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 *GeE*[−] *SprE*[−] by standard assay; Qin *et al.*, 2000; Singh *et al.*, 2005), as well as *gelE* mutants (*GeE*[−] *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 (*GeE*[−], 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

Strain/plasmid	Characteristics/origin	Reference
Strains		
JH2-2	Well-characterized laboratory strain; plasmid-free; <i>gelE</i> ⁺ , lacks <i>fsrA</i> , <i>fsrB</i> ; <i>GeE</i> [−]	Jacob & Hobbs (1974)
TX5441.01	JH2-2(pAT18); Em ^r	This study
TX5441	JH2-2(pTEX5249); <i>GeE</i> ⁺ , Em ^r	This study
TX0006	Endocarditis isolate; medium biofilm producer; lacks <i>fsrB</i> , <i>gelE</i> ; <i>GeE</i> [−]	Mohamed <i>et al.</i> (2004)
TX5453.01	TX0006(pAT18); Em ^r	This study
TX5453	TX0006(pTEX5249); Em ^r	This study
TX0014	Endocarditis isolate; strong biofilm producer; lacks <i>fsrB</i> , <i>gelE</i> ; <i>GeE</i> [−]	Mohamed <i>et al.</i> (2004)
TX5454.01	TX0014(pAT18); Em ^r	This study
TX5454	TX0014(pTEX5249); Em ^r	This study
Plasmids		
pAT18	Shuttle vector; Em ^r	Trieu-Cuot <i>et al.</i> (1991)
pTEX5249	6 kb <i>Pst</i> I– <i>Bgl</i> II fragment containing <i>fsrA</i> , <i>fsrB</i> , <i>fsrC</i> and the first 395 bp of <i>gelE</i> cloned into pAT18; Em ^r	Qin <i>et al.</i> (2000)

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 plates 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 (*esp*⁻ *gelE*⁺, but gelatinase-negative due to the absence of most of the *fsr* locus with truncated *fsrC* 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 (TX5453) 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*.

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.

Acknowledgements

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

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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.

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