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Effects of *Enterococcus faecalis fsr* Genes on Production of Gelatinase and a Serine Protease and Virulence

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Three *agr*-like genes (*fsrA*, *fsrB*, and *fsrC*, for *Enterococcus faecalis* regulator) were found upstream of the previously reported gelatinase gene (*gelE*) and a downstream putative serine protease gene (*sprE*; accession number Z12296) of *Enterococcus faecalis* OG1RF. The deduced amino acid sequence of *fsrA* shows 26% identity and 38% similarity to *Staphylococcus aureus* AgrA (the response regulator of the accessory gene regulator system in the *agr* locus), FsrB shows 23% identity and 41% similarity to *S. aureus* AgrB, and FsrC shows 23% identity and 36% similarity to *S. aureus* AgrC (the sensor transducer of Agr system). Northern blot analysis suggested that *gelE* and *sprE* are cotranscribed and that *fsrB* and *fsrC* are also cotranscribed in OG1RF. Northern blot analysis of *fsrA*, *fsrB*, *fsrC*, *gelE*, and *sprE* insertion mutants showed that *fsrB*, *fsrC*, *gelE*, and *sprE* are not expressed in *fsrA*, *fsrB*, and *fsrC* mutants, while insertion in an open reading frame further upstream of *fsrA* did not effect the expression of these genes, suggesting that *agr*-like genes may be autoregulated and that they regulate *gelE* and *sprE* expression, as further confirmed by complementation of *fsr* gene mutations with a 6-kb fragment which contains all three *fsr* genes in the shuttle vector, pAT18. Testing of 95 other isolates of *E. faecalis* showed that 62% produced gelatinase (Gel^+), while 91% (including all Gel^+ strains) hybridized to a *gelE* probe; 71% (including all Gel^+ strains) hybridized to an *fsr* probe, corroborating the conclusion that both *gelE* and *fsr* are necessary for gelatinase production. Testing of *fsrA*, *fsrB*, and *sprE* mutants in a mouse peritonitis model showed that *sprE* and *agr*-like gene mutants resulted in highly significantly prolonged survival compared to the parent strain OG1RF, a finding similar to what we had previously shown for a *gelE* mutant. These results suggest that *sprE* and *agr*-like genes contribute to the virulence of *E. faecalis* OG1RF in this model.

Enterococci are among the major causes of hospital-acquired infections, including urinary tract infections, bloodstream infections, wound infections, and endocarditis (9). *Enterococcus faecalis* is the most common organism recovered from enterococcal infections and accounted for 85 to 90% of clinical isolates prior to emergence of vancomycin resistance in enterococci (9). However, the mechanisms of pathogenesis of enterococci are not yet well understood. In an attempt to study the pathogenesis of enterococcal infections, we have previously generated an insertion mutation of the gelatinase gene (*gelE*) previously reported by Su et al. (21) in *E. faecalis* OG1RF and tested the mutant in a mouse peritonitis model (19). The time course of survival for animals infected with the *gelE* mutant was significantly prolonged compared to that seen with the parent strain OG1RF. To investigate whether the delayed mortality was caused by inactivation of *gelE* or by a polar effect on expression of downstream gene(s), we partially sequenced *gelE* flanking regions. Sequencing from OG1RF and searches in the enterococcal genome database revealed three open reading frames in the region 5′ of *gelE* which show homology to *agrA*, *agrB*, and *agrC* of *Staphylococcus aureus* and a previously reported gene encoding a putative serine protease (*sprE*, direct submission to database) 3′ of *gelE*.

In *S. aureus*, the *agr*/*hld* locus contains five genes, *agrB*, *agrD*, *agrC*, *agrA*, and *hld* (RNAIII), which are all required for the expression of the *agr* (accessory gene regulator) genes and which encode a quorum-sensing system which regulates the expression of virulence factors (5, 11, 12, 16). *S. aureus agrC* and *agrA* encode a sensor transducer and response regulator of bacterial two-component systems (11), respectively, while *agrD* encodes a pheromone peptide which acts as an autoinducer for sensing of cell density by the Agr system (6). The product of *agrB* seems to be involved in the processing of the peptide encoded by *agrD* (5). The Agr regulatory system in *S. aureus* upregulates the expression of secreted proteins such as alpha-toxin, beta-toxin, delta-toxin, enterotoxin B, toxic shock syndrome toxin 1, and serine protease and downregulates surface proteins such as protein A, coagulase, and fibronectin-binding protein (8, 16). Similar Agr systems have also been found in other staphylococcal species, including *S. epidermidis* (13, 22) and *S. lugdunensis* (5, 25).

To study the functions of these *agr*-like genes in *E. faecalis*, we generated insertion mutations of these genes, studied the expression of these genes and of *gelE* and *sprE* in these mutants, and tested the virulence of these mutants in a mouse peritonitis model. Our results suggest that *E. faecalis agr*-like genes, designated *fsr* for *E. faecalis* regulator, regulate the expression of *gelE* and *sprE* and are important for enterococcal virulence in the mouse peritonitis model.

MATERIALS AND METHODS

Strains and media. The strains and plasmids used in this study are listed in Table 1. *E. faecalis* OG1RF has been described previously (10). Luria-Bertani broth and agar were used for *Escherichia coli* culture, and brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) was used for the growth of *E. faecalis* unless otherwise stated. The concentrations of antibiotics used for selection were: ampicillin (Amp), 50 μg/ml; chloramphenicol (Cm), 10 μg/ml; erythromy-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype	Source or reference
Strain		
OG1RF	Gelatinase and serine protease production positive/(Gel$^+$, Spr$^+$)	10
TX5128	*gelE* transposon mini-γδ insertion mutant of OG1RF, Gel$^-$, Spr$^-$, Kan^r	19
TX5240	*fsrA* insertion mutant of OG1RF, Gel$^-$, Spr$^-$, Kan^r	This study
TX5241	*fsrB* insertion mutant of OG1RF, Gel$^-$, Spr$^-$, Kan^r	This study
TX5242	*fsrC* insertion mutant of OG1RF, Gel$^-$, Spr$^-$, Kan^r	This study
TX5243	*sprE* insertion mutant of OG1RF, Gel$^+$, Spr$^-$, Kan^r	This study
TX5248	*orf1* insertion mutant of OG1RF, Gel$^+$, Spr$^+$, Kan^r	This study
TX5244	TX5240 harboring plasmid TEX5249, Gel$^+$, Spr$^+$, Kan^r Em^r	This study
TX5245	TX5241 harboring plasmid TEX5249, Gel$^+$, Spr$^+$, Kan^r Em^r	This study
TX5246	TX5242 harboring plasmid TEX5249, Gel$^+$, Spr$^+$, Kan^r Em^r	This study
TX5247	TX5128 harboring plasmid TEX5249, Gel$^-$, Spr$^-$, Kan^r Em^r	This study
DH5α	*E. coli* host strain for routine cloning	Stratagene
TX5249	DH5α(pTEX5249)	This study
Plasmids		
pBluescript SK(−)	Cloning vector, Amp^r	Stratagene
pTEX4577	Suicide vector for single cross-over mutagenesis in *E. faecalis*, Kan^r	19
pAT18	Shuttle vector for *E. coli* and *E. faecalis*, Em^r	23
pTEX5249	pTEX5249 was constructed by cloning a 6-kb *Pst*I/*Bgl*II fragment containing *fsrA*, *fsrB*, and *fsrC* into the shuttle vector pAT18, Em^r	This study

cin (Em), 250 μg/ml; kanamycin (Kan), 50 μg/ml; tetracycline (Tet) 12.5 μg/ml for *E. coli*; and Cm, 10 μg/ml; Em, 10 μg/ml; and Kan, 2,000 μg/ml for *E. faecalis*.

DNA techniques. Routine isolation of plasmid DNA from *E. coli* was performed as previously described (3). Large-scale preparation of plasmid DNA from equilibrium centrifugation in CsCl-ethidium bromide gradient, gel electrophoresis, and Southern blot analyses was carried out according to previously described methods (17). Radioactive DNA probes were prepared by random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) according to the protocol supplied. Transformation of *E. faecalis* was accomplished by the method described by Li et al. (7) using a Bio-Rad Gene Pulser. Chromosomal DNA from *E. faecalis* was prepared according to the method described by Wilson (26). PCR amplification of DNA was performed on a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.) using synthetic oligonucleotide primers from Genosys (Genosys Biotechnologies, Inc., Woodlands, Tex.) and *Taq* DNA polymerase from Life Technologies (Gaithersburg, Md.).

DNA sequencing and sequence analysis. Automated sequencing was used to determine nucleotide sequence using the dideoxy-chain termination method (15, 18). PCR sequencing reactions were carried out using the *Taq* DyeDeoxy terminator Cycle Sequencing Kit (ABI, Foster City, Calif.), and the reactions were analyzed by an ABI Model 373A DNA sequencer. DNA sequence analysis was accomplished using the Genetics Computer Group sequence analysis package, version 7.2 (University of Wisconsin, Madison). For the DNA and protein homology search, BLAST sequence comparison programs were applied using GenEMBL and/or SWISS-PROT databases. BESTFIT or GAP was used for comparing two DNA or peptide sequences.

Mutagenesis. Target genes in *E. faecalis* OG1RF were disrupted by using a suicide vector pTEX4577 (19) containing internal fragments of the genes of interest. *E. faecalis* OG1RF was transformed by electroporation, and transformants were selected on BHI agar plates containing kanamycin (2,000 μg/ml). Correct allelic disruption was confirmed by Southern blot or PCR analysis. The primers used to amplify internal fragments of *fsrA*, *fsrB*, *fsrC*, *sprE*, and *orf1* were fsrAF1, 5′-GGG AGC TCT GAT GAT GAT TGA TTG ATG GAC; fsrAR1, 5′-GGG GTA CCA TTA CAA GTG GCA CAC CAG GAC; fsrBF1, 5′-GGG AGC TCT GGA CAA AGT ATT ATC TAA CCG; fsrBR1, 5′-TTG GTA CCC ACA CCA TCA CTG ACT TTT GC; fsrCF1, 5′-GGG AGC TCA TCG TGT GTT AGA AAA TAG C; fsrCR1, 5′-GGG GTA CCA CGA ATC ACA ACC ACT AAG TC; sprEF1 5′-TTG AGC TCC GTT CCT GCC GAA AGT CAT TC; sprER1, 5′-TTG GTA CCG ATT GGG GAA CCA GAT TGA CC; orf1F1, TTG AGC TCC TGG TTG GAA ACT AAT CGC ACG; and orf1R1, GGG GTA CCA GTA TGG CAA AGA AGT TTT AGC (linker sequences of the primers are underlined). Revertants of *fsrC* mutant were screened by scoring the restoration of gelatinase activity and the loss of kanamycin resistance.

Detection of gelatinase and serine protease activities. The production of gelatinase in *E. faecalis* strains was scored using Todd-Hewitt (TH) (Difco Laboratories) agar plates containing 3% gelatin. After overnight incubation at 37°C, colonies that had opaque zones around them were considered gelatinase activity positive. Gelatinase and serine protease activity were detected by gelatin (0.1%) and casein (0.05%) zymogram gels (Novex, San Diego, Calif.), respectively. For electrophoresis of zymogram gels, 10 μl of 20-fold-concentrated supernatant from overnight culture was mixed with 10 μl of 2× sample buffer (0.125 M Tris-HCl [pH 6.8], 25% glycerol, 4% sodium dodecyl sulfate, 0.005% bromophenol blue) and incubated for 10 min at room temperature before loading. Renaturing and developing of zymogram gels were carried out as suggested by the supplier. *S. aureus* protease type XVII-B (Sigma) was used as a positive control for serine protease activity.

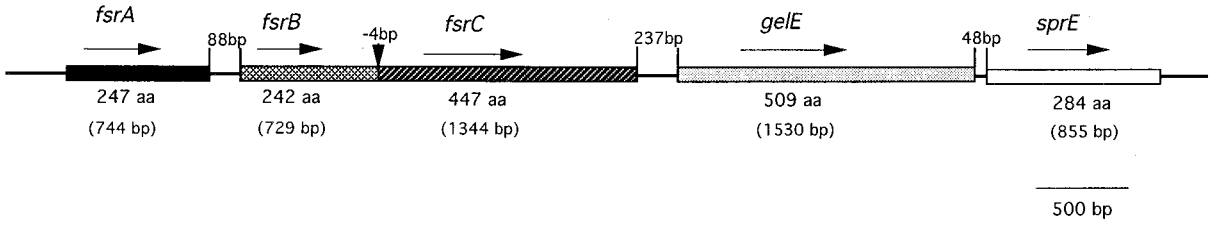
Northern blot analysis and RT-PCR. Total RNA from *E. faecalis* was isolated from cells in exponential phase using RNeasy Mini Kit (Qiagen, Santa Clarita, Calif.) according to the protocol of the supplier with slight modifications. A 6-mg/ml lysozyme solution was used instead of a concentration of 3 mg/ml for the initial lysis step. Total RNA (20 to 40 μg) was treated with 5 U of RQ1 DNase (Promega, Madison, Wis.) for 20 min at 37°C and extracted once with phenol and twice with chloroform-phenol-isoamyl alcohol (25:24:1). Gel electrophoresis, Northern blotting, and hybridization were performed as previously described (17). For reverse transcription PCR (RT-PCR), 50 ng of total RNA was mixed with primers (0.1 μg each), and RT was performed at 37°C for 1 h using Superscript reverse transcriptase (Life Technologies) after heating at 80°C for 5 min. After heat inactivation of the RT mixture at 94°C for 5 min, PCR was performed to amplify cDNA using *Taq* DNA polymerase (Life Technologies). Reactions without reverse transcriptase were used as controls to detect DNA contamination in total RNA. The internal fragments used for mutagenesis of *fsrA*, *fsrB*, *fsrC*, and *sprE* were also used as probes for Northern blot analysis. *gelE* probe for Northern blot analysis was amplified by PCR using gelEF1 (5′-TGG TTG TGA TTC GTT TGT TGG-3′) and gelER1 (5′-TGA ATA AAC TTG TTC TTC TGC G-3′) primers. Two *E. faecalis* autolysin (2) primers, lytF1 (5′-ACA CCA ACC ACA GAA ACT AC-3′) and lytR1 (5′-GGC AAT AAA TTC TGA AGG AC-3′), were used to amplify a 330-bp internal fragment of autolysin as a probe in the control of Northern blot. These two autolysin primers were also used as a positive control in RT-PCR.

Complementation of mutants of *agr*-like genes. To complement the *agr*-like genes *in trans*, a 6-kb *Bgl*II/*Pst*I fragment containing the three *agr*-like genes but only the first 395 bp of the *gelE* gene was cloned into a shuttle vector, pAT18 (Em^r) (23), and transformed into *fsrA*, *fsrB*, *fsrC*, and *gelE* mutants. The Em^r transformants were plated into gelatin containing TH agar plates to determine the complementation of the production of gelatinase activity. The expression of *fsr* and *sprE* genes in the complemented mutants was determined by Northern blot analysis.

Peritonitis model. *E. faecalis* OG1RF and mutants were grown overnight in BHI broth. The cells were harvested by centrifugation, washed once with 0.9% saline, then resuspended in saline to an optical density at 600 nm of 2.2 to 2.8; the CFU of the cell suspensions were determined by plating serial dilutions onto BHI agar plates. Serial dilutions were made in saline and were mixed (1:10) with 50% of sterile rat fecal extract (SRFE) to the desired inocula (19). Groups of 4- to 6-week-old (22 to 25 g), outbred (ICR) female mice were challenged intraperitoneally with different dilutions. A control group of mice was injected with 50% SRFE only. Survival was monitored every 3 to 6 h. Kaplan Meier survival curves and log rank analysis were performed as described previously (19). Results from same-day experiments, when equal inocula or when OG1RF was used at a lower inoculum than a mutant, were compared.

Growth curves. To determine the growth rates of OG1RF and mutants, overnight cultures were diluted 1:20 in BHI and grown at 37°C with shaking. The turbidity was measured at different time points using a Manostat (Manostat, New

A



B

Strain	Size of transcript (kb)				
	fsrA	*fsrB*	*fsrC*	*gelE*	*sprE*
OG1RF	-	2.2	2.2	2.6	2.6
TX5128	-	2.2	2.2	3.8/1.3	-
TX5240	-	-	-	-	-
TX5241	-	-	-	-	-
TX5242	-	-	-	-	-
TX5243	-	2.2	2.2	4.0/2.3	4.0/2.3
TX5248	-	2.2	2.2	2.6	2.6

FIG. 1. Open reading frames and their transcripts in *gelE* flanking regions. (A) Open reading frames. The solid line represents the chromosome, and the genes and open reading frames are indicated by boxes in different shades. The orientation of the genes and open reading frames are indicated by arrows. (B) Summary of the Northern blot results using RNA from different strains and different gene probes. −, No signal in Northern blot analysis.

York, N.Y.). CFU were determined by serial dilution of the cultures at different time points in saline and plating onto BHI agar plates in duplicate.

Colony hybridization. Colony hybridization was carried out as previously described (4) to determine the presence of the genes of interest in clinical isolates. To study the presence of *fsr* genes in clinical isolates, an intragenic *fsrB* fragment was amplified using fsrBF1 and fsrBR1 primers by PCR and used as a probe for colony hybridization. Probes for *gelE* and *sprE* were amplified by PCR using two pairs of primers, primers gelEF1 and gelER1 and primers sprEF1 and sprER1, respectively.

RESULTS

Sequencing and database search of *gelE* flanking regions. Sequencing of the region 3′ of *gelE* of OG1RF revealed an open reading frame of 855 bp (*sprE*) which has been previously deposited in GenBank (accession number Z12296). It encodes a 284-amino-acid peptide which shows homology to bacterial serine proteases. Sequencing and analysis of 2-kb sequence 5′ to *gelE* revealed two open reading frames (Fig. 1A), both oriented in the same direction as *gelE*. The peptide deduced from the first open reading frame (447 amino acids) shows significant homology to histidine kinases of bacterial two-component systems, including AgrC of staphylococci (23% identity and 36% similarity to AgrC of the Agr system in *S. aureus* [accession number AF001783]) (5), and contains residues commonly conserved among these enzymes (Fig. 2). The deduced peptide from the second open reading frame shows 23% identity and 41% similarity to AgrB (accession number AF001782) (5) (Fig. 2), which is unique for the Agr system of staphylococci; thus, we designated them as *fsrC* and *fsrB* (*E. faecalis* regulator; GenBank accession number AF108141). A BLAST search in the *E. faecalis* genome database of The Institute for Genomic Research using this 2-kb sequence 5′ of *gelE* gave a perfect match to a contig and the analysis of the sequence further upstream of this 2-kb region showed one open reading frame (designated *fsrA*) encoding a peptide of 247 amino acids with homology to response regulators of bacterial two-component systems such as AgrA of *S. aureus* (5), including highly conserved N-terminal residues of Asp and Lys (Fig. 1A and 2). Another open reading frame encoding 652 amino acids was found further upstream of *fsrA*, termed *orf1*, which shows homology to bacterial *N*-acetylmuramidases. Putative promoter sequences were found upstream of *fsrA* and *fsrB* but not upstream of *fsrC* or *sprE*. In fact, *fsrB* and *fsrC* overlap by 4 bp. The observation that the open reading frames in the *gelE* 5′ region show homology to all of the *agr* genes except *agrD* of the *S. aureus agr* locus, which functions as a virulence regulator, suggested that this locus in *E. faecalis* may have a similar regulatory function.

Disruption of *fsr* genes and *sprE* and detection of gelatinase and serine protease activities. *E. faecalis* serine protease and *agr*-like genes were disrupted by transforming OG1RF with internal fragments of the target genes in suicide vector pTEX4577 using selection for Kan resistance. All the Kan^r transformants analyzed by Southern blot analysis were found to have the correct insertions in the target genes (data not shown). The resulting mutants are listed in Table 1.

Gelatinase activity was evidenced on TH agar plates containing 3% gelatin and on a gelatin zymogram gel for the parent strain OG1RF and the *sprE* and *orf1* mutants (TX5243 and TX5248) (Fig. 3A), indicating that the disruption of *sprE* and *orf1* did not effect *gelE* expression. However, *fsrA*, *fsrB*, and *fsrC* mutants (TX5240, TX5241, and TX5242) did not show detectable gelatinase activity either on gelatin containing TH agar plates or on a gelatin zymogram gel (Fig. 3A). Similarly, serine protease activity was not detected for *agr*-like gene mutants (Fig. 3B), while OG1RF and the *orf1* mutant had two lysis bands (26 and 20 kDa) on a casein zymogram gel, suggesting a possible regulatory effect or polar effect of *fsrA*, *fsrB*, and *fsrC* on the expression of *gelE* and *sprE*. Neither the *sprE* (TX4243) mutant nor the previously described *gelE* mutant (19) showed serine protease activity on a casein zymogram gel (Fig. 3B),

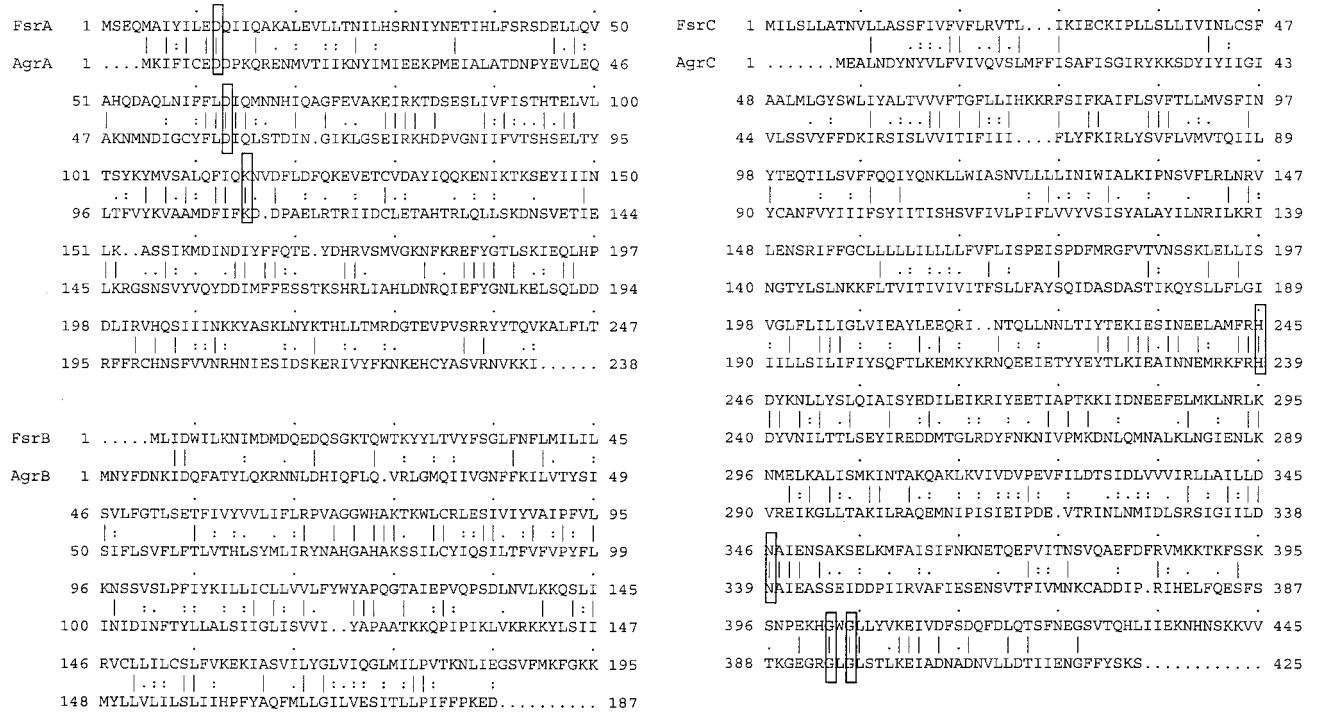


FIG. 2. Sequence alignment of FsrA, FsrB, and FsrC with AgrA, AgrB, and AgrC from *S. aureus*. Identical amino acids are indicated by a symbol as "|", and similar amino acids are indicated by a symbol as ":" or "." between them. Conserved residues in H, N, and G2 blocks of histidine protein kinases and conserved Asp and Lys residues in response regulators of bacterial two-component systems (20) are boxed. The sequences from *S. aureus* were as follows: AgrA, accession number M21854 (14); AgrB, accession number AF001782 (5); and AgrC, accession number AF001783 (5).

suggesting that the insertion in *gelE* has a polar effect on *sprE* expression. The molecular mass of 26 kDa of the higher band shown on the casein gel for OG1RF matches the predicted size of the product of *sprE*. The lower band may be the mature or degraded form of the protease.

Two revertants from the *fsrC* mutant detected by scoring for restoration of gelatinase activity were found to be sensitive to kanamycin and to have restored the gelatinase activity. One of them was subsequently used in the animal experiment.

Northern blot analysis and RT-PCR. Northern blot analysis was conducted to investigate whether the loss of gelatinase activity in the *fsr* gene mutants was caused by polar effects.

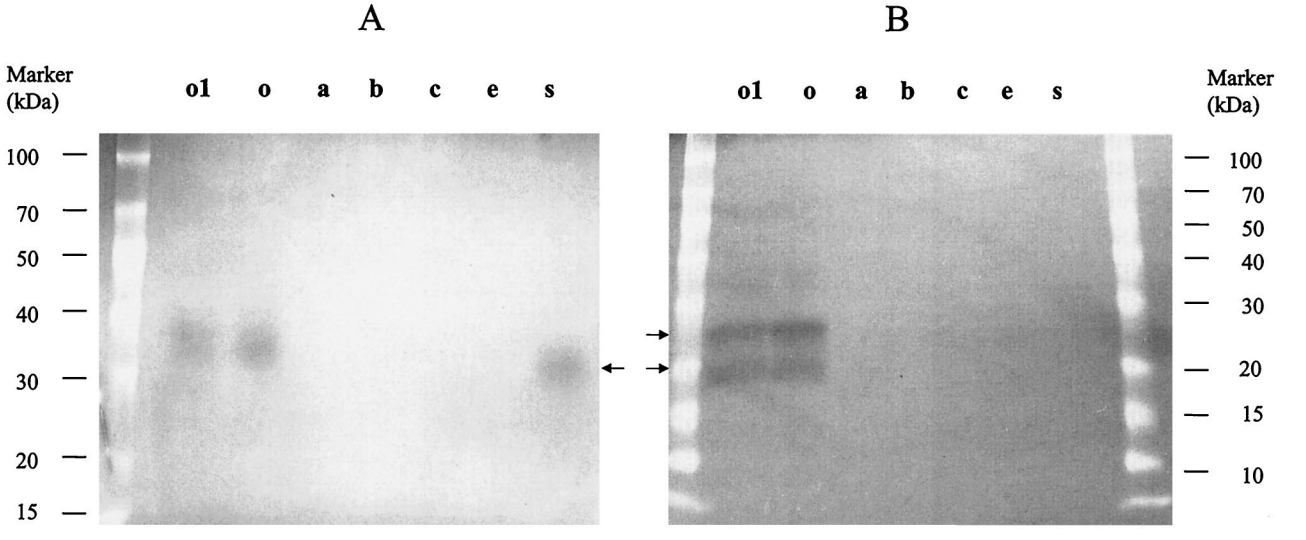


FIG. 3. Gelatinase and serine protease activities in *fsr* and *sprE* mutants. These pictures are the negative images of gelatin and casein zymogram gels. (A) Gelatinase activity of mutants on gelatin zymogram gel. (B) Serine protease activity of mutants on casein zymogram gel. Lanes: o1, mutant with disruption of *orf1*; o, OG1RF; a, mutant with disruption of *fsrA*; b, mutant with disruption of *fsrB*; c, mutant with disruption of *fsrC*; e, mutant with disruption of *gelE*; s, mutant with disruption of *sprE*.

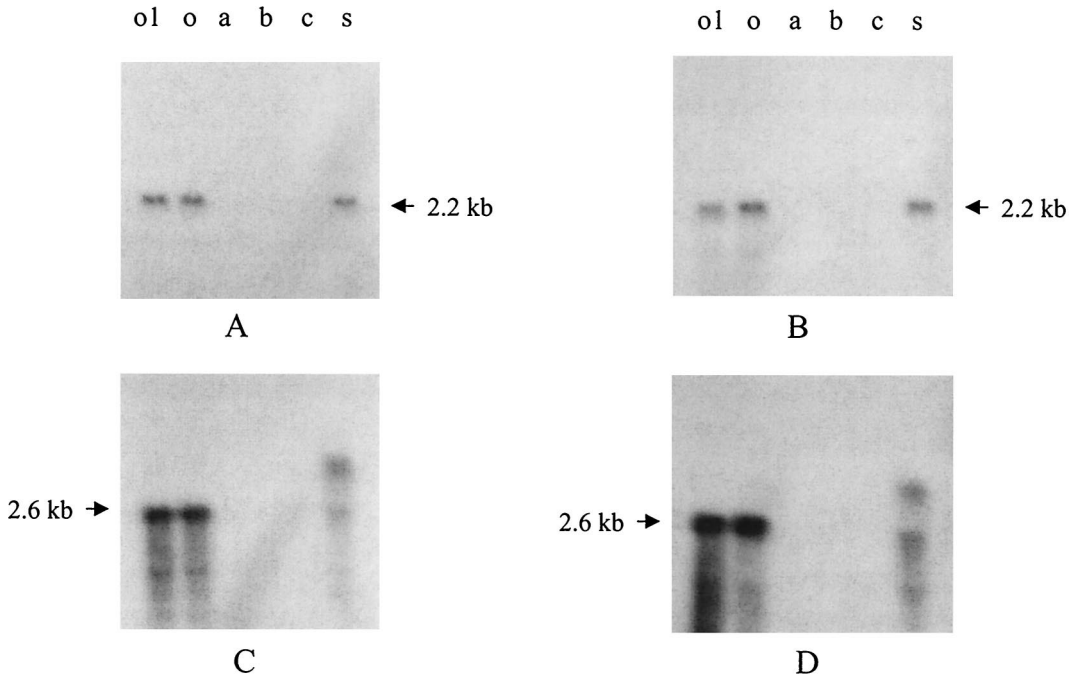


FIG. 4. Northern blot analysis of *fsr* and *sprE* mutants. (A, B, C, and D) Northern blots of mutants using *fsrB*, *fsrC*, *gelE*, and *sprE* probes, respectively. Lanes: o1, mutant with disruption of *orf1*; o, OG1RF; a, mutant with disruption of *fsrA*; b, mutant with disruption of *fsrB*; c, mutant with disruption of *fsrC*; s, mutant with disruption of *sprE*.

Results of blots of total RNA from TX5240, TX5241, TX5242, TX5243, TX5248, TX5128, and OG1RF hybridized with internal fragments of *fsrA*, *fsrB*, *fsrC*, *gelE*, *sprE*, and *orf1* are summarized in Fig. 1B. Hybridization using *fsrB* and *fsrC* probes gave a 2.2-kb band for wild-type OG1RF, *gelE*, *sprE*, and *orf1* mutants (Fig. 4, data not shown for the *gelE* mutant), suggesting that *fsrB* (729 bp) and *fsrC* (1,344 bp) are cotranscribed and that disruption of *orf1*, *gelE*, and *sprE* does not effect the expression of *fsrB* and *fsrC*. Interestingly, expression of *fsrB* and *fsrC* was not detectable in *fsrA*, *fsrB*, or *fsrC* mutants (Fig. 4), suggesting autoregulation of *fsr* genes. A Northern blot was not able to detect expression of *fsrA* or *orf1* in either OG1RF or *fsr* gene mutants. However, RT-PCR showed that *fsrA* was expressed in OG1RF (data not shown), suggesting that *fsrA* is expressed at a low level, which is different from *fsrB* and *fsrC*.

Northern blot analysis using *gelE* and *sprE* probes gave a single 2.6-kb band for OG1RF (Fig. 4), which is approximately 200 bp greater than the sum of sizes of *gelE* (1531 bp) and *sprE* (855 bp), suggesting that *gelE* and *sprE* are cotranscribed. The *gelE* and *sprE* mRNA was not detectable in *fsrA*, *fsrB*, and *fsrC* mutants (Fig. 4), suggesting that these genes may regulate the expression of *gelE* and/or *sprE*. The *gelE* probe hybridized with two bands in the *gelE* and *sprE* mutants (1.3 and 3.8 kb and 2.3 and 4.0 kb, respectively) (Fig. 4, data not shown for the *gelE* mutant). The lower bands were the truncated form of the *gelE* transcript since it only hybridized to the *gelE* probe but not to the kanamycin resistance gene probe from the suicide vector (data not shown), while the higher band was the fusion of the truncated transcript and the readthrough to the insertion sequence since it also hybridized with the kanamycin resistance gene (data not shown). Similarly, the *sprE* probe hybridized with two bands (2.3 and 4.0 kb) in the *sprE* mutant (Fig. 4). No *sprE* mRNA was detected in the *gelE* mutant (data not shown), indicating a polar effect on *sprE* by insertion in *gelE*.

Complementation of *agr*-like genes. To further confirm that abolishing *gelE* and *sprE* expression in *fsr* gene mutants was due to a regulatory effect, not a polar effect, complementation was carried out using a 6-kb *Pst*I/*Bgl*II fragment in shuttle vector pAT18 (pTEX5249) containing *fsrA*, *fsrB*, and *fsrC* genes. *fsrA*, *fsrB*, and *fsrC* mutants which harbored plasmid pTEX5249 (TX5244, TX5245, and TX5246) all produced gelatinase activity as detected by TH agar containing 3% gelatin (data not shown), indicating that *fsr* gene function can be provided *in trans*. Northern blot analysis also showed that *fsrB* and *sprE* were expressed in complemented *fsr* gene mutants (data not shown). No gelatinase activity was detected in the *gelE* mutant harboring pTEX5249 (TX5247). These results further confirm that *fsr* genes positively regulate the expression of the *gelE* and *sprE* genes.

Animal model and growth curves. Using a mouse peritonitis model, we studied the virulence of *fsr* and *sprE* mutants in comparison to OG1RF. In general, the mutants had a similar 50% lethal dose to the parent strain OG1RF. The time course of survival for TX5248 was also similar to that of OG1RF (data not shown), suggesting that *orf1* does not play an important role in enterococcal infection in this animal model. However, the time courses of survival for TX5240 (*fsrA* mutant), TX5241 (*fsrB* mutant), and TX5243 (*sprE* mutant) were significantly prolonged compared to the parent strain OG1RF (e.g., $P < 0.0001$ by log rank for survival at the inocula of 5.5×10^8 CFU and 4.0×10^8 of TX5240 and TX5241 versus 4.0×10^8 CFU of OG1RF [$n = 12$]; $P < 0.0001$ at the inocula of 1.1×10^9 and 8.0×10^8 of TX5240, TX5241 versus 6.1×10^8 of OG1RF [$n = 12$]; $P < 0.0001$ at the inocula of 6.0×10^8 and 1.2×10^9 CFU of TX5243 versus 4.3×10^8 and 6.5×10^8 of OG1RF [$n = 12$]) (Fig. 5). The same highly significant difference was also noted for the *fsrC* revertant compared to the mutants. The delays in lethality were also seen when groups of six animals were used

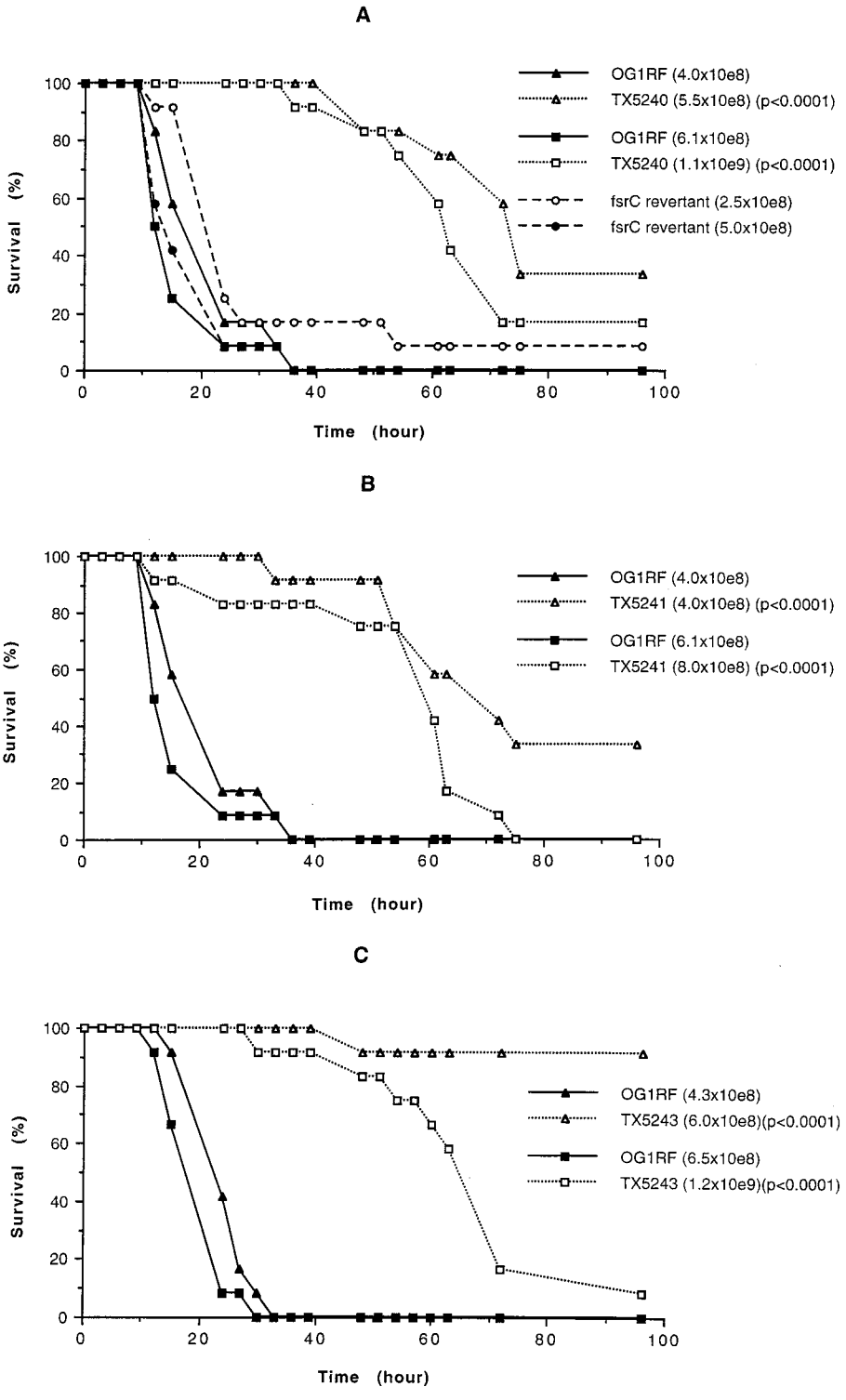


FIG. 5. Kaplan-Meier survival plots of wild-type OG1RF and *fsr* and *sprE* mutants in the mouse peritonitis model. (A) Kaplan-Meier survival plots of OG1RF, the *fsrA* mutant (TX5240) and an *fsrC* revertant. (B) OG1RF and the *fsrB* mutant (TX5241). (C) OG1RF and the *sprE* mutant (TX5243). Twelve mice were tested with each inoculum of each of the strains shown. The *P* value refers to mutant versus OG1RF at the same or a smaller inoculum (▲ versus △ or ■ versus □).

in another trial (data not shown). In separate experiments testing TX5242 (*fsrC* mutant), there was also a trend to delayed death with groups of six mice ($P = 0.0787$, 0.0569, and 0.0835 at inocula of 6.2×10^8, 1.2×10^9, and 2.5×10^9 of TX5242 versus 5.7×10^8, 1.1×10^9, and 2.3×10^9 of OG1RF, respectively); when a single inoculum was tested with 20 mice, the delay was significant ($P = 0.0040$), but the inoculum of OG1RF was slightly higher than TX5242 (4.2×10^9 of TX5242

versus 4.8×10^9 of OG1RF) (data not shown). Because all results for the *fsrC* mutant showed a trend similar to *fsrA* and *fsrB* mutants, these results were not repeated so that additional animals would not be subjected to this lethality assay. The revertant from TX5242 (*fsrC* mutant) whose gelatinase activity was restored was also used as a control in the experiments and the time course to death for the revertant was the same as OG1RF (Fig. 5), indicating that the prolonged survival was not caused by some other mutation that may have occurred during the generation of the mutants.

To make certain that the delay in death in the animal model was not caused by a growth defect of the mutants, we determined the growth rates of *fsr* and *sprE* mutants in comparison to that of OG1RF. The growth rates of the mutants were the same as that of OG1RF (data not shown), which further suggests that *agr*-like genes and *sprE* are virulence factors.

Comparison of colony hybridization results with gelatinase phenotype. Lysates of 152 *E. faecalis* isolates were hybridized with *fsrB*, *gelE*, and *sprE* probes. A total of 105 isolates (~69%) were positive with the *fsrB* probe, while 141 (~93%) isolates were positive with *gelE* and *sprE* probes, respectively. All isolates that hybridized with the *fsrB* probe were also *gelE* and *sprE* probe positive, and *gelE* and *sprE* were both found in the same isolates. Based on these and previous results (4) and retesting of 95 isolates for gelatinase activity and 32 isolates (from the 95 isolates) for the presence of *fsrB* and *gelE* by PCR, 62% (59 of 95), 71% (67 of 95), and 91% (86 of 95) were gelatinase, *fsrB*, and *gelE* positive, respectively. No isolates lacking *gelE* or *fsrB* produced gelatinase, but eight isolates which hybridized to both *gelE* and *fsrB* probes were gelatinase negative.

DISCUSSION

The *agr* loci in *S. aureus* (11, 12, 14), *S. epidermidis* (13, 24), and *S. ludgunensis* (25) contain five genes (*agrA*, *agrB*, *agrD*, *agrC*, and *hld*), whose sequences and organization are similar to each other. The three *agr*-like genes (*fsrA*, *fsrB*, and *fsrC*) that we found in *E. faecalis* OG1RF show significant homology to three *agr* genes (*agrA*, *agrB*, and *agrC*) in staphylococci, although considerably less than is seen among the staphylococcal homologs. FsrA and FsrC resemble response regulators and sensor transducers of bacterial two-component systems, respectively. FsrC was found to have the greatest similarity to AgrC of staphylococci, although FsrA showed greater similarity to some other response regulators than to AgrA. The organization of the *fsr* genes in *E. faecalis* is also different. That is, *fsrA* (the *agrA* analog) is upstream of *fsrB* and *fsrC* (the *agrB* and *agrC* analogs) in *E. faecalis* instead of downstream of these genes as in staphylococci.

In *S. aureus*, *agrB*, *agrD*, *agrC*, and *agrA*, which are autoregulated, are under the control of a promoter designated P2 and *agrA* is also transcribed individually from a different promoter, P1 (11, 12, 14, 24). Our Northern blot and RT-PCR analyses suggest that *fsrB* and *fsrC* are cotranscribed and that *fsrA* may be transcribed from a promoter different from that of *fsrB* and *fsrC*, since its expression level was much lower than that of *fsrB* and *fsrC*. Northern blot analysis of *fsrA*, *fsrB*, and *fsrC* mutants suggests that the *fsr* genes in *E. faecalis* may also be autoregulated since insertion in *fsrC* abolished the expression of *fsrB*, which is upstream of *fsrC*, and disruption of *fsrA* also terminated the expression of *fsrB* and *fsrC*, although the latter two are on a transcript that does not contain *fsrA*. A transcript, RNAIII, which is transcribed from *hld* upstream of *agrB* and encodes delta-hemolysin in the *agr*/*hld* locus in *S. aureus* has been demonstrated to be the effector for the Agr response (12). Insertion mutagenesis of *gelE*, *sprE*, and *orf1* in flanking regions of the *fsr* locus and Northern blot analysis of the mutants indicate that the transcripts of *fsr* flanking regions are not responsible for the autoregulated function of the locus since *fsrB* and *fsrC* were both expressed in these mutants as detected by Northern blot. In addition, no *agrD* or *hld* homolog was found by search in the *E. faecalis* genome database. In different groups of *S. aureus*, which can be divided based on their cross-activation and cross-inhibition of the Agr response (5), the *agrD* homologs (which encode the precursor of an autoinducing peptide of these different groups) show very limited sequence resemblance, suggesting that the peptide inducer of *E. faecalis* would also be different from those of *S. aureus*. Sequence analysis of the putative FsrB of *E. faecalis* and AgrB of *S. aureus* reveals that FsrB is about 50 amino acids longer than AgrB and that ca. 50 amino acids at the C terminus of FsrB do not show sequence homology to AgrB. This raises the possibility that this portion of FsrB, which is approximately the size of the autoinducing peptide precursor encoded by *agrD* in staphylococci, could be the precursor of an autoinducing peptide in *E. faecalis* OG1RF if, indeed, there is a inducer in *E. faecalis*.

We have demonstrated here that the *fsr* locus in *E. faecalis* OG1RF positively regulates the expression of *gelE* and *sprE*, since the expression of both *gelE* and *sprE* was abolished in *fsrA*, *fsrB*, and *fsrC* mutants as detected by Northern blot analysis. Gelatinase and serine protease activities were also undetectable in the *fsr* mutants. Complementation experiments indicate that the elimination of *gelE* and *sprE* expression was not due to a polar effect of the insertions in *fsr* mutants since the expression of *gelE* and *sprE* could be restored by providing the *fsr* genes *in trans*. These results suggest that the *fsr* locus in *E. faecalis* may have a regulator function similar to that of the *agr* locus in *S. aureus*, which positively regulates the expression of secreted proteins. We have not yet tested the effect of the insertions in the *fsr* genes on other surface or secreted proteins in *E. faecalis*.

In a previous study, we had shown that an insertion mutant of *gelE* had a significantly delayed time to death in a mouse peritonitis model (19), which suggests that *gelE* or gene(s) downstream of *gelE* are important for this aspect of the infection. The fact that expression of *sprE* was not detected in the *gelE* mutant and that the gelatinase-positive mutant with an insertion in *sprE* also showed delayed killing indicates that *sprE* is important in the infection in this animal model. However, we still do not know if *gelE* independently influences the outcome of this enterococcal infection. Testing of a *gelE* deletion mutant may provide further information to address this question. In *S. aureus*, an *agr* mutant has been shown to have a significant effect on infection compared to the wild-type strain as shown by a murine arthritis model (1). We found that insertions in the *fsr* genes also caused significant delays in killing in the mouse peritonitis model and that the pattern of delay was similar to that of *gelE* and *sprE* mutants. Since the *agr* locus in *S. aureus* controls the expression of several virulence properties, some of which are activated while some are inhibited (8), the effects of *agr* mutants in animal models may not be attributable to a simple activation or inhibition effect. Further study is needed to address whether the *fsr* genes in *E. faecalis* also regulate other (virulence) factors besides *gelE* and *sprE* and if regulation occurs in a fashion similar to that of *S. aureus agr* system.

It was interesting to note that the presence of *fsrB* was lower than that of *gelE* and *sprE* in our survey of 152 clinical isolates of *E. faecalis* (69% versus 93 and 93%). Of 95 isolates retested for gelatinase in this study, 59 (62%) were gelatinase production positive and all 59 were *fsrB* positive, a finding consistent

with the genetic data obtained with strain OG1RF, indicating that *fsr* genes are also required for the expression of *gelE* gene in *E. faecalis* clinical isolates. No isolates lacking *gelE* produced detectable gelatinase. Whether expression of *gelE* can be elicited in *fsr* lacking strains under other conditions has yet to be determined.

In conclusion, *E. faecalis fsr* genes positively regulate the expression of *gelE* and *sprE* which are important for enterococcal infection in the mouse peritonitis model and these *fsr* genes appear to be autoregulated.

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