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Influence of *femB* on Methicillin Resistance and Peptidoglycan Metabolism in *Staphylococcus aureus*

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The inactivation of FemB by insertion of Tn*551* in the central part of the *femB* open reading frame was shown to increase susceptibility of methicillin-resistant *Staphylococcus aureus* strains toward β-lactam antibiotics to the same extent as did inactivation of *femA*. Strains carrying the methicillin resistance determinant (*mec*) and expressing PBP 2′ were affected to the same extent as were strains selected for in vitro resistance, which did not express PBP 2′. Both *femA* and *femB*, which form an operon, are involved in a yet unknown manner in the glycine interpeptide bridge formation of the *S. aureus* peptidoglycan. FemB inactivation was shown to reduce the glycine content of peptidoglycan by approximately 40%, depending on the *S. aureus* strain. The reduction of the interpeptide bridge glycine content led to significant reduction in peptidoglycan cross-linking, as measured by gel permeation high-pressure liquid chromatography of muramidase-digested cell walls. Maximum peptide chain length was reduced by approximately 40%. It is shown that the complete pentaglycine interpeptide bridge is important for the sensitivity against β-lactam antibiotics and for the undisturbed activity of the staphylococcal cell wall-synthesizing and hydrolyzing enzymes, as was also apparent from electron microscopic examinations, which revealed aberrant placement of cross walls and retarded cell separation, leading to a pseudomulticellular phenotype of the cells for both *femA* and *femB* mutants.

Methicillin resistance in staphylococci is an intrinsic resistance of the cells to virtually all β-lactam antibiotics, including cephalosporins and carbapenems, and does not involve drug destruction (12). The genetic determinant of methicillin resistance (*mec*) carries the structural gene *mecA*, coding for an additional low-affinity penicillin-binding protein, PBP 2′ or 2a (25, 37, 40). PBP 2′ is thought to be the only functional PBP in cell wall synthesis in the presence of otherwise inhibitory concentrations of methicillin (10, 13, 21, 29, 34) and is a prerequisite for methicillin resistance.

mec-mediated methicillin resistance in clinical isolates of *Staphylococcus aureus* is typically heterogeneous in phenotypic expression despite genetic homogeneity (reviewed by Matthews and Stewart [30]). Whereas most cells of a population show only a low resistance level, a minor subpopulation, 1 in 10^4 to 10^8, demonstrates resistance to higher concentrations of methicillin (23). A minority of clinical isolates express homogeneous resistance, whereby the entire population can grow in the presence of high concentrations of the drug (20).

In vitro methicillin resistance selected by serial passages of susceptible strains on increasing concentrations of β-lactam antibiotics, in contrast, is homogeneous. Such in vitro-selected methicillin-resistant (Mc^r) mutants have changes in the binding characteristics of their PBPs (7, 14, 39), but there is no immunological or genetic relationship to the *mec*-determined low-affinity PBP 2′ (2).

The phenotypic level of resistance in *mec*-determined Mc^r strains is not correlated with the quantity of PBP 2′ (1, 6, 24). Other factors besides the *mec* determinant are responsible for the degree to which methicillin resistance is expressed. Four additional genes, termed *fem* (factors essential for the expression of methicillin resistance) (1, 5, 24), not linked to *mec* were mapped on the *S. aureus* chromosome (4). We have shown recently that their inactivation by Tn*551* increases susceptibility of Mc^r strains to β-lactams (27).

The product of *femA* is a 48-kDa protein involved in the pentaglycine cross-bridge formation of the *S. aureus* peptidoglycan (27). This conclusion was subsequently sustained by mass spectrometric analysis of related mutants (11). Whereas the inactivation of *femA* correlates with a 40 to 60% reduction in the interpeptide glycine content of *S. aureus* peptidoglycan, with a reduction in cross-linking and cell wall turnover, and with increased susceptibility to β-lactams, no influence on the synthesis of PBP 2′ was observed. Downstream and adjacent to *femA* lies a second factor, *femB*. *femB* mutants described earlier (4) apparently still retained part of their activity, presumably because they were insertionally inactivated at their outmost carboxy terminus. We show here that *femB* is as important as *femA* for expression of methicillin resistance and that its inactivation also results in lowering of the glycine content of the glycine interpeptide bridges.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The strains used in this study are described in Table 1. They were grown in LB medium (10 g of tryptone [Difco, Detroit, Mich.], 5 g of yeast extract [Difco], and 5 g of NaCl per liter). Growth temperatures were 37°C where not otherwise mentioned and 30°C for strains carrying the temperature-sensitive plasmid pRN3208. Transductions were performed with phage 80a as described earlier (27). Transductants were selected on 20 mg of erythromycin per liter or 10^{-4} M CdCl and screened for the inability to grow on 5 mg of methicillin per liter. MICs were determined according to the National Committee for Clinical Laboratory Standards (32). Population analyses were done by spreading aliquots of overnight cultures on

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TABLE 1. Strains used[a]

Strain	Relevant genotype	Relevant phenotype	Origin or reference
SG511		Mc^s	Strain collection of the Robert Koch Institute, Berlin, Germany
PV1		Mc^r	This study, derived from SG511 by selection for growth on increasing concentrations of penicillin
RN2906	NCTC 8325-4 with pRN3208 [Rep(Ts)]	Em^r Cd^r	33
UT-1	pRN3208 [Rep(Ts)]	Mc^r Em^r Cd^r	This study, by transduction of plasmid pRN3208 from RN2906 by phage 80a into strain PV1 and selection for Cd^r or Em^r
UT-6	Ω2006(*femB*::Tn*551*)	Mc^s Em^r	This study, by insertional inactivation of Mc^r in strain UT-1
UT-32-1	Ω2006(*femB*::Tn*551*)	Mc^s Em^r	This study, by transduction of PV1 with phage 80a grown on UT-6 and selection for Em^r
UT-48-3	SG511, Ω2006(*femB*::Tn*551*)	Mc^s Em^r	This study, by transduction of SG511 with phage 80a grown on UT-6 and selection for Em^r
BB742	SG511, Ω2003(*femA*::Tn*551*)	Mc^s Em^r	27
UT-39-1	Ω(chr::Tn*551*)	Mc^s Em^r	This study, by insertional inactivation of Mc^r in strain UT-1
BB270	NCTC 8325 *mec*	Mc^r	5
UT-34-2	8325 *mec* Ω2006(*femB*::Tn*551*)	Mc^s Em^r	This study, by transduction of BB270 with phage 80a grown on UT-6 and selection for Em^r

[a] Abbreviations: Em, erythromycin; Cd, cadmium; Rep(Ts), temperature sensitive for replication; chr, chromosomal DNA.

increasing concentrations of methicillin. CFU were determined after 48 h of growth at 35°C.

In vitro selection of penicillin-resistant mutants. An overnight culture of *S. aureus* SG511 (0.1 ml, corresponding to 10^9 cells) was spread on LB agar containing increasing amounts of penicillin and incubated at 37°C for up to 2 days. A single colony growing at the highest concentration was isolated, purified, and subjected to the same procedure, with increases in the concentration of the antibiotic. After five passages, the penicillin-resistant strain PV1 was obtained.

Tn*551*-directed mutagenesis. The temperature-sensitive plasmid pRN3208 carrying Tn*551* was transduced into strain PV1, yielding UT-1. Insertional inactivation of methicillin resistance in UT-1 was done by selection for growth in presence of erythromycin at the nonpermissive temperature (1). The colonies obtained were then screened for insertional inactivation of the methicillin resistance.

DNA manipulations. The molecular biological techniques used for nucleic acid manipulations, gel electrophoresis, blotting of DNA, and hybridization were mainly those described by Maniatis et al. (28). Restriction enzymes were obtained from Boehringer Mannheim and used as recommended by the supplier. Probes for hybridizations were a 10.5-kb *Pst*I fragment covering the *femA/B* operon and adjacent sequences, a 2.2-kb *Eco*RV fragment covering *femA*, and a 1.2-kb *Eco*RV fragment specific for *femB* (Fig. 1). For probing with Tn*551*, the *Hpa*I-1 fragment, carrying almost the complete 5.2-kb Tn*551*, was used (19). An internal 1.2-kb *Xba*I-*Pst*I fragment was used for probing *mecA*.

DNA preparations for pulsed-field gel electrophoresis and *Sma*I digests of chromosomal DNA was carried out essentially as described by Goering et al. (17, 18). Electrophoretic runs were performed at 14°C with a CHEF (contour-clamped homogeneous electric field) DRII electrophoresis cell (Bio-Rad, Richmond, Calif.). For the separation of *Sma*I fragments, each run began with 60-s pulses for 15 h followed by 90-s pulses for 9 h.

PBPs. Cell membranes from exponentially growing cells were labeled for 10 min at 30°C with [^{3}H]benzylpenicillin-ethylpiperidinium salt (25 Ci/mmol; Merck, Rahway, N.J.) at a final concentration of 10 μg/ml as described earlier (6). PBPs were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and visualized by fluorography (8, 9).

Isolation of cell walls and peptidoglycan. Bacteria from a 500-ml culture were harvested at an optical density of 0.7 (A_{578}). After suspension in 1 M NaCl, the cells were disintegrated with 130 ml of 0.1-mm glass beads (Braun, Melsungen, Germany) in a cooled cell grinder (Dyno Mill, Bachofen, Switzerland), keeping the temperature below 4°C. After

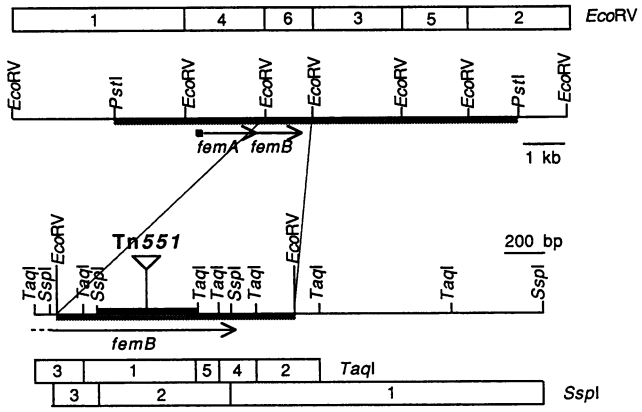


FIG. 1. Restriction map of the *femA/B* region in *S. aureus* BB270. Arrows show the open reading frames encoding *femA* and *femB* which are transcribed on a polycistronic mRNA. The 10.5-kb *Pst*I fragment used as a probe for *femA/B* is indicated by a dotted bar. The bar above the map shows the *Eco*RV fragments labeled by the 10.5-kb *Pst*I probe; they are numbered in descending order of size. The enlarged view of the *femB* open reading frame below shows the cleavage sites of the restriction endonucleases *Taq*I and *Ssp*I. The *Ssp*I-*Taq*I fragment carrying the Tn*551* insert Ω2006 is marked with a dark heavy bar. The bar below shows the *Taq*I and *Ssp*I fragments hybridizing with the 1.2-kb *femB* probe (Fig. 4), which is indicated by a dotted bar. The fragments are numbered according to size as above.

separation of the beads from the suspension by using a sintered glass filter, the suspension was mixed with 250 ml of 1% SDS and heated to 60°C for 30 min to remove membranes and noncovalently bound protein. After centrifugation and four washes with distilled water, the covalently bound cell wall protein (protein A) was removed by incubation with 0.2 mg of trypsin (Sigma) per ml in 0.15 M Tris-HCl buffer (pH 7) for 24 h. The carefully washed cell walls were lyophilized.

Amino acid analysis. Samples for amino acid analysis were prepared as described earlier (27) and analyzed in an LC5001 amino acid analyzer (Biotronic, Maintal, Germany), using ninhydrin as the detection reagent.

Preparation of muropeptides and separation according to size. Isolated cell walls or peptidoglycan (1 to 2 mg) were vortexed with 25 μl of a solution of 25 μg of *Chalaropsis* muramidase (EC 3.2.1.17; prepared essentially as described by Hash and Rothlauf [22]) in 50 mM sodium acetate buffer (pH 4.9). Samples at a final volume of 500 μl were incubated for 16 h at 37°C and subsequently filtered through a 0.2-μm-pore-size filter. Separation of the muropeptides according to the size was achieved by gel permeation high-pressure liquid chromatography (HPLC). Samples were applied to a TSK SW 2000 column (60 by 1.2 cm; Hewlett-Packard, Böblingen, Germany) protected by a 0.2-μm-pore-size filter (Beckman) and eluted with 50 mM Na_2HPO_4 (pH 5) at a flow rate of 0.3 ml/min. A_{206} was determined to detect the eluted compounds. Peaks were identified as monomers, dimers, trimers, etc., by comparison with published data (35, 36).

Electron microscopy. Overnight cultures in 2.5% peptone–0.5% NaCl were diluted twice to an optical density of 0.2 (A_{578}). Bacteria were further grown for 1 h to prepare cells from the logarithmic growth phase or for 4 h to fix cells from the stationary growth phase. Staphylococci were fixed overnight at 4°C with 2.5% glutaraldehyde (Serva, Heidelberg, Germany) in 0.1 M cacodylate buffer. The cells were postfixed for 1 h at room temperature with 1.5% osmium tetroxide (Serva) and 1.65% potassium bichromate (Merck, Darmstadt, Germany) in 0.1 M cacodylate buffer. The samples were then washed with cacodylate buffer and dispersed in 2% agar (Oxoid agar). Small blocks of agar were transferred in 0.5% uranyl acetate (Merck) for 1 h at room temperature. The agar blocks were dehydrated in increasing concentrations of ethanol, embedded in LR White plastic resins (Science Services, München, Germany), and polymerized at 60°C for 24 h. Thin sections were cut with a Reichert OM U3 ultramicrotome, poststained with lead citrate, and viewed with a Philips 400 electron microscope.

RESULTS

Characterization of Mc^r mutant PV1. The β-lactam-resistant (Mc^r) mutant PV1 was isolated from the susceptible *S. aureus* strain SG511 after five passages of in vitro selection for growth on increasing concentrations of penicillin. This mutant was able to grow in the presence of 6 mg of penicillin per ml, whereas the parent strain SG511 was susceptible to concentrations higher than 0.03 mg/liter. PV1 had acquired an intrinsic resistance against β-lactam antibiotics; the methicillin MIC rose to 32 mg/liter, whereas the methicillin MIC for strain SG511 was 1 mg/liter. The gain in resistance to β-lactam antibiotics was paralleled by an increase in the amount of labeled [^{3}H]penicillin bound to PBP 4 (Fig. 2, lanes a and b). Strain PV1 was stable upon storage or growth on nonselective media. The growth rates of PV1 and of the parent strain SG511 were similar, but there were differences in the *Sma*I digests of their chromosomal DNA, as detected by separation on CHEF gels (Fig. 3). PV1 had an additional band in the size range of 135 kb and a change in size of one of the smaller bands (not apparent in Fig. 3).

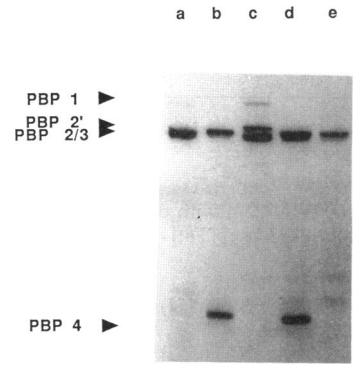


FIG. 2. Fluorography of ^{3}H-labeled PBPs of different Mc^r mutants. Positions of the five PBPs are indicated by arrows. Lanes: a, parent strain (Mc^s SG511); b, in vitro-selected Mc^r PV1; c, Mc^r BB270; d, Mc^s UT-32-1 (*femB* mutant derived from PV1); e, Mc^s UT-39-1 (transductant derived from PV1).

Insertional inactivation of the methicillin resistance in strain PV1. Twenty-eight independent Mc^s clones were obtained after Tn*551*-mediated insertional inactivation of Mc^r strain PV1. Linkage of Tn*551* to inactivation of the methicillin resistance was analyzed by transducing the Tn*551* inserts back into the original Mc^r strain PV1. In only 7 of the 28 insertionally inactivated strains was the Tn*551* insertion clearly linked to the susceptible phenotype. Of these seven strains, four had retained the parental PBP pattern from strain PV1 and three regained the SG511 pattern with a weak labeling of PBP 4 (Fig. 2).

Characterization of the Tn*551* inserts. The seven Tn*551* inserts which were linked to the Mc^s phenotype were characterized further by restriction enzyme analysis and Southern blotting. In all of these seven strains, Tn*551* had inserted into one of the two largest *Sma*I fragments, which migrate close to each other in CHEF gels. To differentiate further between these inserts, *Eco*RV digests were probed with a 10.5-kb *Pst*I fragment, covering the *femA/B* operon and adjacent sequences (Fig. 1), which are known to map in the *Sma*I-A fragment in *S. aureus* NCTC 8325. Two different

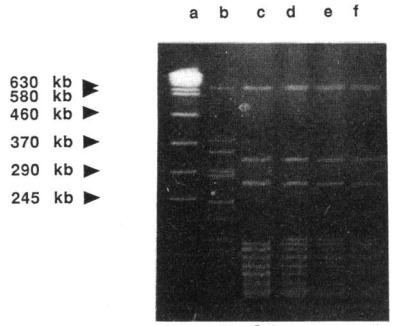


FIG. 3. *Sma*I-digested chromosomal DNA separated by CHEF pulsed-field gel electrophoresis, demonstrating the various *Sma*I fragments of different *S. aureus* species. The electrophoretic runs were carried out at 14°C by 60-s pulses for 15 h followed by 90-s pulses for 9 h. Lanes: a, yeast marker; b, Mc^r BB270; c, Mc^s SG511; d, in vitro-selected Mc^r PV1; e, Mc^s UT-32-1 (*femB* mutant derived from PV1); f, Mc^s UT-39-1 (transductant derived from PV1).

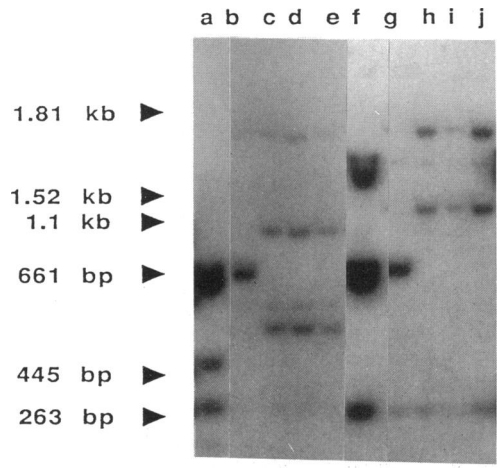
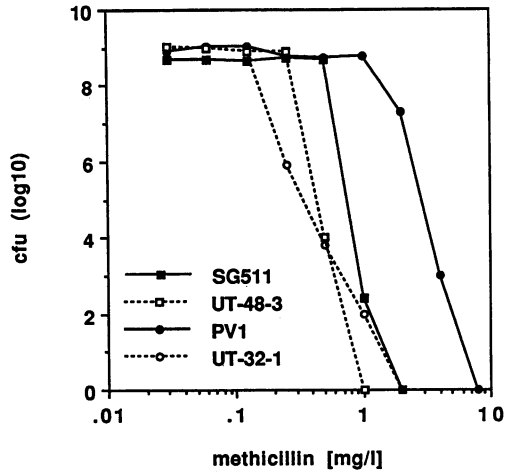


FIG. 4. Fine mapping of the Tn*551* inserts into *femB* of different Mc^s mutants. Lanes: a to e, *Ssp*I digests; f to j, *Taq*I digests, hybridized with the 1.2-kb *Eco*RV fragment covering *femB*; a and f, Mc^r BB270; b and g, Mc^r PV1, in vitro selected; c and h, Mc^s UT-34-2 (*femB* mutant derived from BB270); d and i, Mc^s UT-32-1 (*femB* mutant derived from PV1); e and j, Mc^s UT-48-3 (*femB* mutant derived from SG511).



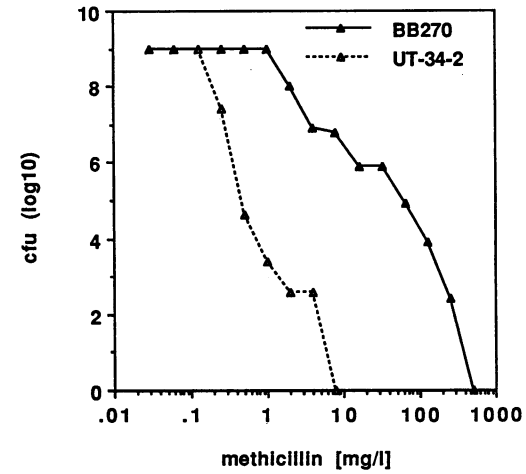


FIG. 5. Influence of insertion Ω2006(*femB*::Tn*551*) on the resistance levels of the population in different Mc^r strains. CFU were determined after 48 h of incubation at 35°C.

classes of Tn*551* integration sites could be detected, which correlated with the differences seen in their respective PBP patterns. In the four strains with the original PBP pattern as in strain PV1, Tn*551* had integrated into the 1.2-kb *Eco*RV fragment carrying *femB*; in the remaining three strains with the weaker PBP 4 band, the Tn*551* insertion sites were not within the 10.5 kb covered by the probe. These three strains were not analyzed further. The insertion sites of Tn*551* in the *femB* region were mapped in more detail by Southern blots. This was possible since the restriction patterns obtained with different multicutting enzymes were identical in the *femB* open reading frames of strains BB270 and SG511.

Hybridizations to *Ssp*I digests showed the Tn*551* integration site on the 661-bp *Ssp*I fragment 2, which disappeared and was replaced by two new fragments. In *Taq*I-digested chromosomal DNA, the Tn*551* insertion could be localized on the 666-bp fragment 1. Two new bands with increased molecular weight appeared instead of fragment 2, corresponding to the left and right junctions after Tn*551* integration (Fig. 4). Since all four inserts yielded the same pattern, one strain, UT-6, was used for further studies. The insertion, called Ω2006(*femB*::Tn*551*), interrupts the *femB* open reading frame in its central part, as shown in Fig. 1.

Characterization of Ω2006(*femB*::Tn*551*). To analyze the effect of Ω2006 on strains carrying the methicillin resistance determinant *mec*, the insertion Ω2006(*femB*::Tn*551*) was further transduced from strain UT-6 into the heterogeneously Mc^r strain BB270. All transductants were susceptible to methicillin. The donor UT-6 and the recipient BB270 were genetically unrelated strains of *S. aureus*, as evident in their respective *Sma*I chromosomal restriction patterns (Fig. 3) and the *Eco*RV restriction site polymorphism in the sequences framing the *femA/B* operon (27). When probed with the 10.5-kb *Pst*I fragment, *femB* transductants with different *Eco*RV and *Hin*dIII restriction patterns were obtained as a result of polymorphism between donor and recipient DNA and different crossover sites of the transduced DNA (data not shown). To have the most isogenic transductant corresponding to BB270, strain UT-34-2, which had restriction patterns identical to those of BB270, was used for further studies.

Physiological characterization of the insertion Ω2006. Inactivation of *femB* was paralleled by an increase in susceptibility to β-lactams. The methicillin MIC of 32 mg/liter for strain PV1 was reduced to 4 mg/liter in the *femB* mutant UT-32-1, obtained by crossing Tn*551* from UT-6 back into PV1. In the *femB* mutant strain UT-34-2, derived from heterogeneous Mc^r strain BB270, the MIC was lowered from 512 to 2 mg/liter. The population analysis (Fig. 5) showed that insertion Ω2006(*femB*::Tn*551*) reduced the resistance level of the in vitro-selected Mc^r strain PV1 as well as that of heterogeneously Mc^r strain BB270, which carries the *mec* determinant. Southern blots showed that Mc^s *femB* transductant UT-34-2 still harbored the *mec* determinant, although the majority of the cells were susceptible. A residual heterogeneity displayed by a small number of cells (10^{-8}) with slightly higher resistance was still apparent.

Amino acid composition and distribution of oligomeric muropeptides of cell walls from related strains differing in expression of *femB*. Inactivation of the structural gene for

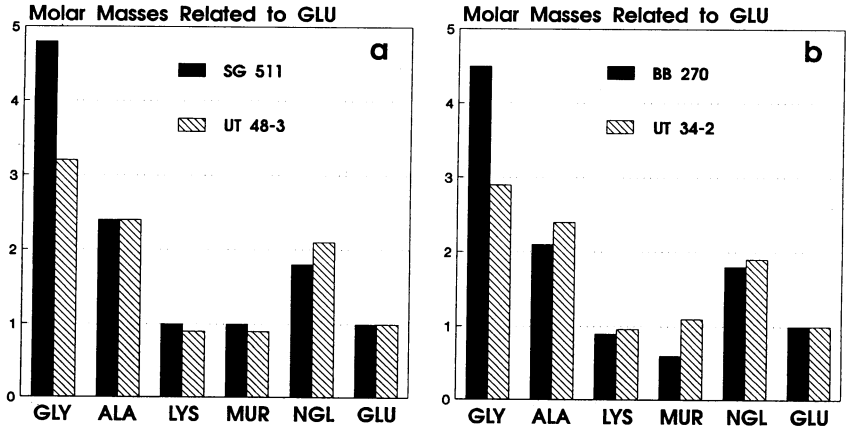


FIG. 6. Peptidoglycan amino compound compositions of two *S. aureus* strains and their respective *femB*-inactivated mutants. The molar masses of each amino compound were normalized with respect to glutamic acid (GLU). A significant difference was found in the glycine (GLY) content, which was reduced by approximately 40% in the *femB* mutants, irrespective the presence of the *mec* determinant. ALA, alanine; LYS, lysine; MUR, *N*-acetylmuramic acid; NGL, *N*-acetylglucosamine.

femB resulted in changes in cell wall composition similar to those described earlier for the mutants inactivated in the control region of the *femA/B* operon. The *femB* mutants UT-48-3 and UT-34-2, regardless of the presence of *mec*, had an approximately 40% reduction in the molar ratio of glycine to glutamic acid compared with the parent strains SG511 and BB270 (Fig. 6). The molar ratios of all other amino acids with respect to glutamic acid were not influenced and correlated with the well-known staphylococcal cell wall composition.

To determine whether the reduction in interpeptide glycine content of both *femA* and *femB* mutants influences the distribution of oligomeric peptides and the degree of cross-linking, muropeptides prepared by glycan chain digestion with *Chalaropsis* muramidase were separated by gel permeation HPLC according to peptide chain length. Two typical examples are shown in Fig. 7 for *femA* and *femB* mutants. Both *femA* and *femB* mutants revealed a clear increase especially of monomeric and dimeric muropeptides at the expense of large oligomers. In particular, the percentage of very large oligomers which could not be resolved as single peaks at short retention times in Fig. 7 was drastically reduced. Maximum peptide chain length decreased from approximately 32 to 19 for the *mec*-carrying strain BB270 versus its *femB* mutant UT 34-2 and for the Mc^s strain SG511 versus its *femA* mutant BB742.

Electron microscopy. The morphology of the *femA* and *femB* mutants is evident from the electron micrographs shown in Fig. 8. Both *femA* and *femB* mutants of *S. aureus* SG511 revealed a suppressed separation of daughter cells, as demonstrated clearly by the pseudomulticellular appearance of their cells (Fig. 8). Both mutants are also impressive because of some highly aberrant placement of cross walls. However, while *femA* strain BB742 created multiple cells, which showed a rather irregular shape with some minicells

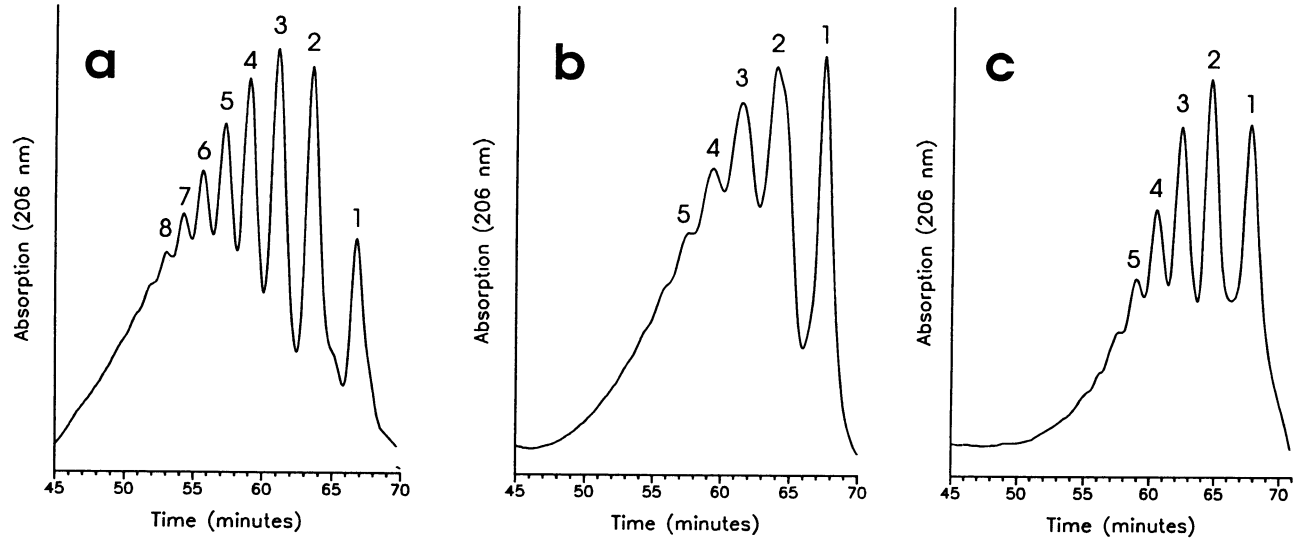


FIG. 7. Comparison between gel permeation HPLC profiles of muramidase-digested cell walls from strain SG511 (a), its *femA* mutant BB742 (b), and its *femB* mutant UT-48-3 (c). Peaks: 1, disaccharide-peptide monomer, 2, bis(disaccharide)-peptide dimer, 3, tris(disaccharide)-peptide trimer; 4 to 8, successive oligomers (tetramer, pentamer, hexamer, etc.).

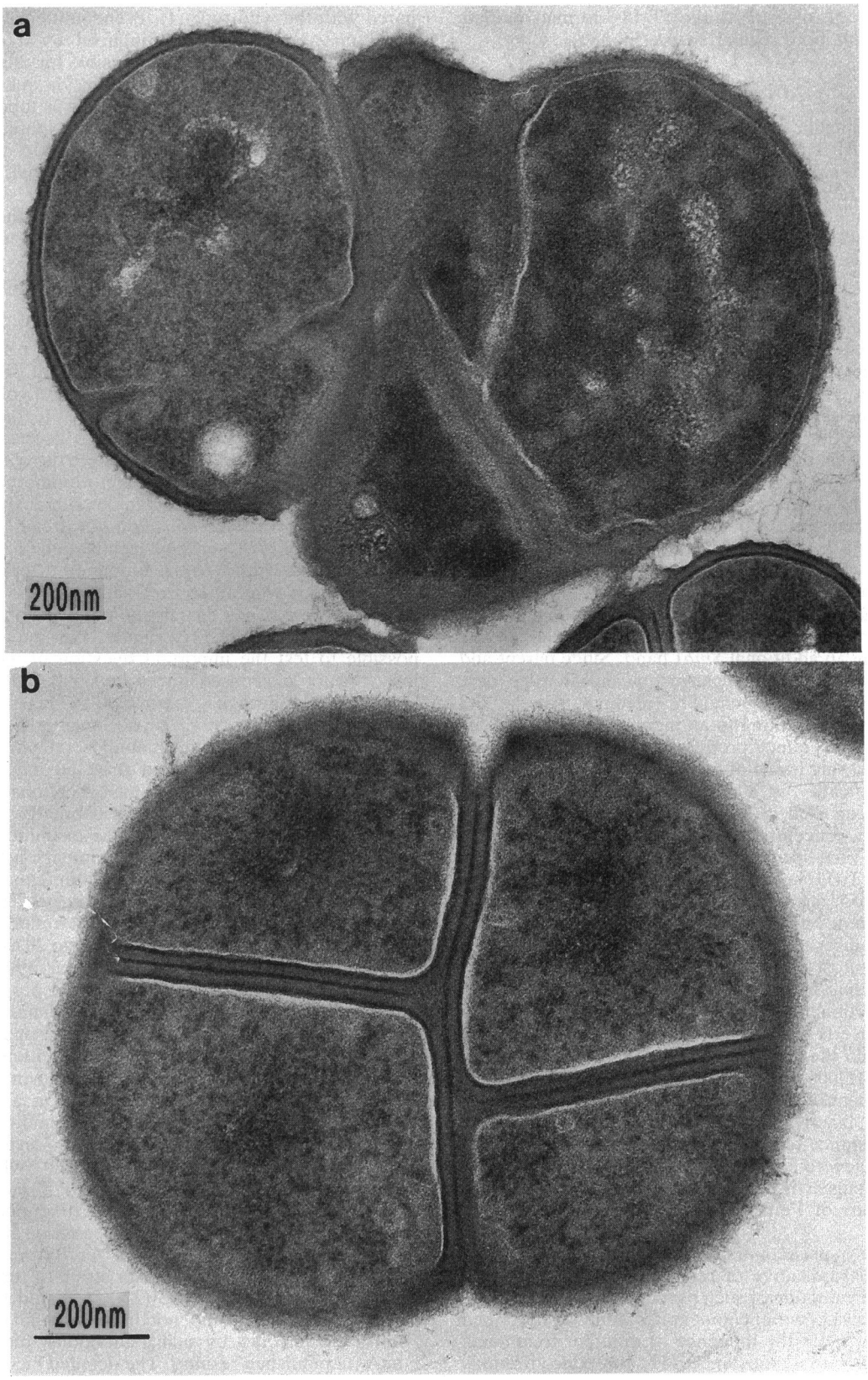


FIG. 8. (a) Thin section of *S. aureus* BB742 (*femA* mutant), showing irregularly formed multiple cells caused by a disturbed initiation of cross walls and delayed separation of daughter cells. (b) Thin section of *S. aureus* UT-48-3 (*femB* mutant). Delayed separation of daughter cells yielded more regularly arranged multiple cells.

(Fig. 8a), most cells of *femB* strain UT-48-3 formed multiple cells with a much more regular shape (Fig. 8b).

DISCUSSION

Intrinsic methicillin resistance in *S. aureus* due to the expression of PBP 2′ is dependent on further genetic factors known as *fem* or *aux* factors (1, 24). It has been speculated (38) that these factors might comprise auxiliary mutations needed for the full expression of high-level methicillin resistance. One of these factors, *femA*, seems to be present in all, susceptible as well as resistant, staphylococci (unpublished results) and is involved in the correct assembly of the staphylococcal pentaglycine interpeptide bridge length of the peptidoglycan moiety (27). The observation revealed that *femA* and subsequently possibly other *fem* factors also play a role in cell wall metabolism. This finding enabled us to use in vitro-selected β-lactam-resistant strains, which did not carry the *mec* determinant, to screen for further mutants in *fem* genes and other genes related to increased β-lactam sensitivity. For this purpose, we used the in vitro-selected, intrinsically resistant SG511 mutant PV1, whose resistance was due to multiple changes in the membrane protein pattern as well as in changes of the penicillin-binding characteristics or amount of PBP 4. These effects were similar to those described earlier in Mc^r strains selected by similar methods (6, 14, 39). Unexpectedly, in addition to these changes, a major rearrangement involving probably a duplication of a substantial part of the chromosome was observed in the mutant, yielding an additional *Sma*I band. Since parent and mutant strain were of identical phage type and ribotype (data not shown), a contamination could be ruled out. Whether the duplication was contributing to an increase in resistance or was merely a side effect produced by the selection for growth on increasing levels of penicillin could not be distinguished. Amplification of a section of chromosomal DNA has also been reported in Mc^r *S. aureus* strains following growth on high concentrations of methicillin (31). In this case, however, *mec*-specific sequences were amplified.

Interestingly Tn*551*-mediated inactivation of Mc^r in strain PV1 yielded Tn*551* insertions in the largest *Sma*I fragments of the chromosome but none in the new *Sma*I fragment. The fact that Tn*551* insertions are probably not random indicates the large portion of strains with identical inserts into the *femB* open reading frame, although they all stemmed from individual insertional inactivation events. In contrast to earlier Tn*551* inserts in *femB* (namely, ΩIII-2 and ΩII-1 [3]), which cleaved off the last 10 to 20 C-terminal amino acids of FemB, insert Ω2006 interrupts the FemB protein in its central part. In an earlier report, we suggested a weaker effect of *femB* inactivation than of *femA* inactivation on methicillin resistance (4). Here we showed that *femB* inactivation has as severe consequences as does *femA* inactivation by Ω2003, suggesting that the earlier *femB* insertions in the terminal part of FemB allowed a residual activity of FemB.

The existence of pseudomulticellular cells of the *femA* and *femB* mutants is indicative of retardation of bacterial cell separation. The induction of such pseudomulticellular staphylococci is a well-known phenomenon which was described earlier for cells under the influence of different treatments such as with polyanionic substances (41), low concentrations of chloramphenicol (16), or certain concentrations of penicillin (15). Investigations of the extent of autolysis of the different Mc^r *S. aureus* strains have shown that the overall autolytic system of the *fem* mutants was not altered compared with the wild type (4). Pseudomulticellular staphylococci may nevertheless be induced by a defect of the autolytic enzymes that are responsible for the separation of daughter cells (16), possibly either via reduction of the amount or via variation of the quality or topology of autolytic enzymes in the cross walls of these mutants. However, the most probable reason for the disturbed cell separation would be an alteration of the wall material, which is the target for the wall autolytic systems.

Since our data have shown a change in the interpeptide bridge of the peptidoglycan, such a variation in the chemical composition and steric configuration of this peptidoglycan part could possibly be involved in the retarded cell separation.

We have described recently that peptidoglycans of *femA*-inactivated strains differ from those of their parent strains by 40 to 60% reduction in the glycine content of the staphylococcal pentaglycine interpeptide bridge involved in peptidoglycan peptide cross-link formation. In these particular mutants, *femB* transcription was also decreased (by up to 90%), since Tn*551* insertion occured in the control region of the *femA/B* operon (27). We therefore determined whether *femB* inactivation caused similar or other alterations with respect to amino acid composition. As already seen for *femA* mutants, the Tn*551* insertional inactivation of *femB* in strains SG511 and BB270 led to the formation of peptidoglycan in mutant strains with an approximately 40% reduction in the glycine content. Thus, inactivation of *femA* and inactivation of *femB* seem to have very similar consequences. It seems possible to test the important question of whether or not these phenotypic consequences are similar only or identical, using the techniques of combined HPLC-mass spectrometric analysis recently described by de Jonge et al. (11).

Molecular model building studies on various bacterial peptidoglycans (26) suggested that the long pentaglycine chain is a prerequisite to reach the exceptionally high values of cross-linking of staphylococcal peptidoglycan compared with the other species. We therefore examined the effect of reduction in interpeptide glycine content on the degree of cross-linking. For *femA* and for *femB* mutants, the degree of cross-linking and the number of glycine residues in the interpeptide bridge were reduced. The reductions in maximal peptide chain length from about 30 to 20 as measured by gel permeation HPLC were also identical in *femA* and *femB* mutants, as was the rise in the relative proportion of the monomeric and low oligomeric peptide chains. The reduced glycine content and thus shortened glycine interpeptide bridge in *femB* mutants led to the assumption that *femB* is almost as important as *femA*, especially when one considers that *femA* and *femB* are transcribed on a polycistronic mRNA under the control of the same promoter. This is also reflected by the population analysis of *femA* (Ω2003) and *femB* (Ω2006) mutants of strain BB270, which revealed almost identical suppression of the PBP 2′-mediated methicillin resistance. The effect was due in the former strain to an over 95% reduction of *femAB* transcription in the latter to FemB inactivation. The residual *femB* transcription was apparently sufficient in the former strain to support complementation with FemA alone (27). It should be noted that inactivation of *femB* did not influence the production of FemA, as detected by using antibodies directed against FemA (unpublished results). The detailed biochemical functions of both proteins, which show considerable amino acid sequence homologies, and their interaction with each other are still not understood. In any case, neither the presumably complete knockout of FemB, as described here, nor the

large reduction of FemA and FemB, as observed in the *femA* mutants described earlier (3, 27), seems to be incompatible with survival of the bacteria, although in both cases methicillin resistance is almost lost. It remains an interesting question whether either a *femA* mutant without any residual FemA activity or a *femA femB* double mutant might be viable.

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