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The Lysostaphin Endopeptidase Resistance Gene (*epr*) Specifies Modification of Peptidoglycan Cross Bridges in *Staphylococcus simulans* and *Staphylococcus aureus*

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Staphylococcus simulans **biovar staphylolyticus produces an extracellular glycylglycine endopeptidase (lysostaphin) that lyses other staphylococci by hydrolyzing the cross bridges in their cell wall peptidoglycans. The genes for endopeptidase (*end*) and endopeptidase resistance (*epr*) reside on plasmid pACK1. An 8.4-kb fragment containing *end* was cloned into shuttle vector pLI50 and was then introduced into *Staphylococcus aureus* RN4220. The recombinant *S. aureus* cells produced endopeptidase and were resistant to lysis by the enzyme, which indicated that the cloned fragment also contained *epr*. Treatments to remove accessory wall polymers (proteins, teichoic acids, and lipoteichoic acids) did not change the endopeptidase sensitivity of walls from strains of *S. simulans* biovar staphylolyticus or of *S. aureus* with and without *epr*. Immunological analyses of various wall fractions showed that there were epitopes associated with endopeptidase resistance and that these epitopes were found only on the peptidoglycans of epr^+ strains of both species. Treatment of purified peptidoglycans with endopeptidase confirmed that resistance or susceptibility of both species was a property of the peptidoglycan itself. A comparison of the chemical compositions of these peptidoglycans revealed that cross bridges in the epr^+ cells contained more serine and fewer glycine residues than those of cells without *epr*. The presence of the 8.4-kb fragment from pACK1 also increased the susceptibility of both species to methicillin.**

Staphylococcus simulans biovar staphylolyticus (35) secretes a glycylglycine endopeptidase that lyses staphylococcal cells by hydrolyzing the polyglycine bridges that cross-link glycopeptide chains in the cell wall peptidoglycans of these organisms (5). A partially purified preparation of this peptidoglycan hydrolase is commercially available as lysostaphin. The lysostaphin endopeptidase gene (*end*) resides on pACK1, the largest of five plasmids in *S. simulans* biovar staphylolyticus (18). The endopeptidase susceptibility of pACK1-cured strain JN351 of *S. simulans* biovar staphylolyticus revealed that an endopeptidase resistance gene (*epr*) is also on pACK1 (17). Furthermore, *epr* expression is regulated, because cells from early-exponential-phase cultures of *S. simulans* biovar staphylolyticus are susceptible to endopeptidase, whereas cells from post-exponential-phase cultures are resistant (28, 31). Endopeptidase is produced as a proenzyme that is activated by an extracellular sulfhydryl protease, which provides time between proendopeptidase production and activation for resistance to be acquired (28).

Modifications of peptidoglycans can affect their sensitivity to peptidoglycan hydrolases. *Bacillus cereus* peptidoglycan is resistant to lysozyme because of the unacetylated amino groups on the majority of its glucosamine residues and can be converted to a lysozyme-sensitive form by acetylation with acetic anhydride (2, 16). Conversely, lysozyme resistance of the peptidoglycans of other organisms is due to O acetylation of amino sugars, and these peptidoglycans can be made lysozyme sensitive by de-O-acetylation with a dilute base (6, 13, 27, 34). Accessory cell wall polymers, such as teichoic acids or lipoteichoic acids, can also affect the susceptibility of bacteria to a number of peptidoglycan hydrolases (1, 4, 7, 12, 14, 21, 22). The present study was undertaken to determine how *epr* confers lysostaphin endopeptidase resistance on staphylococci.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *S. simulans* biovar staphylolyticus (NRRL B-2628) (35) and pACK1-cured strain JN351 (17) were grown aerobically in modified lysostaphin production medium as described previously (32). *Escherichia coli* DH5α was obtained from GIBCO BRL, Gaithersburg, Md. pLI50, an *E. coli*-*Staphylococcus aureus* shuttle vector that specifies ampicillin and chloramphenicol resistance, was kindly provided by John J. Iandolo of Kansas State University. *S. aureus* RN4220, which is defective in one or more restriction systems (29), and derivatives generated in the present study were grown in Tryptic Soy Broth (Difco Laboratories, Detroit, Mich.) at 37°C with shaking at 250 rpm. Methicillin susceptibility was determined by standard assay procedures (3) with 5-μg methicillin disks (Difco).

Wall isolation, fractionation, and modifications. Stationary-phase cultures were harvested by centrifugation at 4°C, and the cells were washed twice with cold saline. The cells were resuspended to a concentration of 50% (wet weight/volume) in cold saline, and 1.0-ml volumes were mechanically disrupted by shaking with 0.1-mm-diameter glass beads for 3 min at 4°C in a Mini-Beadbeater cell disrupter (Biospec Products, Bartlesville, Okla.) to produce crude cell wall preparations. The following methods were used to remove accessory wall polymers and to prepare purified peptidoglycans (15, 19). Trypsinized walls were prepared from crude walls (50 mg/ml) by treatment with trypsin (0.5 mg/ml) for 8 h at 37°C prior to extensive washing with cold deionized water. Trichloroacetic acid (TCA)-extracted walls were prepared from crude walls (50 mg/ml) by treatment with 10% (wt/vol) TCA for 48 h at 4°C followed by extensive washing with cold deionized water. Sodium dodecyl sulfate (SDS)-extracted walls were prepared by boiling crude walls (50 mg/ml) for 30 min in 4% (wt/vol) SDS prior to extensive washing with saline and then with deionized water. Purified peptidoglycans were prepared from cells that had been boiled in 4% (wt/vol) SDS for 30 min by disruption with glass beads and sequential treatment of the crude walls with trypsin, SDS, and TCA as described above. Purified peptidoglycans were N acetylated by treatment with acetic anhydride as described by Heymann et al. (23) or de-O-acetylated by mild base hydrolysis as described by Hayashi et al. (16).

Compositional analysis of peptidoglycans. The purified peptidoglycans were analyzed with an Applied Biosystems (Foster City, Calif.) amino acid analyzer (420A-130A-920A) in the University of Alabama at Birmingham Protein Analysis and Peptide Synthesis Core Facility.

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Endopeptidase assays and susceptibility of cells and cell wall fractions to endopeptidase. Lysostaphin endopeptidase activity was assayed by following the lysis of a suspension of cells (optical density of 0.250 at 620 nm) of *S. aureus* FDA 209P spectrophotometrically as described previously (31). One unit of activity is defined as the amount of enzyme that causes a 50% reduction in turbidity in 10 min at 37°C. The susceptibility of cells and of cell wall fractions was spectrophotometrically determined as described previously (28). Cells or cell wall fractions were resuspended to an optical density of 0.250 at 620 nm. Lysostaphin (Sigma Chemical Co., St. Louis, Mo.) was added to produce a final concentration of 7 U/ml, the suspensions were incubated at 37°C, and the optical density was measured over time. The results were expressed as a percent of the initial optical density after correction for changes determined in controls without enzyme. Endogenous lytic enzymes associated with cells and cell wall preparations were inactivated by boiling for 3 min prior to treatment with exogenous lysostaphin endopeptidase.

Immunological analyses. Antiserum was raised against whole, formalin-killed cells of *S. simulans* biovar staphylolyticus, and cell wall fractions were analyzed immunologically according to the procedures described by Oeding (30). For these analyses, teichoic acid and lipoteichoic acid fractions, removed from crude walls by TCA or phenol extraction, respectively, were further purified as described by Heckels and Virji (19).

RESULTS

Our strategy for cloning *epr* was based on the assumption that *end* and *epr* are close together on pACK1. If the assumption is correct, *end*-containing fragments would be likely to contain *epr* and *end* would provide a positive selection for *epr* in staphylococci. Southern hybridization analysis showed that *Eco*RI yielded an 8.4-kb fragment of pACK1 with 2.8 kb upstream and 4.2 kb downstream from *end*. Therefore, plasmid DNA isolated from *S. simulans* biovar staphylolyticus as previously described (18) was digested with *Eco*RI and ligated into pLI50. Recombinant plasmids were introduced into *E. coli* DH5α cells by electroporation (11). An ampicillin-resistant transformant that produced endopeptidase contained a recombinant plasmid with the expected 8.4-kb insert. This recombinant plasmid was transferred into *S. aureus* RN4220 cells by electroporation (8), transformants were selected for chloramphenicol resistance, and one isolate (strain EE1) was characterized.

Culture supernatants of strain EE1 contained as much endopeptidase activity as did those of *S. simulans* biovar staphylolyticus (7.4 versus 6.8 U/ml, respectively), which was sufficient to lyse the recipient strain, RN4220 (data not shown). Strain EE1 was identified as *S. aureus*, rather than as a chloramphenicol-resistant mutant of *S. simulans* biovar staphylolyticus, because it fermented mannitol and produced coagulase, as did strain RN4220. *S. simulans* biovar staphylolyticus is negative for both of these traits (35). To determine if the endopeptidase resistance of strain EE1 was due to the 8.4-kb fragment, we isolated a spontaneous streptomycin-resistant mutant, strain EE1S, and then cured it by growth at 42°C. An isolate that was streptomycin resistant, coagulase positive, able to ferment mannitol, chloramphenicol susceptible, and endopeptidase negative was obtained, indicating that it was a cured derivative of strain EE1S. This cured strain was as susceptible to endopeptidase as parental strain RN4220 with or without pLI50, while strains EE1 and EE1S were equally resistant (data not shown). Therefore, we concluded that the 8.4-kb fragment contained *epr* in addition to *end*.

Pairs of *S. simulans* biovar staphylolyticus and of *S. aureus* strains with and without *epr* were assayed for susceptibilities to endopeptidase. Viable cells of both pACK1-cured *S. simulans* biovar staphylolyticus JN351 and of *S. aureus* RN4220/pLI50 were susceptible to lysis by the enzyme, whereas viable cells of *S. simulans* biovar staphylolyticus and of *S. aureus* EE1 were resistant (Fig. 1A). In fact, strain EE1 was more resistant than *S. simulans* biovar staphylolyticus. In these assays, cells in the control tubes without endopeptidase showed appreciable lysis, presumably due to endogenous lytic activity. When the cells were boiled prior to the assay to inactivate endogenous lytic enzymes so that a direct assessment of resistance to exogenous endopeptidase could be made, lysis in the controls was greatly reduced and *S. simulans* biovar staphylolyticus and *S. aureus* EE1 were equally resistant (Fig. 1B). Without the activity of the endogenous lytic enzymes, endopeptidase treatment resulted in only about a 50% decrease in the optical density of susceptible cell suspensions.

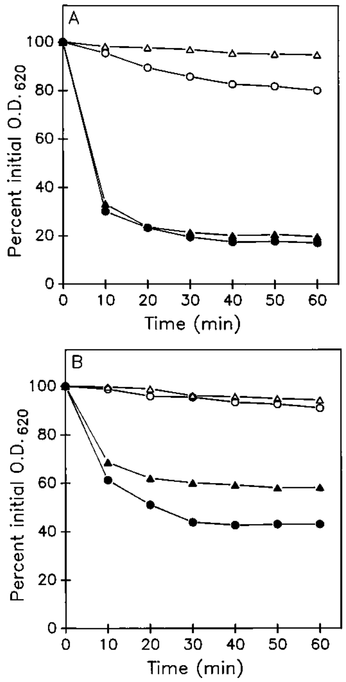

FIG. 1. Relative susceptibilities of viable (A) and heat-treated (B) cells to lysostaphin endopeptidase. ○, *S. simulans* biovar staphylolyticus; ●, *S. simulans* biovar staphylolyticus JN351; △, *S. aureus* EE1; ▲, *S. aureus* RN4220/pLI50. The results shown are from a single experiment. Similar results were obtained upon repetition. O.D.$_{620}$, optical density at 620 nm.

Crude cell wall preparations from the two *epr*$^+$ strains also were resistant to endopeptidase, whereas walls from the two strains without *epr* were sensitive (Fig. 2). As with whole cells, when endogenous lytic enzymes were inactivated by boiling, endopeptidase treatment resulted in about a 50% decrease in the optical density of sensitive cell wall suspensions. To determine if nonpeptidoglycan wall components are required for endopeptidase resistance or sensitivity, samples of crude wall preparations were treated with trypsin, TCA, or boiling SDS to remove proteins, teichoic acids, or lipoteichoic acids, respectively. None of these treatments made resistant walls sensitive or sensitive walls resistant to the enzyme. In fact, purified peptidoglycans prepared from endopeptidase-resistant or -susceptible strains by sequential treatment with trypsin, boiling SDS, and TCA remained as resistant or susceptible to the enzyme as were the crude wall preparations (data not shown).

Serological differences in the walls of strains with and without *epr* also were detected (Table 1). Antiserum specific for pACK1-specified epitopes was prepared by exhaustively absorbing antiserum made against cells of *S. simulans* biovar staphylolyticus with cells of strain JN351. Specificity was demonstrated by the ability of the absorbed antiserum to agglutinate cells of *S. simulans* biovar staphylolyticus but not those of strain JN351. This absorbed antiserum was not able to agglutinate *S. aureus* RN4220/pLI50 cells but was able to agglutinate

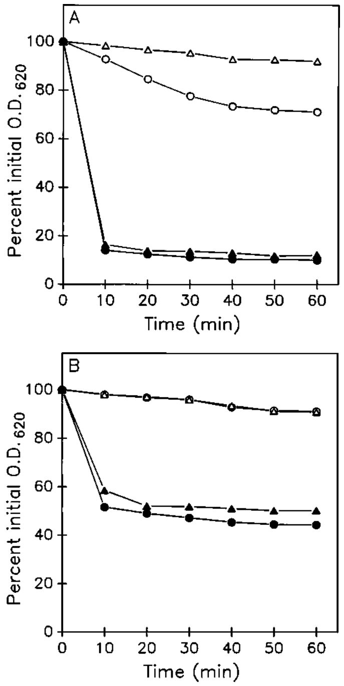

FIG. 2. Relative sensitivities of unheated (A) and heat-treated (B) crude cell wall preparations to lysostaphin endopeptidase. ○, *S. simulans* biovar staphylolyticus; ●, *S. simulans* biovar staphylolyticus JN351; △, *S. aureus* EE1; ▲, *S. aureus* RN4220/pLI50. The results shown are from a single experiment. Similar results were obtained upon repetition. O.D.$_{620}$, optical density at 620 nm.

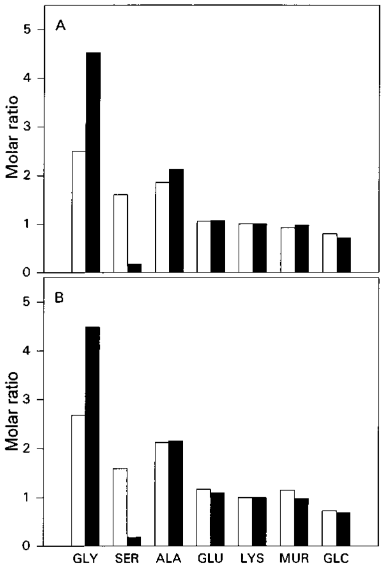

FIG. 3. Peptidoglycan compositions of strains of *S. simulans* biovar staphylolyticus (A) and *S. aureus* (B) with and without *epr*. (A) □, parental strain; ■, strain JN351. (B) □, strain EE1; ■, strain RN4220/pLI50. The amount of each compound is expressed as a molar ratio relative to lysine. GLY, glycine; SER, serine; ALA, alanine; GLU, glutamate; LYS, lysine; MUR, muramic acid; GLC, glucosamine. The values shown are the averaged results from two separate analyses.

cells of strain EE1. The same result was obtained for crude cell walls and purified peptidoglycans from both pairs of organisms. Teichoic acid and lipoteichoic acid fractions extracted from sensitive and resistant *S. aureus* strains exhibited no reaction with unabsorbed antiserum, whereas teichoic acid and lipoteichoic acid fractions from *S. simulans* biovar staphylolyticus yielded bands of identity in Ouchterlony double-diffusion analysis with corresponding fractions from strain JN351. The absorbed antiserum did not react with any teichoic acid or lipoteichoic acid fraction.

TABLE 1. Immunological analysis of cells and cell wall fractions

Cell fraction[a]	Antiserum[b]	Reactivity[c] of:			
		S. simulans biovar staphylolyticus		*S. aureus*	
		Parental	JN351	RN4220/pLI50	EE1
Cells	Unabsorbed	+	+	(+)	+
Cells	JN351 absorbed	+	−	−	+
Walls	Unabsorbed	+	+	(+)	+
Walls	JN351 absorbed	+	−	−	+
TA	Unabsorbed	+^{d}	+^{d}	−	−
TA	JN351 absorbed	−	−	−	−
LTA	Unabsorbed	+^{d}	+^{d}	−	−
LTA	JN351 absorbed	−	−	−	−
PG	Unabsorbed	+	+	(+)	+
PG	JN351 absorbed	+	−	−	+

[a] The fractions used were viable cells (cells), crude cell walls (walls), purified teichoic acids (TA), purified lipoteichoic acids (LTA), and purified peptidoglycans (PG).

[b] Rabbit antiserum made against cells of *S. simulans* biovar staphylolyticus with or without absorption with cells of strain JN351.

[c] Reactivity was measured by agglutination for insoluble samples (cells, crude cell walls, and peptidoglycans) and by Ouchterlony double diffusion for soluble fractions (teichoic acids and lipoteichoic acids). +, positive; (+), weakly positive; −, negative.

[d] Lines of identity between samples of the same fraction.

The data presented above indicated that the peptidoglycans in endopeptidase-resistant and endopeptidase-sensitive strains were different and that these differences were associated with resistance or susceptibility to the enzyme. Therefore, to determine the modification in the peptidoglycans that is specified by *epr*, we tested to see if the presence or absence of acetyl groups on the amino sugars would affect endopeptidase sensitivity. No changes in the endopeptidase resistance or sensitivity of peptidoglycans from cells with or without *epr* after acetylation of any free amino groups were found. Similarly, de-O-acetylation of the amino sugars did not convert sensitive peptidoglycans to resistant or resistant peptidoglycans to sensitive (data not shown).

Analysis of the purified peptidoglycans to determine the amino acid and amino sugar compositions did reveal differences between the strains with and without *epr*. As shown in Fig. 3 and Table 2, *epr*$^{+}$ strains had fewer glycines and more serines in their cross bridges than did strains without *epr*.

Since others have reported an inverse relationship between methicillin resistance and lysostaphin endopeptidase resistance (9, 20, 26), the strains with and without *epr* also were tested for methicillin susceptibility. Both *epr*$^{+}$ strains were more susceptible to methicillin than were the corresponding strains without *epr* (Table 2).

DISCUSSION

We previously showed that the information for resistance of *S. simulans* biovar staphylolyticus to lysostaphin endopeptidase resides on plasmid pACK1 with the endopeptidase gene (17). On the basis of the assumption that *end* and *epr* are close together, a fragment with 2.8 kb preceding and 4.2 kb following *end* was cloned into shuttle vector pLI50. The presence of the

TABLE 2. Peptidoglycan cross bridge composition, lysostaphin endopeptidase sensitivity, and methicillin susceptibility of strains with and without *epr*

Organism	Glycine/serine ratio[a]	Relative sensitivity to endopeptidase[b]	Susceptibility to methicillin[c]
S. simulans biovar staphylolyticus			
Parental	1.6	1.0	116
JN351	26.6	12.4	72
S. aureus			
EE1	1.7	0.9	107
RN4220/pLI50	24.9	11.0	32

[a] Ratios were derived from the data depicted in Fig. 3.

[b] Rate of solubilization (change in OD_{620} in 10 min) of purified peptidoglycans divided by the rate of solubilization of purified peptidoglycan from parental *S. simulans* biovar staphylolyticus.

[c] Diameter in millimeters of the zone of inhibition for a 5-μg methicillin disk. The results shown are the averages of two determinations.

active *end* gene on this fragment provided a positive selection for endopeptidase resistance in *S. aureus*. However, since endopeptidase-resistant mutants of *S. aureus* have been selected by exposure to the enzyme (33, 37), it was necessary to demonstrate that resistance was specified by the recombinant plasmid rather than a mutation in the recipient. The 8.4-kb fragment contained *epr* because it conferred endopeptidase resistance on *S. aureus* and curing restored susceptibility. The ability of *epr* to confer resistance on *S. aureus* raises the possibility that if *epr* became widely disseminated among staphylococci, then the use of lysostaphin in taxonomy (24) or as an antistaphylococcal agent (36) would be compromised.

The availability of matched pairs of strains of both *S. simulans* biovar staphylolyticus and *S. aureus* with and without *epr* has allowed us to show that endopeptidase resistance is due to modification of peptidoglycan rather than modification of teichoic acids, lipoteichoic acids, or proteins. Treatments that removed these accessory wall polymers did not change endopeptidase-resistant cell walls to sensitive or endopeptidase-sensitive walls to resistant. In fact, purified peptidoglycans from both epr^+ strains were as resistant to hydrolysis by exogenous endopeptidase as whole cells or crude cell walls, indicating that *epr* specifies modification of the peptidoglycan itself. Furthermore, peptidoglycans from both strains without *epr* did not react with antiserum rendered specific for pACK1-specified epitopes whereas peptidoglycans from both epr^+ strains did react with this antiserum. Since peptidoglycan from *S. aureus* RN4220/pLI50 did not react with this antiserum and peptidoglycan from strain EE1 did, we conclude that the epitopes that are expressed on *S. aureus* EE1 and on *S. simulans* biovar staphylolyticus peptidoglycans are specified by the 8.4-kb fragment from pACK1.

The presence or absence of acetyl moieties on the amino sugars in the peptidoglycans of some organisms determines their sensitivity or resistance to other peptidoglycan hydrolases (2, 6, 13, 16, 27, 34). If the presence or absence of acetyl groups determined either endopeptidase resistant or susceptibility, then N acetylation or de-O-acetylation would have changed either sensitive peptidoglycans to resistant or resistant peptidoglycans to sensitive. Since neither change occurred, we conclude that the acetylation state of these amino sugars does not affect endopeptidase susceptibility or resistance.

A comparison of the amino acid and amino sugar compositions of the peptidoglycans from strains of *S. simulans* and *S. aureus* with and without *epr* in the present study has revealed that *epr* confers endopeptidase resistance on staphylococcal cells by specifying a change in the cross bridges to contain additional serine and a reduced number of glycine residues. Species of staphylococci that have increased numbers of serine or alanine residues in place of glycine in their peptidoglycan cross bridges have been reported to show reduced susceptibility to lysostaphin endopeptidase (24). In addition, variants of *S. aureus* that have increased resistance to endopeptidase have been found to have reduced numbers of glycines and additional serines in their cross bridges (9, 10, 20, 25, 26, 33, 37). We previously reported that *S. simulans* biovar staphylolyticus has a higher serine content in its peptidoglycan cross bridges than do other strains of *S. simulans* (24, 35). Furthermore, growth of *S. simulans* biovar staphylolyticus under noninducing conditions for endopeptidase production resulted in a slight decrease in the amount of serine in its peptidoglycan cross bridges, and the cells showed a somewhat increased susceptibility to exogenous endopeptidase (31, 33). However, until the strains with and without *epr* used in this study became available, it could not be determined if this unusual cross bridge composition was sufficient to account for the endopeptidase resistance of this organism.

S. simulans biovar staphylolyticus originally was classified as a biovar of *S. simulans* on the basis of its unique ability to produce lysostaphin endopeptidase and its unusual cell wall composition (24, 35). Since the 8.4-kb fragment from pACK1 specifies endopeptidase production and the changes in the wall that are responsible for endopeptidase resistance, this fragment contains all of the information to specify the traits distinguishing this biovar.

We have shown here that the presence of *epr* results in an increased resistance to endopeptidase, a decreased glycine-to-serine ratio in the peptidoglycan cross bridges, and increased susceptibility to methicillin. *femA* and *femB* specify factors essential for expression of methicillin resistance in *S. aureus*, and others have reported that inactivation of either *femA* or *femB* causes increased lysostaphin endopeptidase resistance, decreased glycine content and increased serine content in the peptidoglycan cross bridges, and increased susceptibility to methicillin (9, 20, 26). Therefore, it is attractive to speculate that *epr* negatively affects *femAB* function.

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