Importance of the ebp (Endocarditis-and Biofilm-Associated Pilus) Locus in the Pathogenesis of Enterococcus faecalis Ascending Urinary Tract Infection

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Background. We recently demonstrated that the ubiquitous Enterococcus faecalis ebp (endocarditis- and biofilm-associated pilus) operon is important for biofilm formation and experimental endocarditis. Here, we assess its role in murine urinary tract infection (UTI) by use of wild-type E. faecalis OG1RF and its nonpiliated, ebpA allelic replacement mutant (TX5475).

Methods. OG1RF and TX5475 were administered transurethrally either at an ∼1:1 ratio (competition assay) or individually (monoinfection). Kidney pairs and urinary bladders were cultured 48 h after infection. These strains were also tested in a peritonitis model.

Results. No differences were observed in the peritonitis model. In mixed UTIs, OG1RF significantly outnum-bered TX5475 in kidneys (P < .0033) and bladders (P ≤ .0001). More OG1RF colony-forming units were also recovered from the kidneys of monoinfected mice at the 4 inocula tested (P = .015 to P = .049), and 50% infective doses of OG1RF for kidneys and bladder (9.1 × 10^7 and 3.5 × 10^7 cfu, respectively) were 2–3 log_{10} lower than those of TX5475. Increased tropism for the kidney relative to the bladder was observed for both OG1RF and TX5475.

Conclusion. The ebp locus, part of the core genome of E. faecalis, contributes to infection in an ascending UTI model and is the first such enterococcal locus shown to be important in this site.

Among the various infections with Enterococcus faecalis reported, urinary tract infections (UTIs) are the most common [1–3]. Little is known about the bacterial factors necessary for E. faecalis to cause infections in general, and even less has been reported related to the urinary tract [2, 4, 5]. The presence of Esp, encoded by an acquired gene, was shown to increase the persistence of bacteria in the urinary bladders of mice (with no histological changes) when a parent and its esp mutant were used in an ascending UTI model [5]; however, no difference was found in kidneys. Kau et al. [2], using a similar model, demonstrated that E. faecalis has a preference for the kidneys relative to the bladder and concluded that this model can be used to study the factors involved in the pathogenesis of UTIs.

E. faecalis is also known to form biofilm on such biomedical devices as urinary catheters and central venous catheters, and several genes [6–9] have been shown to be important for biofilm formation among E. faecalis strains, including fva via its effect on gelatinase [7, 10, 11]. Biofilm likely contributes to the high occurrence of enterococci in UTIs in the hospital setting, in part because of the large number of patients who have undergone catheterization and in part because biofilm formation may promote the persistence of bacteria in the urinary tract. Therefore, it is important to
understand the relationship between genes involved in biofilm formation and the pathogenicity of *E. faecalis* in the urinary tract.

We recently identified and characterized the *ebp* (endocarditis- and biofilm-associated pili) operon, which consists of *ebpA*, *ebpB*, *ebpC*, and an associated *srtC* (encoding sortase C) gene that also has an independent promoter [12]. The surface pili these genes encode are immunogenic and pleomorphic in nature, and the role they play in the establishment of biofilm and infective endocarditis in a rat model was shown using the wild-type (WT) *E. faecalis* strain OG1RF and its isogenic, nonpiliated, *ebpA*-deletion mutant (TX5475). Our hypothesis here is that *E. faecalis* Ebp-associated pili may play a role similar to that of the fimbriae of *Escherichia coli* and *Proteus mirabilis* in promoting colonization and adherence to uroepithelium. In the present study, by use of a mouse UTI model, we describe the ability of WT *E. faecalis* OG1RF, which expresses sortase C-dependent pleomorphic surface pili [12], and nonpiliated TX5475 to colonize kidneys and bladders both during mono-infection and in a mixed-infection competition assay. We also tested both strains in a mouse peritonitis model of sepsis.

**MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Bacteria used in the study included WT OG1RF (resistant to rifampicin [RIF; 100 µg/mL] and fusidic acid [25 µg/mL]; ATCC 47077), a well-known plasmid-free isolate used in many laboratories [13]; OG1RF is a medium biofilm producer [7] and has been shown to be pathogenic in various animal models [12, 14–17]. TX5475, a recently described isogenic, nonpiliated, biofilm-deficient, *ebpA*-deletion (polar) mutant of OG1RF in which *ebpA* is replaced with the kanamycin (KAN)–resistance gene, was also used [12]. Brain-heart infusion (BHI) broth (BHI; Difco Laboratories) was used for routine bacterial growth. KAN and heat-inactivated horse serum were purchased from Sigma. The concentrations of antibiotics used in BHI agar (BHA) plates for growth of nonpiliated TX5475 were 2000 µg/mL KAN (hereafter, KAN2000) and 100 µg/mL Rif (hereafter, RIF100).

**UTI model for the competition assay, mono-infection, and determination of ID₅₀.** Female, 4–6-week-old, outbred ICR mice (Harlan Sprague Dawley) with a mean weight of 25 g were used in this study. Preapproved protocol and the guidelines of the Animal Welfare Committee of the University of Texas Health Science Center at Houston were followed throughout the course of the animal experiments. Mice were pre-screened for UTI by culturing urine ∼12 h before inoculation. The urinary bladders of mice were emptied by gently pressing the abdomen before infection. For preparation for administration to mice, bacteria were grown in 10 mL of BHIB plus 40% heat-inactivated horse serum for 10 h at 37°C with gentle shaking. Cells were pelleted for 10 min (11,169 g at 10°C) and were resuspended in 10 mL of 0.9% saline. Further dilutions were also prepared in saline and then plated, to determine the actual inoculum that had been administered. An inoculation method similar to that of a recently described mouse UTI model [2] was used. Sixteen mice were used in the competition assay, and groups of 5 mice/inoculum were used in each of 2 independent monoinfection experiments. In brief, isoflurane-anesthetized mice were infected via intraurethral catheterization (polyethylene catheter, ∼2.5–3 cm long; outer diameter, 0.61 mm; Clay Adams) with 200 µL of the bacterial suspension consisting of an ∼1:1 ratio of WT *E. faecalis* OG1RF to nonpiliated TX5475 in the competition assay; 2 independent experiments, one using 10 mice and the other using 6, were performed, and the results were combined. In 2 independent monoinfection experiments for which results were combined, WT OG1RF was tested using an inocula range of 1 × 10⁹–1 × 10¹⁰, and the nonpiliated TX5475 mutant was tested using an inocula range of 1 × 10⁹–1 × 10¹⁰, to generate data for comparison of the numbers of colony-forming units recovered from the kidneys and bladders of mice as well as for the calculation of ID₅₀. Similar to the method described by Kau et al. [2], we also used an inoculum volume of 200 µL, to facilitate direct delivery of the bacteria to both the bladder and kidneys simultaneously. The urethral catheter was removed immediately after injection of the bacteria, and all of the mice were provided free access to food and water during the course of study. Daily observations were made for any morbidity and mortality in mice. Mice were killed by CO₂ inhalation at 48 h after transurethral challenge. The urinary bladder and kidney pair were excised, weighed, and homogenized in 1 and 5 mL of saline, respectively, and dilutions were plated onto BHA for recovery of bacteria for the monoinfection experiments and onto BHA and BHA+KAN2000 for the mixed-infection experiments, to determine the recovered colony-forming units (in percentages) of WT OG1RF versus nonpiliated TX5475. The detection limit for bacteria in this experiment was 10 cfu. Mice with sterile cultures of kidney and urinary bladder homogenates were considered not to have UTI infection. Identity of the recovered test bacteria from infected organs was confirmed by plating them on bile esculin azide agar (BEA) and BHA+RIF100 plates for WT *E. faecalis* OG1RF and on BHA+KAN2000, BHA+RIF100, and BEA plates for the nonpiliated TX5475. Randomly chosen nonpiliated TX5475 colonies were also tested by polymerase chain reaction (PCR), pulsed-field gel electrophoresis (PFGE) [18], and/or high-stringency hybridization of PFGE gels, to reconfirm the identity of test bacteria.

**Mouse peritonitis model.** *E. faecalis* strains OG1RF and TX5475 were tested using a method that has been published elsewhere [17]. Mice were injected intraperitoneally with appropriate dilutions of premixed bacteria/sterile rat fecal extract and were observed for 5 days. Two-fold dilutions of both test
bacteria in a range of $\sim 1 \times 10^8 - 1 \times 10^9$ cfu were used as the inocula for LD$_{50}$ determination, using 6 mice/group. The LD$_{50}$ was determined by the method of Reed and Muench [19].

**Statistics, ID$_{50}$s, and mean virulence index.** Differences in log$_{10}$ colony-forming unit counts (per gram) of TX5475 and WT OG1RF in tissue (kidney or bladder) of each mouse from the mixed-infection experiments were analyzed for significance by the paired $t$ test. Differences in the log$_{10}$ colony-forming unit counts of TX5475 versus WT OG1RF from the monoinfection experiments were evaluated by the unpaired $t$ test. Fisher’s exact test was used to compare total infection of kidneys and bladders (combining data for all inocula) during monoinfection with OG1RF versus TX5475. Prism for Windows (version 4.00; GraphPad Software) was used for statistical analysis. ID$_{50}$s were calculated using a method that has been published elsewhere [19]. The mean virulence index (MVI) was calculated using the following equation (as described elsewhere for other organisms in mixed infections [12, 15, 20]):

$$MVI = \frac{(GM\text{-}cfu\text{ of OG1RF})_{T=48}/(GM\text{-}cfu\text{ of TX5475})_{T=48}}{(GM\text{-}cfu\text{ of OG1RF})_{T=0}/(GM\text{-}cfu\text{ of TX5475})_{T=0}},$$

where GM$\cdot$cfu is the geometric mean of the colony-forming unit counts and $T$ is the number of hours after infection.

**RESULTS**

**Competition assay.** In the mixed-infection competition assay, approximately equal numbers (as judged by the optical density at 600 nm) of WT *E. faecalis* OG1RF and the TX5475 mutant were administered transurethrally. The actual colony-forming unit counts of WT OG1RF and the TX5475 mutant (combined from 2 experiments) in the inoculum mix were $1.1 \times 10^7$ and $1.2 \times 10^7$, respectively; thus, the percentage of WT OG1RF in the inoculum was 49% (figure 1). WT OG1RF outcompeted the TX5475 mutant in all 16 mice at 48 h after infection, increasing significantly from time zero ($T = 0$) in kidneys and bladders with mean paired differences of 3.1-fold ($P = .0033$) and 179-fold ($P = .0001$), respectively (figure 1). The MVIs of nonpiliated TX5475 relative to WT OG1RF in kidneys and bladders were 0.30 and 0.005, respectively.

**Kidney and bladder monoinfection and ID$_{50}$s.** Because of the possibly that nonpiliated TX5475 colonies may have adhered to or been entrapped by the surface pili of OG1RF and, therefore, may have been retained at the infection site(s) during the course of the competition assay, we also performed monoinfection experiments with 4 equal inocula of each organism. The total number of kidneys infected (38/40 with OG1RF vs. 27/40 with TX5475; $P = .0031$) and the total number of bladders infected (30/40 with OG1RF vs. 18/40 with TX5475; $P = .0115$) (data are combined from all 4 monoinfection inoculum groups) indicated that TX5475 was significantly attentuated in its ability to infect both kidneys and bladders relative to its parent strain, WT OG1RF (table 1). When mice ($n = 10$) were infected with $\sim 1 \times 10^6$, $\sim 1 \times 10^4$, $\sim 1 \times 10^3$, and $\sim 1 \times 10^6$ cfu of WT OG1RF, the geometric mean numbers of colony-forming units recovered from kidneys at 48 after transurethral challenge were $2.1 \times 10^3$, $2.4 \times 10^3$, $1.7 \times 10^3$, and $1.0 \times 10^3$, respectively (figure 2). In mice infected with $\sim 2 \times 10^2$, $\sim 2 \times 10^4$, $\sim 2 \times 10^5$, and $\sim 2 \times 10^6$ cfu of TX5475, the geometric mean numbers of colony-forming units recovered from kidneys were $6.2 \times 10^3$, $3.6 \times 10^3$, $1.3 \times 10^4$, and $8.9 \times 10^4$, respectively (figure 2), showing a $\geq 2$ log$_{10}$ reduction in the geometric mean colony-forming unit counts for TX5475 versus WT OG1RF for the same inoculum (figure 2). The mean $\pm$ SD differences in log$_{10}$ colony-forming unit counts seen in all 4 inoculum groups ($1 \times 10^3$, $1 \times 10^4$, $1 \times 10^5$, and $1 \times 10^6$) between WT OG1RF and nonpiliated TX5475 were 2.5 $\pm$ 1.2 ($P = .0493$), 1.8 $\pm$ 0.9 ($P = .0490$), 2.1 $\pm$ 0.8 ($P = .0156$), and 2.1 $\pm$ 0.9 ($P = .0333$) log$_{10}$ cfu, respectively (figure 2). With respect to bladders after monoinfection, although fewer bladders were infected by TX5475 (table 1), the differences in the colony-forming unit counts were not significant, with mean $\pm$ SD differences of 1.2 $\pm$ 1.2, 0.9 $\pm$ 1.3, and 0.9 $\pm$ 1.1 log$_{10}$ cfu in the $1 \times 10^3$, $1 \times 10^4$, and $1 \times 10^5$ inoculum groups, respectively, and 2.7 $\pm$ 1.4 log$_{10}$ cfu in the $1 \times 10^6$ inoculum.

![Figure 1](https://academic.oup.com/jid/article-abstract/195/11/1671/946460)
group. The results in table 1 also indicate tropism for the kidney, given that 20%–30% of bladders were found to be uninfected in mice whose kidneys were infected with WT OG1RF and that 20%–50% of bladders were found to be uninfected in mice whose kidneys were infected with nonpiliated TX5475. The ID_{50} derived from the monoinfection experiments with WT OG1RF were determined to be 9.1 × 10^4 and 3.5 × 10^3 cfu for kidneys and bladders, respectively, whereas the ID_{50}s with TX5475 for kidneys and bladders were determined to be 1.7 × 10^4 and 1.7 × 10^5 cfu, respectively, showing that ≥2 log_{10} more cells were required to infect 50% of the mice versus WT OG1RF. The ID_{50}s also showed that ∼1 log_{10} fewer colony-forming units of either bacteria were needed to infect 50% of the kidneys than to infect 50% of the bladders.

Mouse peritonitis model. In an experiment using 6 mice/ inoculum, both test bacteria caused mortality at a similar rate in mice (monitored at 3–6-h intervals for 96 h) (data not shown), and mortality was not significantly different by the log rank (Mantel-Cox) test, although there was a small (1.9-fold) decrease in LD_{50} for TX5475.

**DISCUSSION**

_E. faecalis_ was recognized as an important organism associated with UTIs as long ago as the early 1900s, but much needs to be learned about the pathogenesis of this organism in this site. Among gram-negative bacteria, _E. coli_ is the leading cause of community-acquired UTIs [4, 21–25], and _E. coli_ surface fimbriae or pili (type 1, P and related fimbriae, and F1C) are known to mediate initial adherence [5, 26–28]. Recently, it has been shown that the flagella of uropathogenic _E. coli_ contribute to the persistence of the organism in the bladder and kidneys [29]. Among gram-positive bacteria, pili have been recognized in the past few years [30–37], but their role in UTI has not yet been reported. Our recently published study [12] showed the presence of pleomorphic endocarditis and biofilm-associated pili on _E. faecalis_ OG1RF and demonstrated that a nonpiliated ebpA-deletion mutant of OG1RF (TX5475) was significantly attenuated in a rat model of infective endocarditis and formed significantly less biofilm in an in vitro assay. The present study comparing WT OG1RF versus TX5475 was an effort to determine whether the ebp locus played a role in the colonization of kidneys and urinary bladders in a mouse UTI model. The reduced recovery of the TX5475 mutant versus WT OG1RF from kidneys (∼2 log_{10}) and bladders (∼1 log_{10}), evident with all 4 inocula used for monoinfection, confirmed this hypothesis.

Although our results with the ebpA allelic replacement mutant TX5475 (which produces none of the 3 Ebp proteins) [12] unambiguously demonstrated a role for the ebp locus in UTI in this model, we acknowledge that we have not definitively excluded the possibility of an independent contributory effect of its associated sortase C. It is unlikely, however, that srtC played an independent role under the conditions tested, because reverse-transcription PCR at these conditions showed elevated levels of the independent srtC transcript (authors’ unpublished data). Furthermore, complementation of TX5475 with srtC did not restore biofilm formation [12]. Our ongoing studies with different sortase mutants may be helpful in identifying any additional effects of sortase C.

Both test bacteria used for infection in the present study were grown in 40% serum plus BHIB. Serum was chosen for the growth of test bacteria in the present study because our recently published study [12] showed an enhanced level of Ebp when WT _E. faecalis_ OG1RF was grown in 40% horse serum plus BHIB versus growth in BHIB alone. It has been shown previously that growth in serum increased the adherence of tested strains to urinary tract epithelial cells and that clinical UTI isolates more efficiently adhered to urinary tract epithelial cells than to cultured cells by at least 1.5–3-fold, with the greatest increase being observed in UTI strain adherence to Girardi heart cells (8-fold) [38, 39]. Similarly, others have

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Table 1. Tropism for the kidneys by _Enterococcus faecalis_ wild-type strain OG1RF and its isogenic, nonpiliated, ebpA-deletion mutant (TX5475) in a murine model of ascending urinary tract infection.

<table>
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<th>Urinary bladder</th>
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<td>TX5475</td>
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<td>3/10</td>
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<td>1.7 × 10^4</td>
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**NOTE.** Data are proportion of kidneys or bladders infected, unless otherwise specified.

^a_ For total no. of kidneys infected by OG1RF, compared with TX5475 (Fisher's exact test).

^b_ For total no. of urinary bladders infected by OG1RF, compared with TX5475 (Fisher's exact test).
shown the differential expression of several virulence-related genes in *E. faecalis* in response to biological cues in serum and urine [40], and it would be of interest to test growth in urine as well.

An *E. faecalis* protein that has been described previously as influencing bladder colonization is Esp, which is reported to be expressed on the cell surface; the *esp* gene was found significantly more often in strains of human UTI origin than in fecal isolates [5, 41]. A disruption mutation in *esp* reduced colonization of the urinary bladder in a model of ascending UTI [5], although this study failed to demonstrate any difference in bacterial counts in kidneys after infection with the *esp* mutant versus its parent strain, even in mice whose bladders showed differences. Another study that also used the mouse model of ascending UTI was unable to show a role for adhesion or enterococcal binding substance (EBS), either in a competition assay or during monoinfection [4]. Both the *esp* mutant testing and AS/EBS studies [4, 5] noted inconsistent urinary bladder colonization in mice whose kidneys were infected, as was also observed in the present study with both WT OG1RF and TX5475; this is consistent with the findings of a previously published study by Kau et al. [2] demonstrating that *E. faecalis* has greater tropism for the kidneys. The Kau et al. study [2] showed that, when the inoculum volume of *E. faecalis* was increased from 50 to 200 µL (to facilitate direct delivery of the bacteria to both the bladder and the kidneys simultaneously), there was more consistent colonization and recovery of bacteria from >90% of kidneys in mice infected with *E. faecalis*. In preparing for the present study, we also found that only 1 of 6 mice had infected kidneys with no infected bladders when a 50-µL inoculum was used to infect mice, versus 6 of 6 mice with infected kidneys and 3 of 6 mice with infected bladders when a 200-µL inoculum was used (data not shown). Kau et al. [2] suggested that many or most episodes of enterococcal cystitis may be due to seeding from an upper UTI and that asymptomatic bacteriuria with enterococci is often localized to the upper tract rather than the bladder; although we are unaware of data from humans that indicate that upper UTI with enterococci is more common, this is certainly an interesting hypothesis.

Competition assays in which equal numbers of colony-forming units (in a mixed suspension) are inoculated to infect animals have been successfully used in endocarditis models [15, 16, 42] to differentiate the infective capabilities of WT organisms and their isogenic mutants. Kau et al. [2] showed the growth advantage of a pyelonephritis clinical isolate over a laboratory strain of *E. faecalis* in the kidneys, but not in the bladder, in a mouse UTI model and demonstrated the utility of this model to study factors involved in the pathogenesis of UTIs, as it has been with *E. coli* [29]. In the competition assay of the present study, we found significant differences in the numbers of colony-forming units recovered from kidneys (3.1-fold) and bladders (179-fold) for WT OG1RF versus TX5475. However, in all 4 monoinfection inoculum groups, the differences seen in the numbers of recovered colony-forming units for WT OG1RF versus TX5475 from kidneys were much greater (up to 1000-fold) than the differences seen in colony-forming units recovered from kidneys in the competition assay. We suspect that the smaller differences seen in the kidney counts between WT OG1RF and TX5475 in the competition assay might be due to some of the nonpiliated TX5475 cells adhering to the Ebp surface pili of WT OG1RF and remaining at the site of infection sites, and this hypothesis is partially supported by our earlier observation of frequent association of Ebp pili with TSBG [12]. Our previous study also found that anti-Ebp--stained pili were seen on few WT OG1RF cells (<20% of TSBG [tryptic soy broth with 0.25% glucose]--grown and <2% of BHBB-grown cells, respectively), and one possibility is that a low percentage of piliated WT OG1RF cells could be immunologically advantageous to the organism [12]. It is also possible that in vivo conditions increase the production of pili.

In the mouse peritonitis model, TX5475 did not show significant attenuation versus WT OG1RF during the course of infection, although the total mortality showed that ∼2-fold
more cells of TX5475 were needed to cause disease in 50% of the mice. The at most very modest attenuation shown by TX5475 in the peritonitis model versus the high attenuation observed in the UTI model relative to WT OG1RF demonstrates the importance of selecting an appropriate animal model in order to assess the effect of adhesin-related genes. The Ebp proteins have been recognized previously as members of the MSCRAMM (microbial surface component–recognition adhesive matrix molecule) family [43], and a murine UTI model appears to provide suitable in vivo conditions to allow ebp-associated pili of WT OG1RF to attach to and colonize the host renal tissue.

In conclusion, a nonpiliated TX5475 mutant of OG1RF showed marked attenuation versus WT OG1RF in terms of ID50, virulence index, colony-forming unit counts in kidneys after monoinfection, and total numbers of kidneys and bladders colonized in a mouse model of ascending UTI. There was increased tropism of each strain for kidneys versus bladders, and results for bladders also showed attenuation of TX5475 on the basis of ID50 and the results of the competition experiments. The degree of attenuation of the nonpiliated TX5475 is impressive and is consistent with our previous report showing significant reduction in biofilm production and attenuation in a rat model of infective endocarditis [12]. Our previous finding that 100% of 408 strains tested by high-stringency hybridization contain ebp genes indicates that these genes are part of the core E. faecalis genome and suggests that all strains could rely on these pili for colonization and/or infection of tissues. To extrapolate from a report indicating that pilicides target and interfere with pilus-assembly function and disrupt the infection process of uropathogenic E. coli [44], these apparently conserved enterococcal surface pili could be a potential target for new and yet-to-be discovered gram-positive–specific pilicides as a way to treat or prevent enterococcal UTIs in human.

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