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femA, Which Encodes a Factor Essential for Expression of Methicillin Resistance, Affects Glycine Content of Peptidoglycan in Methicillin-Resistant and Methicillin-Susceptible *Staphylococcus aureus* Strains

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***femA* is a chromosomally encoded factor, occurring naturally in *Staphylococcus aureus*, which is essential for the expression of high-level methicillin resistance in this organism. The production of a low-affinity penicillin-binding protein, PBP2a or PBP2′, which is intimately involved with methicillin resistance in *S. aureus*, is not influenced by *femA*. To elucidate a possible physiological function of the 48-kDa protein encoded by *femA*, several related methicillin-resistant, methicillin-susceptible, and Tn*551* insertionally inactivated *femA* mutants were analyzed for possible changes in cell wall structure and metabolism. Independent of the presence of *mec*, the methicillin resistance determinant, all *femA* mutants had a reduced peptidoglycan (PG) glycine content (up to 60% in the molar ratio of glycine/glutamic acid) compared to that of related *femA*$^+$ parent strains. Additional effects of *femA* inactivation and the subsequent decrease in PG-associated glycine were (i) reduced digestion of PG by recombinant lysostaphin, (ii) unaltered digestion of PG by *Chalaropsis* B-muramidase, (iii) reduced cell wall turnover, (iv) reduced whole-cell autolysis, and (v) increased sensitivity towards β-lactam antibiotics. Also, the PG-associated glycine content of a *femA*::Tn*551* methicillin-susceptible strain was restored concomitantly with the methicillin resistance to a level almost equal to that of its *femA*$^+$ methicillin-resistant parent strain by introduction of plasmid pBBB31, encoding *femA*.**

Methicillin-resistant *Staphylococcus aureus* (MRSA) are increasingly responsible for outbreaks of nosocomial infections in countries around the world and have also become established outside the clinical environment, particularly among intravenous drug users (7, 19). It has now been established that the production of an additional penicillin-binding protein, PBP2a or PBP2′, with low affinity for β-lactam antibiotics, is intimately involved in the methicillin resistance mechanism of *S. aureus*.

The methicillin resistance determinant *mec* has been mapped to the *S. aureus* chromosome (17, 30) and contains the structural gene for PBP2a, *mecA* (33). It is believed that PBP2a is essential for cell wall synthesis in the presence of otherwise inhibitory concentrations of methicillin (9, 13, 26) and appears to be highly conserved among unrelated MRSA and methicillin-resistant, coagulase-negative staphylococcal strains (6, 27). Gaisford and Reynolds (11) provided evidence that PBP2a may be involved in the attachment of nascent peptidoglycan (PG) to preexisting cell wall material by an attachment transpeptidase reaction. Although a prerequisite for methicillin resistance, the presence of *mec* is not alone responsible for the degree to which resistance is expressed; it is also known that the level of PBP2a is not directly related to the phenotypic level of resistance (1, 4, 8, 16, 20, 23).

A number of factors essential for methicillin resistance have now been located on the *S. aureus* chromosome which are not linked to the *mec* locus yet which influence the level of resistance (1, 3, 4, 16, 23). One factor, *femA*, has recently been cloned and characterized (2). The product of *femA* is a 48-kDa protein with an unknown function that has no influence on the synthesis of PBP2a.

By analyzing the PG structure of related methicillin-resistant, methicillin-susceptible, and *femA*::Tn*551* methicillin-susceptible *S. aureus* strains, we now demonstrate that inactivation of *femA* correlates with a reduction in the glycine content of *S. aureus* PG. Also, the introduction of plasmid pBBB31 encoding *femA* into a *femA*::Tn*551* methicillin-susceptible strain could restore the level of PG-associated glycine content concomitantly with the level of methicillin resistance possessed by the original parent strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. aureus* strains used in this study are listed in Table 1. All strains were grown at 37°C with shaking in 2.5% Bacto-Peptone (Difco) medium supplemented with 0.5% NaCl. Overnight cultures were diluted in fresh medium to obtain an initial optical density at 578 nm (OD_{578}) of 0.1 and grown to an OD_{578} of 0.8. To ensure exponential growth, the cultures were then diluted again with fresh prewarmed medium to an OD_{578} of 0.1 and regrown to an OD_{578} of 0.6 to 0.7. The cells were harvested by centrifugation, washed with 0.1 M ammonium acetate (pH 6.8), and lyophilized.

Construction of BB742 and molecular biological characterization. BB742 was produced by transduction of SG511 with phage 80 α grown on strain BB308 (*femA*::Tn*551*). Strain SG511 was heated for 2 min at 52°C just prior to transduction to inactivate the restriction system and increase transduction frequency (4a). Transductants were selected on LB plates containing erythromycin at a final concentration of 20 mg/

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TABLE 1. Bacterial strains

Strain	Relevant genotype	Source or reference
BB255	NCTC 8325	1
BB270	NCTC 8325 *mec*	1
BB308	NCTC 8325 *mec* Ω2003 (*femA*::Tn*551*)	1
BB586	NCTC 8325 *mec* Ω2003 (*femA*::Tn551) pBBB31 (*femA*)	2
Col	Col *mec*	16
BB403	Col *mec* Ω2003 (*femA*::Tn*551*)	2
SG511	SG511	Strain collection of the Robert Koch-Institute, Berlin
BB742	SG511 Ω2003 (*femA*::Tn551)	This study

liter. All techniques in molecular biology were performed essentially as described previously (21).

Isolation of PG. A 100-mg amount of lyophilized cells was resuspended in 1 ml of 0.1 M sodium acetate (pH 5.0) containing 1% (wt/vol) sodium dodecyl sulfate (SDS) (Merck, Darmstadt, Germany). The suspension was transferred to a 7-ml Teflon vessel, precooled by liquid nitrogen, and then homogenized for 3 min in a Dismembrator II (Braun, Melsungen, Germany). The homogenate was then resuspended in 250 ml of the above-described buffer and sedimented for 10 min at 12,000 × *g*. The pellet was suspended in 250 ml of 0.1 M Tris-HCl (pH 7.5) containing 1% SDS and incubated for 30 min at 60°C. After being harvested as described above, the pellet was washed in the above-described buffer. Residual nucleic acids and noncovalently bound proteins were removed by incubating the isolated cell walls in 40 ml of a 1:1 (vol/vol) mixture of 0.1 M sodium acetate (pH 5.0) and aqueous phenol (80% [vol/vol]) (Merck) for 60 min at 70°C. The cell walls were then reharvested by centrifugation and washed twice in 0.1 M Tris-HCl (pH 7.5), followed by several washings with distilled water. The cell wall pellet was resuspended in 50 ml of 0.1 M Tris-HCl (pH 7.5) containing 20 μg of trypsin (Sigma, Munich, Germany) per ml and allowed to incubate at 37°C for 15 h. A 50-μl volume of toluene was then added to the cell wall suspension to prevent microbial contamination. The cell walls were washed once with 1% SDS containing 0.5 M NaCl and twice with distilled water. Teichoic acids were removed by suspending the cell walls in 25 ml of 10% (wt/vol) aqueous trichloroacetic acid for 5 h at 37°C. The PG was harvested by centrifugation as described above, washed several times with distilled water, and lyophilized.

PG amino acid analysis. Samples for amino acid analysis were prepared by hydrolyzing isolated PG with 4 M HCl at 100°C for 15 h. Amino acid analyses were performed in an LC5001 amino acid analyzer (Biotronik, Maintal, Germany) by using a sodium citrate buffer system. The amino compounds were detected by means of the ninhydrine reagent.

[^{14}C]*N*-acetylglucosamine labeling of bacterial cells. For cell wall turnover assays, 50 ml of early-log-phase cells (OD_{578}, 0.05) was labeled by adding 0.05 ml of an aqueous solution containing 185 nmol (0.37 MBq) of [^{14}C]*N*-acetylglucosamine (Amersham-Buchler, Braunschweig, Germany). After being shaken at 37°C for 2 h (three to five generations), the labeled bacteria were harvested by membrane filtration, washed twice with fresh, warm peptone broth, resuspended in this medium to an OD_{578} of 0.05 to 0.1, and cultivated for another 2 h, as described above. The labeling period chosen led to a uniform distribution of the label within 80 to 100% of the total wall mass, which ensured correct turnover measurements. Since the generation times were not identical for all of the strains, some strains were labeled for an additional 1 h. During the cultivation, 1-ml samples were withdrawn every 30 min to determine cell wall turnover and OD_{578} measurements. The whole-cell autolysis assay followed the same schedule, except that cells were washed in 0.1 M ammonium acetate (pH 6.8) and resuspended in the same buffer.

Measurements of ^{14}C-labeled material released by whole-cell autolysis and cell wall turnover. Two 0.3-ml portions of each sample were centrifuged for 3 min at 12,000 × *g* in Eppendorf tubes, and 0.2 ml of each supernatant was placed in a scintillation vial for assay of the turnover rates. Boiling of the pellets with 5% SDS failed to solubilize any further significant amounts of the labeled material. Reference samples representing 100% of the incorporated radioactive label were solubilized by adding 0.5 ml of Soluene (Packard Instruments) to 0.1 ml of the bacterial suspensions. The suspensions were then allowed to dissolve overnight at room temperature. An 8-ml volume of Hionic Fluor (Packard Instruments) scintillation cocktail was added to each sample, and radioactivity was measured in a Tri-Carb 1900 CA liquid scintillation counter (Packard Instruments).

Enzymatic digestion of heat-inactivated whole cells. Bacterial autolysins were initially inactivated in whole cells by boiling in a 100°C water bath for 15 min. The cells were then harvested and resuspended in the appropriate assay buffer. Lysostaphin digestions were carried out in 2 ml of 0.2 M Tris-HCl (pH 8.0), and *Chalaropsis* B-muramidase digestions were assayed in 2 ml of 0.05 M ammonium acetate (pH 5). The reactions were initiated by adding recombinant lysostaphin (Applied Microbiology) and *Chalaropsis* B-muramidase (prepared as described by Hash [14]) at final concentrations of 2.5 and 5 μg/ml, respectively. All incubations were carried out at 37°C, and the degradation of the cells, measured as a decrease in OD_{578}, was monitored continuously with a SP1600 spectrophotometer (Pye Unicam, Cambridge, England).

RESULTS

Characterization of SG511 *femA* transductant BB742. *S. aureus* SG511 is a β-lactam-susceptible strain which is genetically unrelated to BB270 and Col. This strain was selected for the transduction experiment because its phenotypic response towards β-lactam antibiotics is especially well characterized (12, 18). Initially, it was difficult to transduce the *femA* mutant genotype of BB308 into SG511, suggesting that these two strains may have different restriction systems. This was overcome by brief heat inactivation of the SG511 restriction system which increased the transduction efficiency about 100-fold to 1.5×10^{-7}. To determine if BB742 had become a *femA* mutant, *Eco*RV chromosomal digests of SG511 and BB742, with BB270 and BB308 as controls, were probed with a 10.5-kb *Pst*I chromosomal fragment known to cover *femA* and the adjacent regions (Fig. 1). The *femA* gene of SG511 (Fig. 2, lane b) and BB270 (Fig. 2, lane d) was located in a 2.2-kb *Eco*RV fragment. Upon insertion of the 5.2-kb Tn*551*-inactivating *femA* in strains BB742 (Fig. 2, lane a) and BB308 (Fig. 2, lane c), the 2.2-kb band disappeared and was replaced by a 7.4-kb band. The increased susceptibility to methicillin-induced lysis of BB742 compared to that of its parent SG511 (Fig. 3) corresponds with the findings of Berger-Bächi et al. (2), where a methicillin-sensitive strain became more sensitive to methi-

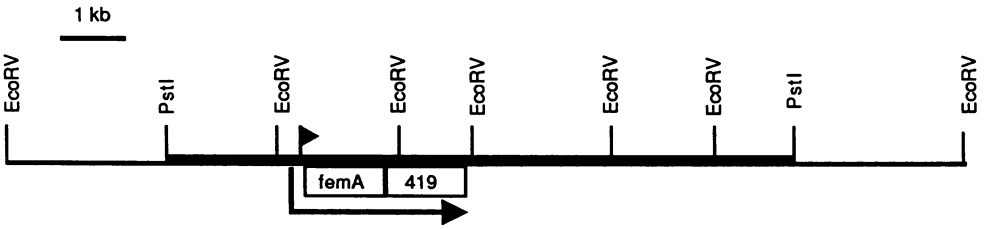

FIG. 1. Restriction map of the *femA* region in *S. aureus* BB270. Boxes show the ORFs encoding *femA* and the adjacent ORF419. Both ORFs are transcribed on a polycistronic mRNA as shown by the arrow (2). The approximate insertion site of Tn*551* is indicated by a flag. The *Pst*I fragment used as a probe in Fig. 2 is indicated by a dark heavy bar.

cillin by the inactivation of *femA*. This corroborated that Tn*551* was inserted into *femA* of BB742. Although the growth of BB742 was inhibited at lower drug concentrations than was the growth of SG511, the subsequent lysis of the cells as measured by the decrease in OD of the culture with time (which seems to be mainly due to postmortem degradation of the cell walls by autolytic processes [12, 18]) was clearly retarded for the *femA* mutants (Fig. 3).

Amino acid composition of PG from related strains differing in their expression of *femA*. To determine if inactivating *femA* caused an alteration in PG structure, PG from all of the strains in this study was isolated and analyzed with respect to amino acid composition. Results of this experiment (Fig. 4) indicated that *femA* mutants BB742, BB308, and BB403, regardless of the presence of *mec*, had a 30 to 60% reduction in the molar ratio of glycine to glutamic acid compared to that of their *femA*$^+$ parent strains. The molar ratios of all other amino acids with respect to glutamic acid were not influenced and agreed with what one would expect on the basis of the well-known staphylococcal PG composition (Fig. 4). Also, strain BB586, derived from BB308 (*femA*::Tn*551*) by introduction of plasmid pBBB31 encoding *femA*, had a glycine content similar to that of BB270 and greater than that of BB308.

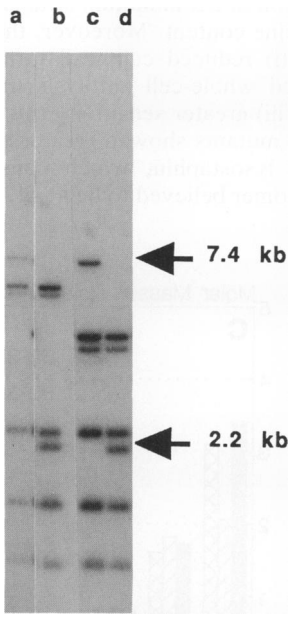

FIG. 2. Southern blot of *Eco*RV-digested chromosomal DNA separated on a 0.9% agarose gel, demonstrating insertion of Tn*551* in the *femA* region. The *Pst*I fragment shown in Fig. 1 was used as a probe. Lanes: a, *femA*::Tn*551*-inactivated strain BB742; b, parent strain SG511 of strain BB742; c, *femA*::Tn*551*-inactivated strain BB308; and d, methicillin-resistant parent strain BB270 of strain BB308. The positions of the 2.2-kb fragment containing *femA* and the 7.4-kb fragment containing the 5.2-kb Tn*551* inserted in *femA* are indicated by arrows.

Effects of inactivating *femA* on cell wall turnover and whole-cell autolysis. Cell wall turnover depends on the activity of the autolytic cell wall hydrolases as well as on the wall structure and was described as occurring in all strains of *S. aureus* so far investigated; during growth in rich media, the turnover rates were found to range from 14 to more than 30% of the total wall mass per generation time (10). It is noteworthy that no significant error arises from part of the wall label being held back in the cytoplasm, since fractionation of the labeled cells by the method of Park and Hancock (24) regularly showed that only less than 5% of the wall label resides in the cytoplasm from which it is lost to the growth medium at a rate of about 5% per generation time (36; see also references 5 and 29). Furthermore, boiling of the cells with 5% SDS did not solubilize any significant amount of the label, an observation that is in agreement with earlier results (29). Therefore, the release rate of the specific cell wall marker of [^{14}C]*N*-acetylglucosamine from growing cells was measured in strains SG511, BB270, Col, and their *femA* mutants BB742, BB308, and BB403, respectively, to determine if inactivating *femA* had an effect on cell wall turnover. All *femA* mutants demonstrated a 30% reduction in cell wall turnover compared with that of their parent strains (Fig. 5). The introduction of *femA* increased the wall turnover rate by 14% in BB568 compared to that in *femA* mutant BB308, but the rate was still about 20% lower than it was in the original parent strain BB270. The results were not changed if cells labeled for 3 h instead of 2 h were used.

As with cell wall turnover, the rate of whole-cell autolysis by *femA* mutants under nongrowth conditions in 0.1 M ammonium buffer (pH 6.8) was lower than that of their parent strains (Fig. 6). Autolysis of the methicillin-susceptible *femA* mutant BB742 was also reduced compared to that of its parent strain SG511. Furthermore, the rate of BB586 whole-cell autolysis was between that of BB308 and that of BB270.

Enzymatic digestion of heat-inactivated whole cells. The results of the turnover and whole-cell autolysis experiments may depend on both altered cell wall structure and any change in amount or activity of the different cell wall autolysins in *femA* mutant strains. To monitor the effects due to altered cell wall glycine content exclusively, wall lytic enzymes were added externally to cells whose own autolytic enzymes had been heat inactivated. Heat-inactivated whole cells of *femA* mutants BB742, BB308, and BB403 lysed at a slower rate and to a lesser extent in the presence of recom-

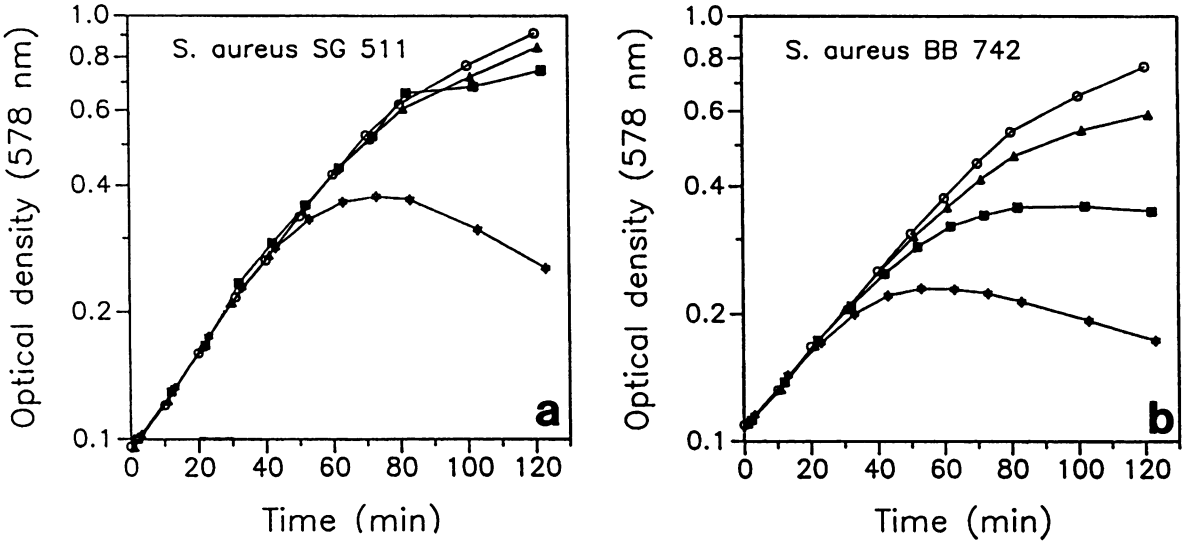

FIG. 3. Increased sensitivity of *femA* mutant BB742 towards methicillin. (a) Parent strain SG511 was affected by 2 μg of methicillin per ml only (★), whereas the cells treated with 0.5 μg methicillin per ml (▲) and 1.0 μg of methicillin per ml (■) grew in a manner very similar to that of the control cells (○). (b) In contrast, *femA* mutant strain BB742 showed a significant deviation from control growth even at a concentration of 0.5 μg/ml. Note that the decrease in OD in strain SG511 at 2.0 μg/ml occurred more rapidly than that in the *femA* mutant strain BB742.

binant lysostaphin than did cells of their respective *femA*$^+$ parents (Fig. 7a). In contrast to the above results, whole cells from *femA* mutants and those from their respective *femA*$^+$ parent strains were lysed at much more similar rates by *Chalaropsis* B-muramidase (Fig. 7b).

DISCUSSION

The production of a novel penicillin-binding protein, PBP2a, which has low affinity for binding β-lactams, is intimately involved in the methicillin resistance mechanism of *S. aureus* (7, 19). Besides PBP2a, another factor which influences the degree of resistance without affecting PBP2a production is *femA* (2–4). The inactivation of *femA* by Tn*551* insertional mutagenesis results in a loss of methicillin resistance (1, 16).

We have now shown that strains with different expression levels of *femA* possess altered PG, particularly with regards to glycine content, independent of *mec*. The Tn*551* insertional inactivation of *femA* in strains SG511, BB270, and Col led to the formation of PG in mutant strains with a 30 to 60% reduction in glycine content. Moreover, these *femA* mutant strains showed (i) reduced cell wall turnover in growing cells, (ii) reduced whole-cell autolysis under nongrowing conditions, and (iii) greater sensitivity towards methicillin.

Further, *femA* mutants showed greater resistance to lysis by recombinant lysostaphin, which consists of a single polypeptide monomer believed to be a polyglycine endopep-

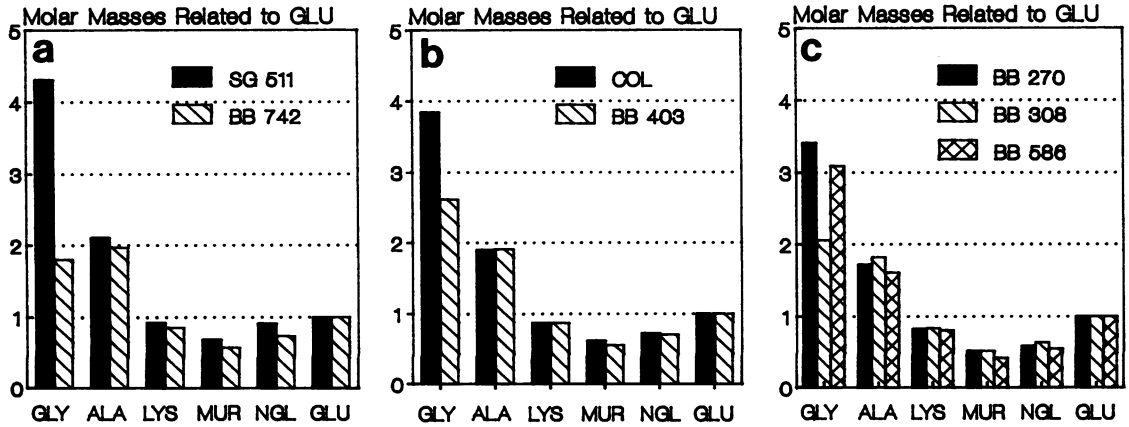

FIG. 4. PG amino compound composition of three *S. aureus* strains and their respective *femA*-inactivated mutants. Symbols: ■, parent strains; ▧, *femA* mutants (Table 1); ▩, *femA* mutant carrying plasmid pBBB31. The molar masses of each amino compound were normalized with respect to glutamic acid (GLU). *N*-Acetylmuramic acid (MUR) and *N*-acetylglucosamine (NGL) are also shown. The only significant difference was found in the glycine content, which was reduced by 30 to 60% in the *femA* mutants, irrespective of the *mec* determinant, which was not present in strains SG511 and BB742. Introduction of plasmid pBBB31, encoding *femA* in BB308 and leading to strain BB586, almost restored the glycine content to the value of BB270 PG (panel c, columns labeled GLY).

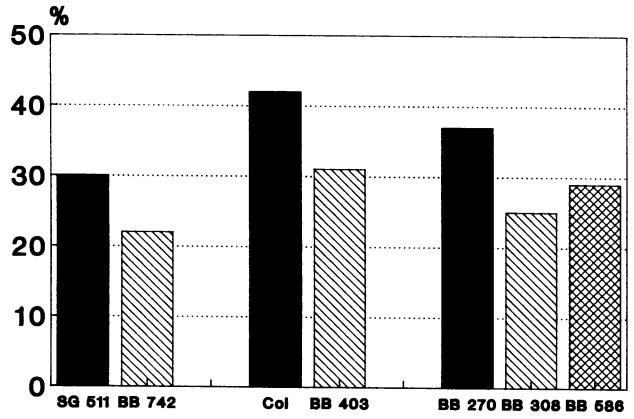

FIG. 5. Reduced wall turnover of *S. aureus* after inactivation of *femA*. Columns represent the relative losses of wall label per generation time during bacterial growth in peptone broth. Symbols: ■, *femA*$^+$ parent strain; ▧, *femA* mutant; ▩, *femA* reintroduced into *femA* mutant.

tidase (25). This is in agreement with the findings of Zygmunt et al. (37), who reported isolating a mutant of *S. aureus* Copenhagen with reduced cell wall glycine content and a concomitant increase in lysostaphin resistance. Related *femA* mutants and *femA*$^+$ parent strains showed similar rates of lysis by *Chalaropsis* B-muramidase which cleaves the β-1,4 linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine of PG. This observation suggested that the inactivation of *femA* did not fundamentally alter the repeating disaccharide backbone structure of PG. However, changes in the degree of *O*-acetylation or in the teichoic acid substitution (which were not examined in this study) could have occurred without affecting the activity of the *Chalaropsis* B-muramidase.

It is conceivable that the lowering of cell wall turnover and whole-cell autolysis in *femA* mutants was caused merely by the reduced glycine content of the PG, i.e., by an alteration in autolysine substrate. Alternatively, a primary abnormality in the autolysins could have led to the abnormal PG composition. However, the first possibility is also supported by the degradation experiments using externally added recombinant lysostaphin, which revealed a significant reduction in degradability of the *femA* mutants, since lysostaphin is believed to be analogous to the autolytic endopeptidase of *S. aureus* (35). If the number of cross-links or the number of glycine residues in the glycine-mediated cross-links, or both, had been changed, the three autolytic activities (32, 34, 35) in *S. aureus* might no longer be able to optimally recognize their substrates.

Assuming that *femA* mutant strains have a shortened glycine interpeptide bridge, one can speculate that *femA* might be a protein involved in the formation of those interpeptide bridges. Bridge formation occurs immediately before the completed PG subunits are transferred to the growing PG (28). The five glycine molecules have to be activated by tRNA, catalyzed by a single Gly-tRNA synthetase, and will be sequentially linked, initially to the ε-amino group of lysine of the stem peptide and then, glycine by glycine, to the amino terminus of the growing interpeptide bridge (15, 22, 31). Thus, since both glycine activation and glycine chain elongation are enzyme-catalyzed processes, *femA* may play a role in these processes.

It is important to note that, in all insertionally *femA*-inactivated strains used, the transposon Tn*551* seemed to be integrated into the promoter or control region of *femA* because low residual transcription of 5 to 10% could be detected in these strains (2). Although this residual activity was not sufficient for high-level methicillin resistance expression, it might be responsible for the residual glycine incorporation into the staphylococcal PG, which still reached at least 40% of the amount found in the *femA*$^+$ strains. The PG-associated glycine content in BB308 was essentially increased to the glycine level of BB270, when pBBB31 was introduced into BB308, providing strain BB586. However, the levels of cell wall turnover and whole-cell autolysis in BB586 were not returned to the levels observed in BB270. This pleiotropic effect could suggest that another factor besides *femA* had been inactivated in BB308. A possible candidate is *femB*, which was mapped earlier (2) downstream of *femA*. Because *femA* and open reading frame 419 (ORF419) (the hypothetical *femB*) are transcribed on a polycistronic mRNA, the Tn*551* insertional inactivation of *femA* also caused a 90% decrease in *femB* transcription. It is also known that *femB* cannot be complemented by pBBB31

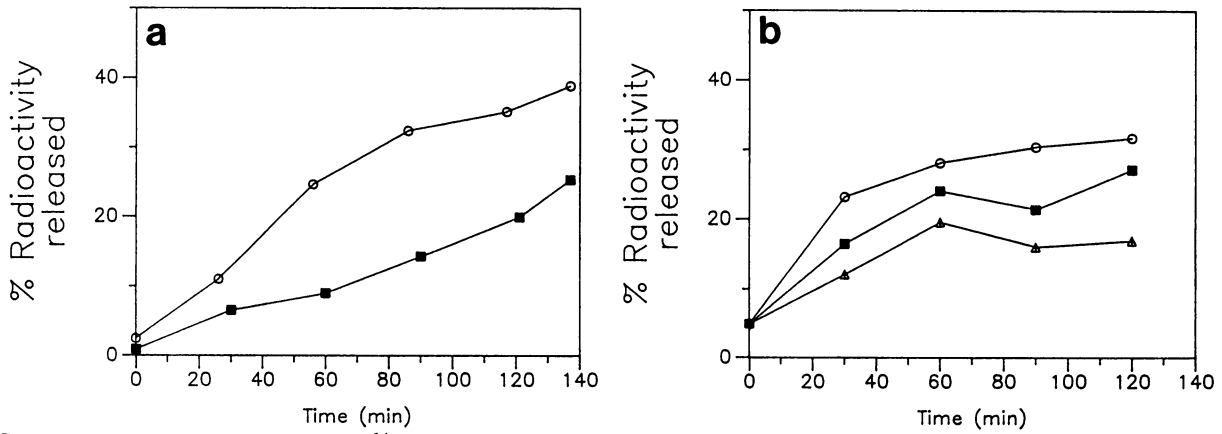

FIG. 6. Release of the cell wall label [^{14}C]*N*-acetylglucosamine from whole staphylococcal cells under nongrowing conditions. (a) Symbols: ○, parent strain SG511; ■, *femA* mutant strain BB742. (b) Symbols: ○, parent strain BB270; △, *femA* mutant strain BB308; ■, strain BB586 (BB308 pBBB31 ([*femA*]).

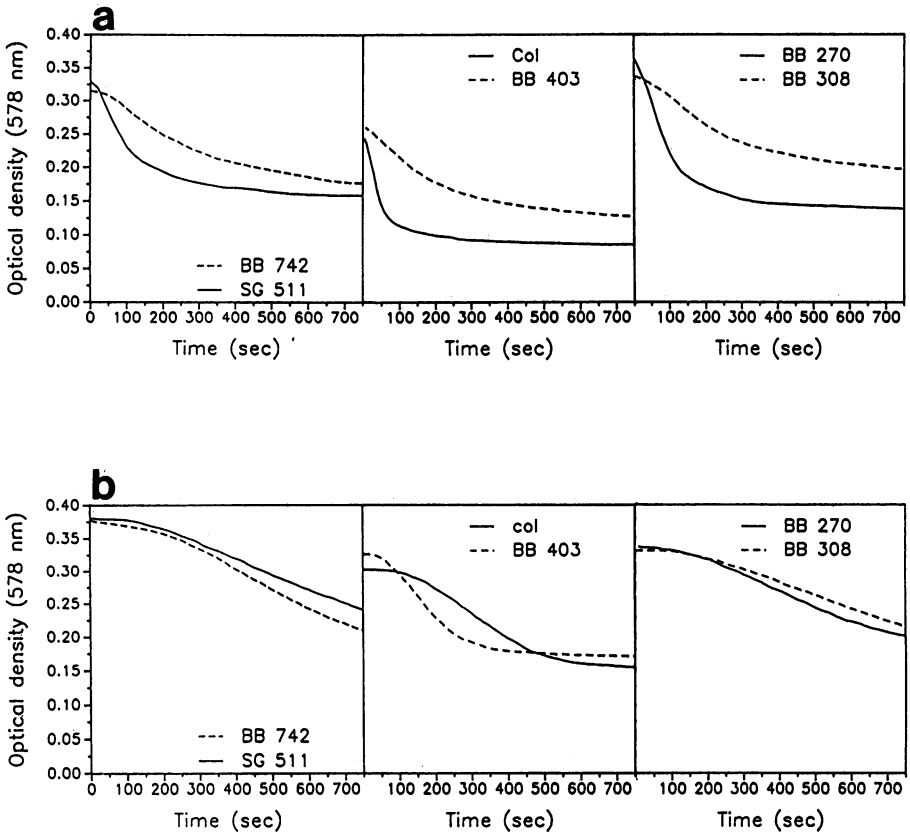

FIG. 7. Degradation of heat-inactivated whole cells by externally added wall lytic enzymes. (a) Degradation by recombinant lysostaphin (glycine endopeptidase); (b) degradation by *Chalaropsis* B-enzyme (muramidase). All *femA* mutants (BB742, BB403, BB308) showed reduced degradability (measured by reduction in OD versus time) compared to that of their parent strains (SG511, Col, BB270), irrespective of the presence or absence of the *mec* determinant. No significant changes were observed in degradation by the muramidase.

(2). The possible inactivation of *femB* in BB742 and BB403 is presently being investigated.

In conclusion, we showed that *femA* is a factor correlated with the amount of glycine in the PG of MRSA as well as in non-MRSA strains. Although it did not significantly impair growth of the bacteria under normal laboratory conditions, *femA* induced increased sensitivity towards β-lactam antibiotics in both methicillin-resistant and methicillin-sensitive strains. It might, therefore, even be considered as an additional target for chemotherapeutic inactivation to combat staphylococcal infections.

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