Published in final edited form as:
J Infect Dis. 2007 June 1; 195(11): 1671–1677. doi:10.1086/517524.

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

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Abstract

Background—We recently showed that the ubiquitous *Enterococcus faecalis ebp* (endocarditis and biofilm-associated pilus) operon was important for biofilm and experimental endocarditis. Here we assess its role in murine urinary tract infection using wild type *E. faecalis* OG1RF and its nonpiliated *ebpA* allelic replacement mutant (TX5475).

Methods—OG1RF and TX5475 were administered transurethrally in a 1:1 ratio (competition assay) or individually (monoinfection). Kidneys and bladders were cultured 48 h postinfection. These strains were also tested in a peritonitis model.

Results—No differences were observed in the peritonitis model. In mixed urinary infection, OG1RF significantly outnumbered TX5475 in kidneys (P=0.0033) and bladders (P=<0.0001). More OG1RF CFU were also recovered from kidneys of monoinfected mice at the four inocula tested (P=0.015 to 0.049) and ID_{50}s of OG1RF for kidney and bladder (9.1×10^1 and 3.5×10^3 CFU, respectively) were two-three $\log_{10}$ lower than with TX5475. Increased tropism for the kidney relative to the bladder was observed by 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.

INTRODUCTION

Among the various infections reported with *Enterococcus faecalis*, urinary tract infections (UTI) 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 persistence of bacteria in the urinary bladders of mice, with no histological changes, when a parent and its *esp* mutant were used in a ascending urinary tract infection model [5]; however, there was no difference found in kidneys. Kau et al. [2], using a similar model, demonstrated

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Potential conflict of interest: None reported

that *E. faecalis* has a preference for the kidney relative to the bladder and concluded that this model can be used to study the factors involved in the pathogenesis of urinary tract infections.

E. faecalis is also known to form biofilm on biomedical devices such 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 *fsr* 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 catheterized patients, and in part because biofilm formation may promote 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* operon (endocarditis and biofilm associated pili) which consists of, *ebpA*, *ebpB*, *ebpC* and an associated *srtC* (encoding sortase C) gene which also has an independent promoter [12]. These surface pili are immunogenic and pleomorphic in nature and their role in the establishment of biofilms and infective endocarditis in a rat model was shown using the wild type (w.t.) *E. faecalis* strain OG1RF and its isogenic nonpiliated Δ*ebpA* (TX5475) mutant. 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 this study, using w.t. *E. faecalis* OG1RF, which expresses sortase C-dependent pleomorphic surface pili [12], and its nonpiliated TX5475, we describe their ability to colonize kidneys and bladders in both monoinfection and a mixed infection competition assay in a murine UTI model. 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 include wild type OG1RF (resistant to rifampicin 100 μg/ml, 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]. A recently described nonpiliated, biofilm-deficient *ebpA* isogenic deletion (polar) mutant of OG1RF, TX5475 with *ebpA* replaced with kanamycin resistance gene was also used [12]. Brain Heart Infusion broth (BHIB) (Difco Laboratories) was used for routine bacterial growth. Kanamycin (KAN) and heat inactivated horse serum were purchased from Sigma. The concentration of antibiotics used in BHI agar (BHIA) plates for the growth of nonpiliated TX5475 was KAN 2000 μg/ml.

UTI model for competition assay, monoinfections and ID_{50} determination

Female, 4- to 6-week-old, outbred ICR mice (Harlan Sprague Dawley) with a mean weight of 25 g were used in this study. Pre-approved protocol and 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 prescreened for UTI by culturing the urine ~12 h prior to inoculation. The urinary bladders of mice were emptied by gently pressing the abdomen before infecting them. For preparation for administration to mice, bacteria were grown in 10 ml of BHIB + 40% horse serum for 10 h at 37°C with gentle shaking. Cells were pelleted for 10 minutes (10,000 rpm at 10°C), and resuspended in 10 ml of 0.9% saline. Further dilutions were also prepared in saline, and plated to determine the actual inoculum that had been administered. An inoculation method similar to a recently described mouse UTI model [2] was used. A group of 16 mice was used in the competition assay while groups of 5 mice per inoculum were used in each of two independent monoinfection experiments with combination of the results. 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 approximately 1:1 ratio of w.t. *E. faecalis* OG1RF:nonpiliated TX5475, respectively, in a competition assay; two independent experiments using 10 and 6 mice, respectively, were performed and the results were combined. In two independent monoinfection experiments, w.t. OG1RF was used in the inocula range of 10^1 - 10^6 while the nonpiliated TX5475 was tested in the inocula range of 10^3 - 10^6 to generate data for comparison of CFU recovered from kidneys and bladder of mice as well as for the calculation of 50% infective dose (ID_{50}). Similar to the method described by Kau et al. [2], we also used an inoculum volume of 200 μl in order to facilitate direct delivery of the bacteria to both bladder and kidneys, simultaneously. The urethral catheter was removed immediately after injecting the bacteria, and all the animals were provided free access to food and water during the course of study. Daily observations were made for any morbidity and mortality in animals. Mice were euthanized by CO_2 inhalation at 48 h after transurethral challenge, the urinary bladder and kidney pair were excised, weighed, homogenized in 1 ml and 5 ml of saline, respectively, and dilutions were plated onto BHIA for recovery of bacteria in case of monoinfections and onto BHIA and BHIA+KAN 2000 μg/ml in the case of mixed infection in order to determine the recovered CFUs (%) of w.t. OG1RF versus nonpiliated TX5475. The detection limit of bacteria in this experiment was 10 CFU. Mice with sterile cultures of kidney and urinary bladder homogenates were considered to have no UTI infection. Identity of the recovered test bacteria from infected organs was confirmed by plating them on Bile Esculin Azide agar (BEA) and BHIA + RIF100 plates for w.t. *E. faecalis* OG1RF and on BHIA + Kan2000, BHIA + RIF100 and BEA plates for the nonpiliated TX5475. Randomly picked nonpiliated TX5475 colonies were also tested by PCR, pulsed-field gel electrophoresis (PFGE) [18] and/or by 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 our previously published method [17]. Mice were injected intraperitoneally with appropriate dilutions of premixed bacteria/sterile rat fecal extract (SRFE) and were observed for 5 days. Two fold dilutions of both test bacteria in a range of ~$1 \times 10^8 - 1 \times 10^9$ CFU were used as the inocula for LD_{50} determination using six mice per group. The LD_{50} was determined by the method of Reed and Muench [19].

Statistics, ID_{50}s, and mean virulence index

The $\log_{10}$ CFU per gram of TX5475 and w.t. OG1RF in tissue of each animal (kidney or bladder) from mixed infection were analyzed for significance by the paired *t* test. Differences in the $\log_{10}$ CFU of TX5475 versus w.t. OG1RF in monoinfections were evaluated by the unpaired *t* test. Fisher's exact test was used to compare the total infection of kidneys and bladders (combining all inocula) in monoinfection with OG1RF versus TX5475. Prism version 4.00 for Windows (GraphPad Software) was used for statistical analysis. The fifty percent infective dose (ID_{50}s) values were calculated by a previously published method [19]. The mean virulence index was calculated using the following equation (as previously described for other organisms in mixed infections [12,15,20]):

$$\text{Mean virulence index} = \frac{(\text{GM} - \text{CFU of OG1RF})_{T=0} / (\text{GM} - \text{CFU of the TX5475 mutant})_{T=0}}{(\text{GM} - \text{CFU of OG1RF})_{T=48} / (\text{GM} - \text{CFU of TX5475 mutant})_{T=48}}$$

where GM-CFU is the geometric mean expressed as CFU.

RESULTS

Competition assay

In the competition experiment, approximately equal numbers (as judged by OD at 600nm) of w.t. OG1RF and TX5475 mutant were administered transurethrally. The actual CFU of w.t. *E. faecalis* OG1RF and the TX5475 mutant (combined from two experiments) in the inoculum mix were 1.1×10^7 and 1.2×10^7, respectively; thus, the percent of w.t. *E. faecalis* OG1RF in the inoculum was 49% (figure 1). W.t. OG1RF outcompeted the TX5475 mutant in all 16 mice at 48 h post infection, increasing significantly from time zero (T = 0) in kidneys and urinary bladder, with mean paired differences of 3.1- fold ($P = 0.0033$) and 179- fold ($P = <0.0001$), respectively (figure 1). The mean virulence indices of nonpiliated TX5475 relative to w.t. OG1RF in kidneys and bladders were 0.30 and 0.005, respectively.

Monoinfection of kidneys and bladders 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 experiment, we also performed monoinfections with four equal inocula of each organism. The total number of kidneys infected (38/40 with OG1RF versus 27/40 with TX5475) (P=0.0031) and total urinary bladders infected (30/40 with OG1RF versus 18/40 with TX5475) (P=0.0115), combining from all four monoinfection inoculum groups indicated that TX5475 was significantly attenuated in its ability to infect both kidneys and bladders versus its parent strain w.t. OG1RF (table 1). CFU counts recovered from animals showed that when mice (n=10) were infected with $1\text{-}4 \times 10^3$, $1\text{-}4 \times 10^4$, $1\text{-}4 \times 10^5$, $1\text{-}4 \times 10^6$ CFU of w.t. OG1RF, the GM-CFU recovered from kidneys at 48 after transurethral challenge were 2.1×10^4, 2.4×10^5, 1.7×10^6, 1.0×10^6, respectively (figure 2). In mice infected with $2\text{-}4 \times 10^3$, $2\text{-}4 \times 10^4$, $2\text{-}4 \times 10^5$, $2\text{-}4 \times 10^6$ CFU of TX5475, the GM-CFU recovered from kidneys were 6.2×10^1, 3.6×10^3, 1.3×10^4, 8.9×10^3, respectively (figure 2), showing ≥two $\log_{10}$ reduction in GM-CFUs of TX5475 versus GM-CFUs of w.t. OG1RF for the same inoculum (figure 2). The mean CFU differences ($\log_{10}$) seen in all four inocula groups (10^3, 10^4, 10^5 and 10^6) between w.t OG1RF versus nonpiliated TX5475 were 2.5 ± 1.2 (P=0.0493), 1.8 ± 0.9 (P=0.0490), 2.1 ± 0.8 (P=0.0156) and 2.1 ± 0.9 (P=0.0333), respectively, (figure 2). In bladders after monoinfection, although fewer bladders were infected by TX5475 (table 1), the CFU differences were not significant with mean differences ($\log_{10}$) of 1.2 ± 1.2, 0.9 ± 1.3 and 0.9 ± 1.1 (in the 10^3, 10^5 and 10^6 inocula group, respectively) and 2.7 ± 1.4 (in the 10^4 inoculum group). The results in table 1 also indicate tropism for the kidney, since 20 – 30% of the urinary bladders were found to be sterile in the same mice whose the corresponding kidneys were infected (OG1RF) and 20 – 50% of the urinary bladders were found to be sterile in the same mice whose kidneys were infected with the nonpiliated TX5475. The ID_{50}s derived from monoinfection experiments using w.t. OG1RF for kidney pairs and urinary bladders were determined to be 9.1×10^1 and 3.5×10^3 CFU, respectively, while the ID_{50}s using TX5475 for kidneys and bladder were determined to be 1.67×10^4 and 1.68×10^5 CFU, respectively, showing that ≥two $\log_{10}$ more cells were required to infect 50% of the mice versus w.t. OG1RF. ID_{50}s also showed that approximately one $\log_{10}$ fewer CFU of either bacteria were needed to infect 50% of the kidney pairs than to infect 50% of the urinary bladders.

Mouse peritonitis model

In an experiment using six mice per inoculum, both test bacteria caused mortality at a similar rate (monitored at 3- 6 h intervals for 96 h) in mice (data not shown) and were not significantly different by statistical analysis using 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 fimbriae) 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 bladder and kidney [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 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. Results of the current study using w.t. OG1RF versus TX5475 was an effort to determine if the *ebp*-locus played a role in colonization of kidneys and urinary bladders in a murine UTI model. The reduced recovery of TX5475 mutant versus w.t. OG1RF from kidneys (≥ 2 $\log_{10}$) and bladders (≥ 1 $\log_{10}$), evident with all four inocula used in monoinfection, confirmed this hypothesis. Although our results with the *ebpA* allelic replacement mutant TX5475 (that produces none of the three Ebp proteins) [12] unambiguously demonstrated a role for the *ebp* locus in urinary tract infection 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 RT-PCR at these conditions showed elevated levels of the independent *srtC* transcript (Nallapareddy et al., unpublished data). Furthermore, the 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 + BHIB. Serum was chosen for the growth of test bacteria in the present study as our recently published study [12] showed an enhanced level of Ebp when w.t *E. faecalis* OG1RF was grown in 40% horse serum + BHIB versus growth in BHIB alone. It has previously been shown that growth in serum increased the adherence of tested strains to urinary tract epithelial cells and that clinical UTI isolates adhered to urinary tract epithelial cells more efficiently than to the cultured cells by at least 1.5- to 3-fold, with the greatest increase being observed in UTI strain adherence to Girardi heart cells (8-fold) [38,39]. Similarly, others have 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 displayed 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 an ascending model of urinary tract infection [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 which also used the ascending model of mouse urinary tract infections was unable to show a role for aggregation substance (AS) or enterococcal binding substance (EBS), either in competition assay or in mono-infection [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 w.t. OG1RF and TX5475; this is consistent with a previously published study of Kau et al. [2] 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 μl to 200 μl, in order 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 this study, we also found that when 50 μl inocula were used to infect the mice, only one of six mice had infected kidneys with no infected bladders versus using 200 μl inocula volume which showed six of six mice with infected kidneys and three of six mice with infected bladder (data not shown). Kau et al. [2] suggested that many or most episodes of enterococcal cystitis may be due to seeding from an upper tract infection 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 tract infection with enterococci is more common, this is certainly an interesting hypothesis.

Competition assays in which equal CFUs (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 w.t. organisms and their isogenic mutants. Kau et al. [2] showed the growth advantage by 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 showed utility of this model to study factors involved in the pathogenesis of urinary tract infections, as it has been with *E. coli* [29]. In the competition assay of the present study, we found a significant differences between recovered CFUs of w.t. OG1RF versus the TX5475 from kidneys (3.1 fold) and bladders (179 fold). However, in all four inoculum groups of monoinfections, the differences seen in recovered CFUs of w.t. OG1RF versus TX5475 from kidneys were much greater (up to 1000 fold) versus the differences seen in recovered CFUs from kidneys in the competition assay. We suspect that the smaller differences seen between kidney counts of w.t. OG1RF and the TX5475 in the competition assay might be due to some of the nonpiliated TX5475 cells adhering to the Ebp surface pili of w.t. OG1RF and remaining at the infected sites and this hypothesis is partially supported by our earlier observation of frequent association of Ebp pili with more than one cell in immunogold electron micrographs [12]. Our previous study also found that anti-Ebp stained pili were seen in few w.t. OG1RF cells (<20% of TSBG- and <2% of BHIB-grown cells, respectively) and one possibility is that a low % of piliated w.t. OG1RF cells could be immunologically advantageous to the organism [12]. It is also possible that in vivo conditions increase production of pili.

In the mouse peritonitis model, TX5475 did not show significant attenuation versus w.t. OG1RF during the course of infection although the total mortality showed ~two fold more cells of TX5475 were needed to cause disease in 50% of the animals. The, atmost, very modest attenuation by TX5475 in peritonitis model versus the high attenuation observed in the UTI model when compared with w.t. OG1RF shows the importance of selecting an appropriate animal model in order to assess the effect of adhesin related genes. The Ebp proteins previously have been recognized as the members of the microbial surface component recognizing adhesive matrix molecules (MSCRAMM) family [43] and a UTI murine model appears to provide suitable in vivo conditions to allow *ebp*-associated pili of w.t. OG1RF to attach and colonize the host renal tissue.

In conclusion, a nonpiliated TX5475 mutant of OG1RF showed marked attenuation versus w.t. OG1RF in terms of ID_{50}, virulence index, CFU in kidneys after monoinfection and total numbers of kidneys and bladders colonized in an ascending mouse UTI model. There was increased tropism of each strain for kidneys versus bladders, and results with bladders also showed attenuation of TX5475 based on ID_{50}s and the competition assays. 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 an infective endocarditis rat model [12]. Our previous finding that 100% of 408 strains tested contain *ebp* genes using high stringency hybridization 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. Extrapolating from a report 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 urinary tract infections in human.

ACKNOWLEDGEMENT

This work was supported in part by NIH grant R37 AI47923 from the Division of Microbiology and Infectious Diseases, NIAID to Barbara E. Murray. We would like to express our sincere thanks to Richard A. Hull, Baylor College of medicine, Houston, TX for helping us learn the urinary tract catheterization techniques for the experimental UTI model. We would also like to express our sincere thanks to Karen Jacques Palaz and Charlene L. Thomson for their technical help.

Financial support: National Institutes of Health grant R37 AI47923 from the Division of Microbiology and Infectious Diseases, NIAID to B.E.M.

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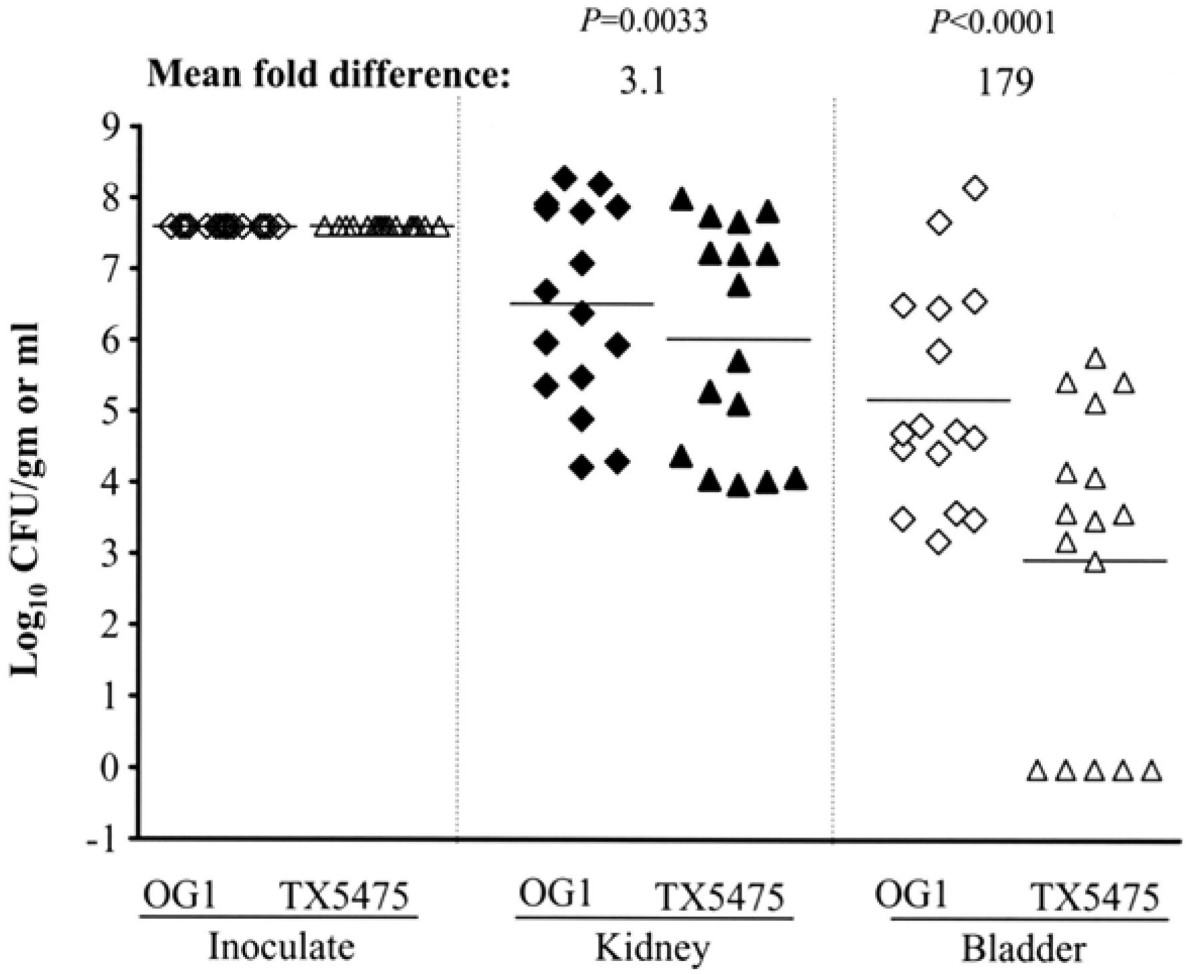

Figure 1.

Mixed infection (competition assay) by w.t. *E. faecalis* OG1RF and nonpiliated TX5475 in the kidneys and urinary bladder of mice (n=16). Data are expressed as $\log_{10}$CFU of w.t. *E. faecalis* OG1RF or TX5475. The $\log_{10}$CFU/gm from both kidneys were combined and averaged. Solid diamonds and triangles represent w.t. *E. faecalis* OG1RF and TX5475, respectively, from kidney homogenates and empty diamonds and triangles represent w.t. *E. faecalis* OG1RF and TX5475, respectively, from urinary bladder homogenates. Horizontal bars represent geometric mean titer. Mean fold difference in CFU are given. Log_{10} CFU were compared for statistical significance using a paired *t* test.

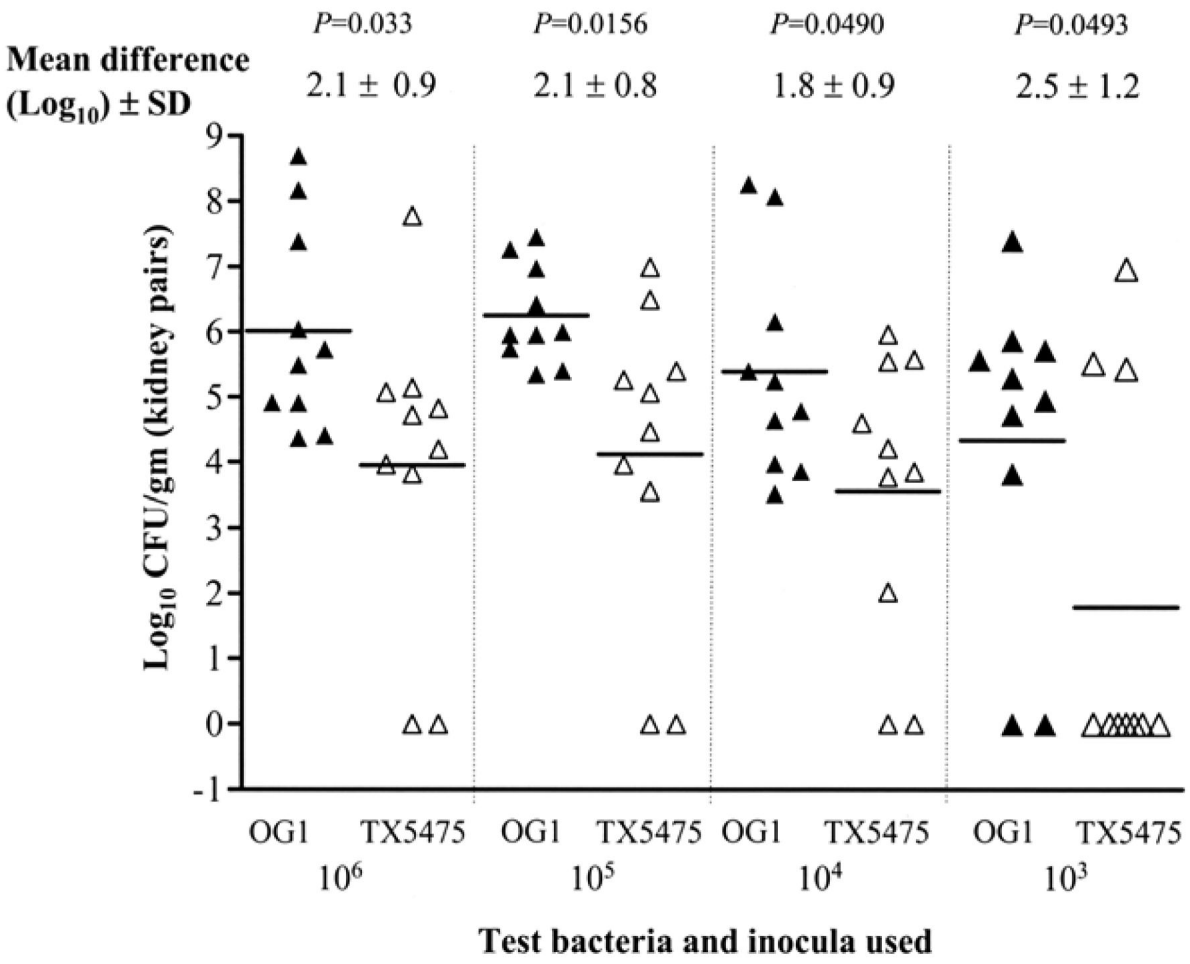

Figure 2.

Monoinfection (10^6, 10^5, 10^4, and 10^3 inocula) using w.t. *E. faecalis* OG1RF or nonpiliated TX5475 in the kidneys of mice (n=10). Data are expressed as $\log_{10}$CFU/gm of w.t. *E. faecalis* OG1RF or TX5475 recovered from kidney homogenates 48 hrs after transurethral challenge. The $\log_{10}$CFU from both kidneys were combined and averaged. Solid and empty triangles represent w.t. *E. faecalis* OG1RF and TX5475, respectively. Horizontal bars represent geometric mean titer. Mean difference in CFU is given as $\log_{10}$ ± standard deviation (SD) for the respective inocula. TX5475 CFUs (recovered from kidneys) were significantly reduced versus OG1RF with all four inocula using unpaired *t* test.

Table 1

Tropism for the kidneys by *E. faecalis* strains OG1RF and its isogenic nonpiliated Δ*ebpA* (TX5475) mutant in a murine model of ascending urinary tract infection

Inocula used	Infected kidney pair and urinary bladder			
	Kidneys		Bladder	
	OG1RF	TX5475	OG1RF	TX5475
10^3	8/10	3/10	5/10	3/10
10^4	10/10	8/10	7/10	3/10
10^5	10/10	8/10	8/10	6/10
10^6	10/10	8/10	10/10	6/10
ID_{50}	9.1×10^1	1.6×10^4	3.5×10^3	1.6×10^5
Total infected	38/40	27/40	30/40	18/40
	P=0.0031[a]		P=0.0115[b]	

[a] *P* values, determined by Fisher's exact test, are for total kidneys infected by OG1RF compared with TX5475;

[b] *P* values are for total urinary bladders infected by OG1RF compared with TX5475