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Characterization of *fsr*, a Regulator Controlling Expression of Gelatinase and Serine Protease in *Enterococcus faecalis* OG1RF

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We have previously identified a locus, *fsr*, a homologue of staphylococcal *agr* loci, which positively regulates the expression of gelatinase and serine protease (encoded by *gelE* and *sprE*, respectively) in *Enterococcus faecalis* OG1RF. The expression of the three genes in the *fsr* locus, *fsrA*, *fsrB*, and *fsrC*, appears to be autoregulated, and we have shown that mutants with insertion disruptions in each of these three genes were significantly attenuated in a mouse peritonitis model compared to the parent strain. In the present study, we showed that *fsrB* and *fsrC* are highly expressed in the postexponential growth phase and that their expression is cell density dependent. Reverse transcriptase PCR using primers covering the intergenic regions in the *fsr*/*gelE* loci confirmed that *fsrB* and *fsrC*, as well as *gelE* and *sprE*, are cotranscribed. We also showed, using a nonpolar *fsrB* deletion mutant, that *fsrB*, the homologue of *agrB* of staphylococci with unknown function, is required for the regulatory function of *fsr*. Primer extension and analysis of transcriptional fusions indicated the presence of promoters immediately upstream of *fsrA*, of *fsrB*, and of *gelE* and that the *fsrB* and *gelE* promoters are *fsr* dependent, while the *fsrA* promoter is an *fsr*-independent weak constitutive promoter. Two conserved 7-bp direct repeats were found immediately upstream of the *fsrB* and *gelE* promoters, similar to the repeats found upstream of P2 and P3 promoters of the *agr* locus; deletions and mutations in the repeated sequences completely abolished the *fsrB* and *gelE* promoter activities, suggesting that the repeats are important for the regulatory function in the *fsrB* and *gelE* promoter regions.

Enterococci are one of the leading causes of nosocomial infections, including urinary tract infections, bloodstream infections, wound infections, and endocarditis (12). In a previous study, we identified a locus, *fsr* (*Enterococcus faecalis r*egulator), which contains three *agr*-like genes (22), immediately upstream of *gelE* (Fig. 1), which encodes a gelatinase (28), in *E. faecalis* strain OG1RF. These three *agr*-like genes, *fsrA*, *fsrB*, and *fsrC*, which show homology to *agrA*, *agrB*, and *agrC*, respectively, in the *agr* (accessory gene regulator) locus of *Staphylococcus aureus*, appeared to be autoregulated and to regulate the expression of *gelE* and *sprE*, a gene encoding a serine protease, which is downstream of *gelE* and which appeared to be cotranscribed with *gelE* (22). Mutants with insertion disruptions in these *fsr* genes showed significant delays in lethality in a mouse peritonitis model compared to wild-type OG1RF (22).

S. aureus agr/*hld* loci temporally control the expression of various virulence factors by positively regulating the expression of secreted proteins such as alpha-toxin, β-toxin, δ-toxin, enterotoxin B, toxic shock syndrome toxin 1, and a serine protease and by negatively regulating the expression of surface proteins such as protein A, coagulase, and fibronectin-binding protein in the postexponential growth phase (3, 7, 8, 10, 23). The *agr* locus in *S. aureus* consists of four genes, *agrA*, *agrB*, *agrC*, and *agrD*, which all appear to be required for the Agr function (15, 16, 19, 23). The expression of *agr* genes is autoregulated, and the expression of *agrB*, *agrC*, and *agrD* is driven by *agr*-dependent promoter P2, while another *agr*-dependent promoter, P3, in the opposite direction from P2, regulates the expression of the P3 transcript (referred to as RNAIII), which is the real effector of the Agr response (15, 16). The expression of *agrA* appears to be driven by a weak constitutive promoter upstream of *agrA* (19). The *agr* genes encode a quorum-sensing system in which an autoinducing peptide encoded by *agrD*, possibly processed and/or secreted by AgrB, functions as an autoinducer to activate the expression of the *agr* genes and RNAIII (5, 6). AgrC and AgrA, which are the sensor transducer and the response regulator of typical bacterial two-component systems, respectively, are thought to sense cell density through the autoinducing peptide and subsequently regulate the expression of virulence properties (5, 15, 16). Based on the cross-activation and cross-inhibition by autoinducing peptides, *S. aureus* strains can be divided into at least three different groups, in which the pheromones from the strains in one group cross-activate the *agr* expression of other strains in that group but inhibit the *agr* expression of strains in the other groups (5).

In this work, we investigated whether *fsrB* of *E. faecalis* is required for *fsr* functions by deletion mutagenesis. We also characterized the *fsr*/*gelE* loci by reverse transcriptase PCR (RT-PCR), Northern blot analysis, primer extension, and gene fusion analyses. Our results suggest that *fsrB* is required for *fsr* functions, that the expression of *fsrA*, *fsrB*, *fsrC*, *gelE*, and *sprE* is driven by three different promoters, and that the expression of *fsrB* and *fsrC* is cell density dependent. Our data also suggest that two 7-bp direct repeats upstream of *fsrB* and *gelE* promoters are important for the regulation of *fsrB* and *gelE* expression.

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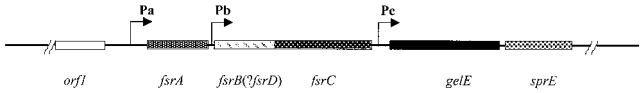

FIG. 1. Open reading frames in *fsr/gelE* loci. Line, chromosome; boxes, genes and open reading frames; ?*fsrD*, possible *agrD* homologue at the 3′ end of *fsrB*; arrows, promoters indicating directions of transcription. Pa, *fsrA* promoter; Pb, *fsrB* promoter; Pe, *gelE* promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Most of the bacterial strains and plasmids used in this study are listed in Table 1. *E. faecalis* OG1RF has been described previously (13); an additional eight gelatinase-positive (Gel^+) *E. faecalis* strains from different clinical sources and geographical areas shown by pulsed-field gel electrophoresis to represent distinct strains were also used. *Escherichia coli* DH5α was used as the host strain for routine cloning. Shuttle vector pTCV-*lac* (20), which contains a promoterless *lacZ*, was used for detection of promoter activity in *E. faecalis*. Luria-Bertani broth and agar were used for *E. coli* culture, and brain heart infusion (BHI; Difco Laboratories, Detroit, Mich.) was used for *E. faecalis* culture unless otherwise stated. The concentrations of antibiotics used for selection were as follows: ampicillin, 50 μg/ml (*E. coli*); erythromycin, 250 (*E. coli*) and 10 μg/ml (*E. faecalis*); kanamycin (KAN), 50 (*E. coli*) and 2,000 μg/ml (*E. faecalis*).

DNA techniques. Routine isolation of plasmid DNA from *E. coli* was performed as previously described (2). Large-scale preparation of plasmid DNA was carried out using the Midi kit or Maxi kit (Qiagen, Valencia, Calif.). Transformation of *E. faecalis* was accomplished by the method described previously by Li et al. (9) using a Gene Pulser (Bio-Rad, Hercules, Calif.). Genomic DNA from *E. faecalis* was prepared according to the method described by Wilson (29). PCR amplification of DNA was performed on a DNA thermal cycler (Perkin-Elmer Corp., Norwalk, Conn.) using synthetic oligonucleotide primers and *Taq* DNA polymerase from Life Technologies (Gaithersburg, Md.). The primers used in the PCR amplification are listed in Table 2.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant properties	Reference or source
Strains		
E. coli		
DH5α	*E. coli* host strain for pBluescript SK(−)	Stratagene
DH5α(pTCV-*lac*)	*E. coli* DH5α containing plasmid pTCV-*lac*	20
TX4577	*E. coli* DH5α containing plasmid pTEX4577	27
E. faecalis		
OG1RF	Gel^+ serine protease positive (Spr^+) Rif^r Fus^r	13
TX5240	OG1RF *fsrA* mutant with pTEX4577 insertion in *fsrA*; Gel^- Spr^- Kan^r	22
TX5241	OG1RF *fsrB* mutant with pTEX4577 insertion in *fsrB*; Gel^- Spr^- Kan^r	22
TX5242	OG1RF *fsrC* mutant with pTEX4577 insertion in *fsrC*; Gel^- Spr^- Kan^r	22
TX5266	OG1RF *fsrB* deletion mutant, deletion from bp 79 to 684 of *fsrB*; Gel^- Spr^-	This study
Plasmids		
pBluescript SK(−)	Cloning vector; Amp^r	Stratagene
pTEX4577	Suicide vector in *E. faecalis* derived from pBluescript SK(−); Kan^r	27
pTCV-*lac*	Shuttle vector containing promoterless *lacZ*; Kan^r Ery^r	20
pTEX5267	pTEX4577 containing *fsrB* flanking regions (917-bp 5′ region: bp −839 to +78, amplified using BDF1 and BDR1 primers; 1,065-bp 3′ region: 45 bp before stop codon to 1,020 bp after stop codon, amplified using DBF2 and DBR2 primers), used for construction of *fsrB* deletion mutant; Kan^r	This study
pTEX5268	*fsrA* promoter cloned upstream of *lacZ* in pTCV-*lac*, from bp −406 to −6 (401 bp, amplified using APRF1 and APRR1 primers) relative to *fsrA* start codon; Kan^r Ery^r	This study
pTEX5269	*fsrB* promoter cloned upstream of *lacZ* in pTCV-*lac*, from bp −110 to −8 (103 bp, amplified using BPRF1 and BPRR1 primers) relative to *fsrB* start codon; Kan^r Ery^r	This study
pTEX5270	*gelE* promoter cloned upstream of *lacZ* in pTCV-*lac*, from bp −218 to −16 (203 bp, amplified using EPRF1 and EPRR1 primers) relative to *gelE* start codon; Kan^r Ery^r	This study
pTEX5298	*fsrB* promoter region (bp −90 to −8 relative to *fsrB* start codon, amplified using BPRF1 and BMP1 primers) cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5299	*fsrB* promoter region (bp −72 to −8 relative to *fsrB* start codon, amplified using BPRF1 and BMP2 primers) cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5300	*fsrB* promoter region (bp −85 to −8 relative to *fsrB* start codon, amplified using BPRF1 and BMP4 primers) cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5301	*fsrB* promoter region (bp −91 to −8 relative to *fsrB* start codon in which bp −70 to −65 were altered from AAGGAA to TTCCTT, amplified using BPRF1 and BMP5 primers) cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5302	*fsrB* promoter region (bp −72 to −8 relative to *fsrB* start codon) with putative *gelE* promoter regulatory region (bp −188 to −170 relative to *gelE* start codon), amplified using BPRF1 and BMP6 primers, cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5303	*gelE* promoter region (bp −188 to −16 relative to *gelE* start codon, amplified using EPRF1 and EMP1 primers) cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5304	*gelE* promoter region (bp −170 to −16 relative to *gelE* start codon, amplified using EPRF1 and EMP2 primers) cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5305	*gelE* promoter region (bp −188 to −16 relative to *gelE* start codon in which bp −167 to −161 were altered from AAGGAA to TTCCTT, amplified using EPRF1 and EMP5 primers) cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study
pTEX5306	*gelE* promoter region (bp −170 to −16 relative to *gelE* start codon) with putative *fsrB* promoter regulatory region (bp −91 to −73 relative to *fsrB* start codon), amplified using EPRF1 and BMP6 primers, cloned upstream of *lacZ* in pTCV-*lac*; Kan^r Ery^r	This study

TABLE 2. Primers

Primer	Length (mer)	Sequence (5′–3′)[a], location, and relevant properties	Reference or source
Used in *fsrB* deletion mutagenesis			
BDF1	31	AAA GAG CTC GAA AGG GAT GAG TGA ACA AAT G, from bp −839 to −818 to start codon of *fsrB*, outer primer used for amplification of *fsrB* 5′ region	This study
BDR1	31	CCG AAT TCC TTT GTC CAT TGT GTT TTT CCT G, from bp 78 to 56 in *fsrB*, inner primer used for amplification of *fsrB* 5′ region	This study
BDF2	31	CCG AAT TCT GGA TGG GAC AAA CTG AAA AAC A, from bp 54 to 23 before *fsrB* stop codon, inner primer used for amplification of *fsrB* 3′ region	This study
BDR2	31	CCG GTA CCT AGC CAA CAA ACG AAT CAC AAC C, from bp 1020 to 999 after *fsrB* stop codon, outer primer used for amplification of *fsrB* 3′ region	This study
Used for cotranscription study			
fsrARTF1	22	ACA ATA CTT CTA ACG CTT TTG C, from bp 70 to 49 after *fsrA* start codon	This study
orf1RTR1	20	TAG TGG CGT GAC CTA CAT TG, from bp 28 to 9 before *orf1* stop codon	This study
fsrBRTF1	21	GAT CTT CCT GAT CCA TAT CCA, from bp 52 to 32 after *fsrB* start codon	This study
fsrARTR1	23	CAA GGC ACT ATT TCT TAC TTA GG, from bp 22 before *fsrA* stop codon to bp 1 after *fsrA* stop codon	This study
fsrCRTF1	21	ACT CGT AAA AAG ACA AAA ACG, from bp 71 to 51 after *fsrC* start codon	This study
fsrBRTR1	20	TGA TGG TGT GGG AAC TAA GC, from bp 103 to 84 before *fsrB* stop codon	This study
gelERTF1	21	AGT ATT GCT TTA TCC TCC CTA, from bp 51 to 71 before *gelE* start codon	This study
fsrCRTR1	21	CTA AAA GTA ATC CAG AAG AGC, from bp 164 to 144 before *fsrC* stop codon	This study
sprERTF1	22	ATC AGA AAC AAA AAA CCA GCA C, from bp 74 to 53 after *sprE* start codon	This study
gelERTR1	19	CTC GTG ATG CGA TGC TTG C, from bp 148 to 130 before *gelE* stop codon	This study
Used for primer extension study			
APE1	22	ACA ATA CTT CTA ACG CTT TTG C, from bp 70 to 49 after *fsrA* start codon	This study
BPE2	21	TAA GCC AGA AAA ATA AAC GGT, from bp 108 to 88 after *fsrB* start codon	This study
EPE2	21	CCA ACA AAG ATG CCT GTA CCT, from bp 50 to 30 after *gelE* start codon	This study
Used for study of promoter activity			
APRF1	32	CGG GAT CCT CTC AAG AAG ATA TAA AAT TAC AC, from bp −6 to −29 to *fsrA* start codon, used for amplification of *fsrA* promoter	This study
APRR1	28	CGG AAT TCG CGA CTC ATT CAA CAG GAA G, from bp −406 to −387 to *fsrA* start codon, used for amplification of *fsrA* promoter	This study
BPRF1	29	CGG GAT CCT CCT CTT CAA GTA TTG CAC TA, from bp −8 to −28 to *fsrB* start codon, used for amplification of *fsrB* promoter	This study
BPRR1	30	CGG AAT TCC AAG GCA CTA TTT CTT ACT TAG, from bp −110 to −89 to *fsrB* start codon, used for amplification of *fsrB* promoter	This study
EPRF1	30	CGG GAT CCT TCC CCA GTT TCC TTT TAT TTC, from bp −16 to −37 to *gelE* start codon, used for amplification of *gelE* promoter	This study
EPRR1	29	CGG AAT TCG CTA TGG TAT TGA GTT ATG AG, from bp −218 to −198 to *gelE* start codon, used for amplification of *gelE* promoter	This study
BMP1	28	CGG AAT TCA GGG AGG GAT AAT GAC TAA T, from bp −90 to −71 to *fsrB* start codon	This study
BMP2	32	CGG AAT TCA TTA AGG AAT TAT CTA TCT ATT AG, from bp −72 to −49 to *fsrB* start codon	This study
BMP4	57	CGG AAT TCG GGA TAA TGA CTA ATT AAG GAA TTA TCT ATC TAT TAG TCG CTA TAT TCG, from bp −85 to −37 to *fsrB* start codon	This study
BMP5	63	CGG AAT TCT AGG GAG GGA TAA TGA CTA ATA TTC CTT TTA TCT ATC TAT TAG TCG CTA TAT TCG, from bp −91 to −37 to *fsrB* start codon, bp −70 to −64 sequence TAA GGA A was changed to ATT CCT T	This study
BMP6	62	CGG AAT TCA GGG AAA AAT GTC GGC TGA TTA AGG AAT TAT CTA TCT ATT AGT CGC TAT ATT CG, from bp −188 to −170 to *gelE* start codon and from bp −72 to −37 to *fsrB* start codon	This study
EMP1	27	CGG AAT TCA GGG AAA AAT GTC GGC TGA, from bp −188 to −170 to *gelE* start codon	This study
EMP2	30	CGG AAT TCA TTA AGG AAT TTA GAT AGT GCC, from bp −170 to −149 to *gelE* start codon	This study
EMP5	55	CGG AAT TCA GGG AAA AAT GTC GGC TGA TTT TCC TTT TTA GAT AGT GCC GGT TAG G, from bp −188 to −142 to *gelE* start codon, bp −167 to −162 sequence AAG GAA was changed to TTC CTT	This study
EMP6	56	CGG AAT TCT AGG GAG GGA TAA TGA CTA ATT AAG GAA TTT AGA TAG TGC CGG TTA GG, from bp −91 to −73 to *fsrB* start codon and bp −170 to −142 to *gelE* start codon	This study

Continued on following page

TABLE 2—*Continued*

Primer	Length (mer)	Sequence (5′–3′)[a], location, and relevant properties	Reference or source
Vlac1	23	GTT GAA TAA CAC TTA TTC CTA TC, flanking pTCV-*lac* cloning sites, used for sequencing inserts in pTCV-*lac*	20
Vlac2	21	CTT CCA CAG TAG TTC ACC ACC, flanking pTCV-*lac* cloning sites, used for sequencing inserts in pTCV-*lac*	20
Others			
lytF1	20	ACA CCA ACC ACA GAA ACT AC, from bp 226 to 245 in *E. faecalis* autolysin gene	This study
lytR1	20	GGC AAT AAA TTC TGA AGG AC, from bp 555 to 536 in *E. faecalis* autolysin gene	This study
gelEF1	21	TGG TTG TGA TTC GTT TGT TGG, from bp −508 to −561 to *gelE* start codon	This study
GBR2	22	TGA CCA GAA CAG ATT CAC TTG G, from bp 9 to 30 before *gelE* stop codon	This study

[a] Linker sequences are underlined.

DNA sequencing and sequence analysis. Automated sequencing was used to determine nucleotide sequence by the dideoxy chain termination method (21, 26). PCR sequencing was 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 inserts in pBluescript SK(−) or in plasmid pTEX4577 (27) were sequenced using T3 and T7 primers. Other primers used for sequencing are listed in Table 2. To determine whether there is sequence variation in 3′ ends of *fsrB*, which shows homology to *agrD* of *S. aureus*, the 3′ ends of *fsrB* from eight different *E. faecalis* strains were amplified by PCR using fsrBF1 and fsrCRTF1 primers (Table 2). The PCR products were purified by a DNA cleanup kit (Promega, Madison, Wis.) and sequenced using the fsrCRTF1 primer.

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, and PILEUP was used for multiple sequence alignment.

Deletion mutagenesis. Primers used for *fsrB* deletion mutagenesis are listed in Table 2. To make a deletion in *fsrB*, 5′ and 3′ flanking regions of *fsrB* were amplified by PCR, ligated together by linkers (*Eco*RI) designed in the two inner primers, and inserted into a mutagenesis vector (27), pTEX4577, using two linkers (with *Sac*I and *Kpn*I recognition sites) designed in the two outer primers. The resulting construct, pTEX5267, was then transformed into OG1RF by electroporation as previously described (9), and single-crossover mutants were selected on BHI-KAN agar plates. The single-crossover mutants were expected to be still gelatinase positive because of the duplication of the flanking regions and putative promoters immediately upstream of *fsrB*. Since this single-crossover event creates duplicated fragments of the regions flanking the target gene, subsequent recombination between these duplicated fragments would lead to the loss of the mutagenesis vector and one copy of the duplicated flanking sequences and give rise to a KAN-sensitive wild type or deletion mutant. To identify *fsrB* deletion mutants, we first plated the cultures of the single-crossover *fsrB* mutant grown overnight without KAN onto Todd-Hewitt agar containing 3% gelatin to score for the loss of gelatinase activity because we predicted that the deletion of *fsrB* would abolish the expression of *gelE*. Colonies that were gelatinase production negative were then scored for the loss of KAN resistance and were further confirmed as deletion mutants by PCR using two primers flanking *fsrB* (primers BDF1 and BDR2) and by sequencing the PCR product. Pulsed-field gel electrophoresis (13) was used to verify that the deletion mutants were not contaminants.

Detection of gelatinase and serine protease activities. The production of gelatinase and serine protease in *E. faecalis* strains was detected by methods previously described by using Todd-Hewitt agar (Difco Laboratories) containing 3% gelatin and zymogram gels containing 0.05% casein (Novex, San Diego, Calif.) and by using 20-fold-concentrated supernatants from overnight cultures (22).

Northern blot analysis and RT-PCR. Isolation of total RNA from *E. faecalis*, Northern blot analysis, and RT-PCR were carried out as previously described by Qin et al. (22). Radioactive DNA probes for Northern blot analysis were prepared using the random-primer DNA labeling system from Life Technologies according to the protocol supplied. Primers used for RT-PCR are listed in Table 2.

Time course of *fsr* and *gelE* expression. To study the time course of *fsr* and *gelE* gene expression in wild-type OG1RF and *fsr* mutants, overnight cultures of OG1RF and the *fsrC* insertion mutant (TX5242) were diluted 1:40 in BHI and incubated at 37°C. Total RNA was isolated from cells harvested at different time points. Northern blotting and hybridization using *fsrC* and *gelE* probes were utilized to determine the expression levels of *fsr* and *gelE* genes.

Cell density-dependent *fsr* expression. Northern blot analysis was used to determine the expression levels of *fsr* and *gelE* genes at different cell concentrations. Cells of OG1RF and the *fsrC* gene disruption mutant (TX5242) from the cultures at early exponential (2 h after inoculation) and postexponential (4 h after inoculation) phases were harvested by centrifugation, resuspended in BHI to the desired cell concentrations, which were determined by measuring the optical density at 600 nm (OD_{600}; 1 OD_{600} unit for an *E. faecalis* culture in BHI = 1.25×10^9 CFU/ml), and then incubated at 37°C for 45 min before isolation of total RNA for Northern blot analysis using *fsrB* and *gelE* genes as probes.

Determination of cotranscription in *fsr*/*gelE* loci. Our previous Northern blot analysis suggested that some genes in the *fsr*/*gelE* loci are cotranscribed (22). To verify these results, RT-PCR using the primers flanking the intergenic region between two adjacent genes was applied to determine the cotranscription of the genes in the *fsr*/*gelE* loci. The primers used in the study of cotranscription are listed in Table 2.

Primer extension analysis and manual DNA sequencing. Primer extension was used to map the 5′ end of the transcripts and to locate putative promoters in *fsr*/*gelE* loci. Primers complementary to sense DNA were designed from the sequences downstream of the start codons of desired genes (Table 2). Primer extension was carried out as previously described (25) with slight modification. In brief, primers were end labeled with ^{32}P using T4 polynucleotide kinase (Life Technologies) and [γ-^{32}P]ATP. For annealing RNA with the primer, 10 to 30 μg of total RNA was mixed with 2×10^6 cpm of ^{32}P-labeled primer and 3 M sodium acetate (pH 4.8) was added to a final concentration of 0.3 M, followed by precipitation with ethanol. The pellet containing the RNA and primer was then dissolved in 30 μl of S1 solution (80% deionized formamide, 40 mM PIPES [piperazine-*N*,*N*′-bis{2-ethanesulfonic acid}] buffer [pH 6.4], 1 mM EDTA, 0.4 M NaCl) and heated for 10 min at 80°C. The mixture was then incubated at 37°C overnight to anneal the primer to the RNA template. The next day, RNA and primer were precipitated by ethanol.

For primer extension, primer extension mixture was prepared by mixing 4 μl of 5× First Strand buffer (Life Technologies; 250 mM Tris-HCl [pH 8.3], 375 mM KCl, 15 mM $MgCl_2$), 2 μl of 50 mM $MgCl_2$, 2 μl of 10 mM deoxynucleoside triphosphates, 1 μl of 20 mM dithiothreitol, 0.5 μl of RNasin (RNase inhibitor; 40 U/μl), 1 μl of Superscript reverse transcriptase (200 U; Life Technologies), and 9.5 μl of diethyl pyrocarbonate-treated water (to a final volume of 20 μl). The hybridized RNA-primer pellet was then dissolved in the primer extension mixture, heated for 5 min at 70°C, and incubated at 37°C for 60 min. The RNA in the reaction mixture was removed by adding 1 μl of 0.5 M EDTA and 0.5 μl of 2-mg/ml DNase-free RNase and incubating for 30 min at 37°C. The cDNA in the reaction mixture was then precipitated with ethanol and resuspended in 4 μl of formamide loading buffer (0.95 ml of deionized formamide, 0.1 ml of 5× Tris-borate-EDTA buffer [25]) and 4 μl of 2× stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanole FF). The primer extension product was analyzed on 5% acrylamide DNA sequencing gel along

with DNA sequencing reactions using the same primer according to the method previously described (25).

A manual DNA sequencing reaction was carried out with a T7 Sequenase 2.0 DNA sequencing kit from Amersham Life Science (Cleveland, Ohio) according to the protocol from the supplier using plasmid pAM-S1 (28) as the DNA template.

Construction of transcriptional fusion and β-galactosidase activity assay. In order to confirm the putative promoters identified from primer extension and to determine the strength and the regulation of these promoters, sequences of putative promoters were amplified by PCR and cloned into transcriptional fusion vector pTCV-*lac* (20) using the *Eco*RI and *Bam*HI restriction sites to generate transcriptional fusion to the promoterless *lacZ* reporter gene in the vector. pTCV-*lac* is a 12-kb broad-host-range shuttle vector containing erythromycin and KAN resistance genes which can be expressed in both gram-positive and -negative organisms and a promoterless β-galactosidase-encoding *lacZ* gene with a gram-positive ribosome binding site. The orientation and sequences of the cloned promoter fragments were confirmed by restriction analysis and DNA sequencing analysis using Vlac1 and Vlac2 primers (Table 2). The primers used in the amplification of wild-type promoters and promoters with mutations are listed in Table 2.

β-Galactosidase activities of *E. faecalis* strains containing pTCV-*lac* or fusion constructs were detected using the method previously described by Poyart and Trieu-Cuot (20). The β-galactosidase activities were calculated using the following formula: units of activity = $1{,}000 \times (OD_{420} - 1.60 \times OD_{550})/(t \times v \times OD_{600})$ (OD_{420} and OD_{550} are the densities measured from the reaction; OD_{600} is the cell density of the culture measured before the β-galactosidase activity assay; t is the time of reaction in minutes; v is the volume of culture used in the assay in milliliters; the light scattering correction factor 1.60 was used instead of 1.75, which is used for *E. coli*, because for *E. faecalis* OD_{420} [light scattering] $\approx 1.60 \times OD_{550}$).

SDS-PAGE gel and 2-D gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as previously described (14). For analysis of proteins in supernatants of *E. faecalis* cultures, supernatants were concentrated 20-fold and dialyzed against 10 mM Tris-HCl (pH 7.4). Two-dimensional (2-D) gel electrophoresis was performed using the method previously described (17). Surface proteins from OG1RF and *fsr gelE* mutants were prepared by the method previously described (30) and 50 or 100 μg of surface proteins was used for each 2-D gel electrophoresis. The proteins in the 2-D gels were visualized by silver staining as previously described (4).

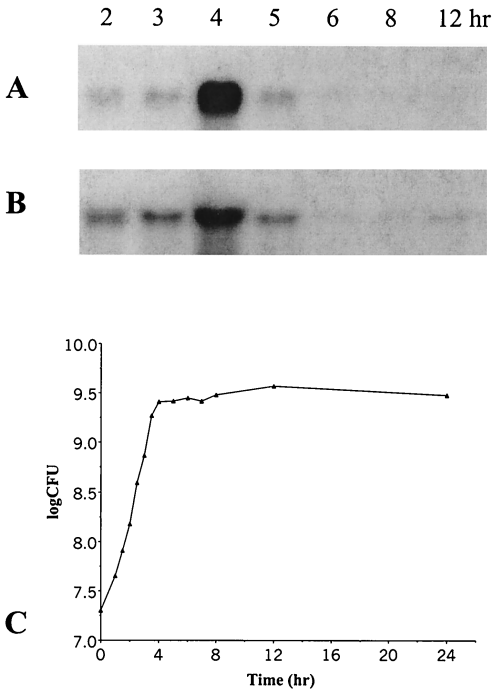

FIG. 2. Northern blot analysis of the time course of *fsrC* and *gelE* expression. (A) Northern blot analysis during growth using an *fsrC* probe. (B) Northern blot analysis during growth using a *gelE* probe. Lanes: total RNA from *E. faecalis* OG1RF cells from 2-, 3-, 4-, 5-, 6-, 8-, and 12-h cultures. RNA isolation, Northern blotting, and hybridization were performed as described in Materials and Methods. (C) Growth curve of OG1RF. Bacterial cells were cultured as described in Materials and Methods. Cell concentrations (CFU per milliliter) were determined by serial dilutions and plating on BHI agar in triplicate.

RESULTS

Time course of *fsr* and *gelE* expression. The expression of *fsr* (using *fsrC* as the examined gene) and *gelE* in wild-type OG1RF and an *fsrC* gene disruption mutant (TX5242; Table 1) (22) during growth was determined by Northern blot analysis. The expression of *fsrC* in OG1RF was at low but detectable levels from 2 to 3 h after inoculation (early and middle exponential phases), peaked at 4 h (postexponential phase), and diminished to undetectable levels after 12 h (Fig. 2A and C). A similar expression pattern of *gelE* was observed in OG1RF (Fig. 2B and C). These results indicate that *fsrC* and *gelE* are highly expressed in postexponential phase in wild-type OG1RF. No *fsrC* or *gelE* expression was detected in different phases during growth in the *fsrC* disruption mutant (data not shown).

To investigate whether the expression of *fsr* genes is cell density dependent as it is for their homologues in *S. aureus* (6), the expression levels of *fsrC* were analyzed at different cell concentrations by Northern blotting. The levels of *fsrC* mRNA increased as the cell concentrations increased from 5×10^7 to 1×10^9 CFU/ml and began to drop after the cell concentration exceeded 1×10^9 CFU/ml, regardless of the growth phase of the bacterial cells used in the experiment (Fig. 3), suggesting that the *fsr* gene expression is cell density dependent.

Determination of gene cotranscription by RT-PCR. Our previous Northern blot results suggested that *fsrB* and *fsrC*, as well as *gelE* and *sprE*, are cotranscribed (22). To further confirm this finding, we applied RT-PCR using the primer pairs that cover the intergenic regions of *fsr*/*gelE* loci. RT-PCR using primer pairs fsrBRTF1 and fsrARTR1 and fsrCRTF1 and fsrBRTR1 encompassing *fsrA*, *fsrB*, and *fsrC* intergenic regions showed amplified bands with the predicted sizes (Fig. 4B and C), indicating that *fsrB* and *fsrC* are cotranscribed and that the transcript from *fsrA* reads through to *fsrB*. Positive amplification was also observed using primer pair sprERTF1 and ge-

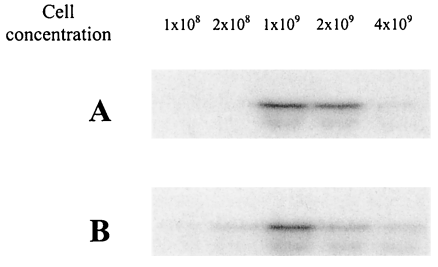

FIG. 3. Northern blot analysis of *fsrC* expression at different cell densities. OG1RF cells from early exponential (2-h) and postexponential (4-h) phases were harvested by centrifugation and resuspended in BHI to desired concentrations and incubated at 37°C for 45 min before RNA was isolated for Northern blot analysis using the *fsrC* probe. (A) OG1RF cells from 2-h culture. (B) OG1RF cells from 4-h culture. The cell concentration (CFU per milliliter) used in each lane is indicated.

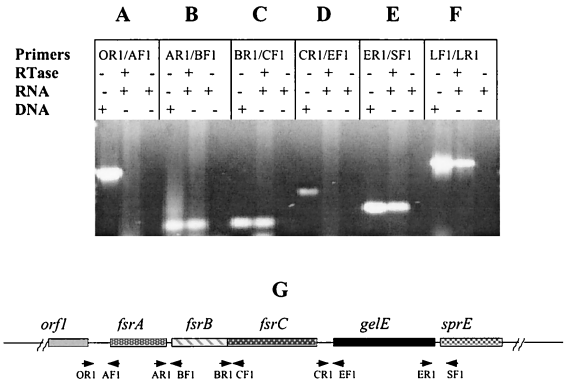

FIG. 4. RT-PCR analysis of gene cotranscription in *fsr*/*gelE* loci. Every three lanes divided by a solid line represents one set of experiments using the same primers. In each set, the first lane shows PCR using chromosomal DNA as the template, which serves as a positive control for PCR, the second lane shows an RT-PCR with (+) RNA and reverse transcriptase (RTase) but without (−) chromosomal DNA; the third lane shows an RT-PCR with RNA but without RTase and chromosomal DNA, which serves as a control to determine the contamination of DNA in RNA samples. (A to E) RT-PCR analysis using primers covering the intergenic regions between *orf1* and *fsrA*, *fsrA* and *fsrB*, *fsrB* and *fsrC*, *fsrC* and *gelE*, and *gelE* and *sprE*, respectively. (F) RT-PCR using two internal primers (lytF1 and lytR1) of *E. faecalis* autolysin, a positive control for RT-PCR. Primers: OR1, orfRTR1; AF1, fsrARTF1; AR1, fsrARTR1; BF1, fsrBRTF1; BR1, fsrBRTR1; CF1, fsrCRTF1; CR1, fsrCRTR1; EF1, gelERTF1; ER1, gelERTR1; SF1, sprERTF1; LF1, lytF1; LR1, lytR1. (G) Diagram illustrating the positions of the primers used in the RT-PCR analysis. Solid line, chromosomal DNA; boxes, genes; arrows, primers.

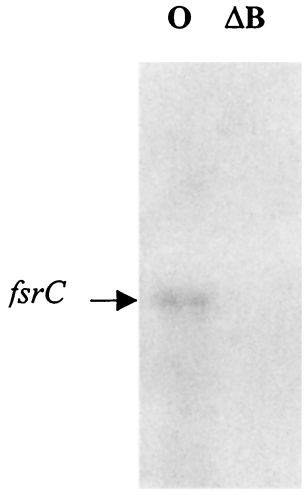

FIG. 5. Northern blot analysis of *fsrB* deletion mutant (TX5266). RNA from postexponential-phase cultures of OG1RF and the *fsrB* deletion mutant (TX5266) was isolated as described in Materials and Methods. Northern blots were probed with the *fsrC* probe. Arrow, band that hybridized with the *fsrC* probe. Lanes: O, OG1RF; ΔB, *fsrB* deletion mutant TX5266 (deleted from bp 79 to 684 in *fsrB*).

lERTR1, which covers the noncoding region between *gelE* and *sprE* (Fig. 4E), indicating the cotranscription of *gelE* and *sprE*. No signal was detected using the primer pairs (orf1RTR1 and fsrARTF1 and fsrCRTR1 and gelERTF1) between *orf1* and *fsrA* and *fsrC* and *gelE* (Fig. 4A and D), suggesting that *orf1* is not on the same transcript as *fsrA* and that transcription of *gelE* is initiated from its own promoter.

Construction and analysis of *fsrB* deletion mutant. In our previous study of *fsr* gene expression in *fsr* insertion mutants, we found that an insertion in *fsrB* abolished the expression of *fsrC*, which is downstream of *fsrB*, and that expression and production of *gelE* and *sprE* were undetectable in an *fsrB* insertion mutant while both were readily detected in wild-type OG1RF (22). It was not clear whether the effect of the insertion in *fsrB* on the regulatory functions of *fsr* was due to the polar effect on *fsrC* or the loss of *fsrB* function. To determine whether *fsrB* is required for the regulatory function of the *fsr* locus, an *fsrB* deletion mutant was generated using suicide vector pTEX4577, containing the flanking regions of *fsrB* (pTEX5267). A KAN-resistant single-crossover *fsrB* mutant was obtained after electroporating pTEX5267 into OG1RF. Two putative deletion mutants were then obtained after growing the single-crossover mutant without KAN and screening about 5,000 colonies for the loss of gelatinase activity and KAN resistance. PCR analysis of the putative deletion mutants using two primers (BDF1 and BDR2) of the flanking regions of *fsrB* gave a shorter product than that resulting from analysis of wild-type OG1RF as expected, and sequencing the PCR product confirmed that these KAN-sensitive clones had the expected deletion in *fsrB* from bp 79 to 684 (data not shown). One of the *fsrB* deletion mutants was designated TX5266.

Gelatinase activity was not detected with TX5266 even after 72 h of incubation at 37°C, and serine protease activity was also not detectable in this *fsrB* deletion mutant on a casein zymogram gel, while both activities were readily detected for wild-type OG1RF (data not shown). Northern blot analysis using an internal fragment of the *fsrC* gene as a probe did not show any detectable signal for the *fsrB* deletion mutant, while OG1RF showed a 2.2-kb band (Fig. 5), suggesting that *fsrB* is also necessary for the regulatory functions of the *fsr* locus.

Mapping 5′ ends of *fsr* and *gelE* transcripts by primer extension. With primers complementary to the sense strands downstream of the start codons of *fsrA*, *fsrB*, and *gelE*, the 5′ ends of *fsrA*, *fsrB*, and *gelE* transcripts were mapped to 9, 23, and 119 bp upstream of *fsrA*, *fsrB*, and *gelE* start codons, respectively, and potential −10 and −35 sequences were identified immediately upstream of the 5′ ends of these transcripts (Fig. 6).

Study of *fsrA*, *fsrB*, and *gelE* promoter activities in wild-type and *fsr* mutant strains. To confirm activity of the *fsrA*, *fsrB*, and *gelE* promoter sequences identified above and to investigate the regulatory functions in these promoter regions, plasmid pTEX5268 (containing the putative *fsrA* promoter), pTEX5269 (containing the putative *fsrB* promoter), and pTEX5270 (containing the putative *gelE* promoter) were constructed. Plasmid pTEX5268 was generated by cloning a 401-bp PCR fragment from the intergenic region between *orf1* and *fsr*A (from −406 to −6 bp relative to the *fsrA* start codon) upstream of the promoterless *lacZ* reporter gene in shuttle vector pTCV-*lac* (20) (Fig. 7A). Similarly, pTEX5269 and pTEX5270 were constructed by fusing 103- and 203-bp fragments covering the intergenic regions between *fsrA* and *fsrB* (from bp −110 to −8 relative to the *fsrB* start codon) and between *fsrC* and *gelE* (from bp −218 to −16 relative to the *gelE* start codon) with the promoterless *lacZ* in pTCV-*lac* (Fig.

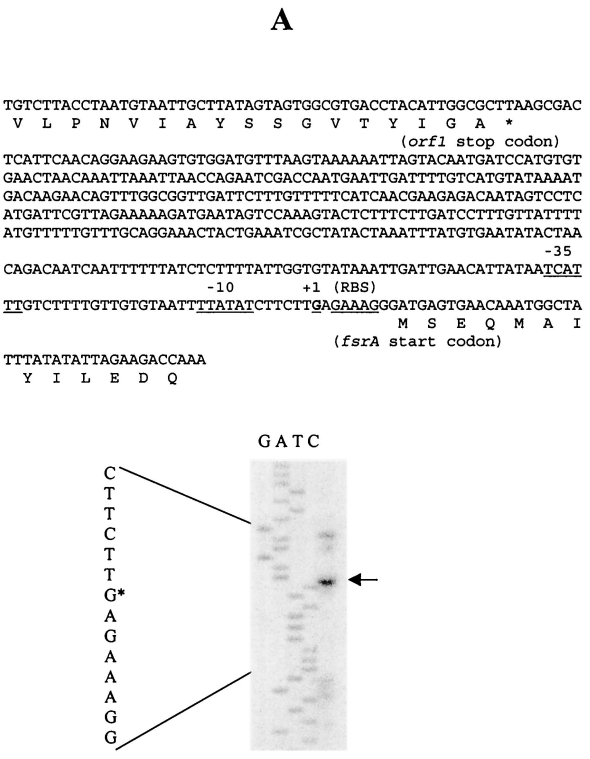

FIG. 6. Determination of the 5′ ends of the *fsrA*, *fsrB*, and *gelE* transcripts by primer extension. Primer extension was carried out as described in Materials and Methods. The primer extension products were run alongside sequencing reactions obtained with the same primers. Arrows, primer extension products in the sequencing gels; asterisks, locations of the 5′ ends of the sequences (left of each gel). The sequences encompassing the 5′ ends of the transcripts are shown on the top of each gel; putative −35, −10, and ribosome binding site (RBS) sequences are underlined, and the transcription start sites (+1) are in boldface and underlined. Primer extensions using primers APE1 (A), BPE2 (B), and EPE2 (C) are shown.

7A). These three constructs, containing putative *fsrA*, *fsrB*, and *gelE* promoters, were introduced into wild-type OG1RF, and the specific β-galactosidase activities in different growth phases were analyzed. The β-galactosidase activities of *fsrB* and *gelE* promoter constructs peaked after 3 to 5 h of growth, while *fsrA* promoter activity remained relatively low and constant (Fig. 7B). The *gelE* promoter (pTEX5270) had the strongest promoter activity compared to *fsrA* and *fsrB* promoters. Based on the β-galactosidase activities, the maximum activity of the *gelE* promoter during growth (at 3 h) was about 3 and 26 times greater than that of *fsrB* (at 5 h) and *fsrA* (at 4 h), respectively.

When the *fsrA*, *fsrB*, and *gelE* promoter fusion constructs were introduced into the *fsr* mutants, only the *fsrA* promoter showed promoter activities in these mutants (Fig. 8), and the *fsrA* promoter activity in each mutant was comparable to its activity in wild-type OG1RF. Neither the *fsrB* nor the *gelE* promoters were active in any *fsr* mutants (Fig. 8), suggesting that the *fsrB* and the *gelE* promoters are *fsr* dependent, while the *fsrA* promoter is an *fsr*-independent constitutive promoter.

Identification of regulatory sequences in *fsrB* and *gelE* promoter regions. Since the promoter activity assay mentioned above and our previous Northern blot analysis indicated that the expression of both *fsrB* and *gelE* is regulated by the *fsr* locus, we analyzed the sequences of the *fsrB*, *gelE*, and *fsrA* promoter regions for possible sequence homology. Sequence alignment revealed two conserved segments immediately upstream of the −35 regions of the *fsrB* and *gelE* promoters (Fig. 9). Within these two homologous regions are two 7-bp imperfect repeats, which are separated by 14 bp (Fig. 9), suggesting

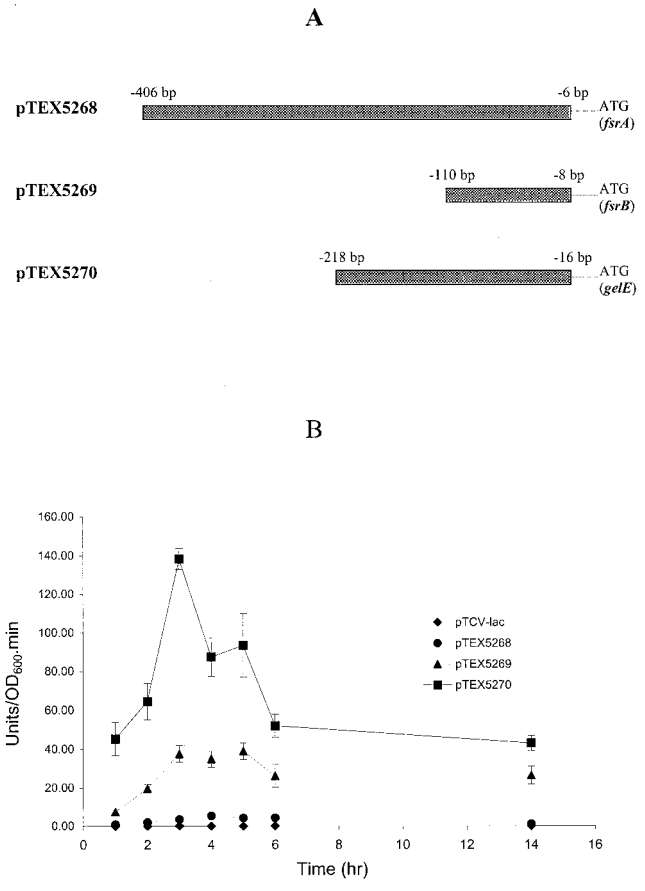

FIG. 7. (A) Constructs of *fsrA*, *fsrB*, and *gelE* promoter *lacZ* fusions. DNA fragments (boxes) containing putative *fsrA*, *fsrB*, and *gelE* promoters were cloned in front of a promoterless *lacZ* in shuttle vector pTCV-*lac*, resulting in plasmids pTEX5268 (putative *fsrA* promoter in pTCV-*lac*), pTEX5269 (putative *fsrB* promoter in pTCV-*lac*), and pTEX5270 (putative *gelE* promoter in pTCV-*lac*), respectively. The distances of each end of the fragments from *fsrA, fsrB*, and *gelE* start codons are indicated. (B) Determination of *fsrA*, *fsrB*, and *gelE* promoter activities of *lacZ* fusion constructs in OG1RF. The β-galactosidase activity assay was performed as described in Materials and Methods. β-Galactosidase activity was represented as units per unit of optical density at 600 nm (OD_{600}) of cells per minute. Error bars, standard deviations.

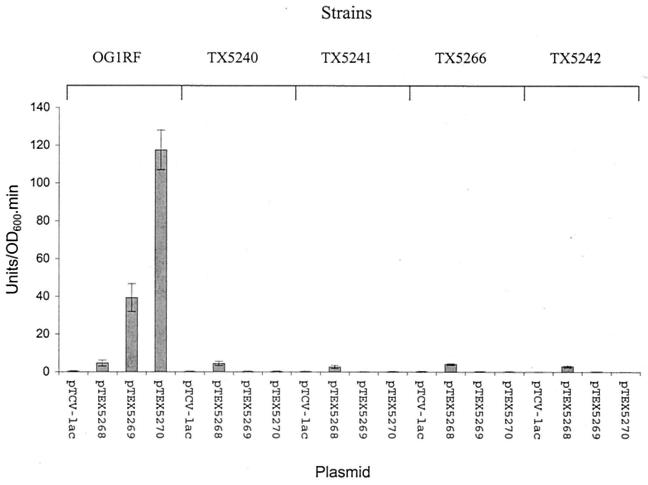

FIG. 8. *fsrA*, *fsrB*, and *gelE* promoter activities in *fsr* mutants. *E. faecalis* cells containing different plasmid constructs grown for 4 h were used for the β-galactosidase activity assay. Error bars, standard deviations. Strains were as follows: TX5240 (*fsrA* gene disruption mutant) (20), TX5241 (*fsrB* gene disruption mutant) (20), TX5242 (*fsrC* gene disruption mutant) (20), TX5266 (*fsrB* deletion mutant). Plasmids in the different strains were as follows: pTCV-*lac* (vector without insert as negative control), pTEX5268 (*fsrA* promoter in pTCV-*lac*), pTEX5269 (*fsrB* promoter in pTCV-*lac*), and pTEX5270 (*gelE* promoter in pTCV-*lac*).

possible regulatory sequences in these regions. The *fsrA* promoter region did not show significant sequence similarity to either the *fsrB* or the *gelE* promoter regions.

In order to investigate the importance of these conserved repeated sequences upstream of *fsrB* and *gelE* promoters, different *lacZ* fusion constructs with deletions or mutations in the repeated sequences of *fsrB* and *gelE* promoters were made in shuttle vector pTCV-*lac* (20) (Fig. 9) and assayed for their β-galactosidase activities in wild-type OG1RF. Constructs pTEX5298 (containing bp −90 to −8 relative to the *fsrB* start codon) and pTEX5303 (containing bp −188 to −16 relative to the *gelE* start codon) (Fig. 9) containing the *fsrB* and *gelE* promoters with just an additional 34-bp sequence immediately upstream of the −35 regions, which include both the repeated sequences, exhibited promoter activities similar to those exhibited by pTEX5269 (containing bp −110 to −8 relative to the *fsrB* start codon; Fig. 7A) and pTEX5270 (containing bp −218 to −16 relative to the *gelE* start codon; 7A), respectively (Fig. 9). This suggested that the *fsrB* and *gelE* promoters with the upstream repeated sequences are sufficient to maintain their promoter activities. However, deletions of the most-upstream repeats (constructs pTEX5299, pTEX5300, and pTEX5304) or mutations in the closer repeats (constructs pTEX5301 and pTEX5305) upstream of the *fsrB* and *gelE* promoters completely abolished the *fsrB* and *gelE* promoter activities (Fig. 9), suggesting that these repeats are important for the regulation of *fsrB* and *gelE* expression. In addition, when the *fsrB* promoter without its own repeated region was fused to the repeated region of the *gelE* promoter (construct pTEX5302), it was not only active but also showed about twofold-higher activity than the *fsrB* promoter with its original repeated sequences (Fig. 9). Similarly, exchange of *fsrB* repeated sequences for those of the *gelE* promoter (construct pTEX5306) also generated a functional *gelE* promoter (Fig. 9), suggesting that these repeated sequences upstream of *fsrB* and *gelE* promoters have a similar regulatory function. None of these fusion constructs exhibited promoter activity in an *fsrC* gene disruption mutant (data not shown), indicating that the regulatory function of the repeated sequences is mediated by *fsr*.

Sequences of 3′ end of *fsrB* from different clinical isolates. To investigate whether there is strain variation in the 3′ end sequence of *fsrB*, a region whose last 50-amino-acid sequence shows homology to that of AgrD of *S. aureus*, the 3′ end of *fsrB* was amplified by PCR from eight distinct Gel^+ strains from different clinical sources and geographical areas and sequenced. DNA sequence analysis of the last 150-bp sequences at the 3′ end of *fsrB* revealed that five strains had identical sequences while three had only single silent-base-pair changes

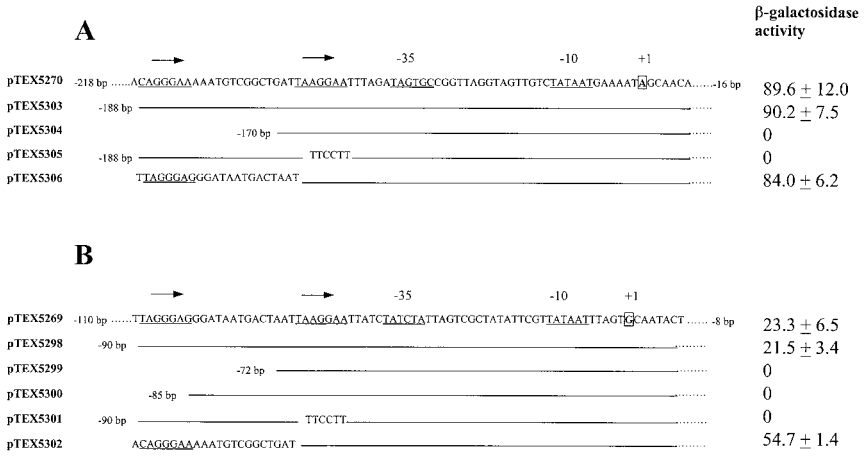

FIG. 9. Schematic map of *fsrB* and *gelE* promoter regions in different *lacZ* fusion constructs and their β-galactosidase activities. (A) Different *gelE* promoter constructs, pTEX5270, pTEX5303, pTEX5304, pTEX5305, and pTEX5306, and their β-galactosidase activities in OG1RF. (B) Different *fsrB* promoter constructs, pTEX5269, pTEX5298, pTEX5299, pTEX5300, pTEX5301, and pTEX5302, and their β-galactosidase activities in OG1RF. The sequences for pTEX5270 and pTEX5269 (top lines of panels A and B) only show the regions containing the *gelE* and *fsrB* promoter and the repeated sequences; the sequences further upstream are not shown. All the *gelE* promoter constructs have the same sequences downstream of the promoter to bp −16 from *gelE* start codon (dots, sequence not shown), and all the *fsrB* promoter constructs have the same sequences downstream of the promoter to bp −8 from the *fsrB* start codon. The position labeled at the end of each line is relative to the *gelE* or *fsrB* start codon. Solid lines (A and B), sequences identical to that of the top sequences (changes in the promoter sequences are shown for constructs pTEX5305, pTEX5306, pTEX5301, and pTEX5302). Repeated sequences upstream of *gelE* and *fsrB* promoters are underlined; putative transcription start sites (+1) are in boldface and boxed; −10 and −35 sequences are also underlined. The β-galactosidase activity of each construct is indicated at the right.

compared to OG1RF (data not shown), indicating that the 3′ ends of *fsrB* are conserved among different gelatinase-producing *E. faecalis* strains. The 150-bp 3′ end sequence of *fsrB* from OG1RF is also identical to this region of *E. faecalis* strain V583 in the TIGR *E. faecalis* genome database.

DISCUSSION

In *S. aureus*, the four *agr* genes (*agrA*, *agrB*, *agrC*, and *agrD*) are all required for the regulatory functions of the *agr* locus (15, 16). We have previously shown by Northern blotting that insertion disruption of the three *agr* homologues in *E. faecalis*, *fsrA*, *fsrB*, and *fsrC*, abolished the expression of *fsr* genes and of *gelE* and *sprE* (22). However, it was not clear whether inactivation of *fsr* regulatory functions by insertion in *fsrB* was caused by the loss of *fsrB* functions or by its polar effect on *fsrC*, which appeared to be cotranscribed with *fsrB*. We have shown here that a nonpolar deletion in *fsrB* in *E. faecalis* which maintained the *fsrB* promoter also led to the elimination of *fsrC* expression and production of gelatinase and serine protease, as demonstrated by Northern blot analysis and zymogram gel analysis, confirming that *fsrB* (the *agrB* homologue) is required for the *fsr* regulatory functions. As corroboration of our previous results (22), it appears that all three *fsr* genes are required for *fsr* functions.

It has been reported that the *agr*/*hld* loci in *S. aureus* consist of three promoters, the P1 promoter, which controls the expression of *agrA* (19), and two divergent but nonoverlapping promoters, P2 and P3, which control the transcription of the P2 transcript (encoding *agrB*, *agrD*, and *agrC*) and the P3 transcript (encoding RNAIII), respectively (15, 16). The P1 promoter is a weak constitutive promoter (19), while P2 and P3 are *agr*-dependent promoters (15). Our primer extension results suggest that a separate promoter is located immediately upstream of *fsrA*, of *fsrB*, and of *gelE*. Gene fusion analysis of these putative promoters with a promoterless *lacZ* in OG1RF and *fsr* mutants indicated that, like the P1 promoter in *S. aureus*, the *fsrA* promoter is a weak and constitutive *fsr*-independent promoter and that *fsrB* and *gelE* promoters are more-active promoters than the *fsrA* promoter and that their expression is *fsr* dependent, as the P2 and P3 promoters are *agr* dependent (15). Our RT-PCR experiments using the primers covering the intergenic regions in *fsr*/*gelE* loci further indicated that *fsrA*, *fsrB*, and *fsrC* and *gelE* and *sprE* are cotranscribed. Analysis of DNA sequences in *fsr*/*gelE* loci revealed one possible rho-independent transcription terminator (24) in the intergenic region between *fsrC* and *gelE* genes (218 bp upstream of *gelE*), which contains two 13-bp inverted repeats, suggesting that transcription from *fsr* genes would not read through to *gelE* and *sprE*. From the promoter fusion analysis results, together with the results of the RT-PCR and previous Northern blot analysis (22), we can conclude that the transcription of *fsrA* starts from the *fsrA* promoter and may read through to *fsrB* and possibly to *fsrC*, that the expression of *fsrB* and *fsrC* is mainly under the control of the *fsrB* promoter, and that the expression of *gelE* and *sprE* is regulated by the *gelE* promoter.

In the *agr*/*hld* loci in *S. aureus*, Morfeldt et al. have previously identified two 7-bp interrupted repeats (separated by 14 bp) upstream of the P3 promoter that are required for the *agr*-dependent expression of the P3 transcript (RNAIII) (11). Similar repeats were found upstream of P2, and the repeats upstream of P2 compete with those of P3 for binding to the SarA protein (11). Similar repeated sequences upstream of the *sapA* promoter, which is required for activation of transcription of *sapA*, the gene encoding a bacteriocin named sakacin A,

has also been found in *Lactobacillus sake* Lb706 (1). By sequence alignment of the intergenic regions between *fsrA* and *fsrB* and between *fsrC* and *gelE*, which contain *fsrB* and *gelE* promoters, respectively, we found two conserved 7-bp repeats, which are also separated by 14 bp, immediately upstream of the *fsrB* and *gelE* promoters. Cloned *fsrB* and *gelE* promoter regions containing their repeated sequences (pTEX5298 and pTEX5303) displayed promoter activities similar to those of cloned *fsrB* and *gelE* promoter regions containing almost the entire intergenic sequences (pTEX5269 and pTEX5270, which had additional 20- and 30-bp sequences upstream of the repeats compared to pTEX5298 and pTEX5303), suggesting that the promoters with the repeated sequences are sufficient to perform the promoter activities. Moreover, deletion or changes of these repeated sequences upstream of *fsrB* and *gelE* completely abolished the promoter activities of the *fsrB* and *gelE* promoters, further indicating that these repeated sequences are required for the *fsr*-dependent regulation of *fsrB* and *gelE* promoters, a mechanism that appears to be similar to that of the Agr system in *S. aureus* (11).

In this study, we also examined the possibility that *fsr* genes regulate the expression of some surface proteins and other secreted proteins besides gelatinase and serine protease. The results of SDS-PAGE analysis of proteins in supernatants from *fsr* mutants and OG1RF showed that the *fsr* mutants had more protein bands in their supernatants than wild-type OG1RF even though a 29-kDa band and a 34-kDa band, presumably the serine protease and gelatinase, were clearly no longer present in the supernatants from the mutants (data not shown). The increase in the number of protein bands in the *fsr* mutants may be due the lack of the two proteases in the supernatants so that proteins released from dead cells or other sources were not degraded by these enzymes, since the supernatant from a *gelE* insertion mutant (TX5128) (22, 27), which did not produce either of these proteases, also showed a pattern of protein bands similar to those from *fsr* mutants. Analysis of surface protein profiles of *fsr* gene disruption mutants and an *fsrB* deletion mutant compared to that of the parental strain, OG1RF, using 2-D gel electrophoresis showed that protein patterns of *fsr* mutants were similar to that of OG1RF and that all the *fsr* mutants had identical protein patterns on 2-D gels (unpublished preliminary data). However, the intensities of at least three spots with the sizes of 44, 33, and 18 kDa on the 2-D gel of *fsr* mutants were greater than the intensities of those of OG1RF, suggesting increases in the production of these proteins in the *fsr* mutant strains. The pattern of surface proteins from a *gelE* insertion mutant (TX5128) (22, 27) was similar to that of surface proteins from OG1RF. Whether *fsr* genes in *E. faecalis* regulate the expression of surface proteins, like their homologues in *S. aureus*, could be further addressed by isolating and partially sequencing these proteins and subsequent studying the expression of the genes encoding these proteins.

Ji et al. have previously shown that the regulatory functions of the Agr system in *S. aureus* are mediated by a secreted oligopeptide pheromone, encoded by *agrD*, which functions as a cell density signal (6). We have not yet identified an AgrD-like peptide in *E. faecalis* even though we identified an *agrD* homologue in the last 150 bp of the 3′ end of *fsrB* by sequence analysis. Nakayama et al. have reported in abstract form the isolation of an 11-amino-acid pheromone, whose sequence matches that of 220 to 230 amino acids of the C terminus of FsrB, from the supernatant of an *E. faecalis* strain and demonstrated that the isolated pheromone could induce the expression of gelatinase in a pheromone concentration-dependent manner (J. Nakayama, Y. Cao, A. D. L. Akkermans, W. M. deVos, and H. Nagasawa, Abstr. 1st Int. ASM Conf. Enterococci, abstr. 21, 2000). The sequence of the pheromone was identical to that of a segment in the C terminus of FsrB. In our present study, we found that the expression of *fsr* genes is cell density dependent, as it is for their homologues in *S. aureus*, and that the expression of *fsr* genes peaked at a cell concentration of 10^9 CFU/ml, a concentration that is about the concentration of cells of *E. faecalis* at postexponential phase, consistent with the time course of *fsr* expression during growth. These results suggest that the regulation of *fsr* gene expression is similar to that of *agr* in *S. aureus*, which is mediated by a quorum-sensing system encoded by *agr* and which is most active in postexponential phase (5, 6, 15).

Strains of *S. aureus* can be classified into at least three different groups based on the cross-activation and inhibition by the autoinducing peptides of the Agr systems (5). For strains studied to date, the autoinducing peptides from strains in the same group are identical and can induce the expression of *agr* genes in the strains of the same group but inhibit *agr* expression in strains from other groups (5). The sequence similarity among the precursors of the autoinducing peptides (encoded by *agrD*) from strains in different groups is very limited (5). As noted above, the last 50 amino acids at the C terminus of FsrB, which show 28% identity and 47% similarity to those of AgrD of *S. aureus*, appear to be the AgrD equivalent in *E. faecalis*. To test whether *E. faecalis* strains that contain the *fsr* locus can also be divided into different groups based on their *fsr* sequences, we partially sequenced the 3′ ends of *fsrB* from eight distinct strains and compared the last 150-bp sequences of *fsrB* from these eight Gel^+ strains with those from strains OG1RF and V583. From the sequence analysis results, we only detected single-base-pair changes with no difference in the deduced amino acid sequences among these 10 strains, suggesting that, unlike the *agr* genes in *S. aureus*, the *fsr* genes in *E. faecalis* are conserved among strains.

In conclusion, the *fsrB* gene as well as *fsrA* and *fsrC* are all required for the regulatory functions of the *fsr* locus. The expression of *fsrA* is under the control of a weak and constitutive promoter, the *fsrA* promoter, and the transcription of *fsrB* and *fsrC* as well as that of *gelE* and *sprE* are regulated by two *fsr*-dependent promoters, the *fsrB* and *gelE* promoters, respectively. Two directly repeated sequences immediately upstream of the *fsrB* and *gelE* promoters are necessary for the activation of *fsrB* and *gelE* promoters in an *fsr*-dependent manner. The expression of *fsr* genes in *E. faecalis* OG1RF is cell density dependent and is most active in the postexponential phase. While these aspects of regulation are similar to those for the *agr* locus of *S. aureus*, unlike *agr*, which is present in all *S. aureus* strains studied (5, 18), these *fsr* genes are found in only some *E. faecalis* strains (22) (but in 100% of gelatinase-producing strains). Analysis of the 3′ end of *fsrB* indicates that this locus is much more conserved than *agr*.

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