INFECTION AND IMMUNITY, Oct. 2002, p. 5647–5650 Vol. 70, No. 10
0019-9567/02/$04.00+0 DOI: 10.1128/IAI.70.10.5647–5650.2002

Virulence Effect of *Enterococcus faecalis* Protease Genes and the Quorum-Sensing Locus *fsr* in *Caenorhabditis elegans* and Mice

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Received 25 March 2002/Returned for modification 14 May 2002/Accepted 4 July 2002

The expression of two *Enterococcus faecalis* extracellular virulence-related proteins, gelatinase (GelE) and serine protease (SprE), has been shown to be positively regulated by the *fsr* quorum-sensing system. We recently developed a novel system for studying *E. faecalis* pathogenicity that involves killing of the nematode worm *Caenorhabditis elegans* and showed that an *E. faecalis fsrB* mutant (strain TX5266) exhibited attenuated killing. We explore here the role of the *fsr*/*gelE-sprE* locus in pathogenicity by comparing results obtained in the nematode system with a mouse peritonitis model of *E. faecalis* infection. Insertion mutants of *fsrA* (TX5240) and *fsrC* (TX5242), like *fsrB* (TX5266), were attenuated in their ability to kill *C. elegans*. A deletion mutant of *gelE* (TX5264) and an insertion mutant of *sprE* (TX5243) were also attenuated in *C. elegans* killing, although to a lesser extent than the *fsr* mutants. Complementation of *fsrB* (TX5266) with a 6-kb fragment containing the entire *fsr* locus restored virulence in both the nematode and the mouse peritonitis models. The *fsr* mutants were not impaired in their ability to colonize the nematode intestine. These data show that extracellular proteases and the quorum-sensing *fsr* system are important for *E. faecalis* virulence in two highly divergent hosts: nematodes and mice.

Enterococci are gram-positive bacteria that are normal inhabitants of the alimentary tract of humans and other animals. They have been recognized as a cause of infective endocarditis for more than a century (17) and have gained prominence over the last two decades as being among the most common pathogens found in hospital-acquired infections, including urinary tract infections, bloodstream infections, and surgical-site infections (27). The increasing importance of enterococci as nosocomial pathogens can be attributed in part to intrinsic and acquired antibiotic resistance (17, 26). Treatment of multidrug-resistant enterococcal infections poses a significant challenge to clinicians (4, 8), and the potential of these organisms to serve as a reservoir for antibiotic resistance genes is of great concern (6, 20, 21). Despite increasing recognition of the clinical importance of enterococcal infections, their pathogenic mechanisms are not well understood (11).

We have recently developed a novel model host-pathogen system for *Enterococcus faecalis* by using the nematode *Caenorhabditis elegans* (7). Adult worms feeding on lawns of *E. faecalis* die over the course of several days in a process that has the hallmarks of an active infection rather than an intoxication; that is, live bacteria colonize and proliferate within the intestine of adult *C. elegans*, causing a persistent and deadly infection. Cytolysin, a well-studied enterococcal virulence factor important in mammalian pathogenesis (recently reviewed by Haas and Gilmore [10]), increases the rate of nematode killing.

E. faecalis gelatinase (GelE) and serine protease (SprE) are two additional putative virulence factors thought to play a role in systemic disease in mammalian hosts (5, 9, 23, 28). GelE is a secreted 30-kDa metalloprotease that shares homology with *Staphylococcus aureus* aureolysin and *Pseudomonas aeruginosa* elastase (29). Insertion disruption of *gelE* significantly attenuates virulence in a mouse peritonitis model (28). The serine protease gene *sprE*, which lies immediately downstream of and is cotranscribed with *gelE*, encodes a secreted 26-kDa serine protease that shares homology with *S. aureus* V8 protease (22, 23). Insertion disruption of *sprE* also attenuates virulence in the mouse peritonitis model (23). Transcription of the *gelE-sprE* operon is positively regulated in a growth-phase-dependent fashion by the *fsr* locus, which shares many similarities with the well-studied *S. aureus agr* regulatory locus (23). The *fsr* locus is composed of three regulatory genes—*fsrA*, *fsrB*, and *fsrC*—located upstream of the *gelE-sprE* operon (23). Based on sequence homology with AgrA and AgrC, FsrA and FsrC likely constitute a classical two-component system, in which FsrC acts as a histidine kinase sensor and FsrA acts as a response regulator. Moreover, the predicted FsrB protein shares homology with AgrB (23), a membrane-bound protein thought to be involved in the production of active, thiolactone-containing, signaling peptide from the AgrD prepheromone (13). FsrB has recently been shown to have a carboxy-terminal extension of approximately 50 amino acids compared to AgrB, and this extension is processed into a lactone-containing, 11-residue peptide pheromone, termed gelatinase biosynthesis-activating pheromone (19, 22).

Previously, we demonstrated that an *fsrB* deletion mutant (TX5266) was attenuated in both the *C. elegans* and the mouse peritonitis model systems (7). In the present study, we further explore the role of *fsrB*, as well as that of *fsrA*, *fsrC*, gelatinase,

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype and/or phenotype[a]	Source or reference
Strains		
OG1RF	Wild-type *E. faecalis* strain; Gel$^+$ Spr$^+$ Rif^r Fus^r	18
TX5128	OG1RF *gelE* transposon mini-γδ insertion mutant; Gel$^-$ Spr$^-$ Rif^r Fus^r Kan^r	28
TX5240	OG1RF *fsrA* insertional mutant; Gel$^-$ Spr$^-$ Rif^r Fus^r Kan^r	23
TX5242	OG1RF *fsrC* insertional mutant; Gel$^-$ Spr$^-$ Rif^r Fus^r Kan^r	23
TX5243	OG1RF *sprE* insertional mutant; Gel$^+$ Spr$^-$ Rif^r Fus^r Kan^r	23
TX5264	OG1RF *gelE* in-frame deletion mutant (bp 252 to 1490), Gel$^-$ Spr$^+$ Rif^r Fus^r	This study
TX5266	OG1RF *fsrB* in-frame deletion mutant (bp 79 to 684), Gel$^-$ Spr$^-$ Rif^r Fus^r	29, 22
TX5266.01	TX5266 harboring plasmid pTEX5249; Gel$^+$ Spr$^+$ Rif^r Fus^r Em^r	This study
TX5266.OS	TX5266 harboring plasmid pAT18; Gel$^-$ Spr$^-$ Rif^r Fus^r Em^r	This study
E007	*E. faecium* clinical isolate	7
Plasmids		
pAT18	*E. coli-E. faecalis* shuttle vector; Em^r	33
pTEX4577	*E. faecalis* suicide vector derived from pBluescript SK(−); Kan^r	28
pTEX5249	pAT18 containing 6-kb *Pst*I/*Bgl*II fragment encoding *fsrA, fsrB,* and *fsrC;* Em^r	23

[a] Abbreviations: Rif, rifampin; Fus, fusidic acid; Kan, kanamycin; Em, erythromycin; Gel, gelatinase; Spr, serine protease; r, resistance.

and serine protease, in *E. faecalis*-mediated nematode killing and compare these findings with those obtained from a mouse peritonitis model.

MATERIALS AND METHODS

Organisms, culture conditions, and assays. The bacterial strains and plasmids used in the present experiments are listed in Table 1. *Enterococcus* strains were grown by using brain heart infusion (Difco Laboratories, Detroit, Mich.) broth and agar at 37°C, supplemented, as appropriate, with rifampin (100 μg/ml), kanamycin (2,000 μg/ml), or erythromycin (10 μg/ml). *Enterococcus* lawns for *C. elegans* killing and intestinal colonization assays were prepared as previously described (7), with the following modification: ciprofloxacin (100 ng/ml) was used as a selective antibiotic against *Escherichia coli* strain OP50, the normal food source for *C. elegans. C. elegans* wild-type strain Bristol N2 was maintained at 15°C on nematode growth medium agar plates spotted with *E. coli* OP50 (2, 14). *C. elegans* killing assays (7), using L4 hermaphrodite nematodes, and the mouse peritonitis model (23), with groups of six outbred ICR female mice for each dose (mean weight of 25 g; Harlan Sprague-Dawley, Houston, Tex.), were carried out as previously described. Nematode alimentary tracts were examined by Nomarski differential interference contrast microscopy by standard techniques (30).

Deletion mutagenesis. To make a deletion in *gelE*, the flanking regions of *gelE* were amplified by PCR with the primer pairs (i) GDF1 (AAA GAG CTC CTA AAA GTG ATT GTT GAT GTG C, from bp 639 to 618 upstream of the start codon of *gelE*) and GDR1 (CCG AAT TCA TCA ACA GTA ACG CCT TCC G, from bp 258 to 239 inside *gelE*) and (ii) GDF2 (AAG AAT TCA TTC AGG TAA ACC AAC CAA GTG, from bp 45 to 24 before the *gelE* stop codon) and GDR2 (TTG GTA CCG ATT ATT TGC CTT CTT TTC AGC, from bp 1047 to 1026 after the *gelE* stop codon) for the 5′ and 3′ regions, respectively (linker sequences are underlined). The amplicons were ligated together by the linker (*Eco*RI) designed in the two inner primers, GDR1 and GDF2, and inserted into the mutagenesis vector pTEX4577 by using the outer linkers (*Sac*I and *Kpn*I) designed in the two outer primers, GDF1 and GDR2. The resulting construct was then transformed into OG1RF by electroporation (15), and single-crossover mutants were selected on brain heart infusion agar plates supplemented with kanamycin. Single-crossover mutants were still gelatinase positive because of the duplication of the flanking regions. Since this single-crossover event creates duplicated fragments of the regions flanking the targeted *gelE* gene, subsequent recombination between these duplicated fragments will lead to the loss of the mutagenesis vector and one copy of the duplicated flanking sequence, giving rise to either the restored wild-type strain or the deletion mutant. After the loss of the mutagenesis vector, both the wild-type and the deletion mutant strains become kanamycin sensitive. To identify the *gelE* deletion mutant, we first plated the culture of the single-crossover mutants of *gelE* grown overnight without kanamycin on Todd-Hewitt (Difco Laboratories) agar containing 3% gelatin (Sigma, Saint Louis, Mo.) to score for the loss of gelatinase activity. Colonies that were gelatinase production negative were then scored for continued serine protease production and for the loss of kanamycin resistance. A selected gelatinase-negative, serine protease-positive, kanamycin-sensitive mutant was further confirmed as being a *gelE* deletion mutant by PCR with the two outer primers, GDF1 and GDR2, and by pulsed field gel electrophoresis (18).

Detection of protease activity. The production of gelatinase in *E. faecalis* was detected by using Todd-Hewitt agar containing 3% gelatin, as previously described (23). The production of serine protease activity in *E. faecalis* was detected by 0.05% casein zymogram gel (Novex, San Diego, Calif.) analysis, as previously described (23).

Statistical analysis. Kaplan-Meier survival estimates determined by using log-rank analysis were performed as previously described for both nematode and mouse survival by using STATA 6.0 for Windows. The data were plotted with GraphPad Prism 3.02 for Windows.

RESULTS AND DISCUSSION

Mutants of the *fsr* quorum-sensing system. To determine whether the previously observed effect of an *fsrB* mutation on *E. faecalis* virulence in the *C. elegans* model was specific to *fsrB* or representative of the entire *fsr* locus, we evaluated the ability of *fsrA* (TX5240) and *fsrC* (TX5242) insertion mutants to kill *C. elegans*. Compared with the wild-type strain OG1RF, strains TX5240 ($P \leq 0.0001$) and TX5242 ($P \leq 0.0001$) were highly defective in their ability to kill *C. elegans*, similar to strain TX5266 (Δ*fsrB*) ($P \leq 0.0001$) (Fig. 1). There were no significant differences in killing among the three *fsr* mutants ($P > 0.05$). As a control, the clinical *E. faecium* isolate E007 demonstrated little nematocidal activity, as has been shown previously (7). These data suggest that enterococci employ quorum-sensing during the pathogenic process in *C. elegans*.

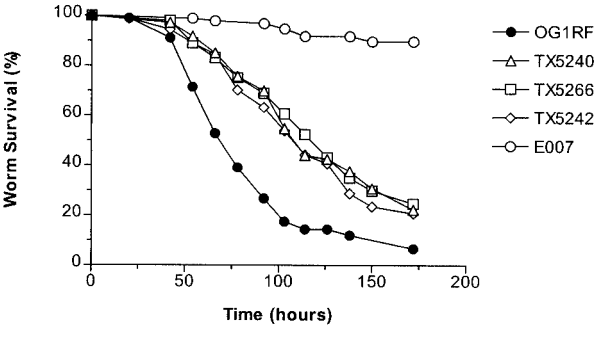

FIG. 1. Survival of *C. elegans* on *fsr* mutants. Kaplan-Meier survival plots of worms feeding on OG1RF and the *fsr* mutants TX5240 (*fsrA*), TX5266 (Δ*fsrB*), and TX5242 (*fsrC*), as well as the *E. faecium* clinical isolate, E007 (negative control). Similar data were obtained in three independent experiments.

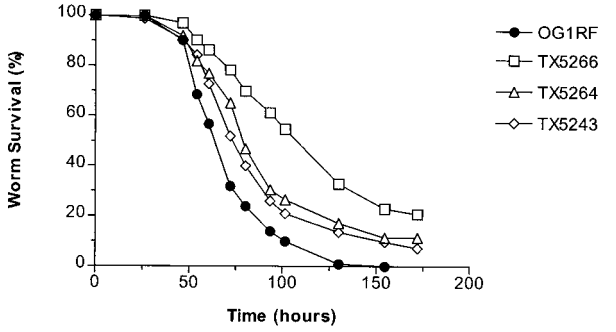

FIG. 2. Survival of *C. elegans* on *gelE* and *sprE* mutants. Kaplan-Meier survival plots of worms feeding on OG1RF, TX5266 (Δ*fsrB*), TX5264 (Δ*gelE*), and TX5243 (*sprE*). Similar data were obtained in three independent experiments.

We had previously obtained similar data showing that the gram-negative human pathogen, *P. aeruginosa*, also employs quorum-sensing during pathogenic processes in both nonvertebrate and vertebrate hosts. Mutants of the quorum-sensing-associated regulators *lasR*, *gacA*, and *mvfR* of *P. aeruginosa* PA14 exhibit reduced virulence in *C. elegans* (16, 31, 32), *Arabidopsis thaliana* (3, 24, 25), and *Galleria mellonella* (*lasR* and *gacA* mutants) (12), as well as in a mouse full-skin-thickness burn model of *P. aeruginosa* infection (24, 25, 32).

Mutants of the protease genes *gelE* and *sprE*. Because neither gelatinase nor serine protease activity are detectable in the *fsr* mutants TX5240, TX5242, and TX5266 (22, 23), we evaluated the contribution of *E. faecalis* gelatinase and serine protease separately to nematocidal activity. A *gelE* in-frame deletion mutant (TX5264) was constructed by allelic replacement (22) and, as shown in Fig. 2, TX5264 ($P \leq 0.0001$) and the previously constructed *sprE* insertion mutant TX5243 (23) ($P \leq 0.0001$) were each found to be attenuated in nematode killing, although to a lesser degree than TX5266. Compared to each other, TX5264 and TX5243 were not significantly different in their ability to kill *C. elegans* ($P > 0.05$) (Fig. 2). Nematode killing by the mini-γδ transposon insertion mutant of *gelE*, TX5128 (28), was more attenuated than the *gelE* in-frame deletion mutant TX5264 ($P \leq 0.0001$) but less attenuated than the *fsrB* deletion mutant TX5266 ($P \leq 0.0001$) (Fig. 3). Since the transposon mutation in *gelE* has a polar effect on the downstream *sprE* gene (as demonstrated by Northern blot, reverse transcription-PCR, and zymographic analyses [22, 23]), these results suggest that GelE and SprE may have additive effects on virulence. However, because TX5266 is even more attenuated than TX5128, these results also raise the possibility that the *fsr* system regulates genes involved in virulence for *C. elegans* in addition to *gelE* and *sprE*.

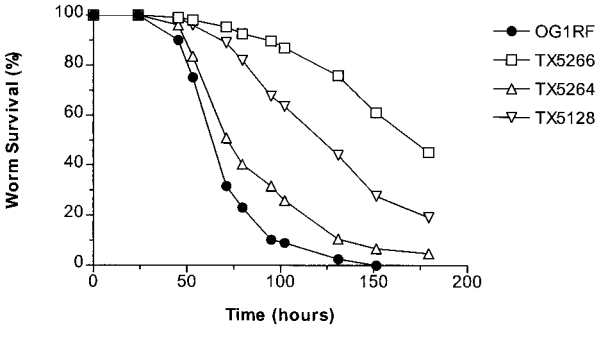

FIG. 3. Survival of *C. elegans* on insertion and deletion *gelE* mutants. Kaplan-Meier survival plots of worms feeding on OG1RF, TX5266 (Δ*fsrB*), TX5264 (Δ*gelE*), and TX5128 (*gelE*::mγδ). Similar data were obtained in three independent experiments.

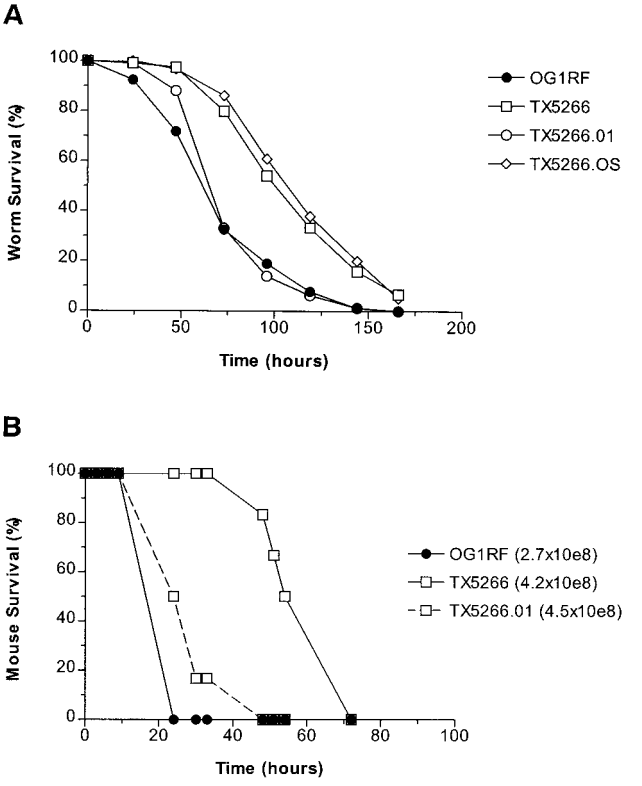

FIG. 4. Complementation of *fsrB in trans* restores virulence in both the nematode and the mouse peritonitis models. (A) Survival plots of worms on OG1RF, TX5266, TX5266 carrying the *fsrABC*+ vector pTEX5249 (TX5266.01), and TX5266 carrying the "empty" vector pAT18 (TX5266.OS). Similar data were obtained in three independent experiments. (B) Kaplan-Meier survival plots of mice infected peritoneally with OG1RF, TX5266 (Δ*fsrB*), and TX5266 carrying the *fsrABC*+ vector pTEX5249 (TX5266.01). TX5266 was significantly attenuated compared to OG1RF at a smaller inoculum ($P = 0.0009$; closed circles versus open squares). TX5266.01 was significantly more virulent than TX5266 at similar inocula ($P = 0.0012$; open dashed squares versus open squares). Similar delays in lethality of TX5266 compared to OG1RF and TX5266.01 were also observed in a separate experiment with one-half of these inocula (data not shown).

Complementation of the *fsrB* mutant. To confirm that the effect on *E. faecalis* virulence in TX5266 is due to the loss of a functional *fsr* system, we complemented the *fsrB* mutant TX5266 with the plasmid pTEX5249, which contains a 6-kb *Pst*I/*Bgl*II fragment encoding *fsrA*, *fsrB*, and *fsrC*. This construct, TX5266.01, demonstrated restored gelatinase production (data not shown). As shown in Fig. 4, TX5266.01 had pathogenicity restored to near-wild-type levels in both the *C. elegans* killing assay and the mouse peritonitis model, indicating that virulence can be complemented with *fsr* function provided in *trans*. As a control, the *fsrB* mutant TX5266 transformed with the pAT18 "empty" vector alone (TX5266.OS)

had no significant difference in *C. elegans* virulence compared to plasmid-free TX5266.

Microscopy. We previously found that live bacteria colonize the alimentary tract of adult *C. elegans* feeding on *E. faecalis*, similar to worms fed *P. aeruginosa* and *Salmonella enterica* (7). Such colonization is not seen when worms are fed *E. coli* or *Bacillus subtilis* (1, 7, 31). A small inoculum of *E. faecalis* is sufficient to colonize the nematode digestive tract and is followed by bacterial proliferation within the gut, leading to marked distention of the intestinal lumen (7). Mutants of the PA14 virulence regulators *gacA* and *lasR* fail to accumulate to a significant degree in the nematode alimentary tract after 24 to 48 h of feeding, suggesting that the establishment and/or proliferation of bacteria within the nematode may be dependent on the quorum-sensing mechanisms of *P. aeruginosa* (31, 32). However, in contrast to the results obtained with quorum-sensing mutants of *P. aeruginosa*, microscopic examination of adult nematodes feeding on lawns of either OG1RF or the *fsrB* mutant TX5266 showed almost identical gut distention with intact bacteria throughout the life span of the worms (data not shown). These data suggest that the *fsr* system does not play a role in *E. faecalis* colonization of the nematode intestine.

Conclusion. The *E. faecalis fsr* system is the second example of a quorum-sensing system that regulates virulence gene expression in bacterial infection of both simple model organisms and mammalian hosts. Quorum sensing may be an important mechanism used by many prokaryotes to adapt to different environments encountered during pathogenesis. Our results also raise the possibility that the *fsr* system in *E. faecalis* regulates virulence genes in addition to *gelE* and *sprE* in this pathogen.

ACKNOWLEDGMENTS

This research was supported by Postdoctoral Research Fellowships for Physicians from the Howard Hughes Medical Institute (C.D.S. and E.M.), by a postdoctoral fellowship from the Irvington Institute for Immunological Research (D.A.G.), by grant AI47923 from the National Institute of Allergy and Infectious Diseases (B.E.M.), and by a grant from Aventis SA (F.M.A. and S.B.C).

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Editor: B. B. Finlay