Diversity of Penicillin-binding Proteins

RESISTANCE FACTOR FmtA OF STAPHYLOCOCCUS AUREUS

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Antibiotic-resistant Staphylococcus aureus is a major concern to public health. Meticillin-resistant S. aureus strains are completely resistant to all β-lactams antibiotics. One of the main factors involved in meticillin resistance in S. aureus is the penicillin-binding protein, PBP2a. This protein is insensitive to inactivation by β-lactam antibiotics such as methicillin. Although other proteins are implicated in high and homogeneous levels of meticillin resistance, the functions of these other proteins remain elusive. Herein, we report for the first time on the putative function of one of these proteins, FmtA. This protein specifically interacts with β-lactam antibiotics forming covalently bound complexes. The serine residue present in the sequence motif Ser-X-X-Lys (which is conserved among penicillin-binding proteins and β-lactamases) is the active-site nucleophile during the formation of acyl-enzyme species. FmtA has a low binding affinity for β-lactams, and it experiences a slow acylation rate, suggesting that this protein is intrinsically resistant to β-lactam inactivation. We found that FmtA undergoes conformational changes in presence of β-lactams that may be essential to the β-lactam resistance mechanism. FmtA binds to peptidoglycan in vitro. Our findings suggest that FmtA is a penicillin-binding protein, and as such, it may compensate for suppressed peptidoglycan biosynthesis under β-lactam induced cell wall stress conditions.

Staphylococcus aureus is one of the leading causes of hospital- and community-acquired infections (1, 2). This microorganism has a remarkable ability to rapidly adapt to antibiotic pressure and acquire resistance (3–6). Extensive use of β-lactam antibiotics has invariably been followed by the development and spread of resistance in S. aureus (7–11). Meticillin, a β-lactam antibiotic designed to circumvent β-lactam-resistant S. aureus, met with resistant strains soon after its introduction into clinics. Meticillin-resistant S. aureus strains pose a serious threat to human health as they have become resistant to almost all the clinically used antibiotics (6, 7, 12). Emergence of intermediate- vancomycin-resistant S. aureus strains (13, 14) has limited the chemotherapeutic treatment options against meticillin-resistant S. aureus (15).

One of the main factors of methicillin resistance in S. aureus is the penicillin-binding protein PBP2a (16). Penicillin-binding proteins (PBPs) are involved in the final steps of peptidoglycan biosynthesis, and they are the targets of the β-lactam antibiotics. It has been suggested that PBP2a takes over the biosynthesis of peptidoglycan in the presence of otherwise lethal doses of β-lactam antibiotics (16). Several studies have demonstrated that additional factors are involved in the expression of high and homogeneous meticillin resistance levels in meticillin-resistant S. aureus (17–30). A few of these additional factors, notably the proteins encoded by the fem genes, have been characterized functionally (17, 30, 31). Among the identified meticillin resistance factors, fmtA in particular has been reported to be part of the cell wall stimulus (32–35), which is a group of genes that is commonly induced upon treatment with cell wall-active antibiotics, such as β-lactams. Furthermore, inactivation of fmtA has been shown to affect peptidoglycan structure (24), suggesting that this gene is an active participant in peptidoglycan biosynthesis under antibiotic-induced cell wall stress conditions. Because β-lactams remain the primary therapeutics for treating bacterial infections, understanding the molecular mechanism of resistance to these antibiotics is essential.

In this study we show for the first time that FmtA is a novel penicillin-binding protein with high resistance to inactivation by β-lactam antibiotics. We propose a mechanism explaining the resistance of FmtA to β-lactams and reveal the potential FmtA role in meticillin resistance and the S. aureus response to cell wall-active antibiotics.

EXPERIMENTAL PROCEDURES

Materials and Chemical Reagents—Antibiotics and other chemicals were purchased from Sigma or Fisher unless otherwise stated. Growth media were purchased from EMD Bioscience. Chromatography media and columns were from GE Healthcare. Escherichia coli NovaBlue and BL21 (DE3) strains pETBlue-1 and pET24a(+) were from Novagen. Bocillin was purchased from Molecular Probes, Inc. The enzymes used for cloning were purchased either from New England Biolabs or Stratagene. The genome of S. aureus strain Mu50 was obtained from ATCC.

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The abbreviations used are: PBP, penicillin-binding protein; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; HPLC, high pressure liquid chromatography; DD peptidases, d-alanyl-d-alanine-carboxypeptidases; nPAGE, native PAGE.
Cloning of the fmtAΔ27 Gene—The DNA sequence data of fmtA was amplified from the genome of S. aureus strain Mu50 (ATCC) by PCR using Pfu Turbo™ polymerase and the oligonucleotide primers 5′-AGC CAT ATG CGA TTA CAG ACG AAG ACA CAT TCT A-3′ (forward) and 5′-AGC TAT ATT GAA CAA TAA CAC CCT TCG-3′ (reverse). Two restriction sites, Ndel and HindIII, were introduced on the forward and reverse primers (the italicized sequence), respectively, to facilitate cloning of fmtA. The fmtA amplicon produced using the above primers did not include the first 81 nucleotides of the gene. This sequence encodes for the signal peptide that was removed to facilitate cytoplasmic expression. We refer to this amplicon as fmtAΔ27.

The fmtAΔ27 gene was cloned into the EcoRV restriction site of the pETBlue-1 vector (Novagen), and a silent mutation was introduced in fmtAΔ27 to remove the Ndel restriction site normally found in fmtA and enable cloning of fmtAΔ27 into the Ndel and HindIII sites of pET24a(+). The mutation was introduced by QuikChange™ site-directed mutagenesis (Stratagene) using two mutagenic primers, 5′-CCT AAG AAA AAC AAG CAC ATG TAT ACG GAT GGG-3′ (forward) and 5′-CCC ATC GTT ATA CAT GTG TTT TGT TGA TTA ATC TCG AGG-3′ (reverse) (mutated bases are italicized). The pETBlue-1::fmtAΔ27 construct was digested with Ndel and HindIII to liberate the insert, which was subsequently ligated into the Ndel and HindIII sites of pET24a(+). E. coli NovaBlue cells were transformed with pET24a(+):fmtAΔ27, and successful cloning was confirmed by DNA sequencing.

An alanine residue (encoded by GCA) was inserted immediately after the N-terminal methionine by QuikChange™ site-directed mutagenesis using the following primers: Dir: 5′-GGA GAT ATA CAT ATG GCA CGA TTA CAG ACG AAG ACA C-3′ and Rev: 5′-GTG TCT TCT TCT GTA ATC GTG CCA TAT GTA TAT CTC C-3′ (the inserted nucleic acids are italicized in both primers). Successful insertion of alanine was confirmed by DNA sequencing, and the final pET24a(+):fmtAΔ27 construct was used to transform an E. coli BL21 (DE3) strain.

Purification of FmtAΔ27—A single colony of E. coli BL21 (DE3) harboring pET24a(+):fmtAΔ27 was used to inoculate 5 ml of Luria Bertani broth (LB) supplemented with 30 μg/ml kanamycin A. The culture was allowed to grow overnight at 37 °C followed by a 200-fold dilution into fresh Terrific Broth medium supplemented with 30 μg/ml kanamycin A, 0.4 M D-sorbitol, and 2.5 mM β-galactopyranoside. The cell culture was grown at 37 °C with shaking to an optical density (OD)600 nm of ~0.6. The cell culture was then cooled down to 25 °C, and protein expression was initiated by the addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1 mM. Subsequently, the cell culture was shaken at 25 °C for 16 h.

Cells were harvested by centrifugation at 10,816 × g for 10 min. The pellet was resuspended in 50 mM phosphate buffer (pH 7.2), and the cytoplasmic content was liberated by sonication. The resulting suspension was centrifuged at 20,199 × g for 1 h at 4 °C. The soluble protein fraction was loaded onto the SP-Sepharose FF column equilibrated with 50 mM phosphate buffer (pH 7.2). FmtAΔ27 was eluted with a linear gradient of 0–1.0 M NaCl in 50 mM phosphate buffer (pH 7.2). Fractions containing FmtAΔ27 were concentrated and loaded onto HiPrep™ butyl FF (16/10) column equilibrated with 50 mM phosphate buffer (pH 7.2) supplemented with 1 M ammonium sulfate. FmtAΔ27 was eluted with a linear gradient of 1.0 to 0 M ammonium sulfate. Fractions containing FmtAΔ27 were concentrated and loaded to a Superdex™ 75 (10/300GL) column equilibrated with 50 mM phosphate buffer (pH 7.2). Gel filtration fractions were analyzed by SDS-PAGE to assess the purity of the isolated protein. FmtAΔ27 was concentrated to 4–5 mg/ml and stored at 4 °C until further use. All the above operations were carried out at 4 °C. The concentration of the purified FmtAΔ27 protein was determined by UV spectrophotometry using an extinction coefficient of ε280 = 56,160 M⁻¹ cm⁻¹ (Cary 100 Bio UV-visible spectrophotometer).

A Superdex™ 75 (10/300GL) column was used to analyze the oligomerization state of FmtAΔ27 in solution. The column was equilibrated with 50 mM sodium phosphate (pH 7.2) and calibrated with a low molecular weight calibration kit (GE Healthcare). The concentration of the protein loaded was 100 μM. The N-terminal sequencing of the isolated protein was achieved by sequential Edman reaction carried out at the Advanced Protein Technology Center, Sick Kids’ Hospital (Toronto, Canada).

Investigation of the Putative Function of FmtA Using a Computational Approach—The amino acid sequence of FmtA was initially aligned by PSI-Blast (position-specific iterated BLAST) (36, 37) against the Swiss-Prot data base and then against the Protein Data Bank data base. The Swiss-Model, First Approach mode (38, 39), was used to build a three-dimensional homology model of FmtA. The Kyte-Doolittle hydropathy prediction method was used to examine the presence of hydrophobic regions in FmtA (40).

Probing the Interaction of FmtAΔ27 with β-Lactams—Acidic 15% polycrylamide gel electrophoresis (nPAGE)3 was used to analyze the interaction of FmtAΔ27 with Bocillin Fl (Bocillin), a fluorescently labeled penicillin G derivative. Acidic conditions in nPAGE were employed due to the high pI of FmtA (∼9.3), and under these conditions FmtAΔ27 was well resolved. Under basic conditions (using a Tris-glycine buffer) the protein did not penetrate the gel.

In concentration-dependent experiments, a typical reaction mixture (15 μl) consisted of 15 μM FmtAΔ27 and Bocillin at concentrations ranging from 0 to 120 μM in 50 mM sodium phosphate (pH 7.0). The reaction mixtures were incubated at 37 °C for 4 h. In the time-dependent experiments, a typical reaction mixture (15 μl) consisted of 15 μM FmtAΔ27 and 120 μM Bocillin incubated in 50 mM sodium phosphate buffer (pH 7.0) for different time intervals. Reaction mixtures were incubated at room temperature and quenched by the addition of the gel electrophoresis sample buffer. Gel imaging was performed using Fluorchem™ FC, and fluorescent bands were quantified using the AlphaEaseFC™ software. As a positive control in these experiments, we used the penicillin binding domain of the transmembrane signal-transducing protein BlaR, BlaR5 (42). Acylation of BlaR5 by Bocillin occurs within seconds (42).
Circular Dichroism (CD) Spectroscopy Studies—Far-UV CD spectra (200 nm-260 nm) of FmtA277 (14 μM in 50 mM sodium phosphate (pH 7.0)) were recorded on a Jasco J-810 instrument using a cuvette with a 1.0-mm light path length. The experiments were carried out at 22 °C in the presence or absence of 150 μM penicillin, ampicillin, carbenicillin, oxacillin, cephalaxin, cephalosporin C, cephalothin, or cephalosporin. Each mixture of protein and β-lactam was incubated for 1 h at 37 °C before the data acquisition. Typically, three scans were averaged and corrected for buffer and β-lactam background signals.

Thermal Denaturation Studies—Thermal denaturation of FmtA277 (14 μM in 50 mM sodium phosphate (pH 7.0)) in the presence or absence of 150 μM penicillin G was examined by monitoring the CD signal at 222 nm at different temperature points that varied from 10 to 100 °C (data were collected every 2 degrees over 30 s).

Tryptophan Fluorescence Quenching Studies—The intrinsic fluorescence emission of FmtA277 (14 μM in 50 mM sodium phosphate (pH 7.0)) was monitored between 300 and 400 nm in the absence and presence of penicillin G (167 mM). The single tryptophan residue of FmtA277, Trp-158, was excited at 295 nm. Spectra were collected at 22 °C in a RF-5301PC spectrofluorophotometer using a cuvette with a 0.4-cm light path length. Each data point was collected over 10 s, and 3 samples were prepared for each experiment. The collected spectra were corrected for the extinction background of buffer and penicillin G.

In another series of experiments we used acrylamide as a quenching reagent of Trp-158 emission. The emission of the protein (12 μM in 50 mM sodium phosphate (pH 7.0)) was recorded at 340 nm (excitation at 295 nm) in the presence of acrylamide at different concentrations (100–350 mM). Similar experiments were carried out with FmtA277 incubated with penicillin G (150 μM, 1 h at 22 °C) before the addition of acrylamide. Three independent sets of experiments were carried out under these conditions. Data were analyzed using the Stern-Volmer equation, \( F/F_0 = 1 + Q \cdot K_{SV} \) (where \( K_{SV} \) is the Stern-Volmer quenching constant, \( Q \) is the concentration of the quencher (acrylamide), and \( F \) and \( F_0 \) are the fluorescence intensities in the absence and the presence of acrylamide, respectively. The values of \( K_{SV} \) were obtained from the linear regression analysis of the experimental data using Grafit 5.0.

Analysis of the Putative Acyl-enzyme Species by Mass Spectrometry—Purified FmtA277 (20 μM) was incubated with or without Bocillin (160 μM) in 50 mM phosphate supplemented with 150 mM NaCl (pH 7.0). Samples were desalted on a C4 reversed-phase HPLC column (Waters Symmetry 300 μm) using a water/CH3CN gradient in the presence of 0.05% trifluorooracetic acid. They were analyzed on a Micromass quadrupole-time-of-flight electrospray ionization mass spectrometer coupled to a Waters 1525μ L HPLC system.

To identify the reactive nucleophile, mixtures of FmtA277 and Bocillin were digested with trypsin (ProteoExtract kit, Calbiochem). Digested protein samples were first desalted and concentrated using a Millipore Zip-Tip®. Peptides were eluted off the tip with 1 μl of 0.1% TFA/4% hydroxy-cinnamic acid matrix (10 mg/ml in 60% acetonitrile and 0.3% trifluorooracetic acid). The solution was spotted on a Perseptive Biosystems MALDI target plate. MS and MS/MS experiments were carried out using an AB/SCIEX QStar XL® hybrid quadrupole/time-of-flight mass spectrometer.

The identity of the unmodified peptide of interest was confirmed by searching the MS and MS/MS spectra against the NCBI non-redundant data base using Matrix Science MASCOT® software. The identity of the modified peptide was also verified by means of MS/MS and submission of the resulting spectrum to MASCOT followed by manual inspection. Error tolerances for both automated and manual searches were 100 ppm for tryptic peptides and 0.2 Da for fragment masses. Several ions corresponding to y fragments with the modification still intact were identified manually.

Binding of FmtA277 to Peptidoglycan—These experiments were carried out following a published protocol with minor modifications (43). Briefly, peptidoglycan from S. aureus RN6390 was isolated as described previously (44). FmtA277 (10, 30, and 60 μg) was incubated with 100 μg of peptidoglycan for 2 h at 4 °C. Two controls were prepared at the same time, 1) FmtA277 (10 μg) incubated in the reaction buffer alone and 2) a mixture of chymotrypsinogen A (10 μg), albumin (10 μg), and ribonuclease A (10 μg) incubated with 100 μg of peptidoglycan. After incubation, all samples were spun down at 353,000 × g to recover peptidoglycan in the pellet. The supernatant containing the unbound proteins was put aside for further analysis. The pellet was washed twice, and the soluble fractions, herein referred to as wash fractions (W1 and W2), were put aside for further analysis. The final pellet, containing the peptidoglycan, was re-dissolved in 2% SDS. All the collected fractions were analyzed by 12.5% SDS-PAGE.

RESULTS

Cloning and Purification of FmtA277—We cloned and purified the untagged mature FmtA protein from the S. aureus strain Mu50. The recombinant protein lacked the first 27 amino acids, which were predicted by the SignalP 3.0 program (45) to form a signal peptide sequence. In addition, an alanine residue was inserted into the sequence of FmtA immediately after the N-terminal methionine to increase the stability of the protein N terminus (N-end Rule in Ref. 46). The protein was expressed in the cytoplasm in a soluble form and was purified to homogeneity in three steps (Fig. 1). Herein we refer to this protein as FmtA277. The identity of the isolated FmtA277 protein was confirmed by N-terminal sequencing. The apparent molecular mass, measured by electrospray ionization mass spectrometry-MS, was 43,031.0 Da, in agreement with the calculated mass (43,031.5 Da).

Oligomerization State of FmtA277—FmtA277 appears as a mixture of monomer and dimer when analyzed by SDS-denaturing PAGE under reducing conditions (Fig. 1A). FmtA277 does not contain cysteine residues; thus, dimerization was not due to the formation of a disulfide bond. We investigated this phenomenon more closely and observed that the dimer:monomer ratio increased with boiling of the protein (data not shown). Dimerization was not observed by nPAGE (Fig. 1B).

The apparent molecular mass of FmtA277 calculated by gel filtration chromatography at pH 7.0 was 43,048 Da (Fig. 1C), indicating that FmtA277 is monomeric under physiological condi-
FmtA, the Fifth Penicillin-binding Protein of S. aureus

FIGURE 1. Polyacrylamide gel electrophoresis of FmtA\textsuperscript{\textregistered}27. A, 12.5% SDS-PAGE. Lane 1, protein marker. Labeled protein bands have molecular masses 83.4 kDa (a), 47.5 kDa (b), and 32.5 kDa (c). Lane 2, purified FmtA\textsuperscript{\textregistered}27 (dimer (d); monomer (m)). B, 10% nPAGE. Lane 1, ribonuclease A (13.7 kDa). Lane 2, chymotrypsinogen A (27 kDa). Lane 3, albumin (43 kDa). Lane 4, albumin (M\textsubscript{r}, 67 kDa). Lane 5, FmtA\textsuperscript{\textregistered}27 (43 kDa). C, calibration of the size exclusion columns (Superdex\textsuperscript{TM} 75 (10/300GL)) using a LMW Calibration Kit (GE Healthcare). The retention volume (V\textsubscript{r}) of purified FmtA\textsuperscript{\textregistered}27 was determined under the same conditions as the protein standards.

tion and that the dimerized species observed by the SDS-PAGE represents an artifact induced by boiling.

Dimerization of FmtA\textsuperscript{\textregistered}27, as observed by SDS-PAGE, could result from non-covalent interactions mediated by the exposure of hydrophobic patches upon boiling (47). We tested this possibility by excising the dimer band from the gel slab, electroeluting the protein, and re-analyzing it by SDS-PAGE. The resulting gel once again showed monomeric and dimeric FmtA\textsuperscript{\textregistered}27 (data not shown), thus, strongly suggesting that formation of the dimer is indeed a non-covalent complex.

Sequence Alignment and Homology Modeling—A search for a putative function of \textit{fmtA} by aligning its sequence against sequenced bacterial genomes resulted in the classification of the protein as a \( \beta \)-lactamase. A closer look at the sequence alignments revealed that FmtA aligned against PBPs and putative class \( \beta \)-lactamases from different organisms. Most of them have not been functionally characterized. Therefore, we restricted the sequence alignment to proteins with known three-dimensional structures. This approach revealed that FmtA has sequence homology to \( \beta \)-allyl-\( \beta \)-alanine-carboxypeptidase from \textit{Streptomyces} R61 (R61 DD-peptidase; \( E = 5 \times 10^{-12} \)) and 908R \( \beta \)-lactamase, a class \( C \) \( \beta \)-lactamase from \textit{Enterococcus cloacae} (\( E = 1 \times 10^{-6} \)) (Fig. 2).

The sequence alignments revealed the presence in FmtA of two of the three conserved motifs of serine active-site PBPs and \( \beta \)-lactamases: SXKK, Y(S)XN, and H(K/R)T(S)G, where X is any amino acid. In FmtA, the first of these motifs has the sequence Ser\textsuperscript{27}-Ala-Gln-Lys (residues in italic represent the non-conserved residues) (Fig. 2). Interestingly, the amino acid that precedes this sequence motif in FmtA is Gly, the same as in R61 DD-peptidase and 908R \( \beta \)-lactamase. The SXKK motif in PBPs and \( \beta \)-lactamases lies at the N terminus of an \( \alpha \)-helix, and this could also be true in FmtA.

The second sequence motif has the sequence Tyr\textsuperscript{211}-Asn-Asp in FmtA. Even though this sequence displays an aspartate instead of the conserved asparagin, it consistently aligns against the Y(S)XN motif of R61 DD-peptidase, 908R \( \beta \)-lactamase, and other PBPs, or class \( C \) \( \beta \)-lactamases in the sequence alignment results.

An interesting feature of FmtA, predicted by the Kyte-Doolittle hydropathy method, is the presence of a hydrophobic-rich region (amino acids 350–370) at the C terminus (data not shown). This hydrophobic-rich region could mediate anchoring of the protein on the outer leaf of the cytoplasmic membrane, which is consistent with a previously reported result (24).

We generated a homology model of FmtA (spanning the amino acids 75–251) based on the crystal structure of R61 DD-peptidase (Protein Data Bank code 1mpl) to examine important predicted structural characteristics. The sequence identity between the two sequences is 28\%, not very high but common among different classes of PBPs and \( \beta \)-lactamases. Despite the relatively low sequence identity, the structural features that define the active site of PBPs and \( \beta \)-lactamases are remarkably the same (48). Hence, the FmtA homology model could be a relevant representation of the FmtA structure. The FmtA homology model superposes well with R61 DD-peptidase structure. The side chains of the residues Ser-127, Lys-130, Tyr-211, and Asp-213 overlap well with their homolog residues in R61 DD-peptidase (Fig. 3). The FmtA homology model does not extend to the C terminus of the protein (amino acids 251–397). In R61 DD-peptidase this region folds into a \( \beta \)-sheet, typical of PBPs and \( \beta \)-lactamases, and houses the KTG (48).

Interaction of FmtA\textsuperscript{\textregistered}27 with \( \beta \)-Lactams—Bocillin, a fluorescently labeled penicillin G derivative, was used to probe the interaction between FmtA\textsuperscript{\textregistered}27 and \( \beta \)-lactams. This reagent has been used routinely to characterize PBPs (42, 49–51). Reaction mixtures of FmtA\textsuperscript{\textregistered}27 with Bocillin were analyzed by SDS-PAGE (Fig. 4A). Under these denaturing conditions both the monomeric and dimeric band of FmtA\textsuperscript{\textregistered}27 were fluorescently labeled (Fig. 4A), suggesting that Bocillin is covalently bound to FmtA\textsuperscript{\textregistered}27, possibly forming a putative acyl-enzyme species. A previous study had failed to show interaction between a His-tagged FmtA and \( \beta \)-labeled penicillin (24).

Partitioning of FmtA\textsuperscript{\textregistered}27 between dimeric and monomeric oligomerization states complicated the quantitative analysis of the interaction with Bocillin. The results from nPAGE experi-
Time-dependent experiments enabled monitoring of the formation of putative acyl-enzyme species between FmtA<sup>27</sup> and Bocillin (Fig. 4D). This process is relatively slow, with up to 40% of the protein forming a covalent complex with Bocillin over 24 h at 22 °C (determined by SDS-PAGE) (Fig. 4D). Under these conditions there is no significant Bocillin turnover, as determined by absorption measurements at 482 nm using a chromophoric β-lactam, nitrocefin (data not shown). The slow rate of the formation of putative acyl-enzyme species implies that FmtA<sup>27</sup> is largely insensitive to inactivation by β-lactams. This could explain why previous efforts were not successful in demonstrating PBP activity for FmtA (24) although the presence of a His<sub>6</sub> tag at the N terminus of FmtA could have affected the activity of the protein.

**CD Spectroscopy and Thermal Denaturation**—Far-UV CD measurements reveal that FmtA<sup>27</sup> undergoes significant conformational changes in the presence of either penicillins or cephalosporins (CD of FmtA<sup>27</sup> with penicillin and cephalosporin C are presented in Fig. 5, and the other antibiotics are presented in supplemental Fig. 2). These changes occur at the secondary structural level and could be initiated by the formation of pre-acylation complex and culminate at the formation of acyl-enzyme species. To investigate the effect these conformational changes have on the overall FmtA<sup>27</sup> stability, we determined the midpoint thermal denaturation of the protein in the presence and absence of penicillin G. These experiments indicate that binding of penicillin G increases the thermodynamic stability of FmtA<sup>27</sup> by 4 degrees, from 48 to 52 °C (data not shown).

**Quenching of the Trp-158 Fluorescence Emission**—The fluorescence emission of tryptophan residues in proteins is highly sensitive to their local environment (54, 55). We monitored the emission spectra of the single tryptophan residue of FmtA<sup>27</sup>, Trp-158, to shed light on the interaction between FmtA<sup>27</sup> and Bocillin turnover, as determined by absorption measurements at 482 nm using a chromophoric β-lactam, nitrocefin (data not shown). The slow rate of the formation of putative acyl-enzyme species implies that FmtA<sup>27</sup> is largely insensitive to inactivation by β-lactams. This could explain why previous efforts were not successful in demonstrating PBP activity for FmtA (24) although the presence of a His<sub>6</sub> tag at the N terminus of FmtA could have affected the activity of the protein.

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**CD Spectroscopy and Thermal Denaturation**—Far-UV CD measurements reveal that FmtA<sup>27</sup> undergoes significant conformational changes in the presence of either penicillins or cephalosporins (CD of FmtA<sup>27</sup> with penicillin and cephalosporin C are presented in Fig. 5, and the other antibiotics are presented in supplemental Fig. 2). These changes occur at the secondary structural level and could be initiated by the formation of pre-acylation complex and culminate at the formation of acyl-enzyme species. To investigate the effect these conformational changes have on the overall FmtA<sup>27</sup> stability, we determined the midpoint thermal denaturation of the protein in the presence and absence of penicillin G. These experiments indicate that binding of penicillin G increases the thermodynamic stability of FmtA<sup>27</sup> by 4 degrees, from 48 to 52 °C (data not shown).
suggested that the microenvironment of Trp-158 is altered as a result of penicillin G binding to FmtA<sup>Δ27</sup>, which is consistent with the CD data (Fig. 5).

We used another technique, tryptophan emission quenching by acrylamide, to further analyze events that occur upon binding of β-lactams to FmtA<sup>Δ27</sup> (56). The solvent accessibility of Trp-158 residue was determined from the Stern-Volmer plots (Fig. 6B). These plots displayed linear trends in the absence and presence of penicillin G, indicating that acrylamide molecules directly contact the indole group of Trp-158 (56). The calculated <i>K</i><sub>SV</sub> value decreased in the presence of penicillin G from 3.0 ± 0.1 to 1.9 ± 0.1 (Fig. 6B), pointing to a decrease in solvent accessibility of the Trp-158 side chain in the presence of penicillin G. These results indicate that β-lactam binding to FmtA<sup>Δ27</sup> affects the tertiary structure of the protein as well.

**Analysis of the Putative Acyl-enzyme Species**—Direct evidence for the formation of a covalent linkage between FmtA<sup>Δ27</sup> and Bocillin was obtained by electrospray ionization MS. Two species were evident under the strongly denaturing electrospray ionization MS conditions, with respective masses of 43,031 ± 2 and 43,671 ± 2 Da (Fig. 7). The first mass corresponds to apoFmtA<sup>Δ27</sup> (expected mass 43,031.5 Da), whereas the second mass is consistent with an acyl-enzyme species formed between FmtA<sup>Δ27</sup> and Bocillin (43,031 + 639 = 43,671 Da). The capability of FmtA<sup>Δ27</sup> to form this covalent species provides strong evidence for the notion that FmtA<sup>Δ2</sup> acts as a PBP.

Having established that β-lactams bind covalently to FmtA<sup>Δ27</sup>, we undertook the identification of the modification site in the protein. A reaction mixture of FmtA<sup>Δ27</sup> and Bocillin was subjected to trypsin digest and analyzed by MALDI-MS. The apofmtA<sup>Δ27</sup> was used as a control. The mass spectrum of FmtA<sup>Δ27</sup> was obtained by electrospray ionization MS. Two ions were confirmed to correspond to the amino acid sequence <i>17</i>TPPTMGFLIGSAQK, carrying the sequence (italicized) motif Ser-X-X-Lys. MS/MS of the m/z 2161.0 ion unambiguously identified Ser-127 as the modification site of FmtA<sup>Δ27</sup> (Fig. 8), thereby identifying this residue as the active-site nucleophile of FmtA<sup>Δ27</sup>.

**In Vitro Binding of FmtA<sup>Δ27</sup> to Peptidoglycan**—FmtA<sup>Δ27</sup> was pulled down by peptidoglycan in <i>in vitro</i> assays (Fig. 9A). This is possible only if FmtA<sup>Δ27</sup> binds to peptidoglycan with relatively high affinity; otherwise, the complex would dissociate during washing steps. In a separate set of experiments, we investigated the dose-dependent binding of FmtA<sup>Δ27</sup> to peptidoglycan. The amount of FmtA<sup>Δ27</sup> recruited to peptidoglycan increased with the increase of FmtA<sup>Δ27</sup> amount in the reaction mixture (Fig. 9A). In contrast, none of the control proteins was pulled down by peptidoglycan (Fig. 9B), which is a strong indication that FmtA<sup>Δ27</sup> binds to peptidoglycan through specific interactions.

**DISCUSSION**

Evidence presented in this study supports the assertion that FmtA is a penicillin-binding protein. We
show for the first time that FmtA$^{27}$ is capable of forming stable acyl-enzyme species with $\beta$-lactams through the active-site nucleophile Ser-127. This residue is part of the sequence motif Ser-$X$-$X$-Lys, which is conserved among the serine-active site PBPs and $\beta$-lactamases.

We identified in FmtA the second conserved motif of PBPs and $\beta$-lactamases, the sequence Ser(Tyr)-$X$-Asn which is also known as the SXN loop. Like R61 DD-peptidase, the first position in this motif is occupied by a tyrosine, Tyr-211. In R61 DD-peptidase this residue activates the active-site nucleophile (57, 58). It is of note that the second conserved residue in the SXN loop in FmtA is occupied by Asp-213. Typically, this position is occupied by Asn in PBPs and $\beta$-lactamases, but the presence of other amino acids such as Val, Ser, or Gly has also been reported (59). The Asn side chain forms hydrogen bonds with the active-site residues and the substrate (60, 61), which can also be facilitated by a protonated aspartate, as in the case of FmtA. Indeed, there are several PBPs that belong to class B of PBPs that carry an Asp at this position (62). The residue at this position in $\beta$-lactamases and PBPs has been proposed to tailor the enzyme substrate specificity, as mutation at this position altered substrate specificity (60, 63). Hence, presence of the Asp at this position in FmtA implies that substrate specificity could be unique to FmtA. Based on the above observations, it is very likely that the sequence motif Tyr$^{211}$-$X$-Asp$^{213}$ imparts in FmtA similar catalytic function as the motif Ser(Tyr)-$X$-Asn does in other PBPs, with Tyr-211 probably being the general base that activates Ser-127 and Asp-213 catalyzing the substrate specificity of FmtA.

The third typical motif of PBPs, namely the KTG box, appears to be absent in FmtA. Lys in this motif or His, as is found in DD peptidases, has been proposed to participate in catalysis and substrate binding in PBPs (64–66). Interestingly, the typical $\beta$-sheet region of PBPs, which houses the KTG box, is not modeled in FmtA. It is possible that FmtA adopts a different fold in this region, and conceivably, it has adopted a different catalytic mechanism from other PBPs.

Several reports have inferred that FmtA is involved with peptidoglycan biosynthesis (24, 32–35), but the function of FmtA is not known. We show that FmtA$^{27}$ is capable of binding to peptidoglycan in vitro with high binding affinity. It also appears that FmtA is a membrane-associated protein, with its C terminus serving as a membrane anchor, a feature that is common for low molecular weight and membrane-bound PBPs with transpeptidase activity (48, 59). In light of our findings that FmtA forms acyl-enzyme species with $\beta$-lactams, it is conceivable that FmtA could be a carboxypeptidase or a transpeptidase.

A previous study by Komatsuzawa et al. (24) reported that inactivation of fmtA causes a reduction in highly cross-linked peptidoglycan components. This report suggests that FmtA could be a transpeptidase that mediates production of highly cross-linked cell wall components. Interestingly, a study by Leski and Tomasz (67) showed that inactivation of a small molecular weight PBP in S. aureus, PBP4, highly sensitive to $\beta$-lactam inactivation, causes a decrease in the highly cross-linked peptidoglycan components. Based on the report by Komatsuzawa et al. (24), a carboxypeptidase activity by FmtA,
proteins that could be involved in regulation of peptidoglycan biosynthesis or its degradation (68). Interestingly, fmtA expression has been reported to increase in the presence of different cell wall-active antibiotics (24, 32–35). These observations support our proposal thatFmtA may directly compensate for peptidoglycan biosynthesis and indirectly prevent cell wall degradation.

The interaction of FmtA\textsuperscript{277} with Bocillin is characterized by a slow rate of formation of the acyl-enzyme species and a binding affinity that is in the range of \(\beta\)-lactam-resistant PBPs (51–53). Each of these observations indicates that FmtA is largely resistant to \(\beta\)-lactam inactivation. This feature of FmtA could explain earlier non-successful efforts to detect penicillin binding activity (24), although the N-terminal-His\textsubscript{6} tag used in that study could have interfered with the protein function.

As in the case of other PBPs, the low binding affinity that FmtA displays for \(\beta\)-lactams should not affect the ability of the protein to bind to peptidoglycan. First of all, the FmtA binding affinity for peptidoglycan can be higher than that measured for Bocillin. Second, the effective concentrations of the peptidoglycan components on the bacterial cytoplasmic membrane are thought to be high (69), and this would overcome the low binding affinity that FmtA might have for peptidoglycan. Also at first glance, the slow acylation rates of FmtA\textsuperscript{277} by Bocillin could be perceived as counterintuitive to the proposal that FmtA represents a PBP. However, the biological efficiency of the proposed FmtA\textsuperscript{277} activity cannot necessarily be inferred from the acylation rate with \(\beta\)-lactams, as they do not represent the native substrate. A good example is PBP2a. Studies have shown that even though \(\beta\)-lactams bind sluggishly to PBP2a (52), the transpeptidation reaction carried out by this protein is as efficient as for other PBPs.

The structural alterations that FmtA\textsuperscript{277} undergoes in the presence of \(\beta\)-lactams may provide clues to the FmtA\textsuperscript{277} resistance mechanism to \(\beta\)-lactam inactivation. The structural changes may be induced by the formation of pre-acylation complex and culminate at the formation of acyl-enzyme species. Similar phenomenon has been observed in PBP2a (52, 70) and the penicillin-binding domain of the transmembrane signal transducer BlaR1 protein (42, 71). The latter protein is involved in the regulation of \(\beta\)-lactamase expression in \textit{S. aureus} (71). It appears that FmtA\textsuperscript{277} is thermodynamically more stable in the bound state than in the apo state. We speculate that initial binding of \(\beta\)-lactam (pre-acylation complex) to FmtA may not be favorable due to steric clashes with the active site residues, e.g., resulting from a closed conformation of the active site in the apo state. As the pre-acylation complex approaches the transition state, local conformational changes in the active site may

It has been reported that inactivation of fmtA causes \textit{S. aureus} to lyse more rapidly in the presence of Triton X-100 (Triton X-100 is used to solubilize away membrane-associated proteins) (24). This suggests that FmtA may be involved in regulation of the activity of a peptidoglycan hydrolyase, conceivably by recruiting the enzyme to peptidoglycan. This is in agreement with the current knowledge that PBPs, besides their direct role in peptidoglycan synthesis, may interact with other...
facilitate the transition state to proceed toward acyl-enzyme species, e.g. active site opens up. Once the covalent linkage has been established the protein becomes permanently trapped in an altered and stable conformation. The proposed mechanism is supported by the low binding affinity ofFmtA for β-lactams and slow acylation rates. In vivo, this mechanism would enableFmtA to discriminate between the native substrate and surrogate substrates, such as β-lactams. A similar mechanism has also been proposed for PB2a; in its apo state, PB2a has been suggested to participate in peptidoglycan cross-linking, whereby PB4 mediates production of highly cross-linked peptidoglycan components (67). PB2 is unique in that it is the only bifunctional PBP ofS. aureus capable of carrying out transpeptidation and transglycosylation functions (76, 77). All the native PBPs are sensitive to β-lactam inactivation, although PB2 has been associated with resistance to β-lactam antibiotics, albeit indirectly. The glycosyltransferase domain of PB2 coordinates with PB2 to synthesize a functional peptidoglycan in the presence of β-lactam antibiotics (16).FmtA is the only native PBP intrinsically resistant to β-lactam inactivation. It is tempting to speculate that in the presence of β-lactam antibiotics, all the other native PBPs ofS. aureus are inactivated,FmtA works together with PB2 to synthesize a functional cross-linked peptidoglycan. Conceivably, FmtA takes over the PB4 function in facilitating the biosynthesis of highly cross-linked peptidoglycan components. This proposal is in agreement with observations that inactivation of fmtA reduces methicillin resistance and peptidoglycan cross-linking (24). It is also consistent with the recent transcriptomics studies that have identified fmtA as an active participant of theS. aureus response to cell wall-active antibiotics (24, 32–35).

In summary, our study provides for the first time highlights to the biological function and biochemical properties ofFmtA and new insights into the role ofFmtA in methicillin resistance and overallS. aureus response to cell wall-active antibiotics. FmtA unique features, such as resistance to β-lactam inactivation and regulation of its gene expression in the presence of cell wall-active antibiotics, warrant further investigation of the role of this protein in peptidoglycan biosynthesis.

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