Enterococcus faecalis tropism for the kidneys in the urinary tract of C57BL/6J mice

Andrew L. Kau  
*Washington University School of Medicine in St. Louis*

Steven M. Martin  
*Washington University School of Medicine in St. Louis*

William Lyon  
*Washington University School of Medicine in St. Louis*

Ericka Hayes  
*Washington University School of Medicine in St. Louis*

Michael G. Caparon  
*Washington University School of Medicine in St. Louis*

See next page for additional authors

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Andrew L. Kau, Steven M. Martin, William Lyon, Ericka Hayes, Michael G. Caparon,* and Scott J. Hultgren*

Department of Molecular Microbiology, Washington University, St. Louis, Missouri

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Enterococcus faecalis is a gram-positive bacterium that can cause a variety of nosocomial infections of which urinary tract infections are the most common. These infections can be exceptionally difficult to treat because of drug resistance of many E. faecalis isolates. Despite their troublesome nature, little is known about the host or bacterial factors necessary for E. faecalis to cause disease in the urinary tract. Using a mouse model of urinary tract infection, we have shown that E. faecalis is capable of persisting in the kidneys of mice for at least 2 weeks. In contrast, bacterial titers from the bladders of the same mice were inconsistent and tended to be much lower than those recovered from the kidney. This preference for the kidney over the bladder is also observed in other clinical E. faecalis strains. Histologic examination of bladder and kidney tissues demonstrated that E. faecalis induced an inflammatory response in the kidney but not in the bladder. This inflammatory response was TLR2 independent and did not induce inflammatory markers typically associated with uropathogenic Escherichia coli. Using a competition assay, we demonstrated that a pyelonephritis clinical isolate had a growth advantage over a laboratory strain of E. faecalis in the kidneys but not in the bladders of mice. Taken together, these results demonstrate that E. faecalis has tropism for the kidneys in the urinary tracts of mice and that this system can be used to study factors involved in the pathogenesis of urinary tract infections.

Enterococcus faecalis, while normally a gut commensal, is a frequent cause of many serious human infections, including urinary tract infections, endocarditis, bacteremia, and wound infections. Among the diseases that E. faecalis causes, urinary tract infections are the most common, responsible for approximately 110,000 cases yearly, many of which are nosocomial. Infections with E. faecalis can be especially troublesome to treat because of their frequent resistance to multiple antibiotics, including vancomycin, a drug of last resort for many gram-positive infections (for a review, see reference 8).

Both rat and mouse model systems have been used to study factors involved in the pathogenesis of E. faecalis in the urinary tract. Studies by Guze and colleagues showed that E. faecalis has a growth advantage over other enterococcal species in rat kidneys in a hematogenous inoculation pyelonephritis model (18). Another model system was used to show that pyelonephritis caused by Pseudomonas aeruginosa was aggravated by coinfection with E. faecalis, as determined by histological changes in the kidney (30). In this model, ligation of the urethra was used to induce retrograde reflux of bacteria into the kidney, increasing the susceptibility of the mouse to infection. In a bladder catheterization model of urinary tract infection, the Esp (enterococcal surface protein) adhesin was found to increase persistence in the urinary bladder of mice, although no histological changes were observed (26). Another study was unable to demonstrate a critical function for aggregation substance, a well-characterized enterococcal adhesin, in a murine model of cystitis (12). Thus, a robust murine cystitis model of E. faecalis infection has yet to be established, most likely due to complicating host and bacterial factors reflecting the adaptations enterococcus has evolved to coexist with its host. In order to more effectively devise strategies and therapies to prevent and treat enterococcal urinary tract infections, an infection model that can give a sensitive readout of virulence factors must be developed.

In contrast, many of the host-pathogen interactions critical for bladder infections caused by uropathogenic Escherichia coli, the most common etiologic agent of urinary tract infections, have been well-defined in murine models. Uropathogenic E. coli initially interacts with bladder epithelium through the expression of type 1 pili, which mediate adhesion and invasion into the uroplakin-expressing umbrella cells on the surface of the bladder (19). The invasion of E. coli into the urinary epithelium results in expression of a variety of cytokines, induction of apoptosis in infected cells, and a massive neutrophil inflammatory response. This response is mediated primarily by Toll-like receptor 4, which has been demonstrated to respond to lipopolysaccharide (22, 23). The interaction of E. coli with the mouse kidney epithelium during pyelonephritis is less well characterized, but the importance of P pili is well established as an initial mediator of adherence in other animal models (21). Similar to bladder infections, acute E. coli–mediated pyelonephritis in mice is characterized by a strong neutrophil immune response (4).

In this study, we describe the tropism of E. faecalis for the kidneys in C57BL/6J mice. The consequences of this tropism were investigated by characterizing the host response to E.
strains other than 0852 were contributed by Thomas Hooten and Walter Stamm.

37°C in brain heart infusion (BHI) medium (Difco) without antibiotics. Clinical laboratories and C57BL/6 TLR2

E. faecalis
E. faecalis
mice,

0852
E. faecalis

were 36 to 48 weeks in age at the time of inoculation. Wild-type 12-week-old and

recoveries of

These experiments demonstrated that there was no substantial difference in the

m-thick sections were prepared. Sections were deparaffinized using Hemo De

Quantitative real-time PCR. Methods were as described in Mysorekar et al. (20) with the following modifications. Female C57BL/6J/mice were inoculated

Student's one-tailed
test for correlated samples using the logarithmic values at

and mixed in equal volumes to make the mixed inoculum. The BP78 to OG1X ratio was determined by plating the mixed inoculum on medium selective for each strain; 48 h after infection, mice were sacrificed and their bladders and kidneys were collected and homogenized as described above. The homogenate was then plated onto either BHI agar supplemented with streptomycin, to select for OG1X, or tetracycline, to select for BP78. The competition index was calculated similarly to Freret et al. using OG1X as the reference strain (3). Briefly, the competition index = [([CFU of BP78/CFU of OG1X recovered from mice]/

TABLE 1. Strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Resistance(s)</th>
<th>Source</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NU14</td>
<td>E. coli</td>
<td>Str&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Cystitis isolate</td>
<td>7, 16</td>
</tr>
<tr>
<td>OG1X</td>
<td>E. faecalis</td>
<td>Str&lt;sup&gt;t&lt;/sup&gt;, Tet&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Gelatinase-negative, plasmid-free strain derived from an oral isolate by nitrosoguanidine mutagenesis</td>
<td>9, 10</td>
</tr>
<tr>
<td>0852</td>
<td>E. faecalis</td>
<td>Tet&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Urinary tract isolate, not otherwise specified</td>
<td>This study</td>
</tr>
<tr>
<td>B1223</td>
<td>E. faecalis</td>
<td>Tet&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Cystitis isolate</td>
<td>This study</td>
</tr>
<tr>
<td>B1384</td>
<td>E. faecalis</td>
<td>Cystitis isolate</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>P1505</td>
<td>E. faecalis</td>
<td>Tet&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Pyelonephritis isolate</td>
<td>This study</td>
</tr>
<tr>
<td>BP78</td>
<td>E. faecalis</td>
<td>Tet&lt;sup&gt;t&lt;/sup&gt;, Str&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Pyelonephritis isolate</td>
<td>This study</td>
</tr>
<tr>
<td>BP250</td>
<td>E. faecalis</td>
<td>Tet&lt;sup&gt;t&lt;/sup&gt;</td>
<td>Pyelonephritis isolate</td>
<td>This study</td>
</tr>
</tbody>
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faecalis and contrasting these differences to the response induced by uropathogenic E. coli.

**MATERIALS AND METHODS**

**Strains and growth conditions.** A summary of all the strains used in this study, their origins, and relevant drug resistances are shown in Table 1. For infection of mice, E. faecalis strains were grown statically overnight (typically 12 to 15 h) at 37°C in brain heart infusion (BHI) medium (Difco) without antibiotics. Clinical strains other than 0852 were contributed by Thomas Hooten and Walter Stamm (University of Washington) and categorized as either cystitis or pyelonephritis strains using clinical criteria.

**Animals.** Female wild-type C57BL/6 mice were obtained from Jackson Laboratories and C57BL/6 TLR2/E. coli mice were a gift of S. Akira (29). TLR2

**E. faecalis**

was most likely due to the clearance of nonadherent bacteria from the bladder but noticeably higher titers in the kidney. Based on this observation, we used a 200-μl inoculum volume to intentionally induce retrograde reflux of the inoculum from the bladder into the kidney (11).

The ability of E. faecalis 0852 to persist in bladders and kidneys over a 2-week period is shown in Fig. 1. Fifteen minutes after inoculation, bacterial titers were high in both the bladder and the kidney. E. faecalis did not persist in the bladder, as bacterial titers decreased dramatically after 15 min, and many bladders were sterile at later time points. In contrast, recovery of bacteria from the kidneys remained steady over the first 12 h; 24 h after inoculation, bacterial levels in the kidney decreased but nevertheless persisted over a 2-week period. The conclusion from these studies was that E. faecalis persisted at higher titers in the kidney than in the bladder over a 2-week period. The large dropoff in bladder titers between 15 min and 6 h was most likely due to the clearance of nonadherent bacteria from the bladder by mechanical forces of urine flow and other innate defenses. The persistence of E. faecalis in the kidneys over this time period indicated that the bacteria in the kidney were able to establish residence capable of evading innate defenses. Consistent with this hypothesis was the finding that 16% of mice had recoverable titers of 0852 in the kidneys despite having sterile bladders; conversely, no mice had recoverable bacterial titers in the bladder if their kidneys were

In order to study E. faecalis cystitis, we inoculated 50 μl of 10<sup>6</sup> CFU of an E. faecalis strain, 0852, from a diagnosed urinary tract infection transurethrally into female C57BL/6J mice. This protocol led to inconsistent recovery of bacteria from the bladder but noticeably higher titers in the kidney. Based on this observation, we used a 200-μl inoculum volume to intentionally induce retrograde reflux of the inoculum from the bladder into the kidney (11).

RESULTS

E. faecalis can cause a reproductive infection in C57BL/6J mouse kidneys. In order to study E. faecalis cystitis, we inoculated 50 μl of 10<sup>6</sup> CFU of an E. faecalis strain, 0852, from a diagnosed urinary tract infection transurethrally into female C57BL/6J mice. This protocol led to inconsistent recovery of bacteria from the bladder but noticeably higher titers in the kidney. Based on this observation, we used a 200-μl inoculum volume to intentionally induce retrograde reflux of the inoculum from the bladder into the kidney (11).

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Commercially available affinity matrix-based kit (RNeasy kits, Qiagen). cDNA was generated using random hexamers and quantitative real-time PCR was performed as described (20) using the Bio-Rad iCycler. Primers for amplification of glyceraldehyde-3-phosphate dehydrogenase, Mip-2, and Socs-3 are as previously described (20).

**Competitive infection.** Cultures of OG1X and BP78 were resuspended in PBS and mixed in equal volumes to make the mixed inoculum. The BP78 to OG1X ratio was determined by plating the mixed inoculum on medium selective for each strain; 48 h after infection, mice were sacrificed and their bladders and kidneys were collected and homogenized as described above. The homogenate was then plated onto either BHI agar supplemented with streptomycin, to select for OG1X, or tetracycline, to select for BP78. The competition index was calculated similarly to Freret et al. using OG1X as the reference strain (3). Briefly, the competition index = [([CFU of BP78/CFU of OG1X recovered from mice]/

(CFU of BP78/CFU of OG1X present in initial inoculum)].
sterile (Fig. 1B, squares). Given the variability of CFU per bladder, we hypothesized that bacteria recovered from the bladder at later time points may represent bacteria shed from the kidney. Taken together, the persistence of 0852 in the kidney and its clearance from the bladder suggest that **E. faecalis** has tropism for the kidneys in the urinary tract of C57BL/6J mice.

Multiple **E. faecalis** clinical isolates display tropism for the kidney in the urinary tract of C57BL/6J mice. To determine if the tropism of **E. faecalis** for the kidney in C57BL/6J mice is specific to strain 0852 or whether it represents a general feature of this species, we inoculated mice with five different strains of **E. faecalis** and harvested their bladders and kidneys for titers 48 h after infection (Fig. 2). Two of these strains, B1223 and B1384, were cystitis isolates, and three others, P1503, BP78, and BP250, were pyelonephritis isolates. Consistent with what had been observed with 0852, all of the strains persisted in the kidney to higher levels than in the bladder. Only three mice of the 29 in the group infected with cystitis-derived isolates had more bacteria in the bladder than in the kidney. While this finding may represent an adaptation of the cystitis isolates to have increased adherence to bladder epithelium, the kidney still appeared to have the larger bacterial burden in the majority of mice.

**Transurethral inoculation of 0852 results in inflammation in the kidneys but not the bladder.** Kidneys and bladders were subjected to histological analysis at various time points following inoculation with **E. faecalis** 0852. Hematoxylin and eosin staining revealed that there was little difference between infected and uninfected bladders at any of the time points examined (6 h, 24 h, 2 days, and 4 days; 24-h time point shown in Fig. 3). In contrast, **E. coli** induces cystitis, as measured by the disruption of the bladder epithelium, marked edema, and recruitment of numerous neutrophils at the same time point (4, 14, 19).

Kidney sections from **E. faecalis**-infected mice, however, showed an inflammatory infiltrate in the renal pelvis. The inflammation was most consistently evident at 24 h after inoculation but was also seen at other time points as small, isolated collections of inflammatory cells. The level of inflammation at 24 h in the kidney was variable, sometimes appearing quite extensive, with inflammatory cells lining the entire pelvis (Fig. 3b and 3e), but small, localized patches of inflammation were present.
also observed along the pelvis (as in Fig. 3c and 3f). The cellular infiltrate in the kidneys is primarily monocytic, as determined by histologic features. This is in contrast to the mostly neutrophilic infiltrate seen in *E. coli* pyelonephritis (4). The results of the histologic analysis of the bladder and kidney demonstrate that *E. faecalis* can consistently cause pathology in the kidney but not the bladder, further confirming the tropism of *E. faecalis* for the mouse kidney.

Immunohistochemistry was also utilized to demonstrate the presence of enterococcal antigen within the infected kidney. Using the rabbit Lancefield group D antibody, we showed staining in association with areas of inflammation at 24 h postinfection, demonstrating that the inflammatory cells were recruited in response to the presence of *E. faecalis* rather than damage to the kidney parenchyma by the inoculation procedure (Fig. 4).

**Inflammatory markers induced by uropathogenic *E. coli are not upregulated in the bladder in response to *E. faecalis*. The**
interaction of uropathogenic *E. coli* with the bladder epithelium results in the expression of a variety of proteins involved in epithelial renewal and immune function (20). Among these, the proinflammatory marker Mip-2, the mouse orthologue to human interleukin-8, and Socs-3, a modulator of cytokine induction, were highly upregulated. We investigated whether *E. faecalis* induced a similar response in the urinary tracts of mice.

*E. faecalis* 0852- or sham-infected mouse bladders and kidneys were harvested 6 h or 24 h after inoculation, and RNA was collected in order to quantify the relative levels of Mip-2 and Socs-3 by quantitative reverse transcription-PCR. The relative induction of Mip-2 and Socs-3 after infection with NU14 (a clinical uropathogenic *E. coli* isolate) or *E. faecalis* 0852 was investigated (Fig. 5); 24 h after infection with either *E. coli* NU14 or *E. faecalis* 0852, NU14 induced Mip-2 and Socs-3 176-fold and 15-fold, respectively, over *E. faecalis* in the bladder. In the kidney, Mip-2 and Socs-3 were also more strongly induced by *E. coli* NU14 but not to the same magnitude (24-
Kidney infections as a model system to study pathogenesis of OG1X in the kidneys of mice.

Bladders and kidneys of mice equally well when infected sep- 

cision. As shown in Fig. 7a, OG1X and BP78 colonized the faecalis, the clinical strain BP78 and the laboratory strain OG1X were inoculated either separately or in a mixed suspension. As shown in Fig. 7a, OG1X and BP78 colonized the bladders and kidneys of mice equally well when infected sep-

FIG. 5. Quantitative reverse transcription-PCR of inflammatory markers of uropathogenic E. coli cystitis strain Mip-2 and Socs-3. The bladders and kidneys of uropathogenic E. coli- or E. faecalis-infected mice were collected 6 h or 24 h after infection and the RNA was harvested. Quantitative reverse transcription-PCR was performed for Mip-2 and Socs-3 expression levels, and data are normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. Results are shown as induction relative to a PBS-inoculated control. Induction by uropathogenic E. coli strain NU14 (black bars) was consistently higher than that by E. faecalis 0852 (white bars) in both the bladder and kidneys.

ievably. As shown in Fig. 7a, OG1X and BP78 colonized the bladders and kidneys of mice equally well when infected sep-

Two recent studies used an ascending model of urinary tract infections similar to that used for uropathogenic E. coli to investigate the function of the Esp and aggregation substance adhesins in the pathogenesis of E. faecalis urinary tract infection. A role for aggregation substance has not yet been elucidated, whereas mutations in esp reduced colonization of the bladder. An interesting caveat in these studies was that approximately 30% of the mice had sterile bladders even when challenged with wild-type E. faecalis (12, 26). These results highlight the need to elucidate the host-pathogen interactions required to maintain robust urinary tract infections with enterococci. Interestingly, both studies noted significant colonization of the kidney tissue despite using a volume of inoculum designed to deliver bacteria only to the bladder.

In the present study, we discovered that E. faecalis has tropism for the kidneys in female C57BL/6J mice. This finding was made using a model where the inoculation volume of E. faecalis was increased in order to facilitate direct delivery of

FIG. 6. Effect of TLR2 on the recovery of E. faecalis 0852 from the kidneys of mice. C57BL/6J TLR2-deficient mice 36 to 48 weeks old (w.o.) or C57BL/6J wild-type mice 12 weeks old or 52 weeks old were inoculated with E. faecalis 0852 and sacrificed 24 h after infection, and the CFU per pair of kidneys were enumerated. TLR2-deficient mice respond comparably to wild-type mice to E. faecalis kidney infection.

arately, as determined 48 h after infection. However, in the mixed-infection model, BP78 showed a competitive advantage over OG1X of approximately 10-fold in the kidney, but there was no discernible difference in the bladder (Fig. 7b).

DISCUSSION

Enterococci are usually perceived as commensal bacteria that coexist with their host under most circumstances as part of the normal flora. While not regarded as particularly virulent organisms, enterococci can be significant agents of urinary tract infections in the hospital setting, where patients’ defenses can be compromised by catheterization, immune deficiencies, or both. Given that E. faecalis is an opportunistic pathogen, animal model systems to study ascending urinary tract infections have been difficult to develop but will be essential for understanding the molecular basis of enterococcal disease.

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umbrella cells lining the lumen of the bladder have specialized properties, including an ability to assemble uroplakins on their surface. Uroplakins form impermeable plaques that coat the luminal surface of the bladder (15). Cultured epithelial cells are not terminally differentiated, do not assemble uroplakins into plaques, and lack other distinguishing features of superficial umbrella cells, such as their large surface area and pentagonal shape (27). Thus, tropism is best studied in an animal model such as the one described here.

_E. faecalis_-mediated urinary tract infection appears to occur via a strikingly different mechanism than _E. coli_-mediated urinary tract infection. In a typical _E. coli_ urinary tract infection, the bacteria invade the superficial umbrella cells and replicate to high levels, forming intracellular biofilms, a process that induces a TLR4-mediated cytokine response that recruits neutrophils to the site of infection (1, 22). In enterococcal urinary tract infection, there is little to no inflammation in the bladder. Furthermore, neutrophils represented only a minority of the inflammatory cells targeted to the site of infection, which typically is in the kidney. We found that TLR2, which is hypothesized to play a similar role in the innate response to gram-positives as TLR4 does for gram-negative pathogens (28, 29), does not play a substantial role in the innate response to _E. faecalis_ in the urinary tract. Several observations reinforce this hypothesis. The presence or absence of TLR2 had no significant impact on the recovery of _E. faecalis_ 24 h after infection. A luciferase reporter cell line expressing TLR2 was not stimulated by enterococci (32). Finally, there is substantial evidence that the host uses TLR2-independent pathways to stimulate inflammatory cascades in response to gram-positive infection (2, 5, 17). The recent discovery of TLR11, a Toll-like receptor that specifically recognizes uropathogens, suggested an additional innate mechanism used by the host to respond to enterococci (32).

All six _E. faecalis_ strains tested in this study demonstrated the same tropism for the kidney, suggesting that most _E. faecalis_ strains contain the core set of virulence factors necessary to cause disease in the kidney. When inoculated separately, OG1X, a commonly used laboratory strain of _E. faecalis_ that lacks the recently described enterococcal pathogenicity island (25) and plasmid elements, and BP78, a clinical pyelonephritis isolate, persisted at similar levels over a 48-h period. This implies that OG1X has the essential molecular elements necessary to persist in the kidney. However, in a mixed-competition experiment, BP78 had a survival advantage of approximately 10-fold, suggesting that uropathogenic _E. faecalis_ isolates have additional factors that lead to increased fitness in the urinary tract. Thus, mixed-infection experiments proved to be the most sensitive readout of additional virulence factors that enhance persistence in the kidney.

This work raises numerous questions related to human disease. The ability of enterococci to cause disease in the kidney is well established. The diagnosis of upper versus lower urinary tract infection caused by enterococci, as with gram-negative bacilli, is generally based on signs and symptoms. Thus, the presence of fever and flank pain, with or without lower tract symptoms of dysuria, frequency, and urgency suggest upper tract infection (13). Lower tract symptoms without upper tract symptoms are assumed to represent bladder infection, although it has been reported that about 30% of women with
symptoms of bladder infection have silent upper tract infection (13). It will be important to determine whether the findings described in this work can be extended to humans. If enterococci have a strong tropism for the kidney, it is possible that many or most episodes of enterococcal cystitis are due to seeding from an upper tract infection. It is also possible that asymptomatic bacteriuria with enterococci is often localized to the upper tract rather than the bladder. The observation that enterococci fail to elicit a strong induction of inflammatory cytokines in our murine model is consistent with this hypothesis. If enterococcal urinary tract infections are often associated with upper tract infection, there may be treatment implications, since upper tract infection is usually treated for a longer duration than bladder infections (6).

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