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Purification and Molecular Characterization of Glycylglycine Endopeptidase Produced by *Staphylococcus capitis* EPK1

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A novel staphylolytic enzyme, ALE-1, acting on *Staphylococcus aureus*, was purified from a *Staphylococcus capitis* EPK1 culture supernatant. The optimal pH range for staphylolytic activity was 7 to 9. ALE-1 contains one Zn^{2+} atom per molecule. Analysis of peptidoglycan fragments released by ALE-1 indicated that the enzyme is a glycylglycine endopeptidase. The effects of various modulators were determined, and we found that *o*-phenanthroline, iodoacetic acid, diethylpyrocarbonate, and Cu^{2+} reduced the staphylolytic activity of ALE-1. β-Casein, elastin, and pentaglycine were poor substrates for ALE-1. Molecular cloning data revealed that ALE-1 is composed of 362 amino acid residues and is synthesized as a precursor protein which is cleaved after Ala at position 35, thus producing a mature ALE-1 of 35.6 kDa. The primary structure of mature ALE-1 is very similar to the proenzyme form of lysostaphin. It has the modular design of an N-terminal domain of tandem repeats of a 13-amino-acid sequence fused to the active site containing C-terminal domain. Unlike lysostaphin, ALE-1 does not undergo processing of the N-terminal repeat domain in broth culture. *ale-1* is encoded on the plasmid. Protein homology search suggested that ALE-1 and lysostaphin are members of the novel Zn^{2+} protease family with a homologous 38-amino-acid-long motif, Tyr-X-His-X_{11}-Val-$X_{12/20}$-Gly-$X_{5\text{-}6}$-His.

A number of bacterial species produce peptidoglycan hydrolases that preferentially lyse staphylococcal species. They include *Pseudomonas* (5–7, 28, 35, 40), *Aeromonas* (10, 25, 35, 68), *Clostridium* (36), *Actinomyces* (1), *Streptomyces* (64, 70), *Chalaropsis* (17), *Flavobacterium* (27), and *Staphylococcus* (45, 46, 56) species. The physiological functions of these enzymes remains largely unknown. Lysostaphin is a staphylolytic enzyme with a molecular weight of 25,000 secreted by only one strain of *Staphylococcus simulans* bv. *staphylolyticus* (45, 46). Although its catalytic properties are not well characterized, it is apparent that lysostaphin hydrolyzes glycylglycine bonds in interpeptide bridges of *Staphylococcus aureus* peptidoglycan (24). Herein, we describe the purification and molecular characterization of the novel staphylolytic enzyme ALE-1, which is produced by *Staphylococcus capitis* EPK1 that was originally isolated from a contaminated nutrient agar plate (56). We show that ALE-1 is a glycylglycine endopeptidase that is structurally and functionally related to lysostaphin and is a member of the novel Zn^{2+} protease family.

MATERIALS AND METHODS

Bacterial strains and plasmid. *S. capitis* EPK1 (56) and 10 clinically isolated *S. capitis* strains were used. Clinical strains were obtained from normal human skin. DNA from *S. capitis* EPK1 was cloned in *Escherichia coli* JM109 (69). Subcloning of the ALE-1 gene containing DNA fragment was carried out with pUC19 as vector and *E. coli* XL1-Blue (4). Heat-killed *S. aureus* FDA209P was used for the staphylolytic assay. *Staphylococcus* and *Escherichia* were grown in Trypticase soy broth (TSB) (Becton Dickinson Microbiology Systems, Cockeysville, Md.) and Luria-Bertani (LB) broth (5 g of yeast extract, 10 g of polypeptone, 10 g of NaCl per liter [pH 7.2]), respectively. When necessary, ampicillin was added to a final concentration of 50 μg/ml.

Materials and chemicals. To assay staphylolytic activity, *S. aureus* FDA209P or *S. capitis* EPK1 was the substrate. TSKgel Blue-5PW, TSKgel HA1000, TSKgel G3000 SW_{XL}, and TSKgel Phenyl 5PW RP were purchased from Tosoh, Tokyo, Japan. Lysostaphin was purified to homogeneity from a commercially obtained specimen (Sigma Chemical Co., St. Louis, Mo.) as described previously (52). All restriction enzymes, T4 DNA ligase, Klenow fragment of DNA polymerase I, and bacterial alkaline phosphatase were from Toyobo Co., Ltd., Osaka, Japan; ribonuclease was from Sigma Chemical Co. Isopropylthio-β-D-galactoside (IPTG) was from Wako Pure Chemical Industries, Ltd., Osaka, Japan; 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), proteinase K, and Agarose LO3 were from Takara Shuzo Co., Kyoto, Japan. Other materials and chemicals used were from commercial sources.

Preparation of CCF. *S. capitis* EPK1 exponentially growing in TSB was inoculated into 3 liters of the same fresh medium and incubated with continuous agitation by a rotary shaker for 18 h at 37°C until the cells reached the stationary phase. The culture was centrifuged at 10,000 × *g* for 30 min at 4°C, and then the supernatant was filtered through a membrane (pore size, 0.22 μm; Nihon Millipore Kogyo K.K., Yonezawa, Japan). Concentrated culture filtrate (CCF) was prepared by 80% saturated ammonium sulfate precipitation of the culture filtrate.

Purification. CCF dialyzed against 10 mM phosphate buffer (pH 7.0) (buffer 1) was applied to a TSKgel Blue-5PW column (7.5 by 75 mm) which was equilibrated with buffer 1. The column was washed with buffer 1 until most of the unbound proteins passed through. Bound proteins were eluted with a linear gradient from buffer 1 to 10 mM phosphate buffer containing 1 M NaCl (pH 7.0) at a flow rate of 1 ml/min. High-performance liquid chromatography (HPLC) was performed at room temperature. The active fractions (8 ml) were dialyzed against buffer 1 at 4°C. The dialyzate was applied to TSKgel HA1000, which was equilibrated with buffer 1. After washing with 1 to 2 column volumes of buffer 1, the sample was eluted with a linear gradient from buffer 1 to 0.5 M phosphate buffer (pH 7.0) at a flow rate of 1 ml/min. The active fractions (3 ml) were dialyzed against buffer 1 at 4°C. The dialyzate (1 volume) was mixed with cold acetone (7 volumes) and placed at −20°C for 30 min. The mixture was centrifuged at 12,000 × *g* for 30 min at 4°C, and the precipitate was dried in vacuo. The dried material was solubilized in 0.1 M phosphate buffer containing 0.1 M sodium sulfate (pH 7.0) (buffer 2). The solubilized sample was loaded onto a TSKgel G3000 SW_{XL} and eluted with buffer 2 at a flow rate of 0.5 ml/min. The active fractions (1.5 ml) were dialyzed against buffer 1 at 4°C. The purity of the enzyme was analyzed with a TSKgel phenyl 5PW-RP column. The solvent system was a linear gradient from A to B for 30 min. H_2O-CH_3CN-5% trifluoroacetic acid for A was 90:10:1, and for B it was 40:60:1 (vol/vol).

Assay of staphylolytic activity and enzymatic specificity. Heat-killed cells of *S. aureus* FDA209P were used as the standard substrate for the staphylolytic enzyme unless otherwise noted. In some experiments, viable *S. aureus* FDA209P or heat-killed or viable *S. capitis* EPK1 was used as the substrate. Lytic activity was assayed by following the rate of decrease in the turbidity of the cell suspension as described previously (53). Cells were suspended in 0.1 M Tris-HCl (pH 8.5) (1

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mg [dry weight]/ml) unless otherwise noted. An appropriate dilution of the test specimen was mixed with 2 ml of the cell suspension, and then the mixture was incubated at 37°C and the rate of decrease in turbidity was measured at 595 nm in a spectrophotometer. One unit of enzyme was defined as the amount of protein that decreased the A_{595} from 0.25 to 0.125 in 60 min. The SDS-extracted cell wall of *S. aureus* FDA209P was prepared as described previously (53) and washed extensively with 10 mM phosphate-buffered saline (PBS) (pH 7.0). The nature of the lytic enzyme was determined by analyzing the reaction products. The washed SDS-extracted cell wall was suspended in 0.1 M phosphate buffer (pH 7.0) (5 mg/ml) and incubated at 37°C with the purified enzyme in a total volume of 4 ml. Samples were removed at intervals to measure the turbidity and concentrations of reducing sugars and free amino groups. The appearances of reducing sugars, N-terminal amino groups, and C-terminal amino groups in soluble fragments of the enzymatically hydrolyzed *S. aureus* cell wall were determined by a modified Park-Johnson procedure (59) and the Ghuysen procedure using 2,4-dinitrofluorobenzene (15). Hydrazinolysis was performed by incubating dried test samples with anhydrous hydrazine for 6 h at 100°C. 2,4-Dinitrophenyl (DNP)-amino acids were analyzed by thin-layer chromatography. The plate was first developed with *n*-butanol–1% ammonia for 2.5 h at room temperature. After drying, the plate was developed with chloroform-methanol-acetic acid (85:14:1 [vol/vol/vol]) for 45 min at 4°C.

Effect of pH and modulators on staphylolytic activity. The effect of pH on the staphylolytic activities was determined under standard staphylolytic assay conditions using 0.1 M Tris-maleate at pH 4.74, 0.1 M Tris-HCl at pH 7.0, 0.1 M Tris-HCl at pH 8.5, 0.025 M diethanolamine-HCl at pH 9.5, and 0.025 M diethanolamine-HCl at pH 10.5. The effects of several inhibitors on the staphylolytic activities were determined after an incubation with purified enzymes for 10 min at room temperature, followed by addition of the heat-killed cells suspended in 0.1 M Tris-HCl at pH 8.5. The final concentrations of the inhibitors were phosphoramidon at 20 μM, *o*-phenanthroline at 10 mM, EDTA at 10 mM, phenylmethylsulfonyl fluoride (PMSF) at 1 mM, benzamidine at 12.8 mM, dithiothreitol at 10 mM, iodoacetic acid at 0.1 mM, and diethylpyrocarbonate at 5 mM. The effect of diethylpyrocarbonate was determined in 0.1 M phosphate buffer (pH 8.5).

Assay of proteolytic activity. General proteolytic activities and a preference for glycine-rich peptides were measured using β-casein (Sigma Chemical Co.), elastin-Congo red (Sigma), and pentaglycine (Sigma), respectively, as described previously (28, 40). To measure caseinolytic activity, 10 μl of β-casein solution (1 mg/ml in 25 mM ethanolamine-HCl buffer [pH 9.5]) was incubated with ALE-1 or lysostaphin at 37°C for 1 h. Thereafter, an aliquot of the mixture was resolved by SDS-polyacrylamide gel electrophoresis (PAGE) (12% polyacrylamide gel), and then the gel was stained with Coomassie blue. For elastolytic activity, elastin-Congo red suspension (4.6 mg/ml in 50 mM Tris-HCl, 0.5 mM $CaCl_2$ [pH 7.5]) was shaken with various concentrations of ALE-1 or lysostaphin at 37°C for 2 h. After centrifuging of the suspension at 9,000 × *g* for 10 min, the A_{495} of the supernatant was measured. Pentaglycine hydrolysis was measured by thin-layer chromatography as described previously (28). Standards including glycine, diglycine, triglycine, tetraglycine, and pentaglycine were from Sigma Chemical Co. Pentaglycine (4 mM) was solved in 20 mM Tris-HCl (pH 8.5). Peptide solution (50 μl) was incubated with 1.5 μg of ALE-1 or lysostaphin at 37°C for 5 h. After the incubation, 5 μl of aliquots was analyzed by thin-layer chromatography on PE SIL G (0.25 mm; Whatman Ltd., Kent, England) with a mixture of butanol-acetic acid-water (4:1:1) as the solvent. After chromatography, the plates were sprayed with 0.2% ninhydrin in ethanol and heated at 80°C.

Amino-terminal sequence determination. About 33 μg of purified protein was concentrated by precipitation with acetone. The precipitate was washed with acetone several times, separated by SDS-PAGE, and then electrotransferred to Trans-Blot membrane (polyvinylidene difluoride membrane; Bio-Rad Laboratories, Hercules, Calif.) by using 30 mM Tris-borate buffer containing 0.02% SDS (pH 8.5) (33). After staining with 0.1% Coomassie brilliant blue R-250 and destaining with 50% methanol, the band was excised and washed with distilled water. The amino-terminal sequence of the purified enzyme was determined with a Shimadzu Gas Phase Protein Sequencer PSQ-1. A homology search of the 10-amino-terminal-residue sequence was performed at the National Center for Biotechnology Information by using the BLAST network service.

Determination of zinc content in the purified enzyme. Zinc levels were determined with a Zeeman-effect atomic absorption spectrometer (Hitachi 170-70; Nissei Sangyo, Co., Tokyo, Japan). The data are averages of three determinations of various dilutions of the sample. All solutions were prepared in plastic ware with Milli-Q-water (Millipore).

Antiserum. The purified enzyme emulsified with an equal volume of Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.) (500 μg of protein per ml) was injected into the footpads of rabbits (weighing 2 kg). On day 1 and at 2 and 4 weeks, the rabbits were injected intravenously with 100 μg of the purified enzyme in PBS. Antiserum was obtained 5 weeks after the first injection. The antiserum was diluted 2,000-fold against purified enzyme for immunoblotting.

Preparation of DNA. *S. capitis* EPK1 grown to midlogarithmic phase in 100 ml of TSB was harvested by centrifugation, washed with 100 mM Tris-HCl–10 mM EDTA–150 mM NaCl, pH 7.5, and suspended in 5 ml of the same buffer containing lysostaphin (60 μg/ml). After 1 h of incubation at 37°C, proteinase K was added and the suspension was incubated for 30 min at 37°C. To the lysate 0.5 ml of 5% SDS–50% ethanol was added, and the suspension was shaken for 20 min at room temperature. The suspension was extracted three times with an equal volume of phenol equilibrated with 10 mM Tris-HCl, pH 8.0. The nucleic acid was precipitated from aqueous phase by the addition of 2 volumes of ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol, and dissolved in TE (10 mM Tris-HCl–1 mM EDTA, pH 8.0). The yield was 150 μg of DNA. Chromosomal and plasmid DNA of *S. capitis* was obtained by CsCl centrifugation. Plasmid DNA was isolated from *E. coli* by alkaline SDS extraction of cell lysate (2).

Cloning and DNA sequencing. Routine DNA manipulations were carried out essentially as described previously (43). *S. capitis* EPK1 DNA was partially digested with *Hin*dIII and separated by agarose gel electrophoresis. Five- to 8-kbp fragments were recovered from the agarose gels with Gene-clean Kit II (Bio 101). Ligation of the DNA fragments into the dephosphorylated *Hin*dIII site of pUC19 was followed by electrotransformation into *E. coli* JM109. Ampicillin-resistant transformants containing recombinant plasmids, as indicated by the inactivation of *lacZ′*, were used for screening. To select clones expressing staphylolytic activity, agar plates containing heat-killed *S. aureus* were used as described elsewhere (8, 20, 41, 55). *E. coli* cells were plated on TSB solidified with 1.5% (wt/vol) agar containing heat-killed *S. aureus* FDA209P (0.5 mg [dry weight]/ml), lysozyme (4 mg/ml), and IPTG. After 2 days the plates were examined for the appearance of halos around the colonies. Transformants with clear halos were grown to late logarithmic growth phase in LB in the presence of IPTG and collected by centrifugation. The resulting pellet suspended in 10 mM PBS (pH 7.0) was disrupted with bath sonicator. Staphylolytic enzyme profiles of the cell extract from sonicated samples were analyzed by zymography (31, 51). Deletion of DNA fragment using *Exo*III was carried out with a Deletion kit (Takara). DNA sequence was determined by the dideoxy chain-termination method (44) with T7 DNA polymerase (Pharmacia LKB Biotechnology Inc., Björkgatan, Sweden) or Thermo Sequenase (Amersham International plc, Middlesex, United Kingdom) by using an automated DNA sequencing system (ALFred; Pharmacia). Both DNA strands were sequenced by using vector-derived primers.

Southern transfer, DNA hybridization, and PCR. After agarose electrophoresis, DNA was transferred to Hybond-N membranes (Amersham) in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]). The membranes were washed with 5× SSC for 5 min at room temperature, and the DNA was cross-linked to Hybond-N membranes by UV illumination. Probe labelling and hybridization were carried out with the ECL labelling and detection system according to the instructions of the manufacturer (Amersham). PCR was performed to construct N-terminal truncated ALE-1 with *Taq* DNA polymerase according to the instructions of the manufacturer (Toyobo). Primers were designed as 5′-GGGGATCCGCTGCTCAATCT-3′ and 5′-GGGAATTCCCCTTCGTGTTG-3′, which contain *Bam*HI and *Eco*RI sites, respectively. The amplified fragment digested with *Bam*HI and *Eco*RI was cloned into pUC19.

Other procedures. SDS-PAGE, Western blotting (immunoblotting), and isoelectric focusing proceeded as described previously (53). Protein was immunodetected by using Renaissance 4CN plus (Dupont NEN, Boston, Mass.). Zymography was performed as described previously (31, 51). Protein concentrations were determined with the BCA protein assay reagent (Pierce, Rockford, Ill.), with bovine serum albumin as the standard (49). Curing of the plasmid was performed using ethidium bromide at 42°C as described previously (65).

Nucleotide sequence accession number. The nucleotide sequence data identified in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number D86328.

RESULTS

Purification. Dye ligand affinity columns have been used to purify several bacteriolytic enzymes, including endo-β-*N*-acetylglucosaminidase (53) and *N*-acetylmuramyl-L-alanine amidase (54) from *S. aureus*, *N*-acetylmuramyl-L-alanine amidase from *Bacillus subtilis* (50), and glycylglycine endopeptidase (lysostaphin) from *S. simulans* bv. *staphylolyticus* (52). We therefore used Cibacron blue affinity gel HPLC for the first step of purification. Staphylolytic activity of the CCF bound to the TSKgel Blue 5PW column was eluted by increasing the NaCl concentration of the buffer. The active fraction was applied to hydroxyapatite HPLC with TSKgel HA1000. The staphylolytic activity bound to the column and was eluted as a single major peak. The active fractions were concentrated by acetone precipitation and subjected to gel permeation HPLC with a TSKgel G3000 SW_{XL} column. The staphylolytic activity eluted with the first symmetrical peak as shown in Fig. 1. The homogeneity of the purified staphylolytic enzyme preparation was analyzed by means of reverse-phase HPLC, and a single peak was recognized (not shown). The purified enzyme migrated as a single protein band on electrophoresis, and silver

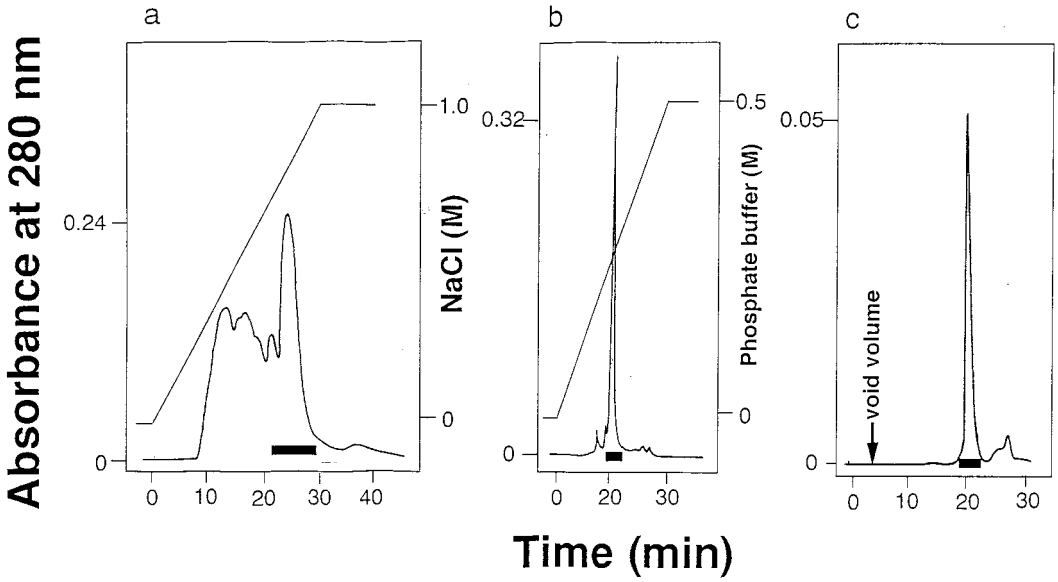

FIG. 1. Purification of staphylolytic enzyme by blue dye ligand affinity HPLC, hydroxyapatite HPLC, and gel permeation HPLC. (a) A concentrated culture filtrate was eluted through a blue dye ligand affinity column (TSKgel Blue-5PW run at room temperature at 60 ml/h). Solvent A was 0.01 M phosphate buffer (pH 7.0), solvent B was 0.01 M phosphate buffer containing 1 M NaCl (pH 7.0), and the gradient was 0 to 100% solvent B over 30 min starting at time zero. (b) Active fractions from TSKgel Blue-5PW were pooled and eluted through a hydroxyapatite column (TSKgel HA1000 run at room temperature at 60 ml/h). Solvent A was 0.01 M phosphate buffer (pH 7.0), solvent B was 0.5 M phosphate buffer (pH 7.0), and the gradient was 0 to 100% solvent B in 30 min starting at time zero. (c) Active fractions from the TSKgel HA1000 were loaded onto a gel permeation HPLC column (TSKgel G3000 SW_{XL} run at room temperature at 30 ml/h). The solvent was 0.1 M phosphate buffer containing 0.1 M sodium sulfate (pH 7.0). Horizontal bar for all panels, pooled fraction with staphylolytic activity.

staining showed the estimated molecular mass as 48,000 (Fig. 2a). The enzyme was most likely a single polypeptide, since it migrated in the SDS gel with the same mobility in the presence or absence of a reducing agent. Isoelectric focusing by agarose gel electrophoresis (pH 3 to 10) suggested that the isoelectric point of the enzyme was 9.0. Amino-terminal sequence determination of the purified enzyme identified the following 10 residues: STKVDAPKVE. Computer-assisted analysis of the deduced sequence failed to identify any homologous sequences among proteins in the DNA and protein databases, indicating that this is a novel protein. We designated this protein ALE-1. Zymography using a polyacrylamide gel containing *S. aureus* revealed a single bacteriolytic band corresponding to ALE-1 (Fig. 2b). The homogeneity of purified ALE-1 was also confirmed by immunoblotting using antiserum against purified ALE-1, and a single peroxidase-positive band had a mobility corresponding to that of the enzyme (Fig. 2c). The antiserum against ALE-1 cross-reacted with lysostaphin (Fig. 2c). Preimmune serum did not react with any protein on the blot at all (not shown). A summary of purification is shown in Table 1. We investigated ALE-1 in the culture supernatants of 10 clinically isolated *S. capitis* strains. ALE-1 was not detected in culture supernatants of any strain by using anti-ALE-1 serum after blotting (data not shown). For further characterizations, ALE-1 was purified from several batches of CCF according to the procedure described above and used.

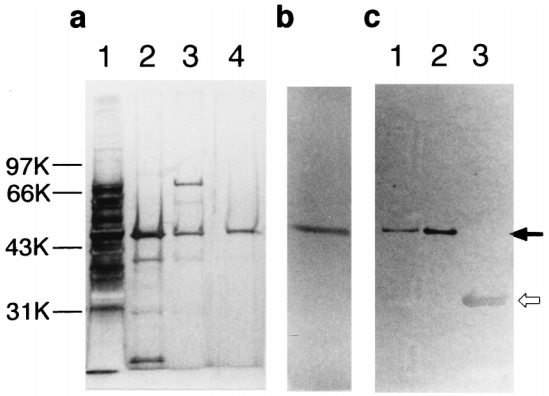

FIG. 2. SDS-PAGE, bacteriolytic profile, and Western blots of the staphylolytic enzyme. (a) Samples at various stages of purification were resolved by SDS-PAGE in a 10% gel and then stained with silver. Lanes: 1, CCF of *S. capitis* EPK1 culture; 2, material eluted from TSKgel Blue-5PW; 3, active fraction eluted from TSKgel HA1000; 4, purified enzyme eluted from TSKgel G3000 SW_{XL}. The gel was stained with silver. M_r values (10^3) of the standard are indicated by arrows. (b) The purified enzyme was resolved on an SDS-polyacrylamide gel (12%) containing *S. aureus* FDA209P, and then bacteriolytic activity was analyzed as described elsewhere (51). (c) Western blotting of CCF, purified enzyme, and lysostaphin was performed as described previously (53). Immunodetection was performed according to the manual supplied with Renaissance 4CN plus (Dupont NEN). Lanes: 1, CCF of *S. capitis* EPK1 culture; 2, purified enzyme (0.9 μg of protein); 3, lysostaphin (5 μg of protein).

Mode of staphylolytic action. The lytic activity of ALE-1 was assessed with *S. aureus* FDA209P or *S. capitis* EPK1 (Fig. 3). ALE-1 lysed viable and heat-killed *S. aureus* cells dose dependently. On the other hand, ALE-1 showed little lytic activity

TABLE 1. Summary of purification

Sample	Protein (μg)	Sp act (U/μg)[a]	Purification (fold)	Yield (%)
CCF	153,120	0.001	1	100
Blue-5PW	496	0.25	250	75.1
HA1000	172	0.32	320	32.8
G3000SW_{XL}	156	0.35	350	32.7

[a] Lytic activity was assayed by following the rate of decrease in the turbidity of the heat-killed *S. aureus* cell suspension as described in Materials and Methods. One unit of enzyme was defined as the amount of protein that decreased the A_{660} from 0.25 to 0.125 in 60 min.

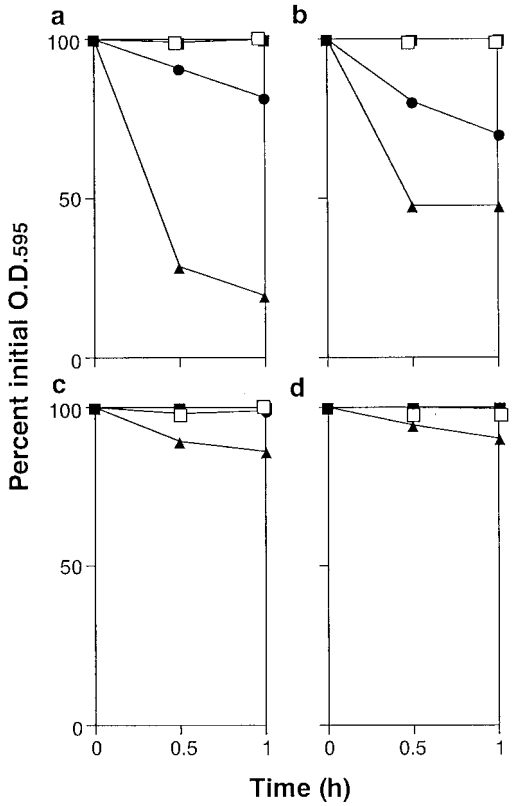

FIG. 3. Bacteriolytic activities of purified staphylolytic enzyme determined by turbidimetry. Purified enzyme was incubated with a 2-ml cell suspension of viable (a) or heat-killed (b) *S. aureus* FDA209P or viable (c) or heat-killed (d) *S. capitis* EPK1 in 0.1 M Tris-HCl (pH 8.5). *S. capitis* EPK1 cells were prepared from a culture in logarithmic growth phase. The decrease in turbidity was measured at 595 nm in a spectrophotometer. Symbols: □, control; ■, 1 μg/ml; ●, 10 μg/ml; ▲, 100 μg/ml.

toward either heat-killed or viable *S. capitis* EPK1. Similar results were obtained with lysostaphin (not shown). The appearance of new free amino groups and reducing sugars during enzymatic hydrolysis of the *S. aureus* cell wall by ALE-1 was monitored. An increase in the concentration of amino groups suggested that ALE-1 is an endopeptidase (Fig. 4). To determine the N-terminal amino acids generated during lytic action, the supernatant of the reaction mixture was incubated with 2,4-dinitrofluorobenzene and the incubation was followed by hydrolysis with 4 N HCl. Thin-layer chromatography revealed DNP-glycine (data not shown). Hydrazinolysis of the reaction mixture followed by 2,4-dinitrophenylation also resulted in the appearance of DNP-glycine by thin-layer chromatography (data not shown). These results indicated that the ALE-1 is a glycylglycine endopeptidase.

Zinc content in ALE-1. Lysostaphin has been reported as a zinc enzyme containing one zinc atom per molecule (60). Because of the antigenetical and functional similarities of ALE-1 to lysostaphin, we determined whether ALE-1 contains a zinc atom. Analysis with a Zeeman-effect atomic absorption spectrometer revealed that the calculated zinc content of ALE-1 was 0.94 mol/mol of protein (M_r of ALE-1 is estimated as 35,600 from molecular cloning data).

Characterization of staphylolytic activities. The pH optimum of the ALE-1 was assessed under standard lytic assay conditions with buffers ranging from pH 4.74 to 10.5. The optimal pH was between 7 and 9, which was distinct from that of lysostaphin. We studied the effect of various modulators on the ALE-1 activity (Table 2) and found that *o*-phenanthroline, iodoacetic acid, and diethylpyrocarbonate inhibited the activity. Others, including serine protease and thiol protease inhibitors, were inactive. Similar results were obtained with lysostaphin. At concentrations of 10 mM, Cu^{2+} completely inhibited the activity of ALE-1 and partially inhibited (39%) the activity of lysostaphin. On the other hand, Fe^{2+} inhibited lysostaphin but not ALE-1 activity at a concentration of 10 mM. The effect of Zn^{2+} over 5 mM was not measurable due to precipitation. Zn^{2+} at 1 mM had little effect on the staphylolytic activities of ALE-1 and lysostaphin. Na^+ and NH_4^+ stimulated ALE-1 activity but had no effect on lysostaphin. We investigated whether β-casein, elastin, and pentaglycine could be substrates for ALE-1, since these substrates are efficiently hydrolyzed by the staphylolytic proteases produced by *Pseudomonas aeruginosa* (28, 40). Up to 65 μg of ALE-1 or lysostaphin per ml did not show a decrease in band intensity of β-casein nor the appearance of any minor bands resulting from the hydrolysis of β-casein. Elastin is very rich in Gly (one-third of the protein), so we tested the elastolytic activity of the ALE-1 or lysostaphin using elastin-Congo red. Up to a concentration of 200 μg/ml, ALE-1 or lysostaphin did not increase the Congo red concentration in the supernatant of the reaction mixture over 2 h. A ninhydrin-positive spot was found at the position of pentaglycine, and a trace amount was located at the positions of triglycine and tetraglycine even after a 5-h incubation with 1.5 μg of enzyme, indicating that neither ALE-1

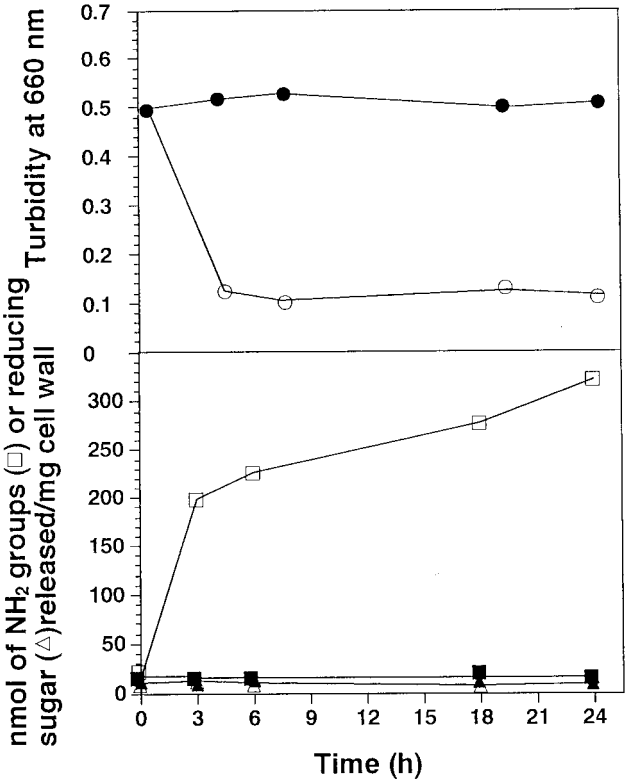

FIG. 4. Time course of digestion of *S. aureus* FDA209P cell wall with purified ALE-1. The purified cell wall of *S. aureus* (5 mg/ml) was suspended in 0.1 M phosphate buffer (pH 7.0) and incubated at 37°C with 190 μg of purified ALE-1 in a total volume of 3.5 ml. Samples were removed at intervals to measure turbidity (○ and ●), the amounts of free amino groups (□ and ■), and reducing sugars (△ and ▲). Solid symbols represent the control (no enzyme).

TABLE 2. Effects of various modulators on the staphylolytic activities of ALE-1 and lysostaphin[a]

Modulator	Concn (mM)	Remaining staphylolytic activity (%)	
		ALE-1	Lysostaphin
None		100	100
1,10-Phenanthroline	10	6.3	17.2
EDTA	10	94.2	100.9
Phosphoramidon	0.02	117	105
PMSF	1	99.1	103.4
Benzamidine	12.8	91	90.3
Dithiothreitol	10	81.2	96.6
Iodoacetic acid	0.1	0	2.8
Diethylpyrocarbonate	5	4.5	0
Mg^{2+}	10	93.3	87
Mn^{2+}	10	110.8	77
Cu^{2+}	10	0	38.8
Fe^{2+}	10	113.3	8.2
Na^{+}	10	150.8	108.4
NH_4^{+}	10	153.3	103.3
Ca^{2+}	10	85.6	82.4

[a] The effects of several modulators on the staphylolytic activities were determined after incubation with 1 U of purified enzyme for 10 min at room temperature, followed by addition of the heat-killed *S. aureus* cells as described in Materials and Methods. The mixture was incubated at 37°C for 1 h, and the rate of decrease in turbidity was measured at 660 nm with spectrophotometer. Results were presented as the relative percent remaining staphylolytic activity.

nor lysostaphin significantly hydrolyzed pentaglycine under these experimental conditions.

Cloning of the ALE-1 gene. After 2-day incubation, six colonies among 800 transformants were found to form a halo around the colonies. Cell extracts of these six clones were assayed for staphylolytic activity by zymography. All extracts of the clones revealed a staphylolytic band, which was slightly larger than ALE-1, in the gel containing *S. aureus* FDA209P. Furthermore, Western blotting analysis demonstrated that all bands reacted with antiserum raised against ALE-1. The recombinant plasmids of these clones contained DNA inserts of 2 kbp in common. One of the recombinant plasmids was designated pTF1 (Fig. 5). To determine the minimum amount of DNA required for the staphylolytic activity, a deletion series of the *Hin*dIII fragment was constructed. Derivatives were cloned into pUC19. Bacteria carrying pTF11, pTF12, and pTF15 expressed staphylolytic bands (Fig. 5). On the other hand, those carrying pTF13, pTF14, or pTF16 did not show any staphylolytic bands (Fig. 5). Fragments expressing staphylolytic activities were sequenced by using either the universal or the reverse sequencing primer. The nucleotide sequencing revealed one potential open reading frame (ORF), which was devoid of the amino terminus of the ORF. Therefore the pTF1 DNA was used as a probe to screen restriction digests of chromosomal DNA from *S. capitis* EPK1, and a 6-kbp *Eco*RI fragment was identified. The 6-kbp *Eco*RI fragment was cloned into the *Eco*RI site of pUC19 to generate pTF2. By probing *Hin*cII digests of the 6-kbp *Eco*RI fragment with pTF1 DNA, a 3.5-kbp *Hin*cII fragment was identified and cloned into pUC19 to generate pTF3. Both pTF2 and pTF3 clones expressed staphylolytic bands which revealed electrophoretic mobility similar to that of ALE-1 (not shown). The *Exo*III deletions of the *Hin*cII fragment of pTF3 were carried out in both directions. Expression analysis together with restriction mapping suggested that the 3.5-kbp fragment contained the entire ORF (Fig. 5). Six subclones were used for sequencing of the flanking region of *Hin*dIII fragment.

Nucleotide and deduced amino acid sequence of the ALE-1. The location of the ORF is shown in Fig. 5, and the nucleotide sequence for 1,540 bp of DNA including the entire ORF together with the deduced amino acid sequence is shown in Fig. 6. A Shine-Dalgarno sequence, AGGAGGT, which is similar to those of *S. aureus* showing canonical homology with 16S RNA of *B. subtilis* (37), is observed 9 nucleotide upstream of the putative start codon. A possible candidate for promoter sequence is present upstream of the ORF. The sequences TTGATA (positions 160 to 165) and TACATA (positions 183 to 188) might in *E. coli* function as a −35 and a −10 promoter region, respectively. The termination codon TGA occurs 1,086 bp from the initiation codon. A putative rho-independent terminator that consists of two stem-loop structures is present downstream from the stop codon at bases 1337 to 1379 and 1392 to 1432. The sequenced fragment has a G+C content of 35.1 mol%, which is in agreement with the G+C content determined for *S. capitis* (31 to 36 mol%) (47).

A region of the predicted amino acid sequence, positions 36 to 45, has a perfect match with N-terminal sequence determined from purified ALE-1. The sequence starting from the ATG codon shows the presence of an NH_2-terminal extension of 35 amino acid residues prior to the determined NH_2 terminus of ALE-1, which is assumed to be a signal sequence since ALE-1 is an extracellularly secreted protein of *S. capitis* EPK1. The extended sequence shown here has characteristics of a

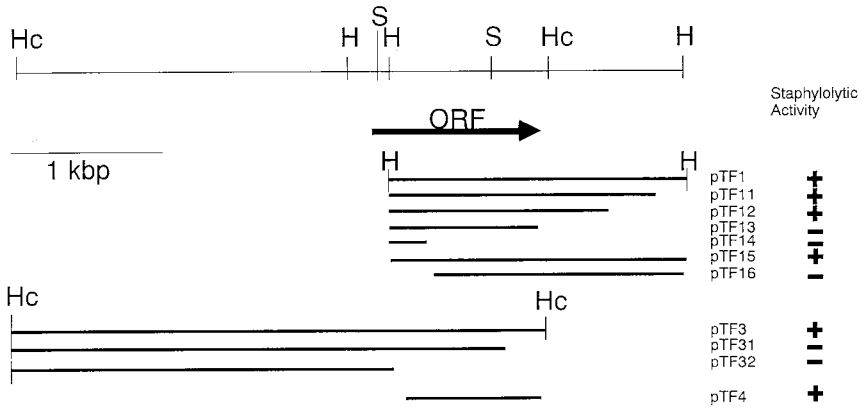

FIG. 5. Genetic organization of the *ale-1* gene and phenotypes of recombinant plasmids. Thick arrow shows the ORF and the direction of transcription. H, *Hin*dIII; Hc, *Hin*cII; S, *Sau*3A1. Extracts of *E. coli* XL1Blue cells carrying each plasmid were examined for staphylolytic activity by zymography: +, lytic band; −, no lytic band.

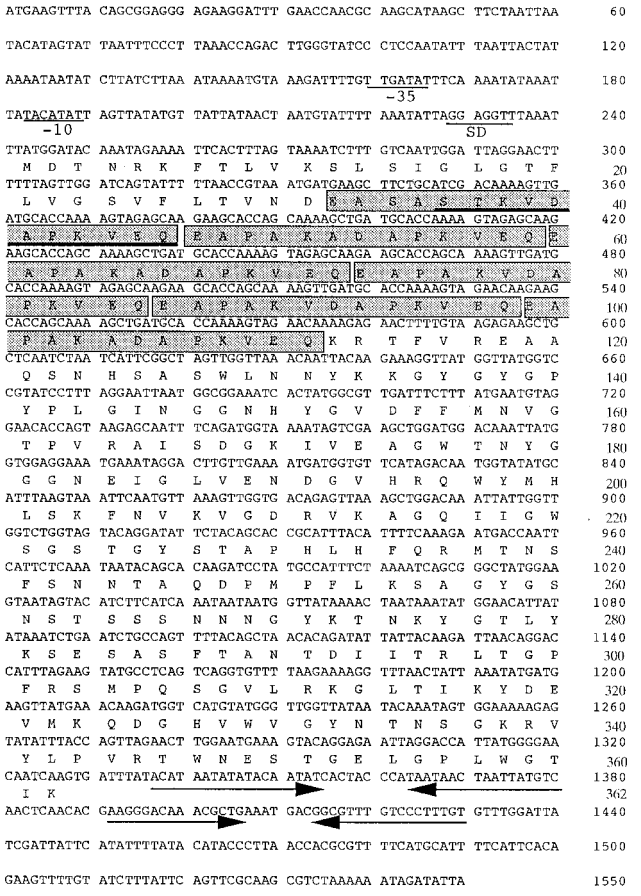

FIG. 6. Nucleotide sequence and deduced amino acid sequence of the *S. capitis* EPK1 *ale-1* gene. A possible candidate for promoter sequences (−35 and −10 regions) and the putative ribosome binding site (SD; Shine-Dalgarno sequence) are shown. Inverted repeat sequences are indicated by arrows. The six repeated amino acid sequences are boxed and shaded. The thick underline corresponds to amino acid sequence deduced from analysis of purified ALE-1 with protein sequencer.

typical bacterial signal sequence; it contained three positively charged amino acids within its NH_2-terminal region followed by a hydrophobic region, and the putative signal cleavage site predicted by the method of Heijne (19) corresponded to the determined NH_2 terminus of purified ALE-1. *ale-1* encodes a protein of 362 amino acids with a deduced molecular weight of 39,306. Cleavage of the putative signal sequence would result in a protein with a molecular mass of 35,596 Da. The entire protein has a predicted isoelectric point (pI) of 9.61, and the protein with its signal sequence cleaved has a predicted pI of 9.62, a value that was similar to that obtained by isoelectric focusing of purified ALE-1.

In the N-terminal part of ALE-1, six tandemly repeating sequences beginning at position 32 and ending at position 111 are present. Each repeat consists of 13 amino acids. The amino acid sequence was perfectly conserved from repeats 2 to 6, and the first repeat revealed a 5-amino-acid substitution from the beginning.

Protein homology search revealed that ALE-1 has a striking homology with lysostaphin, glycylglycine endopeptidase produced by *S. simulans* bv. *staphylolyticus* (45, 46), and identity of 50% was found between overall sequences (Fig. 7). These enzymes have a similar modular design. The overall identity of the multiple-repeat domain in ALE-1 (Glu-32 to Gln-111) and lysostaphin (Glu-33 to Thr-222) is 23% at the amino acid level. On the other hand, the identity of the putative functional domain corresponding to positions 199 to 362 in ALE-1 is 83% at the amino acid level. Lysostaphin was shown to undergo processing of the N-terminal repeat sequence, and the mature form retains staphylolytic activity. A DNA fragment corresponding to ALE-1 with a truncated N-terminal repeat sequence starting from Ala-119 which corresponds to the N-terminal amino acid of mature lysostaphin (Ala-119 to Lys-362) was constructed by PCR and then cloned into pUC19 to generate pTF4. Extracts of *E. coli* carrying pTF4 revealed a staphylolytic band with a low molecular mass (not shown).

Significant amino acid similarity to the region from positions 198 to 233 was also observed with *Lysobacter enzymogenes* β-lytic metalloendopeptidase (66), *Achromobacter lyticus* β-lytic protease (32), *P. aeruginosa* LasA (28, 48), *E. coli* OrfU (26), H10409 of *Haemophilus influenzae* (13), *Vibrio cholerae* TagE (29), LppB of *H. somnus* (58), NlpD of *H. influenzae* (13), *P. aeruginosa* (57) *Salmonella typhimurium* (42), *Yersinia enterocolitica* (23), *E. coli* (22, 30), *P. putida*, and *Synecococcus elongatus* Orf1 (Fig. 8). Among the amino acid sequences, Gly-160, Tyr-198, His-200, Val-212, Gly-225, and His-233 in ALE-1 were conserved in all 16 protein sequences. A region of considerable similarity was also found at the C-terminal amino acid sequence with *S. aureus* amidase (63) (Fig. 8).

Lysostaphin is synthesized and secreted as a 42-kDa proenzyme, which is later processed to form a mature 25-kDa enzyme in culture supernatant (20, 41). This processing mainly occurs during the stationary-growth phase (41). Therefore we performed zymography of the staphylolytic enzyme in culture supernatant of *S. capitis* EPK1 at different growth stages by zymography and immunoblotting with anti-ALE-1 serum. A single staphylolytic band corresponding to ALE-1 was observed in all culture supernatants tested at every growth stage (not shown). This was further confirmed by immunoblotting, in which a single peroxidase-positive band corresponded to ALE-1 was observed at all growth stages (not shown). These results indicated that ALE-1 does not undergo further proteolytic processing in broth culture.

ALE-1 is plasmid encoded. A lysostaphin gene has been located on a large plasmid which carries the β-lactamase gene hybridizing to a fragment of *S. aureus* pI258 (41). We therefore tested whether *ale-1* is encoded on a plasmid. Southern blot analysis using the *Hin*dIII fragment of pTF1 as probe demonstrated that the gene was present on a large plasmid (not shown). Treatment of *S. capitis* EPK1 with ethidium bromide at a high temperature resulted in loss of the production of ALE-1 in some clones. The loss of the production correlated with the disappearance of the plasmid DNA band hybridizing with the *Hin*dIII fragment of pTF1 (not shown). This indicated that *ale-1* is encoded on the plasmid and that the gene is not essential for the growth of the bacteria.

DISCUSSION

In this article, we describe the purification and molecular characterization of the glycylglycine endopeptidase, ALE-1, produced by *S. capitis* EPK1. Both ALE-1 and lysostaphin revealed staphylolytic activity, as they hydrolyzed the internal glycylglycine sites of *S. aureus* peptidoglycan. However, β-casein, elastin, and pentaglycine were poor substrates for both enzymes when compared with these substrates for the pseudomonal staphylolytic enzymes LasA and LasD, which have wider substrate specificities, including pentaglycine and β-casein (28, 40). Park et al. have demonstrated that lysostaphin is elastolytic, but the activity levels are low with relatively slow kinetics

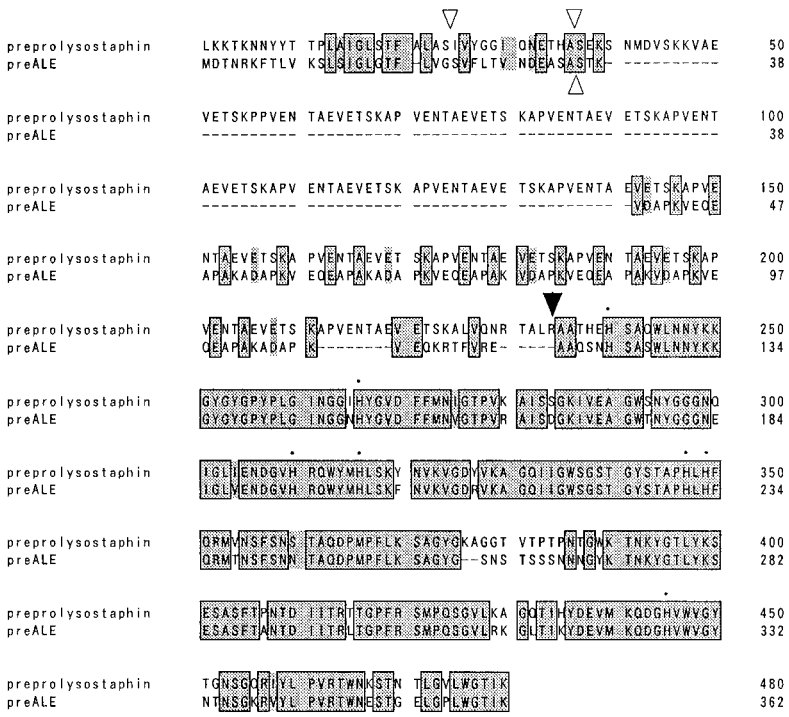

FIG. 7. Comparison of the deduced amino acid sequences of the ALE-1 described here and the lysostaphin produced by *S. simulans* bv. *staphylolyticus*. Since the amino acid sequences deposited as lysostaphin by Recsei et al. (41) and by Heinrich et al. (20) are essentially the same, except for the number of N-terminal 13-amino-acid repeats, although both groups used the same strain, we tentatively used the sequence (41) for comparison. Identical residues are boxed and shaded. Broken lines represent breaks introduced to maximize homology. ▽, proposed signal peptide cleavage sites of preprolysostaphin (41); △, signal peptide cleavage site of ALE-1; ▼, cleavage site for processing of prolysostaphin; ●, conserved His residues.

(39). Our data support their findings. ALE-1 and lysostaphin may have a narrow substrate specificity and require a peptidoglycan structure for enzyme action.

The ALE-1 gene from *S. capitis* EPK1 encodes a proenzyme with an M_r of 39,306 with an N-terminal signal sequence followed by six tandem repeats of a 13 amino acid sequence. Inconsistency between the molecular mass of mature ALE-1 calculated from the deduced amino acid sequence (35.6 kDa) and that based on SDS-PAGE analysis (48 kDa) may be due to the tandem repeats with many N-terminal glutamic acid residues, which result in lower rates of binding of SDS to the protein as suggested for slower electrophoretic mobility of the proenzyme form of lysostaphin (41). In support of this, ALE-1 with a truncated N-terminal repeat domain electrophoresed as a 28-kDa protein in SDS-PAGE, which is comparable to its calculated molecular mass (26.7 kDa) from its deduced amino acid sequence (data not shown).

ALE-1 and the proenzyme form of lysostaphin have very similar primary structures (Fig. 7). Each consists of a multiple-repeat N-terminal domain fused to an active site containing a C-terminal domain. Unlike lysostaphin, ALE-1 does not undergo further processing of the multiple-repeat N-terminal domain for an unknown reason. Although the numbers of N-terminal repeats are different (six repeats in ALE-1 and 14 [20] or 7 [41] repeats in lysostaphin), both repeats are composed of 13 amino acids as a unit which contains charged and nonpolar amino acids with a potential α-helix structure. Two-dimensional representations of the α-helical structure of repeats in ALE-1 and prolysostaphin revealed similar periodic clusters of charged amino acids as shown in Fig. 9. Multiple-repeat domains in peptidoglycan hydrolases have been suggested to be involved in binding to ligands in the cell wall (3, 14, 38, 67). We observed that some ALE-1 is associated with the cell surface of the producing strain and some is extracted with 3 M LiCl treatment, suggesting its ionic interaction with the cell surface (not shown). By analogy, the multiple-repeat N-terminal domains of ALE-1 and lysostaphin may also be involved in binding to cell surface ligands, and periodic clusters of charged amino acids in the putative α-helical structure may be important for the association of the enzymes with ligands.

The inhibition of staphylolytic activities of both ALE-1 and lysostaphin with diethylpyrocarbonate or iodoacetic acid suggested that a histidine residue(s) is important for the staphylolytic activity of ALE-1 as well as lysostaphin (39). Comparison of the amino acid sequence with lysostaphin revealed that seven His residues at positions 124, 150, 194, 200, 231, 233, and 327 are conserved. His at positions 124, 194, 200, 231 and 233 is present among the proposed catalytic domain of lysostaphin (63) and moreover, His at positions 200 and 233 is thoroughly conserved among 16 proteins sharing a homologous 38-amino-acid region in the domain (Fig. 8). Histidine residues in various proteins are ligands to zinc and are important for enzyme catalytic activity (9, 21, 61). Among the proteins with a homologous 38-amino-acid region, ALE-1, lysostaphin (24, 62), and LasA (28) are endopeptidase and exhibited staphylolytic activity. Lysotaphin has been reported to contain one zinc atom in the molecule (60). Atomic absorption spectrometry revealed that ALE-1 contains one zinc atom per molecule. LasA is suggested to be a metalloenzyme (40). β-Lytic metalloprotease produced by *L. enzymogenes* ATCC29487 and *A. lyticus* revealed lytic activity toward gram-positive bacteria (32). Both enzymes are suggested to be Zn^{2+} enzymes (32). Among 16 proteins with a homologous 38-amino-acid region, seven belong to the NlpD/LppB region of gram-negative bacteria. Al-

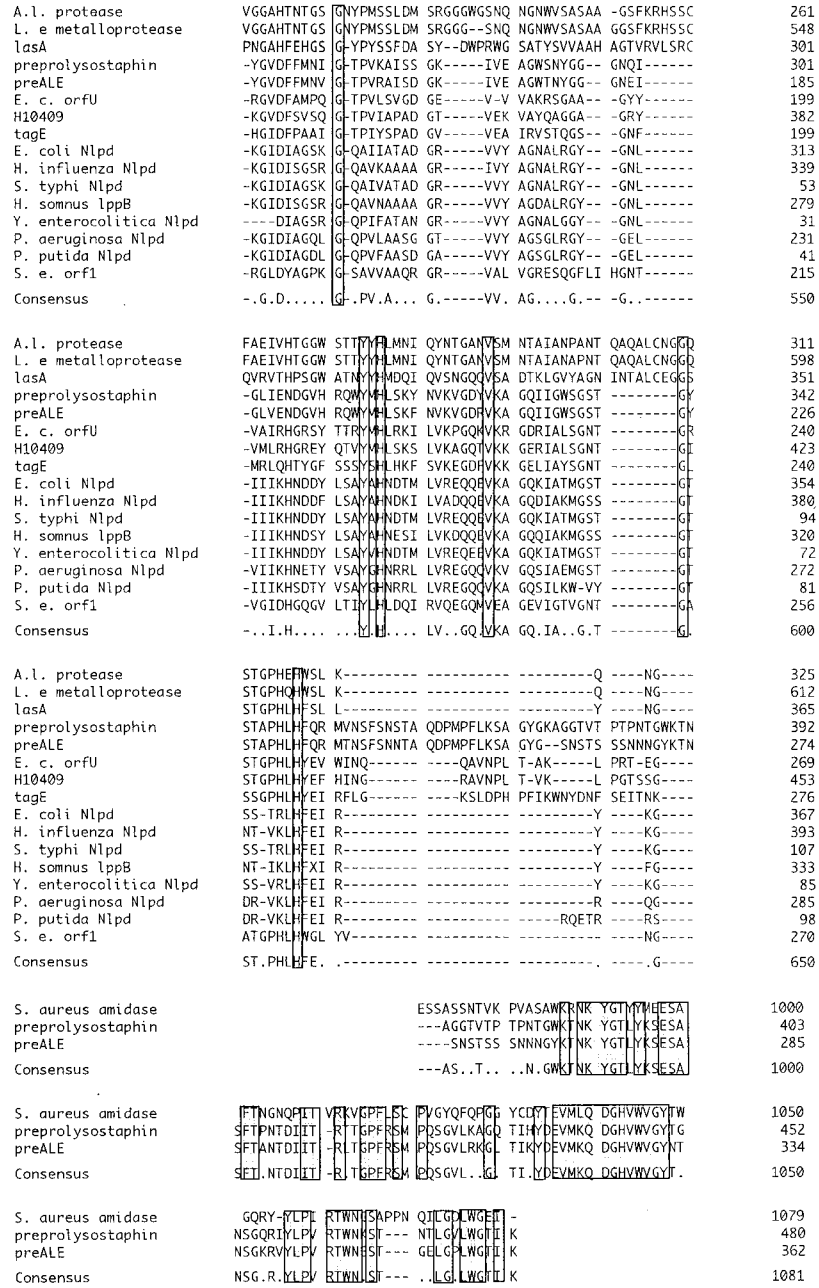

FIG. 8. Amino acid sequence alignment of the portion of ALE-1 with the other proteins with significant similarity. In the alignment, the numbers after the protein sequence in each case refer to the protein domain in which that stretch of sequence resides. Conserved amino acids are boxed. Abbreviations (GenBank accession numbers in parentheses): A. 1. protease, β-lytic metalloprotease from *A. lyticus* (P27458); L. e. metalloprotease, β-lytic metalloprotease from *L. enzymogenes* (P00801); lasA, LasA from *P. aeruginosa* (A33661); preprolysostaphin, lysostaphin precursor (X06121, M15686); H10409, *H. influenzae* hypothetical protein (P44693); E.c. orfU, *E. coli orfU* (P24204); tagE, *V. cholerae* ToxR-activated gene, *tagE* (JC2569); Nlpd of *E. coli* (P33648), *H. influenzae* (P44833), *S. typhi* (X81641), *H. somnus* (P36685), *Y. enterocolitica* (U16152), *P. aeruginosa* (P45682), and *P. putida* (X91546); S. e. orf1, *S. elongatus* orf1 (D13173), *S. aureus* amidase, *S. aureus N*-acetylmuramyl-L-alanine amidase encoded in *lytA* (P24556).

though the physiological function of these NlpD/LppB is unclear, the fact that overproduction of NlpD in *E. coli* resulted in the formation of bulges and eventual cell lysis suggests that NlpD may have some cell wall lytic function (30). Taken together, these results suggest that the proteins with the conserved motif of Tyr-X-His-X_{11}-Val-$X_{12/20}$-Gly-$X_{5\text{-}6}$-His may conform to a novel bacterial Zn^{2+} protease family.

Li et al. pointed out that His-Glu-His at positions 122 to 124 in β-lytic protease of *A. lyticus*, which corresponds to positions 231 to 233 in ALE-1, is similar to the His-X-His sequence serving as the Zn^{2+} ligand in carbonic anhydrase and suggested that the sequences serve as possible ligands to Zn^{2+} in β-lytic protease of *A. lyticus* (32). Indeed, His-234 in ALE-1 is conserved in 16 proteins, but His-231 is not (Fig. 9a). Recently, Gustin et al. have shown that a LasA with a substitution of His-354 to Ala-354 lost staphylolytic activity (16). On the other hand, Park et al. speculated that the N-terminal Ala-Ala-Thr-His-Glu sequence of lysostaphin is involved in the Zn^{2+} bind-

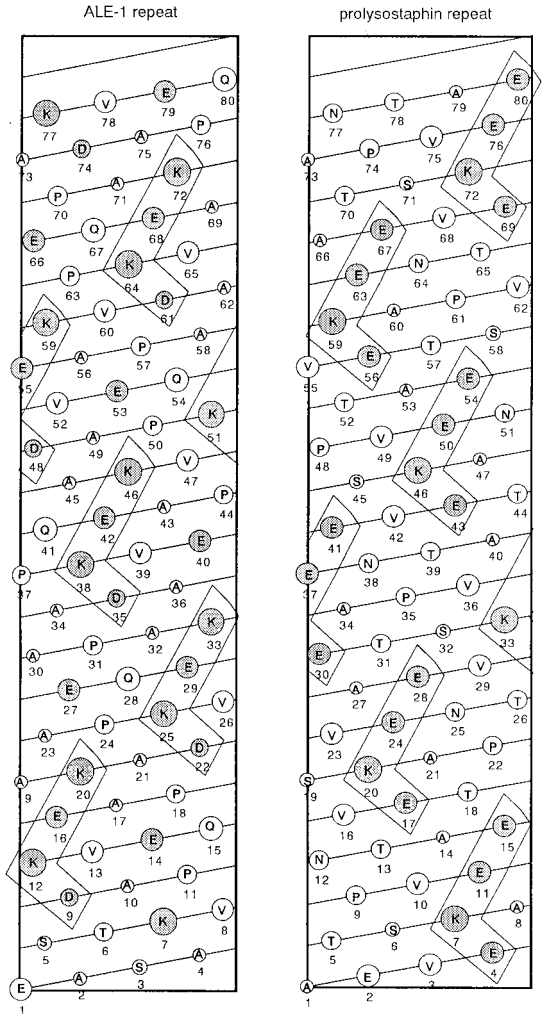

FIG. 9. Two-dimensional representations of α-helical structure of repeats in ALE-1 and prolysostaphin. Eighty residues of amino acid repeats of ALE-1 (32 to 111) and prolysostaphin (40 to 130) were lined up as two-dimensional representations of α-helix along the long axis. The diagonal line connecting the left and right sides represents a 360° turn of the α-helix (100° per residue). The relative amino acid scale was displayed according to hydrophobicity (34). Charged amino acids are shaded, and clusters of charged amino acids are boxed.

ing domain based on the homology with a catalytic domain of mammalian matrix metalloproteinases, with His and Glu critical to the enzyme activity (39). However, this sequence is not well conserved in ALE-1, as shown in alignment results (Fig. 8). Site-directed mutagenesis of His residues in ALE-1 and studies of the variant enzyme activity might help us to understand which His is important for the enzyme activity as well as the ligand to a Zn^{2+}.

The purified ALE-1 revealed little lytic activity toward *S. capitis* EPK1, the strain which produced it. Moreover, active ALE-1 was recovered from the cell surface of *S. capitis* EPK1. This is reasonable, since otherwise the strain would self-destruct. Heath et al. have demonstrated the presence of a genetic element that confers lysostaphin resistance to *S. simulans* bv. *staphylolyticus* peptidoglycan (18). The presence of the gene for endopeptidase resistance (*epr*) causes increased resistance to lysostaphin by decreasing the glycine-to-serine ratio in the peptidoglycan cross bridges (11). On the other hand, cell wall teichoic acid and lipoteichoic acid are implicated as endogenous regulators of peptidoglycan hydrolase activity (12). These factors may also contribute to the insensitivity of *S. capitis* EPK1 to ALE-1. How *S. capitis* EPK1 escapes from the staphylolytic action of ALE-1 remains to be elucidated.

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