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Characterization of Peptidoglycan in *Fem*-deletion Mutants of Methicillin-resistant *Staphylococcus aureus* by Solid-State NMR†

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Abstract

Compositional analysis of the peptidoglycan (PG) of a wild-type methicillin-resistant *Staphylococcus aureus* and its *fem*-deletion mutants has been performed on whole cells and cell walls using stable-isotope labeling and rotational-echo double-resonance NMR. The labels included [1-¹³C, ¹⁵N] glycine and L-[ε-¹⁵N]lysine (for a direct measure of the number of glycyl residues in the bridging segment), [1-¹³C]glycine and L-[ε-¹⁵N]lysine (concentration of bridge-links), and D-[1-¹³C]alanine and [¹⁵N]glycine (concentrations of cross-links and wall teichoic acids). The bridging segment length changed from 5.0 glycyl residues (wild-type strain) to 2.5±0.1 (*FemB*) with modest changes in cross-link and bridge-link concentrations. This accurate *in situ* measurement for the *FemB* mutant indicates a heterogeneous PG-structure with 25% monoglycyl- and 75% triglycyl-bridges. When the bridging segment was reduced to a single (1.0±0.1) glycyl residue (*FemA*), cross-linking decreased by more than 20%, resulting in a high concentration of open N-terminus glycyl segments.

Keywords

Fem factors; magic-angle spinning; rotational-echo double resonance; transglycosylase; transpeptidase

Methicillin-resistant *Staphylococcus aureus* (MRSA) is the leading pathogen for nosocomial infections (1). The mechanism of β-lactam antibiotic resistance in MRSA is related to the chromosomal *mec* determinant containing gene *mecA* (2), which encodes for the low-affinity penicillin-binding protein PBP2a (alternatively referred to as PBP2') that participates in cell-wall biosynthesis (3). The level of β-lactam antibiotic resistance in MRSA also depends on *fem* (factors essential for methicillin resistance) factors (4–6), also called *auxiliary* factors

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Supporting Information Available. (i) Growth curves of the bacteria used in this report in defined medium (SASM); (ii) ¹⁵N{¹³C} REDOR spectra of whole cells of wild-type *S. aureus* (BB255), *FemA*, and *FemB*, enriched with L-[ε-¹⁵N]lysine and [1-¹³C]glycine or [¹⁵N]glycine and D-[1-¹³C]alanine; (iii) ¹³C{¹⁵N} REDOR spectra of cell-wall isolates enriched with [¹⁵N]glycine and D-[1-¹³C]alanine; (iv) complete accounting of cell-wall D-alanine, experimental deconvolution of the normalized 75-MHz carbonyl-¹³C spectra of wild-type *S. aureus* (BB255), and *FemB* (UT 34-2) and *FemA* (UK 17) mutants labeled by [¹⁵N]glycine and D-[1-¹³C]alanine; (v) ¹⁵N{¹³C} REDOR spectra of whole cells of wild-type *S. aureus* (BB255) and its *FemX* mutant (SR 18) grown in media containing 1% D-(+)-glucose or D-(+)-xylose, and labeled by L-[ε-¹⁵N]lysine and [1-¹³C]glycine; (vi) ¹³C{¹⁵N} REDOR spectra of whole cells of wild-type *S. aureus* (BB255) and its *FemX* mutant (SR 18) labeled by L-[ε-¹⁵N]lysine and [1-¹³C]glycine; (vii) ¹⁵N{¹³C} REDOR spectra of whole cells of wild-type *S. aureus* (BB255) grown in media containing 1% D-(+)-glucose or D-(+)-xylose, and labeled by L-[ε-¹⁵N]lysine and [1-¹³C]glycine; (viii) description of the D-alanine quantization in cell-wall isolates labeled by [¹⁵N]glycine and D-[1-¹³C]alanine; (ix) description of the *FemX* growth experiments; (x) sensitivity of the bacteria used in this report to lysostaphin and methicillin; (xi) tabulated selected REDOR dephasing values for both main text and supporting material. This material is available free of charge via the Internet at <http://pubs.acs.org>.

(7), that are unrelated to the *mec* determinant (2). *Fem* factors are known to affect β -lactam antibiotic resistance in MRSA indirectly through cell-wall metabolism (4–6). Some of these *fem* factors include *femX*, *femA*, and *femB*, which encode for the proteins *FemX*, *FemA*, and *FemB*, respectively. These *Fem* proteins are involved in peptidoglycan (PG) biosynthesis by enabling the sequential addition of glycines to the lysyl side chain of the PG stem of lipid II, the membrane-associated PG precursor which is attached to the lipid transporter C₅₅ (pyrophosphoryl-undecaprenol). *FemX* (8,9) presumably catalyzes the addition of the first glycyl unit, *FemA* (10–12), second and third units, and *FemB* (13), fourth and fifth glycyl units to complete the pentaglycyl bridge structure of the PG unit in lipid II. Complete *femAB* inactivation results in a *FemAB* null mutant (14–16), which has a similar structure to that of *FemA*.

Lipid II is the essential precursor required for transglycosylation, the incorporation of a PG unit to the growing linear glycan chain. The chemical structure of a repeating PG unit in *S. aureus* (Scheme 1, right panel) consists of a disaccharide, *N*-acetylglucosamine (GlcNAc) plus *N*-acetylmuramic (MurNAc) acid, and a pentapeptide stem (L-Ala-D-iso-Gln-L-Lys-D-Ala-D-Ala), with an attached pentaglycyl bridging segment (17). Transpeptidase transforms linear glycan chains to a three-dimensional meshed PG lattice by forming cross-links (2). Each cross-link is a peptide bond formed between the terminal glycine of the bridging segment of one glycan chain, and the D-alanine (the 4th amino acid) of the stem of an adjacent glycan chain. The terminal D-alanine (the 5th amino acid) of the stem is cleaved by transpeptidase upon formation of a cross-link. A schematic representation of a meshed PG structure for *S. aureus* and its *fem*-deletion mutants is shown in Scheme 1 (left panel).

The deletion of *femX* in *S. aureus* is lethal (8,9), whereas the deletion of *femA* and *femB* results in biosynthesis of precursors with PG-bridge segments consisting of mono- and triglycyl segments, respectively (4–6). Shortened bridging segments result in reduced separation between the adjacent glycan chains (Scheme 1). Hence the PG-tertiary structures of *fem*-deletion mutants are likely to differ from that of wild-type *S. aureus*. These variations are consistent with reduced sensitivity to lysostaphin digestion (4,5), a glycine-glycine endopeptidase which selectively cleaves the PG bridges. Therefore, the use of *S. aureus* and its isogenic *fem*-deletion mutants provides a unique opportunity to investigate the role of bridge length on the PG-tertiary structure. The bridges are systematically varied from penta- to tri- to mono-glycyl segments, while other enzymatic steps in PG biosynthesis are constant.

Our long-range goal is to understand the effect of bridge length on the 3-dimensional PG lattice in *S. aureus*. This will be accomplished using solid-state NMR in two steps: (i) determination of the PG composition of whole cells of a wild-type *S. aureus* (18) and its *femX* (8,9), *femA*-deletion (10–12), and *femB*-deletion (13) mutants (Table S1 in the supporting material); and (ii) investigation of the PG-tertiary structure of these bacteria. In this report, we describe the use of solid-state NMR to characterize the composition of intact cell walls in wild-type *S. aureus* and its *fem*-deletion mutants by determining the number of glycines in the PG-bridge, the degree of bridge-linking (ρ_{BL}), the degree of cross-linking (ρ_{CL}), and the average cross-link density (ρ). The analysis of PG composition of intact cell walls of these bacteria is the necessary first step for the subsequent investigation of PG-tertiary structure in which ¹⁹F-labeled glycopeptides are used as molecular NMR probes to examine structural trends in the same intact cell walls, to provide new insights into the assembly of the PG lattice, and to understand the mode of action of drugs that bind to the cell-wall lattice (19,20).

Experimental Section

Growth and Labeling of Whole Cells of Wild-type, FemA, and FemB *S. aureus*

Starter cultures of the wild-type *S. aureus* (BB255) (18) and its *FemA* (UK 17) (10–12), *FemB* (UT 34-2) (13) mutants, listed in Table S1 in the supporting material, were prepared by inoculating 5 mL of trypticase soy broth (TSB) in a test tube with a single colony obtained from a nutrient agar plate. The starter cultures were shaken at 200 rpm in an Environ-Shaker (Lab-Lines Instruments, Inc., Melrose Park, IL), maintained overnight at 37 °C, but not aerated. The overnight starter culture (1% final volume) was added to 2 L sterile *S. aureus* standard medium (SASM), six 1 L flasks each containing 330 mL. SASM, as described earlier by Tong et al. (21), contained the following on a per-liter basis: 10 g of D(+)-glucose; 1 g each of $K_2HPO_4 \cdot 3 H_2O$, KH_2PO_4 , and $(NH_4)_2SO_4$; 0.2 g of $MgSO_4 \cdot 7 H_2O$; 10 mg each of $MnSO_4 \cdot H_2O$, $FeSO_4 \cdot H_2O$, and NaCl; 5 mg each of adenine, cytosine, guanine, uracil, and xanthine; 2 mg each of thiamine-HCl (vitamin B₁), niacin (vitamin B₃), and calcium pantothenate (vitamin B₅); 1 mg each of riboflavin (vitamin B₂), pyridoxine-HCl (vitamin B₆), inositol, $CuSO_4 \cdot 5 H_2O$, and $ZnSO_4 \cdot 7 H_2O$; 0.1 mg each of biotin (vitamin B₇) and folic acid (vitamin B₉); and 0.1 g of all 20 common amino acids. The pH of SASM was adjusted to 7.0 with 1 M KOH, followed by sterile filtration (0.22 µm membrane).

The natural-abundance amino acids in SASM were replaced by either [1-¹³C, ¹⁵N]glycine and L-[ε-¹⁵N]lysine, or [1-¹³C]glycine and L-[ε-¹⁵N]lysine, or [¹⁵N]glycine and D-[1-¹³C]alanine, to incorporate specific ¹³C and ¹⁵N labels (Cambridge Isotope Laboratories, Inc. and Isotec) in the glycyI bridging segments, bridge-links, and cross-links, respectively, in the PG of whole cells (Scheme 1).

The cells were harvested at log-phase at an optical density O.D. of 0.6 at 660 nm by centrifugation at 10,000 g for 10 min at 4 °C in a Sorvall GS-3 rotor. Cell pellets were rinsed twice with 300 mL of ice-cold 40 mM triethanolamine hydrochloride (pH 7.0, adjusted with 1 M NaOH). The rinsed pellets were resuspended in 15 mL of the same buffer followed by rapid freezing and lyophilization. The resulting lyophilized whole-cells harvested from 2 L growth typically weighed 400 mg. Growth curves of the wild-type *S. aureus* (BB255) and its *FemA* (UK 17), *FemB* (UT 34-2) mutants in SASM are shown in Figure S1 in the supporting material.

Growth and Labeling of Whole Cells of FemX *S. aureus*

Because the inactivation of *fmbB* (postulated *femX* gene) is lethal (9), the *fmbB*-deletion mutant (SR 18) contains a temperature-sensitive plasmid with the *fmbB* gene under the regulation of a xylose promoter with erythromycin-resistance (8). To ensure the integration of the temperature-sensitive plasmid, all SR 18 growths were carried out at 42 °C in the presence of 10 µg/mL erythromycin (Sigma-Aldrich) in both solid and liquid media. The whole-cell samples were prepared by inoculating 1 L SASM (three 1 L flasks, each containing 330 mL) with an overnight starter culture of SR 18 grown in LB Broth supplemented with either 0.5% D(+)-glucose or D(+)-xylose. SASM contained either 1% D(+)-glucose or D(+)-xylose as the sole carbon source to enhance or to repress the *fmbB* expression, respectively. The natural abundance glycine and lysine in SASM were replaced by labeled [1-¹³C]glycine and L-[ε-¹⁵N]lysine. Whole-cells were harvested at OD_{660 nm} 0.6 and subsequently prepared and lyophilized as described earlier (20,21). The resulting lyophilized whole cells harvested from 1 L growth typically weighed 200 mg. The growth curve of SR 18 in SASM is provided in the supporting material as Figure S1.

Isolation of Peptidoglycan

Cell-wall isolates were prepared from lyophilized whole cells as previously described (20, 21). Briefly, lyophilized cells from the 2 L of log-phase growth were suspended in 100 mL of

sterile 0.025 M potassium phosphate buffer (pH 7.0), boiled for 30 min, and then chilled on an ice bath. To the cell suspension, DNase I (type II; from bovine pancreas, Sigma-Aldrich, 1 mg per 100 mg dry cell weight) was added and the mixture was transferred to a 250 mL Bead-Beater (Biospec Products, Bartlesville, OK) chamber containing one-third 0.5-mm diameter glass beads. Cell disruption employed ten 1 min cycles separated by 1 min cooling periods at 0 °C. Glass beads were separated from the broken cells with a coarse sintered glass funnel (20 µm). The cells were washed with 1 L of 10 mM EDTA. Centrifugation of the filtrate at 10,000 g for 1 h at 4 °C provided crude cell walls.

Cell-wall isolates for NMR analysis were obtained from a suspension of the crude cell-wall pellet in a minimum amount of sterile 10 mM triethanolamine hydrochloride buffer (pH 7.0, adjusted with 1 M NaOH), which was added dropwise with stirring to 100 mL of boiling 4% sodium dodecyl sulfate (SDS). After boiling for 30 min, the suspension was allowed to cool for 2 h with stirring, after which it was allowed to stand unstirred overnight at room temperature, and sedimented by centrifugation at 38,000 g for 1 h at room temperature in a Sorvall SS-34 rotor. Walls were rinsed with 100 ml of buffer at least four times, with centrifugation after each rinse, until no SDS could be observed. The pellet was resuspended in 60 mL of 0.01 M Tris buffer, pH 8.2, containing 1 mg per 100 mg dry cell weight DNase I and 3.2 mg per 100 mg dry cell weight of trypsin (type II-S; from bovine pancreas, Sigma-Aldrich) and α -chymotrypsin (type II; from bovine pancreas, Sigma-Aldrich). The suspension was incubated at 37 °C and shaken at 150 rpm in an Environ-Shaker for 16 h and then sedimented at 38,000g for 1 h at 20 °C and washed at least four times with buffer, with centrifugation after each rinse. Cells were then resuspended in a minimum amount of 12 ml of the same buffer followed by rapid freezing and lyophilization. The resulting cell-wall isolates weighed approximately 150 mg.

Transmission Electron Microscopy

Overnight starter cultures of wild-type BB255, *FemA*, and *FemB* mutants of *S. aureus* grown in TSB were used to inoculate 40 mL of sterile SASM (1% final volume) in 125 ml flasks. Cells were grown at 37 °C and shaken at 200 rpm in an Environ-Shaker and were harvested at log-phase at an OD_{660 nm} 0.2 was reached by centrifugation at 2,750 g for 20 min at 4 °C (Eppendorf Centrifuge 5810R). For ultrastructural analysis, bacteria were fixed in 1 ml of 2% paraformaldehyde / 2.5% glutaraldehyde (Polysciences Inc.) in 100 mM phosphate buffer, pH 7.2 for 3 h at room temperature. Samples were washed in phosphate buffer and post-fixed in 1% osmium tetroxide (Polysciences Inc.) for 1 h. Samples were then rinsed extensively in distilled water prior to enbloc staining with 1% aqueous uranyl acetate (Ted Pella Inc.) for 1 h. Following several rinses in distilled water, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc.), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc.).

Solid-State NMR Measurements

Experiments were performed at 7 T (300 MHz for protons), and at 12 T (500 MHz for protons) provided by 89-mm bore Oxford (Cambridge, U.K.) and Magnex (Oxfordshire, U.K.) superconducting solenoids, respectively. Experiments performed at 7 T used a four-frequency transmission-line probe (22) having a 14-mm long, 9-mm inner-diameter sample coil, and a Chemagnetics/Varian magic-angle spinning ceramic stator. Samples were spun in Chemagnetics/Varian 7.5-mm outer-diameter zirconia rotors at 5 kHz, with the speed were under active control and maintained to within ± 2 Hz. Experiments were done at room temperature using a Chemagnetics CMX-300 spectrometer operating at 30 MHz for ¹⁵N and 75 MHz for ¹³C. Radio-frequency pulses were produced by 1-kW Kalmus, ENI, and American

Microwave Technology power amplifiers, each under active control (23); π -pulse lengths were 10 μ s for ^{13}C and ^{15}N . Proton-carbon and proton-nitrogen matched cross-polarization transfers were at 50 kHz for 2 ms. Proton dipolar decoupling was 105 kHz during data acquisition.

Experiments performed at 12 T used a six-frequency transmission-line probe having a 12-mm long and 6-mm inner-diameter sample coil and a Chemagnetics/Varian ceramic spinning module. Samples were spun using a thin-wall Chemagnetics/Varian (Fort Collins, CO/Palo Alto, CA) 5-mm outer-diameter zirconia rotor at 7143 Hz, with the speed under active control and maintained to within ± 2 Hz. A Tecmag Libra pulse programmer (Houston, TX) controlled the spectrometer. Two-kW American Microwave Technology power amplifiers were used to produce radio-frequency pulses for ^{15}N (50 MHz) and ^{13}C (125 MHz). The ^1H (500 MHz) radio-frequency pulses were generated by 2-kW Creative Electronics tube amplifiers driven by a 50-W American Microwave Technology power amplifier. All amplifiers were under active control (23). The π -pulse lengths were 8 μ s for ^{13}C and 9 μ s for ^{15}N . Proton-carbon matched cross-polarization transfers were made in 2 ms at 62.5 kHz. Proton dipolar decoupling was 100 kHz during data acquisition, and TPPM of the ^1H radio frequency (24) was used throughout both dipolar evolution and decoupling periods.

Rotational-echo double resonance (REDOR) is a solid-state NMR technique used to recouple heteronuclear dipolar interactions under magic-angle spinning (MAS) (25,26). REDOR experiments are performed in two parts: once with dephasing pulses (S) and once without (S_0). In the first part of the experiment, the dephasing pulses are off and magic angle spinning spatially averages chemical-shift and dipolar anisotropic interactions to produce a signal of full intensity. In the second part of the experiment, π pulses are applied on the dephasing channel to reintroduce the dipolar coupling between spins and produce a reduction in echo-signal intensity. The difference in signal intensity ($\Delta S = S_0 - S$) is directly related to the internuclear distance between the observed and dephasing spins. For the 1.2-kHz one-bond ^{13}C - ^{15}N dipolar coupling in a labeled peptide bond, total dephasing is observed after $8T_1$ (1.60 ms) with 5-kHz MAS, or after $12T_1$ (1.68 ms) with 7143-Hz MAS. The effect of a second weaker coupling is negligible because of dipolar truncation (27). The uncertainty associated with the REDOR dephasing measurements was estimated by integrals of ΔS (28). For all ΔS spectra, the uncertainty was less than 1%. REDOR spectra typically consisted of 4098 acquisition scans for ^{13}C and 20,000 acquisition scans for ^{15}N .

Results

Transmission Electron Micrographs

Transmission electron micrographs (TEM) of ultrathin sections of wild-type *S. aureus* (BB255) and *FemA* (UK 17) and *FemB* (UT34-2) mutants are shown in Figure 1. The cell-wall thickness of wild-type and *FemB* mutant was in the range of 20 nm (Figure 1, left and middle). Only the *FemA* mutant exhibited partial cell-wall thickening with a maximum thickness measured at 67 nm, indicated by the arrows in Figure 1 (top right panel). The *FemA* mutant also exhibited diffused staining at cross walls thickened in a range of 50–70 nm. This suggested an accumulation of immature peptidoglycan at the septum in association with aberrant growth. Deformed cell morphologies and aberrant growths with multiple septum formation were observed in both *fem*-deletion mutants (arrows).

Glycine Content in Whole Cells

The 75-MHz $^{13}\text{C}\{^{15}\text{N}\}$ REDOR spectra of whole cells of wild-type *S. aureus* and its *fem*-deletion mutants, grown on SASM medium labeled by [$1\text{-}^{13}\text{C}$, ^{15}N]glycine and L- $[\epsilon\text{-}^{15}\text{N}]$ lysine, after 1.60 ms dipolar evolution, are shown in Figure 2. Signals from the sugars in the PG glycan backbone appear around 70 ppm (29), and from the aliphatic carbons including the

lipids from the membrane, between 20–40 ppm (30). The peak at 149 ppm is from [1-¹³C] glycine metabolism in purine biosynthesis (31). The similarities of the wild-type and mutant natural-abundance ¹³C spectral profiles, as well as the similar intensities of purine peaks, suggest that no significant cytoplasmic compositional variations are present in the *fem*-deletion mutants. However, the large decrease in the 171-ppm intensities in the *S*₀ spectra of *FemB* and *FemA* compared to that in wild-type *S. aureus* shows qualitatively a reduction in the number of glycines in the bridge structures.

Quantitative measurements of glycine content in PG bridging segments of wild-type and *fem*-deletion mutants were made using REDOR NMR to select glycyI peptide bonds. Only ¹³C-¹⁵N spin pairs in peptide bonds contribute to the 171-ppm ¹³C{¹⁵N} Δ*S* after 1.60 ms of dipolar evolution (Figure 2, top). These ¹³C-¹⁵N spin pairs include the glycyI carbonyl-¹³C of [1-¹³C, ¹⁵N]glycine that forms a peptide bond with the ¹⁵N of another [1-¹³C, ¹⁵N]glycine, or an isopeptide bond with L-[ε-¹⁵N]lysine (Scheme 2). Hence, the 171-ppm peak intensity in the Δ*S* spectrum is directly proportional to the glycine content of the PG-bridge structure. For wild-type *S. aureus*, there are five ¹³C-¹⁵N peptide bonds in the PG-bridge structure that contribute to Δ*S*. Four ¹³C-¹⁵N peptide bonds are from four glycines in the glycine-glycine sequence, and the fifth is from the glycine-lysine (isopeptide bond) of the bridge link (Scheme 2). Because PG bridges shorter than five glycines constitute only a minor fraction in wild-type strains (32), the 171-ppm peak intensity in the Δ*S* spectrum of *S. aureus* BB255 was normalized to 5.0 (Figure 2, top inset). Thus, the corresponding glycine content in the PG of the *FemB* and *FemA* mutants is 2.5±0.1 and 1.0±0.1 glycine-equivalent units (GEU), respectively.

Although glycine-glycine peptide bonds in proteins also contribute to Δ*S*, this contribution is relatively small compared to that arising from the PG. First, the cytoplasmic dry weight is only two-thirds of the cell-wall dry weight (33). Second, the low frequency of glycine-glycine occurrences, estimated at 0.5% in protein (34), is minimal compared to the high frequency of glycine-glycine occurrences in PG of more than 50%. The relative protein contribution to Δ*S* increases in *fem*-deletion mutants due to the reduced number of glycines in the PG, but in all strains the glycine-glycine contribution from protein to Δ*S* remain below the uncertainty associated with the Δ*S* measurement, which is less than 1%. The relative glycine incorporation into protein is estimated by subtracting Δ*S* (glycine exclusively in PG) from *S*₀ (total glycine content), and is approximately 42% for BB255, 54% for *FemB*, and 72% for *FemA*.

Bridge-links in Cell-Wall Isolates

The 50-MHz ¹⁵N{¹³C} REDOR spectra of cell-wall isolates of wild-type *S. aureus* and its *fem*-deletion mutants labeled by [1-¹³C]glycine and L-[ε-¹⁵N]lysine are shown in Figure 3 (left). The ε-¹⁵N-lysyl amide resonance is at 92 ppm and identifies a bridge-link to a PG glycine, while the ε-¹⁵N-lysyl amine peak at 9 ppm is from a stem with no bridge attached. We assume that the incorporation of labeled lysine into undigested cell-wall protein is small compared to incorporation in PG (29). Thus, the ratio of the 92- to 9-ppm peak integrals in the *S*₀ spectrum is related to the degree of bridge-linking, ρ_{BL}, by

$$\frac{I(S_0^{\text{amide}})}{\alpha I(S_0^{\text{amine}})} = \frac{\rho_{\text{BL}}}{1 - \rho_{\text{BL}}}, \text{ with } \alpha = 1.2. \quad (1)$$

The correction factor α was used to account for the differences in rotating-frame cross-polarization dynamics between amides and amines in lyophilized whole cells and cell walls (35,36). These differences did not vary for BB255, *FemB*, and *FemA*. The integral of each peak was obtained as a function of cross-polarization contact time (35,36). The values of ρ_{BL} for all

strains are listed in Table 1. The errors are those of measuring the integrals which we estimate at $\pm 2\%$.

Cross-links in Cell-Wall Isolates

The 30-MHz $^{15}\text{N}\{^{13}\text{C}\}$ REDOR spectra of cell-wall isolates of wild-type *S. aureus* and its *fem*-deletion mutants labeled by $[^{15}\text{N}]$ glycine and D- $[1-^{13}\text{C}]$ alanine are shown in Figure 3 (right). The wild-type *S. aureus* and its *FemB* mutant have a similar amide (~ 92 ppm) to amine (~ 9 ppm) S_0 ratio. In contrast, the *FemA* mutant shows a significant increase in the amine-peak intensity, with an integrated amide-to-amine ratio of approximately 2:1. Therefore, *FemA* has a much higher percentage of glycyl amine, consistent with an increase in uncross-linked (open) monoglycyl bridges in its PG. Open bridges are the likely source of the shoulder observed in the ^{13}C ΔS spectrum of *FemA* (Figure 2, top inset, blue).

The percentage of cross-linking, ρ_{CL} , was determined by comparing the S_0 amide-to-amine ratio for $[^{15}\text{N}]$ glycine in isolated cell walls for all strains (Figure 3, bottom right). This is more direct than using the $^{15}\text{N}\{^{13}\text{C}\}$ $\Delta S/S_0$ which requires an accounting of the number of glycines contributing to S_0 , the isotopic enrichment of D-Ala, and any leakage of D- $[1-^{13}\text{C}]$ alanine label via pyruvate to $[1-^{13}\text{C}]$ glycine. (Such scrambling was minimal because the resulting ^{13}C labeling of aliphatics in the cell-wall ^{13}C spectra was not observed, Figure S3, left panel, supporting material.)

The values of ρ_{CL} listed in Table 1, were obtained from

$$\frac{I(S_0^{\text{amide}})}{\alpha I(S_0^{\text{amine}})} = \frac{\rho_{\text{CL}} + (N - 1)}{1 - \rho_{\text{CL}}}, \quad (2)$$

where N is the experimentally determined average number of glycines in the PG-bridging segments for different strains. The glycyl amide peak in the S_0 spectra represents the cross-linked bridge, $I(S_0^{\text{amide}})$, and the glycyl amine peak represents the uncross-linked bridges $I(S_0^{\text{amine}})$ in the PG. The factor α (equal to 1.2) accounted for differences in rotating-frame cross-polarization dynamics between amides and amines (35,36). The errors in the ρ_{CL} 's are $\pm 2\%$ (see above).

Isotopic Enrichment of Glycine and D-Alanine

The metabolic scrambling of labeled lysine is minimal (21,29), hence the ϵ - ^{15}N (98%) isotopic enrichment is high and did not vary between strains. Isopeptide bonds between ϵ - ^{15}N of lysine and glycyl carbonyl- ^{13}C are only present in PG. The maximum $^{15}\text{N}\{^{13}\text{C}\}$ ΔS (observed after about 1.6 ms dipolar evolution) was used to estimate the ^{13}C isotopic enrichment of the carbonyl- ^{13}C of glycine, $f(^{13}\text{C})$, of approximately 73% for all strains (Table 1). The enrichments measured from cell-wall isolates (Figure 3, left) were comparable to those inferred from measurements done on whole cells (Figure S2). Dilution of incorporated $[1-^{13}\text{C}]$ glycine is due to the endogenous glycine biosynthesis which also affected the ^{15}N isotopic enrichment of $[^{15}\text{N}]$ glycine and $[1-^{13}\text{C}, ^{15}\text{N}]$ glycine (Table 1).

In the $^{15}\text{N}\{^{13}\text{C}\}$ ΔS spectra of Figure 3 (top right), only the $[^{15}\text{N}]$ glycyl amide of the terminal $[^{15}\text{N}]$ glycine that is cross-linked to the D- $[^{13}\text{C}]$ alanine (Scheme 1) contributes to the 92-ppm peak during 1.60 ms of dipolar evolution. The 4-bond penultimate glycyl contribution is negligible. The ^{13}C isotopic enrichment of D- $[1-^{13}\text{C}]$ alanine, $f(^{13}\text{C})$, is proportional to the dephasing value (ΔS^{amide}) divided by the scaled intensity of S_0 at 92 ppm, $I(*S_0^{\text{amide}})$, and normalized by N ,

$$f(^{13}\text{C}) = \frac{1}{N} \frac{I(\Delta S^{\text{amide}})}{I(*S_0^{\text{amide}})} \quad (3)$$

The observed S_0 intensity at 92 ppm (S_0^{amide}) contains contributions from the [^{15}N]glycyl amides of both open and cross-linked bridges. The contribution from the [^{15}N]glycyl amides of open bridges is proportional to the number of [^{15}N]glycyl amides in the open bridges ($N-1$), times the intensity of S_0 at 9 ppm (S_0^{amine}) so that

$$I(*S_0^{\text{amide}}) = I(S_0^{\text{amide}}) - (N-1)I(S_0^{\text{amine}}) \quad (4)$$

The estimated ^{13}C isotopic enrichments, $f(^{13}\text{C})$, of incorporated D-[1- ^{13}C]alanine for all strains using equations (3) and (4) are listed in Table 1. The results of the D-alanine quantification in cell-wall isolates (Figures S3 and S4) and a description of this analysis are presented in the supporting material.

Whole Cells of *S. aureus* Fem Mutant

The interesting *FemX* mutant, which was investigated to have a complete overview of the *fem*-deletion mutants, is not part of our aim to understand the effect of bridge length on the 3-dimensional PG lattice in *S. aureus*. Results of experiments performed on *FemX* (Figures S5 to S7) and a brief analysis are presented in the supporting material.

Discussion

Transmission Electron Micrographs

The typical cell-wall thickness of wild-type *S. aureus* (BB255) measured by TEM is around 20 nm (37). The wild-type strain exhibited normal cell division without irregularities. However, in *FemA* (UK 17) and *FemB* (UT34-2) mutants, irregular cell divisions were observed with “pseudomulticellular” (37) morphology. Multiple division planes in *FemA* are shown in Figure 1 (right, bottom). A premature septation associated with the formation of multiple, disorganized, and incomplete cross walls and an accumulation of PG material at the septum were also observed for both *fem*-deletion mutants. The partial thickening of the cross wall and the outer cell wall was the result of presumably immature (uncross-linked) PG accumulating at the septum. The splitting system is absent in *fem*-deletion mutants during cell division, which is in agreement with the literature (37). The loss of ability to divide in *fem*-deletion mutants (37) is reflected in an unusually long lag-phase growth in both defined and complex media (Figure S1 in the supporting material).

Glycine Content of Cell Walls

The spectral profiles of ^{13}C -natural abundance peaks in the REDOR S_0 spectra (Figure 2, bottom) are similar, indicating that *fem*-inactivation did not significantly alter cytoplasmic composition. Reduction in the carbonyl- ^{13}C peak intensity at 171 ppm in *fem*-deletion mutants confirmed qualitatively that labeled glycine incorporation in whole cells was reduced. This reduction is primarily due to less glycine incorporation in the PG-bridge structure by the mutants, as measured directly by $^{13}\text{C}\{^{15}\text{N}\}$ ΔS intensities (Figure 2, top). The GEUs for whole cells of *FemB* and *FemA* are 2.5 ± 0.1 and 1.0 ± 0.1 , respectively. These measurements are comparable to the values reported by LC-MS analysis of cell-wall isolates, which are 2.6–2.9 for the *FemB* mutant (13) and 1.2 for the *FemA* mutant (10). However, the NMR measurements are not dependent on external calibration factors and are therefore inherently more accurate.

The *in situ* measurement of 2.5 ± 0.1 GEU for the *FemB* mutant indicates a heterogeneous PG-structure with 25% monoglycyl- and 75% triglycyl-bridges. This result is independent of a sampling bias and is a global average for whole cells.

Bridge-Links and Cross-Links

The reduction of PG-bridge lengths from 5.0 (BB255) to 2.5 ± 0.1 (*FemB*) to 1.0 ± 0.1 (*FemA*) GEU, is associated with a decrease in the degree of cross-linking (ρ_{CL}) from 80 to 75 to 62%, respectively. The degree of bridge-linking (ρ_{BL}) remained stable, from 94 to 92 to 91%, respectively, consistent with the notion that the *FemA* and *FemB* proteins are not involved in the attachment of the first glycine in the PG-bridge. The average degree of cross-link density (ρ), the product of ρ_{BL} and ρ_{CL} , therefore remained relatively stable for the wild-type strain and *FemB*, decreasing from 75% to 70%, but dropped to 57% for *FemA*. We believe that these cross-link densities are more accurate than estimates based on the LC-MS analysis of cell-wall digests, which are complicated by LC peaks representing multiple muropeptides of varying ionization efficiencies. This is a particularly acute problem in the LC-MS analysis of *S. aureus* because the high degree of cross-linking results in a wide range of mass fragments in the cell-wall digests.

The shortening of the bridge in *FemB* is associated with only modest changes in ρ_{CL} and ρ , although these changes could still be important. We speculate that a conformational change from a compact helical conformation observed for the pentaglycyl-bridge structure in *S. aureus* (21) to a more extended bridge conformation in *FemB*, could compensate for the shortening of the bridge. The only major difference in the results of Table 1 between the wild-type strain and *FemB* is the reduction of the isotopic enrichment of incorporated D-[1- ^{13}C] alanine, from 75% in the wild-type strain to 26% in *FemB*. The cause of this reduction is unclear; however, the TEM images (Figure 1) show an accumulation of PG material at the septum implying increased PG-biosynthesis. This places a high demand on D-alanine, possibly accompanied by an increase in endogenous alanine biosynthesis and a dilution of exogenous D-[1- ^{13}C]alanine. A similar dilution was also observed for *FemA* where the D-[1- ^{13}C]alanine isotopic enrichment was reduced to 37%.

Combination of NMR and LC-MS

To investigate PG-tertiary structure of *S. aureus*, we propose a strategy of combining NMR analysis of intact cell walls with LC-MS analysis of digested cell walls. This strategy was first employed successfully in the characterization of the PG of *Enterococcus faecium* (29). Conditions for the gentle enzymatic digestion of the glycan sugars were found such that subsequent accurate-mass measurements of unfragmented products led to a determination of total cross-linking in agreement with the solid-state NMR results. Because the usual prolonged period to ensure complete digestion could be avoided, structural modifications of the glycan sugars of the unfragmented products were still in place. These modifications are essential to an understanding of the complete cell-wall architecture of *S. aureus* and its *fem*-deletion mutants. We intend to use the compositions of Table 1 to help establish the least perturbative conditions for cell-wall digestions of wild-type methicillin-resistant *S. aureus* and its *fem*-deletion mutants.

Cell-wall Composition and Architecture

A significant difference in cross-linking is observed between *FemB* and *FemA* mutants, a reduction from 70% to 57% (Table 1). Nevertheless, for *FemA* to achieve an average cross-link density as high as 57% with a short monoglycyl bridge, a rearrangement of cell-wall architecture seems likely. We believe that the transition from the tertiary PG-structure in *FemB* to that in *FemA* represents a fundamental difference in the cell-wall structure of Gram-positive bacteria with long bridges like (wild-type) *S. aureus*, and those with short bridges like

E. faecium (29) This difference in tertiary-PG structure certainly affects cell-wall biosynthesis and metabolism, and may also affect cell morphology, drug permeability, and sensitivity to antimicrobial agents that target PG-biosynthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

C₅₅, pyrophosphoryl-undecaprenol
 CPMAS, cross-polarization magic-angle spinning
FEM, factors essential for methicillin resistance
 GEU, glycine-equivalent unit
 lipid II, N-acetylglucosamine-N-acetyl-muramyl-pentapeptide-pyrophosphoryl-undecaprenol
 MAS, magic-angle spinning
 MIC, minimum inhibitory concentration
 MRSA, methicillin-resistant *S. aureus*
 PBP, penicillin-binding protein
 PG, peptidoglycan
 REDOR, rotational-echo double resonance
 SASM, *Staphylococcus aureus* standard medium
 SSB, spinning-side band
 TEM, transmission electron micrograph
 TSB, trypticase soy broth

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BB255 (wild-type)

FemB

FemA

Figure 1.

Transmission electron micrographs of whole-cells of *S. aureus* grown in defined medium (SASM) and harvested at O.D. 0.2 at 660 nm. (Left) wild-type BB255. (Middle) *FemB* (UT34-2). (Right) *FemA* (UK 17). The arrows indicate morphological irregularities for the mutant strains. Average cell size for the mutants did not exceed that of the wild-type strain.

L-[ϵ - ^{15}N]Lys and [1 - ^{13}C , ^{15}N]Gly Whole Cells

Figure 2.
 $^{13}\text{C}\{^{15}\text{N}\}$ REDOR spectra after $8-T_r$ dipolar evolution (1.60 ms) obtained at 75 MHz with 5-kHz MAS of whole cells of wild-type *S. aureus* (BB255, black), and *FemB* (UT 34-2, red) and *FemA* (UK 17, blue) mutants labeled by [1 - ^{13}C , ^{15}N]glycine and L-[ϵ - ^{15}N]lysine. The full-echo spectra (S_0) are at the bottom of the figure, and the REDOR differences ($\Delta S = S_0 - S$, where S is the dephased echo) are shown at the top. Natural-abundance carbon peaks appear between 20–70 ppm in the S_0 spectrum (bottom inset). The 171-ppm peak intensity in the ΔS spectrum (top inset) of wild-type *S. aureus* BB255 was normalized to a five glycine-equivalent unit (GEU). The ^{13}C shoulder for *FemA* (top inset) is assigned to glycy l amines of open bridging segments with terminal NH_2 groups. The spectra were scaled according to

sample weight and total number of scans; ^{13}C chemical shifts are with respect to external tetramethylsilane TMS.

Figure 3.
 $^{15}\text{N}\{^{13}\text{C}\}$ REDOR spectra of cell-wall isolates of wild-type *S. aureus* (BB255, black) and its *FemB* (UT 34-2, red) and *FemA* (UK 17, blue) mutants labeled by (left) L- $[\epsilon\text{-}^{15}\text{N}]$ lysine and $[1\text{-}^{13}\text{C}]$ glycine, and (right) ^{15}N glycine and D- $[1\text{-}^{13}\text{C}]$ alanine. The full-echo spectra (S_0) are at the bottom of the figure, and the REDOR differences ($\Delta S = S_0 - S$, where S is the dephased echo) are shown at the top. The spectra on the left were obtained at 50 MHz with 7143-Hz MAS. After an evolution time of 1.68 ms, the amide peak at ~92 ppm is due to a lysyl bridge-link, and the amine peak at ~9 ppm is from an $[\epsilon\text{-}^{15}\text{N}]$ lysyl residue that is not bridge-linked. The spectra on the right were obtained at 30 MHz with 5-kHz MAS. After an evolution time of 1.60 ms, the amide peak at ~90 ppm is due to a glycyl-D-alanyl cross-link, and the amine

peak at ~8 ppm is from a terminal glycyI bridging segment that is not cross-linked. ^{15}N chemical shifts are with respect to external ammonium sulfate $(\text{NH}_4)_2\text{SO}_4$.

Scheme 1.

(Left) Schematic representation of an idealized cell-wall peptidoglycan of *Staphylococcus aureus* and its *fem*-deletion mutants. A four unit peptide stem (triangles) having the sequence, L-Ala-D-iGln-L-Lys-D-Ala, is attached to every second sugar of the glycan backbone (open circles). Cross-linking between glycans occurs through glyceryl side chains (dark circles) connecting the carbonyl-carbon of D-Ala of the fourth position of one stem to the ε-nitrogen (in an isopeptide bond) of L-Lys of the third position of another. The glycine content in the side chain varies from pentaglycyl (*S. aureus*), triglycyl (*FemB*), monoglycyl (*FemA*), to an accumulation of unsubstituted precursors with no bridges (*FemX*). (Right) Chemical structure of the peptidoglycan stems in the peptidoglycan of *Staphylococcus aureus* and its *fem*-deletion mutants.

Scheme 2.

Distribution of labels in the cell-wall peptidoglycan of a wild-type *Staphylococcus aureus*, and its *FemB* and *FemA* mutants, grown in defined medium containing [1-¹³C, ¹⁵N]glycine and L-[ε-¹⁵N]lysine.

Table 1
Composition of methicillin-resistant wild-type *S. aureus* (BB255), and its *FemB* (UT 34-2) and *FemA* (UK 17) Mutants

System	N^a GEU	$f^{13C}b$ Gly	$f^{15N}c$ Gly	$f^{13C}d$ D-Ala	$\rho_{BL}e$	$\rho_{CL}f$	ρ^g
BB255	5.0 ^b	0.73	0.73	0.73	0.94	0.80	0.75
<i>FemB</i>	2.5±0.1	0.73	0.73	0.26	0.92	0.75	0.70
<i>FemA</i>	1.0±0.1	0.73	0.73	0.37	0.91	0.62	0.57

^a N : average number of glycyl residues in PG-bridge structure.
^b ^{13}C enrichment of incorporated $[1-^{13}C]$ glycine.
^c ^{15}N enrichment of incorporated $[^{15}N]$ glycine.
^d ^{13}C enrichment of incorporated D- $[1-^{13}C]$ alanine determined by using equations (3) and (4)
^e ρ_{BL} bridge-link degree is determined by using equation (1).
^f ρ_{CL} cross-link degree is determined by using equation (2).
^g average degree of cross-linking is $\rho = \rho_{BL} \cdot \rho_{CL}$. The estimated error is $\pm 5\%$ based on the $\pm 2\%$ errors for ρ_{BL} and ρ_{CL} .
^h The wild-type *S. aureus* BB255 was normalized to a five glycine-equivalent unit (GEU).