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# Revised Model for *Enterococcus faecalis fsr* Quorum-Sensing System: the Small Open Reading Frame *fsrD* Encodes the Gelatinase Biosynthesis-Activating Pheromone Propeptide Corresponding to Staphylococcal AgrD▽†

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**Gelatinase biosynthesis-activating pheromone (GBAP) is an autoinducing peptide involved in *Enterococcus faecalis fsr* quorum sensing, and its 11-amino-acid sequence has been identified in the C-terminal region of the 242-residue deduced *fsrB* product (J. Nakayama et al., Mol. Microbiol. 41:145–154, 2001). In this study, however, we demonstrated the existence of *fsrD*, encoding the GBAP propeptide, which is in frame with *fsrB* but is translated independently of *fsrB*. It was also demonstrated that FsrB′, an FsrD segment-truncated FsrB, functions as a cysteine protease-like processing enzyme to generate GBAP from FsrD. This revised model is consistent with the staphylococcal *agr* system.**

The staphylococcal *agr* system is the best-understood cyclic peptide-mediated quorum-sensing system among gram-positive bacteria. In the *agr* system, an autoinducing peptide (AIP) is generated from its propeptide, AgrD, by its processing enzyme, AgrB, and is then sensed by a two-component regulatory system comprising a membrane histidine kinase, AgrC, and a response regulator, AgrA (10, 14, 18, 22, 23). The cognate gene cluster consisting of these four components has been identified in the genome databases of *Listeria*, *Clostridium*, *Lactobacillus*, and *Bacillus* spp., suggesting that cyclic peptide-mediated quorum sensing is widespread among gram-positive bacteria (14, 18, 21).

The *fsr* system in *Enterococcus faecalis* controls the expression of pathogenicity-related extracellular proteases, gelatinase, and a serine protease via a quorum-sensing mechanism (11, 16, 17), and recent studies have suggested that it also regulates biofilm formation (7, 15) and other genes important for virulence (2). The *fsr* quorum-sensing system also mediates a cyclic peptide named gelatinase biosynthesis-activating pheromone (GBAP), although the ring is formed by lactone instead of the thiolactone found in other gram-positive AIPs (10, 11, 21). However, a small open reading frame corresponding to staphylococcal *agrD* has not been identified in the nucleotide sequence of the *fsr* gene cluster (16). The 11-amino-acid sequence of GBAP was identified in the C-terminal part of the 242-residue deduced *fsrB* product (11). As shown in Fig. 1, the N-terminal part of FsrB (189 residues) shows sequence similarity to staphylococcal AgrB, and the remaining C-terminal part of FsrB (53 residues) appears to be a GBAP propeptide like AgrD. Based on these observations, we have suspected that FsrB autoprocesses its C-terminal part to generate GBAP, which is a unique biosynthetic mechanism compared with those of other cyclic AIPs.

In the present study, we demonstrate the existence of a small open reading frame, *fsrD*, corresponding to *agrD*, which is carried in frame with *fsrB* but is translated independently of *fsrB*. Here we propose a revised *fsr* system model sharing a common mechanism of AIP biosynthesis with the thiolactone-mediated quorum-sensing systems of staphylococci and probably other gram-positive bacteria.

**Construction of a nisin-inducible expression system for wild-type and mutant *fsrBD* strains.** The *E. faecalis* strains and plasmids used in this study are listed in Table 1 and Table 2, respectively. The primers used for PCR amplification are listed in Table S1 in the supplemental material. Genetic maps of plasmids and chromosomes related to this study are illustrated in Fig. 2.

Throughout this study, the nisin-controlled expression system was employed (5, 8, 9). This system uses a nisin-controlled two-component regulatory system encoded by *nisRK* to control the expression of wild-type or mutant *fsr* genes under the *nisA* promoter. The *fsrBD* segment was amplified from a gelatinase-positive *E. faecalis* strain, OG1SP (11), by PCR using primers FSRB5 and FSRB3. The amplified fragment was digested with NcoI and PstI and then translationally fused to pNZ8048 (9) digested with the same two restriction enzymes. From the resultant plasmid, the *fsrBD* segment together with the *nisA* promoter region and the transcriptional terminator region was amplified by PCR with primers NZ8048P and NZ8048T, and

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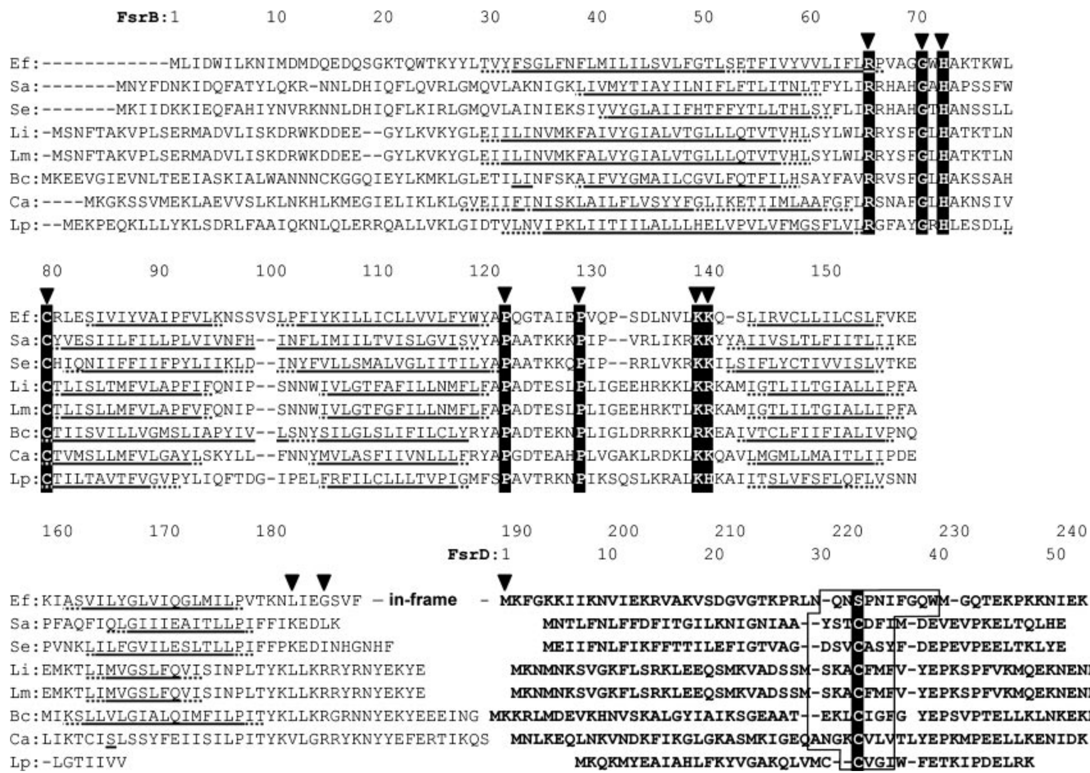


FIG. 1. Deduced amino acid sequences and alignments of FsrB and FsrD with AgrBs, AgrDs, and their homologs. Ef, *Enterococcus faecalis* (GenBank accession no. AAF14219); Sa, *Staphylococcus aureus* (accession no. CAA36781 and CAA36782); Se, *Staphylococcus epidermidis* (accession no. AAC38295 and ACC38294); Li, *Listeria innocua* (accession no. CAC95274 and CAC95275); Lm, *Listeria monocytogenes* (accession no. CAC98263 and CAC98264); Bc, *Bacillus cereus* (accession no. ZP_00237848 and ZP_00237849); Ca, *Clostridium acetobutylicum* (accession no. AAK78063 and AAK78064); Lp, *Lactobacillus plantarum* (accession no. NP_786783 and NP_786782). Dashes indicate gaps in the alignment. Conserved residues are indicated by black shading. Dotted and solid underlines indicate the predicted transmembrane segments showing scores higher than 1.7 and 2.2, respectively, using the dense alignment surface method (http://www.sbc.su.se/~miklos/DAS/) (4). Inverted triangles indicate the positions where site-directed mutagenesis was performed. Sequences of AIPs (for *E. faecalis*, *S. aureus*, *S. epidermidis*, and *L. plantarum*) and putative AIPs (for the corresponding positions for *L. innocua*, *L. monocytogenes*, and *C. acetobutylicum*) are enclosed in boxes. The *fsrB* [568]ATG codon for Met-190 (also for Met-1 in *fsrD*) is directly connected in frame to the *fsrB* codon for Phe-189.

the amplified fragment was cloned into a pGEM-T vector (Promega, Madison, WI). The resultant plasmid, pQU1100, was linearized with PstI and ligated with pNZ9530 linearized with PstI and carrying *ery*, *nisRK*, and pAMβ1 origins of replication (8). A shuttle plasmid, pQU2100, carrying nisin-inducible *fsrBD* was eventually obtained (Fig. 2). In order to generate site-directed deletions or mutations in *fsrBD*, pQU1100 was used as a parental plasmid.

For the deletion of the C-terminal GBAP-encoding region (the *fsrD* segment), inverse PCR was performed with primers FSRB′I5 and FSRB′I3, and the amplified product was then digested with SpeI and self-ligated. The resultant plasmid was linearized with PstI and ligated into pNZ9530 linearized with PstI, resulting in pQU2200 (Fig. 2). Site-directed mutagenesis was performed using a Quick Change II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) or inverse PCR (the primers used are listed in Table S1 in the supplemental material). Each mutated gene was fused with PstI-linearized pNZ9530, as described above. For the construction of pQU2300, inverse PCR was performed using primers FSRDI5 and FSRDI3 with pQU1100 as a template, and the PCR product was then phosphorylated by T4 polynucleotide kinase (Toyobo, Tokyo, Japan) and self-ligated.

Each plasmid was introduced into *E. faecalis* by electroporation (3), and transformants were selected by using erythromycin (50 μg/ml) for pNZ9530 derivatives or chloramphenicol

TABLE 1. *E. faecalis* strains used in this study

| Strain | Genotype | Phenotype | Reference or source |
|---|---|---|---|
| OG1SP | *gelE*$^+$ *sprE*$^+$ *fsrA*$^+$ *fsrB*$^+$ *fsrC*$^+$ | GBAP and gelatinase positive | 11 |
| OU510 | *gelE*$^+$ *sprE*$^+$ *fsrA*$^+$ *fsrB* (amber mutation at the position corresponding to Leu-65) *fsrC*$^+$ | GBAP and gelatinase negative; sensitive to GBAP | Urine isolate (Okayama University Hospital) |
| OU598 | *fsrA fsrB fsrC* (23.9-kb deletion involving the *fsr* gene cluster) *gelE*$^+$ *sprE*$^+$ | GBAP and gelatinase negative; insensitive to GBAP | 12 |

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TABLE 2. Plasmids used in this study

| Plasmid | Gene construction | Reference or source |
|---|---|---|
| pNZ8048 | *nisA* promoter + NcoI site + multiple cloning site + terminator, pSH71 replicon, *cam*, translational fusion vector for nisin-controlled expression | 9 |
| pNZ9530 | *nisR* and *nisK* (both expressed from rep promoter), pAMβ1 replicon, *ery* | 8 |
| pQU1100 | *fsrB* associated with a *nisA* promoter upstream and a transcriptional terminator downstream cloned into a pGEM-T vector | This study |
| pQU2100 | Chimera of pQU1100 and pNZ9530 | This study |
| pQU2101 | pQU2100 derivative carrying *fsrB* with the deletion of $^{568}$ATG | This study |
| pQU2102 | pQU2100 derivative carrying *fsrB* with a mutation of $^{568}$ATG to $^{568}$GCA | This study |
| pQU2103 | pQU2100 derivative carrying *fsrB* with a mutation of $^{556}$GGA to $^{556}$AAA | This study |
| pQU2104 | pQU2100 derivative carrying *fsrB* with the deletion of $^{548}$T | This study |
| pQU2200 | pQU2100 derivative carrying *fsrB′* that is a 3′-end truncation of *fsrB*, lacking in the *fsrD* region | This study |
| pQU2231 | pQU2200 derivative carrying *fsrB′* coding for R66A mutation | This study |
| pQU2241 | pQU2200 derivative carrying *fsrB′* coding for G71A mutation | This study |
| pQU2251 | pQU2200 derivative carrying *fsrB′* coding for H73F mutation | This study |
| pQU2261 | pQU2200 derivative carrying *fsrB′* coding for C80A mutation | This study |
| pQU2271 | pQU2200 derivative carrying *fsrB′* coding for P123A and P130A mutations | This study |
| pQU2281 | pQU2200 derivative carrying *fsrB′* coding for K140A and K141A mutations | This study |
| pQU2300 | *fsrD* translationally fused to pNZ8048 | This study |

(20 μg/ml) for pNZ8048 derivatives. Each *E. faecalis* transformant was grown in Todd-Hewitt broth (Oxoid, Hampshire, United Kingdom) with or without nisin (25 ng/ml; Sigma, St. Louis, MO) at 37°C.

**Demonstration of the existence of *fsrD*.** As shown in Fig. 1, the C-terminal extension of FsrB is similar in length to those of staphylococcal AgrDs and the homologues of other gram-positive bacteria. Based on this alignment, it was suspected that there may be a small open reading frame, *fsrD*, starting from $^{568}$ATG, corresponding to the Met-190 position of *fsrB*.

In order to address this possibility, a nisin-controlled expression system for *fsrBD* was constructed using GBAP- and gelatinase-negative *E. faecalis* OU598, which carries the 23.9-kb chromosomal deletion involving the *fsr* gene cluster, as shown in Fig. 2 (12). OU598 was transformed with pQU2100 carrying the entire *fsrBD* gene segment under a nisin-inducible promoter, and GBAP activity in the culture supernatant was measured after nisin induction. Significant GBAP activity was detected, as shown in Fig. 3. The production of GBAP was also confirmed by liquid chromatography-mass spectrometry (LC-MS) analysis of the culture supernatant (21). As shown in Fig. 4, a peak was detected at the same retention time as that for the chemically synthesized GBAP (13). As shown in the inset of Fig. 4, the mass spectrum of this peak showed the same isotopic distribution at the same *m*/*z* position as the synthetic GBAP.

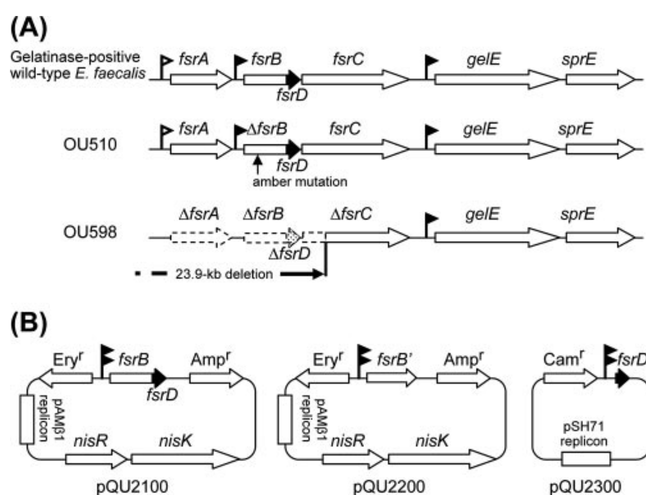


FIG. 2. (A) Genetic map of the *fsr* gene cluster and the downstream regions in gelatinase-positive wild-type *E. faecalis* and in two gelatinase-negative isolates, OU510 and OU598. White and black flags represent constitutive and GBAP-inducible promoters, respectively. Black arrows indicate *fsrD.* (B) Genetic map of the plasmids used in this study. Double flags indicate the nisin-inducible promoter.

Starting with pQU2100, $^{568}$ATG was deleted or modified to GCA, corresponding to Ala, resulting in pQU2101 or pQU2102, respectively (Fig. 3). Each plasmid was introduced into OU598, and the GBAP activity in each culture supernatant was assayed. Neither OU598(pQU2101) nor OU598 (pQU2102) showed significant levels of GBAP activity (Fig. 3). The loss of GBAP production was also confirmed by the LC-MS analysis of the OU598(pQU2101) culture supernatant as shown in Fig. 4. These results indicate that the putative $^{568}$ATG start codon is necessary for the production of GBAP.

Site-directed mutagenesis was also performed in order to change the putative ribosomal binding site, $^{556}$GGAAG$^{560}$, to $^{556}$AAAAG$^{560}$, resulting in plasmid pQU2103. The GBAP activity in the culture supernatant of nisin-induced OU598 (pQU2103) was greatly decreased. These results demonstrate that *fsrD* is translated from $^{568}$ATG. Furthermore, a frameshift mutation was introduced several base pairs upstream of the putative ribosomal binding site to generate a TAA stop codon corresponding to the original position of *fsrB* Leu-183, resulting in pQU2104. OU598(pQU2104) showed significant GBAP activity. This indicates not only the existence of *fsrD* but also the fact that the whole translated product of *fsrB* (amino acids M1 to K242) is not necessary for the production of GBAP and that the C-terminally truncated FsrB functions to produce mature GBAP from FsrD. To further demonstrate this notion, *fsrD* and a 3′-end-truncated *fsrB* corresponding to amino acids M1 to F189 of FsrB, henceforth termed *fsrB′*, were expressed

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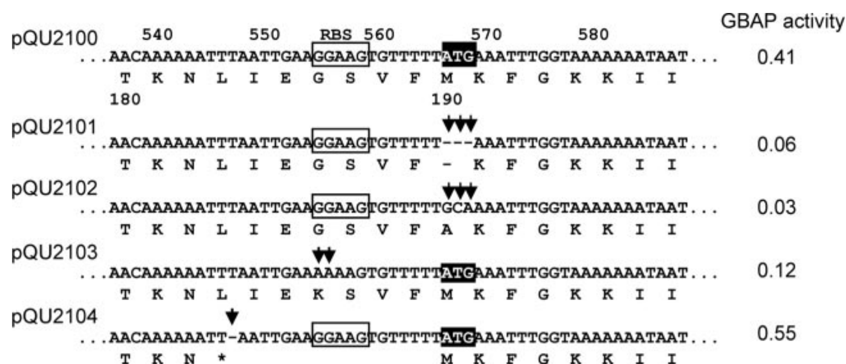


FIG. 3. Site-directed mutagenesis of pQU2100 in *E. faecalis* OU598. The nucleotide sequences around the mutagenesis site are indicated by vertical arrows, and the GBAP activities of each transformant are shown. Black shading indicates the putative start codon of *fsrD*. Open boxes indicate the putative ribosomal binding site of *fsrD*. The GBAP activity of each transformant was measured as described previously (12), except that *E. faecalis* OU510 was used as a responder strain instead of OG1SP and gelatinase was induced for 5 h instead of 3 h. The GBAP activity is represented by the induced gelatinase activity (change in $A_{540}$) determined by two independent experiments.

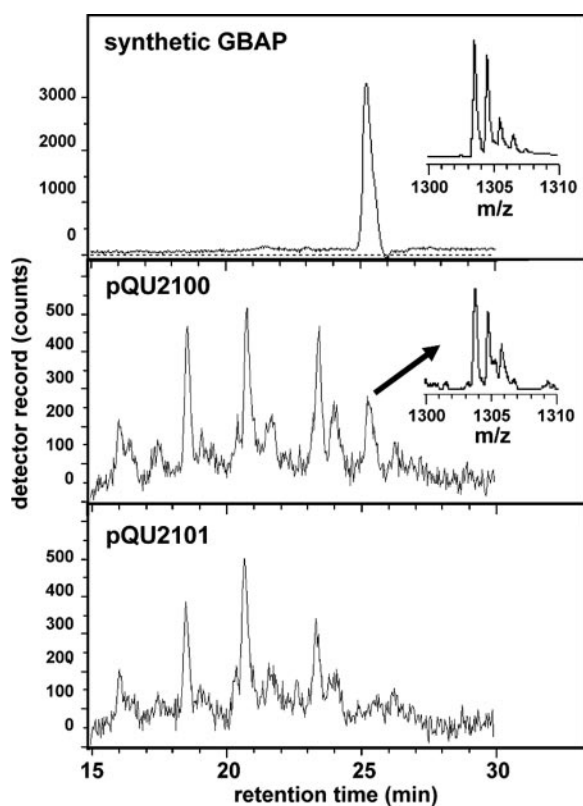


FIG. 4. LC-MS analysis of culture supernatants of OU598 (pQU2100) and OU598(pQU2101). After the strains were cultured in a chemically defined medium (200 ml) (21) for 2 h, nisin was added to a final concentration of 25 ng/ml, and the strains were then cultured for another 5 h. The culture supernatants were partially purified by using a Spe-pak octyldecyl silane cartridge column (720 mg; Waters Co., Milford, Mass.) according to a procedure previously described (12, 21) and were then injected into a LC-MS device (Accutof T100LC; JEOL, Tokyo, Japan; LC column, Agilent Zorbax Eclipse XDB-C8 [2.1 by 150 mm]). The column was eluted with a linear gradient of acetonitrile (24% to 50% in 26 min) in an 0.05% trifluoroacetic acid aqueous solution, and the eluates at a protonated molecular mass of GBAP (*m*/*z*,1303.7) were monitored. The mass spectra of the peaks indicated by an arrow are shown in insets. As a standard, the synthetic GBAP was monitored by using the same LC-MS system.

from two different plasmids, pQU2200 and pQU2300, respectively, and GBAP production was examined. As expected, OU598 carrying both plasmids produced a high level of GBAP (change in optical density at 540 nm, 1.41), whereas the OU598 strain carrying either pQU2200 or pQU2300 showed no GBAP activity. The high level of GBAP production in OU598 (pQU2200,pQU2300) can be explained by the high copy number of pQU2300 compared to the low copy number of pQU2100.

In order to determine whether *fsrD* is indeed translated from the chromosomal *fsrBDC* operon, a complementation test was performed using pQU2200 and *E. faecalis* OU510 as the host. OU510 is a clinical isolate having an amber point mutation at the chromosomal *fsrB* codon corresponding to Leu-65 (Fig. 2), which causes the loss of GBAP production and leads to the gelatinase-negative phenotype. When pQU2200 was introduced into OU510, gelatinase activity was recovered, as shown in Fig. 5, suggesting that the loss of GBAP biosynthesis was complemented by the expression of *fsrB′*. The recovery of GBAP biosynthesis indicated that *fsrD* was translated from the chromosomal *fsrBDC* operon of OU510 and that the translated product was processed to GBAP by FsrB′.

**Site-directed mutagenesis of *fsrB′*.** There are six perfectly conserved residues and one well-conserved double basic residue among the AgrB family proteins (Fig. 1). Starting with the nisin-inducible *fsrB′* expression plasmid pQU2200, site-directed mutagenesis was performed with these conserved residues. Both Pro-123 and Pro-130 residues were replaced with alanine, resulting in pQU2271. The conserved double basic residues Lys-140 and Lys-141 were also replaced with alanine, resulting in pQU2281.

OU510 carrying each mutated plasmid was cultured with nisin, and gelatinase activity in the culture supernatant was measured in order to confirm GBAP production (Fig. 5). We also examined gelatinase production on agar medium containing gelatin, in which a turbid halo was observed around gelatinase-positive colonies. All mutants except OU510(pQU2281) lost gelatinase activity in liquid culture. Interestingly, OU510 (pQU2271) showed low gelatinase activity on solid medium, while it did not show any activity in liquid culture. Considering that quorum sensing would be more sensitive in colonies grow-

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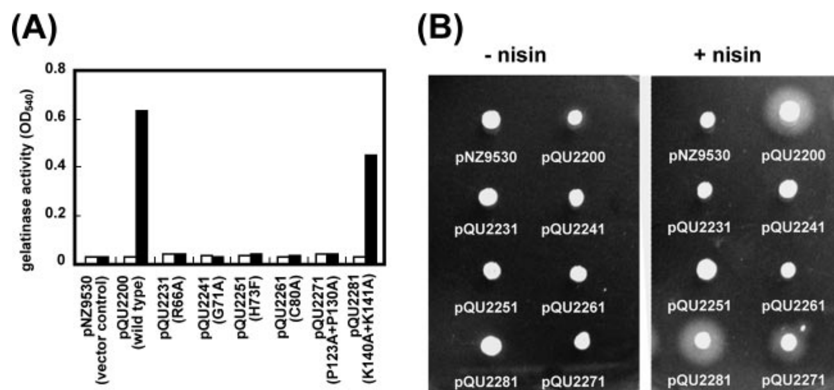


FIG. 5. Gelatinase activity of nisin-induced *E. faecalis* OU510 carrying each plasmid. (A) Gelatinase activity in liquid culture. Each strain was cultured in medium with (black bars) or without (white bars) nisin for 5 h, and the culture supernatant was subjected to an Azocoll assay, as described previously (11). The experiment was performed in duplicate, and average values were plotted. (B) Gelatinase activity on solid agar medium. Overnight liquid cultures (0.5 μl) of each strain were spotted and grown on agar medium containing 3% gelatin with (+) or without (−) nisin overnight, cooled at 4°C for 1 h, and photographed.

ing on solid surfaces than in planktonic cells growing in liquid culture, it appears that OU510(pQU2271) produces trace amounts of GBAP, which are able to induce gelatinase production only in colonies on solid medium.

These results suggest that the four conserved residues (Arg-66, Gly-71, His-73, and Cys-80) are indispensable for GBAP biosynthesis and that Pro-123 and Pro-130 have a crucial role in the efficient biosynthesis of GBAP. Histidine and cysteine are the most likely catalytic residues in cysteine proteases. Indeed, a recent study demonstrated that AgrB had endopeptidase activity against AgrD and identified two amino acid residues corresponding to His-73 and Cys-80 in FsrB that were essential for this activity (18, 23). As suspected for AgrB, FsrB′ is likely to have a cysteine protease-like function to process FsrD.

As shown in Fig. 1, AgrB family proteins, including FsrB′, show similarities in both their predicted transmembrane topology profiles and in their amino acid sequences (4). The cluster of four conserved residues in FsrB′ (Arg-66, Gly-71, His-73, and Cys-80), which is essential for the production of GBAP both in liquid medium and on solid agar medium, is located between the two predicted transmembrane segments and would be located on the cytoplasmic side. Moreover, AgrB-PhoA fusion analysis in a previous study indicated that the two cysteine protease-like residues are located on the inner surface of the cytoplasmic membrane (22).

Taken together, the above-described results suggest that the processing of FsrD is performed inside the cell, similarly to the processing of AgrD and other cyclic autoinducing peptides in gram-positive bacteria. The other three conserved residues, Pro-123, Pro-130, and Lys-141, are also located in the intersegment region between the two predicted transmembrane segments. However, the AgrB-PhoA fusion analysis of an earlier report considers the hydrophobic region of AgrB (Ile-104 to Ala-124) to be an extracellular loop and the hydrophilic region containing the two prolines and one lysine to be a transmembrane region (22). The authors have suggested that this high-energy configuration might play a crucial role in the processing and exporting of the AgrD peptide, and this also might be the case for FsrB′.

Whether the translation of *fsrB* reads through to *fsrD* or terminates ahead of *fsrD* in wild-type *E. faecalis* is uncertain. There is a run of six adenines (nucleotide positions 540 to 545 in Fig. 3) in the proximal upstream region of the *fsrD* start codon, which might lead to programmed or spontaneous translational frameshifting, as previously found for *Escherichia coli* insertion sequence elements and *trpR* (1, 6, 19, 20). If this is the case, the translation of *fsrB* terminates upstream of *fsrD* and results in FsrB′. Further studies are required to address this possibility. There is also the possibility that GBAP is partially yielded from the C-terminal fragment generated by the proteolytic processing of FsrB. However, the data presented here (Fig. 3 and 4) showed that the breakdown of *fsrD* translation resulted in the loss of GBAP production, suggesting that GBAP is generated mostly from FsrD, not from FsrB. Taken together, the data strongly suggested that the *E. faecalis fsr* system consists of four components, as found for the staphylococcal *agr* system and other gram-positive bacterial genomes: an AIP propeptide (FsrD), a cysteine protease-like processing enzyme (maybe FsrB′ but not FsrB), and two-component sensory proteins (FsrC and FsrA).

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