Influence of the fsr locus on biofilm formation by Enterococcus faecalis lacking gelE

Although enterococci are normal commensals of the gastrointestinal tract of humans and many mammals, they have emerged as important pathogens causing nosocomial infections, including urinary tract, bloodstream, wound and surgical-site infections, in addition to their long-recognized importance as a cause of infective endocarditis (Murray, 1990). The binding of Enterococcus faecalis to heart valves and to various biomaterials (Joyanes et al., 1999; Toledo-Arana et al., 2001; Mohamed et al., 2004; Seno et al., 2005) and medical devices (Keane et al., 1994; Dautle et al., 2003; Sandoe et al., 2003) is the presumed initiating factor that then allows subsequent formation of a biofilm. Biofilm formation in E. faecalis has been reported to be influenced by various genes such as esp, initially reported by Toledo-Arana et al. (2001). Gelatinase, which strongly influences virulence in models of peritonitis, endocarditis (Singh et al., 1998, 2005), endophthalmitis (Engelbert et al., 2004) and in vitro translocation (Zeng et al., 2005), has been reported to influence biofilm formation (Mohamed et al., 2003, 2004; Hancock & Perego, 2004; Kristich et al., 2004). Other E. faecalis genes such as epa, atn (Mohamed et al., 2004), bop (Hufnagel et al., 2004), salA and salB (Mohamed et al., 2006) have also been shown to influence biofilms. Biofilm production has been shown to be regulated by quorum-sensing systems in several important pathogens, including fsr in E. faecalis, which was shown to have a pronounced effect on biofilms (Hancock & Perego, 2004; Mohamed et al., 2003, 2004; Pillai et al., 2004).

The fsr locus, a homologue of the staphylococcal agr loci, global regulators of virulence and metabolism (Dunman et al., 2001), positively regulates the expression of gelatinase and serine protease in E. faecalis (Qin et al., 2000) and autoregulates expression of the fsrB and fsrC genes (Nakayama et al., 2001; Qin et al., 2001). Most strains of E. faecalis are gelE⁺, although only about 60% of them have the fsr locus and are gelatinase producers by standard assay (Roberts et al., 2004). We have shown recently that all three fsr mutants (phenotypically Gele⁻ SprE⁻) showed decreased biofilm production (Mohamed et al., 2003, 2004), a finding confirmed by Hancock & Perego (2004); the latter also showed that the introduction of fsrABC and fsrABC( gelE( sprE) into strain FA2-2 (Gele⁻, with point mutations in the fsrB and fsrC loci) resulted in gelatinase production and increased biofilm production (Hancock & Perego, 2004). However, our initial results had also suggested that fsr may have a role, in addition to activating gelatinase-mediated biofilm formation, as the fsr mutants formed slightly more biofilm than the gelatinase-serine protease double mutant TX5128 (Mohamed et al., 2004). In order to study the effect of fsr on biofilm formation by E. faecalis, independent of activation of its gelatinase production, we used E. faecalis clinical isolates lacking gelE and fsr as hosts in this study.

Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Characteristics/origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JH2-2</td>
<td>Well-characterized laboratory strain; plasmid-free; gelE⁺, lacks fsrA, fsrB; Gele⁻</td>
<td>Jacob &amp; Hobbs (1974)</td>
</tr>
<tr>
<td>TX5441.01</td>
<td>JH2-2(pAT18); Em'</td>
<td>This study</td>
</tr>
<tr>
<td>TX5441</td>
<td>JH2-2(pTEX5249); Gele⁺, Em'</td>
<td>This study</td>
</tr>
<tr>
<td>TX0096</td>
<td>Endocarditis isolate; medium biofilm producer; lacks fsrB, gelE; Gele⁻</td>
<td>Mohamed et al. (2004)</td>
</tr>
<tr>
<td>TX5453.01</td>
<td>TX0096(pAT18); Em'</td>
<td>This study</td>
</tr>
<tr>
<td>TX543</td>
<td>TX0096(pTEX5249); Em'</td>
<td>This study</td>
</tr>
<tr>
<td>TX0014</td>
<td>Endocarditis isolate; strong biofilm producer; lacks fsrB, gelE; Gele⁻</td>
<td>Mohamed et al. (2004)</td>
</tr>
<tr>
<td>TX5454.01</td>
<td>TX0014(pAT18); Em'</td>
<td>This study</td>
</tr>
<tr>
<td>TX545</td>
<td>TX0014(pTEX5249); Em'</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pAT18</td>
<td>Shuttle vector; Em'</td>
<td>Trieu-Cuot et al. (1991)</td>
</tr>
<tr>
<td>pTEX5249</td>
<td>6 kb Pet–BglII fragment containing fsrA, fsrB, fsrC and the first 395 bp of gelE cloned into pAT18; Em'</td>
<td>Qin et al. (2000)</td>
</tr>
</tbody>
</table>
The bacterial strains and plasmids used in this study are listed in Table 1. For biofilm experiments, bacteria were first grown overnight in tryptic soy broth plus 0.25% glucose (TSBG) with or without erythromycin, as appropriate, and then in TSBG for biofilm formation. Hosts lacking fsr and gelE that produced strong, medium and weak biofilms from our culture collections and JH2-2 were used as recipients, pTEX5249 (pAT18 carrying fsrABC) and its appropriate control, pAT18, were introduced by electroporation and plated on Todd–Hewitt (TH) agar with 25 μg erythromycin ml⁻¹.

Erythromycin-resistant colonies carrying pTEX5249 were identified by PCR and mini-plasmid preparations. Gelatinase production was tested at 72 h on TH agar containing 3% gelatin (Qin et al., 2000). Biofilm formation and adherence to a polystyrene surface were assessed quantitatively as well as by phase-contrast microscopy, as described previously (Mohamed et al., 2004).

We first introduced pTEX5249 into JH2-2 (esp₂ gelE¹), but gelatinase-negative due to the absence of most of the fsr locus with truncated fsc and a 23.9 kb deletion; Nallapareddy et al., 2005) and found gelatinase production and increased biofilm production (53%) compared with the respective controls, similar to results reported by Hancock & Perego (2004) with FA2-2. We then tested the strong biofilm producer TX0014 (biofilm OD₅₇₀ > 2.0) (lacking fsr, gelE and esp) after the introduction of pTEX5249 (TX5454) and found that biofilm formation was significantly reduced (41%; P < 0.0001, Student's t-test) compared with the wild-type and control TX5454.01 (TX0014 containing pAT18) (Fig. 1a). Similarly, when we tested the medium biofilm producer TX0006 (biofilm OD₅₇₀ of 1–2), after the introduction of fsr genes, resulting in TX5453, this strain also showed a significant decrease (29%; P < 0.0001, Student's t-test) in biofilm formation relative to its respective controls, TX0006 and TX5453.01 (Fig. 1a). By phase-contrast microscopy, the fsr-containing strains displayed either no evidence of (TX5433) or fewer (TX5454) microcolonies with empty areas of plastic surface and reduced biofilm production (Fig. 2). TX5453 and TX5454 harbouring the fsr locus also showed less initial attachment than fsr-lacking strains (Fig. 1b), indicating that fsr negatively controls initial adherence, a prerequisite of biofilm formation. We also tested a weak biofilm producer, TX0238, but were unable to recover pTEX5249-containing derivatives from it; none of our other fsr/ gelE-lacking strains produced weak biofilm.

Under some conditions, agr mutants of Staphylococcus aureus (Vuong et al., 2000) and Staphylococcus epidermidis (Vuong et al., 2003) have been shown previously to display enhanced biofilm formation and increased adhesion to epithelial cells (Vuong et al., 2004) compared with a corresponding isogenic agr wild-type, indicating that agr can suppress biofilm formation. Our results suggest that fsr has an effect, independent of proteases, on biofilm formation in E. faecalis and that this effect is in the same direction as that of agr of staphylococci. In summary, the introduction of the fsr locus into gelE-lacking strains resulted in reduced initial attachment, no microcolonies and/or fewer clusters of cells and a reduced amount of biofilm production relative to the parental strains, indicating that the fsr locus has an additional role, independent of gelatinase production, that leads to decreased initial attachment followed by reduced biofilm production in E. faecalis.

**Acknowledgements**

This work was supported by NIH grant R37 AI47923 from the Division of Microbiology and Infectious Diseases, NIAID, to B. E. M.

Jamal A. Mohamed¹,² and Barbara E. Murray¹,²,³

Division of Infectious Diseases, Department of Internal Medicine¹, Center for the Study of Emerging and Reemerging Pathogens³ and Department of Microbiology and Molecular Genetics³, University of Texas Medical School, Houston, TX 77030, USA

Correspondence: Barbara E. Murray (bem.asst@uth.tmc.edu)

---

**Fig. 1.** (a) Biofilm formation by fsr-lacking and fsr-containing hosts at 24 h. The experiment was performed three times in quadruplicate and the OD₅₇₀ (mean ± SD) is shown. (b) Initial attachment to a polystyrene surface. The experiment was performed twice and five fields were counted in each experiment. The number of bacteria per field (mean ± SD) is shown.


Fig. 2. Phase-contrast microscopic analysis of biofilm structure after 24 h in TSBG. (a) TX0014, (b) TX5454.01, (c) TX5454, (d) TX0006, (e) TX5453.01, (f) TX5453.


