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Molecular Diversity of a Putative Virulence Factor: Purification and Characterization of Isoforms of an Extracellular Serine Glutamyl Endopeptidase of *Enterococcus faecalis* with Different Enzymatic Activities

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A previously identified gene *sprE* of *Enterococcus faecalis* strain OG1 was shown to encode an extracellular serine protease that appears to belong to the glutamyl endopeptidase I staphylococcal group. A single form of SprE with a molecular mass of 25 kDa and a pH optimum between 7.0 and 7.5 was isolated from culture supernatant of wild-type *E. faecalis* strain OG1RF (TX4002); this form was apparently generated by cleavage of the Ser^{-1}-Leu^{1} and Arg^{230}-Leu^{231} peptide bonds of the secreted zymogen. In contrast, the culture supernatant of the gelatinase-null mutant, TX5264, with a nonpolar deletion of *gelE* which encodes the *E. faecalis* gelatinase, was found to contain several forms of SprE proteolytically processed on both the N and C termini; in addition to a full-length zymogen and a truncated zymogen, three mature forms of the SprE proteinase, Leu^{1}-Ala^{237}, Ser^{-1}-Glu^{227}, and Leu^{1}-Glu^{227}, were identified. As with the V8 proteinase of *Staphylococcus aureus*, the closest homologue of SprE, all of the active forms cleaved specifically Glu-Xaa peptide bonds but with substantially different efficiencies, while none was able to hydrolyze peptide bonds with Asp in the P1 position. The most active of all these enzyme forms against several substrates, including human fibrinogen and β-chain insulin, was the Ser^{-1}-Glu^{227} ($^{-1}$S-SprE) isolated from TX5264; $^{-1}$S-SprE, in contrast to other forms of SprE, was unstable at 37°C, apparently due to autodegradation. In conclusion, our results demonstrate that *sprE* encodes a highly specific serine-type glutamyl endopeptidase, the maturation of which is dependent on the presence of gelatinase. In the absence of gelatinase activity, the aberrant processing of pro-SprE results in the appearance of a "superactive" form of the enzyme, $^{-1}$S-SprE.

Enterococci, often viewed primarily as human commensals and even used as probiotics, have become problematic nosocomial pathogens, at least in part because of their increasing resistance to many antibiotics and their ability to infect the growing pool of severely debilitated and/or immunocompromised patients who undergo prolonged antibiotic therapy (27, 37–39). Several groups have recently undertaken a search for enteroccocal virulence factors in an effort to devise new solutions to the problems caused by these bacteria (20, 25). Included among these may be enterococcal proteinases, as enzymes of this class have been previously suggested to be important virulence factors for other bacterial pathogens. Examples include the V8 proteinase of *Staphylococcus aureus* involved in septicemia (2, 14, 44) and its homologue GluSE from *S. epidermidis*, found to be important for slime production and, consequently, biofilm formation by this bacterium in vitro (36, 43). Furthermore, the cysteine endopeptidase SpeB of *Streptococcus pyogenes* (8, 9, 16, 29–31) and proteases of *Porphyromonas gingivalis* (3, 4, 24, 42, 45), *Yersinia* spp. (22, 28, 54–56), and *Pseudomonas aeruginosa* (10, 17, 23) have all been implicated as virulence factors.

Enterococcus faecalis has long been known to produce gelatinase (coccolinase; EC 3.4.24.30) (GelE) (1, 21, 32, 38, 51, 58), a 30-kDa extracellular metalloendopeptidase encoded by the *gelE* gene (58). Downstream from *gelE*, an open reading frame called *sprE*, coding for a putative serine protease (GenBank accession No Z12296), was identified (57). While gelatinase activity and the *gelE* gene have been utilized in a number of studies, including epidemiological ones (11, 18, 26, 35, 61–63), and in animal models of infection (15, 53), suggesting a possible role in microbial virulence and host response (33), until recently, little has been done to investigate *sprE* and the possible role of the predicted SprE protein or the presence of any other proteolytic activities in *E. faecalis*. Qin et al. described the *fsr* locus, a regulatory system of *E. faecalis* (41, 47, 48) that is homologous to the *S. aureus agr* locus (49) that encodes a quorum sensing system regulating cotranscription of *gelE* and *sprE* from the common promoter (47, 48). The deduced amino acid sequence of SprE shows a high degree of similarity to those of staphylococcal glutamyl endopeptidases, including V8 (49% similarity, 27% identity) (66) and GluSE (49% similarity, 26% identity) (43), but this predicted enzyme has not been purified or characterized.

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An array of *E. faecalis* OG1RF disruption and deletion mutants in the *fsr* and *gelE* loci has been previously made, and their proteolytic activity and virulence phenotypes have been tested in zymography (48) and animal infection models, respectively. Strains disrupted in *fsr* and a polar mutant of *gelE*, which produce neither GelE nor SprE, were considerably attenuated in a mouse peritonitis model (48, 53). Also, an isogenic mutant of *sprE* which, in comparison to the parental strain, is deficient in caseinolytic activity, was significantly less virulent in the same model (53). Finally, the pathogenic potentials of a nonpolar *gelE* deletion mutant (GelE $^{-}$SprE^{+}), an isogenic *sprE* knockout, and a *gelE sprE* double mutant (46, 52) were compared using a model of *Caenorhabditis elegans* killing (19). In this model, the first two mutant strains were each attenuated to the same degree, and this attenuation was significantly less profound than in the case of the mutant lacking both enzymes (52).

These studies, as well as the similarity of SprE to V8 of *S. aureus*, suggested that *sprE* might code for a extracellular glutamic acid-specific serine endopeptidase that may possibly be engaged in pathogenic processes related to *E. faecalis* infections. The aim of this study was to characterize the activity of the enzyme predicted by the *sprE* gene.

MATERIALS AND METHODS

Bacterial strains. *E. faecalis* OG1RF (TX4002) is a well-characterized plasmid-free, GelE- and SprE-producing strain (40, 48), and *E. faecalis* TX5264 is its isogenic mutant, with an in-frame deletion of the *gelE* gene that preserves *sprE* expression under the control of the *fsr* system and *gelE* promoter (46, 52). *E. faecalis* TX5243, an isogenic mutant of OG1RF with a disruption in *sprE*, and *E. faecalis* TX5128, with an insertion disrupting *gelE* (thou producing none of the proteinases), were used as SprE-negative controls in the initial characterization of proteolytic activity (47, 48).

Reagents. All reagents used in procedures described below were purchased from Sigma (Sigma Chemical Company, St. Louis, Mo.), unless otherwise indicated, and were of at least analytical grade.

Bacterial cultivation. The logarithmic starter culture with cell density corresponding to an optical density at 600 nm of 0.6 to 0.7 in brain heart infusion broth (Becton Dickinson, Franklin Lakes, N.J.) was diluted 1:20 into Todd-Hewitt broth (Becton Dickinson) and cultured overnight with vigorous shaking at 37°C. Usually 10 liters of the culture was used.

Proteinase purification. All purification steps were performed at 4°C except for the fast-protein liquid chromatography (FPLC), which was run in room temperature. All FPLC columns were from Amersham Biosciences, Piscataway, N.J. Bacteria were harvested at the late stationary phase by centrifugation, and the cell-free culture fluid was collected. Proteins from the supernatant were precipitated with cold acetone (Fisher Scientific, Hampton, N.H.) (technical grade) at a 60% final concentration at −20°C (dry ice-ice bath), collected by centrifugation (6,000 × *g*, 30 min, −4°C), redissolved in 0.2 M Tris-HCl (Fisher Scientific)–5 mM $CaCl_2$ (usually about 100 ml), and extensively dialyzed overnight (4°C) with 50 mM Tris-HCl–5 mM $CaCl_2$ (pH 7.6) (buffer A) and with a 10-kDa molecular mass cutoff membrane (Millipore, Billerica, Mass.), with three additional changes of buffer. The dialyzed sample was mixed with drained DE-52 cellulose (Whatman, Kent, United Kingdom) equilibrated in the same buffer and incubated for 30 min with occasional stirring, and the chromatography matrix was washed extensively with equilibrium buffer to remove nonbound proteins. A column was formed, and bound proteins were eluted at a flow rate of 120 ml/h with a NaCl gradient (0 to 0.5 M) in buffer A, with fractions (9 ml) analyzed for proteolytic activity against azocasein as described before (6). Active fractions were pooled and concentrated by ultrafiltration (Amicon PM-10 membrane; Millipore), dialyzed against 25 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol (Bis-Tris), 150 mM NaCl, 5 mM $CaCl_2$, 0.02% NaN_3 (pH 6.8) (buffer B), and clarified by centrifugation (28,000 × *g*, 30 min). The resulting sample was applied in 20-ml portions, corresponding to 10 liters of starting supernatant, onto a Sephadex G-150 (Serva, Heidelberg, Germany) column (5.3 by 105 cm) equilibrated with buffer B and run at a flow rate of 30 ml/h. Fractions (9 ml) were analyzed for proteolytic activity and enzyme activity, pooled, concentrated, dialyzed against buffer A, and applied to a Resource Q FPLC column equilibrated with the same buffer. The column was developed at a flow rate of 5 ml/min, first with equilibration buffer and then with 100 ml of a 0 to 600 mM NaCl linear gradient in buffer A. Again, proteolytically active fractions (5 ml) were pooled and concentrated, dialyzed against buffer A, and applied at a flow rate of 1 ml/min onto a MonoQ HR 5/5 FPLC column equilibrated with the same buffer. Bound proteins were eluted with 50 ml of a 0 to 500 mM NaCl linear gradient in buffer A, fractions (0.5 ml) were analyzed for proteolytic activity, and distinct activity peaks were pooled separately. This step yielded a highly pure preparation of each of the various forms of SprE.

Protein determination, electrophoretic techniques, Western blotting, and N-terminal sequence determination. Protein concentrations were determined using a BCA reagent kit (Pierce, Rockford, Ill.) with bovine serum albumin (BSA) as a standard. The enzyme purification was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% separating gel by use of a Tris-HCl–Tricine {*N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine} buffer system, according to the method of Schagger and von Jagow (50). All SDS-PAGE reagents were from Bio-Rad, Hercules, Calif. To demonstrate that the purified protein was a serine proteinase, 2 μg of each purified SprE form was incubated with biotinylated diisopropyl fluorophosphate (DFP; obtained by courtesy of B. F. Cravatt, The Scripps Research Institute, La Jolla, Calif.) (final concentration, 0.5 μM) in 0.1 M Bis-Tris (pH 7.0)–1 mM $CaCl_2$ for 15 min in 37°C, resolved in SDS-PAGE, and electrotransferred onto a nitrocellulose membrane (Bio-Rad), according to the method of Towbin and coworkers (60). After nonspecific binding sites were saturated with BSA, the protein bands labeled with biotin were detected by reaction with streptavidin-conjugated alkaline phosphatase (Pierce), followed by development using a 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium mixture as a color reagent (Bio-Rad).

For N-terminal sequence analysis, proteins resolved by SDS-PAGE were electroblotted onto polyvinylidene difluoride (Bio-Rad) membranes by use of 10 mM 3-(cyclohexylamino)propanesulphonic acid (CAPS) (pH 11)–10% methanol (34). After staining with Coomassie blue G250, the blot was air dried and protein bands were excised and subjected to amino-terminal sequence analysis by Edman degradation with a model 491 protein sequencer (Applied Biosystems, Foster City, Calif.) by use of a program designed by the manufacturer.

Gelatin-casein zymography. Zymography analysis using gelatin or casein gels was performed at each step of the proteinase purification. Samples were treated with Laemmli SDS-PAGE sample buffer for 30 min at 37°C and subjected to electrophoresis at 4°C on 12% polyacrylamide gels with 10 mg of gelatin or casein (Becton Dickinson)/ml incorporated into the gel. In inhibition studies, samples were pretreated with 10 mM DFP before incubation with sample buffer. Following electrophoresis, the gel was washed twice with 2.5% (wt/vol) Triton X-100 to remove SDS and then incubated in buffer A at 37°C for 2 h. The zymography was developed in 0.1% amido black, with clear zones indicating proteolytic digestion of the incorporated protein.

Molecular mass determination. The molecular mass of the native proteinase was estimated by gel filtration on a Sephadex G-150 column calibrated with protein standards (Bio-Rad) and by SDS-PAGE using low-molecular-weight electrophoresis standards (Amersham Biosciences) as a reference. The precise molecular mass of the SprE forms was determined using matrix-assisted laser desorption ionization (MALDI), with mass spectra acquired by a Vestec MALDI–time-of-flight mass spectrometer (MALDI-TOF; Perspective Biosystems, Hertford, United Kingdom). The analysis was performed at the Mass Spectroscopy Facility core facility (University of Georgia, Athens) according to the manufacturer's instructions.

Enzyme specificity. The proteolytic specificities of different forms of SprE were determined using oxidized insulin β-chain as a substrate. Briefly, 0.1 mg of insulin β-chain was incubated with individual SprE forms at an enzyme/substrate molar ratio of 1:100 for 4 or 16 h in 0.1 mM Bis-Tris (pH 7.0) at 37°C. The reaction was then stopped by freezing samples at −20°C. The samples were analyzed by MALDI-TOF as described above, and the cleavage site(s) of the insulin β-chain were inferred from the molecular masses of proteinase-generated peptides.

Enzyme activity assays and kinetic analysis. All *p*-nitroanilide (*p*Na) peptide substrates were purchased from Bachem, King of Prussia, Pa. Glutamyl endopeptidase activity was routinely measured at different stages of purification with succinyl (Suc)-Ala-Ala-Pro-Glu-*p*NA (0.5 mM) as a substrate in 0.2 M HEPES (pH 7.5) at 37°C. The optimal conditions for substrate hydrolysis (buffer, pH, and ionic strength) were set using purified forms of SprE. On the basis of this analysis, 0.1 M Bis-Tris (pH 7.0) was chosen for further enzyme characterization. The assay was performed in a total volume of 0.2 ml in microplates, and the initial turnover rate (milliunits of optical density/min) was recorded using a microplate reader (Spectramax; Molecular Devices, Sunnyvale, Calif.) at 405 nm.

The enzyme activity on other substrates (each at 0.5 mM concentration), including H-Gly-Glu-*p*Na, benzyloxycarbonyl (Z)-Leu-Leu-Glu-*p*Na, Z-Phe-Leu-Glu-*p*Na, Suc-Ala-Ala-Ala-*p*Na, Suc-Ala-Ala-Pro-Asp-*p*Na, Suc-Ala-Ala-Pro-Phe-*p*Na, Suc-Ala-Ala-Pro-Arg-*p*Na, and Suc-Ala-Ala-Pro-Val-*p*Na, was assayed in the same way. K_m and V_{max} values were determined by hyperbolic regression of the kinetic data obtained with Suc-Ala-Ala-Pro-Glu-*p*NA at 37°C in optimal buffering conditions and with the software package Hyper (version 1.02) obtained from J. S. Easterby (University of Liverpool, Liverpool, United Kingdom). A total of 1 μg of the proteinase (final concentration, 0.2 μM) was incubated with serial double dilutions of the substrate in final concentrations from 2.5 to 0.0195 mM. The measurements were repeated twice.

To investigate the SprE sensitivity to inhibition by several diagnostic inhibitors specific for different catalytic classes of peptidases, the purified enzyme was preincubated with DFP (Calbiochem, San Diego, Calif.), leupeptin (Calbiochem), 3,4-dichloroisocumarin (3,4-DCI; Calbiochem), tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), phenylmethylsulphonyl fluoride (PMSF), 2-Phe-Phe$(OPh)_2$, t-butyloxycarbonyl (Boc)-Val-Pro-Val$(OPh)_2$, EDTA, *o*-phenanthroline, or *N*-(L-3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl-amido(4-guanidino)butane (E-64) at a suitable concentration of inhibitor for 15 min at 37°C, a substrate was added, and residual activity was recorded.

Degradation of human fibrinogen by SprE. The degradation of human fibrinogen (Fbg; essentially plasminogen free) by SprE forms was determined electrophoretically as described above. After incubation of 25 μg of the protein in 0.1 M Bis-Tris (pH 7.0) with SprE in a molar ratio of 100:1 and a volume of 100 μl, aliquots of 20 μl were collected at 30 min, 1 h, 4 h, and 16 h, frozen, and then resolved by SDS-PAGE. Fbg incubated without SprE served as a negative control.

SprE autodegradation and stabilization assays. The autodegradation of the SprE forms was analyzed using SDS-PAGE after incubation of the enzyme in 0.1 M Bis-Tris (pH 7.0) at 37°C for 30 min, 1 h, 4 h, and 16 h. As controls, enzyme samples stored on ice or pretreated with 10 mM DFP were used. The effect of GelE (purified according to the method by Makinen et al.) (32), BSA, and the other forms of SprE on the stability of $^{-1}$S-SprE was also tested. Briefly, $^{-1}$S-SprE was incubated in 0.1 M Bis-Tris (pH 7.0) at 37°C for 2, 4, and 16 h alone or in the presence of tested proteins. At specific time points, aliquots were removed from the incubation mixture and assayed for residual $^{-1}$S-SprE activity with Suc-Ala-Ala-Pro-Glu-*p*NA as a substrate to determine the time course of SprE inactivation during incubation. The molar ratio of $^{-1}$S-SprE to each of the proteins was 2:1 except for BSA, for which the ratio 1:5 was used. The pH influence on $^{-1}$S-SprE stability was assayed after 1 h of preincubation of the proteinase at 37°C in the series of buffers with pH ranging from 4.2 to 9.0, and the activity of the pretreated samples against Suc-Ala-Ala-Pro-Glu-*p*NA was measured in 0.1 M Bis-Tris (pH 7.0) at 37°C over 30 min.

In vitro maturation of SprE zymogens. The susceptibility of different forms of SprE zymogens to proteolytic processing by the other forms of SprE and GelE was determined. Zymogens were preincubated with peptidases in 0.1 M Bis-Tris (pH 7.0) at 37°C for different time durations of 30 min to 16 h at a molar ratio of zymogen to proteinase of 5:1. Samples were then analyzed for SprE activity (zymography and with Suc-Ala-Ala-Pro-Glu-*p*Na), zymogen processing, and/or degradation (SDS-PAGE). Zymogens incubated alone, or in the presence of 10 mM DFP, served as controls.

RESULTS

Initial characterization of proteinase activity secreted by *E. faecalis* strains. Using early-stationary-phase cultures, zymography with casein- or gelatin-containing gels revealed a proteolytic band with an apparent molecular mass of 25 kDa, in the supernatant from both the wild-type strain OG1RF (TX4002) and its *gelE* deletion mutant (TX5264) but not in the supernatant from the isogenic *sprE* knockout mutant (TX5243) or from the *gelE sprE* double mutant (TX5128; data not shown). In contrast to the results seen with DFP, which completely inhibited enzyme activity, pretreatment of samples with EDTA or *o*-phenanthroline had no effect. However, when the synthetic chromogenic substrate Suc-Ala-Ala-Pro-Glu-*p*NA was used, activity was found only with the *gelE* mutant (TX5264).

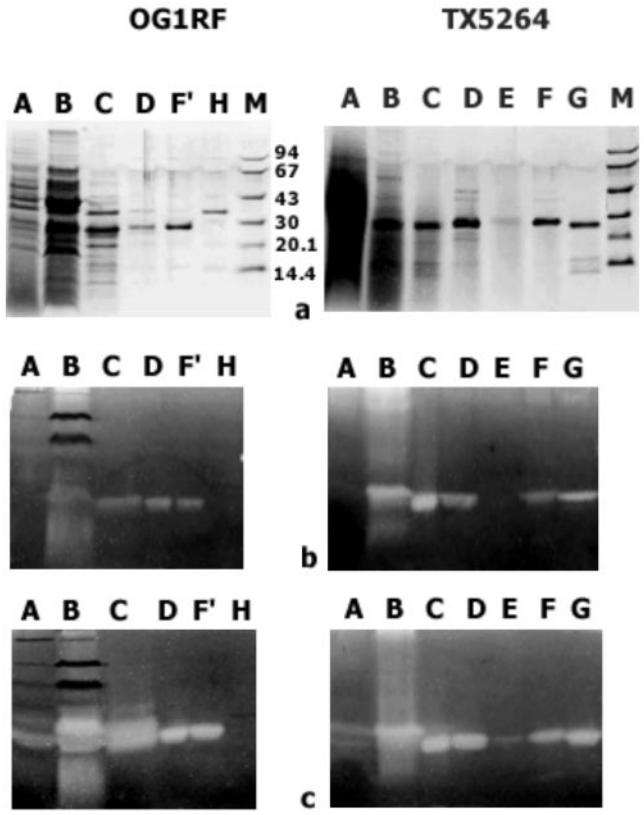

FIG. 1. SDS-PAGE (a) and gelatin (b) and casein (c) zymography analysis of the SprE-containing fractions obtained at successive steps of the SprE purification from the wild-type *E. faecalis* strain OG1RF and the gelatinase deletion mutant (TX5264). Lane As, the acetone precipitate of the culture supernatant; lane B, the protein pool after the DE-52 chromatography; lanes C and D, pooled fractions after Sephadex G-150 and SourceQ column chromatography, respectively. Lanes E to G show the results obtained with the TX5264 mutant panel alone. *Lane E*, zymogen fraction; lane F, ^{1}L-SprE (Leu^{1}-Ala^{237}); lane G, $^{-1}$S-SprE (Ser^{-1}-Glu^{227} with residual Leu^{1}-Glu^{227}). Lanes F′ and H show the results obtained with the wild-type strain OG1RF panel alone. Lanes F′, pooled fractions after Mono Q column ^{1}L-SprE (Leu^{1}-Arg^{230}); lane H, purified GelE. Lanes M, molecular mass standard (Low Molecular Range; Bio-Rad).

SprE purification. Acetone precipitation of the culture supernatants of the OG1RF strain and the *gelE* deletion mutant (TX5264) was followed by batch anion-exchange chromatography and gel filtration chromatography. Figure 1 shows the purification steps, the purity of collected fractions in SDS-PAGE (Fig. 1a), and their caseinolytic (panel b) and gelatinolytic (panel c) activities in zymography. The activity measured with Suc-Ala-Ala-Pro-Glu-*p*NA was detected and recovered only in the case of the acetone precipitate of the TX5264 mutant and not in the case of the wild-type strain. Table 1 summarizes the purification scheme and its efficiency for glutamic acid-specific endopeptidase activity, as measured by utilization of Suc-Ala-Ala-Pro-Glu-*p*Na. Around 65% of the initial activity was recovered, and the specific activity was increased more than 1,400 times.

Final high-resolution separation of proteins was obtained by MonoQ FPLC (Fig. 2). In contrast to the results seen with the wild-type strain-derived sample, which yielded a single protein

TABLE 1. Table of purification of SprE from *E. faecalis* TX5264 gelatinase-negative mutant

Fraction	Method	Volume (ml)	Amount (mg)	Whole activity (unit[a])	Specific activity (unit[a]/mg)
A	60% acetone	575	3,414.535	2,460	1.417
B	De-52	68	298.6013	2,063.2	13.626
C	G-150	26	30.25052	2,045.2	153.416
D	SourceQ	24	7.941545	1,972.8	248.415
E	MonoQ	3	0.033977	0	0
F	MonoQ	1.5	0.197286	18.3	92.759
G	MonoQ	2.0	0.178497	1,591.4	20,143.240

[a] Initial turnover rate of Suc-Ala-Ala-Pro-Glu-*p*Na (milliunits of optical density/min).

peak on a MonoQ column (Fig. 2a), the material from the *gelE* mutant (TX5264) was resolved into several distinct peaks (Fig. 2b). Each peak was subjected to rechromatography on a MonoQ column, which in the case of fraction G yielded two subfractions, referred to as G1 and G2. Proteins purified in this manner were analyzed for N-terminal amino acid sequence, precise molecular mass, and proteolytic activity.

Identification of SprE forms. For the wild-type strain OG1RF, the Edman degradation of proteins present in the peak of the fraction F′ (Fig. 1 and 2) revealed a single peptide sequence matching exactly that of the processed mature form of the SprE proteinase N terminus (LLDPEDRRQE; ^{1}L-SprE) (numbering of N-terminal residues of the SprE forms is according to Fig. 3) as inferred from the alignment of SprE with V8 and GluSE (Fig. 3). The same N-terminal sequence was also found in the peak F characteristic of the TX5264 mutant and in the G1 subfraction of the triple peak G (Fig. 1 and 2). A sequence with an additional N-terminal amino acid (SLLDPEDRRQ; $^{-1}$S-SprE) was identified in its subfraction G2. Finally, analysis of the fraction E revealed two peptide sequences in equimolar amounts, EYIVPAESHS and KRSLLDPEDR . The comparison with the deduced amino acid sequence of the pre-proprotein derived from the *sprE* gene revealed that the first sequence starts one residue downstream of the putative signal peptide; this is referred to as $^{-15}$E-SprE or the long zymogen. The other one was found to match the N terminus of the mature proteinase and is referred to as $^{-3}$K-SprE or the short zymogen (Fig. 3). For the proteolytic activity analysis, these two forms of the zymogen were resolved by two rechromatography cycles on the MonoQ column, and only $^{-3}$K-SprE had some activity in the casein zymography gel (consistent with the data shown in Fig. 1c, lane E, and Fig. 4h, lane E).

Forms ^{1}L-SprE and $^{-1}$S-SprE were characterized further by mass spectroscopy analysis. By matching molecular mass with the deduced amino acid sequence of SprE and N-terminal sequences of the individual forms, it was apparent that, in addition to the N-terminal processing results, the forms were also differentially truncated at the C terminus. One form came from the wild-type strain OG1RF (Leu^{1}-Arg^{230}), and three came from the *gelE* deletion mutant TX5264 (Leu^{1}-Ala^{237}, Leu^{1}-Glu^{227}, and Ser^{-1}-Glu^{227}), as summarized in Table 2 and Fig. 3.

Activity of the SprE forms on synthetic substrates. Among all of the isolated forms of the SprE proteinase, only $^{-1}$S-SprE showed amidolytic activity on Suc-Ala-Ala-Pro-Glu-*p*NA. Using this activity, we were able to calculate the following kinetic values: $K_m = 1.027 \pm 0.1529$ mM and $V_{max} = 31.99 \pm 2.158$. Of the several other *p*-nitroanilide-derived peptides tested, only Z-Phe-Leu-Glu-*p*NA was cleaved, and the efficiency was 35% in comparison to the Suc-Ala-Ala-Pro-Glu-*p*NA hydrolysis results. The pH optimum of $^{-1}$S-SprE for hydrolysis of Suc-Ala-Ala-Pro-Glu-*p*NA was found to be in the range from 7.0 to 7.5 and was inversely dependent on the ionic strength of the buffer. At 1 M NaCl, the activity was only 20% of that in the buffer alone.

Specificity of the SprE forms on oxidized insulin β-chain. The specificity of different SprE proteinase forms in cleaving Glu-Xaa peptide bonds was confirmed by the mass spectroscopy analysis of SprE-generated fragments of the insulin β-chain. It should be noted that the insulin β-chain contains only two Glu and no Asp residues. As expected, this peptide was cleaved by all forms specifically at the Glu^{13} ↓ Ala^{14} and Glu^{21} ↓ Arg^{22} peptide bonds, although at different rates. In the case of the ^{1}L-SprE forms (Leu^{1}-Arg^{230}, Leu^{1}-Ala^{237}), only the Glu^{13} ↓ Ala^{14} bond was cleaved after the 2-h incubation. The second cleavage at the Glu^{21} ↓ Arg^{22} bond occurred slowly and was still only partial even after overnight incubation. Significantly, no variation in the cleavage rate was observed among the ^{1}L-SprE variants. In contrast, $^{-1}$S-SprE cleaved both peptide bonds after the 2-h incubation. This difference in the cleavage rates apparently reflected the higher enzymatic activity of $^{-1}$S-SprE in comparison to the ^{1}L-SprE forms of SprE. The activity of the $^{-1}$S-SprE and ^{1}L-SprE forms was also compared on human fibrinogen. $^{-1}$S-SprE was once again the most active, and there were differences between the digestion patterns obtained with ^{1}L-SprE purified from the wild-type strain OG1RF (Fig. 4a, lane F′) and from the *gelE* deletion mutant TX5264 (Fig. 4a, lane F) after incubation for 30 min. However, all forms of SprE were effective in degradation of fibrinogen after prolonged incubation (4 and 16 h).

Inhibition profile. Inhibition of $^{-1}$S-SprE by common proteinase inhibitors was investigated using Suc-Ala-Ala-Pro-Glu-*p*NA as a substrate. Enzyme activity was shown to be sensitive to the presence of DFP, which resulted in complete inhibition at a 0.3 mM concentration. At the same concentration, 3,4-dichloroisocoumarin inhibited the activity to 62%. Other inhibitors of serine-cysteine proteinases, including PMSF, leupeptin, TPCK, and TLCK, had only marginal effects on proteinase activity, while no inhibition was found with E-64, pepstatin, and *o*-phenanthroline or EDTA, which are inhibitors of cysteine-, aspartyl- and metalloproteinases, respectively. DFP was also found to be the most effective inhibitor of caseinolytic and gelatinolytic activity of all SprE forms, as assayed by zymography analysis (Fig. 4b and c). In this assay, the other

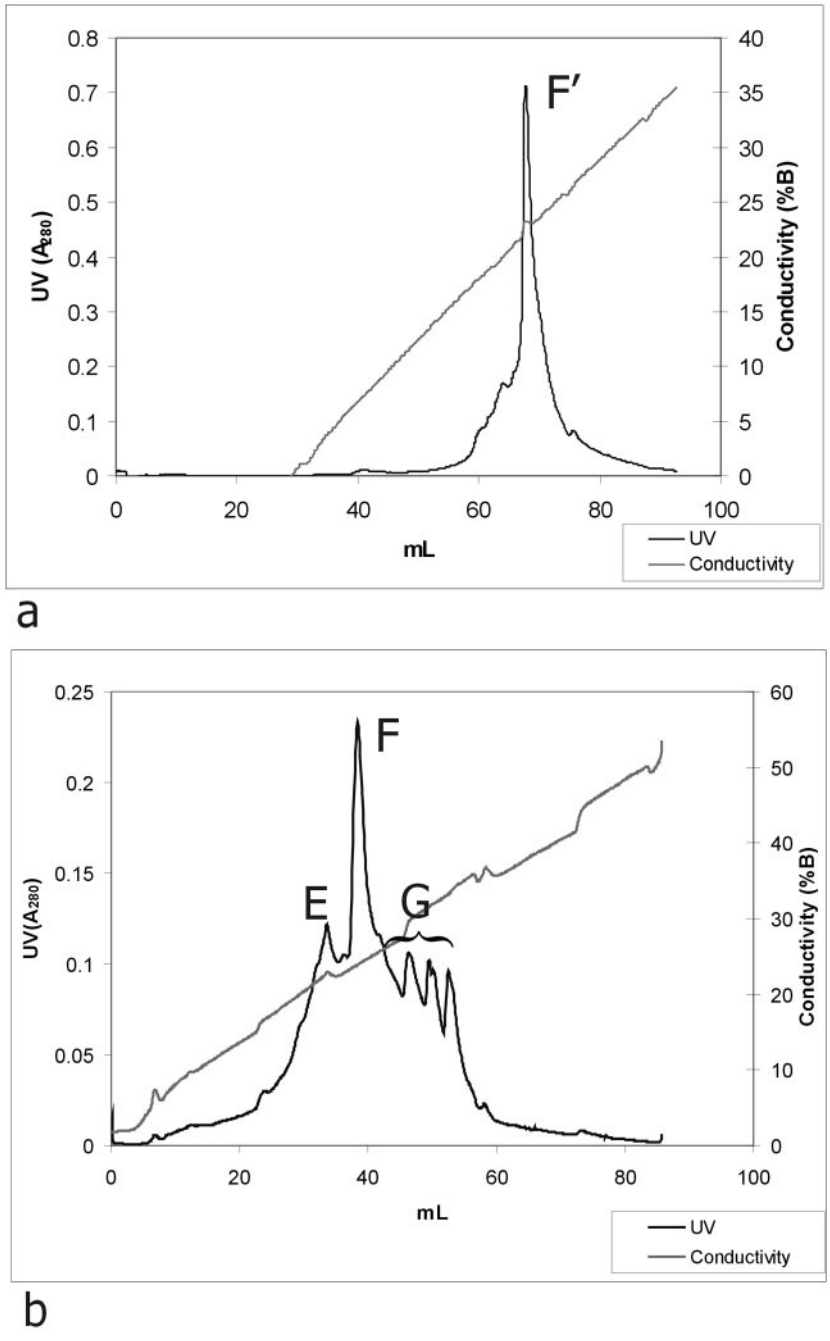

FIG. 2. Chromatography of SprE on the MonoQ column for the wild-type strain OG1RF (a) and the GelE-null mutant TX5264 (b). Protein peaks are marked as follows: F′, Leu^{1}-Arg^{230}; E, SprE zymogens; F, Leu^{1}-Ala^{237}; G, Ser^{-1}-Glu^{227} with residual Leu^{1}-Glu^{227}.

serine proteinase inhibitors were much less effective, while the inhibitors of the other proteinase classes did not have any effect on the proteolytic activity of $^{-1}$S-SprE. In addition, studies of binding of biotinylated DFP to the native proteinase revealed that DFP bound effectively to all the forms but the zymogens (Fig. 4d and e).

Comparison of the proteolytic activity of SprE forms. Despite the lack of amidolytic activity against synthetic substrates, the ^{1}L-SprE variants digested both gelatin and casein in zymography, with activity comparable to $^{-1}$S-SprE (Fig. 1, lanes F and F′ versus lane G). In contrast, the activity of the zymogens (fraction E) was much weaker and visible only with casein. This residual activity was associated with the presence of the short zymogen, possessing a three-amino-acid extension at the N terminus ($^{-3}$K-SprE). The full-length zymogen ($^{-15}$E-SprE) was proteolytically inert. In addition to the zymography analysis, which gives semiqualitative results, the general proteolytic activity was compared using azocasein as a substrate. In this assay, the specific activity of the ^{1}L-SprE forms was about 150 times lower than that of $^{-1}$S-SprE. As expected, the full-length

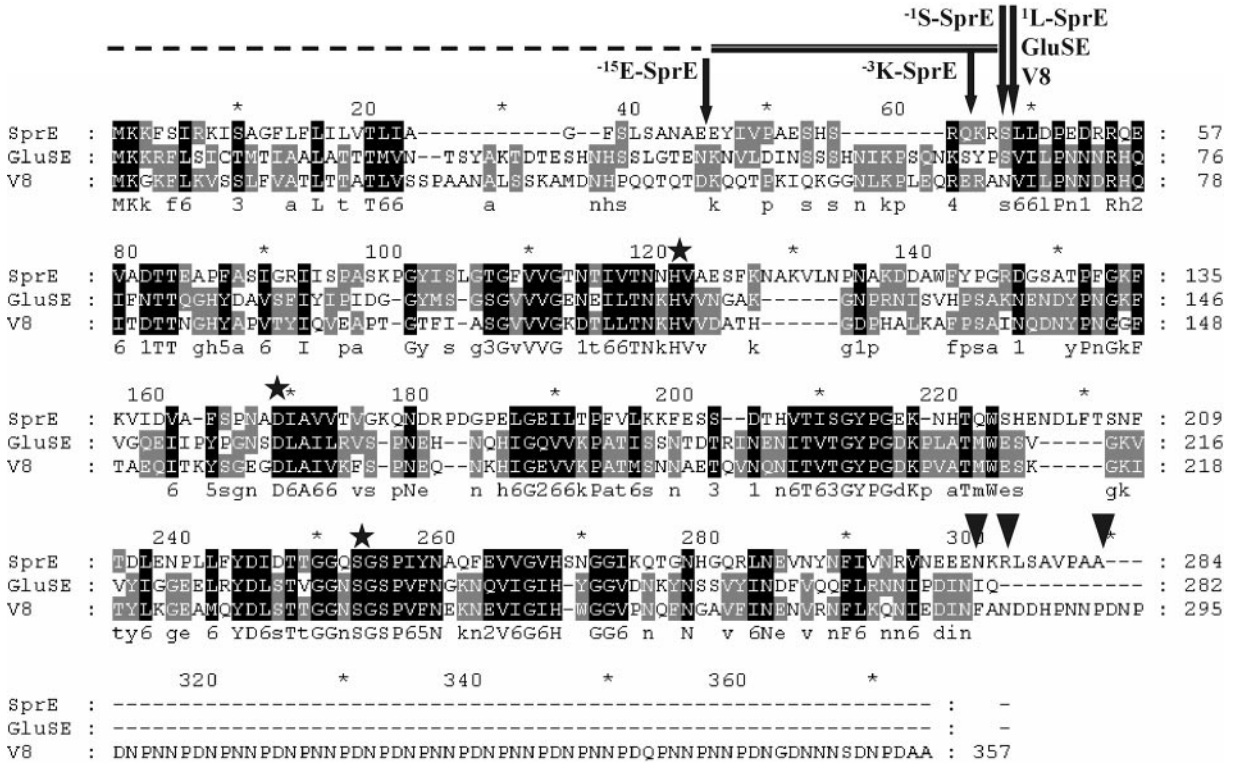

FIG. 3. Alignment of the amino acid sequence deduced from the *sprE* gene (GenBank accession no. Z12296) with those of glutamyl endopeptidases of *S. aureus* V8 (Swissprot accession no. P04188) and *S. epidermidis* ATCC 14990 GluSE (deduced from *gseA* gene; GenBank accession no. AB096695) obtained with MegAlign 5.0 software (DNAStar, Inc., Madison, Wis.). The consensus signal sequence is marked with a bold broken line; the profragment is marked with a double line. The catalytic triad residues of His117, Asp159, and Ser235 (the numbering is according to the V8 sequence) are indicated by solid asterisks. The activation cleavage sites of the proenzymes are marked by down-pointing arrows. The truncation sites at the C terminus of SprE are indicated by arrowheads.

zymogen was inactive whereas its truncated version ($^{-3}$K-SprE) showed trace activity with azocasein. At the same time, there was no difference in this assay between the three ^{1}L-SprE forms.

Stability of SprE forms. All forms of SprE were stable at −70°C for at least 6 months, but it was noticed that at 4°C, $^{-1}$S-SprE slowly lost activity and that after 4 weeks, this activity dropped to 50% of the initial level. Under the same conditions, the ^{1}L-SprE forms were stable. Since the difference in proteolytic activity between the $^{-1}$S- and ^{1}L- SprE forms may be due to their inactivation during incubation at 37°C, we compared the levels of enzyme stability at this temperature. In contrast to the ^{1}L-SprE forms, which were stable for up to 16 h of incubation, $^{-1}$S-SprE underwent almost complete autoproteolysis by 4 h (Fig. 4f). The degradation was prevented by the treatment of the enzyme with DFP. In addition, the assay performed with Suc-Ala-Ala-Pro-Glu-*p*NA revealed that $^{-1}$S-SprE was fairly stable when preincubated in pH 5.0 to 6.0, showing 90% of residual activity preserved versus less than 80% after the preincubation in pH 7.0, despite its low amidolytic activity in low pH. Both these observations suggested that $^{-1}$S-SprE is very prone to autodegradation, which apparently also occurs during the purification procedure, since the two weaker, lower-molecular-mass bands present in the purified sample of the enzyme (Fig. 1a, lane G) were found by N-terminal sequencing to be fragments of the SprE polypeptide cleaved at Glu-Xaa peptide bonds (cleavages Glu^{16} ↓ Ala^{17} and Glu^{201} ↓ Asn^{202}).

The autodegradation of $^{-1}$S-SprE correlated well with the loss of amidolytic activity by the enzyme during incubation at 37°C. After the 4-h incubation, the activity against Suc-Ala-Ala-Pro-Glu-*p*NA was reduced to 30% and completely disappeared after 16 h. The decrease in activity could not be prevented by addition of carrier proteins such as BSA or other forms of the enzyme. In contrast, the ^{1}L-SprE forms remained active up to 16 h incubation, as determined by the zymography analysis.

Proteolytic processing of different SprE forms. The presence of the aberrantly processed multiple forms of the SprE proteinase in the *gelE* deletion mutant suggests that gelatinase is involved in processing of proSprE in the wild-type strain. To test this hypothesis, we purified GelE from OG1RF, which, to our surprise, although highly active on azocasein, did not have any gelatinolytic or caseinolytic activity in zymography gels (Fig. 1, lanes H). This metalloproteinase was incubated for different periods ranging from 30 min to 16 h with the purified zymogen fraction of SprE (that consisted of $^{-15}$E-SprE and $^{-3}$K-SprE) (Fig. 4g and h, lanes E). The disappearance of the larger zymogen band after incubation with GelE (Fig. 4g and h, lanes E/H), lack of a strong band in casein zymography, and lack of any activity in the gelatin zymography (data not shown) indicated that GelE converted

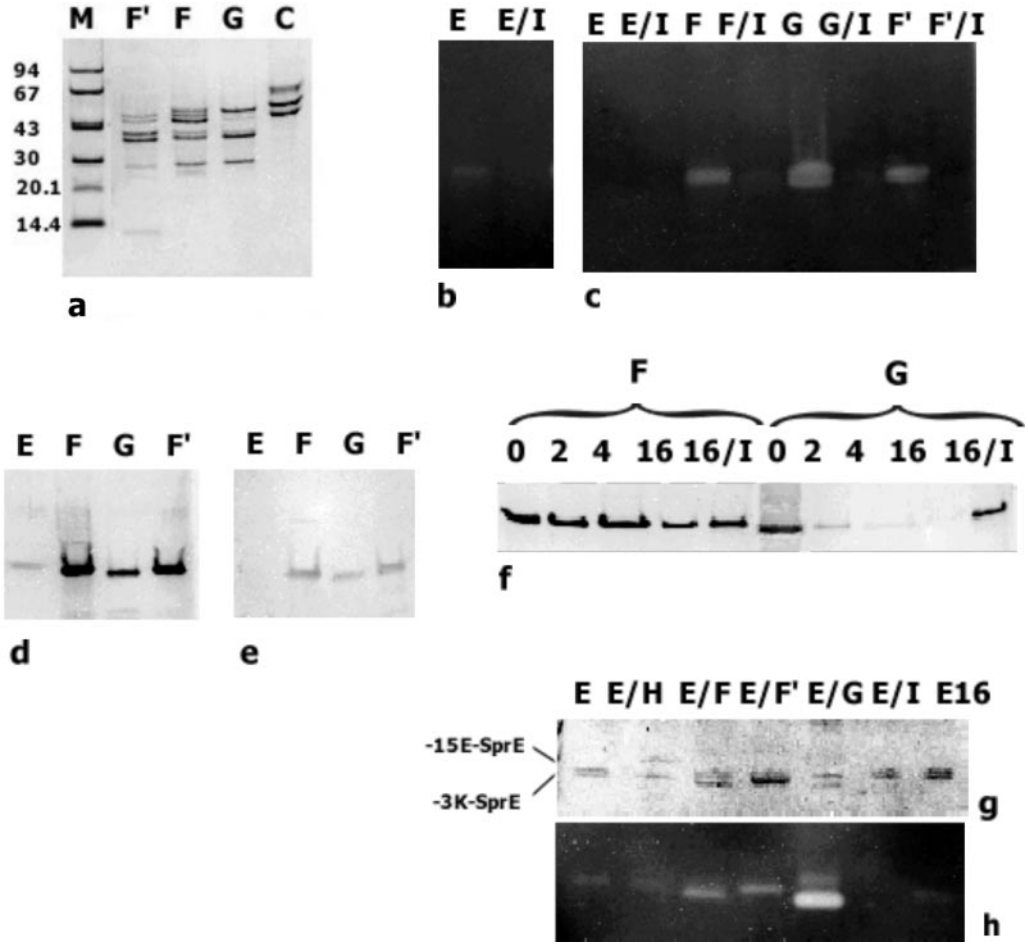

FIG. 4. SDS-PAGE analysis of human fibrinogen (Fbg) digested by mature SprE forms at 30 min of incubation (a). (b and c) Inhibition of the SprE proteolytic activity by DFP as shown by the casein (b) and gelatin (c) zymography analysis of all SprE forms. (d and e) SDS-PAGE (d) and Western blotting with biotinylated DFP (e) of all SprE forms. (f) Comparison of the stability of ^{1}L-SprE, marked as F, and $^{-1}$S-SprE, marked as G, isolated from the GelE-negative mutant TX5264 in SDS-PAGE following their incubation at 37°C. (g and h) Processing of the SprE zymogen fractions containing $^{-15}$E-SprE and $^{-3}$K-SprE by incubation for 16 h under optimal conditions at a molar ratio of 5:1 with *E. faecalis* proteinases followed by SDS-PAGE (g) and zymography on casein gel (h). E, F, and G refer to zymogens, ^{1}L-SprE, and $^{-1}$S-SprE from TX5264 mutant, respectively, and F′ refers to ^{1}L-SprE from OG1RF. C, negative control (the undigested Fbg); M, molecular mass standard (low molecular range; Bio-Rad); I, inhibitor (DFP) incubated with the particular form of SprE; H, GelE, purified from OG1RF by the method described in reference 32; / (slash), common incubation. Numbers refer to the time of incubation (in hours).

only the long zymogen to the short one. Similar results were obtained when the zymogens were treated with mature ^{1}L-SprE and $^{-1}$S-SprE forms, Leu^{1}-Ala^{237}, Leu^{1}-Glu^{227}, and Ser^{-1}-Glu^{227} (Fig. 4g and h, lanes F, F′ and G, respectively). The possibility of zymogen autoactivation was excluded in experiments in which the zymogen fraction was incubated alone under optimal conditions (Fig. 4g and h, lane E16) or in the presence of DFP (Fig. 4g and h, lane E/I). Finally, we tested whether GelE could truncate the C terminus of the full-length ^{1}L-SprE form (Leu^{1}-Ala^{237}) isolated from the *gelE* mutant strain TX5264. Again, no change in molecular mass of ^{1}L-SprE was observed.

TABLE 2. Prediction of the primary structure of the SprE forms on the basis of N-terminal amino acid sequence and MALDI-TOF mass spectroscopy analysis

Strain	SprE form (fraction)	N-terminal sequence	Experimentally determined molecular mass (Da)	Theoretical molecular mass of the inferred fragment (Da)	Predicted structure
TX4002	^{1}L-SprE (F′)	LLDPE	25,216	25,215.7	Leu^{1}-Arg^{230}
TX5264	^{1}L-SprE (F)	LLDPE	25,826	25,525.5	Leu^{1}-Ala^{237}
	^{1}L-SprE (G1)	LLPDE	24,820	24,817.3	Leu^{1}-Glu^{227}
	$^{-1}$S-SprE (G2)	SLLDP	24,902	24,904.4	Ser^{-1}-Glu^{227}

DISCUSSION

The aim of this study was to purify and characterize the nongelatinase extracellular proteolytic activity of *E. faecalis*, as observed by means of gelatin and casein zymography in both *E. faecalis* wild-type OG1RF (TX4002) and its GelE-lacking mutant (TX5264). Cumulatively, our data indicate that this activity may be assigned to SprE, which is encoded by a gene located immediately downstream from *gelE*. Our results indicated that the *sprE* gene product purified from a culture supernatant of *E. faecalis* is a serine proteinase with a molecular mass of 25 kDa, with specificity towards glutamic acid at position P1. The mature enzyme was found to exist in four different forms, as confirmed by N-terminal sequencing and MALDI-TOF (three forms of ^{1}L-SprE, Leu^{1}-Arg^{230}, Leu^{1}-Ala^{237} and Leu^{1}-Glu^{227} and a single form of $^{-1}$S-SprE, Ser^{-1}-Glu^{227}), of which only one, Leu^{1}-Arg^{230}, was purified from the wild-type OG1RF strain and the remaining ones were obtained from the GelE-null mutant TX5264. Sequence analysis of these SprE forms indicated that they all were derived from the same initial translation product of the *sprE* gene, which is present in a single copy in the OG1RF genome (46, 48) and the *E. faecalis* V583 genome (http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gef). The inhibition profile of $^{-1}$S-SprE and the other mature forms confirmed the results of amino acid sequence comparisons showing that SprE is similar to V8 and GluSE proteinases from *S. aureus* and *S. epidermidis*, respectively (46, 48).

The comparative activity analysis demonstrated a much higher catalytic efficiency of $^{-1}$S-SprE versus that of the ^{1}L-SprE variants. With all the substrates tested, this form was from several to more than a thousand times more active. These effects were most probably due to the single amino acid residue at the N terminus, since it is known that docking of an N-terminal residue in a specific site on a molecule of a chymotrypsin family of serine protease affects substrate binding sites of the enzyme (7). Apparently, additional serine residues in the $^{-1}$S-SprE formed through the effect on substrate binding site configuration enables the enzyme to bind and cleave the amido bond in the synthetic substrate Suc-Ala-Ala-Pro-Glu-*p*Na. Although the active SprE fraction purified from TX5264, apart from $^{-1}$S-SprE, also contained a C-truncated form, Leu^{1}-Glu^{227}, this form, like Leu^{1}-Arg^{230} from OG1RF, was not active with respect to the synthetic substrate utilized in this study. The C-terminal sequence of the enzyme seems to be important for the activity as well. Different patterns of human fibrinogen digestion by two ^{1}L-SprE forms, Leu^{1}-Arg^{230} and Leu^{1}-Ala^{237}, indicated clearly their different specificity towards particular Glu-Xaa bonds present in that substrate. Thus, the slight sequence differences on both termini between the SprE forms had a profound effect on their proteolytic activity.

The negligible rate of hydrolysis of Suc-Ala-Ala-Pro-Asp-*p*NA by $^{-1}$S-SprE versus the good activity of the enzyme against Suc-Ala-Ala-Pro-Glu-*p*NA was similar to that of GluSE of *S. epidermidis* (36) and argues for the fact that SprE does not tolerate an Asp residue at the P1 site. On the other hand, the lack of turnover of Z-Leu-Leu-Glu-*p*NA, in contrast to Z-Phe-Leu-Glu-*p*NA results, strongly indicated that enzyme specificity was modulated by the S3 subsite substrate-binding pocket, which apparently could not accommodate a branched aliphatic chain of the Leu residue. This narrow specificity of SprE is surprising, since with the exception of the preference for Glu at the P1 position, its close homologues, V8 and GluSE (12, 36, 57), are rather indiscriminant. It is even more interesting, however, that none of the other forms of the SprE proteinase was able to cleave any of the tested synthetic substrates, even though they were active on protein substrates such as insulin, fibrinogen, and casein.

Taken together, all the data presented above indicated that SprE should be classified into the glutamyl endopeptidase I staphylococcal group (57). In the sequence alignment (Fig. 3) of three members of the group, V8, GluSE and SprE, the Asp-Asn-Pro C-terminal extension of V8 is not found in either GluSE or SprE. However, it was shown that this sequence is not necessary for enzymatic activity or for protein folding (65). Although the propeptide sequences of the enzymes are very different (as observed already in the alignment of V8 versus GluSE)(43), the generally high similarity of the mature proteinase sequences suggests that they originated from a common ancestor. This hypothesis is supported further by the fact that in *S. aureus* and *E. faecalis*, the expression of V8 and SprE is controlled by the homologous regulatory systems Agr and Fsr, respectively (41, 47, 49). In addition, the maturation process of at least two of these enzymes relies in some way on neutral metalloendopeptidase activities (5, 13), as discussed below.

In the course of purification, the SprE proenzymes ($^{-15}$E-SprE and $^{-3}$K-SprE) were retrievable only from the *gelE* mutant TX5264. On the basis of both this observation and the fact that *S. aureus* V8 is activated by aureolysin, a broad-spectrum extracellular neutral metalloendopeptidase from the thermolysin family (5, 13), a key role in SprE maturation was considered likely to be played by the GelE metalloproteinase. We hypothesized that gelatinase might be responsible for the direct maturation of the zymogens into ^{1}L-SprE (cleavage $^{-3}$KRS $\downarrow$ L), particularly since the specificity of purified GelE for cleavage between serine and leucine-isoleucine residues has been demonstrated previously (32). The GelE treatment of the zymogens, however, resulted only in the hydrolysis of the long zymogen ($^{-15}$E-SprE) to the shorter one ($^{-3}$K-SprE), even though the site of this cleavage ($^{-5}$RQ $\downarrow$ KR) is not particularly specific for gelatinase (13, 32). Moreover, two of the ^{1}L-SprE forms (Leu^{1}-Ala^{237} and Leu^{1}-Glu^{227}) existed as well in the GelE-negative mutant. Since GelE may process other surface and/or secreted proteins of *E. faecalis* (64), which in turn may act on SprE zymogens, it is possible that other factors participate in the proSprE processing. The influence of gelatinase may be necessary but not direct, and in the absence of GelE another proteolytic pathway(s) is almost certainly operative.

On the other hand, SprE is not involved in GelE processing, since the *sprE* insertion mutant TX5243 produces gelatinase (48), which has a proper N terminus and molecular weight (M. Kawalec, B. E. Murray, and J. Potempa, unpublished results). The *gelE* and *sprE* coexpression (48) may indicate some specific advantage to *E. faecalis* that comes from the effect of GelE on SprE maturation into ^{1}L-SprE and not the "superactive" variant of the enzyme, $^{-1}$S-SprE. Waters and coworkers analyzed the role of GelE in determination of cellular chain length, supernatant pheromone levels, and degradation of mis-

folded surface proteins in *E. faecalis* in vitro. They observed abnormal growth and morphology of cellular chains in mutant TX5264 and attributed these phenomena to the absence of GelE (64). The results obtained in this work allow us to speculate that the presence of $^{-1}$S-SprE could be involved in the effects as well.

Interestingly, the ^{1}L-SprE from the wild-type strain was processed not only at the N terminus (cleavage at $^{-3}$KRS ↓ L) but at the C terminus (^{229}KR ↓ LS) as well. Leu^{1}-Ala^{237}, one of the two ^{1}L-SprE forms from the *gelE* mutant, appeared to be processed only at the N terminus, whereas the second, Leu^{1}-Glu^{227}, was also truncated at the C terminus (^{225}EEE ↓ NKRLS). The coexisting superactive form $^{-1}$S-SprE was a result of another N-terminal cleavage ($^{-3}$KR ↓ SL) and the same C-terminal processing event as that with the Leu^{1}-Glu^{227} form of the enzyme. It is possible that this C-terminal cleavage was due to $^{-1}$S-SprE itself, which therefore would be able to both process Leu^{1}-Ala^{237} into Leu^{1}-Glu^{227} and to autoprocess. The unique autoprocessing ability of $^{-1}$S-SprE was demonstrated in experiments which proved that $^{-1}$S-SprE, but not ^{1}L-SprE, undergoes autodegradation. Thus, the variety of the SprE forms, observed both in the wild-type strain and in the *gelE* mutant, resulted from numerous proteolytic cleavages at both termini, which could be due to different enzymes, including one of the forms itself.

The "superactivation" cleavage site Arg-Gln-Lys-Arg ↓ Ser and, to a lesser degree, the C-terminal truncation site may meet the requirements of specificity of host proprotein convertases from the SPC family of proteases (furins) (59). It is tempting to speculate that in the human digestive tract or sites invaded by *E. faecalis*, the released SprE zymogen is activated by furin-type enzymes associated with the cell surface of many host cells. This would lead to the generation of powerful proteolytic activity and, subsequently, to considerable local damage to the adjoining tissue. However, the *gelE* mutant TX5264 turned out to be attenuated equally to the *sprE* mutant in the *C. elegans* model (52), which could suggest that, despite its in vitro superactivity, $^{-1}$S-SprE has no extraordinary virulence potential. The reason for this may be inadequacy of the model, i.e., the lack of an essential substrate, for demonstration of the unique properties of $^{-1}$S-SprE (a substrate with an appropriate availability of Glu-Xaa bonds) or lack of biological stabilizers protecting the SprE form from autodegradation. Indeed, we have found that the $^{-1}$S-SprE activity was stable in the presence of aminopeptidase M (Kawalec et al., unpublished). The other possibility could be fitness problems resulting from the already discussed morphological abnormalities of the mutant (52). There is also lack of a proper mutant control for the $^{-1}$S-SprE testing, as there is no strain that would produce only ^{1}L-SprE and not GelE. It would be interesting to elucidate whether the $^{-1}$S-SprE exists in naturally occurring *E. faecalis* strains in spite of the results of earlier works, in which the cotranscribed *gelE* and *sprE* genes always coexisted (52).

In conclusion, we were able to isolate different forms of the SprE serine glutamylendopeptidase, which is a putative virulence factor of *E. faecalis*. The finding of profound activity differences relying on the extra amino acid at the N terminus of $^{-1}$S-SprE, the superactive form, must be stressed in light of knowledge of the homologous enzymes modus operandi. Moreover, we made first steps to investigate the in vitro maturation of the SprE zymogens. Further in vitro and in vivo studies of the purified enzyme can now be utilized to explore the possible relationship of the SprE proteinase to *E. faecalis* virulence. In addition, it would also be of interest to fully explore the SprE maturation pathway and enzyme(s) involved in it, especially at the site of infection, and to understand the conformational changes that cause the dramatic differences in enzyme activity between $^{-1}$S-SprE and ^{1}L-SprE forms.

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