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NOTES

Contribution of Gelatinase, Serine Protease, and fsr to the Pathogenesis of Enterococcus faecalis Endophthalmitis

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Gelatinase and serine protease were found to contribute in concert to pathogenesis in a rabbit model of endophthalmitis. However, a mutant defective in the fsr regulator was observed to be more attenuated than a mutant rendered defective in the expression of gelatinase and serine protease as the result of a polar transposon insertion into the former. This increased attenuation suggests that there are possible additional pleiotropic effects of the defect in fsr on expression of traits contributing to the pathogenesis of enterococcal infection.

Enterococci are gram-positive intestinal commensals of humans and other animals and are leading causes of nosocomial infections and subacute endocarditis (5, 13). The emergence of multidrug-resistant enterococci poses a formidable therapeutic challenge (5, 6, 10, 11). Efforts to identify enterococcal virulence factors with a view towards finding new therapeutic targets have led to the discovery of two quorum-regulated systems that contribute to enterococcal pathogenesis in several disease models (4, 15).

One of these quorum-sensing systems regulates cytolysin (4), a bipartite toxin produced by cytolytic strains of Enterococcus faecalis that contributes to virulence in all models tested (2, 3, 7, 9, 19), including endophthalmitis (9, 19). It was recently demonstrated that intraocular infection with the noncytolytic enterococcal strain OG1RF also follows a malignant course where quorum concentrations generated in vitro may be present upon infection. In addition, the endophthalmitis model provides an exquisitely sensitive infection system in which the expression of traits contributing to the pathogenesis of enterococcal infection can be monitored as the enterococcal quorum develops (14), which measures the electrical responses of the visual cells in the retina in reaction to light; many other parameters of infection can be monitored as the enterococcal quorum develops (9).

Because an fsrB mutant which was shown to be defective in the production of both gelatinase and serine protease was attenuated in virulence in the endophthalmitis model (14), it was of interest to determine whether this attenuation was caused by the loss of one protease or the other, or perhaps both. In this report we demonstrate that both gelatinase and serine protease contribute to virulence but that the combined loss of both protease activities does not fully account for the degree of attenuation observed for the fsr mutant; this finding suggests an additional contribution to virulence of one or more yet unidentified factors regulated by the fsr quorum-sensing system.

All strains were propagated in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.). For OG1RF and derivatives, the medium was supplemented with rifampin (25 µg/ml) and fusidic acid (10 µg/ml). For establishing endoph-
thalmitis, bacteria were diluted to approximately 10^3 CFU/ml in phosphate-buffered saline and injected intravitreally as described below. Enumeration of organisms at the time of inoculation and after recovery from the vitreous was accomplished by plating duplicate serial dilutions on BHI agar (8) with selective antimicrobials as appropriate. The bacterial strains used are summarized in Table 1.

Experimental endophthalmitis was induced in female New Zealand White rabbits (weight, 1.75 to 2.25 kg), as previously described (9). The animals underwent general anesthesia by intramuscular administration of 35 mg of ketamine per kg of body weight (Ketaved; Phoenix Scientific Inc., St. Joseph, Mo.) and 5 mg of xylazine per kg (Rompun; Bayer Corp., Shawnee Mission, Kans.). The rabbits’ eyes were dilated with topical 1% tropicamide and 2.5% phenylephrine hydrochloride. After paracentesis and the withdrawal of 0.1 ml of aqueous humor from the anterior chamber to reduce the intraocular pressure and prevent reflux of the inoculum, 100 CFU of the strain of interest suspended in 0.1 ml of sterile saline was injected into the midvitreous cavity with a sterile insulin syringe. Experiments were conducted at least in duplicate to demonstrate reproducibility of results. Animals were housed and cared for in accordance with the Association for Research in Vision and Ophthalmology regulations (http://www.arvo.org/AboutArvo/animalst.asp).

To detect potential differences in the intraocular growth rates of mutant and wild-type *E. faecalis* strains, the number of organisms in the vitreous at 12, 36, and 48 h after infection was determined. Eyes were enucleated at the indicated time points, and the anterior eye segment encompassing the cornea, iris, and ciliary body was separated from the posterior segment by a circumferential cut along the pars plana and the retina, with the attached vitreous entirely scraped out of the remaining scleral cup with a no. 10 scalpel blade. This material was homogenized by bead beating with 1.0-mm-diameter glass beads and a BeadBeater (Biospec Products, Bartlesville, Okla.) for 1 min at maximum speed, and duplicate serial 10-fold dilutions of homogenates were plated on BHI agar. After 24 h of incubation at 37°C, colonies were counted, and concentrations were expressed as CFU per milliliter of intraocular sample.

*E. faecalis* OG1RF and isogenic mutant strains with various levels of expression of gelatinase and serine protease were assessed by ERG for their abilities to affect retinal responsiveness to light stimulus. After general and topical anesthesia was administered as described above, and after pharmacologic dilation and dark adaptation, the B-wave amplitudes (trough of A wave to peak of B wave) in response to a bright flash (flash intensity of 700 cd/m^2^ in a Ganzfeld illumination sphere) were assessed simultaneously for the infected right eye and the saline-injected left eye (EPIC-2000 visual electrodagnostic system; LKC Technologies, Gaithersburg, Md.). ERGs were performed at 12, 24, 36, and 48 h postinfection. Percent retinal function was defined as the ratio of the B-wave amplitude of the infected eye to the B-wave amplitude of the contralateral saline-injected eye.

All values represent the arithmetic means ± standard errors of the means. A two-tailed Student *t* test for unequal variances was used for statistical comparisons between groups. A *P* value of <0.05 was considered significant.

Eyes infected with wild-type and mutant *E. faecalis* strains were enucleated for histopathological analysis 48 h postinfection. The sclera anterior to the superior rectus muscle was marked with tattoo ink to ensure uniform orientation of the specimen during preparation of sections. Eyes were fixed in 4% paraformaldehyde for at least 24 h. Five serial sagittal sections were prepared and stained with hematoxylin and eosin. Since pathological findings were largely confined to the inferior portion of the retina, and inflammatory and structural changes elsewhere in the eye were subtle in comparison with changes resulting from infection with cytolytic *E. faecalis* or more virulent organisms such as *S. aureus* (1, 9), only this area was evaluated and assigned a score from 0 (normal) to 4 (most severe) as follows: 0, normal appearance; 1, cystoid changes and few infiltrates; 2, moderate infiltrate and photoreceptors recognizable; 3, retinal layers still discernible, marked inflammatory infiltrate, and no recognizable photoreceptors; 4, no discernible retinal layers and massive inflammatory infiltrate.

The intraocular growth kinetics of *E. faecalis* OG1RF, TX 5266 (ΔfsrB, phenotypically GelE^- SprE^-), TX 5264 (OG1RF GelE^- SprE^-), TX 5243 (OG1RF GelE^- SprE^-), and TX 5128 (OG1RF which possesses a polar mini yō insertion in gelE, which also reduces expression of sprE below detectable levels [15, 18], but has a functional fsr system) are depicted in Fig. 1. In vivo growth levels were similar for all strains studied. A steady increase in bacterial numbers was seen through 36 h postinoculation; the numbers of CFU reached a maximum at this time and declined slightly thereafter.

Figure 2 illustrates the effect of infection on retinal function as measured by ERG. As previously reported (14), infection with *E. faecalis* OG1RF resulted in a significantly greater reduction of B-wave amplitude than infection with the ΔfsrB deletion mutant TX 5266 at the 24-h (*P* < 0.001), 36-h (*P* < 0.001), 48-h (*P* < 0.001), 72-h (*P* < 0.001), and 96-h (*P* < 0.001) postinfection. Percent retinal function was defined as the ratio of the B-wave amplitude of the infected eye to the B-wave amplitude of the contralateral saline-injected eye.

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th><em>E. faecalis</em> strain</th>
<th>Phenotype</th>
<th>Genotype and resistance phenotype</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OG1RF</td>
<td>WT</td>
<td>Wild-type strain; Rif^- Fus^-</td>
<td>12</td>
</tr>
<tr>
<td>TX 5266</td>
<td>ΔfsrB</td>
<td>OG1RF ΔfsrB in-frame deletion mutant with deletion of bp 79 to 684</td>
<td>16</td>
</tr>
<tr>
<td>TX 5264</td>
<td>GelE^- SprE^-</td>
<td>OG1RF gelE in-frame deletion mutant; Rif^- Fus^- Em^-</td>
<td>17</td>
</tr>
<tr>
<td>TX 5243</td>
<td>GelE^- SprE^-</td>
<td>OG1RF sprE insertional mutant; Rif^- Fus^- Em^-</td>
<td>15</td>
</tr>
<tr>
<td>TX 5128</td>
<td>GelE^- SprE^-</td>
<td>OG1RF polar insertional mutant in gelE with expression of sprE below detectable levels; Rif^- Fus^- Em^-</td>
<td>18, 15</td>
</tr>
</tbody>
</table>

Abbreviations: WT, wild type; Rif, rifampicin; Fus, fusidic acid; Em, erythromycin; GelE, gelatinase; SprE, serine protease.
Infection with E. faecalis OG1RF resulted in a rapid loss of retinal responsiveness to light of about 85% at 24 h and a nearly complete loss by 36 h. In contrast, eyes infected with the fsrB deletion mutant TX 5266 retained over 90% of retinal function at 24 h, and retinal function did not decrease below 40% of the control value over the course of the entire experiment. Interestingly, infection with strains TX 5264 and TX 5243, which are deficient in production of gelatinase or serine protease individually, showed a course of disease that was nearly superimposable onto that caused by the parent strain. The TX 5264 (GelE−SprE−)-infected eyes retained better visual function (24%) at 24 h than the eyes infected with TX 5243 (GelE−SprE−) or OG1RF (both below 10%), a difference that was statistically significant (P < 0.05) and reproducible in repeat experiments but transient.

In contrast to the gelE and sprE single mutants, a gelE sprE double mutant (TX 5128; GelE−SprE−) defective in production of both gelatinase and serine protease was significantly attenuated in the rabbit endophthalmitis model. TX 5128 resulted in infections characterized by a loss of retinal function of 51% at 24 h postinfection. Importantly, this loss of retinal function was significantly greater than the loss of 8% observed for infection caused by the fsrB deletion mutant TX 5266 (P < 0.05), which is also phenotypically defective in GelE and SprE. By 36 h, retinal function in eyes infected with the double knockout strain TX 5128 (GelE−SprE−) had decreased to 22% of preoperative levels, which was a significantly greater retention of function than that observed in eyes infected with mutants defective in only gelatinase (TX 5243 GelE−SprE−) or serine protease (TX 5243 GelE−SprE−) production (P < 0.01) but a significantly greater loss than that observed in eyes infected with the fsrB deletion mutant TX 5266 (P < 0.005), which had retained 51% of retinal function. At 48 h, retinal function in the eyes infected with the mutant defective in both gelatinase and serine protease production (TX 5128) was undetectable and similar to that of eyes infected with the mutants defective in either gelatinase or serine protease production only, whereas the eyes infected with the fsrB deletion mutant TX 5266 still retained 41% of retinal function. Histopathological slides were ranked according to severity, and the median severities of individual groups were compared. This approach was necessary because histopathological changes within the individual groups were variable, and changes overall were less extensive than those observed with cytolytic E. faecalis or more virulent organisms such as S. aureus (1, 9). In most eyes, the bacteria and the inflammatory infiltrate appeared to be concentrated in the inferior portion of the globe. The most dramatic histopathological changes were observed in the retina just below this accumulation of neutrophils and bacteria. As was observed previously (14), eyes infected with E. faecalis OG1RF showed marked vitreal poly-
morpheonuclear infiltrate, cystoid changes in the ganglionic cell layer, decreased nuclear density of the inner and outer nuclear layers, and subretinal polymorpheonuclear infiltrate; the majority of eyes showed an overall loss of structural integrity within 48 h. In contrast, most of the eyes injected with TX 5266 (OG1RF; GelE/SprE) mutants showed only mild vitreal polymorpheonuclear infiltrate, preserved structure of all retinal layers, and no subretinal inflammatory infiltrate by 48 h, features which were similar to those of saline-injected control eyes (representative slides are shown in Fig. 3a to c).

Interestingly, infection with TX 5128 (GelE⁻/SprE⁻) resulted in less severe histopathological changes after 48 h than those observed in the eyes infected with wild-type OG1RF, even though retinal function in the eyes infected with TX 5128 was undetectable at that time. In particular, inflammatory infiltration was less marked (representative slide shown in Fig. 3d), and complete loss of structural integrity could be observed only in 1 out of 10 specimens. These histopathological changes were never seen in the TX 5266 (OG1RF ±fsrB)-infected eyes.

The median severity of disease as reflected in the stained sections of the eyes infected with mutants defective in the expression of gelatinase (TX 5264; GelE⁻/SprE⁻) or serine protease (TX 5243; GelE⁺/SprE⁻) was greater than that observed for the TX 5266 (OG1RF ΔfsrB) or TX 5128 (GelE⁻/SprE⁻) mutants. The majority of eyes infected with TX 5243 or TX 5243 demonstrated moderate to marked vitreal infiltration and various degrees of disintegration of retinal structure (Fig. 3e and f). A score of 0 to 4 was assigned to each histopathological slide according to the standardized grading system described above, and the results of this analysis are depicted in Fig. 4. At 48 h after infection, statistical significance could be demonstrated for the difference between wild-type OG1RF and every mutant studied (P < 0.02), corroborating the results obtained by ERG. However, the statistically significant differences for retinal function observed with ERG between the different mutants at 24 and 36 h did not translate into statistically significant differences in histopathological appearance at 48 h (Fig. 3 and 4) or 24 h (data not shown).

The E. faecalis fsr system is known to regulate two known proteases, a gelatinase and a serine protease (15). A structural relationship between the enterococcal fsr system and the global regulatory agr system in S. aureus has been observed based on sequence similarities between the two (15). The S. aureus agr system in concert with sar has been demonstrated to govern the expression of at least 19 exoproteins. This system has been shown to be involved in the pathogenesis of S. aureus infections, including endophthalmitis (1). It was previously demonstrated that in experimental enterococcal endophthalmitis, a mutation in the enterococcal fsr locus mitigated the course of retinal-function loss (14). In the present study, we sought to determine the individual contribution of the two proteases known to be regulated by fsr. We found that only when both proteases were knocked out simultaneously could a distinct reduction in severity be demonstrated. Infection with a mutant defective in the expression of sprE but which produces gelatinase showed a course of disease that was indistinguishable from that caused by the wild-type strain at all time points. Infection with a mutant defective in gelatinase (TX 5264; GelE⁺/SprE⁻) led to a nominally but reproducibly attenuated course of disease as measured by ERG at 24 h but otherwise rapid and complete loss of this residual function 12 h later. In contrast to the results obtained with mutants defective singly in production of either protease, infection with a mutant defective in production of both GelE and SprE (TX 5128; GelE⁻/SprE⁻) was significantly attenuated early; however, retinal-function loss was complete by 48 h. These data suggest that serine protease and gelatinase have redundant activities in the pathogenesis of endophthalmitis. Similar observations have been made in a C. elegans killing assay in which the strains with single mutations in either gelatinase or serine protease were observed to be only nominally less toxic than wild-type OG1RF (17).

The course of disease observed in eyes infected with the mutant TX 5128, which is defective in both extracellular proteases as the result of a polar insertion in gelE (15), is significantly different from that observed for infection with TX 5266 (OG1RF ΔfsrB). This difference suggests that either the polar effect of the mini γδ insertion on sprE expression is not complete, even though sprE activity is reduced below levels that are detectable in vitro (15, 18), or that there are other pleiotropic effects of the deletion within fsrB which relate to the expression of other traits contributing to the pathogenesis of enterococcal infection.

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