Mechanism and Suppression of Lysostaphin Resistance in Oxacillin-Resistant Staphylococcus aureus

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The potential for the development of resistance in oxacillin-resistant Staphylococcus aureus (ORSA) to lysostaphin, a glycylglycine endopeptidase produced by Staphylococcus simulans biovar staphyloyticus, was examined in vitro and in an in vivo model of infection. Following in vitro exposure of ORSA to subinhibitory concentrations of lysostaphin, lysostaphin-resistant mutants were identified among all isolates examined. Resistance to lysostaphin was associated with a loss of resistance to β-lactams and a change in the muropeptide interpeptide cross bridge from pentaglycine to a single glycine. Mutations in femA, the gene required for incorporation of the second and third glycines into the cross bridge, were found following PCR amplification and nucleotide sequence analysis. Complementation of lysostaphin-resistant mutants with pBBB31, which encodes femA, restored the phenotype of oxacillin resistance and lysostaphin susceptibility. Addition of β-lactam antibiotics to lysostaphin in vitro prevented the development of lysostaphin-resistant mutants. In the rabbit model of experimental endocarditis, administration of a low dose of lysostaphin for 3 days led predictably to the appearance of lysostaphin-resistant ORSA mutants in vegetations. Co-administration of nafcillin with lysostaphin prevented the emergence of lysostaphin-resistant mutants and led to a mean reduction in aortic valve vegetation counts of 7.5 log₁₀ CFU/g compared to those for untreated controls and eliminated the isolation of lysostaphin-resistant mutants from aortic valve vegetations. Treatment with nafcillin and lysostaphin given alone led to mean reductions of 1.35 and 1.65 log₁₀ CFU/g respectively. In ORSA, resistance to lysostaphin was associated with mutations in femA, but resistance could be suppressed by the co-administration of β-lactam antibiotics.

Lysostaphin, a 27-kDa endopeptidase produced by Staphylococcus simulans, has potent antistaphylococcal activity (7, 11, 12, 13, 22, 23, 24, 25, 30). In previous experiments we have demonstrated that lysostaphin is highly active against both oxacillin-resistant Staphylococcus aureus (ORSA) and vancomycin-intermediate-susceptible S. aureus (VISA) (4, 19). In the rabbit model of endocarditis caused by either ORSA or VISA strains, treatment with lysostaphin reduced mean aortic valve vegetation counts by >8.0 log₁₀ CFU/g compared to those for untreated controls (4, 19). No lysostaphin-resistant mutants were found in infected vegetations following treatment with high doses of lysostaphin. However, lysostaphin-resistant mutants can easily be selected in vitro when S. aureus is exposed to low concentrations of lysostaphin (30).

Resistance to lysostaphin has previously been described among ORSA strains with alterations in the formation of the pentaglycine cross bridge (26). Current evidence suggests that the pentaglycine cross bridge is formed in S. aureus under the control of three separate genes. fmhB encodes a protein factor responsible for the addition of the first glycine to the ε-amino group of lysine of the stem peptide and appears to be an essential gene (21, 29). femA and femB encode factors that catalyze the successive addition of the second through fifth glycines (1, 9, 14, 17). femA null mutants generated by either chemical mutagenesis or transposon insertion develop resistance to lysostaphin as well as a hypersusceptibility to β-lactam antibiotics associated with the formation of a cross bridge composed entirely of monoglycines instead of the normal pentaglycines (6, 26). Lysostaphin, which acts as a glycylglycine endopeptidase, is unable to cleave the cross bridge of femA null mutants. Although these femA null mutants were selected by chemical mutagenesis, we hypothesized that the resistance that develops following exposure of S. aureus to low (subinhibitory) concentrations of lysostaphin could also be caused by the selection of femAB mutants.

In the study described here we sought to systematically assess the development of lysostaphin resistance in ORSA exposed to low doses of the enzyme both in vitro and in vivo and its relationship to FemAB activity. In addition, we wanted to find out if the hypersusceptibility of lysostaphin-resistant mutants to β-lactam antibiotics could be exploited to both prevent resistance and generate synergistic activity when both lysostaphin and β-lactams were administered in combination.

MATERIALS AND METHODS

Bacterial strains and plasmids. The ORSA isolates tested were taken from the collection maintained at the Medical College of Virginia campus of Virginia Commonwealth University as described previously (2). Mu3, a vancomycin-intermediate-resistant ORSA strain and Mu50, a VISA, ORSA strain, were the kind gift of K. Hiramatsu (15, 27). The femA-expressing plasmid pBBB31, a low-copy-number plasmid containing the entire femA gene complex and promoter, was the kind gift of B. Berger-Bachi (1). For complementation experiments, pBBB31 was introduced into the lysostaphin-resistant strains by transduction with phage 80a, as described previously (3).
Antimicrobial susceptibility testing. MICs were determined by the broth microdilution method in cation-adjusted Mueller-Hinton broth (Becton Dickinson, Cockeysville, Md.) according to NCCLS standards (18). Lysostaphin MICS were determined in the presence of 0.1% bovine serum albumin (Sigma) to prevent the adsorption of lysostaphin to polystyrene microtiter wells, as described previously (4). The MIC was the lowest concentration of antibiotic that yielded no visible growth after incubation at 37°C for 24 h.

Checkerboard synergy testing was performed by the microdilution method in microtiter trays with cation-adjusted Mueller-Hinton broth. Combinations of lysostaphin and oxacillin were tested at concentrations of 0.015 to 16 and 0.125 to 512 μg/ml, respectively. Microtiter plates were incubated at 37°C and read at 24 and 48 h. The fractional inhibitory concentration (FIC) index was calculated by adding the FICs (MIC of drug A in combination with drug B/MIC of drug A alone) of lysostaphin and oxacillin. An FIC index of ≤0.5 was defined as synergy, an FIC index of >0.5 to 4.0 was defined as additive or indifferent, and an FIC index of >4.0 was defined as antagonism. The checkerboard test results represent the averages of duplicate tests.

Lysostaphin-resistant mutants were generated following overnight incubation in Mueller-Hinton broth containing one-quarter to one-half the MIC of lysostaphin, as determined by broth microdilution testing. Following overnight incubation at 37°C, bacteria were plated on Mueller-Hinton agar containing lysostaphin (8 μg/ml). The frequency of resistance development was defined as the number of colonies growing on lysostaphin-containing agar divided by the number of colonies growing on Mueller-Hinton agar containing no antibiotics.

Growth curve assays were performed in 50 ml of cation-adjusted Mueller-Hinton broth inoculated with the test organisms at a starting concentration of 5 × 10^5 CFU/ml. Lysostaphin was used at a concentration (0.03 μg/ml) that represents the MIC and one-half the MIC for test organisms 27615 and 27225, respectively. Oxacillin was used at a concentration of 1 μg/ml. Bacterial counts were enumerated at 0, 1, 4, and 24 h by plating 0.1-ml aliquots of serial 10-fold dilutions onto Mueller-Hinton agar containing no antibiotics, 8 μg of lysostaphin per ml, or 6 μg of oxacillin per ml.

Experimental infection. The rabbit model of aortic valve endocarditis, as described previously (20), was used to evaluate antibiotic treatment regimens. Seventy-two hours after transcarotid placement of a polyethylene catheter across the aortic valve, rabbits were injected intravenously through the marginal ear vein with 1 ml of an overnight culture containing 10^7 CFU of the test organism, ORSA 27619 (4, 5, 19). Blood samples for culture were obtained 24 h later and the rabbits were randomly assigned to either one of the following treatment groups: lysostaphin (AMBI, Tarrytown, N.J.) given at 1 mg/kg of body weight intravenously (i.v.) twice a day (BID), lysostaphin given at 1 mg/kg i.v. BID plus nafcillin (Bristol-Meyer Squibb, Princeton, N.J. given at 200 mg/kg intramuscularly (i.m.) BID, or no treatment (control group). For comparison, treatment groups were compared to rabbits receiving nafcillin at 200 mg i.m. three times a day (TID), which has been tested previously (5). This dose has previously been shown to be ineffective in the treatment of experimental endocarditis due to oxacillin-resistant S. aureus 27619, and as such the data from the previous series are presented for comparison. Surviving animals were killed by i.v. administration of pentobarbital after a total of 3 days of antibiotic treatment. Rabbits with negative blood cultures at 24 h were excluded from subsequent analysis. To reduce the possibility of antibiotic carryover, rabbits were not killed until at least 18 h after administration of the last antimicrobial dose. The heart and kidneys were removed aseptically from each rabbit. Aortic valve vegetations were removed from each rabbit’s heart and weighed, and serial dilutions of vegetation homogenates were made. Kidneys were examined, and areas of abscess or infarct were removed, weighed, homogenized in saline, and serially diluted. Tissue homogenates were also plated onto Mueller-Hinton agar containing lysostaphin (16 μg/ml) in order to screen for resistant subpopulations. Cultures were read after 48 h. Titers of bacteria were expressed as log_{10} CFU per gram of vegetation or kidney tissue. Sterile vegetation and kidney cultures contained ≤2 and ≤1 log_{10} CFU/g, respectively (the limit of detection).

Inclusion criteria. For the final analysis, animals that fulfilled the following criteria were included: (i) positive blood culture at 24 h, (ii) survival for at least 24 h of antibiotic treatment, (iii) proper placement of the catheter across the aortic valve at necropsy with macroscopic evidence of aortic valve endocarditis (visible vegetations), and (iv) aortic valve vegetation and kidney tissue that yielded pure cultures of the test organism.

Statistical analysis. The mean numbers of bacteria per gram of vegetation and kidney tissue in all treatment groups were compared by analysis of variance. Sterile aortic valve and kidney cultures were entered as 2 and 1 log_{10} CFU/g, respectively (the limit of detection). The Student-Newman-Keuls test was used to adjust for multiple comparisons. For analysis of the sterilization of tissue cultures, we used Fisher’s exact test. A P value of <0.05 was considered statistically significant for all tests.

Analysis of muropeptide composition. Isolated cell walls were prepared as described previously (20). Lysolphilized peptidoglycan was digested with mutanolysin (Sigma), and the resulting muropeptides were reduced into their muramidol derivatives. Separation of muropeptides was achieved by reversed-phase high-pressure liquid chromatography with a Waters 626 system and the conditions described previously (20). Muropeptides were detected at 206 nm.

DNA sequencing. The entire femA structural gene and sequences 327 bp 5' to the structural gene region of each strain were amplified by PCR with the following primers: 5'-AAATCTAAACCGCGTGAAGG-3' and 5'-TATCCAAAGTT GTGAAAACC-3'. The nucleotide sequences of femA were determined by direct sequencing of specific amplified PCR products obtained from genomic template DNA prepared with the Genomic Qiaqen-tip kit (Qiagen, Valencia, Calif.). Sequencing of the PCR fragments was performed by the dye-deoxy chain termination procedure on an ABI 3737 automatic sequencer with the ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit with Ampli-Taq DNA polymerase FS (Perkin-Elmer, Applied Biosystems Division, Foster, Calif.).

RESULTS

Development of lysostaphin resistance and antimicrobial susceptibilities of lysostaphin-resistant mutants. Overnight incubation in the presence of subinhibitory concentrations of lysostaphin (one-quarter to one-half the MIC) selected for lysostaphin-resistant mutants among all oxacillin-resistant S. aureus strains tested (Table 1). The frequency of resistance development ranged between 5.3 × 10^{-7} and 1.0 × 10^{-7}. All resistant mutants had decreased susceptibility to lysostaphin, with lysostaphin MICs ranging from 2 to 512 μg/ml (5- to 15-fold increases) by broth microdilution testing. Resistance to lysostaphin was associated with a loss of resistance to oxacillin.

### TABLE 1. Antimicrobial susceptibilities of lysostaphin-resistant mutants

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC (μg/ml) for parent isolate</th>
<th>Mutant designation</th>
<th>MIC (μg/ml) [fold increase in resistance] for lysostaphin-resistant mutants</th>
<th>Frequency of resistance development</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lysopestaphin</td>
<td>Oxacillin</td>
<td>Lysopestaphin</td>
<td>Oxacillin</td>
</tr>
<tr>
<td>450M</td>
<td>0.015</td>
<td>16</td>
<td>450MLR</td>
<td>2 (8)</td>
</tr>
<tr>
<td>27253</td>
<td>0.03</td>
<td>16</td>
<td>27253LR</td>
<td>32 (10)</td>
</tr>
<tr>
<td>27225</td>
<td>0.06</td>
<td>512</td>
<td>27225LR</td>
<td>64 (10)</td>
</tr>
<tr>
<td>27615</td>
<td>0.03</td>
<td>512</td>
<td>27615LR</td>
<td>4 (7)</td>
</tr>
<tr>
<td>Mu3</td>
<td>0.015</td>
<td>1,024</td>
<td>Mu3LR</td>
<td>512 (15)</td>
</tr>
<tr>
<td>Mu50</td>
<td>0.015</td>
<td>32</td>
<td>Mu50LR</td>
<td>512 (15)</td>
</tr>
<tr>
<td>27619</td>
<td>0.03</td>
<td>128</td>
<td>27619LR</td>
<td>512 (14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>27619B</td>
<td>8 (3)</td>
</tr>
</tbody>
</table>

* Derived in vivo.
among all mutants. In all cases, oxacillin resistance was abolished upon establishment of the lysostaphin resistance phenotype, with oxacillin MICs decreasing from 16 to 1,024 μg/ml to 0.25 to 1 μg/ml, representing 5- to 11-fold decreases in oxacillin susceptibility (Table 1).

The reversion to the oxacillin susceptible phenotype among lysostaphin-resistant mutants suggested that coadministration of β-lactams with lysostaphin could prevent the development of lysostaphin-resistant mutants. We examined this possibility in growth curve experiments, the results of which presented in Fig. 1 and 2.

In tests with the two oxacillin-resistant S. aureus strains, 27615 (Fig. 1A) and 27287 (Fig. 1B) in the presence of lysostaphin (at the MIC and one-half the MIC, respectively), lysostaphin inhibited growth for 6 h, but the development of lysostaphin-resistant mutants was seen by 24 h, with frequencies of resistance of $2.5 \times 10^{-2}$ for ORSA 27615 and $3.3 \times 10^{-5}$ for ORSA 27287. Coadministration of oxacillin at a concentration of 1 μg/ml completely abolished the development of lysostaphin-resistant mutants. Testing of three additional ORSA strains (strains 450M, 27285, and 27223) demonstrated results identical to those described above, with the complete suppression of the development of lysostaphin-resistant mutants following overnight incubation in lysostaphin (0.03 to 0.06 μg/ml) and oxacillin (1 to 5 μg/ml), while incubation in the presence of lysostaphin alone led to the development of resistant mutants among all strains tested. The suppression of lysostaphin resistance by oxacillin was dose dependent. For the majority of strains (strains 450M, 27615, and 27285), complete suppression of lysostaphin-resistant mutants could be achieved with oxacillin at 1 μg/ml. In tests with ORSA 27223, complete suppression of lysostaphin-resistant mutants was achieved with the coadministration of oxacillin at 5 μg/ml (Fig. 2). At this concentration, there were still viable lysostaphin-susceptible cells at 24 h, but growth in the presence of oxacillin at 10 μg/ml and lysostaphin at 0.0625 μg/ml resulted in complete sterilization of the culture (Fig. 2B).

Results of microdilution checkerboard testing demonstrated significant synergy with the combination of oxacillin and lysostaphin in tests with all strains used in growth curve experiments, with FIC indices ranging between 0.009 and 0.3125 (data not shown). Similar results were seen with a number of other β-lactams including ceftriaxone, ceftazidime, and cefazolin (data not shown).

Development and suppression of lysostaphin resistance in vivo. In previous experiments, we were unable to document the presence of lysostaphin-resistant mutants following a 3-day treatment trial of ORSA endocarditis in the rabbit model of experimental aortic valve endocarditis (4). As the doses of lysostaphin used in these experiments were large and a majority of vegetations were sterile, we hypothesized that lysostaphin-resistant mutants might be able to develop with exposure to lower doses of lysostaphin. Using the rabbit model of experimental endocarditis, we treated rabbits with lower doses of lysostaphin and screened for the development of lysostaphin-resistant mutants among the surviving bacterial populations in aortic valve vegetations. Following the establishment of endocarditis with the ORSA strain 27619, rabbits were treated for 3 days with lysostaphin at 1 mg/kg i.v. BID, a dose substantially lower than that tested previously (5 to 15 mg/kg/day). The rabbits were also treated with the combination of lysostaphin (1 mg/kg i.v. BID) and nafcillin (200 mg/kg i.m. BID) to examine the effect of β-lactam exposure on the development of lysostaphin-resistant mutants. Animals were also compared to those that have received treatment with nafcillin alone given at a dose of 200 mg/kg i.m. TID, which was previously shown to be ineffective in the treatment of ORSA 27619 (5).

The results were similar to those seen in the in vitro experiments. Lysostaphin-resistant mutants could be demonstrated
in five of seven rabbits exposed to lower doses of lysostaphin (Table 2). The frequency of resistance among vegetation material ranged from 0 to \(10^{-6}\). No lysostaphin-resistant mutants could be demonstrated among rabbits treated with the combination of lysostaphin and nafcillin. In addition, the rabbits treated with the lysostaphin-nafcillin combination had mean \(\log_{10}\) vegetation counts (2.52 \(\pm\) 1.89 CFU/g) significantly lower than those for lysostaphin-treated rabbits (8.40 \(\pm\) 1.22 CFU/g) or controls (10.05 \(\pm\) 0.88 CFU/g) \((P < 0.05)\). The mean reduction in bacterial counts compared to those for the controls of 7.53 \(\log\) 10 CFU/g is comparable to those seen in previous tests with lysostaphin given at 15 mg/kg/day (8.5 \(\log\) 10 CFU/g) \((4)\).

The oxacillin susceptible phenotype was seen among all lysostaphin-resistant mutants harvested from vegetation material. Further testing was completed with one of these lysostaphin-resistant mutants, designated 27619B (a mutant derived in vivo). Pulsed-field gel electrophoresis confirmed that 27619B was identical to the parent strain, 27619. Lysostaphin resistance was again associated with a loss of oxacillin resistance (oxacillin MIC, 0.5 \(\mu\)g/ml; lysostaphin MIC, 2 \(\mu\)g/ml) in 27619B.

Muropeptide compositions of lysostaphin-resistant mutants. The peptidoglycan compositions of several lysostaphin-resistant mutants were analyzed and compared to those of the parent strains. The five strains analyzed included 27619, 27619LR (derived by passage), 27619B (derived in vivo), Mu50, and Mu50LR (derived by passage). Whereas lysostaphin-susceptible strain 27619 showed the normal bell-shaped staphylococcal muropeptide pattern and contained a large amount of highly cross-linked wall material and the pentaglycine-modified muropeptide M4 as the main monomeric species, the patterns of lysostaphin-resistant mutant 27619LR derived in vitro and mutant 27619B derived in vivo showed reductions in the amounts of highly cross-linked wall material.

**FIG. 2.** Influence of oxacillin on the development of lysostaphin-resistant mutants. The growth curve for ORSA 27223 is shown in panel A (OD 600, optical density at 600 nm), with the corresponding time kill curve shown in panel B. ■, ORSA 27223, no antibiotics; ●, lysostaphin only at 0.0625 \(\mu\)g/ml; ●, lysostaphin at 0.0625 \(\mu\)g/ml plus oxacillin at 5 \(\mu\)g/ml; ★, lysostaphin at 0.0625 \(\mu\)g/ml plus oxacillin at 10 \(\mu\)g/ml.

**TABLE 2.** Treatment of experimental ORSA endocarditis with lysostaphin and nafcillin and the development of lysostaphin resistance

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Aortic valve vegetations</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (\pm) SD (\log_{10}) CFU/g</td>
<td>No. sterile/no. treated</td>
</tr>
<tr>
<td>Control</td>
<td>10.05 (\pm) 0.88</td>
<td>0/7</td>
</tr>
<tr>
<td>Lysostaphin BID</td>
<td>8.40 (\pm) 1.22(^a)</td>
<td>0/7</td>
</tr>
<tr>
<td>Nafcillin TID(^c)</td>
<td>8.70 (\pm) 0.77</td>
<td>0/8</td>
</tr>
<tr>
<td>Lysostaphin plus nafcillin BID</td>
<td>2.52 (\pm) 1.89(^b)</td>
<td>3/8</td>
</tr>
</tbody>
</table>

\(^a\) \(P < 0.05\) compared to control group alone (Student-Newman-Keuls test)

\(^b\) \(P < 0.05\) compared to control group and lysostaphin BID group (Student-Newman-Keuls test)

\(^c\) As determined in previous studies \((5)\).
Concomitantly, the monoglycine-substituted muropeptide M2 was the main monomeric species (Fig. 3). Furthermore, the peptidoglycan composition analysis of lysostaphin-resistant mutant Mu50LR (derived by passage) also revealed monoglycine interpeptide bridges instead of pentaglycine bridges (data not shown). The results of the muropeptide analysis for all lysostaphin-resistant mutants were similar to those seen among previously described femA null mutants (6, 26).

**Complementation.** The formation of a muropeptide composed of monoglycine cross bridges, which generates lysostaphin resistance, has previously been reported following the inactivation of femAB (6, 26). To test this hypothesis several lysostaphin-resistant mutants were complemented with femA-carrying plasmid pBBB31. pBBB31 is a low-copy-number plasmid that contains the entire femA gene under the control of its own promoter. Following the introduction of pBBB31 into lysostaphin-resistant mutants 27619LR and 27619B, both strains regained the phenotype of lysostaphin susceptibility and oxacillin resistance, identical to that of parent strain 27619.

**Sequence analysis and locations of femA mutations in lysostaphin-resistant mutants.** The DNAs of four lysostaphin-resistant mutants were sequenced to determine if there were any abnormalities in the femAB gene. They included three mutants derived in vitro (mutants 450MLR, 27615LR, and 27169LR) and mutant 27619B derived in vivo. No differences in the predicted amino acid sequences of FemB were seen for any of the isolates. The nucleotide sequences of the femA and femB structural genes of 450MLR were identical to those of 450M. However, the putative Shine-Dalgarno region had a 1-bp change. The remaining three isolates had changes within the femA gene. Strain 27619LR had a 4-bp substitution after base pair 890, which introduced a premature translational termination at amino acid 301. Strain 27615LR had a 1-bp deletion that caused a premature translational termination of FemA at amino acid 138. Strain 27169B demonstrated a 6-bp deletion of nucleotides 151 to 156 that caused a two-amino-acid deletion and a one-amino-acid change without premature translational termination (50K51E52V).

**DISCUSSION**

Lysostaphin is a potent staphylolytic agent with activity against both oxacillin-resistant and -susceptible S. aureus strains as well VISA strains. As a glycylglycine endopeptidase, lysostaphin cleaves between the second and third glycines of the pentaglycine cross bridge of all S. aureus strains. We have previously demonstrated that lysostaphin is an effective treatment for experimental aortic valve endocarditis due to ORSA and VISA (4, 19). However, the activity of lysostaphin could be compromised by the development of resistance, an issue that has been incompletely addressed in the past.

In the study described in this report, we demonstrate that resistance to lysostaphin can occur both in vitro and in an in vivo model of infection following prolonged exposure to low concentrations of lysostaphin. Following overnight incubation in the presence of one-half the MIC of lysostaphin, ORSA isolates developed mutants with reduced susceptibility to lysostaphin at frequencies ranging from $5.3 \times 10^{-1}$ to $1.0 \times 10^{-7}$. Resistance to lysostaphin in mutants derived both in vitro and in vivo was associated with three characteristics: increased susceptibility to β-lactams, mutations in femA, and an altered...
muropeptide structure in which the normal pentaglycine cross bridges were replaced with a single glycine.

These results are not unexpected. Previous work has shown that alterations in the \textit{femAB} operon, which affects the glycine content of the muropeptide cross bridge, have a direct impact on lysostaphin susceptibility. Current evidence suggests that the pentaglycine cross bridge is formed in \textit{S. aureus} under the control of three separate genes, \textit{fmhB}, \textit{femA}, and \textit{femB}, that encode factors that catalyze the successive addition of five glycines. \textit{femA} null mutants generated by either chemical mutagenesis or transposon insertion have the same antimicrobial phenotype (lysostaphin resistance, oxacillin susceptibility) and cell wall structure seen among the lysostaphin-resistant mutants generated in the present study (26). In the present study, demonstrated alterations in \textit{femA} included mutations that lead to premature translational termination as well as alterations in the putative Shine-Dalgarno site that may have altered transcription (mutant 450MLR). Both of these alterations have been described with mutants obtained by chemical mutagenesis (26). In mutant 27619B, there was an alteration in amino acids 49 to 51. This may be the first documentation of a functional domain of FemA since complementation with pBBB31, which encodes an intact \textit{femA}, restored lysostaphin susceptibility as well as oxacillin resistance. The \textit{femAB} gene products continue to be potentially attractive targets for chemotherapeutic inactivation since alterations in \textit{femAB} induce increased sensitivity to \beta-lactam antibiotics in \textit{S. aureus} (8, 16).

Resistance to lysostaphin in the present study appeared to be due only to alterations in the activity of FemA and not to other alterations of cross-bridge formation, as determined by the ability of \textit{femA} complementation to restore the wild-type phenotype. The incorporation of increased amounts of amino acids other than glycine into the cross bridge can also confer resistance to lysostaphin. Among the coagulase-negative staphylococci, serine and alanine are often found in the cross bridge, explaining the decreased susceptibility to lysostaphin in these species. In \textit{S. simulans}, the organism that produces lysostaphin, the presence of the \textit{lf} (lyostaphin immunity factor) gene causes the incorporation of increased amounts of serine into the third and fifth positions of the cross bridge, protecting itself against the lytic action of lysostaphin (10, 28). \textit{epr}, found in \textit{Staphylococcus capitis}, produces changes in the cross bridge identical to those produced by the \textit{lf} gene. \textit{lf} and \textit{epr} both have high degrees of homology to \textit{femA} and \textit{femB}, and recent evidence indicates that they act together with \textit{femA} to increase the level of incorporation of serine into the cross bridge (10).

In the present study, resistance to lysostaphin emerged both in vitro and in vivo following exposure to the enzyme. This resistance development was easily circumvented by the coadministration of \beta-lactam antibiotics. There are several possible explanations for this observation. First, there could be selective killing of developing lysostaphin-resistant mutants that emerge due to their hypersusceptibility to \beta-lactam antibiotics. The \beta-lactam hypersusceptibility in these \textit{femA} mutants has not yet been explained, although it has been speculated that PBP 2a has a substrate requirement which does not include muropeptides that contain the monoglycine cross bridges seen in \textit{femA} mutants. PBP 2a, a penicillin-binding protein (PBP) with a low affinity for \beta-lactams, must perform cell wall transpeptidation activity following exposure of staphylococci to \beta-lactams when the remaining four PBPs are inactivated. However, PBP 2a appears to have a specific requirement for pentaglycine muropeptide monomers for efficient cross-linking to occur. In the setting of lysostaphin resistance due to alterations in \textit{femA}, only monomeric muropeptides with a single glycine are available for cross-linking, and these are poor transpeptidation substrates for PBP 2a. Second, exposure to \beta-lactams could cause increased levels of activation and transcription of the \textit{femAB} operon, reducing the probability of spontaneous \textit{femA} mutations. An increased level of \textit{femAB} transcription has been observed following oxacillin-induced growth (A. Rosato and G. L. Archer, unpublished data). Finally, the combination of lysostaphin and \beta-lactams appears to be synergistic, a phenomenon that appears to be independent of the development of lysostaphin resistance. This synergism was demonstrated in both broth microdilution checkerboard tests and the rabbit model of experimental aortic valve endocarditis. In the rabbit model of experimental endocarditis due to ORSA 27619, treatment with either lysostaphin or nafcillin alone was associated with a modest reduction in the mean log$_{10}$ vegetation counts compared to those for the controls (1.65 and 1.39 log$_{10}$ CFU/g, respectively). In contrast, the combination of lysostaphin and nafcillin resulted in a mean reduction in vegetation counts of 7.5 log$_{10}$ CFU/g. This reduction is similar to those seen in previous trials with lysostaphin alone, even though a much lower dose was used in the present study (2 versus 15 mg/kg/day). These data suggest that therapeutic trials with lysostaphin should include nafcillin or oxacillin to both suppress resistance development and promote synergy.

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