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Cell Wall Monoglycine Cross-Bridges and Methicillin Hypersusceptibility in a *femAB* Null Mutant of Methicillin-Resistant *Staphylococcus aureus*

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The *femAB* operon is involved in the formation of the characteristic pentaglycine side chain of the staphylococcal peptidoglycan. Allele replacement of the *femAB* operon with the tetracycline resistance determinant *tetK* in a methicillin-resistant *Staphylococcus aureus* strain resulted in impaired growth, methicillin hypersusceptibility, and lysostaphin resistance. The usual pentaglycine cross-bridges were replaced by monoglycine bridges exclusively, and cross-linking of the peptidoglycan strands was drastically reduced. Complementation of the *femAB* null mutant by either *femA* or *femAB* resulted in the extension of the cross-bridges to a triglycine or a pentaglycine, respectively. This finding suggests that FemA is responsible for the formation of glycines 2 and 3, and FemB is responsible for formation of glycines 4 and 5, of the pentaglycine side chain of the peptidoglycan precursor. Moreover, it can be deduced that addition of the first glycine must occur by a *femAB*-independent mechanism.

The chromosomal *femAB* operon belongs to the *Staphylococcus aureus* housekeeping genes and is found in all *S. aureus* strains (15, 18), and *femAB* alleles similar in organization and sequence were identified in *Staphylococcus epidermidis* and *Staphylococcus haemolyticus* (1). The operon arose by gene duplication and codes for two similar cytoplasmic proteins that are produced mainly during the exponential phase (7, 16). FemA and FemB are involved in a yet unknown way in the formation of the pentaglycine side chain that is attached to the L-lysine of the peptidoglycan stem peptide (7, 14, 21). This long and flexible pentaglycine peptide allows the high cross-linking between the peptide moiety of the single peptidoglycan strands observed in *S. aureus* cell walls. While FemB-less cells are able to form only triglycine cross-bridges, the cell wall composition of leaky *femAB* mutants suggests that FemA might be responsible for the addition of the second and third glycine residues (9, 14). A *femAB* null mutant would be expected to attach only a single glycine to the stem peptide, and it was questionable if such a mutant would be still viable.

The consequences of either *femB* inactivation or lowering of *femAB* transcription are pleiotropic. Besides the reduction of the glycine content of the cell walls, overall peptidoglycan cross-linking and cell wall turnover are reduced, there is an aberrant cell septum formation, and cell separation is retarded (13, 14, 21). Methicillin resistance in *S. aureus* is abolished, and β-lactam susceptibility in susceptible strains is increased. The production of the low-affinity penicillin-binding protein PBP2′ in methicillin-resistant (Mc^r) strains, the prerequisite for methicillin resistance, is thereby not affected (6), suggesting that PBP2′ is unable to function properly in *femAB* mutants. This makes FemA and/or FemB suitable targets for inhibitors which could restore the efficacy of β-lactam antibiotics against Mc^r strains. The construction of a *femAB* null mutant is the first step in unravelling the biological functions of FemA and FemB.

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MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Tables 1 and 2. The strains were grown in LB (10 g of tryptone [Difco, Detroit, Mich.] and 5 g of yeast extract [Difco] per liter) plus 5 g of NaCl per liter at 37°C except when specified otherwise. Where appropriate, antibiotics were used at the following concentrations: ampicillin, 50 mg/liter; chloramphenicol, 25 mg/liter; and tetracycline, 5 mg/liter when selecting for the tetracycline resistant (Tc^r) plasmid and 2 mg/liter when selecting for the chromosomally integrated *tetK* marker. Phage-mediated transductions were performed as described earlier (5).

Susceptibility tests. The MICs of antibiotics were determined by the E-test (AB Biodisk, Solna, Sweden) (3). MICs of lysostaphin (Sigma) were determined by microbroth dilution in Mueller-Hinton medium (Difco) in a final volume of 100 μl inoculated with 10^4 cells per well and incubated for 24 h.

DNA manipulations. DNA manipulations were performed generally as described in references 2 and 22. DNA was extracted from *S. aureus* by lysing a 30-ml overnight culture in 3 ml of 0.1 M Tris-HCl–0.1 M EDTA–0.15 M NaCl (pH 7.5) containing 200 μg of lysostaphin and 1 mg of lysozyme. The lysis was completed after 30 min of incubation at 37°C by addition of 300 μl of 10% (wt/vol) sodium dodecyl sulfate in 50% ethanol. After extraction of the lysis mix with 1 ml of phenol equilibrated at pH 8, the DNA was spooled out from the supernatant on a glass rod, rinsed in ethanol, and resuspended in 2 ml of TE buffer (10 mM Tris-HCl–1 mM EDTA [pH 8] containing 5 μg of RNase). The cell walls of the *femAB* null mutants were lysed with 500 U of achromopeptidase in 3 ml of 0.01 M NaCl–0.01 M Tris-HCl (pH 8). Subsequent steps of DNA isolation were as described above except that the DNA was precipitated with 2 volumes of ethanol after the phenol extraction. Enzymes to manipulate the DNA were from Boehringer Mannheim and used according to the manufacturer's recommendations. DNA probes to analyze the mutants were the 3-kb *Bcl*I fragment spanning the *femAB* operon, the 10.5-kb *Pst*I fragment containing the *femAB* region isolated from plasmid pBBB13 (7) (Fig. 1), the 1-kb *Pst*I-*Xba*I fragment internal to *mecA* (26) isolated from pBBB62, and the *Hin*dIII fragment from plasmid pCW59 containing *tetK* (28).

Allele replacement of *femAB*. The 5.4-kb *Kpn*I insert of plasmid pBBB18 (7) which contains the *femAB* operon (Fig. 1) was cloned into the *Kpn*I cloning site of the temperature-sensitive shuttle vector pTS1, yielding plasmid pAS120. Plasmid pAS120 was propagated in *dcm dam Escherichia coli* JM110 to prevent methylation of the two *Bcl*I sites, which were located at the 3′ ends of the *trpA* and *femB* genes (Fig. 1). This 3-kb *Bcl*I fragment, starting within *trpA* and spanning virtually the complete *femAB* operon, was replaced in pAS120 by blunt-end ligation with a Klenow enzyme-filled 2.3-kb *Hin*dIII fragment containing the Tc^r determinant *tetK* isolated from plasmid pCW59, to produce plasmid pAS122 (Fig. 1). Plasmid pAS122 was then introduced first by electroporation

TABLE 1. Strains used

Strain	Relevant genotype	Relevant phenotype	Source or reference
E. coli JM110	*dam dcm*		Stratagene
S. aureus			
RN4220	NCTC8325-4 r^-	Restriction negative, transformable by electroporation	20
BB255	NCTC8325	Mc^s	
BB270	NCTC8325 *mec*	Mc^r	4
BB308	NCTC8325 *mec* Ω2003*femAB*::Tn*551*	Mc^s Em^r[a]	7
AS167	NCTC8325 *mec* Ω2003*femAB*::Tn*551*, pBBB31(*femA*)	Mc^r Em^r Cm^r	This study, backcross of pBBB31 into BB308
AS123	NCTC8325 r^-, pAS122	Cm^r Tc^r	This study, by electroporation of pAS122 into RN4220
AS145	NCTC8325 *mec* Δ*femAB*::*tetK*	Mc^s Tc^r lysostaphin^r	This study, by allele replacement of *femAB*
AS158	NCTC8325 *mec* Δ*femAB*::*tetK*	Mc^s Tc^r lysostaphin^r	This study, by backtransduction of Δ*femAB*::*tetK* from AS145 into BB270
AS164	NCTC8325 *mec* Δ*femAB*::*tetK*, pBBB31(*femA*)	Mc^s Tc^r Cm^r lysostaphin^r	This study, by transduction of pBBB31 into AS145
AS170	NCTC8325 *mec* Δ*femAB*::*tetK*	Mc^s Tc^r Cm^s lysostaphin^r	This study, by spontaneous curing of pBBB31 from AS164
AS163	NCTC8325 *mec* Δ*femAB*::*tetK*, pBBB64(*femAB*)	Mc^r Tc^r Cm^r lysostaphin^s	This study, by transduction of pBBB64 into AS145
AS169	NCTC8325 *mec* Δ*femAB*::*tetK*	Mc^s Tc^r Cm^r lysostaphin^r	This study, by spontaneous curing of AS163 from its plasmid

[a] Em, erythromycin.

into restriction-negative *S. aureus* RN4220 as an intermediary host, selecting for Tc^r transformants at 28°C, resulting in strain AS123. Plasmid pAS122 was then transduced with phage 85 from strain AS123 into Mc^r strain BB270. Single transductants containing pAS122 were purified and grown overnight at 28°C in the presence of tetracycline, then diluted 1:100 in LB, and grown for three growth cycles at 43°C in the absence of tetracycline to promote integration of pAS122 into the chromosome. Tc^r colonies were tested for loss of the plasmid-borne chloramphenicol resistance (Cm^r) marker. These Tc^r Cm^s colonies were used to inoculate microtiter plates containing geometrically increasing concentrations of lysostaphin (Sigma, Munich, Germany) from 0.008 to 256 μg/ml. From those wells with the highest lysostaphin concentration where growth occurred, single colonies were purified in the presence of 8 μg of lysostaphin per ml and analyzed. Strain AS145, an Mc^s lysostaphin-resistant mutant, was finally chosen as representative for the *femAB* allele replacement.

Muropeptide analysis. (i) Preparation of murein. Preparation and analysis of muropeptides were done essentially as described previously (19). Briefly, bacteria were grown in 500 ml of brain heart infusion medium (Difco) and harvested at an optical density at 600 nm of 0.7. Cells were resuspended in 10 ml of 1 M NaCl and broken with glass beads (0.1 mm), using a cell grinder for 5 min at 4°C. Glass beads were separated by filtration and washed with 0.5% sodium dodecyl sulfate. The collected cell suspension was incubated at 60°C for 30 min to remove noncovalently bound components. The cell walls were isolated by centrifugation and washed three times with water. Protein A was removed by incubation with 0.2 mg of trypsin per ml in 1 M Tris-HCl (pH 7.0) for 24 h at 37°C. Samples were centrifuged, washed several times with buffer and water, and then resuspended in 1 ml of 40% (wt/vol) aqueous hydrofluoric acid for 18 h at 4°C to remove teichoic acids. Purified murein was isolated by centrifugation, washed with water, and lyophilized.

(ii) Preparation and reduction of muropeptides. The lyophilized murein (1.3 mg) was degraded with 25 μg of *Streptomyces globisporus* mutanolysin (Sigma) per ml in 0.3 ml of sodium citrate buffer (pH 7.0) containing 4 mM $MgCl_2$ and 0.02% sodium azide at 37°C overnight. Samples were adjusted to pH 4.0 with phosphoric acid and incubated at 100°C for 5 min. Immediately after the addition of 175 μl of 1.5 M sodium borate buffer (pH 9.0), 10 mg of solid sodium borohydride was added followed by incubation at room temperature for 15 min to reduce the muropeptides to the corresponding muramitol derivatives (25). Excess borohydride was destroyed by the addition of 20% H_3PO_4. To hydrolyze remaining *O*-acetyl groups present at the muramic acid residues, samples were adjusted to pH 12 with 4 N NaOH and incubated at 37°C for 1.5 h. The muropeptide solution was then adjusted to pH 2.5 with 4 N HCl prior to

TABLE 2. Plasmids used[a]

Plasmid	Vector and plasmid insert	Relevant properties	Reference or origin
pCW59	*S. aureus* cloning vector	Cm^r Tc^r	28
pTS1	*E. coli-S. aureus ts* shuttle vector	Ap^r (*E. coli*); Cm^r, *ts* replicon in *S. aureus*	24
pBBB13	*E. coli* cloning vector pSP64, 10.5-kb *Pst*I fragment of BB270 covering *femAB*	Ap^r	7
pBBB18	*E. coli* cloning vector pTZ18R, 5.5-kb *Kpn*I subfragment of pBBB13 covering *femAB*	Ap^r	7
pBBB31	*E. coli-S. aureus* shuttle vector pHV33, 2.3-kb *Eco*RV fragment carrying *femA*	Ap^r (*E. coli*); Cm^r (*S. aureus*)	7
pBBB62	*E. coli* cloning vector pTZ18R, 4-kb *Hin*dIII fragment of BB270 covering *mecA*	Ap^r	26
pBBB64	*E. coli-S. aureus ts* shuttle vector pGC2, *Pst*I-*Eco*RI 5.2-kb fragment carrying *femAB*	Ap^r (*E. coli*); Cm^r (*S. aureus*)	7
pAS120	*E. coli-S. aureus ts* shuttle vector (*femAB*)	Ap^r (*E. coli*); Cm^r (*S. aureus*)	This study, 5.4-kb *Kpn*I fragment of pBBB18 (7) cloned into the *Kpn*I site of pTS1
pAS122	*E. coli-S. aureus ts* shuttle vector (Δ*femAB*::Tc^r)	Ap^r (*E. coli*); Cm^r Tc^r (*S. aureus*)	This study, 2.35-kb *Hin*dIII fragment of pCW59 containing *tetK* cloned into the *Bcl*I sites of pAS120, deleting *femAB*

[a] Ap, ampicillin; *ts*, temperature-sensitive.

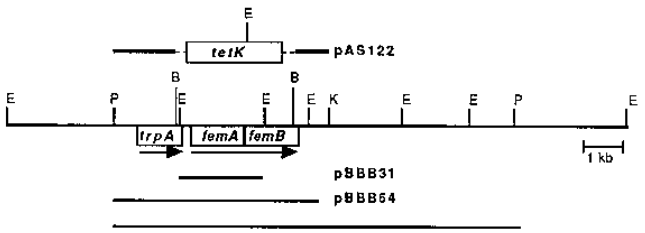

FIG. 1. Map of the *femAB* region and plasmids. The orientations and locations of the *trpA* open reading frame and the *femAB* operon are shown. The bottom line shows the 10.5-kb *Pst*I fragment used as a probe. Restriction enzyme abbreviations: B, *Bcl*I; E, *Eco*RV; K, *Kpn*I; P, *Pst*I.

centrifugation (13,000 × *g*, 10 min) and filtration (0.22-μm-pore-size filter) to remove insoluble contaminations.

(iii) Fractionation of muropeptides by HPLC. Muropeptides were separated by reversed-phase high-pressure liquid chromatography (HPLC) (8, 11). The HPLC system (Waters) consisted of a 600S controller, a 626 pump, a model 717 autosampler, and a 996 photodiode array detector. Millenium software was used for recording. The column (3-μm Hypersil ODS, 4.0- by 250-mm CompAx-peek; precolumn, 14 by 4.0 mm; Knauer, Berlin, Germany) was eluted at 45°C with a linear gradient from 5% methanol in 50 mM sodium phosphate (pH 2.5) containing 0.0005% sodium azide to 30% methanol in 50 mM sodium phosphate (pH 2.8) within 210 min. Muropeptides were detected at 206 nm and identified by amino acid analysis and mass spectrometry data obtained previously (25).

Amino acid analysis. Lyophilized murein (1 mg) was hydrolyzed in 6 N HCl at 166°C for 1 h. Samples were subjected to an amino acid analyzer (Biotronic LC 5000) after drying.

RESULTS

Construction of *femAB* null mutants. Tn*551* insertion in the chromosome is not completely random. Preferential integration sites for Tn*551* within the *femAB* operon were found to be either in the nontranslated part of the mRNA, resulting in a polar effect on *femAB* transcription, or in the 3′ end of the *femA* or *femB* open reading frame, removing the last few C-terminal amino acids, but not excluding some residual activity of FemA or FemB (7). With the exception of the insertion Ω2005*femB*::Tn*551*, which inactivated FemB completely (14), all inserts were leaky. The aim of this study was therefore to construct and characterize a *femAB* null mutant.

The classic approach of allele replacement was chosen. Making use of the two *Bcl*I sites on the 5.4-kb *Kpn*I fragment (Fig. 1), located upstream of *femA* within the 3′ end of *trpA* and within the 3′ end of *femB*, the *femAB* operon was removed and replaced by the Tc^r determinant *tetK*. This construct was cloned into the temperature-sensitive shuttle vector pTS1, yielding plasmid pAS122. Plasmid pAS122 was electroporated in RN4220 and subsequently transduced into Mc^r *S. aureus* BB270. Integration of plasmid pAS122 into the chromosome of strain BB270 was forced by repeated growth of the transductants at the nonpermissive temperature. We searched for the rare double-crossover event in which *femAB* would be replaced by *tetK* and the plasmid would be eliminated. These strains were expected to be susceptible to chloramphenicol and to have become susceptible to methicillin. Repeated growth at the nonpermissive temperature, however, yielded only Campbell-type integrations still containing the complete plasmid integrated via the left or right homologous sequences in the chromosome as well as a functional *femAB* operon. Some rare Cm^s clones were found; however, they were still Mc^r and contained the *femAB* operon, as verified by Southern hybridizations. They had a plasmid integration either to the left or to the right of *femAB* plus some rearrangements or deletions resulting in loss of chloramphenicol resistance. Since we expected that *femAB* null mutants would become less susceptible to lysostaphin, a glycyl-glycine-specific endopeptidase, the Cm^s mutants were screened after the cycling at high temperature for growth in microtiter plates containing increasing concentrations of lysostaphin. From the well with the highest lysostaphin concentration where growth still occurred, mutants were isolated, purified on plates containing 8 μg of lysostaphin per ml, and screened subsequently for Tc^r and Cm^s. All mutants that were Tc^r, Cm^s, and lysostaphin resistant were found to be also susceptible to methicillin, a first indication that they might be the correct *femAB* null mutants. One representative mutant, strain AS145 was kept for further analysis.

Analysis and characterization of the *femAB* null mutant. Cell walls of strain AS145 were resistant to degradation by lysostaphin and had to be digested with achromopeptidase to isolate the DNA. Probing chromosomal *Pst*I digests with *mecA* confirmed that the putative *femAB* null mutants still contained the *mec* determinant (data not shown), indicating that their methicillin susceptibility was presumably due to impairment of *femAB*. Hybridization with the substituted *Bcl*I fragment covering *femAB* showed that the *femAB* operon was indeed absent in the mutants (Fig. 2a), represented here by strain AS145, whereas in strains BB270 and BB255, the 2.2-kb *Eco*RV fragment and the two framing but weakly labelled fragments of 4.4 and 1.2 kb were visible. Finally, hybridization with the chromosomal 10.5-kb *Pst*I fragment, which covers the sequences surrounding the *femAB* operon, showed that no major rearrangements except for the exchange of *femAB* by *tetK* had occurred within this region. The three *Eco*RV bands affected by the substitution (1.2, 2.2, and 4.4 kb) disappeared in AS145 (Fig. 2b), whereas we detected two new bands of 5.6 and 1.6 kb due to the *tetK* insertion that hybridized with the *tet* probe, as shown in Fig. 2c.

The doubling time of strain AS145 in LB medium rose from 36 min for the parent BB270 to 48 min for the mutant. Whereas a stationary culture of strain BB270 contained mostly separated single cells, strain AS145 formed large multicellular

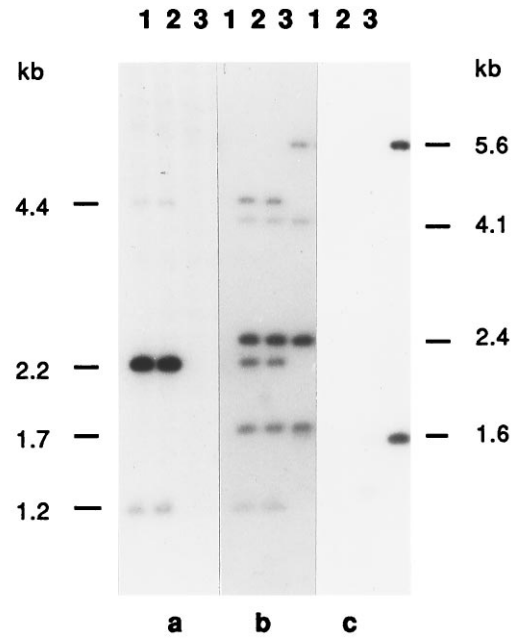

FIG. 2. Hybridization of *Eco*RV digests with different probes. The probes were the *Bcl*I fragment covering *femA* (a), the 10.5-kb *Pst*I fragment covering the *femA* region (b), and the *Hin*dIII fragment containing *tetK* (c). Lanes: 1, strain BB255; 2, strain BB270; 3, strain AS145.

TABLE 3. Effect of *femAB* on MICs of lysostaphin and different β-lactams

Strain	MIC (mg/liter)						
	Lysostaphin	Ampicillin	Methicillin	Oxacillin	Imipenem	Cefotaxime	Cefoxitin
BB255	0.015	0.094	1	0.19	0.023	1	2
BB270	0.03	6	16	64	0.25	>256	24
AS145	64	0.016	0.19	0.047	0.004	0.016	0.094
AS158	16	0.023	0.5	0.064	0.008	0.19	0.050
AS164	16	1.5	2	4	0.064	12	6
AS163	0.03	3	32	128	0.75	>256	24
AS170	64	<0.016	0.19	0.064	0.003	<0.023	0.19
AS169	32	0.023	0.5	0.092	0.004	0.25	0.5

aggregates, suggesting that cell separation was impaired. Electron micrographs of exponentially growing cells revealed thickened cell septa and aberrant cross-wall positioning in AS145 (data not shown).

The MICs of all β-lactams analyzed, methicillin, oxacillin, cefoxitin, cefotaxime, and imipenem, were drastically reduced in AS145 and dropped even below those for the isogenic susceptible strain BB255. In parallel, MICs of lysostaphin increased from 0.03 mg/liter in BB270 and 0.015 mg/liter in BB255 to 64 mg/liter in AS145 (Table 3).

To rule out that eventual secondary mutations not linked to the *femAB* locus might have occurred during the genetic manipulations, Δ*femAB*::*tetK* was backcrossed by phage 80α from AS145 into BB270. The transductants, represented here by strain AS158, had virtually the same phenotype as AS145 (Table 3), corroborating the linkage of β-lactam susceptibility and lysostaphin resistance with the *femAB* deletion.

Complementation of the *femAB* null mutant by *femA* or *femAB*. The *femA*-carrying plasmid pBBB31 was transduced into the *femAB* null mutant AS145. The transductant strain AS164 was susceptible to β-lactams at slightly increased MICs and had an increased resistance to lysostaphin (Table 3). This finding was in accordance with data for chromosomal *femB* mutants which produce FemA but no FemB (14). The functionality of FemA in AS164 was confirmed by backcrossing pBBB31 from AS164 into BB308 (7), a polar *femAB* mutant producing only 10% of FemA, where it restored methicillin resistance as expected (data not shown). Moreover, spontaneous curing of the plasmid from AS164 yielded strain AS170, which was phenotypically identical to AS145.

In contrast, complementation of AS145 with pBBB64 containing the complete *femAB* operon increased methicillin resistance and restored lysostaphin susceptibility in AS163 (Table 3). Spontaneous curing of the plasmid from AS163 by overnight growth in nonselective medium in the absence of chloramphenicol produced the original β-lactam-susceptible, lysostaphin-resistant AS145 phenotype again, as represented here by AS169.

Cell wall composition of the *femAB* null mutants. Amino acid analysis was performed on isolated cell wall murein. The molar ratio of glycine to glutamic acid residues of the parent strain BB270 was slightly more than 4, whereas the value dropped below 1 in the *femAB* null mutant AS145 (Table 4) due to a drastic reduction in cell wall glycine content. Complementation of strain AS145 with the *femA*-containing plasmid pBBB31 increased the ratio to about 2.4 in strain AS164, indicating a partial restoration of the glycine content by FemA, whereas nearly complete complementation to wild-type levels was obtained with the *femAB*-carrying plasmid pBBB64 in AS163.

Muropeptide analysis of the *femAB* null mutants. Muropeptide profiles of the different strains were established by reversed-phase HPLC. Strain BB270 had the characteristic muropeptide pattern of wild-type *S. aureus* strains, with the highest peak in the dimeric fraction and a high degree of cross-linking revealed by the large amount of oligomers (Fig. 3). In striking contrast, the *femAB* null mutant AS145 showed the highest peak in the monomeric fraction and a strongly reduced amount of oligomeric muropeptides, pointing to a significant reduction in the overall murein cross-linking (Fig. 3). Complementation with *femA* resulted in AS164 in an intermediary pattern, where the main monomeric and dimeric peaks were of the same level (Fig. 3), whereas complementation with the *femAB* operon in AS163 (Fig. 3) yielded a pattern virtually indistinguishable from that of the parent strain BB270. The degree of muropeptide cross-linking, calculated by quantification of the relative amounts of monomers, dimers, trimers, and oligomers in the muropeptide pattern, reached in BB270 a high value of about 71%; this value was drastically reduced to 57% by the *femAB* deletion of AS145 but could be partially restored to 68% by FemA in AS164 and was even higher in the *femAB*-complemented AS163 than in BB270 (Table 5).

A detailed resolution and identification of the monomer and dimer fractions is shown in Fig. 4. All peaks indicated were identified by comparison with standard samples based on mass spectometry (25) and/or by amino acid analysis. In the wild-type strain BB270, the major muropeptide contained a pentaglycine moiety (M4) (for nomenclature, see Fig. 4e), and only small peaks of minor muropeptides with mono- or triglycine residues (M2, M3) could be detected. In contrast, a completely different muropeptide pattern was observed in the *femAB* deletion mutant AS145. All muropeptides showed a drastic shortening of the glycine interpeptide bridge. Both the main monomeric and dimeric peaks of the *femAB* null mutant were substituted with a monoglycine (M2, D2), as confirmed by amino acid analysis. Also, the muropeptides containing stem tetrapeptides instead of stem pentapeptides (M2*, M7*) as-

TABLE 4. Cell wall compositions of parent and mutant strains[a]

Amino acid	Molar mass ratio, glycine/glutamic acid			
	BB270	AS145	AS164	AS163
Ser	0.05	0.02	0.03	0.01
Glu	1.00	1.00	1.00	1.00
Gly	4.27	0.94	2.43	3.77
Ala	2.43	2.11	1.86	1.90
Lys	0.83	0.86	0.87	0.79

[a] Cell walls were isolated and subjected to amino acid analysis as described in Materials and Methods. BB270, parent strain; AS145, isogenic *femAB* null mutant; AS164, the mutant complemented with the *femA*-carrying plasmid pBBB31; AS163, the mutant complemented with the *femAB*-carrying plasmid pBBB64.

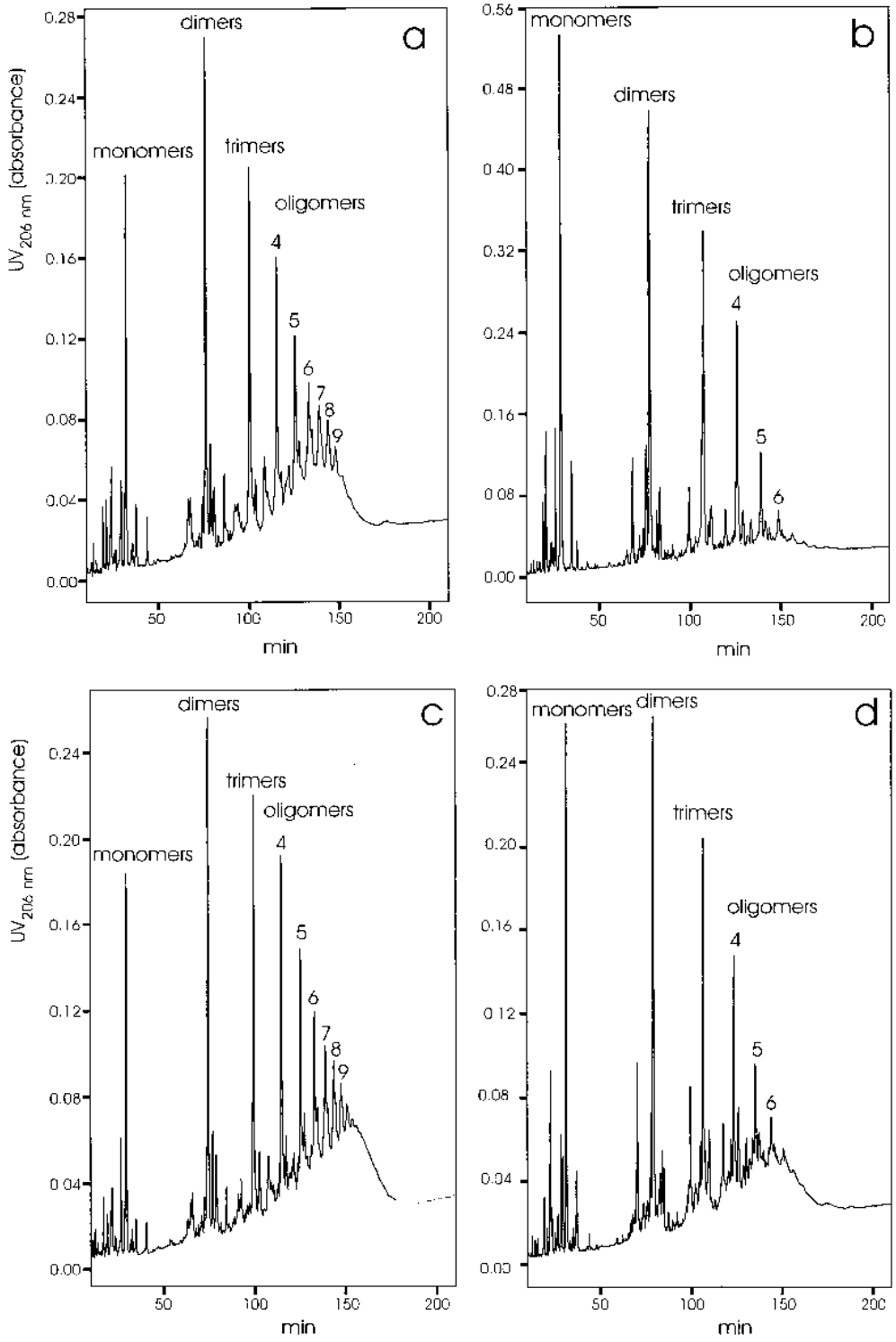

FIG. 3. Muropeptide pattern. Cell walls were isolated and digested with muramidase and subjected to reversed-phase HPLC. (a) Parent strain *S. aureus* BB270; (b) *femAB* null mutant AS145; (c) strain AS163 (AS145 complemented with *femAB* plasmid pBBB64); (d) strain AS164 (AS145 complemented with *femA* plasmid pBBB31). The following fractions are indicated: monomers, corresponding to un-cross-linked material (10- to 50-min retention time); dimers, corresponding to two cross-linked muropeptides (50 to 95 min); trimers, corresponding to three cross-linked muropeptides (95 to 115 min); and oligomers, corresponding to more than three muropeptides cross-linked to each other (115 to 210 min). The numbers indicate the numbers of cross-linked muropeptides.

sumed to be degradation products due to an endopeptidase activity were modified with shortened glycine bridges. The occurrence of these muropeptides and the main dimeric structure D2, modified with one glycine, showed that cross-bridge formation between two muropeptides with one single glycine was feasible in *S. aureus* and that there must exist a cell-specific endopeptidase which in contrast to lysostaphin is able to cleave these bonds. In addition, compared to the profile for the par-

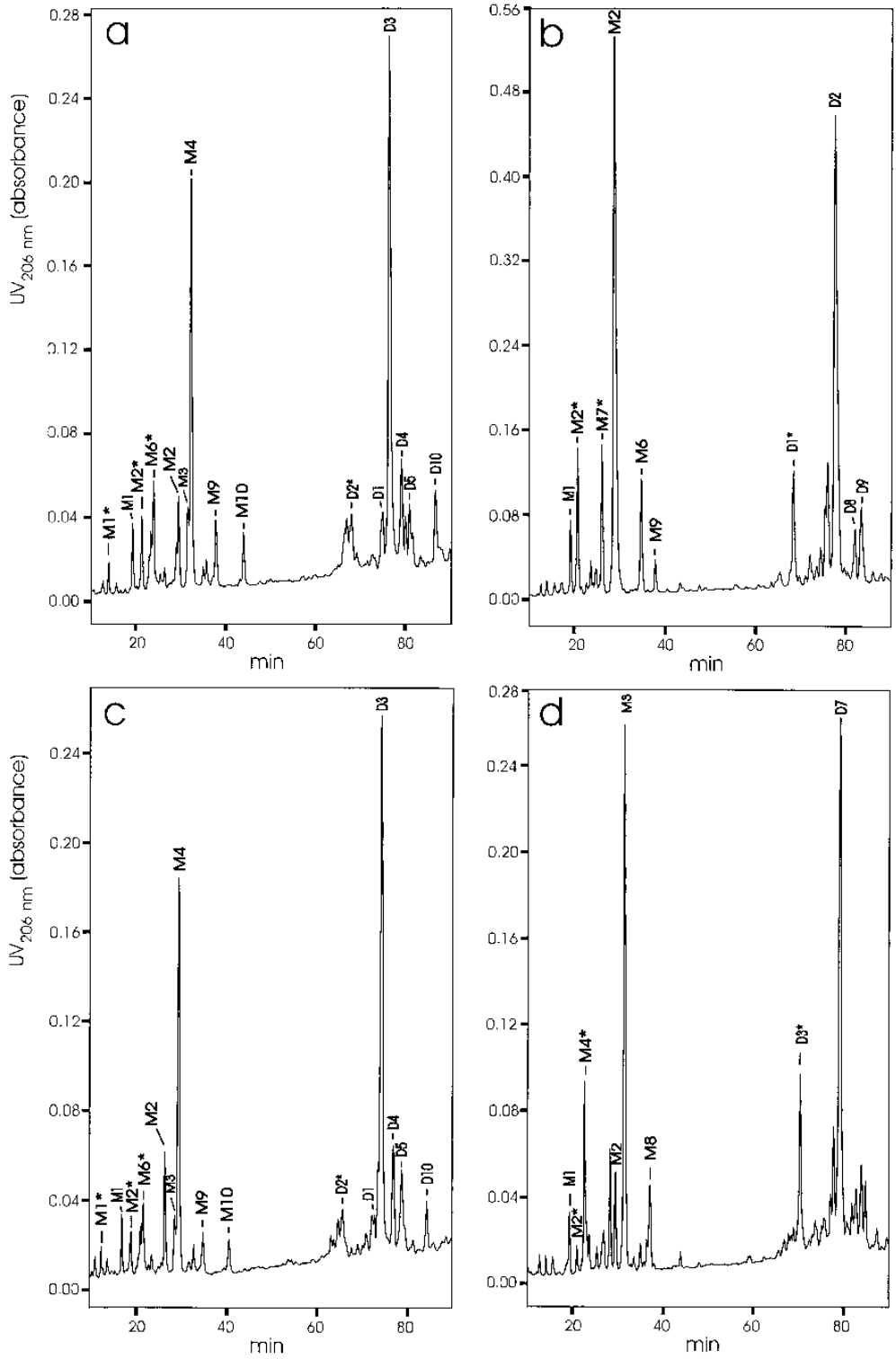

FIG. 4. Detailed view of monomeric and dimeric muropeptide profiles of different *S. aureus* strains. Muropeptides were prepared as for Fig. 3. Peaks were either identified by their retention times and compared with standard samples or identified by amino acid analysis (M2 and D2 of strain AS145). The nomenclature for the muropeptide structures (19) is shown in panel e.

ent strain BB270, peak M6, containing a glutamic acid instead of glutamine in position 2 of the stem peptide, modified with one glycine appeared. No muropeptides with tri- or pentaglycine residues (M3, M4, M10) could be identified in this strain. Since neither the monomeric nor the dimeric fractions of strain AS145 contained muropeptides with more than one glycine, a finding supported by the amino acid composition of the whole cell wall material (Table 4), we assumed that the same mono-

e

Monomers	Structure	Abbreviations
M1	M.P.	M., monomer; P., stem-pentapeptide
M1*	M.T.G1/2	T., stem-tetrapeptide; G, glycine residue; 1/2, one glycine per two possible attachment sites (either lys in position 3 or ala in position 4)
M2	M.P.G1	monomer pentapeptide modified with one glycine
M2*	M.T.G2/2	see M1*
M3	M.P.G3	monomer pentapeptide modified with three glycines
M4	M.P.G5	monomer pentapeptide modified with five glycines
M4*	M.T.G4/2	see M1*
M6	M.Pn.G1	see M2; n, glutamic acid instead of glutamine in position 2
M6*	M.T.G6/2	see M1*
M7*	M.Tn.G2/2	see M1*
M8	M.Pn.G3	see M6
M9	M.Pn.G5 and M.P.A1	A, alanine; two muropeptides with identical retention time
M10	M.P.G4A1	see M4 and M9
Dimers		
D1	D.P.G5+T.G1 or D.P.G1+T.G5	dimer from M4 and M2
D1*	D.2xT.G1/3	dimer of M2
D2	D.P.G1+T.G1	dimer of M2
D2*	D.2xT.G11/3	dimer of M4
D3	D.P.G5+T.G5	dimer of M4
D3*	D.2xT.G7/3	dimer of M3
D4	D.P.G5+T.P3	dimer of M3 and M4
D5	D.Pn.G5+T.G5 or D.P.G5+Tn.G5	dimer from M4 and M9
D7	D.P.G3+T.G3	dimer of M3
D8	D.P.G1+Tn.G1	dimer of M2 and M6
D9	D.P.G1+T.A1	dimer of M2 and M9
D10	D.P.G5+T.G4A1	dimer of M4 and M10

* degradation products produced by an endopeptidase

FIG. 4—*Continued.*

TABLE 5. Percent muropeptides and overall degree of cross-linking in parent and mutant *S. aureus* strains

Strain	% Muropeptide[a]				CL[b] (%)
	Monomer	Dimer	Trimer	Oligomer	
BB270	10.0	17.0	14.4	58.6	70.9
AS145	20.1	25.4	20.0	34.5	57.2
AS164	11.6	18.1	17.6	52.7	68.3
AS163	7.4	14.0	17.6	61.0	73.7

[a] Sum of the peak areas of the indicated fractions.
[b] The cross-linking (CL) value was calculated as follows: 0.5 × dimer(%) + 0.67 × trimer(%) + 0.9 × oligomer(%) (10).

glycine interpeptide bridge was present in the higher oligomers. Finally, it is important to mention that the relative amounts of unmodified muropeptides containing no glycine (e.g., M1) remained constant in the *femAB* mutant compared to the parent strain BB270, indicating that *femAB* is not involved in the addition of the first glycine.

Upon complementation of the *femAB* null mutant AS145 with *femA* (Fig. 4), the major monomeric (M3) and dimeric (D7) peaks were composed of triglycine-substituted muropeptides, while no pentaglycine could be found, indicating that FemA was involved in the attachment of glycine residues 2 and 3. Complementation with the entire *femAB* operon restored in AS163 the original peptidoglycan composition with a pentaglycine bridge as in the parent strain BB270.

Interestingly, no intermediary muropeptide structures containing either two or four glycine residues attached to an uncross-linked stem pentapeptide could be detected in any of the strains. Peaks labelled with asterisks in Fig. 4 were considered degradation products of an endopeptidase cleaving within the glycine interpeptide bridge, thereby assuming that no carboxypeptidase activity was present. They contained a stem tetrapeptide due to the loss of the alanine residue in position 5, suggesting that they had been used in a transpeptidation reaction and subsequently cleaved from the oligomeric structure. In contrast to un-cross-linked material, muropeptides with two or four glycine residues could be detected in these degradation products.

DISCUSSION

The *femAB* operon is known to be involved in the formation of the staphylococcal peptidoglycan pentaglycine cross-bridge. Whereas *femB* mutants produce triglycine cross-bridges (14), the only *femAB* mutants available either were leaky and still expressed 10% of the original FemA (16) producing a peptidoglycan containing mono-, tri-, and pentaglycine cross-bridges (9) or were obtained by chemical mutagenesis (19), leaving unanswered the question of whether a *femA* null mutant would survive. We have shown here for the first time that complete inactivation of the *femAB* operon by allele replacement leads to a viable mutant containing exclusively monoglycine cross-bridges. The molar ratio of glycine to glutamine as well as the muropeptide analysis indicated unambiguously a shortening of the bridge from five to one glycine and a significant reduction in cross-linking. The loss of the glycyl-glycine cross-bridges led to lysostaphin resistance. The transpeptidases and the cells' own autolytic activities were apparently able to function sufficiently well to allow growth, although reduced, with this monoglycine cross-bridge.

From these results, it can be deduced that the addition of the first glycine residue to the stem peptide takes place independently of FemA and FemB. A hypothetical protein FemX has been suggested to function in this process (19). The exact role

of FemA and FemB in the biosynthesis of the interpeptide bridge has still to be demonstrated. The glycine residues are hooked onto the lipid-linked murein precursors facing the cytoplasm. Because of the absence of uncross-linked muropeptides carrying two or four glycine residues, a mechanism that involves attachment of the first glycine by FemX, the second and third glycines by FemA, and the fourth and fifth glycines by FemB can be proposed. Glycyl-tRNAs are substrates in this process. Besides the glycyl-tRNA involved in protein synthesis, *S. aureus* contains three additional glycyl-tRNAs assumed to be involved in cell wall synthesis (12). How FemA and FemB interact with them is unknown. The proposed stepwise attachment of diglycine building blocks to the side chain seems in apparent contrast to earlier experiments demonstrating that glycine residues are added one by one by cell wall-specific glycyl-tRNAs to the growing side chain (17, 23). However, these earlier experiments were done in vitro, whereas our findings reflect the situation in vivo. Because of no sequence similarity of FemA and FemB with known tRNA synthetases, it seems unlikely that they belong to this group of enzymes. It is possible, however, that they have some scaffolding activity or chaperone effect.

Loss of the *femAB* operon caused lysostaphin resistance due to the shortening of the glycine interpeptide bridge; additionally, it caused methicillin hypersusceptibility, despite the presence of PBP2′ in the Mc^r *S. aureus*. Inactivation of *femAB* seemed to act synergistically with β-lactams. Growth of an Mc^r strain in the presence of β-lactams or shortening of the glycine cross-bridges by *femAB* inactivation leads to a reduction in overall peptidoglycan cross-linking. In both situations, lowering of the cross-linking is not sufficient to destabilize the murein network in a lethal manner. However, PBP2′ combined with *femAB* inactivation cannot prevent β-lactam-induced cell lysis. In this case, the cross-linking might be reduced below a critical value, and as a consequence, lysis is caused by the action of the murein hydrolases.

Many surface proteins are anchored via the free amino group of un-cross-linked pentaglycine side chains to the staphylococcal cell wall (27). It would be of interest to investigate how the shortened cross-bridges and the resulting reduced cross-linking affect cell wall sorting of these proteins and the pathogenesis of *S. aureus.*

In summary, *S. aureus* has a remarkable ability to cope even with a severely shortened glycine cross-bridge; the price, however, is a highly increased β-lactam susceptibility. This makes the FemA and FemB proteins potential targets for drugs that might restore the activity of the entire group of β-lactams against Mc^r *S. aureus*. Another intriguing question is whether *S. aureus* would be able to survive inactivation of the hypothetical *femX* and to cross-link peptidoglycan strands containing no glycine cross-bridges.

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