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Comparison of OG1RF and an Isogenic *fsrB* Deletion Mutant by Transcriptional Analysis: the Fsr System of *Enterococcus faecalis* Is More than the Activator of Gelatinase and Serine Protease†

Agathe Bourgogne,[1,2] Susan G. Hilsenbeck,[3] Gary M. Dunny,[4] and Barbara E. Murray[1,2,5]*

Division of Infectious Disease, Department of Medicine,[1] Center for the Study of Emerging and Reemerging Pathogens,[2] and Department of Microbiology and Molecular Genetics,[5] University of Texas Medical School, Houston, Texas 77030; Breast Center, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030[3]; and Department of Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455[4]

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The FsrABC system of *Enterococcus faecalis* controls the expression of gelatinase and a serine protease via a quorum-sensing mechanism, and recent studies suggest that the Fsr system may also regulate other genes important for virulence. To investigate the possibility that Fsr influences the expression of additional genes, we used transcriptional profiling, with microarrays based on the *E. faecalis* strain V583 sequence, to compare the *E. faecalis* strain OG1RF with its isogenic mutant, TX5266, an *fsrB* deletion mutant. We found that the presence of an intact *fsrB* influences expression of numerous genes throughout the growth phases tested, namely, late log to early stationary phase. In addition, the Fsr regulon is independent of the activity of the proteases, GelE and SprE, whose expression was confirmed to be activated at all three time points tested. While expression of some genes (i.e., ef1097 and ef0750 to -757, encoding hypothetical proteins) was activated in late log phase in OG1RF versus the *fsrB* deletion mutant, expression of ef1617 to -1634 (*eut-pdu* orthologues) was highly repressed by the presence of an intact Fsr at entry into stationary phase. This is the first time that Fsr has been characterized as a negative regulator. The newly recognized Fsr-regulated targets include other factors, besides gelatinase, described as important for biofilms (BopD), and genes predicted to encode the surface proteins EF0750 to -0757 and EF1097, along with proteins implicated in several metabolic pathways, indicating that the FsrABC system may be an important regulator in strain OG1RF, with both positive and negative effects.

Enterococcus faecalis is adapted to survive, persist, and proliferate in a wide range of environments as different as the gastrointestinal tract, heart valves, water, and soil. To do so, it is likely that *E. faecalis* has developed various mechanisms of adaptation. Examples include transcriptional regulators, such as *hypR* (47) or *efaR* (25); two-component systems (*etaRS* [46], *croRS* [9], *vanSR* [11], and RR1-13 [18]); and cell-cell signaling systems, including pheromone-inducible plasmid transfer (for a review, see reference 7), the Cyl system (8, 16), and the FsrABC system (30, 31, 34, 35).

The *fsrABC* operon, a homologue of *agrABCD* in *Staphylococcus aureus*, was originally shown by Qin et al. to activate, at the transcriptional level, the expression of two genes, *gelE* and *sprE*, coding for a metalloprotease and a serine protease, in addition to *fsrBC* (34, 35). Nakayama et al. and Qin et al. subsequently purified and characterized the FsrABC system pheromone as an 11-residue peptide lactone, produced from the C-terminal portion of the *fsrB* gene product and reaching peak levels at entry into stationary phase (ENT-stat) (30, 31, 35). Studies have also shown that, in the majority of the strains studied, a *gelE*$^+$ genotype with a GelE$^-$ phenotype is associated with a 23.9-kb deletion, from ef1841 through part of ef1820 (ef1820 is *fsrC*). This deletion is found in many distinct clinical strains, as well as in isolates from healthy volunteers (32, 37). While Nakayama et al. found a correlation between the clinical origin of isolates and a Gel$^+$ phenotype, Roberts et al., studying a larger panel of strains, did not see a statistically significant relationship (32, 37). Nonetheless, in the OG1RF background, the FsrABC system and gelatinase have been shown to be important for virulence in different animal models, with *fsr* and *gelE* mutants showing attenuation in a mouse peritonitis model (34), in *Caenorhabditis elegans* (43), in a rabbit endophthalmitis model (14, 29), in an *Arabidopsis thaliana* plant model (21), and, more recently, in an endocarditis model (44). Different outcomes have been observed in different assays when *fsrABC* or *gelE-sprE* mutants have been compared with the parental strain. In biofilm formation (18, 27) and translocation experiments (54), the double-protease mutant exhibits strong attenuation, and *fsrABC* mutants have not shown additional reduction. However, in the *C. elegans* model (43) and in the rabbit model of endophthalmitis (14, 29), an *fsrBC* mutant was more attenuated than a *gelE-sprE* mutant, indicating that the FsrABC system may regulate other genes important for virulence.

A number of groups have used microarray analysis to investigate complex regulatory cascades by taking snapshots during growth or by comparing strains. For example, in *S. aureus*, which shares a number of homologues with *E. faecalis* (e.g., Fsr

* Corresponding author. Mailing address: Division of Infectious Disease, Center for the Study of Emerging and Re-emerging Pathogens, MSB 2.112, University of Texas Medical School, 6431 Fannin St., Houston, TX 77030. Phone: (713) 500-6745. Fax: (713) 500-6766. E-mail: BEM.asst@uth.tmc.edu.

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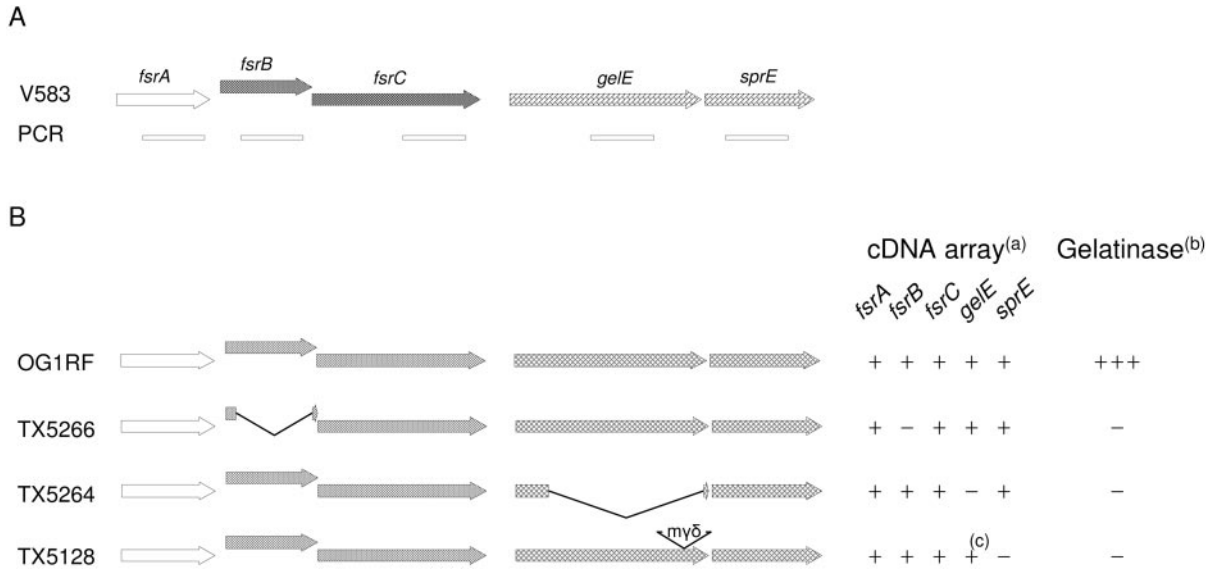

FIG. 1. Characterization of the strains used in this study. (A) Genetic organization of the *fsr* and *gelE* loci in V583 and locations of the PCR products of these genes used to construct the microarray. (B) Genotype and phenotype of the OG1RF parental and isogenic strains used in this study. TX5266 and TX5264 are both in-frame deletion mutants in which the deleted sequence contains the portion used for the microarray (*fsrB* and *gelE*, respectively). TX5128 is a *gelE* disruption mutant in which the minitransposon, mγδ, is inserted in the 3′ end of the gene. (a) Expression pattern using the V583 array. (b) Gelatinase activity. (c) Although the gene is truncated and the protein inactive, due to the location of the PCR primers, *gelE* transcript was detected in TX5128 by microarray.

and Agr [for a review, see reference 6] and Ace and Cna [53]), Dunman et al. described a complex regulatory network controlled by *agr* and *sar* (13), followed by studies looking at the *rot* (40) and *sigB* (3) regulons, all using transcriptional profiling. Recently, the targets of another quorum system, which was shown to regulate the expression of *agr*, were also examined using a microarray (23), thus adding another layer of complexity. However, much less is known about regulatory cascades in enterococci.

In this study, we present microarray-based data from three distinct genetic backgrounds (an *fsrB* in-frame deletion mutant, a *gelE* in-frame mutant, and a polar insertion mutation in *gelE*) compared to the parental strain, OG1RF. Our results show that the *E. faecalis* FsrABC system positively and negatively regulates the expression of numerous genes between late exponential growth phase and early stationary phase (EAR-stat). In addition, the FsrABC system regulon is independent of the expression of *gelE* and *sprE*.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study were OG1RF (28) and its isogenic deletion mutants TX5266 (Δ*fsrB*) (35), TX5264 (Δ*gelE*) (43), and the *gelE* insertion mutant TX5128 ($GelE^-$ $SprE^-$) (36). The details of the *fsr-gel* loci of these strains and the PCR fragments of these genes that were used for the array are shown in Fig. 1. Because of a chaining phenotype of TX5266 (unpublished data) and of TX5128 (48) compared to OG1RF, growth was assessed by the optical density at 600 nm and not by CFU. All strains were grown routinely in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 150 to 200 rpm or on BHI agar at 37°C with kanamycin (2 mg/ml) when needed for mutant selection.

RNA isolation and labeling. RNA was extracted from cells grown in BHI broth with shaking at 37°C, conditions known to promote *fsrB* expression and used previously in investigations of the expression of *fsrABC* and *gelE* (34, 35). Briefly, after being cultured overnight, the cells were diluted so that the starting optical density at 600 nm was 0.05. Following 3 h (late log phase), 4 h (ENT-stat), and 5 h (EAR-stat) of incubation (Fig. 2A), cells were collected for RNA isolation. RNA was extracted from cultures using RNAwiz (Ambion, Austin, TX) according to the protocol provided by the supplier. Typically, between 15 and 30 μg of RNA was obtained per ml of culture. RNA was labeled with the FairPlay Microarray Labeling Kit (Stratagene, La Jolla, CA) using the manufacturer's protocol, with one exception. After the annealing step with poly(T), 1 μl of random primers (Invitrogen, Rockville, Md.) was added, and the sample was incubated again at 70°C for 10 min before being cooled on ice until it was ready to use. The appropriate samples, one labeled with Cy3 and the other with Cy5, were mixed and dried to completion using a speed vacuum.

Slides and hybridization. PCR products and slide processing were described by Aakra et al. (1). After being printed, the slides were rehydrated with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and dried using a heat block at 140°C for 15 s. Before use, the slides were prehybridized in 5× SSC, 0.1% sodium dodecyl sulfate (SDS), 1% bovine serum albumin for 1 h and washed with H_2O and then with propanol before being dried using a centrifuge at 1,000 × *g* for 2 min. The dried probes were resuspended in 30-μl volumes (50% formamide, 5× SSC, 0.1% SDS, and 40 μg Cot1 DNA/ml). This mixture was heated for 10 min at 95°C, cooled on ice, and then added to a prehybridized slide under a coverslip. The slide was then placed at 42°C overnight in a sealed hybridization chamber (Corning 2551; Fisher Scientific Co., Pittsburgh, PA), which was humidified with 20 μl of 5× SSC. The arrays were then washed at room temperature, once in a solution of 2× SSC-0.1% SDS for 10 min, once in 0.1× SSC-0.1% SDS for 10 min, and once in 0.1× SSC for 2 min, followed by a final quick wash in double-distilled H_2O.

The array was made with PCR products amplified with V583 genomic DNA (gDNA) using primers selected from the V583 genome sequence (33). To assess the percentage of genes detectable using the V583 array, we performed hybridization with gDNA from OG1RF and V583 using the 3DNA Array 900DNA kit (Genisphere, Hatfield, PA) as described in the manufacturer's protocol.

Data acquisition and statistics. Hybridized microarray slides were scanned (GenePix Pro 5.0; Axon Instruments, Inc.) with independent excitation of the fluorophores Cy3 and Cy5 at 10-μm resolution. The signal and background fluorescence intensities were calculated for each DNA spot using the segmentation method of the GENPIXPRO software (Molecular Devices Corp., Union City, CA). After the local background of each spot intensity was subtracted, the ratios of intensities for Cy3- to Cy5-labeled probes were determined for each DNA spot.

To allow appropriate statistical analysis of the results, RNA preparations from at least three independent cultures were tested for each set of strains. For each

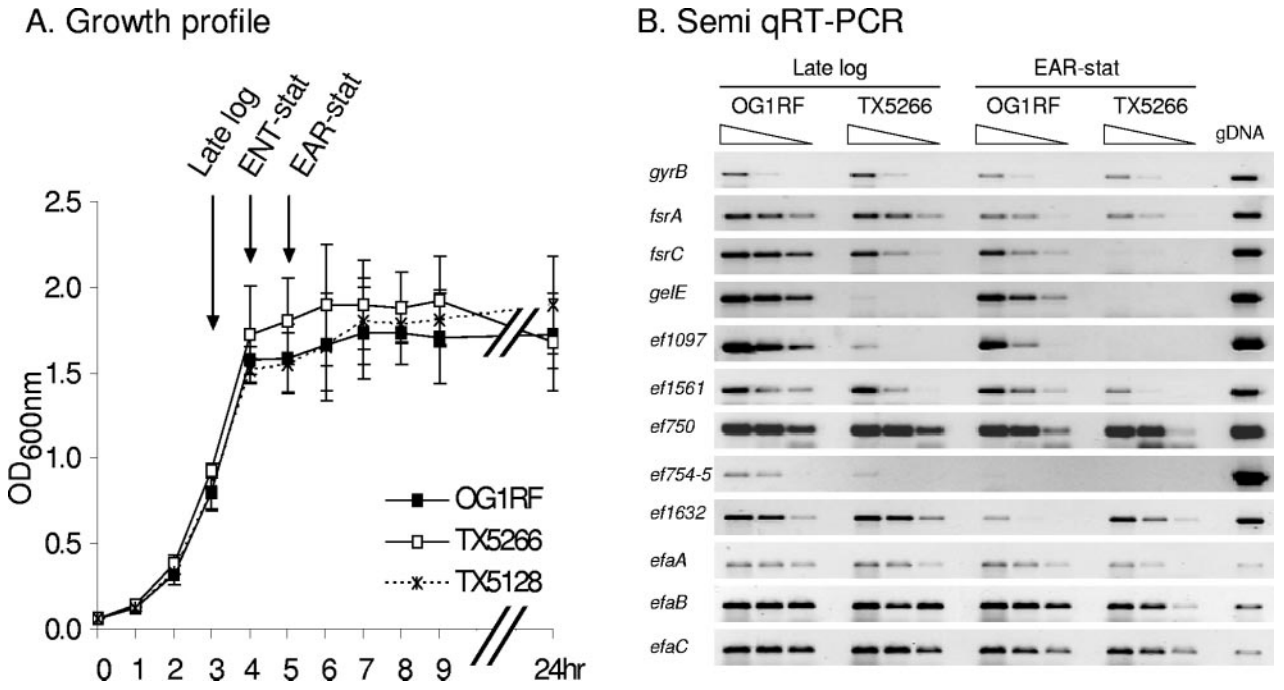

FIG. 2. Growth profile of *E. faecalis* in BHI and semiquantitative RT-PCR. (A) The means and standard deviations were calculated from three independent cultures grown in parallel. OG1RF is the parental strain, while TX5266 is the in-frame *fsrB* deletion mutant, and TX5128 is a *gelE* disruption mutant. The arrows indicate when samples were taken for microarray analysis. Hour 3 corresponds to late log phase, hour 4 to ENT-stat, and hour 5 to EAR-stat. (B) Semiquantitative RT-PCR analysis of *gyrB*, *fsrC*, *gelE*, ef1097, ef1561, ef0750, ef754 to -755, ef1632, *efaA*, *efaB*, and *efaC* showing RT-PCR products from RNA extracted from OG1RF or TX5266 at hour 3 (late log phase), and hour 5 (EAR-stat). The three lanes for each RNA represent undiluted cDNA and two 10-fold dilutions of cDNA before the PCR. The gDNA used as a control for the PCR was extracted from OG1RF.

hybridization, RNA was obtained from cultures of the OG1RF parent strain and the mutant strains grown in parallel. Each RNA preparation was used in at least two separate dye swap hybridizations (one with parent-Cy3/mutant-Cy5 and the other with parent-Cy5/mutant-Cy3).

The results for each mutant were analyzed separately, as described previously (4). After quantitation and global normalization using the average spot intensity, $\log_e$ ratios of OG1RF to the Δ*fsrB* mutant, OG1RF to the Δ*gelE* mutant, or OG1RF to the *gelE* insertion mutant were calculated for each spot. For each open reading frame (ORF), $\log_e$ ratios for each replicate culture were calculated by averaging $\log_e$ ratios for spots ($n = 5$) within a chip that met quality criteria and their averaging dye-swap hybridizations. Unacceptable spots were those with no signal or those associated with a slide problem, such as dust or a scratch. ORFs were considered significantly regulated if (i) the overall change was at least twofold (i.e., the absolute value of the average $\log_e$ ratio was greater than 0.693) and (ii) the *P* value from a one-sample *t* test, testing whether the grand mean $\log_e$ ratio was different from 0.0, was significant at the 0.05 level or better.

Sequence analysis and results available online. We used the following resources to characterize the predicted products of genes of interest: the KEGG website (http://www.KEGG.com), BLAST (http://www.ncbi.nlm.nih.gov/BLAST), SMART (http://smart.embl-heidelberg.de), TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), and PredictProtein (http://www.embl-heidelberg.de/predictprotein/predictprotein.html).

A file containing the induction and *P* values for all regulated ORFs (>2-fold regulation; $P < 0.05$) from experiments comparing OG1RF to the TX5266 Δ*fsrB* strain is available in the supplemental material.

Semiquantitative RT-PCR. RNA was extracted as described above from two independent cultures. Twenty micrograms of RNA was treated twice with DNase-Free solution (Ambion) according to the protocol of the supplier. For the reverse transcription (RT)-PCR, 10 μg of RNA was mixed with 2 μl of random decamers (Invitrogen; 50 μM) for a final volume of 18.5 μl in water. The mixture was heated to 70°C for 5 min and then immediately placed on ice. A master mixture comprised of 6 μl of 5× Superscript II buffer, 2 μl of deoxynucleoside triphosphate mixture (2.5 mM each), 3 μl of 0.1 M dithiothreitol, and 1 μl of Superscript II reverse transcriptase (Invitrogen; 200 U/μl) was added to the RNA solution (final volume, 30 μl), and the mixture was incubated at 42°C for 2 h. A control sample that contained RNA and all of the RT components except the Superscript II reverse transcriptase was prepared simultaneously. The PCRs were then performed using undiluted RNA control sample, serial dilution of cDNA (1, 1:10 and 1:100), and a gDNA template control. The list of primers is available in the supplemental material.

Mapping of ef1097 transcriptional start site. Total RNA of *E. faecalis* OG1RF was isolated from late-log growth phase (3 h) and purified as described previously. The transcriptional start point of ef1097 was determined using the RACE 5′/3′ kit (Roche, Indianapolis, IN) according to the manufacturer's instructions.

RESULTS AND DISCUSSION

Overview of the Fsr regulon. In this study, we quantified the genomewide transcriptional profile of OG1RF and its isogenic Δ*fsrB* mutant (Fig. 1) in three different growth phases, namely, late log phase (3 h after the cultures were started), at ENT-stat (4 h), and at EAR-stat (5 h) (Fig. 2A). It is known that in broth culture during this period, the FsrABC system regulates the *gelE-sprE* operon, which codes for gelatinase and serine protease, factors important for virulence, in addition to the *fsrBC* operon, by a quorum-sensing mechanism at the level of transcription. As shown in Fig. 3A, B, and C, four genes are markedly influenced by the deletion of *fsrB* in all three phases tested. In late log phase, expression of *fsrB*, *fsrC*, *gelE*, and *sprE* was, respectively, 480-, 44-, 725-, and 503-fold higher in OG1RF than in TX5266, the *fsrB* mutant. This increase in expression in the presence of an intact *fsrB* gene was maintained through early stationary phase with 118-, 33-, 215-, and 166-fold-enhanced transcription in the parental strain for the same genes. Considering the relative expression in the parent

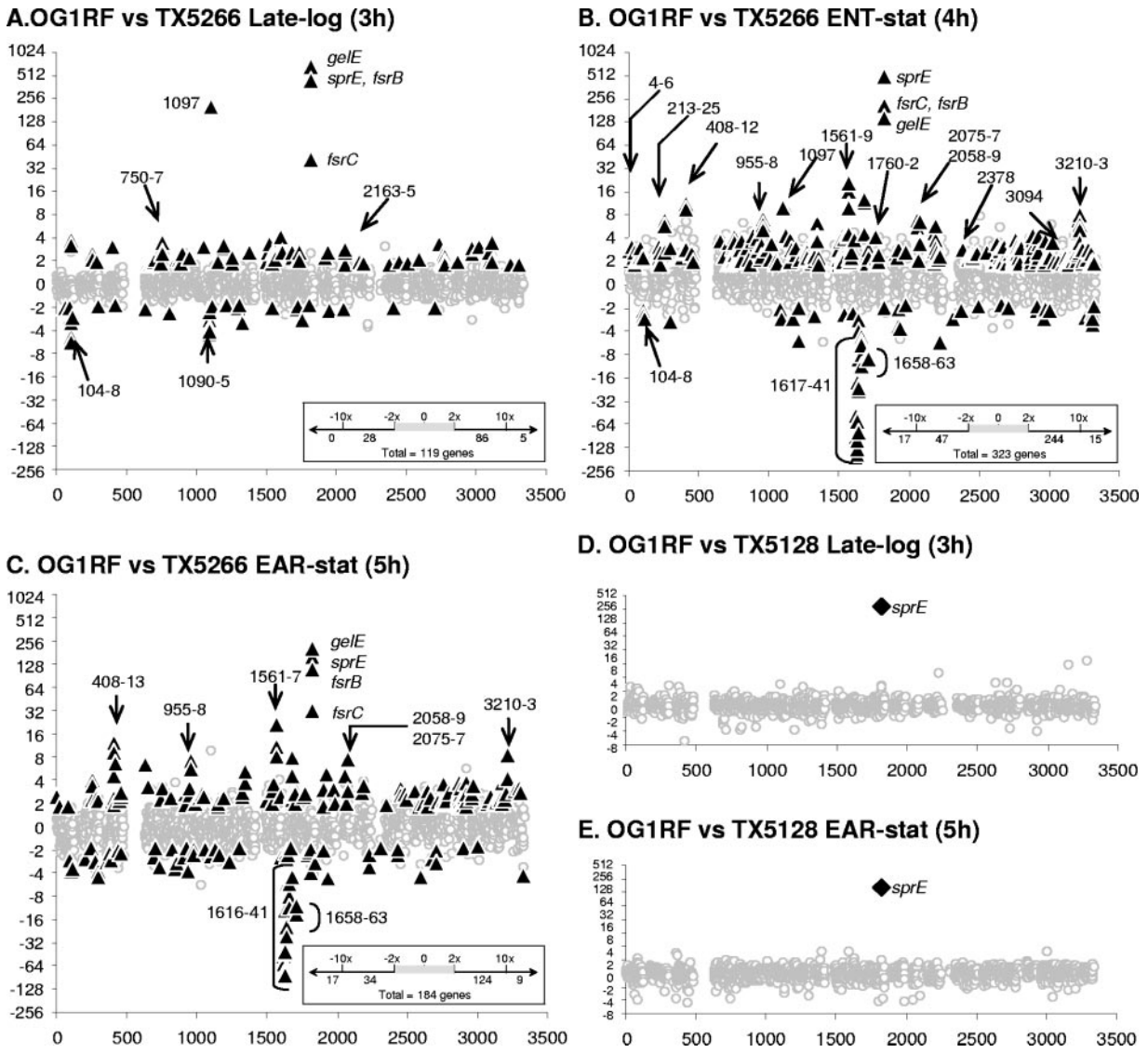

FIG. 3. Alteration in gene expression between OG1RF and TX5266 (*fsrB* in-frame deletion) and between OG1RF and TX5128 (*gelE* disruption mutant). Each panel represents an average of microarray results obtained with RNA preparations from at least three different cultures. The genes are represented if at least one average ratio per RNA preparation was available for statistical analysis. For all panels, the *x* axis represents the gene identification number as annotated by TIGR (NCBI ID, NC_004668). The *y* axis indicates the change (*n*-fold); the change was considered positive when the expression was higher in OG1RF than in the mutant. The gray circles correspond to nonsignificantly affected genes. The black triangles (A, B, and C) correspond to genes that were regulated at least twofold with a *P* value of <0.05 for OG1RF versus TX5266, while the black diamonds (D and E) represent genes that were regulated at least twofold with a *P* value of <0.05 for OG1RF versus TX5128. The arrows indicate specific genes of interest ("ef" has been omitted). In the boxes in the right lower corners of panels A to C are shown the numbers of regulated genes and their classification into four categories: >10-fold repression, between 2- and 10-fold repression, between 2- and 10-fold activation, and >10-fold activation.

versus the *fsrB* mutant, we would classify all four genes as being *fsrB* dependent (the expression levels of these genes in the *fsrB* deletion mutant were below the detection level of the microarray). However, with undiluted cDNA and RT-PCR (Fig. 2B), the *fsrC* primers could be shown to amplify a product in TX5266 (in late log phase [3-h] and EAR-stat [5-h] cultures), confirming the basal expression presumably due, at least in part, to the *fsrA* promoter (35). In contrast, during these two phases, the *fsrC* product was detected with OG1RF cDNA diluted 1:100. Our data corroborate published studies by Qin et al. (35) showing that *fsrBC* is autoregulated and is critical for the expression of the *gelE-sprE* operon, while *fsrA* is constitutively expressed at a low level (detected by microarray in all three phases), but neither *fsrB* nor growth phase regulated.

At least two published studies have suggested that the regulon of the FsrABC system should be greater than just activation of the *gelE-sprE* operon; that is, an *fsrB* mutant was more attenuated than a *gelE-sprE* double mutant in a *C. elegans* model (43) and in a rabbit model of endophthalmitis (14, 29). Consistent with these results, we found, in late log phase, that 119 genes were affected by the deletion of *fsrB* (at least twofold difference in expression between OG1RF and TX5266; $P < 0.05$),

although only one additional gene was found to be *fsrB* dependent, ef1097 (214×; $P = 0.004$) (Fig. 3A), while others generally showed a two- to sixfold difference (see the supplemental material). At entry into stationary phase (Fig. 3B), 323 genes were statistically significantly regulated, 259 activated, and 64 repressed by the Fsr system. One set of genes, activated two- to threefold, encodes proteins likely to be growth phase dependent, suggesting the possibility that, although the growth curves appear similar between OG1RF and TX5266 (Fig. 2A), slight differences in growth profile may have led to statistically significant gene expression differences, as described by Sapolsky in his chronotranscriptome study of *Bacillus subtilis* (41). In EAR-stat (5 h), these putatively growth phase-dependent genes were no longer *fsrB* regulated (Fig. 3B and C). However, 109 genes remained *fsrB* regulated in EAR-stat, with an additional 76 new genes also showing evidence of *fsrB* regulation (Fig. 3C). Among these 185 genes, the expression levels of 9 were >10-fold higher in OG1RF than in TX5266. These genes belong to three loci: the *fsrABC-gelE-sprE* locus, four genes of the ef1561 to -1567 operon (with an average induction for all genes of ca. 13-fold), and ef0411, a member of the ef0408 to -0413 operon (with an average induction of 10-fold). On the other hand, the expression of 17 genes increased >10-fold in the absence of *fsrB* (TX5266) compared to OG1RF. Among these 17 genes, 14 showed no detectable expression in the parent strain, while high levels of expression were detected in the mutant (homologues of the *eut-pdu* genes [ef1617 to -1635]) (Fig. 2B and 3C). This is the first time that the FsrABC system has been suggested as a negative regulator, although whether this is a direct or indirect effect remains to be elucidated.

The Fsr regulon is independent of gelatinase activity. The FsrABC system has been recognized as the *gelE-sprE* regulator. The *gelE-sprE* operon codes for proteases that, once secreted and activated, may have a number of effects that could cause altered cell physiology; for example, a *gelE* disruption mutant of OG1RF is known to show extensive chaining (48). It is also possible that proteases might release or degrade a second autoinducer, and this might be responsible for some of the differences seen between OG1RF and the *fsr* mutants, particularly in EAR-stat. To examine this possibility, we compared the transcriptome of OG1RF with TX5264, a *gelE* deletion mutant, at two different growth phases (late log phase and EAR-stat). No difference was observed, except for *gelE* itself (the deletion includes the sequence of the PCR fragment used for the array) (Fig. 1B). Since Kawalec et al. (22) showed that absence of gelatinase activity results in aberrant processing of pro-SprE and the appearance of a "superactive" form of the enzyme, we also examined the *gelE* disruption mutant, TX5128 ($GelE^-$ $SprE^-$) in these two growth phases (late log phase and EAR-stat). As shown in Fig. 3D and E, no gene was significantly affected (as defined by a ratio of >2 and a *P* value of <0.05) in the disruption mutant (TX5128) versus OG1RF between late log phase and EAR-stat, except the *sprE* gene, which showed a >200-fold ($P < 0.05$) decrease in TX5128 compared to OG1RF. These results are consistent with those of Qin et al., who found that *sprE* is not expressed in a *gelE* insertion mutant due to the downstream polar effect of the insertion (35). The *gelE* mRNA level is not altered in the microarray analysis, because the *gelE* primers used to create the array are upstream of the disruption (Fig. 1A and B). When OG1RF and TX5128 were compared, the variance of the ratio of nonsignificantly affected genes (Fig. 3) was 0.078 at 3 h (late log phase) and 0.071 5 h (EAR-stat), confirming that there are few differences in the expression of other genes between the parent and the $GelE^-$ $SprE^-$ mutant. In contrast, the variance ranged from 0.069 in late log phase to 0.213 in ENT-stat and 0.200 in EAR-stat when OG1RF was compared with TX5266, the Δ*fsrB* mutant.

The importance of the proteases in virulence has been proven in different models, even if the mechanism is not well understood. Our microarray results imply that (i) the Fsr regulon is not controlled via a mechanism dependent on GelE and/or SprE activity under our conditions and (ii) the lack of protease activity does not lead to significant changes in gene expression by microarray analysis in the two growth phases studied. These results, albeit not performed with in vivo-grown organisms, suggest that the decrease in virulence seen with a *gelE-sprE* mutant strain is not due to a secondary effect on the expression of other genes. Additional studies will be needed to characterize the targets of GelE and SprE and their specific effects on biofilm formation and translocation and in heart valves and/or vegetations.

Influence of Fsr on regulatory cascades. Qin et al. characterized a consensus sequence necessary for expression of the *fsrBC* and *gelE-sprE* operons in their promoter area (35). Using in silico analysis with the sequence [ATCG]AGG[GA]A[AG]\w {13 to 16 bp}[ATCG]AAGGA[AG], we found eight additional representations of this consensus sequence in front of potential ORFs (ef0126, ef0138, ef0198, ef0562, ef1839, ef1890, ef2702, and ef3132). None of these genes appeared to be affected by the *fsrB* deletion under the conditions used. In addition to *fsrBC* and *gelE-sprE*, ef1097 (ef1097 is described below) was the only other gene that was also *fsrB* dependent for its expression (i.e., no detectable expression in TX5266, while expression was present in OG1RF) (Fig. 3A and B). We determined the transcriptional start point of ef1097 by 5′ rapid amplification of cDNA ends-PCR and found that the A that is 24 bp upstream of the ATG codon corresponds to the +1 of the transcript. An alignment between *gelE*, *fsrB*, and ef1097 (Fig. 4B) shows that the ef1097 promoter area differs slightly from the previously predicted consensus sequence. A less stringent consensus sequence (AGG[AG]{17 bp}A[AG]GGA) was not found upstream of other *fsrB*-regulated genes. Now that we have a better understanding of the extent of the Fsr regulon, additional work will be needed to define the *fsrB* consensus sequence and what other elements are required for the other highly *fsrB*-regulated genes.

As potential candidates for members of secondary regulation pathways, we found that 25 homologues of known regulatory proteins (identified by The Institute for Genomic Research [TIGR] annotation) that were *fsrB* regulated throughout the different growth phases listed in Table 1. In late log phase, four genes coding for potential regulatory proteins showed higher expression in OG1RF versus TX5266, while two genes were less expressed in the parental strain than in the *fsrB* mutant. At ENT- and EAR-stat, expression of two genes, ef1632 and ef1633, could not be detected in OG1RF, while they were expressed at a high level by TX5266. These two genes appear to code for a putative two-component system, of which EF1632 would be the histidine kinase and EF1633 the response regulator (HK17 and RR17) (17). These two genes

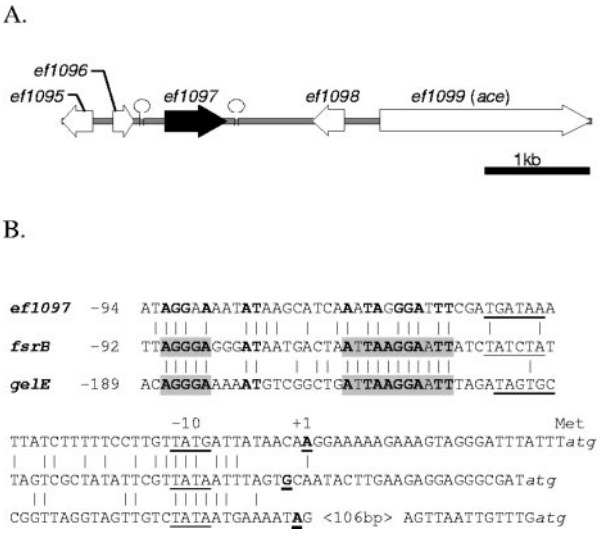

FIG. 4. ef1097 locus and transcriptional start site. (A) Genetic organization of the ef1097 chromosomal region. (B) Sequence alignment between ef1097, *gelE*, and *fsrB* promoter regions. Putative −10 and −35 sequences are underlined, and transcription start sites (+1) are boldface and underlined. The two boxes characterized by Qin et al. (36) as essential for expression are shaded. Common nucleotides between ef1097, *fsrB*, and *gelE* promoters are boldface. The stem loops indicate a putative transcriptional terminator.

are included in the *eut-pdu* locus. In *Salmonella enterica*, this locus encodes enzymes necessary for the transport and degradation of phosphatidylethanolamine, and expression of the *eut-pdu* operon is activated by *eutR* in response to the simultaneous presence of ethanolamine plus adenosylcobalamin (38, 42). In the V583 genome, no homologue was found for *eutR*. It should be of interest to see if EF1632 and EF1633 share a similar function with EutR of *S. enterica*. Inactivation of EF1633, also known as HK17, in the JH2-2 background did not show an effect on antibiotic resistance, biofilm formation, or environmental stress (including osmolarity, oxidative stress, low pH, heat, and detergents) (19). On the other hand, this work was done in a JH2-2 background, a strain known as a natural *fsrB* mutant due to a 23.9-kb deletion (32).

The fact that Fsr regulates genes coding for potential regulatory proteins may explain why we found fewer *fsrB*-regulated genes in late log phase than were observed at the later time points (ENT- and EAR-stat); that is, the increase in regulated genes may be, to some extent, the consequence of secondary regulatory cascades. Agr in *S. aureus*, which is in the same family of regulators as Fsr, shares some regulatory pathways with other regulators, like SarA, but also directly modulates the expression of other regulatory proteins, such as PyrR (13).

The FsrABC system is an important regulator of "food" supplies. As would be expected with a quorum-sensing system, the Fsr regulon changes dramatically with entry into stationary phase. As can be seen in Fig. 3B and C and in Table 2, there

TABLE 1. Regulatory proteins influenced by *fsrB* deletion

ID[a]	Putative function	Late log		ENT-stat		EAR-stat	
		Change (*n*-fold)[b]	*P*[c]	Change (*n*-fold)	*P*	Change (*n*-fold)	*P*
EF0097	Regulatory protein (PfoR family)	4	0.05				
EF0301	Transcriptional regulator (GntR family)	−2	0.02				
EF0372	RR13[d]					2	0.02
EF0927	HX09[d]					−2	0.02
EF1051	HK10[d]	3	<0.01				
EF1306	Heat-inducible transcription repressor HrcA			2	<0.01		
EF1515	Transcription antiterminator BglG	3	<0.01				
EF1522	RNA polymerase sigma-70 factor			3	0.01		
EF1525	Ferric uptake regulator			2	0.02		
EF1569	Transcriptional regulator; PSR protein			5	0.02		
EF1632	HX17[d]			−18	0.01	−12	0.01
EF1633	RR17[d]			−24	0.01	−21	<0.01
EF2063	Transcriptional activator (AraC family)					5	0.04
EF2218	RR01[d]			2	0.02		
EF2417	Ferric uptake regulator			2	0.05		
EF2426	Transcriptional regulator (GntR family)			2	0.01		
EF2703	Transcriptional regulator	−2	0.03				
EF2711	Transcriptional regulator (AraC family)			−2	0.04	−3	0.02
EF2767	Transcriptional regulator	2	0.01	3	<0.01		
EF2911	DNA-binding response regulator			2	0.02	2	0.04
EF3196	RR02[d]			2	0.01		
EF3214	Sigma-54-dependent transcriptional regulator			4	<0.01		
EF3216	Transcriptional regulator; putative			3	0.02		
EF3308	Transcriptional activator			−2	0.02		
EF3309	Transcriptional antiterminator (BglG family)			−3	0.01		

[a] EF numbers and their putative functions are from the V583 genome sequenced by TIGR (NCBI ID, NC_004668).

[b] The change represents mRNA expression levels in OG1RF relative to those in the *fsrB* mutant at specific growth phase time points and corresponds to averages of experiments done with three independent cultures. Minus indicates that the expression was lower in the wild type than in the *fsrB* mutant (TX5266).

[c] The *P* value from a one-sample *t* test, testing whether the grand mean $\log_e$ ratio was different from 0.0, was significant at the 0.05 or better level for each of the genes tested.

[d] HK, histidine kinase; RR, response regulator (17).

TABLE 2. Metabolic pathways regulated by Fsr

ID[a]	Gene name or definition[b]	Change[c]		
		Late log	ENT-stat	EAR-stat
Arginine and proline metabolism				
EF0104	*arcA*	−5	−3	
EF0105	*argF-1*	−6	−2	
EF0106	*arcC-1*	−3	−3	−4
PTS system				
EF0411	Mannitol-specific IIBC components		12	13
EF0412	*mltF*		10	9
EF0413	*mtlD*			7
EF0955	*bopC*/*malM*[d]		6	
EF0956	*bopB*/*malB*		7	6
EF0957	*bopA*/*malP*		6	7
EF0958	*mall*		5	6
EF3210	Putative PTS system, IIA component		7	3
EF3211	PTS system, IIB component		8	9
EF3212	PTS system, IIC component		6	5
EF3213	PTS system, IID component		5	4
Phenylalanine, tyrosine, and tryptophan biosynthesis				
EF1561	*aroE*		5	9
EF1562	P-2-dehydro-3-deoxyheptonate aldolas	2	17	24
EF1563	*aroB*	3	24	22
EF1564	*aroC*	2	21	9
EF1565	Prephenate dehydrogenase	2	11	13
EF1566	*aroA*		11	11
EF1567	*aroK*		4	8
EF1568	Prephenate dehydratase		10	
EF1569	*psr*		5	
Glycerophospholipid metabolism				
EF1616	CoA-binding domain protein			−2
EF1617	Hypothetical protein		−87	−49
EF1618	*eutH*		−63	−32
EF1619	Putative *pduN*		−51	−59
EF1620	Hypothetical protein		−95	−63
EF1621	Hypothetical protein		−96	−92
EF1622	Hypothetical protein		−181	−45
EF1623	Microcompartment protein		−61	−45
EF1624	Putative aldehyde dehydrogenase		−165	−77
EF1625	Microcompartment protein family		−8	−6
EF1627	*eutC*		−139	−77
EF1629	*eutB*		−288	−86
EF1630	Chaperonin? (frameshift)		−107	−43
EF1632	Sensor nistidine kinase		−18	−12
EF1633	Response regulator		−24	−21
EF1634	*eutS*		−85	−27
EF1635	Putative *pduU*		−18	−7
EF1637	ATP:cob(I)alamin adenosyltransferase		−9	
EF1638	*pduv*		−22	
Valine, leucine, and isoleucine degradation				
EF1658	*bkdC*		−5	
EF1659	*bkdB*		−7	−8
EF1660	*bkdA*		−8	−11
EF1661	*bkdD*		−6	
EF1662	*buk*		−11	
EF1663	*ptb*		−6	−6

[a] Only those operons in which at least one gene was at least fivefold Fsr regulated and where a metabolic function was attributed using the KEGG database are represented. EF numbers and their putative functions are from the V583 genome sequenced by TIGR (NCBI ID, NC_004668).
[b] The gene name or definition was obtained using the KEGG database.
[c] See note *b* in Table 1; in all cases, the *P* value was <0.05.
[d] *bop* nomenclature is used in *E. faecalis* type 9 strain (2, 20), while *mal* is used in the JH2-2 background (24).

is a cluster of genes that was found to be highly repressed in the presence of *fsrB* upon entry into stationary phase (4 h) and that remained repressed in early stationary phase (5 h). These genes can be divided into two sets. One set corresponds to ef1617 to ef1638 (*eut-pdu* orthologues); as mentioned earlier, the expression of these genes was noted to be turned off at ENT-stat by the presence of an intact *fsrB* (Fig. 2B). For the second set of genes (ef1658 to -1663), expression was only partially repressed in the parent strain. However, the level of regulation remained substantial, with a 5- to 11-fold decrease for ef1658 to -1663 expression in OG1RF compared to TX5266 at ENT- and EAR-stat.

In *Salmonella*, use of ethanolamine as a carbon and nitrogen source may be important, since this compound is a constituent of an abundant species of lipid present in the intestinal tract: phosphatidylethanolamine (51). In *S. enterica*, the *eut*/*pdu* operon contains the genes encoding the ethanolamine metabolosome: the complex containing the enzymatic machinery necessary for the degradation of ethanolamine to acetyl-coenzyme A (CoA) (5). Based on the KEGG database and BLAST searches, it seems that OG1RF carries all the genes needed for the expression of the ethanolamine metabolosome in its equivalent of the *eut*/*pdu* operon, namely, ef1617 to ef1634, which appeared to be highly expressed at late log phase by both the parental and the *fsrB* mutant strains. At ENT-stat and EAR-stat, the FsrABC system appeared to turn off the *eut*/*pdu* operon, suggesting the possibility that, when detecting a limitation for some essential nutrients (glycerophospholipid), OG1RF cells redirect their metabolism to avoid the use of phosphatidylethanolamine as a carbon and nitrogen source.

Following the same theme, we observed enhanced transcription (4- to 24-fold) of ef1561 to -1567 in OG1RF versus TX5266 from ENT-stat (Fig. 3B and C and Table 2), also seen by semiquantitative RT-PCR (Fig. 2B). This operon codes for enzymes predicted to be necessary for phenylalanine and tyrosine synthesis. This regulation profile is reversed in the case of ef1658 to -1663, genes coding for enzymes implicated in isoleucine, valine, and leucine degradation pathways (reduced transcription, at ENT-stat, by 5- to 11-fold in OG1RF versus TX5266 for all the genes of the operon and 8- to 11-fold for ef1659 to -1660 and ef1663 at EAR-stat). The repression of the *eut-pdu* operon, described above, should also lead to the conservation of methionine by reducing its use as a methyl donor. It has been shown that methionine, tryptophan, histidine, and isoleucine are essential for all the *E. faecalis* strains tested, while arginine, glutamate, glycine, leucine, and valine are important for OG1RF yet appear to be essential for JH2-2 (28). We can postulate that strain JH2-2, a natural *fsrB* mutant, is more sensitive to the absence of some or all of these amino acids than OG1RF because its FsrABC system is not functional to protect at least leucine and valine from degradation.

Finally, at both ENT-stat and EAR-stat, the expression of three phosphotransferase (PTS) systems was activated in the presence of *fsrB* (ef0408 to -0413 [~9-fold], ef0955 to -0958 [~6-fold], and ef3210 to -3213 [~7-fold]). Based on the KEGG website, each of these PTS systems appears to be sugar specific, with EF408 to -412 specific for mannitol, EF0955 to -0958 for maltose, and EF3210 to -3213 for mannose. Interestingly, the ef0954 to -0958 locus has been described in two different papers: (i) as *bopABCD* (ef0957 to -0954) (20) and (ii) as *malT* (ef0958) and *malPBMR* (ef0957 to -0954) (24). Both *malT* (ef0958) and *malP* (ef0957/*bopA*) appear to be essential for maltose transport and utilization in strain JH2-2 (24) and, using *E. faecalis* type 9 strain, *bopD* (ef0954/*malR*, a LacI family transcriptional regulator), but not *bopABC*, appears to be important for biofilm formation and for bacteremia in mice (20). These data suggest that Fsr plays a positive role in biofilm production by at least two independent mechanisms: through the activation of gelatinase production (18, 27) and through the activation of *bopABCD* expression (20).

Factors (potentially) important for virulence. In addition to GelE and SprE, we also found that the FsrABC system of OG1RF regulates at least one other well-described factor important for OG1RF virulence, namely, EfaA. EfaA is a 37-kDa dominant antigen in infective endocarditis caused by *E. faecalis* (2, 26) and is part of what is predicted to be an ABC-type transporter, with EfaA being the lipoprotein component. *efaA* is the third gene of the *efaCBA* operon. *efaB* (ef2075) and *efaA* (ef2076) expression was activated in OG1RF three- to eightfold ($P < 0.05$) compared to the *fsrB* mutant in ENT-stat and EAR-stat (Fig. 2B and 3B and C), while no statistically significant results were obtained for the first gene (ef2074). All three genes were expressed, but not Fsr regulated, in late log phase. In strain JH2-2, expression of this operon is Mn^{+} dependent and regulated via EfaR, a DNA binding protein (25). In OG1RF, the Fsr effect on *efaA* and *efaB* expression appeared to be independent of the expression of *efaR*, because although expressed, *efaR* was not regulated under any of our conditions. It would be of interest to investigate if another regulator of JH2-2 (which lacks *fsrABC*) influences the expression of genes that are part of the Fsr regulon of OG1RF, similar to what was shown recently in *Pseudomonas aeruginosa*, where the regulon of MvfR modulates some of the *lasRI*/*rhlRI*-regulated genes without directly affecting the *lasRI* or *rhlRI* systems (12).

The FsrABC system also regulates ef2058 to -2059, coding for orthologues of *cydC* and *cydD* (components of an ABC-type transporter required for assembly of cytochrome *bd* in *Escherichia coli* [10] and in *Bacillus subtilis* [52]). In *Shigella flexneri* (49, 50), as in *Brucella abortus* (39), this ABC transporter is critical for intracellular survival and full bacterial virulence. ef2058 to -2059 are part of an operon (ef2061 to -2058) in which the expression level was constant for the two first genes of the operon (ef2061 and -2060) while the expression level of the two downstream genes (ef2059 and -2058) was affected in the Fsr mutant as early as late log phase. These two genes were expressed threefold higher in late log phase and six- to sevenfold higher at ENT-stat, and at EAR-stat, *cydD* was expressed fivefold higher in the parent strain than in TX5266 (Fig. 3B and C). As with the *efa* operon, this is the second time that only the downstream genes of an operon appeared to be Fsr activated. We can postulate that the Fsr system may have an effect on mRNA stability.

As mentioned previously and as shown in Fig. 2B, only one other gene besides the *fsr* and *gel* loci was dependent on the presence of an intact *fsrB* gene for its expression in late log phase to ENT-stat: ef1097. In OG1RF, expression of ef1097 was very high in late log phase, decreased significantly at ENT-stat (4 h), and finally was undetectable at EAR-stat (5 h) by microarray. This gene is localized 2 kb upstream of ef1099 (Fig. 4A), a gene encoding Ace, a well-characterized collagen adhe-

sin (32). ef1097, like *ace*, is present in clinical as well as in food isolates (P. Serror, personal communication). The gene codes for a putative membrane protein (170 amino acids) of unknown function that shares some homology with uncharacterized plasmid proteins from *Streptococcus pyogenes* (42% identity; 65% similarity) and *Corynebacterium jeikeium* (25% identity; 50% similarity). As with the ef0750 insertion mutant, no obvious difference in growth rate or in cellular physiology was observed when an ef1097 insertion mutant was compared with OG1RF in BHI broth culture, and no difference in pathogenicity in the *C. elegans* worm model was observed (D. Garsin, personal communication).

Of interest, too, is an operon including eight genes, ef0750 to -0757, that was found to be activated in the presence of an intact *fsrB* by two- to fourfold ($P < 0.01$) in late log phase and at ENT-stat. The level of ef0750 to -0757 expression decreased between late log phase and ENT-stat, although the level of regulation of the genes with detectable intensity remained stable (~4-fold). At EAR-stat, the level of expression reached an undetectable level with each strain by microarray, but ef0750 expression was still detected by RT-PCR. The expression of ef0755 to -0757 compared to ef0750 to -0754 was 10 times lower, likely due to the presence of a weak termination loop (Fig. 2B and data not shown). In the V583 genome, five operons were found with an organization similar to that of ef0750 to -0757. Two of these five operons were found to be expressed in OG1RF, although only the ef0750 to -0757 operon was regulated by the FsrABC system under our conditions. The presence of the *fsrB*-regulated complex ef0750 to -0757, encoding surface and secreted proteins, with at least four paralogous systems in *E. faecalis* but none in other species, is intriguing.

Conclusion. The Fsr system, known to be important for virulence in several animal models, is critical for expression of the *gelE-sprE* operon. In this study, we characterized the Fsr regulon in broth culture in three successive phases of growth (late log phase, at entry into stationary phase, and 1 h after entry into stationary phase). Although we were unable to study all OG1RF genes (10 to 15% of the OG1RF genes identified in reference 15 are not present in the V583 genome, and only 75% of the V583 PCR products printed on the array appeared positive with OG1RF genomic DNA) (data not shown), the extent of the Fsr regulon establishes that the FsrABC system is an important general regulator in OG1RF. From the other Agr-like systems studied, it appears that Agr-like systems act as significant regulators in pathogenic species (Agr in *S. aureus* and now Fsr in *E. faecalis*) and as limited regulators in nonpathogenic organisms (Lam in *Lactobacillus plantarum*) (45).

Fsr activates and represses numerous genes: besides *gelE* and *sprE*, the strongest activation effect (undetectable expression in the mutant) was seen in late log phase with ef1097, which encodes a putative membrane protein of unknown function, while the strongest repression effect (undetectable expression in the parent) was seen at ENT-stat and EAR-stat with ef1617 to -1638 (*eut* and *pdu* locus). New findings concerning *fsrB*-regulated factors potentially important for virulence, such as ef2058 to -2059 (*cydCD*), ef0954 to -0957 (*bopABCD*), or ef0750 to -0757 (putative membrane complex), may help elucidate additional pathogenicity mechanisms independent of the function of GelE and SprE of *E. faecalis*.

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