interacts with teichoic acids (25). These observations led to the proposal that FmtA may be a PBP (25, 26). *S. aureus* has four native PBPs. PBP1 and PBP2 are essential enzymes (27). PBP1 and PBP4 are transpeptidases, and PBP2 is a bifunctional enzyme. The function of PBP3 remains elusive. PBP2 and PBP4 are involved in the synthesis of highly cross-linked peptidoglycan (28).

The interaction of FmtA with teichoic acids (26) is peculiar, considering that teichoic acids have been found to play roles in the temporal and spatial regulation of AtlA and PBP4 (29, 30). There are two types of teichoic acids in *S. aureus*. Teichoic acids that are bound to the outer leaflet of the cytoplasmic membrane are referred to as lipoteichoic acids (LTAs), whereas teichoic acids that are bound to peptidoglycan are referred to as wall teichoic acids (WTAs) (31, 32). LTAs are phosphate-rich polyglycerol polymers, and WTAs are phosphate-rich polyribitol polymers (Fig. 1) (33, 34). Their biosynthetic pathways differ. LTA synthesis takes place in the periplasm, and WTA synthesis takes place in the cytoplasm (35–37). Both polymers are postsynthetically glycosylated with *N*-acetylglucosamine and/or esterified with D-alanine, as shown in Fig. 1 (32, 38, 39). Modification of WTA with *N*-acetylglucosamine takes place in the cytoplasm, whereas modification with D-Ala takes place in the extracellular milieu (38, 40).

The biological function of LTAs in *S. aureus* is not well understood, but they have been shown to interact with cell division proteins (41). It has also been suggested that LTAs are involved in osmoprotection of the cell (32). WTAs have been shown to act as scaffolds for many proteins, such as exogenous proteins required for phage infection (42) and endogenous proteins required for cell growth and division, such as AtlA (29, 43), PBP4 (30), and FmtA (26). Decoration of teichoic acids with D-Ala and α- or β-O-N-acetyl-D-glucosamine (GlcNAc) has been implicated in many processes. The D-Ala content of teichoic acids affects the bacterial surface charge by adding a positive charge to an otherwise negatively charged polyol-phosphate backbone of teichoic acids (44) and has been implicated in *S. aureus* virulence (45–47), attachment to artificial surfaces, initiation of biofilm formation (48, 49), cell autolysis (29, 34, 50), and susceptibility to cationic peptide antibiotics and vancomycin (47, 51). Glycosylation of WTAs with an α- or β-anomer of GlcNAc has also been reported to be important for methicillin resistance and horizontal gene transfer between bacterial pathogens (37, 52).

FmtA exhibits very weak D,D-carboxypeptidase activity. It was proposed that FmtA might require binding to a ligand for activation (26). In light of our previous finding that FmtA interacts with teichoic acids, we aimed to evaluate the role of teichoic acids on the D,D-carboxypeptidase activity of FmtA. We found that FmtA acts as an esterase and removes D-Ala groups from WTAs and LTAs. Deletion of *fmtA* led to a 7-fold increase in the D-Ala con-

tent of WTAs, and complementation of the *fmtA* deletion mutant (Δ*fmtA*) with wild-type *fmtA* restored the D-Ala content to the level in WTAs from wild-type *S. aureus*. These findings have important implications for two aspects of D-Ala modification of teichoic acids. First, it has been proposed that the D-Ala of LTAs serves as the D-Ala source for D-alanylation of WTAs (33), but no study has shown how D-Ala is removed from LTA. Although it has been reported that D-Ala can spontaneously be removed from LTA, these processes are very slow (53). Second, studies have shown that the D-Ala content in teichoic acids is affected by a number of environmental factors, such as salt concentration, pH, and temperature (54–56), which suggests that, under certain environmental conditions, D-Ala from teichoic acids must be removed (54). Our results suggest that FmtA modulates D-alanylation of teichoic acids in *S. aureus*.

RESULTS

FmtA has D-amino esterase activity toward teichoic acids. D-Ala is a small molecule that lacks optical properties to enable its monitoring by classical spectrophotometric methods, such as the use of a UV-visible (UV-Vis) spectrophotometer or fluorimeter. For this purpose, we used a fluorescence-based coupled enzyme assay to assess the D,D-carboxypeptidase activity of FmtA, measured as release of free D-Ala, in the absence and presence of WTA. The amount of free D-Ala measured in the reaction mixtures, which contained the tripeptide $N\alpha,N\epsilon$-diacetyl-Lys-D-Ala-D-Ala (6 mM), WTA (0.1 $\mu g/\mu l$ to 0.3 $\mu g/\mu L$), and FmtA, was 3- to 4-fold higher than in the reaction mixtures lacking WTA (Fig. 2A). This increase in free D-Ala was also observed in the absence of the tripeptide, indicating that WTA was the source of the free D-Ala in the assay.

WTAs are D-alanylated at the C-2 position of ribitol (Fig. 1). To exclude the possibility that WTAs are an adventitious source of free D-Ala in our assays, we performed the assay using WTAs isolated from an *S. aureus* strain in which the *dltABCD* operon was deleted (Δ*dlt* WTAs). This operon is responsible for incorporating D-Ala into WTAs (51). D-Ala release was not observed in the presence of Δ*dlt* WTAs (Fig. 2B) suggesting that WTAs are the source of D-Ala and that FmtA may remove D-Ala from WTAs. PBP4 and PBP2a activities toward WTA was also investigated, but no hydrolysis activity was detected (see Fig. S1A and B in the supplemental material).

We used proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) to further investigate the activity of FmtA on WTAs. The release of free D-Ala was identified by the appearance of a new resonance signal at 1.47 ppm in the ^{1}H-NMR spectrum of WTA and the disappearance of the resonance peak at 1.63 ppm that corresponds to bound D-Ala (Fig. 3A and B). In the absence of FmtA, free D-Ala was not detected after incubation for 2 days (Fig. 3C). To investigate the specificity of this FmtA activity, we incubated WTAs with heat-denatured FmtA, and no significant D-Ala release was detected (data not shown).

We also investigated the activity of FmtA on LTAs by ^{1}H-NMR. LTA modification with D-Ala occurs on C2 of glycerol (57). Removal of the D-Ala from LTA is identified by the disappearance of the resonance peak at 1.63 ppm (bound D-Ala) and the appearance of the free D-Ala resonance peak at 1.47 ppm. In the absence of FmtA, there was no significant removal of D-Ala from LTA (Fig. 4).

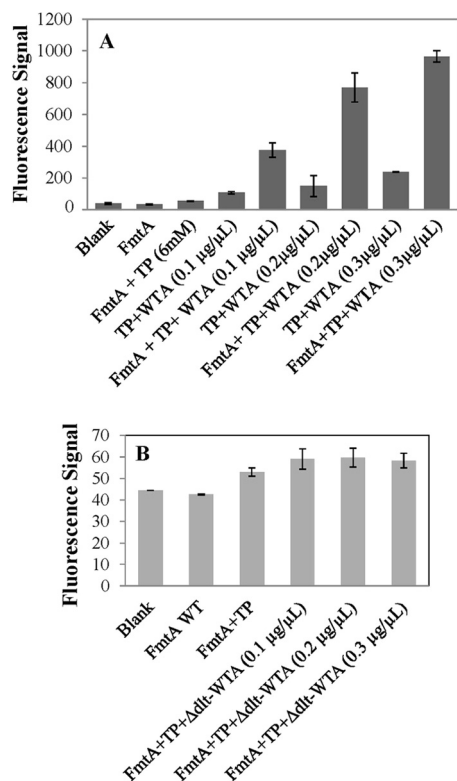To investigate whether the esterase activity of FmtA is related

FIG 1 A schematic view of the cell surface of *S. aureus*. Highlighted are the cytoplasmic membrane, peptidoglycan (PG), lipoteichoic acid (LTA), and wall teichoic acid (WTA). The general chemical structures of LTA and WTA are shown.

to the presence of the conserved PBP motifs in FmtA, we investigated the activity of two PBPs, PBP2a (a nonnative PBP from *S. aureus*) and PBP4 (a native PBP from *S. aureus*) toward WTAs. Both PBP2a and PBP4 have been shown to have transpeptidase activity (58–60) and to interact with WTAs, although the interaction of PBP4 with WTA is weaker than with FmtA (26) (see Fig. S2 in the supplemental material). We found that PBP2a and PBP4 did not have significant activity toward WTAs (Fig. 5); these data agree with our fluorescence data (see Fig. S1 in the supplemental material).

Kinetics of FmtA esterase activity. The NMR studies described above provided the necessary means of monitoring of free

D-Ala, which otherwise lacks any special optical properties, and the much-needed sensitivity for our experimental settings, where we are limited by the amount of WTA isolated from *S. aureus*, about 1 mg per liter of cell culture. In addition, the use of the 700 MHz NMR enabled us to use reaction volumes as low as 250 μl and employ pseudo-first-order rate kinetics in characterization of the rate constant of D-Ala removal from WTA by FmtA.

The rate of FmtA esterase activity was monitored by ^{1}H-NMR. The amount of free D-Ala released during the reaction was quantified and normalized to the GlcNAc resonance peak, as the GlcNAc content of WTA does not change over time. The normalized free D-Ala amounts measured at different time points were used to

FIG 2 Analysis of D-Ala removal from the tripeptide $N\alpha,N\varepsilon$-diacetyl-Lys-D-Ala-D-Ala (TP, 6 mM) by FmtA (10 μM) in the absence or presence of WTA (0.1 μg/μl, 0.2 μg/μl, 0.3 μg/μl) (A) or in the presence of Δdlt WTA (B). Free D-Ala was measured using a fluorescence-based coupled enzymatic assay. Error bars represent standard deviations from three independent experiments.

construct progress curves in which the data were fitted to pseudo-first-order rate kinetics. The observed pseudo-first-order rate constant (k_{obs}) was 0.57 ± 0.01 h^{-1} with 10 μM FmtA and 5 mg/ml WTA. The reaction rates increased with increased enzyme concentrations as indicated from the calculated k_{obs} values, which increased from 0.46 h^{-1} with 5 μM FmtA to 1.11 h^{-1} with 20 μM FmtA ([WTA] = 5 mg/ml) (Fig. 6A). In contrast, FmtA reaction rates increased with the decrease of WTA concentrations. The k_{obs} value increased 3-fold, to 1.3 ± 0.2 h^{-1}, when the WTA concentration was reduced from 5 mg/ml to 0.5 mg/ml (Fig. 6B). The k_{obs} value measured at 0.5 mg/ml WTA is the highest pseudo-first-order rate constant that we could measure; as such, it can serve as an approximation for the apparent first-order rate constant of the esterase reaction catalyzed by FmtA, although it is an underestimation of the true first-order rate constant for this reaction. From this k_{obs} value, we estimated the time required to hydrolyze 50% of the D-alanyl ester bonds in WTA (half time [$t_{1/2}$]) to be 32 min.

The reduction in FmtA esterase activity found with increased WTA concentrations could be a result of structural changes in FmtA upon WTA binding. Indeed, an earlier investigation of the interaction of FmtA with WTA by circular dichroism revealed that the secondary structural elements of FmtA were altered extensively in the presence of high WTA concentrations (26). We have observed that high concentrations of WTA (≥10 mg/ml) cause aggregation of FmtA. This may be due to a salting-out effect produced by the highly negatively charged WTAs.

To investigate the significance of the conserved PBP motif

SXXK for the esterase activity of FmtA, we constructed two variants of FmtA by replacing Ser127 and Lys130 with Ala. By determining the ratios of the observed pseudo-first-order rate constants of the mutants and wild-type FmtA, we found that FmtA-Ser127Ala and FmtA-Lys130Ala had 16% of the activity of wild-type FmtA (Fig. 6C). The seemingly high remaining activity of FmtA mutant could result from relative instability of ester moieties in slight acidic or basic aqueous solutions and binding of the WTA to FmtA; i.e., the topology of the FmtA active site may introduce enough constraints to the carbonyl C of the D-Ala bound to WTA to lead to its increased electrophilicity and attack by weak nucleophiles such as water molecules in long incubation times.

To investigate whether FmtA can catalyze esterification of WTA by D-Ala, we set up several assays. We incubated D-Ala (6 mM) with ribitol (10 mM) or with Δdlt WTAs (2.5 mg/ml) and monitored the reaction by ^{1}H-NMR in the absence and presence of FmtA (20 μM). In addition, LTA (0.5 mg/ml) and Δdlt WTA (2.5 mg/ml) were incubated together in the absence and presence of FmtA (20 μM) to investigate whether D-Ala released from LTA can be transferred to WTA by FmtA. Lastly, we incubated D-Ala with ATP (5 mM), MgCl$_2$ (10 mM), dithiothreitol (DTT) (1 mM), and Δdlt WTA in the absence and presence of FmtA (20 μM). None of the above conditions led to the incorporation of D-Ala to WTA (data not shown).

Specificity of FmtA esterase activity. To determine the substrate specificity of FmtA, we tested several carboxylesterase substrates, including p-nitrophenyl butyrate (p-NPB) and p-nitrophenyl acetate (p-NPA). FmtA showed very low esterase activity on p-NPB and p-NPA as assessed by continuous spectrophotometric assay (data not shown). We also used ^{1}H-NMR to monitor hydrolysis of p-NPB and p-NPA and detected very little activity on p-NPB and p-NPA (data not shown). In addition, we investigated potential α-amino-acid esterase and aminopeptidase activities in FmtA with D-alanine methyl ester and L-alanine p-nitroanilide, respectively. However, we did not observe any catalytic activity against these substrates. Carboxylesterases have been reported to catalyze the hydrolysis of short-chain aliphatic and aromatic carboxylic ester compounds (61) suggesting that FmtA may be specific for the D-Ala ester attached at the C-2 of ribitol-5-phosphate and glycerol-3-phosphate, which are the repeating units of teichoic acids.

Assessment of FmtA esterase activity in cells. To investigate the significance of our findings in *S. aureus*, we constructed three *S. aureus* RN4220 mutant strains (see Text S1 in the supplemental material). The *fmtA* deletion ($\Delta fmtA$) strain was constructed using a pMAD vector (62). The complementation strain was created by cloning *fmtA* into a pMK4 vector (63) and introducing the construct into *S. aureus* RN4220. We also constructed an *fmtA* conditional mutant (CM) in which *fmtA* expression is under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter, P_{spac} (P_{spac}-*fmtA*), using a pMUTIN integration vector (Bacillus Genetic Stock Center, The Ohio University).

We isolated WTAs from *S. aureus* RN4220 and the $\Delta fmtA$, complemented $\Delta fmtA$, and P_{spac}-*fmtA* strains in the presence or absence of 0.5 mM IPTG. WTAs from each strain were analyzed by ^{1}H-NMR. The amount of D-Ala attached to ribitol relative to the amount of *N*-acetylglucosamine attached to ribitol was determined by integrating the resonance peaks of *N*-acetylglucosamine at 2.08 ppm and D-Ala at 1.63 ppm. Deletion of *fmtA* resulted in a 7-fold increase in D-alanylation of WTAs (Table 1; Fig. 7). Inter-
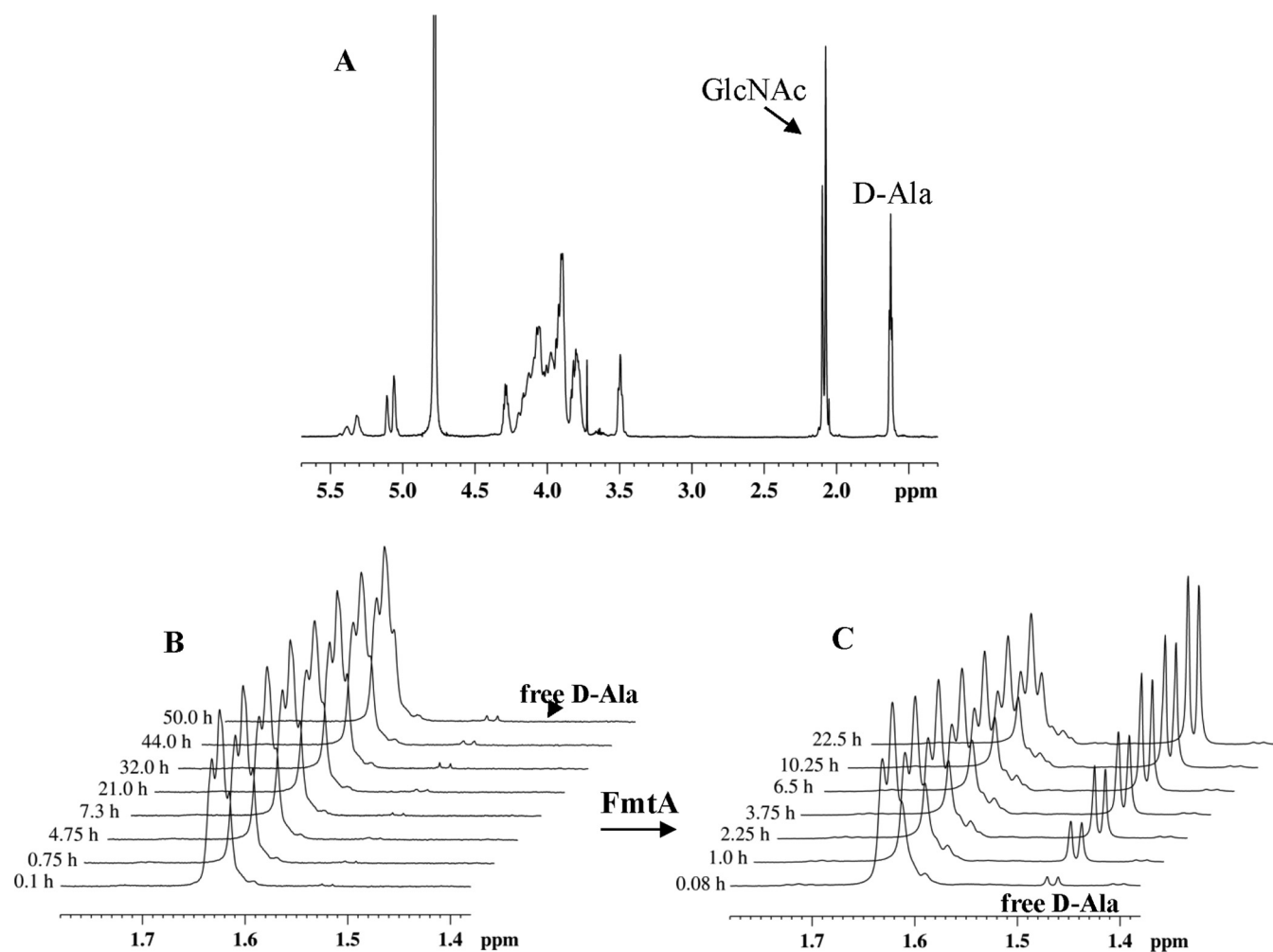
FIG 3 (A) ^{1}H-NMR spectra of WTA isolated from *S. aureus* RN4220. (B) ^{1}H-NMR spectra centered at 1.60 ppm in the absence of FmtA. (C) ^{1}H-NMR spectra centered at 1.60 ppm in the presence of FmtA. Experiments were carried out in 10 mM sodium phosphate (pH 7.2) at 25°C with 5 μg/μl of WTA.

estingly, this increased D-alanylation was also seen in WTAs isolated from the P_{spac}-*fmtA* strain in the absence of IPTG (4.5-fold increase), but in the presence of IPTG, the WTAs exhibited wild-type levels of D-alanylation. WTAs isolated from the complemented Δ*fmtA* strain also exhibited wild-type levels of D-alanylation.

DISCUSSION

We discovered that FmtA has esterase activity specific to teichoic acids. We determined that the pseudo-first-order rate constant of the FmtA esterase activity was 1.3 h^{-1} at 5 μM FmtA and 0.5 mg/ml WTA. The time that it would take FmtA to remove 50% of the D-Ala from teichoic acid was estimated using the above observed pseudo-first-order rate constant, and it was determined that $t_{1/2}$ does not exceed 32 min. Taking into account that *S. aureus* doubles every 30 min, we can conclude that the esterase activity of FmtA is significant and relevant *in vivo*. Evidence for alanyl turnover in *S. aureus* LTAs has been reported by Haas et al., and their study determined that the $t_{1/2}$ of alanyl turnover in *S. aureus* LTA was 37 min (64), which is in remarkable agreement with our estimated $t_{1/2}$. By comparing the kinetics of alanyl turnover in *S. aureus* LTA to the kinetics of alanyl turnover in a base-catalyzed

reaction (53), Haas et al. suggested that D-alanyl removal from LTA is likely to be an enzyme-catalyzed process (64). Our study suggests that FmtA is the enzyme that catalyzes the alanyl turnover of teichoic acids. The FmtA esterase activity that we observed *in vitro* strongly correlates with our *in vivo* data in which WTA isolated from *S. aureus* Δ*fmtA* had a higher D-Ala content than WTA isolated from wild-type *S. aureus*. Complementation of the Δ*fmtA* mutant with wild-type *fmtA* restored the D-Ala level to wild-type.

The presence of FmtA esterase activity toward teichoic acids and the absence of FmtA esterase activity toward small-molecule substrates of amidases, peptidases, and carboxylesterases suggest that FmtA has D-amino esterase activity, is specific for D-amino acids and may recognize the positive charge on the amino group of D-Ala; this D-amino esterase nomenclature is in line with the nomenclature of D,D-carboxypeptidases and D-aminopeptidases. Microbial esterases, commonly referred to as carboxylesterases, constitute a large group of enzymes that hydrolyze short-chain aliphatic and aromatic carboxylic ester compounds. They have been classified into eight families based on the their conserved sequence motifs and biological properties (65), and their esterase activity has primarily been attributed to the GXSXG motif. However, in family VIII esterases, represented by EstB from *Burkhold-*
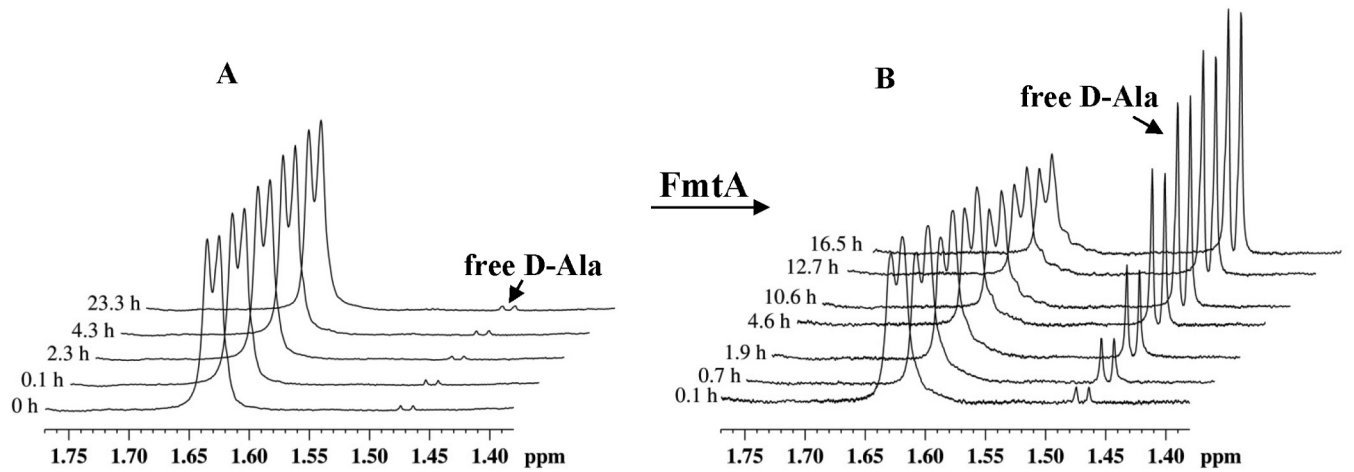
FIG 4 ^{1}H-NMR spectra of LTA from *S. aureus* (Sigma). ^{1}H-NMR spectra were centered at 1.80 ppm in the absence (A) and presence (B) of 10 μM FmtA. Experiments were carried out in 10 mM sodium phosphate (pH 7.2) at 25°C with 5 μg/μl of WTA.

eria gladioli, the esterase activity is associated with a conserved SXXK motif, which is found in the serine active site of PBPs and β-lactamases (66), despite the presence of the GXSXG motif. Notably, these enzymes show sequence and structural similarities to class C β-lactamases and D,D-carboxypeptidase R61. EstB contains two of the three conserved motifs found in the serine active site of PBPs and β-lactamases, SXXK and SYN, whereas the third conserved KTG motif is replaced with WGG. However, EstB does not have β-lactamase or D,D-carboxypeptidase activity (66). EstB is not an exception in this respect; there are a large number of enzymes that exhibit a wide range of activities, such as D,D-

endopeptidases (67), D,L-endopeptidases (68), D-amino acid amidases (69–71), D-aminopeptidases (72, 73), and D-esterases (66, 74), that also share the conserved motifs and structural folds found in the serine active sites of PBPs and β-lactamases but do not function as PBPs and β-lactamases (75). These enzymes are referred to as penicillin-recognizing enzymes (PRE) (75, 76). Many structural studies on PREs have shown that the divergence in their activities may be attributed to the presence of a core enzyme surrounded by specific structural modulators (69, 71, 77).

Our previous studies showed that FmtA has primary structure similarities with class C β-lactamases and D,D-carboxypeptidase R61 and has an overall structural fold similar to that of D,D-carboxypeptidase R61 (25); thus, FmtA can be considered a member of family VIII of esterases. However, FmtA does not have typical carboxylesterase activity. This lack of activity toward short-chain aliphatic carboxylic ester compounds indicates that FmtA recognizes the positive charge on the amino group of D-alanine. Recognition of the charge on an amino acid is a common structural feature of D-amino acid amidases (DAA), D-aminopeptidases (DAP), and D,D-carboxypeptidases. DAA and DAP recognize the positive charge on the amino group of D-Ala, whereas D,D-carboxypeptidases recognize the negative charge on the carboxylic group of D-Ala. Moreover, structural and mutagenesis studies on DAP from *Ochrobactrum anthropi* have revealed that this enzyme can be converted to a D,D-carboxypeptidase by removing two structural elements that interact with the amino group of D-Ala and introducing a structural element from D,D-carboxypeptidases that is known to interact with the carboxylic group of D-Ala (69). In the case of FmtA, the lack of activity toward a typical carboxylesterase substrate suggests that FmtA may have binding specificity for the glycerol-3-phosphate and ribitol-5-phosphate repeating units of teichoic acids.

The function of FmtA as a D-amino esterase of teichoic acids requires FmtA to be located in the extracellular milieu where the teichoic acids are located. Moreover, the D-Ala content, and as a result, the charge state of teichoic acids is expected to be dynamic and dependent on the environment, the growth state, and lifestyle (i.e., growth, division, planktonic or biofilm state) of the bacteria (31, 54). As such, the dynamic modulation of the charge state of
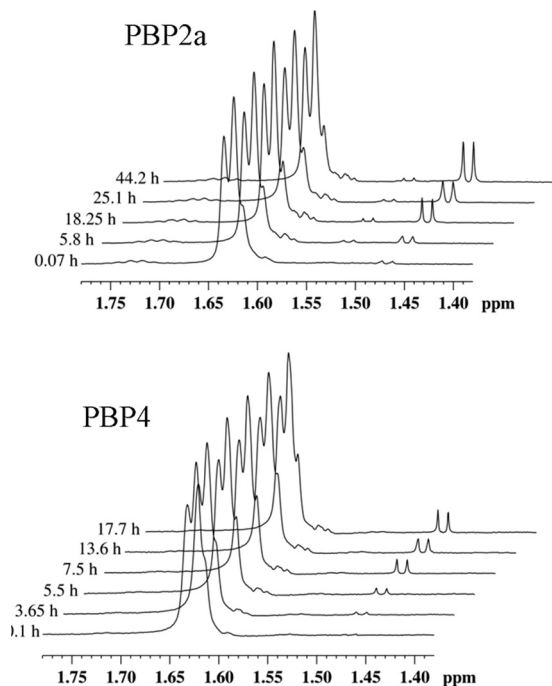

FIG 5 ^{1}H-NMR spectra of WTA isolated from *S. aureus* RN4220. ^{1}H-NMR spectra were centered at 1.60 ppm in the presence of 10 μM PBP2 and 10 μM PBP4 at various incubation times. Experiments were carried out in 10 mM sodium phosphate buffer (pH 7.2) at 25°C with 5 μg/μl of WTA.
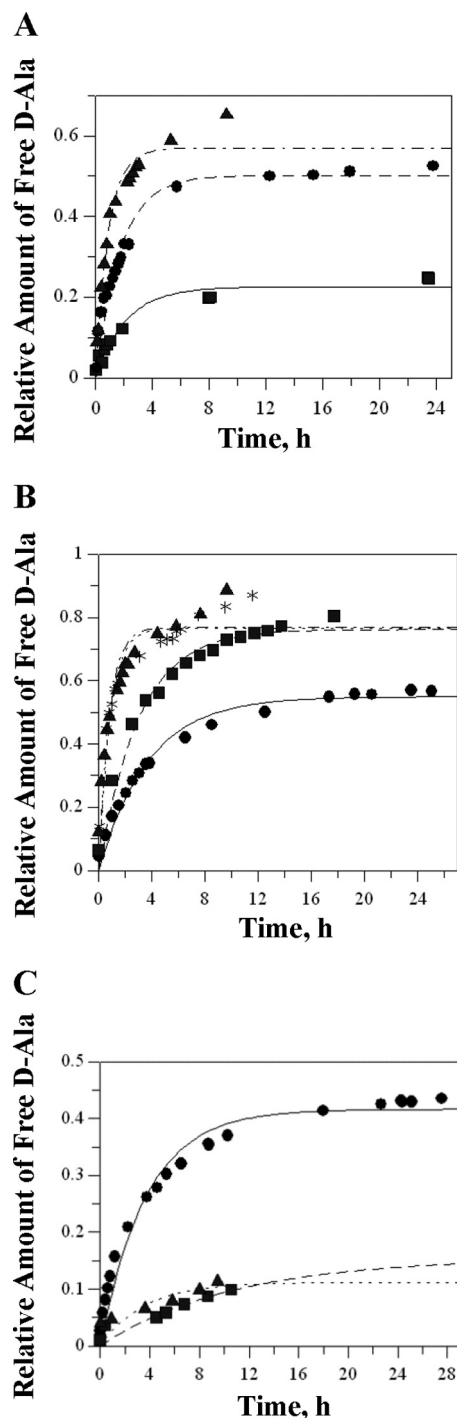
A

B

C

FIG 6 Kinetics of D-Ala release from WTA by FmtA. (A) D-Ala release was monitored in the presence of different FmtA concentrations (5 μM [squares], 10 μM [circles], and 20 μM [triangles]). (B) D-Ala release was monitored in the presence of different WTA concentrations (0.5 μg/μl [asterisks], 1 μg/μl [triangles], 2.5 μg/μl [squares], and 5 μg/μl [circles]). (C) Effects of the FmtA-Ser127Ala and FmtA-Lys130Ala mutations on the removal of D-Ala from WTA (circles, wild-type FmtA; triangles, FmtA-Ser127Ala FmtA; squares, FmtA-Lys130Ala).

TABLE 1 Quantitative analysis of the relative amount of D-Ala on WTAs isolated from various *S. aureus* strains[a]

Strain or description	Relative integration (GlucNac/D-Ala)
RN4220	0.63 ± 0.03
Δ*fmtA*	4.0 ± 0.8
P_{spac}-*fmtA*	2.6 ± 0.5
P_{spac}-*fmtA* + IPTG	0.6 ± 0.1
Δ*fmtA*::pMK4:*fmtA*	0.5 ± 0.1

[a] Values are the averages of three independent measurements and the errors represent the standard deviations.

teichoic acids requires temporal regulation of the D-Ala removal. The requirement for localization of FmtA in the extracellular milieu is met by FmtA, as earlier findings show that FmtA is an extracellular protein (20) and that green fluorescent protein (GFP)-labeled FmtA, which lacked the first 27 N-terminal amino acid residues, considered to constitute the signal peptide (25), localized to the cell division septum of *S. aureus* (26). The requirement for temporal regulation of D-Ala content of teichoic acids is also met by FmtA, as the expression of *fmtA* has been shown to be tightly regulated (24, 78), and we recently demonstrated that *fmtA* is controlled by the globular regulatory protein SarA (79). Of note, the involvement of SarA in the regulation of *fmtA* is in agreement with the esterase function of FmtA. Both SarA and FmtA have independently been shown to be involved in *S. aureus* biofilm formation (80–84). Further, *S. aureus* biofilm formation is dependent on the major autolysin AtlA (85, 86), activity of which is closely associated with the D-Ala content of WTA (29, 43).

Haas et al. showed that D-Ala removed from LTA can be incorporated into WTA (64). In addition, Reichmann et al. showed that WTA isolated from *S. aureus* lacking LTA contained a low level of D-Ala (87). The inference from these studies is that LTA may serve as the donor of D-Ala for D-alanylation of WTA. Because FmtA has esterase activity, FmtA may function to remove D-Ala from LTA so that it is available for uptake by WTA (31). The question that subsequently arises is that of how D-Ala is incorporated into WTA. It is generally accepted that the *dltABCD* operon is involved in the *de novo* esterification of LTA, but not WTA, with D-Ala (87). The esterase activity of FmtA raises the question of whether FmtA may function as a transesterase. As a transesterase, FmtA would remove D-Ala from LTA and transfer it to WTA. The major concern with this proposal comes from recent findings that show little
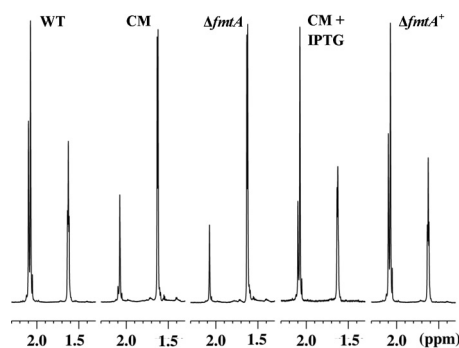

FIG 7 ^{1}H-NMR spectra of WTA isolated from *S. aureus* RN4220 (WT) and the P_{spac}-*fmtA* mutant (CM), the Δ*fmtA* mutant, the P_{spac}-*fmtA* mutant (CM) with 0.5 mM IPTG, and the complemented Δ*fmtA* (Δ*fmtA*$^+$) strain. ^{1}H-NMR spectra were centered at 1.80 ppm.

spatial overlap between mature LTA and WTA polymers in the extracellular milieu. *S. aureus* LTAs are composed of approximately 25 glycerol-3-phosphate repeating units and are anchored to the membrane, and they do not stretch beyond the peptidoglycan layer (32, 41). In contrast, WTAs are composed of approximately 40 ribitol-5-phosphate repeating units, are anchored to peptidoglycan, and stretch well beyond the peptidoglycan (31). It could be argued that D-alanylation of WTA may occur prior to the attachment of WTA to peptidoglycan while it is anchored in the outer leaflet of the lipid bilayer and positioned close to LTA at the cell division septum. However, studies indicate that D-alanylation of WTA is not a one-time event in the life cycle of the cell. We did not observe any FmtA transesterase activity in this study. Further studies are needed to elucidate the mechanism of D-Ala incorporation into WTA.

Teichoic acids are involved in *S. aureus* cell division, cell wall synthesis, biofilm formation, and attachment to artificial surfaces and colonization. Although the role of teichoic acids in many of these processes remains elusive, glycosylation and/or esterification of the polyol-phosphate backbone of teichoic acids is seen as a factor in establishing interactions with bacterial extracellular proteins, host receptors, and phage receptors involved in the above-described processes (29, 30, 37, 44, 45, 52, 88). However, D-alanylation goes beyond transforming teichoic acids into recognition modules on the cell surface. It has been suggested that the intrinsic negative charge of the teichoic acid backbone functions as a bacterial cell surface charge and/or pH determinant (89); hence, D-alanylation of teichoic acids may be a mechanism through which bacteria modulate their surface charge and/or pH. In light of the FmtA enzymatic activity that we identified, we propose that FmtA may provide the cell with the ability to temporally and spatially regulate their cell surface charge by removing D-Ala from LTA so that it is available to WTA in response to certain conditions and by removing D-Ala from WTA to allow the cell to reset its surface charge to a previous condition.

MATERIALS AND METHODS

Materials and reagents. Growth media were purchased from EMD Bioscience. Enzymes (trypsin, DNase, RNase, horseradish peroxidase, and D-amino acid oxidase) and chemicals (Tris, cytochrome *c*, and LTA) were purchased from Sigma (Oakville, Canada) and Thermo-Fisher (Whitby, Canada) unless otherwise stated. Amplex red (AR) was purchased from Molecular Probes, Inc. Oligonucleotides were acquired from Sigma.

Isolation and purification of FmtA, FmtA-Ser127Ala, and FmtA-Lys130Ala. The *fmtA* gene from *S. aureus* strain Mu50 was cloned into pET24a(+) using the NdeI and HindIII restriction sites (25). The cloned *fmtA* lacked the first 81 nucleotides, corresponding to the 27-amino-acid signal peptide. The predicted conserved active-site residues in the SXXK motif in FmtA, Ser127 (encoded by TCA) and Lys130 (encoded by AAA), were mutated to alanine with the Quik-Change site-directed mutagenesis kit (Agilent). Specifically, pET24a(+)::*fmtA* was used as the template, and amplification was carried out using *Pfu* Turbo DNA polymerase and a pair of mutagenic primers for each substitution. The primer sets used were DirS127A (5′ CGATGTTTTTAATAGGT*GC*AGCTCAAAAATTTTC 3′)-RevS127A (5′ GAAAATTTTTGAGC*TGC*ACCTATTAAAAACATCG 3′) and DirK130A (5′ GGTTCAGCTCAA*GC*ATTTTCAACAGGGTTAC 3′)-RevK130A (5′ GTAACCCTGTTGAAAA*TGC*TTGAGCTGAACC 3′) (mutations are in italics). The nucleotide sequences of the variants were verified by DNA sequencing (Core Facility, York University). *E. coli* BL21(DE3) cells were then transformed with the mutagenized vectors. Protein synthesis, isolation, and purification were performed as described previously (25).

Enzyme assays. The D,D-carboxypeptidase activity of FmtA was assessed using a fluorescent enzyme-coupled assay as described previously (90). The esterase activity of FmtA was investigated by a continuous spectrophotometric assay using *p*-NPB or *p*-NPA as the substrate. The standard reaction mixture contained *p*-NPB or *p*-NPA (1 mM), FmtA, PBP4 or PBP2a (5 μM), and 50-mM sodium phosphate (pH 7.0) in a final volume of 0.5 ml and was incubated at room temperature. The reaction was initiated by the addition of substrate. Hydrolysis of *p*-NPB or *p*-NPA was monitored spectrophotometrically for formation of *p*-nitrophenol, which is detected at 405 nm ($\varepsilon = 0.2$ mM^{-1} cm^{-1}).

Investigation of the enzymatic activity of FmtA by NMR. WTAs were isolated from various strains (RN4220, $\Delta fmtA$, complemented $\Delta fmtA$, and CM) using trichloroacetic acid as described by Fridman et al. (91). LTA (*S. aureus*) was purchased from Sigma. Teichoic acids were lyophilized and resuspended in 100% D$_2$O to a concentration of 5 to 10 mg/ml and were analyzed by NMR. One-dimensional (1D) ^{1}H NMR spectra were collected at 25°C on a Bruker AV III 700 MHz spectrometer (operating frequencies of 700.28 MHz for ^{1}H NMR and 176.096 MHz for ^{13}C NMR). The spectrometer was controlled with TOPSPIN version 3.2 software and equipped with a 5-mm ^{1}H/^{13}C/^{15}N cryoprobe. For quantitative 1D ^{1}H spectra, 1D ^{1}H T$_1$ analysis was completed using the inversion recovery experiment. The enzymatic activity of FmtA toward teichoic acids was investigated using reaction mixtures containing 10 μM FmtA unless stated otherwise, various concentrations of WTA, 5 mg/ml LTA, and 10 mM sodium phosphate (pH 7.0). Residual solvent suppression was completed by excitation sculpting using the standard library pulse program. The progress of the reactions was monitored by recording the ^{1}H-NMR spectra of the teichoic acids at various time intervals at 25°C.

To investigate the substrate specificity of FmtA by NMR, several esterase substrates, including *p*-NPB, *p*-NPA, and D-Ala-methyl ester, were dissolved in 300 μl of 10 mM sodium phosphate buffer (pH 7.0) (at a final concentration of 1 mM) and were incubated with FmtA (10 μM) and 300 μl of deuterium oxide. The *p*-NPA and *p*-NPB reactions were monitored by NMR for the appearance of peaks at 8.15 ppm and for the disappearance of peaks at 8.35 ppm at various time intervals at 25°C. The D-Ala methyl ester reactions were monitored for the appearance of peaks at 1.46 ppm and for the disappearance of peaks at 1.53 ppm.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.02070-15/-/DCSupplemental.

Text S1, PDF file, 0.1 MB.
Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.1 MB.

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