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NOTES

The *Enterococcus faecalis fsrB* Gene, a Key Component of the *fsr* Quorum-Sensing System, Is Associated with Virulence in the Rabbit Endophthalmitis Model

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We used a rabbit endophthalmitis model to explore the role of *fsrB*, a gene required for the function of the *fsr* quorum-sensing system of *Enterococcus faecalis*, in pathogenicity. A nonpolar deletion mutant of *fsrB* had significantly reduced virulence compared to wild type. Complementation of mutation restored virulence. These data corroborate the role of *fsrB* in *E. faecalis* pathogenesis and suggest that the rabbit endophthalmitis model can be used to study the in vivo role of quorum sensing.

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 (16) and are among the most common pathogens found in hospital-acquired infections, including vision-threatening endophthalmitis (3, 6, 9). The mechanisms of pathogenesis of enterococcal infections are not yet well understood (8), and the development of multidrug resistance in enterococci has made some enterococcal infections difficult or impossible to treat (14, 15).

Quorum sensing is a cell density-dependent regulatory system that controls a variety of group behaviors in bacteria (7, 21). In *Enterococcus faecalis*, the *fsr* system positively regulates the expression of gelatinase and serine protease in a cell density-dependent manner, similar to the well-studied regulation of toxins by the *Staphylococcus aureus agr* quorum-sensing regulatory locus (10, 18). Qin et al. have characterized three genes in the *fsr* regulatory locus, *fsrA*, *fsrB*, and *fsrC*. Using a nonpolar deletion mutant in *fsrB*, the same workers showed that *fsrB* is required for the regulatory function of the Fsr system (19, 20). The expression of the *fsr* genes in *E. faecalis* OG1RF is cell density dependent and is most active in the postexponential phase of growth (17, 19). Recent work has shown that the gelatinase biosynthesis-activation pheromone is actually encoded in the 3′ portion of *fsrB*, within the *fsr* gene cluster (17, 19, 20).

Previously, we have demonstrated that an *fsrB* nonpolar deletion mutant (TX5266) was attenuated not only in a mouse peritonitis model but also in a novel *E. faecalis* pathogenesis system that utilizes the nematode *Caenorhabditis elegans* as a model alternative host (5). In the present study, we further explored the role of *fsrB* in an established model of rabbit endophthalmitis, which provides unique opportunities to study the evolution of enterococcal disease by direct observation and through sensitive electrophysiologic measures of organ function (11). Our results suggest that quorum sensing may be particularly important in this closed-space infection.

Strains were propagated in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.). For OG1RF and mutant derivatives, the medium was supplemented with rifampin (25 μg/ml) and fusidic acid (10 μg/ml). For the endophthalmitis model, before intravitreal inoculation cells were diluted in phosphate-buffered saline, and each inoculum was ca. 10^2 CFU. Enumeration of organisms at the time of inoculation and after recovery from the vitreous was accomplished by plating on BHI agar, with selective antimicrobials (as detailed in Table 1) and Bacto-agar (1.5% [wt/vol] [Difco]). The strains used are summarized in Table 1. To confirm that the effect on *E. faecalis* virulence in TX5266 was due to loss of a functional *fsr* system, we complemented the Δ*fsrB* mutant TX5266 with plasmid pTEX5249, which contains a 6-kb *Pst*I/*Bgl*II fragment encoding *fsrA*, *fsrB*, and *fsrC*. This construct, TX5266.01, demonstrated restoration of gelatinase (data not shown); gelatinase production was assayed on Todd-Hewitt medium containing 3% gelatin (catalog no. 0143-17-9; Difco).

Experimental endophthalmitis. New Zealand White female rabbits (weight, 1.75 to 2.25 kg) were used for our experiments. Infection was induced, and electroretinography (ERG) was evaluated as previously described (2, 11). Animals were housed and cared for, in accordance with the Association for Research

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or phenotype[a]	Reference
Strains		
OGIRF	Wild-type *E. faecalis* strain; Gel^+ Spr^+ Rif^r Fus^r	20
TX5266	OG1RF *fsrB* in-frame deletion mutant, deletion from bp 79 to 684 of *fsrB*; Gel^- Rif^r Fus^r	19
TX5266.01	TX5266 harboring plasmid pTEX5249; Gel^+ Spr^+ Rif^r Fus^r Em^r	This study
Plasmid		
pTEX5249	pAT18 shuttle vector containing 6-kb *Pst*I/*Bgl*II fragment encoding *fsrA, fsrB*, and *fsrC*; Em^r	20

[a] Abbreviations: Rif, rifampicin; Fus, fusidic acid; Kan, kanamycin; Em, erythromycin; Gel, gelatinase

in Vision and Ophthalmology regulations, at the Dean A. McGee Eye Institute, Oklahoma City, Okla. For evaluation of intraocular growth, a group of rabbits was sacrificed at 12, 36, and 48 h after infection, and the number of organisms in each homogenized sample was determined by plating duplicate serial 10-fold dilutions on BHI-agar. After 24 h incubation at 37°C, colonies were counted and concentrations were expressed as CFU per milliliter of intraocular sample. For histology, eyes were fixed in 4% paraformaldehyde for 24 h. Five serial sections were prepared and stained with hematoxylin and eosin (H-E). Pathological interpretations were made with the investigator blinded as to the nature of the infecting organism.

For statistical analysis, all values represent the mean ± standard error of the mean. A two-tailed Student's *t* test for unequal variances was used for statistical comparisons between groups. A *P* value of <0.05 was considered significant.

Intraocular growth of *E. faecalis*. The rates of intraocular growth of *E. faecalis* OG1RF, the Δ*fsrB* deletion mutant, and the complemented mutant TX5266.01 are depicted in Fig. 1a. The data reveal similar in vivo growth kinetics for all three strains. A steady increase in CFU was seen through 36 h postinoculation, at which time CFU counts reached a plateau and remained constant for the duration of the experiment.

Loss of retinal function (ERG). As shown in Fig. 1b, infections caused by *E. faecalis* OG1RF resulted in a significantly greater reduction of B-wave amplitude than the *fsrB* deletion mutant at time points 24 h ($P < 0.001$), 36 h ($P < 0.001$), and 48 h ($P < 0.005$). While infection with *E. faecalis* OG1RF resulted in a rapid functional loss of about 85% by 24 h and practically complete visual loss by 36 h, eyes infected with the *fsrB* deletion mutant retained 100% visual function at 24 h and did not decrease to below 50% over the whole course of the experiment. The complemented mutant TX5266.01 caused a virtually identical course of functional loss compared to the wild-type organism.

Histopathology. Within 36 to 48 h, eyes injected with *E. faecalis* OG1RF showed marked vitreal polymorphonuclear infiltrate, cystoid changes in the ganglionic cell layer, decreased nuclear density of the inner and outer nuclear layers, mild subretinal polymorphonuclear infiltrate, and overall loss of structural integrity. In contrast, even after 48 h, eyes injected with the Δ*fsrB* strain showed only mild vitreal polymorphonuclear infiltrate, preserved structure of all retinal layers, and no subretinal inflammatory infiltrate (representative slides are shown in Fig. 2). The histopathologic data from the group of rabbits infected with the complemented mutant were identical to those noted in the group injected with the wild type. When loss of B-wave response was 100% (for the *fsrB* deletion mutant, this time point was after the time points noted on Fig. 1b), histologic examination revealed the same degree of destruction in all three groups.

Gram-positive bacteria often use small peptide "pheromones" as cell-to-cell communication signals to mediate quorum sensing (4, 7, 17). These communication signals are secreted from growing cells and accumulate outside the growing

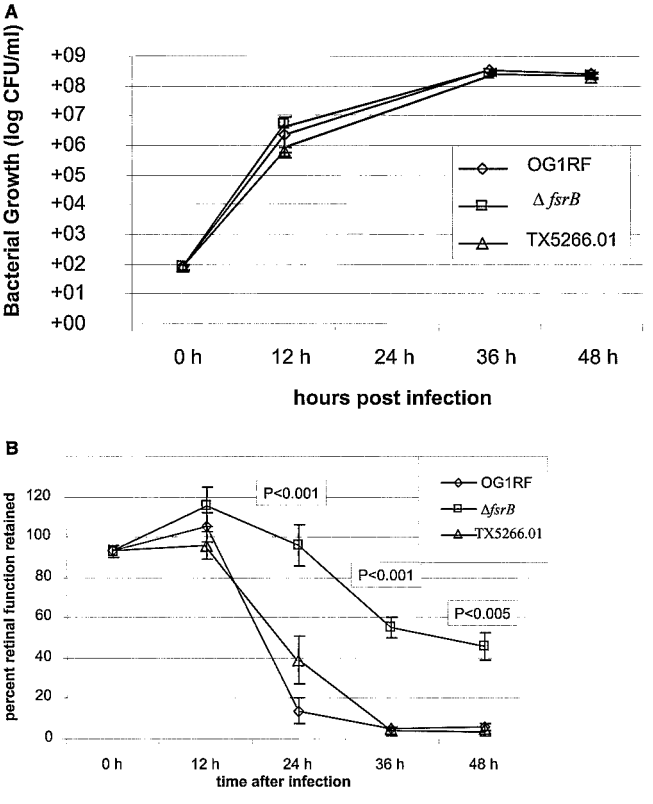

FIG. 1. (a) Growth of wild-type *E. faecalis* strain OG1RF, the nonpolar deletion mutant Δ*fsrB*, and the complemented strain TX5266.01 in vitreo. (b) Retinal function after intraocular infection with *E. faecalis* OG1RF, the nonpolar deletion mutant Δ*fsrB* (TX5266), and the complemented strain TX5266.01. Rabbits ($n = 12$) were injected with ca. 10^2 CFU, and retinal function was assessed at 12, 24, 36, and 48 h after injection. Percent retinal function was defined as follows: (B-wave amplitude of the infected eye)/(B-wave amplitude of the saline-injected contralateral eye). The *P* values shown are derived from a two-tailed Student's *t* test for unequal variances comparing Δ*fsrB* to OG1RF; there was no statistical difference between the group that received OG1RF and the TX5266.01 group. Error bars represent the standard errors of the means.

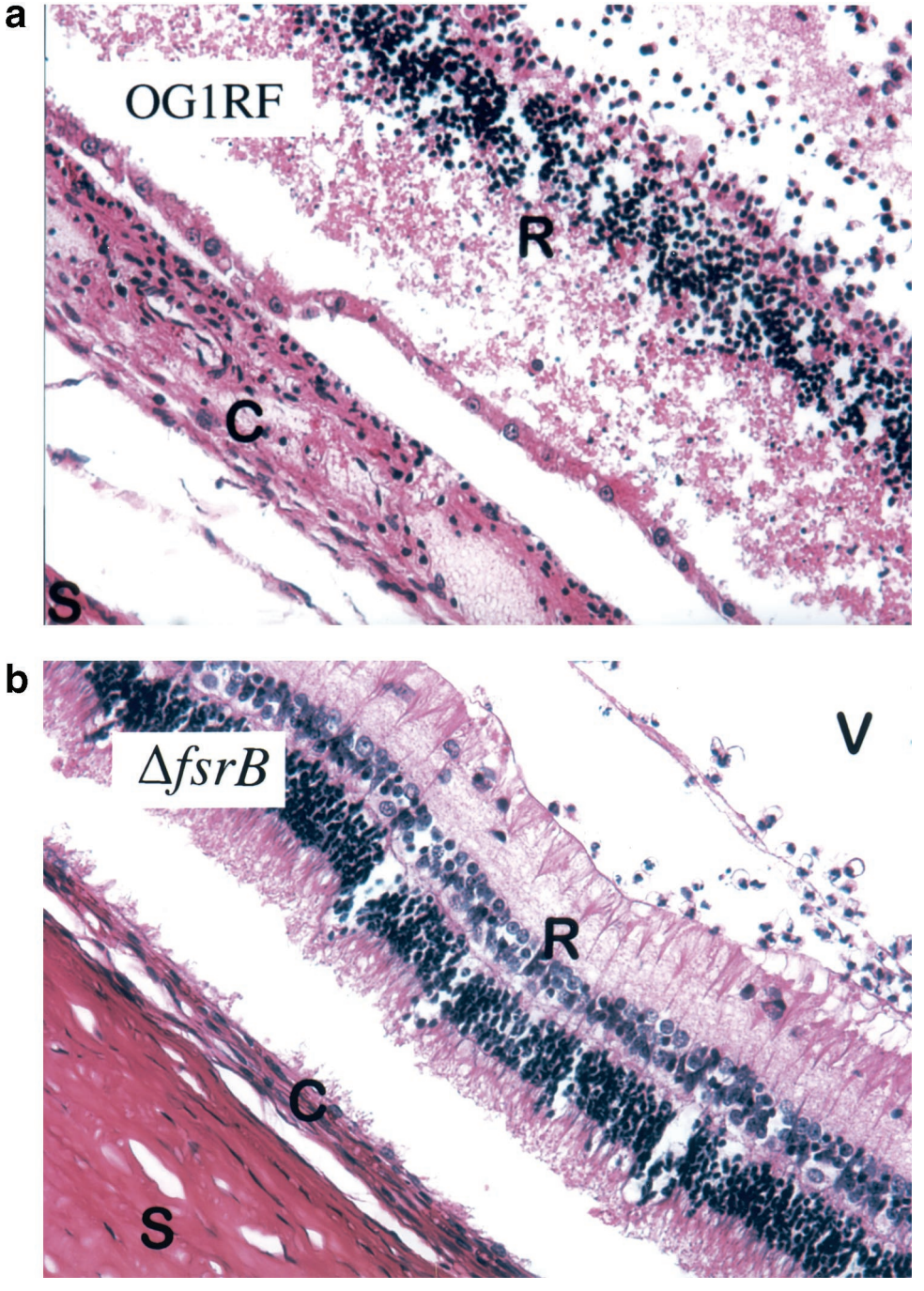

FIG. 2. Thin-section histopathology (representative slides; H-E stain). (A) Infection with *E. faecalis* OG1RF after 48 h, showing marked vitreal polymorphonuclear infiltrate, cystoid changes in the ganglionic cell layer, decreased nuclear density of inner and outer nuclear layers, mild subretinal polymorphonuclear infiltrate, and overall loss of structural integrity. (B) Infection with the Δ*fsrB* strain after 48 h, showing mild vitreal polymorphonuclear infiltrate, structure of all retinal layers preserved, and no subretinal inflammatory infiltrate. V, vitreous; R, retina; C, cornea; S, sclera.

cells. When the concentration of the signal compound reaches a threshold level, a bacterial sensor is switched on and the expression of certain genes is activated. As a consequence of this mechanism, species- or strain-specific group behavior is controlled in response to a certain cell density (7, 17, 19). The *E. faecalis fsr* regulatory system appears to be functionally as well as structurally related to the staphylococcal *agr* locus in that it regulates two known proteases and possibly other factors associated with virulence. The *S. aureus agr* regulates expression of at least 19 exoproteins that are potentially important in the pathogenesis of endophthalmitis (1). In the rabbit endophthalmitis model, a strain of *S. aureus* defective in expression of the global regulatory locus *agr* consistently resulted in a slower loss of B-wave response than in the wild-type strain

(1). The one known enterococcal toxin, the cytolysin, also mediates the precipitous loss of organ function in the endophthalmitis model (13) and renders this infection refractory to existing antibiotic or antiinflammatory treatment (12). The cytolysin was recently shown to be regulated by a novel autoregulated quorum system (7) distinct from the *fsr* system. The present study demonstrates that *fsr* regulates additional virulence traits that contribute to the pathogenesis of enterococcal infection in the eye.

FsrB is predicted to be a membrane protein with multiple transmembrane segments (similar to AgrB in staphylococci) and also shows partial similarity to transporter proteins, such as the putative glutamate transporter of *Borrelia burgdorferi* (23% identity over 101 amino acid residues) and the integral membrane component of an ABC transporter protein in *Escherichia coli* (32% identity over 75 amino acid residues) (17). FsrB may control the production of gelatinase in response to gelatinase biosynthesis-activation pheromone accumulation outside the cell.

Enterococci are an important cause of postoperative endophthalmitis, associated with significant loss of vision, and enterococcal infections have the second worst visual sequelae among all causes of endophthalmitis (6, 9). Study of enterococcal pathogenesis in a rabbit model of endophthalmitis may be particularly relevant for the evaluation of a quorum-sensing system such as Fsr. A very low number of *E. faecalis* CFU can be used to establish infection (for example, in this study each eye was injected with ca. 10^2 CFU). Thus, in the rabbit endophthalmitis model, the quorum develops under in vivo conditions, as opposed to the high inocula used in most other models where quorum concentrations are present immediately upon injection. In addition, the endophthalmitis model provides an exquisitely sensitive infection system in which organ function can be directly assessed (by ERG) and the effects of the infection can be monitored as the enterococcal quorum develops. Previous reports have outlined the importance of the *fsrB* gene in enterococcal virulence in a mouse peritonitis model as well as in a nonmammalian model of infection (5, 19). Herein, we show that deletion of the *fsrB* gene significantly decreases virulence in an endophthalmitis model as well. As in every other animal model, results from this study cannot necessarily be extrapolated to the variety of human infections caused by this important pathogen. The unique physiology of the intraocular space, the tissue specificity of bacterial adherence and possible virulence factors, and the relative absence of humoral factors and mechanical clearance mechanisms should be taken into consideration. However, previous data from the mouse peritonitis (19) and *C. elegans* (5) models of enterococcal infection, in conjunction with the present data in the rabbit endophthalmitis model, suggest that *fsrB* is important in *E. faecalis* pathogenicity across a broad range of both local and systemic infections. This study, therefore, provides additional support for the conclusion that many features of bacterial virulence are conserved across a variety of different model systems.

In conclusion, the experimental endophthalmitis model is a sensitive model for assessment of *E. faecalis* quorum sensing. The Fsr system plays a significant role in the virulence of *E. faecalis* in this and other disease models, and it may provide an attractive target for development of new antimicrobial agents.

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E. Mylonakis and M. Engelbert contributed equally to this work.

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