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# Enterococcal Biofilm Formation and Virulence in an Optimized Murine Model of Foreign Body-Associated Urinary Tract Infections<sup>∇†</sup>

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**Catheter-associated urinary tract infections (CAUTIs) constitute the majority of nosocomial UTIs and pose significant clinical challenges. Enterococcal species are among the predominant causative agents of CAUTIs. However, very little is known about the pathophysiology of *Enterococcus*-mediated UTIs. We optimized a murine model of foreign body-associated UTI in order to mimic conditions of indwelling catheters in patients. In this model, the presence of a foreign body elicits major histological changes and induces the expression of several proinflammatory cytokines in the bladder. In addition, in contrast to naïve mice, infection of catheter-implanted mice with *Enterococcus faecalis* induced the specific expression of interleukin 1 $\beta$  (IL-1 $\beta$ ) and macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) in the bladder. These responses resulted in a favorable niche for the development of persistent *E. faecalis* infections in the murine bladders and kidneys. Furthermore, biofilm formation on the catheter implant *in vivo* correlated with persistent infections. However, the enterococcal autolytic factors GelE and Atn (also known as AtlA), which are important in biofilm formation *in vitro*, are dispensable *in vivo*. In contrast, the housekeeping sortase A (SrtA) is critical for biofilm formation and virulence in CAUTIs. Overall, this murine model represents a significant advance in the understanding of CAUTIs and underscores the importance of urinary catheterization during *E. faecalis* uropathogenesis. This model is also a valuable tool for the identification of virulence determinants that can serve as potential antimicrobial targets for the treatment of enterococcal infections.**

Catheter-associated urinary tract infections (CAUTIs) are the most common complications resulting from the use of indwelling urinary catheters (25, 70). CAUTIs account for 40% of all nosocomial infections (48) with more than one million cases diagnosed annually in hospitals and nursing homes in the United States, generating approximately \$600 million in medical expenditures every year (26, 69, 70). The pathophysiology of CAUTIs results from the disruption of the normal mechanical and antimicrobial defenses of the bladder and the injuries ensuing from urinary catheterization, which render the bladder environment vulnerable to microbial adhesion, multiplication, and dissemination within the urinary tract (15, 47, 49, 51, 78). Furthermore, indwelling urinary catheters provide an additional surface for microbial attachment and biofilm formation, which is a major component of the pathophysiology of CAUTIs and other chronic device-associated persistent infections (4, 6, 12, 14, 37, 46, 68). Once formed, biofilms provide a favorable milieu for microbial survival within the host as the organisms are shielded from the host immune response, as well as antibiotics and antimicrobial agents (36, 67, 81), leading to chronic or recurrent infections that are difficult to treat. If

untreated, CAUTIs can lead to more severe diseases such as acute pyelonephritis, bacteremia, urosepsis, and in some cases, death (38, 77). The high incidence of CAUTIs and their medical and economic challenges underscore the need for a better understanding of CAUTI pathogenesis.

While community-acquired UTIs are most commonly due to uropathogenic *Escherichia coli* (UPEC), UPEC represent only 50% of bacterial isolates derived from patients with CAUTIs (34). The *Enterococcus* species, especially *Enterococcus faecalis* and *Enterococcus faecium*, account for 15% to 30% of CAUTIs (38) and are now considered the third leading cause of hospital-acquired UTIs (20, 68). The ability of many enterococcal isolates to produce biofilms (41, 60, 73) and the increasing microbial resistance to antibiotics, including vancomycin, pose significant challenges for the treatment of enterococcal infections (11, 80).

Several rodent models have been developed to study *E. faecalis* pathogenesis in the urinary tract in single and mixed infections with other Gram-negative pathogens (21, 29, 43, 61, 75). These models involve the transurethral inoculation of bacterial suspensions into the bladders of healthy animals or streptozocin-induced diabetic animals (56) and have been shown to be primarily useful for the study of *E. faecalis*-mediated pyelonephritis. The experimental conditions defined by these models are inadequate for the investigation of persistent enterococcal CAUTI, since the bacteria are readily cleared from the bladder and fail to establish chronic cystitis (29, 56, 61). Nonetheless, data obtained from these models have implicated some enterococcal factors in UTI pathogenesis, including the

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TABLE 1. Bacterial strains used in this study

Bacterial strain	Relevant antibiotic resistance <sup>a</sup>	Description	Reference
<i>E. faecalis</i> strains			
OG1RF (wild type)	Rif <sup>25</sup> Fus <sup>25</sup>	Parental OG1RF strain	16
OG1RFΔ <i>atn</i>	Rif <sup>25</sup> Fus <sup>25</sup>	Strain OG1RF with an in-frame deletion of <i>atn</i>	16
OG1RFΔ <i>srtA</i>	Rif <sup>25</sup> Fus <sup>25</sup>	OG1RF with an in-frame deletion of <i>srtA</i>	16
OG1RFΔ <i>srtA</i> C	Rif <sup>25</sup> Fus <sup>25</sup> Kan <sup>500</sup>	Complemented strain OG1RFΔ <i>srtA</i> expressing a wild-type copy of <i>srtA</i> from the <i>rofA</i> promoter on pABG5	16
OG1RFΔ <i>gelE</i>	Rif <sup>25</sup> Fus <sup>25</sup>	OG1RF with an in-frame deletion of <i>gelE</i>	70
<i>E. coli</i> UTI89		Uropathogenic <i>E. coli</i> cystitis isolate	44

<sup>a</sup> The superscript numbers indicate the concentrations (in μg/ml) of antibiotics to which the strains are resistant. Rif, rifampin; Fus, fusidic acid; Kan, kanamycin.

enterococcal surface protein Esp (61), the pilus-associated sortase C (SrtC) (30), and the endocarditis and biofilm-associated pilus (Ebp) (65). However, well-characterized adhesins and biofilm determinants often associated with enterococcal UTI isolates, like aggregation substance (AS) and the housekeeping sortase A (SrtA) (17, 27, 33), were reported to be dispensable for virulence in the urinary tract (27, 30). Since these conclusions are drawn from models where persistent infections cannot be established, it is imperative to reexamine the existing paradigm in an animal model that better mimics the transition of *E. faecalis* from a commensal organism to a virulent pathogen in the urinary tract.

In this study, we optimized a rodent model of foreign body-associated UTI, developed in rats by Kurosaka et al. (35) and adapted for mice by Kadurugamuwa et al. (28), to investigate the pathophysiology of *E. faecalis*-mediated CAUTIs. We report that the presence of silicone catheter implants causes major physiological changes in the bladder, which becomes predisposing to *E. faecalis* biofilm formation, and is associated with persistent enterococcal cystitis and pyelonephritis. We further demonstrate that biofilm formation on the silicone implants and the development of successful enterococcal UTI are independent of autolytic factors GelE and Atn, also known as AtlA (13), but requires the presence of the housekeeping sortase A (SrtA). Overall, this optimized murine model is well suited to identify host and enterococcal factors critical for pathogenesis in the urinary tract which will provide a better understanding of the mechanisms underlying the pathophysiology of CAUTIs.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** All strains used in this study and their characteristics are listed in Table 1. Unless otherwise specified, *Enterococcus faecalis* experiments were performed using an overnight culture grown in brain heart infusion broth (BHI) (Becton Dickinson, Franklin Lakes, NJ) from a single colony of *E. faecalis* grown on BHI agar plates supplemented with the appropriate antibiotics. Liquid cultures were grown statically at 37°C for 18 h. For *E. coli*, bacteria from a single colony from a Luria-Bertani (LB) (Becton Dickinson) agar plate were inoculated into LB broth, grown statically for 24 h, and subcultured for another 24 h before use.

**Mouse strain.** Six- to 7-week-old female wild-type C57BL/6Ncr mice purchased from the National Cancer Institute (NCI) were used in this study. Experiments were performed following 1 week of adaptation in the animal facility. All studies and procedures were approved by the Animal Studies Committee at the Washington University School of Medicine.

**Murine implantation procedure.** The experimental model for foreign body-associated UTI (28, 35) was adapted for the study of *E. faecalis* with the following modifications. Polyethylene tubing (PE10 tubing with an outside diameter of 0.6

mm and inner diameter of 0.28 mm; Becton Dickinson, MD) and platinum-cured silicone tubing (SIL025 tubing with an outside diameter of 0.64 mm and inside diameter of 0.30 mm; Braintree Scientific, Inc., MA) were sterilized with 70% ethanol and air dried. A 7-mm-long segment of PE10 tubing was fitted onto a 30.5-gauge sterile needle (Becton Dickinson). A 4- to 5-mm-long straight segment of SIL025 tubing was then placed on top of the PE10 segment. The assembly was then UV sterilized for 24 h. Seven- to 8-week-old female wild-type C57BL/6Ncr mice were anesthetized with inhaled isoflurane and placed on their backs. The periurethral area was sterilized with 100% ethanol and 10% povidone-iodine solution. The needle with both PE10 and SIL025 tubing was gently inserted in the urethral opening. The long segment (PE10) was delicately advanced with tweezers until the shorter segment (SIL025) was released into the bladder. Subsequently, the needle with the PE10 tubing was removed, leaving the silicone implant in the murine bladder. The recovery of straight silicone implants was greater than 95% at 24 h, and it was approximately 75% 7 days following implantation (see Fig. S1 in the supplemental material).

**Bacterial infection and CFU determination.** Bacterial cells were collected from overnight cultures by centrifugation at  $8,000 \times g$  for 5 min and resuspended in  $1 \times$  phosphate-buffered saline ( $1 \times$  PBS) to an approximate optical density at 600 nm of 1 corresponding to approximately  $7 \times 10^8$  CFU/ml. Seven- to eight-week-old female C57BL/6Ncr mice with or without a silicone implant were anesthetized by inhalation of isoflurane and infected by transurethral catheterization as previously described (23, 44, 59) with 50-μl inocula of PBS or bacterial suspensions ( $\sim 2 \times 10^7$  to  $3.5 \times 10^7$  CFU) immediately following implantation. To quantify bacteria present on implants and in mouse organs, mice were sacrificed at desired time points by cervical dislocation after anesthesia inhalation, and the bladders and kidneys were aseptically harvested. Subsequently, the silicone implant was retrieved from the bladder when present, placed in  $1 \times$  PBS, sonicated for 10 min, and then vortexed at maximum speed for 3 min. The bladder and both kidneys from each mouse were homogenized in  $1 \times$  PBS. For bacterial enumeration on implants and tissues, samples were serially diluted and plated on BHI supplemented with the appropriate antibiotics or LB plates. CFU were enumerated after 24 h of incubation at 37°C. Experiments were performed at least twice with five mice per strain per condition.

**Cytokine profiling.** Bladder homogenates from animals with or without implants and mock infected or infected with *E. faecalis* for 24 h were microcentrifuged at 14,000 rpm for 5 min, and supernatants were frozen at  $-80^\circ\text{C}$  until the time of the assay. Assays were carried out according to the manufacturer's protocols using the Bio-Plex Pro mouse cytokine 23-plex assay kit from Bio-Rad Laboratories (Hercules, CA).

**Histopathology and immunohistochemistry.** For histological analyses, the bladders were fixed in methacarn (60% methanol, 30% chloroform, and 10% glacial acetic acid) for 1 to 2 h and dehydrated in 70% ethanol overnight at 4°C. They were then embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E) for light microscopy. For immunofluorescence examination, unstained bladder sections were deparaffinized with xylene (twice, for 10 min each time), rinsed in 100% isopropanol (three times, for 5 min each time), and washed with running deionized water for 5 min. This step was followed by a  $1 \times$  PBS wash (three times for 5 min). The tissue sections were then blocked statically in 5% fetal bovine serum in PBS (FBS-BB) for 30 min at room temperature. Primary antibody raised in rabbit against *Streptococcus* group D antigen (Lee Laboratories) and primary antibody raised in goat against mouse uroplakin III (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) were added to FBS-BB at 1:1,000 and 1:500 dilutions, respectively, and incubated at room temperature for 1 h. After a 5-min PBS wash (three times, for 5 min each time), tissue sections

were incubated with Alexa Fluor 555-labeled donkey anti-rabbit antibody and Alexa Fluor 488-labeled donkey anti-goat antibody, each diluted 1:1,000 in FBS-BB for 30 min at room temperature. After the tissues were washed with PBS (once for 5 min), they were counterstained with TOPRO-3 (Molecular Probes, Eugene, OR) at 1:1,000 dilution in 1× PBS (second wash for 5 min) to reveal nuclear morphology. After a third PBS wash for 5 min, immunostained tissues were mounted using Prolong Gold Antifade (Molecular Probes) and visualized by immunofluorescence microscopy (IFM). IFM was performed with an LSM 510 Meta laser scanning confocal microscope (Carl Zeiss, Thornwood, NY) using a 63× oil immersion objective. Images were acquired using the LSM Image Examiner software (Carl Zeiss, Thornwood, NY).

**Scanning electron microscopy.** Retrieved silicone implants were fixed in 4% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M cacodylate and prepared for scanning electron microscopy (Hitachi Ltd., Tokyo, Japan).

**Statistical methods.** Comparisons among groups were conducted by the Mann-Whitney U test using GraphPad Prism (GraphPad software, version 4.03). Values below the limit of detection (LOD) (40 or 20 CFU for organs and 80 CFU for implants) were assigned the appropriate LOD value for statistical analyses. All tests were two tailed, and a *P* value of less than 0.05 was considered significant. Colonization and infection were defined as organs/implants with bacterial titers above the limit of detection at 24 hours postinfection (hpi).

## RESULTS

***E. faecalis* establishes persistent infections in the bladders and kidneys in an optimized murine model of CAUTI.** In order to study the pathophysiology of *E. faecalis*-mediated CAUTI, we introduced 4- to 5-mm-long pieces of silicone tubing into the bladders of C57BL/6Ncr female mice. Silicone is a common material used in Foley catheters in humans (18, 57). Following catheter implantation, mice were immediately infected with  $2 \times 10^7$  to  $3.5 \times 10^7$  CFU of *E. faecalis* strain OG1RF by transurethral catheterization. In mock infection studies with phosphate-buffered saline (PBS), no bacteria were recovered from implants or organs (data not shown). Twenty-four hours after animals with implants were infected with strain OG1RF, bacteria were recovered from the implants at a median value of  $9.76 \times 10^5$  CFU (Fig. 1A). Bacterial titers on the abiotic surfaces increased significantly over the course of the infection (7 days) to a median value of  $8.64 \times 10^6$  CFU/ml. In animals with implants, strain OG1RF reaches a median titer of  $3.2 \times 10^6$  CFU/ml 24 hpi in the bladders, which is significantly higher ( $P = 0.0011$ ) than bacterial titers ( $2.7 \times 10^3$  CFU/ml) recovered from the bladders of animals with no implants (Fig. 1B). Similarly, bacterial titers from the kidneys increased significantly from 24 hpi to 7 days postinfection (dpi) in implanted animals (Fig. 1C). *E. faecalis* OG1RF was readily cleared from the bladders and the kidneys within 48 hpi in the absence of implants (Fig. 1B and C). In addition, bacterial titers were significantly lower in tissues derived from animals that lost their implants during the course of infection than in tissues from animals where the implants were retrieved (Fig. 2), with the exception of bladder titers at 48 hpi ( $P = 0.0737$ ) and kidneys titers at 72 hpi ( $P = 0.130$ ). These data suggest that the presence of the implants contributes to increased bacterial colonization and persistence in the urinary tract. In contrast, bladder implants did not enhance the colonization of uropathogenic *E. coli* UTI89 strain, a well-studied isolate from a human with cystitis (45) and with strong tropism to the bladder (see Fig. S2 in the supplemental material).

**Implanting tubing into the bladder induces histological and immunological changes in the murine bladder.** Urinary catheterization causes disruption of the protective layers of the uroepithelium and causes histological changes (16, 51). In an-

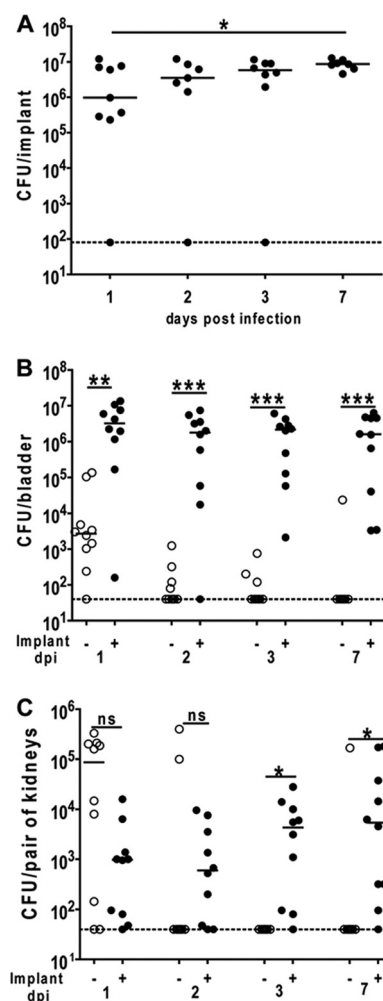


FIG. 1. *E. faecalis* establishes persistent colonization of silicone implants, bladders, and kidneys in a murine model of CAUTI. (A) Bacterial titers in logarithmic scale (log scale) from silicone implants retrieved from female C57BL/6Ncr mice infected with OG1RF for 24, 48, and 72 hpi (1, 2, and 3 dpi) and 7 dpi. (B and C) Graphs represent bacterial titers from homogenized bladders (B) and kidneys (C) from animals without implants (–) (○) or animals with implants (+) (●) infected with strain OG1RF for 1, 2, 3, or 7 dpi. In panels A to C, the horizontal broken line represents the limit of detection for viable bacteria. Each symbol represents the value for an individual mouse from two independent experiments with five mice for each condition. The horizontal bar represents the median value for each group of mice. Values that are significantly different by the Mann-Whitney U test are indicated as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ . Values that are not significantly different (ns) by the Mann-Whitney U test are indicated.

imals with implants, both infected and mock-infected bladders were edematous, distended, and sometimes hemorrhagic throughout the experimental period (data not shown). Compared to mock-infected bladders without implants (Fig. 3A and D), mock-infected bladders with implants revealed mucosal hyperplasia, submucosal edema with polymorphonuclear leukocyte (PMN) infiltration into the lamina propria and epithelium, as well as urothelial sloughing at 24 hpi (Fig. 3B and E). The histology of the uroepithelium following infection of implanted bladders with *E. faecalis* OG1RF was similar to that of



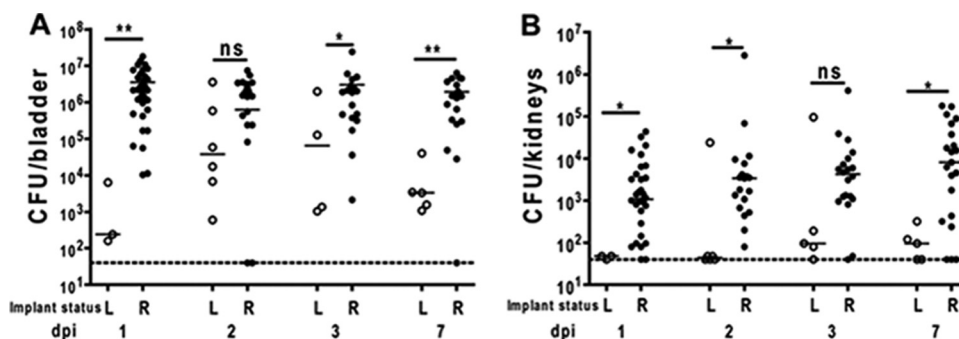


FIG. 2. Implant loss from murine bladders correlates with less severe infection of the bladder and kidney. Graphs represent bacterial titers in log scale recovered from the bladders (A) and kidneys (B) of animals that have lost the implants (L) (○) or retained the implants (R) (●) at the indicated time of sacrifice. The experiment was done at least three times with five mice in each experiment. The horizontal broken line depicts the limit of detection. The horizontal bar represents the median value for each group of mice. Values that are significantly different by the Mann-Whitney U test are indicated as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0005$ . Values that are not significantly different ( $P > 0.05$ ) (ns) by the Mann-Whitney U test are indicated.

mock-infected implanted animals (Fig. 3C and F). In contrast, nonimplanted bladders infected with strain OG1RF showed no major histological changes and appeared similar to the bladders of mock-infected nonimplanted animals (data not shown) as has been shown previously with other *E. faecalis* strains (29).

Damage to the uroepithelium in bladders with implants could also be observed by immunofluorescence microscopy using anti-urolakin III antibody, which stains the luminal surface of the terminally differentiated superficial umbrella cells of the bladder. Comparison of bladder sections at 24 hpi showed discontinuity of urolakin staining of bladders with implants (Fig. 3H) versus continuous epithelial staining of bladders without implants (Fig. 3G), indicating the loss of superficial umbrella cells in the former. Using an antibody against the Lancefield streptococcal group D antigen to stain the enterococcal organisms in histology sections, we found bacterial staining in the bladder lumen of infected animals with implants. The bacteria were found in association with the surface of the urolakin-positive and urolakin-negative uroepithelium and occasionally found in the submucosal areas (Fig. 3I; see Fig. S3 in the supplemental material). In contrast, very few bacteria were observed in the bladders of nonimplanted animals 24 hpi (data not shown). No labeling was observed in mock-infected animals with or without implants (Fig. 3G and H), reaffirming the specificity of the antibody staining. These data suggest that implantation with a foreign body elicits major architectural changes in the bladder associated with activation of the host immune response, which predisposes the bladder to enterococcal colonization.

**Implantation leads to the upregulation of specific inflammatory markers in the murine bladder.** To investigate how the host immune status is altered in the presence of a foreign body, we analyzed the levels of expression of 23 cytokines from whole-bladder homogenates from mice with and without implants after the mice were inoculated with PBS or *E. faecalis* OG1RF. Of the 23 cytokines examined, only interleukin 6 (IL-6), granulocyte colony-stimulating factor (G-CSF), and keratinocyte-derived cytokine (KC) were consistently expressed in implanted animals at least 2-fold above the levels of non-implanted mock-infected (PBS-inoculated) animals at 24 hpi (Fig. 4). Conversely, granulocyte-monocyte colony-stimulating

factor (GM-CSF) and macrophage inflammatory protein 1 $\alpha$  (MIP-1 $\alpha$ ) showed at least twofold decreased expression in implanted animals compared to nonimplanted animals 24 hpi (Fig. 4). *E. faecalis* infection of the implanted animals slightly enhanced the expression of IL-6, IL-12(p40), G-CSF, and KC and specifically induced the expression of IL-1 $\beta$  and MIP-1 $\alpha$  in implanted animals compared to implanted mock-infected animal controls (Fig. 4). *E. faecalis* OG1RF infection of nonimplanted animals resulted in no significant changes in the expression of the cytokines tested compared to mock infection (see Fig. S4 in the supplemental material). These findings indicate that the silicone implant elicits a specific inflammatory response in the bladder that is enhanced upon bacterial challenge with strain OG1RF.

***E. faecalis* produces biofilm on the surface of the silicone implant in vivo.** To determine whether bacteria recovered from silicone implants are within biofilms, we examined 15 implants retrieved 72 hpi from murine bladders from three independent experiments by scanning electron microscopy (SEM). The implants from mock-infected mice were coated on the outside and in the lumen with host factors found in the urine as previously reported (58; data not shown). A similar appearing film was also observed on 12 implants retrieved from animals infected with *E. faecalis* OG1RF. In approximately 70% of the silicone implants retrieved from infected animals, bacteria were readily observed in clumps both on the outer surface (Fig. 5A and B) or filling the entire lumen (Fig. 5C to F). The coccoid microorganisms were observed embedded in what appeared to be an extracellular matrix (Fig. 5E and F). The reason for the absence of biofilms from the remaining implants is not certain, but it could be due to sample processing prior to visualization, since those animals also exhibited significantly enhanced infection. These findings indicate that enterococcal biofilms are produced *in vivo* on indwelling silicone material during the course of the infection.

***E. faecalis* biofilm formation and virulence in the urinary tract occur independently of autolytic factors but require sortase A.** *E. faecalis* biofilm production on abiotic surfaces *in vitro* has been shown to depend in part on the autolytic factors GelE and Atn as well as the housekeeping sortase SrtA (17, 33, 71). Thus, we created in-frame deletions in each of these genes and

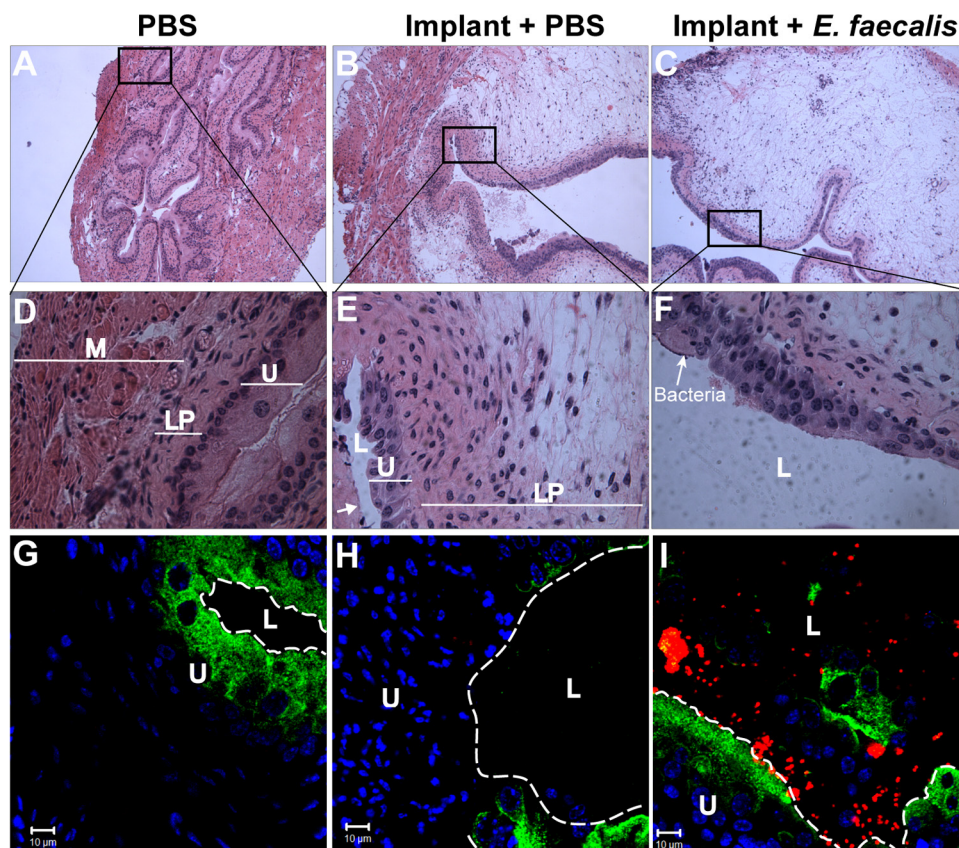


FIG. 3. Implantation-mediated histological changes are associated with enterococcal colonization of the murine bladder. (A to F) H&E staining of bladder sections derived from animals without implants inoculated with PBS (A and D), animals with implants inoculated with PBS (B and E), or animals with implants infected with *E. faecalis* OG1RF (C and F) 24 hpi observed with a light microscope at magnifications of  $\times 40$  (A to C) and  $\times 63$  (D to F). Areas in black boxes on panels A, B, and C are magnified in panels D, E, and F, respectively. Severe edema, hyperplasia, urothelial sloughing, and immune cell infiltration characterize bladders of implanted animals with or without bacterial challenge. The white arrow in panel E indicates damage to the uroepithelium. Abbreviations: U, uroepithelium; LP, lamina propria; L, lumen; M, muscularis. (G to I) Representative laser scanning confocal microscopic (CLSM) images of bladder sections from PBS-treated nonimplanted, PBS-treated implanted, and OG1RF-infected implanted mice 24 hpi immunolabeled with goat anti-mouse uroplakin III (green), rabbit anti-Lancefield group D antigen (red), and counterstained with TOPRO-3 nuclear dye (blue) reveal alterations to the uroepithelium of implanted bladders and the presence of bacteria in the bladder lumen and on the uroepithelial surface. The white broken line separates the lumen (L) from the uroepithelium surface (U). Bars = 10  $\mu$ m.

tested their virulence in the CAUTI model. When introduced into animals with implants, *E. faecalis* OG1RF $\Delta$ gelE colonized the implants, bladders, and kidneys as well as or better (significant increase in implant titers;  $P = 0.0279$ ) than the parental strain OG1RF 24 hpi (Fig. 6A to C). The absence of the major autolysin Atn did not alter the colonization of implants or urinary tissues relative to the wild-type strain (Fig. 6D to F). Moreover, strain OG1RF $\Delta$ atn was able to persist in the bladder and kidney tissues (see Fig. S5 in the supplemental material) and on the implants (Fig. 6D) as well as the wild-type OG1RF strain did (Fig. 6D). However, infection of implanted mice with a previously characterized *srtA*-deficient OG1RF strain (OG1RF $\Delta$ srtA) caused a  $10^5$  decrease in bacterial titers recovered from the implants (Fig. 7A) and an approximately  $10^3$  decrease in CFU recovered from both bladders and kidneys compared to infection with the wild-type strain (Fig. 7B and C). Infecting mice with implants with OG1RF $\Delta$ srtAc, a complemented strain ectopically expressing a wild-type copy of *srtA*, partially rescued wild-type colonization levels of implants and organs (Fig. 7A to C). The lack of full complementation is

not entirely understood, but it may be due to plasmid loss during infection based on higher colony counts in the absence of antibiotic. Similar findings were observed at 48 hpi (data not shown). Electron microscopic examination of the implants revealed very few OG1RF $\Delta$ srtA cells present on the surface or in the lumen of retrieved implants 72 hpi in contrast to the complemented, *atn*-deficient, and wild-type strains that heavily colonized the implants (Fig. 7D). Comparable results were obtained when biofilms were grown *in vitro* in human urine under fluid flow (data not shown). These results indicate that unlike GelE and Atn, SrtA or more specifically, a SrtA-dependent substrate(s) is critical for biofilm production and virulence in the urinary tracts of animals with implants.

## DISCUSSION

Enterococci, normally commensal bacteria of the human oral cavity and gastrointestinal tract, have become important opportunistic pathogens in hospital settings. While not historically regarded as an important uropathogen in uncomplicated



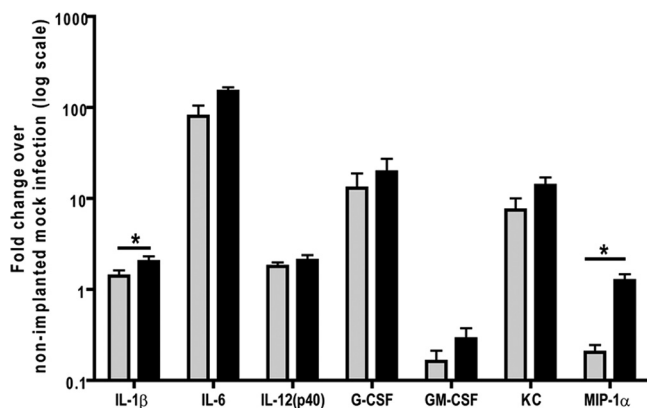


FIG. 4. Implantation and *E. faecalis* infection elicit the production of specific cytokines in the bladder. Graphs represent cytokine expression in the whole bladder of animals with implants inoculated with PBS or *E. faecalis* OG1RF at 24 hpi. Cytokines reported are expressed with at least twofold difference relative to mock-infected animals with no implants. The presence of implants induces the specific expression of IL-6, G-CSF, and KC and causes the downregulation of GM-CSF and MIP-1α. In contrast, *E. faecalis* elicits the specific expression of IL-1β, IL-12(p40), and MIP-1α at 24 hpi. Data represent a representative experiment performed four times with five mice for each condition in the experiment. Values are shown as the means plus standard errors of the means (error bars). Values that are significantly different ( $P < 0.05$  by the Mann-Whitney U test) are indicated (\*).

community-acquired cystitis, *E. faecalis* is now considered a major agent of hospital-acquired UTIs where catheterization and indwelling medical devices can negatively impact patients' defenses against pathogens (20). However, the lack of a robust animal model of enterococcal UTI has impeded the study of this pathogen within the urinary tract. In previously established models of ascending UTIs, *E. faecalis* fails to establish persistent bladder colonization and primarily displays tropism to the kidneys (21, 29, 43, 56, 61, 75). The absence of persistent infections in these murine models reflects the profile of *E. faecalis* in community-acquired UTIs where this bacterium represents less than 5% of clinical isolates (55). As an opportunistic pathogen, *E. faecalis* requires changes in bladder homeostasis following urinary catheterization in order to achieve successful infection of the urinary tract.

In this study, we investigated the pathophysiology of *E. faecalis*-mediated CAUTIs using a murine model of foreign body-associated UTI that was modified from previous studies (28, 35). Our results indicate that the presence of silicone tubing in the murine bladder elicits histopathological and immunological changes that are similar to the effects seen in patients with spinal cord injuries and postsurgical patients with indwelling urinary catheters (8, 16, 51, 76). Furthermore, we show that *E. faecalis* forms biofilms on the silicone implants and is able to persist at high titers in the bladders and kidneys of animals with implants.

The striking difference in bladder colonization and virulence from previous models is a direct consequence of implantation, which provides an abiotic surface for biofilm production. Biofilms have been implicated in the establishment of chronic infections, including CAUTIs (6, 74). Our data indicate that biofilm production on the surface of the implants is associated with persistent enterococcal cystitis. Bacteria within biofilms

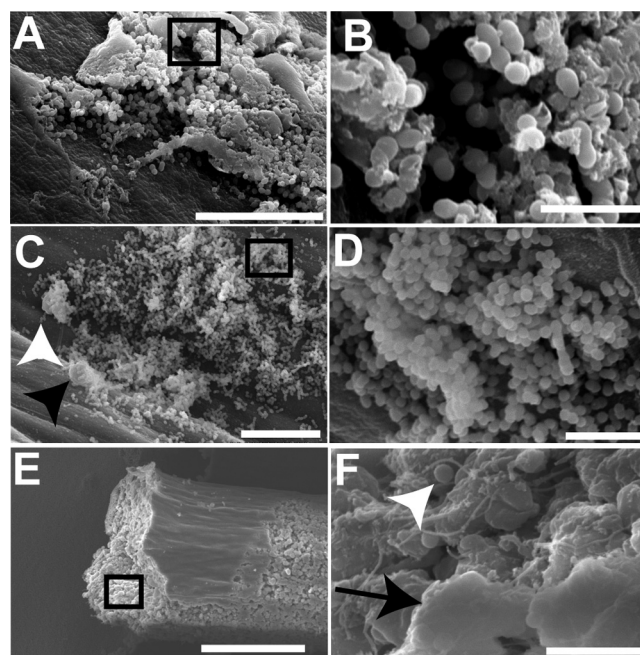


FIG. 5. *E. faecalis* produces biofilms on the surfaces of silicone implants *in vivo*. Representative scanning electron micrographs of silicone implants retrieved from murine bladders infected with *E. faecalis* OG1RF at 72 hpi showing bacterial biofilms on the outer surface (A and B) and filling the lumen (C and F) of the silicone implant. Bacteria (white arrowhead) are often associated with host cells, indicated by the black arrowhead in panel C. Both bacteria and host immune cells can also be found embedded in a thick extracellular material, indicated by the black arrow, in the lumen of the silicone implant 72 hpi (E and F). Areas in black boxes on panels A, C, and E are magnified in panels B, D, and F, respectively. Bars, 20 μm (A and C), 5 μm (B, D, and F), and 100 μm (E).

may continuously seed the bladder, preventing effective clearance by the host and leading to persistent cystitis.

Atn (17, 71) and GeIE (52, 71, 72) have been shown to be important in *E. faecalis* biofilm formation. Atn has been shown to be a mediator of autolysis and DNA release, facilitating attachment to abiotic surfaces, thus promoting biofilm formation *in vitro* (17, 33, 41, 53). The extracellular protease GeIE (1, 39) and the serine protease (SprE) mediate Atn-dependent DNA release during enterococcal biofilm formation (71, 72). GeIE has been associated with virulence in several models of infection (19, 22, 52, 62, 66). When introduced into mice with silicone implants to assess their contribution to CAUTI, both *atn*- and *gelE*-deficient mutants were as virulent as the wild-type strain. Thus, under these conditions, urinary tract colonization and biofilm formation on the implant did not require Atn or GeIE. While these factors are important determinants of DNA-dependent biofilm production *in vitro*, the ability of *E. faecalis* to colonize the implants and promote infection in the absence of both Atn and GeIE argues that extracellular DNA may not be an important contributor of the extracellular matrix of enterococcal biofilms *in vivo* in the urinary tract or that its release may occur in an Atn/GeIE-independent manner. Consistent with this finding was the observation that gelatinase was not expressed in 71/163 clinical isolates (42) of *E. faecalis*, and the presence of *gelE* did not correlate with gelatinase expres-



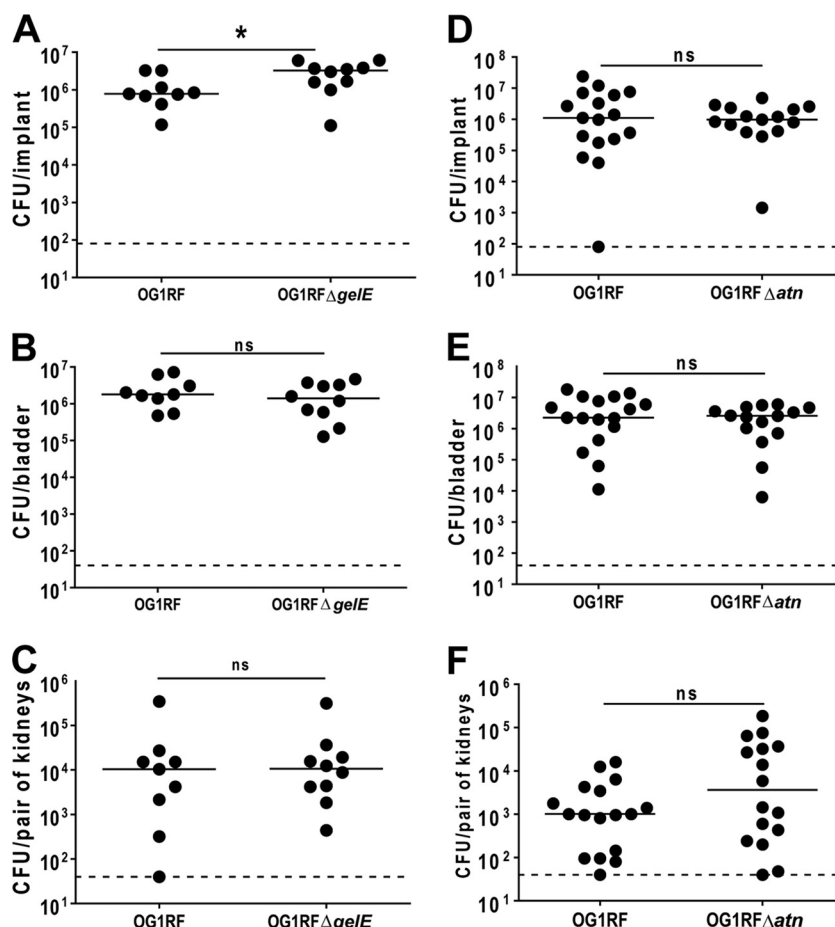


FIG. 6. Autolytic factors are not required for *E. faecalis* virulence in the urinary tract. (A to F) Graphs show the numbers of CFU of *E. faecalis* OG1RF and isogenic in-frame *gelE*- and *atn*-deficient mutants recovered from retrieved implants (A and D), bladders (B and E), and kidneys (C and F) from animals 24 hpi following infection with either strain. Horizontal dashed lines represent the limits of detection for viable bacteria. Each symbol represents the value for an individual mouse, and each experiment was done twice with four or five mice for each strain. Only data from animals with implants at the time of sacrifice are represented. The horizontal bars indicate the median values for groups. Values that are statistically significantly different ( $P < 0.05$ ) by the Mann-Whitney U test are indicated (\*). Values that are not statistically significantly different ( $P > 0.05$ ) by the Mann-Whitney U test are indicated (ns).

sion (7) or their ability to form biofilms *in vitro* (42). However, a recent study by Arciola et al. (1) found a correlation with a high level of expression of GelE and the ability to form biofilms in enterococci from orthopedic implant infections, indicating that the factors utilized by enterococci in forming biofilms may depend on the substrate and/or site of infection. Identification of the major components of the extracellular matrix of *in vivo* enterococcal biofilms will lead to a better understanding of CAUTIs and will be of valuable interest for designing therapeutics that promote the prevention and disruption of these structures.

Sortases have also been implicated in enterococcal biofilm production (17, 30, 33). Recent studies have shown that SrtA, a transpeptidase that anchors LPXTG-containing surface proteins to the cell walls of Gram-positive bacteria (40), plays a major role during *in vitro* biofilm development (17, 30, 33). Deletion of *srtA* in *E. faecalis* prevented adherence and subsequent biofilm growth on abiotic surfaces (17). Although Kemp et al. (30) reported that disruption of *srtA* did not significantly affect virulence in a murine model of ascending UTI, our data

reveal that SrtA is critical in the establishment of CAUTI. The *srtA*-deficient mutant is unable to colonize the implants *in vivo* and is significantly attenuated in CAUTI. These findings further underscore the importance of biofilm production during *E. faecalis* pathogenesis and strongly suggest that SrtA-dependent substrates may be required for attachment to abiotic surfaces and/or to damaged uroepithelium *in vivo* during the establishment of infection. It will be of interest to identify SrtA-dependent substrates that are critical in CAUTI. For example, surface proteins known as MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) have been identified in *E. faecium* and *E. faecalis* (50, 63, 64). MSCRAMMs, some of which are putative SrtA-dependent substrates, can bind to host cells via interactions with host extracellular matrix proteins such as collagen and fibrinogen. The disruption of urothelial surfaces, as the result of catheterization, may expose factors that SrtA-dependent substrates, such as MSCRAMMs, can recognize to promote attachment and colonization.

Additionally, catheter-mediated abrasions to bladder epithe-

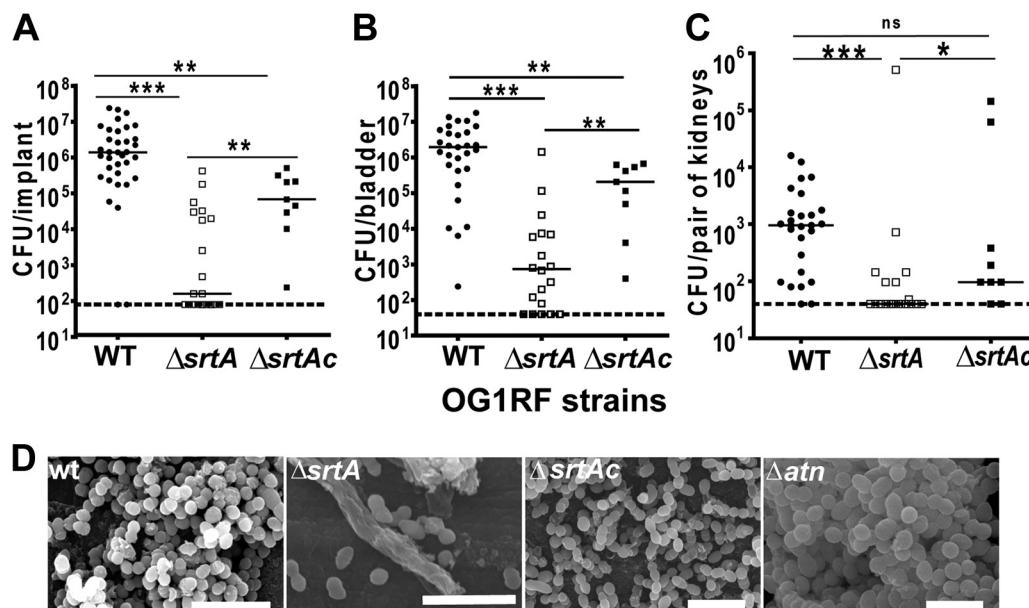


FIG. 7. Sortase A is critical for implant colonization and virulence in the urinary tract. (A to C) Graphs show bacterial titers from implants (A), homogenized bladders (B), and kidneys (C) from mice with implants infected for 24 h with *E. faecalis* OG1RF (wild type [WT]) (●), OG1RF $\Delta srtA$  (□), or OG1RF $\Delta srtAc$  (■). Thick horizontal broken lines represent the limits of detection for viable bacteria. Each symbol is the value for an individual mouse, and each experiment was done at least twice with five mice for each strain. Only data from animals with implants at the time of sacrifice are represented. The horizontal bar indicates the median value for each group of mice. Values that are statistically significantly different by the Mann-Whitney U test are indicated as follows: \*,  $P < 0.05$ ; \*\*,  $P < 0.005$ ; \*\*\*,  $P < 0.0001$ . Values that are not statistically significantly different ( $P > 0.05$ ) by the Mann-Whitney U test are indicated (ns). (D) Representative scanning electron micrographs of silicone implants retrieved from murine bladders 72 hpi with *E. faecalis* OG1RF (WT), OG1RF $\Delta srtA$ , OG1RF $\Delta srtAc$ , and OG1RF $\Delta atn$  showing massive bacterial colonization for the wild type and *atn*-deficient mutant, but very few OG1RF $\Delta srtA$  cells are present in the lumen of the implants. Bars, 5  $\mu$ m for strains OG1RF (WT), OG1RF $\Delta srtA$ , and OG1RF $\Delta srtAc$  and 2.5  $\mu$ m for strain OG1RF $\Delta atn$ .

lial cells have been previously shown to induce the production of lactate dehydrogenase and small amounts of IL-6 and IL-8 (2). In this report, we also show that the presence of these implants elicits the expression of specific inflammatory markers in the bladders, including granulocyte colony-stimulating factor (G-CSF), keratinocyte-derived cytokine (KC) or CXCL1 (chemokine [C-X-C motif] ligand 1), and IL-6, whereas GM-CSF and MIP-1 $\alpha$  (chemokine [C-C motif] ligand 3 [CCL3]) expression was decreased. All the above cytokines are produced by macrophages, neutrophils, and epithelial cells and are important in the recruitment of PMNs and other immune cells to sites of infection (3, 5, 31, 32, 54). The presence of *E. faecalis* in animals with implants specifically elicits the production of IL-1 $\beta$  and MIP-1 $\alpha$  during CAUTI. These cytokines, produced by neutrophils and other immune cells, are important mediators of the inflammatory response to microbial infections (5, 9). In particular, IL-1 $\beta$  was shown to be released during UPEC-mediated acute cystitis (24). The CAUTI model will be useful in unraveling the balance and interplay between inflammation and bacterial colonization and persistence.

The efficient bacterial clearance and the significantly lower titers recovered from the urinary tracts of mice without implants and/or with the loss of implants further corroborate the importance of a foreign body in bacterial persistence. Furthermore, our results indicate that the presence of a silicone implant in the bladder allows *E. faecalis* to successfully ascend to the kidneys and establish persistent infection in animals with implants. The onset of acute pyelonephritis has also been re-

ported in postmortem studies of the elderly with indwelling catheters at the time of death (79). In the clinical setting, the removal of infected indwelling urinary catheters is one of the most effective methods used to resolve bacteriuria and CAUTIs (74, 78). However, removing the indwelling medical device, even combined with long course of antibiotic treatment (74), may not be sufficient for complete resolution of the infection, especially with the rise in antibiotic resistance observed in nosocomial settings (11, 20). In addition, implant removal upon infection in other device-associated infections like prosthetic valve endocarditis is not in itself efficacious (10, 12) and thus may not be a suitable therapeutic approach in all instances. Understanding the pathogenesis of CAUTIs may lead to new and better ways to treat and prevent these diseases.

The optimized murine model of foreign body-associated UTI presented here is highly relevant for the investigation of the mechanisms underlying *Enterococcus*-mediated CAUTIs, since it couples biofilm production to enterococcal virulence during *E. faecalis* uropathogenesis. This murine model is a valuable and robust tool in the identification and characterization of novel biofilm determinants and bacterial virulence factors and host responses pertinent to the pathogenesis of *E. faecalis* in the urinary tract and the mechanisms underlying enterococcal ascension to the kidneys. Importantly, our optimized model will serve as an ideal platform for testing potential therapeutics against enterococcal infections and antibiofilm compounds.

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