JOURNAL OF BACTERIOLOGY, July 1997, p. 4311–4318 Vol. 179, No. 13
0021-9193/97/$04.00+0

epr, Which Encodes Glycylglycine Endopeptidase Resistance, Is Homologous to *femAB* and Affects Serine Content of Peptidoglycan Cross Bridges in *Staphylococcus capitis* and *Staphylococcus aureus*

MOTOYUKI SUGAI,* TAMAKI FUJIWARA, KOUJI OHTA, HITOSHI KOMATSUZAWA, MASARU OHARA,† AND HIDEKAZU SUGINAKA

Department of Microbiology, Hiroshima University School of Dentistry, Hiroshima 734, Japan

Received 29 January 1997/Accepted 25 April 1997

***Staphylococcus capitis* EPK1 produces a glycylglycine endopeptidase, ALE-1 (M. Sugai, T. Fujiwara, T. Akiyama, M. Ohara, H. Komatsuzawa, S. Inoue, and H. Suginaka, J. Bacteriol. 179:1193–1202, 1997), which hydrolyzes interpeptide pentaglycine chains of cell wall peptidoglycan of *S. aureus*. Characterizations of the enzyme activity and cloning of *ale-1* revealed that ALE-1 is very similar to prolysostaphin produced by *S. simulans* bv. *staphylolyticus*. Strain EPK1 is resistant to lysis by ALE-1 and by lysostaphin. A gene that renders the cells resistant to glycylglycine endopeptidase (*epr*) was found 322 bp upstream of and in the opposite orientation to *ale-1*. The deduced amino acid sequence of *epr* showed similarities to FemA and FemB, which have been characterized as factors essential for methicillin resistance of *S. aureus*. Inactivation of either *femA* or *femB* causes decreased resistance to methicillin, increased resistance to lysostaphin, and decreased glycine content in the interpeptide chains of peptidoglycan. Therefore, *femAB* is suggested to be involved in the addition of glycine to pentapeptide peptidoglycan precursor. *S. aureus* with *epr* on a multicopy plasmid had phenotypes similar to those of *femAB* mutants except that it did not alter resistance level to methicillin. These results suggest that *epr* and *femAB* belong to the protein family involved in adding amino acids to the pentapeptide peptidoglycan precursor and that *epr* is involved in the addition of serine to the pentapeptide.**

We recently purified a glycylglycine endopeptidase, ALE-1, from the culture supernatant of *Staphylococcus capitis* EPK1 (51). Characterization of ALE-1 enzyme activity suggested its similarity to lysostaphin, a glycylglycine endopeptidase produced by *S. simulans* bv. *staphylolyticus* (41, 42). Molecular cloning of the *ale-1* gene further showed that the primary structure of mature ALE-1 is very similar to that of the proenzyme form of lysostaphin (51). Like *end*, the gene for lysostaphin in *S. simulans* bv. *staphylolyticus*, *ale-1* is located on a large plasmid of *S. capitis* EPK1 (17, 34, 51). *S. capitis* EPK1 is resistant to lysis by purified ALE-1 (51); otherwise, the enzyme would be detrimental to the cells.

Robinson et al. have demonstrated that *S. simulans* bv. *staphylolyticus* is resistant to lysis by lysostaphin (35) and that the resistance is conferred by modifying the amino acid composition of interpeptide chains in cell wall peptidoglycan by increasing the serine content and decreasing the glycine content (9, 35). The gene involved in the modification of peptidoglycan, designated *epr* (endopeptidase resistance), was shown to be located on a large plasmid, pACK1, together with *end* (9, 16). *S. aureus* cells transformed with a plasmid containing the 8.4-kbp DNA fragment from pACK1 produced lysostaphin and were resistant to lysis by lysostaphin, which indicated that the DNA fragment contained *epr* as well as *end* (9). However, the molecular cloning and characterization of *epr* in *S. simulans* bv. *staphylolyticus* have not been described. We attempted to determine whether *S. capitis* EPK1 is similar in genetic organization to *S. simulans* bv. *staphylolyticus*, protecting against its own product, ALE-1. Herein, we describe the identification, cloning, and sequencing of the *epr* gene from *S. capitis* EPK1. We show that *epr* of *S. capitis* EPK1 is homologous to *femAB*, which has been implicated in the biosynthesis of pentaglycine interpeptide chains of *S. aureus* (10, 19, 26, 30, 48).

* Corresponding author. Mailing address: Department of Microbiology, Hiroshima University School of Dentistry, Kasumi 1-2-3, Minami-ku, Hiroshima 734, Japan. Phone: 81 82 257 5637. Fax: 81 82 257 5639. E-mail: sugai@ipc.hiroshima-u.ac.jp.

† Present address: Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD 20814-4799.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Tables 1 and 2 except the following: *S. hominis* CCM27327, *S. delphini* DSM207717, *S. kloosii* DSM206767, *S. schleiferi* N880033, *S. caprae* CCM35737, *S. arlettae* DSM206727, *S. chromogenes* CCM33877, *S. hycus* CCM23687, and *S. equorum* DSM206747, obtained from Jean Freney; and *S. capitis* ATCC 27840, *S. cohni* ATCC 29994, *S. haemolyticus* ATCC 29970, *S. intermedius* ATCC 29663, *S. saccharolyticus* ATCC 14953, *S. simulans* ATCC 27848, *S. warneri* ATCC 27836, *S. xylosus* ATCC 27971, *S. lugdenensis* ATCC 438097, and *S. felis* ATCC 491687, from the American Type Culture Collection. Other strains were from our laboratory stock. Manipulation of DNA in *Escherichia coli* was carried out with pUC19 (60) as the vector and *E. coli* XL1-Blue (6). Shuttle vector pGC2 (45), kindly supplied by Tadashi Oshida, was used when gene expression was attempted in *S. aureus. Staphylococcus* and *Escherichia* strains were grown in Trypticase soy broth (TSB; Becton Dickinson Microbiology Systems, Cockeysville, Md.) and Luria-Bertani broth (5 g of yeast extract, 10 g of polypeptone, 10 g of NaCl per liter [pH 7.2]), respectively. When necessary, ampicillin (50 μg/ml) or chloramphenicol (50 μg/ml) was added for selection or maintenance of plasmid. To examine the production and secretion of staphylolytic activity, *S. aureus* cells were plated on brain heart infusion agar (Difco Laboratories, Detroit, Mich.) containing heat-killed *S. aureus* FDA209P (0.5 mg [dry weight]/ml). After a 2-day incubation at 37°C, the plate was examined for the appearance of halos around the colonies as described previously (54).

Materials and chemicals. All restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase I, and bacterial alkaline phosphatase were from Boehringer Mannheim, Tokyo, Japan. Lysostaphin, mutanolysin, methicillin, and

TABLE 1. Strains used

Strain	Relevant characteristics	Source or reference	MIC[a]			
			ALE-1	Lysostaphin	DMPPC	VCM
S. aureus						
RN4220	8325-4 r^-	R. Novick	100	0.2	<0.5	0.5
COL	COL *mec*	27	—	0.1	1,024	1
BB270	NCTC8325 *mec*	30	—	0.8	512 (512)[b]	—
BB308	NCTC8325 *mec* (*femA*::Tn*551*)	3	—	3.2	4 (32)	—
UT-34-2	NCTC8325 *mec* (*femB*::Tn*551*)	19	—	6.4	4 (16)	—
TF1	RN4220 pGC2	This study	100	0.2	1	1
TF2	RN4220 pTFS3	This study	—	50	—	—
TF3	RN4220 pTFS31	This study	—	100	—	—
TF4	RN4220 pTFS32	This study	—	100	—	—
TF5	RN4220 pTFS33	This study	—	0.4	—	—
TF6	RN4220 pTFS34	This study	—	0.2	—	—
TF7	RN4220 pTFS5	This study	—	>500	—	—
TF8	RN4220 pTFS6	This study	>500	>500	0.5	1
TF9	COL pGC2	This study	—	0.8	512	1
TF10	COL pTFS6	This study	—	100	512	1
TF11	NCTC8325 *mec* pGC2	This study	—	0.4	512 (1,024)	1
TF12	NCTC8325 *mec* pTFS6	This study	—	100	512 (1,024)	1
TF13	NCTC8325 *mec* (*femA*::Tn*551*) pGC2	This study	—	3.2	4 (32)	1
TF14	NCTC8325 *mec* (*femA*::Tn*551*) pTFS6	This study	—	100	16 (512)	1
TF15	NCTC8325 *mec* (*femB*::Tn*551*) pGC2	This study	—	0.8	4 (16)	1
TF16	NCTC8325 *mec* (*femB*::Tn*551*) pTFS6	This study	—	100	4 (4)	1
S. capitis						
EPK1	*ale-1*	51	>500	100	—	—
EPK2	Cured of *ale-1*-encoding plasmid	This study	100	0.4	—	—
E. coli XL1-Blue	*rec1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZM15* Tn*10* (Tet^r)]	6				

[a] DMPPC, methicillin; VCM, vancomycin; —, not tested.
[b] MIC after 48 h of incubation.

vancomycin were from Sigma Chemical Co., St. Louis, Mo. Lysostaphin was further purified to homogeneity before use as described previously (50). ALE-1, 51-kDa endo-β-*N*-acetylglucosaminidase (GL), and 62-kDa *N*-acetylmuramyl-L-alanine amidase (AM) were purified to homogeneity from the culture supernatant of *S. capitis* EPK1 (51) or *S. aureus* FDA209P (52, 53) as described previously. EXPRE^{35}S^{35}S protein labeling mix (1,000 Ci/mmol) was purchased from DuPont-NEN Research Products, Boston, Mass. Other materials and chemicals used were from commercial sources.

Assay for endopeptidase resistance. Exponentially growing bacterial cells in TSB were cultured in TSB containing either lysostaphin or ALE-1 with standing at 37°C to analyze the endopeptidase resistance of the cells. To determine the endopeptidase resistance level, small portions (10^6 CFU) of exponentially growing culture were added to serial twofold dilutions of either lysostaphin or ALE-1 in TSB (150 μl) in two steps on a microtiter plate (Becton Dickinson and Co.). The endopeptidase concentration was within the range of 0.05 to 500 μg/ml. The plates were incubated at 37°C for 16 h, and the MICs of endopeptidases were determined by monitoring cell growth through observation of the well bottoms.

MIC and population analysis. MICs of methicillin and vancomycin were determined by a microdilution method as described previously (25) except that in some cases the incubation time was 48 h instead of 24 h. To determine the resistance level to methicillin more precisely, population analysis profiles were determined as described previously (38). Briefly, aliquots of overnight culture (10^9 CFU) were plated on tryptic soy agar containing various concentrations of methicillin (from 0.5 to 1,024 μg/ml), and colonies were counted after a 48-h incubation at 37°C.

DNA manipulations. Routine DNA manipulations, DNA digestion with restriction enzymes, DNA ligations, gel electrophoresis, Southern blotting of DNA and hybridization, and DNA sequencing were performed essentially as described

TABLE 2. Plasmids used

Plasmid	Vector	Cloning site	Relevant properties	Reference or source
pUC19			*E. coli* cloning vector	60
pCR2.1			*E. coli* cloning vector for PCR products	Invitrogen
pGC2			*S. aureus-E. coli* shuttle vector	45
pTFS3	pGC2	*Hin*cII	3.5-kbp *Hin*cII fragment of pTF3 (51)	This study
pTFS31	pGC2	*Hin*cII/*Eco*RI	3.2 kbp *Hin*cII-*Eco*RI fragment of pTF31 (3′ deletion fragment of pTF3 *Hin*cII insert)	This study
pTFS32	pGC2	*Hin*cII/*Eco*RI	2.5-kbp *Hin*cII-*Eco*RI fragment of pTF32 (3′ deletion fragment of pTF3 *Hin*cII insert)	This study
pTFS33	pGC2	*Hin*cII/*Eco*RI	2.3-kbp *Hin*cII-*Eco*RI fragment of pTF33 (3′ deletion fragment of pTF3 *Hin*cII insert)	This study
pTFS34	pGC2	*Hin*cII/*Hin*dIII	2.2-kbp *Hin*cII-*Hin*dIII fragment of pTF3 insert	This study
pTFS5	pGC2	*Nhe*I/*Eco*RI	1.7-kbp *Nhe*I-*Eco*RI fragment of pTF32 insert	This study
pTFS6	pGC2	*Eco*RI	1.65-kbp PCR product	This study
pBBB13	pSP64	*Pst*I	10.5-kbp *Pst*I fragment of BB270 chromosome containing *femAB*	3

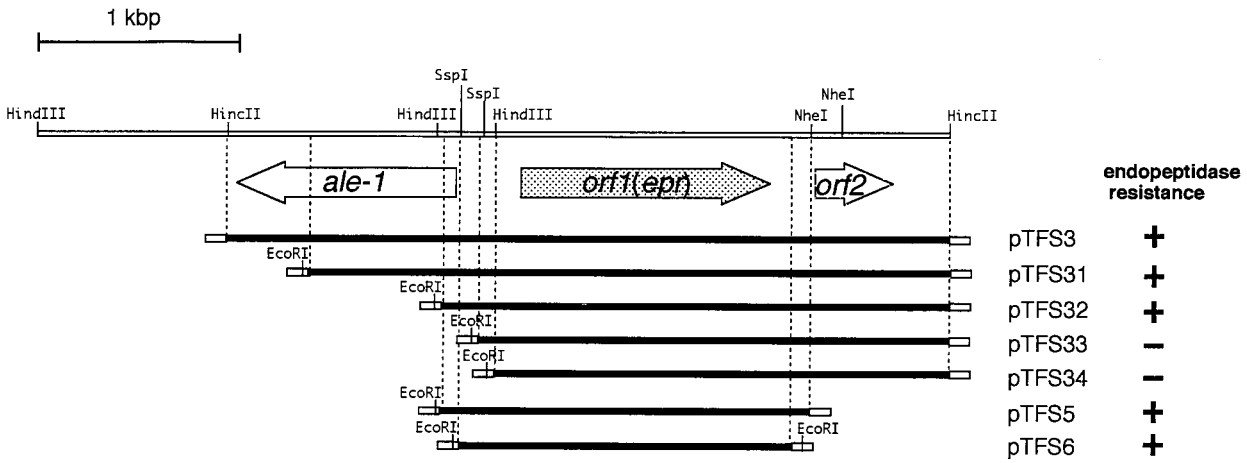

FIG. 1. Genetic organization of *epr*, *orf2*, and *ale-1* and phenotypes of recombinant plasmids. Thick arrows represent ORFs and directions of transcription. Black bars indicate DNA from *S. capitis* EPK1, and white bars indicate vector DNA. Endopeptidase resistance was examined by growing cells containing recombinant plasmid in the presence of various concentrations of lysostaphin at 37°C for 48 h: −, lysostaphin MIC was less than 1 μg/ml; +, lysostaphin MIC was more than 1 μg/ml.

previously (39, 46, 51). Purification of chromosomal DNA from staphylococcal cells was performed as described previously (51), with modification as follows. To lyse staphylococcal cells, lysostaphin (120 μg/ml) and mutanolysin (60 μg/ml) were used instead of lysostaphin alone for coagulase-negative staphylococci. Hybridization was performed by means of an enhanced chemiluminescence (ECL) procedure (ECL direct labeling kit or 3′-oligolabeling kit; Amersham Life Science, Buckinghamshire, United Kingdom). DNA sequences of both strands were determined by the dideoxy-chain termination method (40) with an Auto-Read sequencing kit (Pharmacia Biotech, Tokyo, Japan). A nested set of deletions for sequencing was constructed by using exonuclease III and mung bean nuclease (Takara Kilosequence deletion kit; Takara Biomedicals, Tokyo, Japan) according to the method of Henikoff (18).

PCR. PCR reagents were from Perkin-Elmer (Norwalk, Conn.), and PCR was performed with the GeneAmp PCR System 2400 (Perkin-Elmer). Primers were supplied by Greiner Japan Co. (Tokyo, Japan). To generate PCR fragments containing the entire *epr* gene with the putative promoter region, the following primers were used: 5′-AAATTTAAACCTCCTAATA-3′ and 5′-GCCAGCTTG TTGGGATACTC-3′. The amplified fragment was cloned into pCR2.1 vector by using an Original TA Cloning kit (Invitrogen Co., San Diego, Calif.). The cloned DNA fragment was checked by DNA sequencing, cut with *Eco*RI, and cloned into the *Eco*RI site of pGC2. To generate PCR fragments containing a portion of *epr*, the following primers were used: 5′-TCCAATTATCCAAAACTGA-3′ and 5′-TTTTGTAAATAAAGGGTCTAA-3′. The amplified fragment was purified and used as the probe for Southern hybridization.

Transformation of *S. aureus* by electroporation. For transformation of *S. aureus*, *S. aureus* RN4220 was used as the primary acceptor for recombinant plasmid. Exponentially growing cells of *S. aureus* were harvested at an optical density at 660 nm of 0.6, washed twice, resuspended in 1/100 volume of ice-cold 0.5 M sucrose, and kept on ice for 15 min. To 0.04 ml of cell suspension was added 0.1 to 0.2 μg of plasmid DNA. A pulse of 25 μF and 2.2 kV was delivered by an Electro Cell Manipulator 600 (BTX Electroporation system, BTX Inc., San Diego, Calif.). The cells were spread on selective tryptic soy agar containing chloramphenicol (50 μg/ml). The recombinant plasmid was subsequently transduced from the transformant by phage 80α (47) to the appropriate recipient strain as described previously (2).

In vitro transcription and translation. In vitro synthesis of proteins from plasmid DNA templates was performed by using the Linked T7 transcription-translation system (Amersham Life Science) with EXPRE^{35}S^{35}S protein labeling mix according to the manufacturer's protocol.

Amino acid analysis of peptidoglycan. Peptidoglycan was isolated as described previously (11). Samples for amino acid analysis were prepared by hydrolyzing peptidoglycan with 4 N HCl at 100°C for 15 h. Samples were then analyzed by an L-8500 amino acid analyzer (Hitachi Ltd., Tokyo, Japan) with ninhydrin detection.

Susceptibility of heat-inactivated cells to bacteriolytic enzymes. The susceptibility of heat-inactivated cells to bacteriolytic enzymes was tested by zymography (24) using sodium dodecyl sulfate-polyacrylamide gel electrophoresis on polyacrylamide gels containing heat-inactivated staphylococci (0.5 mg [dry weight] of cells/ml) (28, 49). Lysostaphin, 51-kDa GL, and 62-kDa AM were used as bacteriolytic enzymes. To detect their activities, 15% gels were used for lysostaphin and 62-kDa AM, and a 7.5% gel was used for 51-kDa GL. Ten microliters of serially diluted enzyme sample was applied to the wells. After electrophoresis, the gels were washed with distilled water for 30 min with constant agitation and incubated at 37°C in 0.1 M sodium phosphate buffer (pH 6.8). After a 12-h incubation, visible bands were detected with an immunoviewer, and the minimal concentration that showed a visible band was defined as the minimal bacteriolytic dose (MBD) (24).

Nucleotide sequence accession number. The nucleotide sequence data presented in this report will appear in the DDBJ, EMBL, and GenBank nucleotide sequence database under accession no. AB000222.

RESULTS

Cloning of the *epr* gene. DeHart et al. reported that *epr* is encoded very close to the gene for lysostaphin in *S. simulans* bv. *staphylolyticus* (9). Both were shown to reside on an 8.4-kbp fragment. While cloning a gene for *ale-1* in *S. capitis* EPK1, we obtained a 3.5-kbp *Hinc*II fragment containing *ale-1* and cloned it into pUC19 to generate pTF3 (51). We tried to determine whether the fragment contained *epr*. Thus, the 3.5-kbp *Hinc*II fragment of pTF3 was cloned into the *Hinc*II site of shuttle vector pGC2 to generate pTFS3. pTFS3 was transferred into *S. aureus* RN4220 by electroporation, transformants were selected for chloramphenicol resistance, and one isolate was designated TF2. Similarly, RN4220 carrying pGC2 was obtained as a control and designated as TF1. MICs of lysostaphin for TF2 and TF1 were determined. As shown in Table 1, TF2 was more resistant to lysostaphin than TF1. When TF2 was spread on brain heart infusion agar containing heat-killed *S. aureus* FDA209P and incubated at 37°C for 24 h, all colonies of TF2 were surrounded by halos due to the lysis of *S. aureus* FDA209P, while no halos were observed around the colonies of TF1. This result indicated that TF2 produced and secreted ALE-1 extracellularly. On the other hand, attempts to express ALE-1 in *S. aureus* by transformation with pGC2 containing *ale-1* alone were not successful (not shown). This result further indicated that TF2 became resistant to glycylglycine endopeptidase. A restriction map of the 3.5-kbp *Hinc*II fragment was established (Fig. 1). To determine the minimum amount of DNA required for the endopeptidase resistance, several transformants with subcloned DNA fragments of the 3.5-kbp *Hinc*II fragment were generated. Deletions of the 3.5-kbp *Hinc*II fragment, 3.2-, 2.5-, and 2.3-kbp *Hinc*II-EcoRI fragments, and 2.2-kbp *Hinc*II-*Hin*dIII fragment were cloned into pGC2 to generate pTFS31, pTFS32, pTFS33, and pTFS34, respectively. These plasmids were then transferred into RN4220 by electroporation, and transformants were designated TF3, TF4, TF5, and TF6, respectively. Assays to deter-

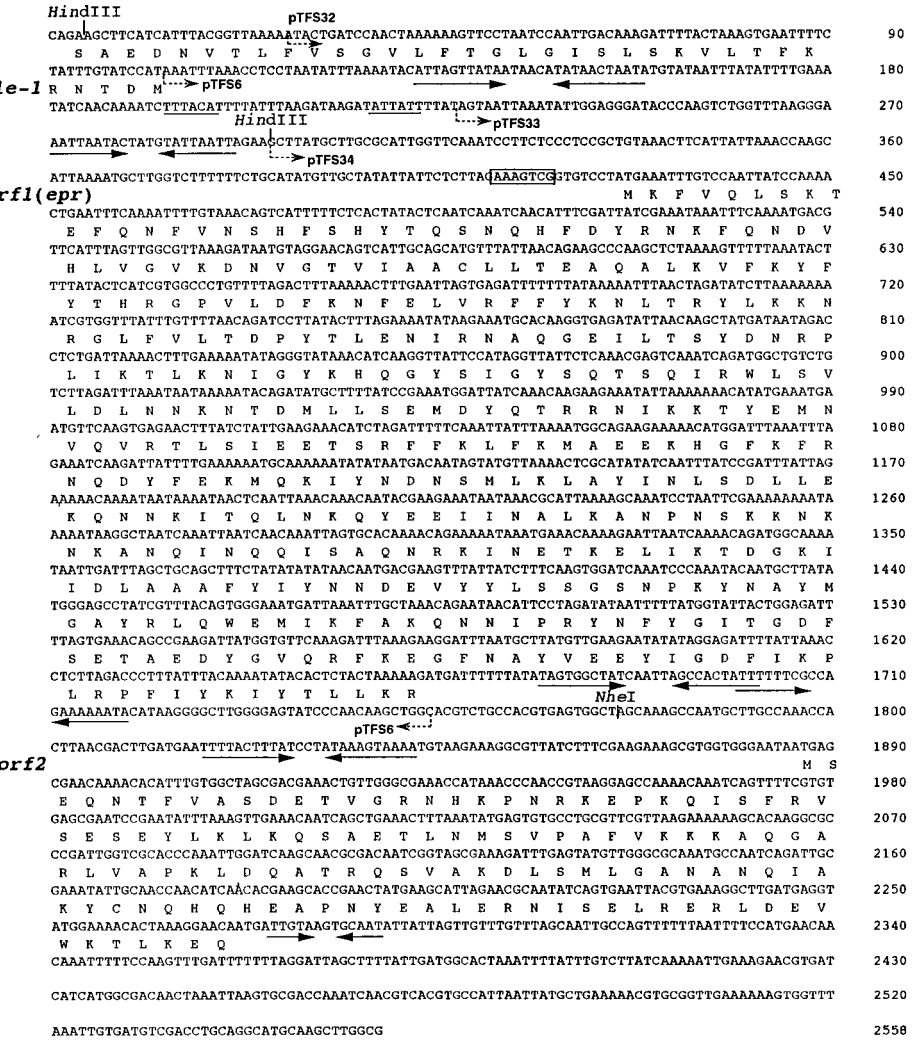

FIG. 2. DNA sequence analysis in the region of the *Hin*dIII-*Hin*cII site of the cloned DNA fragment. Two complete ORFs defining *epr* and *orf2* were found. A possible candidate for promoter sequences (−35 and −10 regions) (underlined) and a putative ribosome binding site for *epr* (boxed) are indicated. Palindromic sequences are indicated by arrows.

mine the MIC of lysostaphin revealed that TF3 and TF4 expressed endopeptidase resistance whereas TF5 and TF6 did not (Table 1). Fragments expressing endopeptidase resistance were sequenced by using either the universal or the reverse sequencing primer. The nucleotide sequencing revealed two potential new open reading frames (ORFs), *orf1* and *orf2*, which were in the same transcriptional direction but on different frames (Fig. 1). The *ale-1* ORF was on the opposite DNA strand to *orf1*. These findings suggested that the 5′ flanking region of the 2.2-kbp *Hin*dIII-*Hin*cII DNA fragment is necessary for an endopeptidase resistance phenotype whereas *orf2* is not necessary. A 1.7-kbp *Eco*RI-*Nhe*I fragment from insert DNA of pTF32 was subcloned into pGC2 to obtain pTFS5. The plasmid was transferred into RN4220 by electroporation, and the transformant was designated TF7. An assay to determine the MIC of lysostaphin revealed that TF7 was highly resistant to endopeptidase (Table 1). Therefore, we designed a primer set for amplifying the DNA fragment containing all of *orf1* and the flanking DNA region which contains the putative promoter sequence, as described later. PCR products were cloned into pCR2.1 in *E. coli* XL1-Blue and then subcloned into pGC2 to obtain pTFS6. The plasmid was transferred into RN4220 by electroporation, and the transformant was designated TF8. An assay to determine the MICs of lysostaphin revealed that TF8 was highly resistant to lysostaphin as well as ALE-1 (Table 1), and *orf1* was thus designated *epr.*

Nucleotide sequence of *epr*. Figure 2 shows the nucleotide sequence of the 2.5-kbp DNA fragment in pTFS32. *epr* starts with an ATG codon at nucleotide 426 and ends with a TGA codon at nucleotide 1665. A Shine-Dargarno sequence, AAA GTCG, which is similar to those of *S. aureus* in showing canonical homology with 16S RNA of *Bacillus subtilis* (32), was observed eight nucleotides upstream of the putative start codon. A possible candidate for a promoter sequence was present upstream of the *epr* ORF. The sequences TTTCACA (positions 194 to 199) and TATTATT (positions 217 to 223) might be −35 and −10 promoter regions, respectively. In the region between *epr* and *ale-1*, two palindromic sequences were identified. In the region downstream of *epr*, we found a stem-loop structure followed by multiple thymidine residues, resembling a rho-independent termination region. *epr* can code for a 413-amino-acid polypeptide with a calculated molecular weight

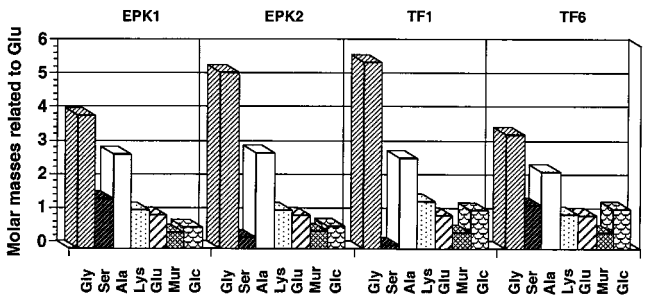

FIG. 3. Peptidoglycan amino compound composition of *S. capitis* EPK1 (EPK1), its derived strain without *epr* (EPK2), *S. aureus* RN4220 (TF1), and *S. aureus* RN4220 with *epr* (TF6). The amount of each compound is expressed as a molar ratio relative to glutamic acid (Glu). Gly, glycine; Ser, serine; Ala, alanine; Lys, lysine; Glu, glutamic acid; Mur, muramic acid; Glc, glucosamine.

of 48,990. The entire protein has a predicted isoelectric point of 9.93. In vitro protein labeling experiments using pTFS6 were carried out. The product of pTFS6, which was not found in pGC2, in the in vitro system was a protein band with an M_r of 46,000, comparable to the molecular weight of the protein encoded by *epr* (data not shown).

When the nucleotide and deduced amino acid sequences of *epr* were compared with those in databases in the BLAST and FASTA network search service (DDBJ), a strong similarity was found with the amino acid sequences of FemA and FemB of *S. aureus* (3) (accession no. M23918). When the protein sequences of FemA and FemB were aligned with that of Epr, we found identity at 162 positions (36%) with FemA and identity at 149 positions (33%) with FemB of the total 413 amino acid residues of Epr. The hydropathy plots of these proteins are strikingly similar (not shown).

Amino acid composition of cell walls of strains with or without *epr* expression. To determine whether the presence of *epr* caused an alteration in peptidoglycan structure, peptidoglycan was isolated from *S. capitis* EPK1, *S. capitis* EPK2 (a derivative of strain EPK1 lacking a plasmid containing *ale-1*), *S. aureus* TF1, and *S. aureus* TF8, and then the amino acid compositions were compared. As shown in Fig. 3, a relative decrease of serine and an increase of glycine in the molar ratio were observed in EPK2 compared with EPK1. On the other hand, TF8 had fewer glycines and more serines than TF1.

Bacteriolytic enzyme susceptibility of heat-inactivated cells. The lysostaphin-resistant phenotype and changes in amino acid composition of peptidoglycan of TF8 strongly suggested that the presence of *epr* altered the amino acid composition of cross bridges in *S. aureus* peptidoglycan. To elucidate the effect of *epr* overexpression on peptidoglycan, we further analyzed the cell wall structure by determining susceptibilities to various bacteriolytic enzymes with different bond specificities, including lysostaphin, 51-kDa GL, and 62-kDa AM, by using zymography as described in Materials and Methods. These enzymes are known to cleave Gly-Gly (22), GlcNAc-MurNAc (52), and MurNAc-Ala (53) bonds, respectively, in the peptidoglycan structure of *S. aureus*. Zymography is a highly sensitive method for detecting bacteriolytic enzymes (28, 49, 55) and is especially useful for enzymes, such as 51-kDa GL or 62-kDa AM, with lytic activity toward *S. aureus* unmeasurable by conventional turbidimetry without using a large quantity of the enzyme (53). When TF8 cells were used as the substrate for zymography, the MBD of lysostaphin was eight times higher than that when TF1 was used as the substrate (Table 3). On the other hand, MBDs of 51-kDa GL and 62-kDa AM in the TF8-containing gel were similar to those in the TF1-containing gel (Table 3). When comparing the results of susceptibility to lysostaphin, we found the difference in MBDs between cells with and without *epr* to be less than the difference in MICs. This might be due to the difference in assay systems.

Hybridization. The findings of the protein homology search of *epr* suggested that *S. capitis* EPK1 contains a gene closely related to *femAB*. *S. aureus* strains with inactivated *femA* or *femB* exhibited lysostaphin resistance and decreased glycine content of cross bridges (3, 19, 26, 30, 48). In contrast, in *S. capitis* EPK1 and *S. aureus* carrying a plasmid containing *epr*, glycine content was decreased and serine content was increased. This result suggested that *epr* and *femAB* belong to the same gene family but have distinct functions. A lysostaphin-resistant phenotype is prevalent in coagulase-negative staphylococci (62), and its relationship to substitution of glycine residues with serine in interpeptide chains of peptidoglycan has been suggested (43, 61). Therefore, we screened DNAs from various *Staphylococcus* species, including *S. aureus* and several coagulase-negative staphylococci, by using a probe generated by PCR. This probe represented the DNA encoding a portion of *epr* between the designed primers as described in Materials and Methods. The findings obtained from 26 strains are shown in Fig. 4. This probe hybridized to an 8.3-kbp *Eco*RI fragment of *S. capitis* EPK1 and to similar-size fragments in strains of other coagulase-negative staphylococci. Variations in molecular mass of the DNA fragment hybridizing with the probe were observed in several strains. In *S. capitis* EPK1, the probe weakly hybridized to DNA fragments with molecular masses of less than 8.3 kbp. Loss of the 8.3-kbp *Eco*RI fragment in *S. capitis* EPK2, which is devoid of a plasmid carrying *epr* and *ale-1*, suggests that *epr* was present only on the plasmid. To our surprise, the probe hybridized to a fragment the same size as the 8.3-kbp *Eco*RI fragment of *S. capitis* EPK1 in *S. aureus* strains, although it did not hybridize to a DNA fragment containing the *femAB* region.

Effect of overexpression of *epr* in *S. aureus* on susceptibility to methicillin and vancomycin. *femA* and *femB* were initially identified as genes essential for methicillin resistance in methicillin-resistant *S. aureus* (MRSA) (3). Transposon mutagenesis of either *femA* or *femB* increased the susceptibility of methicillin-susceptible *S. aureus* (MSSA) and MRSA to methicillin, and this sensitization effect was markedly apparent in MRSA (3, 19, 30). These mutants showed increased resistance to lysostaphin, decreased glycine content, and a slight increase in serine content in the peptidoglycan cross bridges (19, 30). On the other hand, *S. haemolyticus* strains expressing increased levels of resistance to glycopeptide antibiotics were shown to contain additional serine in place of glycine in interpeptide chains of the peptidoglycan (5). The presence of *epr* inducing similar changes in amino acid content in peptidoglycan struc-

TABLE 3. Susceptibilities of heat-inactivated staphylococcal cells to various bacteriolytic enzymes

Strain	MBD (ng)[a]		
	Lysostaphin	62-kDa AM	51-kDa GL
COL	0.1	2.5	25
TF1	0.1	2.5	25
TF8	0.8	2.5	25

[a] The susceptibilities of heat-inactivated staphylococcal cells (COL, TF1, or TF8) to the bacteriolytic enzymes lysostaphin, 62-kDa AM, and 51-kDa GL were tested by zymography as described in Materials and Methods. After electrophoresis, the gels were washed with distilled water for 30 min and incubated at 37°C in 0.1 M sodium phosphate buffer (pH 6.8). After a 12-h incubation, the minimal concentration that showed a visible band was defined as the MBD.

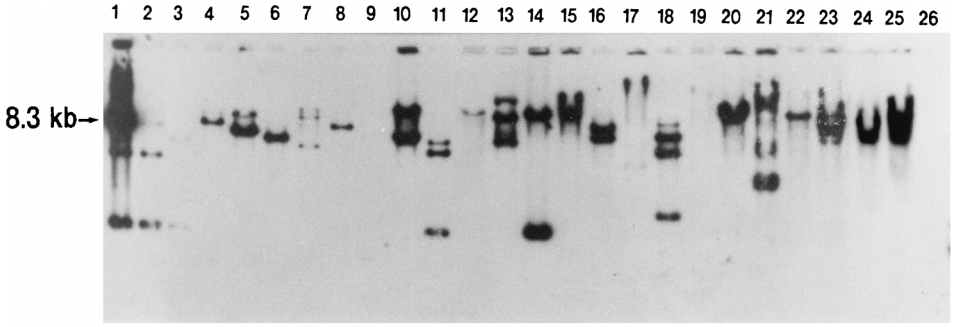

FIG. 4. Hybridization of the *epr* fragment to DNA fragments of various staphylococcal strains. Southern hybridization analyses were carried out by using ECL labeling and a detection kit (Amersham). Lanes contained chromosomal DNAs digested with *Eco*RI or plasmid digested with *Pst*I and separated on a 0.7% agarose gel. Lanes: 1, *S. capitis* EPK1; 2, *S. capitis* EPK2; 3, *S. epidermidis* M-5-1; 4, *S. saprophyticus* BCL1; 5, *S. capitis* ATCC 27840; 6, *S. cohnii* ATCC 29994; 7, *S. haemolyticus* ATCC 29970; 8, *S. intermedius* ATCC 29663; 9, *S. saccharolyticus* ATCC 14953; 10, *S. simulans* ATCC 27848; 11, *S. warneri* ATCC 27836; 12, *S. xylosus* ATCC 27971; 13, *S. hominis* CCM27327; 14, *S. lugdenensis* ATCC 438097; 15, *S. delphini* DSM207717; 16, *S. kloosii* DSM206767; 17, *S. schleiferi* N880033; 18, *S. caprae* CCM35737; 19, *S. arlettae* DSM206727; 20, *S. chromogenes* CCM33877; 21, *S. hycus* CCM23687; 22, *S. equorum* DSM206747; 23, *S. felis* ATCC 491687; 24, *S. aureus* FDA209P; 25, *S. aureus* RN4220; 26, pBBB13 containing *femAB.*

ture in *S. aureus* suggested that *epr* might affect the susceptibility of *S. aureus* to methicillin and/or vancomycin. MRSA strain COL (27) was transformed with pGC2 and pTFS6 to generate TF9 and TF10, respectively. MSSA (TF1 and TF8) and MRSA (TF9 and TF10) strains were tested for susceptibilities to methicillin and vancomycin. No differences were found in sensitivities to methicillin and vancomycin between the strains with *epr* and the corresponding strains without *epr* (Table 1). Resistance levels of TF9 and TF10 to methicillin were further assessed by population analysis, and no differences were found between them (not shown). We next tried to determine whether *epr* is able to complement methicillin resistance of *femA* and *femB* MRSA mutants. MRSA strain BB270 and its *femA* and *femB* mutants, BB308 and UT-34-2, were transformed with either pGC2 or pTFS6. Transformants were tested for susceptibilities to methicillin and lysostaphin. As shown in Table 1, we confirmed that *femA* and *femB* mutants were more resistant to lysostaphin than the parent strain BB270, although their resistance levels were much lower than that of *S. aureus* with *epr* on multicopy plasmid pGC2. In the presence of *epr*, the *femA* mutant became highly resistant to lysostaphin and partially restored its resistance to methicillin compared with TF13. This effect was markedly apparent after 48 h of incubation, probably due to the relatively slow growth of the transformants. In contrast, the *femB* mutant with *epr* became highly resistant to lysostaphin but maintained its sensitivity to methicillin.

DISCUSSION

Several mechanisms have been proposed for the resistance of cell wall peptidoglycan to peptidoglycan hydrolases. For example, O acetylation or unacetylation of amino sugars in peptidoglycan makes the cells resistant to lysozyme and other peptidoglycan hydrolases (1, 7, 12, 15, 29, 37, 56). Accessory cell wall polymers, such as lipoteichoic acid (4, 8, 13, 20, 21), teichoic acid (14, 59), and teichuronic acid (36), are implicated as endogenous inhibitors of peptidoglycan hydrolases and are suggested to protect peptidoglycan by associating with peptidoglycan hydrolases. In *S. simulans* bv. *staphylolyticus*, modification of the amino acid composition in peptidoglycan interpeptide chains was found to confer on the cells resistance to its product, lysostaphin (9, 35), and the genetic element involved in this modification was designated *epr* (9). Possibilities of involvement of additional polymers, such as teichoic acid or lipoteichoic acid, and acetyl moieties of the amino sugars in lysostaphin resistance were excluded (9). By analogy, we suggest that the alteration of the amino acid composition in peptidoglycan interpeptide chains of *S. capitis* EPK1 confers on the cells resistance to ALE-1. The mechanism of this resistance appears to be specific for glycylglycine endopeptidase since the susceptibilities of cells to bacteriolytic enzymes with different bond specificities were not affected by the presence of *epr* (Table 3).

Protein homology search revealed that the deduced amino acid sequence of the *epr* gene of *S. capitis* EPK1 is similar to that of *femAB* of *S. aureus*. Since matching amino acid residues are distributed all over the molecule, they seem to be derived from a common ancestal gene. Although they have amino acid sequence similarities, their functions appear to contrast. In staphylococcal strains with *epr* on a multicopy plasmid, serine content was increased and glycine content was decreased in peptidoglycan interpeptide chains. On the other hand, in *S. aureus* strains with inactivated *femA* or *femB*, glycine content was decreased in peptidoglycan interpeptide chains (3, 19, 26, 30, 48). In *S. aureus*, the pentaglycine chain is introduced by sequential addition of glycine from Gly-tRNA to the ε-amino group of the L-lysine residue of the pentapeptide in the lipid-linked intermediate (23, 31, 57). This process does not require ribosome and is distinct from protein synthesis in which amino acids are added at the carboxyl terminus. In *S. epidermidis*, interpeptide chains contain glycine and L-serine with a characteristic that the initial substituent of the ε-amino group of the L-lysine residue is glycine (58). The two are transferred from Gly-tRNA and Ser-tRNA, respectively (33, 58). Detailed analysis of the peptidoglycan structure of a *femA* mutant revealed an accumulation of mono-glycyl-substituted muropeptide and several species of muropeptides with substitution by serine in the second or fourth positions of the interpeptide chains (10). This result suggested that the biosynthetic block in the *femA* mutant occurs after the addition of the first glycine residue to the interpeptide chain. Further studies suggested that *femA* is involved in the addition of second and third glycines to the first glycine, and *femB* is involved in the addition of fourth and fifth glycines, of the interpeptide pentaglycine chain of the peptidoglycan precursor (26, 48). Our results suggest that gene products of *femAB* and *epr* belong to a protein family involved in adding amino acids to the pentapeptide peptidoglycan precursor and that *epr* is involved in adding serine to the pen-

tapeptide. Further studies are clearly required to elucidate the exact biochemical functions of these proteins.

Southern hybridization analysis revealed that most of the coagulase-negative staphylococci tested possess *epr* sequences or sequences similar to that of *epr*. An unexpected finding was that *S. aureus* possesses a DNA fragment hybridizing with *epr*. It is not likely that this fragment contains *femAB* since a DNA fragment containing *femAB* did not hybridize with *epr* under the same experimental conditions (Fig. 4). In some transposon mutants of *femA*, a decrease in glycine content was accompanied by a slight increase in serine content of interpeptide bridges (10). The presence of serine in the interpeptide bridges of some *S. aureus* strains has been reported (44). *S. aureus* might contain an *epr* homolog, the expression of which is somehow suppressed under normal growth conditions.

Unlike the *femA* or *femB* mutant of MRSA, MRSA with *epr* did not show a lowered resistance to methicillin (Table 1). *S. aureus* with *epr* showed no change in vancomycin MIC (Table 1). These findings suggest that the alteration in amino acid composition (i.e., increase in serine content and decrease in glycine content) is not the direct cause of alteration in susceptibility of *S. aureus* to methicillin or vancomycin. It should be noted that the sum of glycine and serine contents in *femA* and *femB* mutants was much lower than that of the parent strain (10, 19, 26, 30, 48). On the other hand, the sum of glycine and serine contents in *S. aureus* expressing *epr* was constant, about 5 mol per mol of glutamic acid (Fig. 3). These results suggest that shortening of interpeptide bridges is an important factor in lowering the susceptibilities of MRSA to methicillin and affects peptidoglycan cross-linking and cell wall turnover. Complementation experiments of *femA* and *femB* mutants with *epr* suggest that *epr* has a functional relationship to *femA* in regard to methicillin resistance (Table 1).

ACKNOWLEDGMENTS

We thank B. B. Bächi, J. Freney, R. Novick, and A. Tomasz for generous gifts of plasmids and bacterial strains and T. Oshida for pGC2.

This work was supported by a grant-in-aid for scientific research (0755715) from the Ministry of Education, Science, Sports and Culture of Japan.

ADDENDUM IN PROOF

Recently, it has been reported that *Staphylococcus simulans* bv. *staphylolyticus* possesses the *lif* gene, which is located in the opposite direction to that of the lysostaphin gene (G. Thumm and F. Götz Mol. Microbiol. **23:**1251–1265, 1997). The expression of *lif* in *S. carnosus* led to an increase in the serine/glycine ratio of the interpeptide bridges of peptidoglycan.

REFERENCES

1. **Araki, Y., T. Nakatani, K. Wakayama, and E. Ito.** 1972. Occurrence of *N*-nonsubstituted glucosamine residues in peptidoglycan of lysozyme-resistant cell walls of *Bacillus cereus*. J. Biol. Chem. **247:**6312–6322.
2. **Asheshov, E. H.** 1966. *Staphylococcus aureus* strains in the "52, 52A, 80, 81 complex." Nature (London) **209:**638–639.
3. **Berger-Bächi, B., L. Barberis-Maino, A. Strässle, and F. H. Kayser.** 1989. *FemA*, a host-mediated factor essential for methicillin resistance in *Staphylococcus aureus*: molecular cloning and characterization. Mol. Gen. Genet. **219:**263–269.
4. **Bierbaum, G., and H.-G. Sahl.** 1987. Autolytic system of *Staphylococcus simulans* 22: influence of cationic peptides on activity of *N*-acetylmuramoyl-L-alanine amidase. J. Bacteriol. **169:**5452–5458.
5. **Billot-Klein, D., L. Gutmann, D. Bryant, D. Bell, J. van Heijenoort, J. Grewal, and D. M. Shlaes.** 1996. Peptidoglycan synthesis and structure in *Staphylococcus haemolyticus* expressing increasing levels of resistance to glycopeptide antibiotics. J. Bacteriol. **178:**4696–4703.
6. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue: a high efficiency plasmid transforming recA *Escherichia coli* strain with beta-galactosidase selection. BioTechniques **5:**376–379.
7. **Clarke, A. J.** 1993. Extent of peptidoglycan O acetylation in the tribe *Proteeae*. J. Bacteriol. **175:**4550–4553.
8. **Cleveland, R. F., J.-V. Höltje, A. J. Wicken, A. Tomasz, L. Daneo-Moore, and J. D. Shockman.** 1975. Inhibition of bacterial wall lysins by lipoteichoic acids and related compounds. Biochem. Biophys. Res. Commun. **67:**1128–1135.
9. **DeHart, H. P., H. E. Heath, L. S. Heath, P. A. Leblanc, and G. L. Sloan.** 1995. The lysostaphin endopeptidase resistance gene (*epr*) specifies modification of peptidoglycan cross bridges in *Staphylococcus simulans* and *Staphylococcus aureus*. Appl. Environ. Microbiol. **61:**1475–1479.
10. **de Jonge, B. L. M., T. Sidow, Y.-S. Chang, H. Labischinski, B. Berger-Bächi, D. A. Gage, and A. Tomasz.** 1993. Altered muropeptide composition in *Staphylococcus aureus* strains with an inactivated *femA* locus. J. Bacteriol. **175:**2779–2782.
11. **de Jonge, B. L. M., Y.-S. Chang, D. Gage, and A. Tomasz.** 1992. Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. J. Biol. Chem. **267:**11248–11254.
12. **Dupont, C., and A. J. Clarke.** 1991. Dependence of lysozyme-catalyzed solubilization of *Proteus mirabilis* peptidoglycan on the extent of *o*-acetylation. Eur. J. Biochem. **195:**763–769.
13. **Fischer, W., P. Rösel, and H. U. Koch.** 1981. Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*. J. Bacteriol. **146:**467–475.
14. **Guidicelli, S., and A. Tomasz.** 1984. Attachment of pneumococcal autolysin to wall teichoic acids, an essential step in enzymatic wall degradation. J. Bacteriol. **158:**1188–1190.
15. **Hayashi, H., Y. Araki, and E. Ito.** 1973. Occurrence of glucosamine residues with free amino groups in cell wall peptidoglycan from bacilli as a factor responsible for resistance to lysozyme. J. Bacteriol. **113:**592–598.
16. **Heath, H. E., L. S. Heath, J. D. Nitterauer, K. E. Rose, and G. L. Sloan.** 1989. Plasmid-encoded lysostaphin endopeptidase resistance of *Staphylococcus simulans* biovar *staphylolyticus*. Biochem. Biophys. Res. Commun. **160:**1106–1109.
17. **Heath, L. S., H. E. Heath, and G. L. Sloan.** 1987. Plasmid-encoded lysostaphin endopeptidase gene of *Staphylococcus simulans* biovar *staphylolyticus*. FEMS Microbiol. Lett. **44:**129–133.
18. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
19. **Henze, U., T. Sidow, J. Wecke, H. Labischinski, and B. Berger-Bächi.** 1993. Influence of *femB* on methicillin resistance and peptidoglycan metabolism in *Staphylococcus aureus*. J. Bacteriol. **175:**1612–1620.
20. **Herbold, D. R., and L. Glaser.** 1975. Interaction of *N*-acetylmuramic acid L-alanine amidase with cell wall polymers. J. Biol. Chem. **250:**7231–7283.
21. **Höltje, J. V., and A. Tomasz.** 1975. Lipoteichoic acid: a specific inhibitor of autolysin activity in Pneumococcus. Proc. Natl. Acad. Sci. USA **72:**1690–1694.
22. **Iversen, O., and A. Grov.** 1973. Studies on lysostaphin. Separation and characterization of three enzymes. Eur. J. Biochem. **38:**293–300.
23. **Kamiryo, T., and M. Matsuhashi.** 1972. The biosynthesis of the cross-linking peptides in the cell wall peptidoglycan of *Staphylococcus aureus*. J. Biol. Chem. **247:**6306–6311.
24. **Komatsuzawa, H., M. Sugai, C. Shirai, J. Suzuki, K. Hiramatsu, and H. Suginaka.** 1995. Triton X-100 alters the resistance level of methicillin-resistant *Staphylococcus aureus* to oxacillin. FEMS Microbiol. Lett. **134:**209–212.
25. **Komatsuzawa, H., J. Suzuki, M. Sugai, Y. Miyake, and H. Suginaka.** 1994. The effect of Triton X-100 on the in-vitro susceptibility of methicillin-resistant *Staphylococcus aureus* to oxacillin. J. Antimicrob. Chemother. **34:**885–897.
26. **Kopp, U., M. Roos, J. Wecke, and H. Labischinski.** 1996. Staphylococcal peptidoglycan interpeptide bridge biosynthesis: a novel antistaphylococcal target? Microb. Drug Res. **2:**29–41.
27. **Kornblum, J., B. J. Hartman, R. P. Novick, and A. Tomasz.** 1986. Conversion of a homogenously methicillin-resistant strain of *Staphylococcus aureus* to heterogenous resistance by Tn*551*-mediated insertional inactivation. Eur. J. Clin. Microbiol. **5:**714–718.
28. **LeClerc, D., and A. Asselin.** 1989. Detection of bacterial cell wall hydrolases after denaturing polyacrylamide gel electrophoresis. Can. J. Microbiol. **35:**749–753.
29. **Losardt, I. M., and H. Y. Neujahr.** 1975. Lysis of modified walls from *Lactobacillus fermentum*. J. Bacteriol. **124:**73–77.
30. **Maidhof, H., B. Reinicke, P. Blümel, B. Berger-Bächi, and H. Labischinski.** 1991. *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. J. Bacteriol. **173:**3507–3513.
31. **Matsuhashi, M., C. P. Dietrich, and J. L. Strominger.** 1967. Biosynthesis of the peptidoglycan of bacterial cell wall. J. Biol. Chem. **242:**3191–3206.
32. **Novick, R. P.** 1990. The staphylococcus as a molecular genetic system, p. 1–37. *In* R. P. Novick (ed.), Molecular biology of staphylococci. VCH Publishers, New York, N.Y.

33. **Petit, J. F., J. L. Strominger, and D. Söll.** 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. VII. Incorporation of serine and glycine into interpeptide bridges in *Staphylococcus epidermidis*. J. Biol. Chem. **243:**757–767.
34. **Recsei, P. A., A. D. Gruss, and R. P. Novick.** 1987. Cloning, sequence, and expression of the lysostaphin gene from *Staphylococcus simulans*. Proc. Natl. Acad. Sci. USA **84:**1127–1131.
35. **Robinson, J. M., J. K. Hardman, and G. L. Sloan.** 1979. Relationship between lysostaphin endopeptidase production and cell wall composition in *Staphylococcus staphylolyticus*. J. Bacteriol. **137:**1158–1164.
36. **Robson, R. L., and J. Baddiley.** 1977. Role of teichuronic acid in *Bacillus licheniformis*: defective autolysis due to deficiency of teichuronic acid in a novobiocin-resistant mutant. J. Bacteriol. **129:**1051–1058.
37. **Rosenthal, R. S., J. K. Blundell, and H. R. Perkins.** 1982. Strain-related differences in lysozyme sensitivity and extent of O-acetylation of gonococcal peptidoglycan. Infect. Immun. **37:**826–829.
38. **Ryffel, C., A. Strässle, F. H. Kayser, and B. Berger-Bächi.** 1994. Mechanisms of heteroresistance in methicillin-resistant *Staphylococcus aureus*. Antimicrob. Agents Chemother. **38:**724–728.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
41. **Schindler, C. A., and V. T. Schuhardt.** 1964. Lysostaphin: a new bacteriolytic agent for the *Staphylococcus*. Proc. Natl. Acad. Sci. USA **51:**414–421.
42. **Schindler, C. A., and V. T. Schuhardt.** 1965. Purification and properties of lysostaphin—a lytic agent for *Staphylococcus aureus*. Biochim. Biophys. Acta **97:**242–250.
43. **Schleifer, K. H.** 1986. Gram-positive cocci, p. 999–1103. *In* P. H. A. Sneath, N. S. Mair, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology. Williams & Wilkins, Baltimore, Md.
44. **Schleifer, K. H., and O. Kandler.** 1972. Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriol. Rev. **36:**407–477.
45. **Skinner, S., B. Inglis, P. R. Matthews, and P. R. Stewart.** 1988. Mercury and tetracycline resistance genes and flanking repeats associated with methicillin resistance on the chromosome of *Staphylococcus aureus*. Mol. Microbiol. **2:**289–297.
46. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
47. **Stewart, P. R., H. G. Waldron, J. S. Lee, and P. R. Matthews.** 1985. Molecular relationships among serogroup B bacteriophages of *Staphylococcus aureus*. J. Virol. **55:**111–116.
48. **Stranden, A. M., K. Ehlert, H. Labischinski, and B. Berger-Bächi.** 1997. Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a *femAB* null mutant of methicillin-resistant *Staphylococcus aureus*. J. Bacteriol. **179:**9–16.
49. **Sugai, M., T. Akiyama, H. Komatsuzawa, Y. Miyake, and H. Suginaka.** 1990. Characterization of sodium dodecyl sulfate-stable *Staphylococcus aureus* bacteriolytic enzymes by polyacrylamide gel electrophoresis. J. Bacteriol. **172:**6494–6498.
50. **Sugai, M., T. Akiyama, Y. Miyake, E. Ishida, and H. Suginaka.** 1990. Rapid purification method of lysostaphin for analysis of cell-wall proteins. J. Microbiol. Methods **12:**133–138.
51. **Sugai, M., T. Fujiwara, T. Akiyama, M. Ohara, H. Komatsuzawa, S. Inoue, and H. Suginaka.** 1997. Purification and molecular characterization of glycylglycine endopeptidase produced by *Staphylococcus capitis* EPK1. J. Bacteriol. **179:**1193–1202.
52. **Sugai, M., H. Koike, Y.-M. Hong, Y. Miyake, R. Nogami, and H. Suginaka.** 1989. Purification of a 51 kDa endo-β-*N*-acetylglucosaminidase from *Staphylococcus aureus*. FEMS Microbiol. Lett. **61:**267–272.
53. **Sugai, M., H. Komatsuzawa, T. Akiyama, Y.-M. Hong, T. Oshida, Y. Miyake, T. Yamaguchi, and H. Suginaka.** 1995. Identification of endo-β-*N*-acetylglucosaminidase and *N*-acetylmuramyl-L-alanine amidase as cluster-dispersing enzymes in *Staphylococcus aureus*. J. Bacteriol. **177:**1491–1496.
54. **Sugai, M., H. Komatsuzawa, K. Ooku-Inomata, Y. Miyake, E. Ishida, and H. Suginaka.** 1994. Isolation and characterization of *Staphylococcus aureus* mutants which form altered cell clusters. Microbiol. Immunol. **38:**995–999.
55. **Sugai, M., H. Komatsuzawa, S. Tomita, T. Akiyama, Y. Miyake, and H. Suginaka.** 1991. Detection of a staphylococcal endo-β-*N*-acetylglucosaminidase using polyacrylamide gels. J. Microbiol. Methods **13:**11–16.
56. **Swim, S. C., M. A. Gfell, C. E. I. Wilde, and R. S. Rosenthal.** 1983. Strain distribution in extents of lysozyme resistance and O-acetylation of gonococcal peptidoglycan determined by high-performance liquid chromatography. Infect. Immun. **42:**446–452.
57. **Thorndike, J., and J. T. Park.** 1969. A method for demonstrating the stepwise addition of glycine from transfer RNA into the murein precursor of *Staphylococcus aureus*. Biochem. Biophys. Res. Commun. **34:**642–647.
58. **Tipper, D. J.** 1969. Structures of the cell wall peptidoglycans of *Staphylococcus epidermidis* Texas 26 and *Staphylococcus aureus* Copenhagen. II. Structure of neutral and basic peptides from hydrolysis with the Myxobacter al-1 peptidase. Biochemistry **8:**2192–2202.
59. **Tomasz, A., M. Westphal, E. B. Briles, and P. Fletcher.** 1975. On the physiological function of teichoic acids. J. Supramol. Struct. **3:**1–16.
60. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
61. **Zygmunt, W., and P. A. Tavormina.** 1972. Lysostaphin: model for a specific enzymatic approach to infectious disease. Prog. Drug Res. **16:**309–333.
62. **Zygmunt, W. A., H. P. Browder, and P. A. Tavormina.** 1968. Susceptibility of coagulase-negative staphylococci to lysostaphin and other antibiotics. Appl. Microbiol. **16:**1168–1173.