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**Subinhibitory antibiotic therapy alters recurrent urinary tract infection pathogenesis through modulation of bacterial virulence and host immunity**

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The capacity of subinhibitory levels of antibiotics to modulate bacterial virulence has recently been brought to light, raising concerns over the appropriateness of low-dose therapies, including antibiotic prophylaxis for recurrent urinary tract infection management. However, the mechanisms involved and their relevance in influencing pathogenesis have not been investigated. We characterized the ability of antibiotics to modulate virulence in the uropathogens *Staphylococcus saprophyticus* and *Escherichia coli*. Several antibiotics were able to induce the expression of adhesins critical to urothelial colonization, resulting in increased biofilm formation, colonization of murine bladders and kidneys, and promotion of intracellular niche formation. Mice receiving subinhibitory ciprofloxacin treatment were also more susceptible to severe infections and frequent recurrences. A ciprofloxacin prophylaxis model revealed this strategy to be ineffective in reducing recurrences and worsened infection by creating larger intracellular reservoirs at higher frequencies. Our study indicates that certain agents used for antibiotic prophylaxis have the potential to complicate infections.

**IMPORTANCE** Antibiotics are the mainstay treatment for bacterial infections; however, evidence is emerging that argues these agents may have off-target effects if sublethal concentrations are present. Most studies have focused on changes occurring in vitro, leaving questions regarding the clinical relevance in vivo. We utilized a murine urinary tract infection model to explore the potential impact of low-dose antibiotics on pathogenesis. Using this model, we showed that subinhibitory antibiotics prime uropathogens for adherence and invasion of murine urothelial tissues. These changes in initial colonization promoted the establishment of chronic infection. Furthermore, treatment of chronically infected mice with subtherapeutic ciprofloxacin served to exacerbate infection. A part of these changes was thought to be due to suppression of mucosal immunity, as demonstrated through reductions in cytokine secretion and migration of leukocytes into the urinary tract. This work identifies novel risk factors associated with antibiotic therapy when dosing strategies fall below subtherapeutic levels.
inappropriate antibiotic treatment may be a contributing factor. The objective of our study was to explore this phenomenon in the context of a subinhibitory dosing regimen, namely, antibiotic prophylactic management for recurrent urinary tract infection (rUTI).

UTIs are very common, accounting for 10.5 million outpatient and emergency room visits during 2007 in the United States alone (22). They are a significant cause of morbidity in women throughout their life span and in infant boys and older men, with serious sequelae, including treatment failure, frequent recurrences, pyelonephritis with sepsis, and renal damage in young children (23, 24); however, upwards of 44% of women will experience a recurrence, with ~10% of these caused by a relapse of the original index episode strain (25). Such recurrent or persistent infections are generally managed by using long-term, low-dose prophylactic regimens. In general, UTIs can be readily treated with short-course antibiotic therapy (~3 to 7 days). However, following therapy, recurrent or persistent infections may develop that are often managed using long-term, low-dose prophylactic regimens. While prophylaxis is usually effective in reducing symptoms during its application (26), it does not alter the long-term risk of recurrence, as infection rates return to pretreatment levels following therapy (27) and pathogens become more resistant to future treatment (28). Thus, it is critical to understand the effectiveness of antimicrobial prophylaxis in managing recurrent infections and whether the benefits of such treatments outweigh the risks of promoting resistance and potential increased virulence.

Uropathogenic *Escherichia coli* (UPEC) and *Staphylococcus saprophyticus* collectively cause ~95% of all uncomplicated UTIs (23, 29) and are often associated with recurrent and chronic disease necessitating long-term prophylaxis management. Type 1 fimbriae and uro-adherence factors (Uafs) are critical in establishing UPEC and *S. saprophyticus* UTIs, respectively (30, 31). In particular, type 1 fimbriae are important in promoting the invasion of superficial umbrella cells on the luminal surface of the bladder epithelium and formation of intracellular bacterial communities, whereby UPEC rapidly replicates within the cytoplasm during acute infection to establish a foothold in the bladder (32–37). While the fate of intracellular *S. saprophyticus* is unknown, intracellular UPEC proliferation results in the formation of large, multicellular aggregates, dubbed intracellular bacterial communities (IBCs) (32). Within this niche, uropathogens are able to subvert host immune cells and rapidly build up in numbers, thus facilitating the progression toward chronic infection in mice. In addition, UPEC has been shown to be able to survive antibiotic therapy by forming dormant quiescent intracellular reservoirs (QIRs) that can serve as seeds for recurrent infection (38–41).

We hypothesized that subinhibitory antibiotic-dependent virulence modulation and immune suppression in *vitro* would translate to actual changes in disease outcome *in vivo*. Thus, we investigated various classes of subinhibitory antibiotics for their capacity to influence bacterial virulence traits in two uropathogenic clinical isolates, *S. saprophyticus* 15305 and *E. coli* UTI89. We also attempted to characterize the signaling networks involved with these responses in UPEC by using expression analyses. Changes in virulence and pathogenesis were assessed *in vivo* by inoculating ciprofloxacin-primed mice (who had received 1/4 the minimal inhibitory dose of ciprofloxacin) with *S. saprophyticus* or UPEC and determining acute and chronic infection characteristics, including bacterial persistence within the urinary tract and establishment of intracellular bacterial reservoirs. The effect of inadequate (with 1/50 the empirical therapeutic dose) ciprofloxacin dosing in chronically infected or resolved mice was also investigated, in addition to characterizing immune responses in this host. Lastly, the efficacy of prophylaxis was assessed for its ability to prevent persistent UTIs and clear UPEC from host urine and tissues in addition to the intracellular niche.

**RESULTS**

**Subinhibitory antibiotics induce adhesin expression and biofilm formation in uropathogens.** Challenge of both *S. saprophyticus* and UPEC with subinhibitory ciprofloxacin, ampicillin, or gentamicin resulted in denser biofilm production (Fig. 1A). In addition, enhanced planktonic aggregation was observed with both organisms (see Fig. S1 in the supplemental material). For UPEC, motility was suppressed (Fig. 1B), as determined in a soft agar motility assay, and type 1 fimbriation was increased, as determined via a guinea pig erythrocyte hemagglutination assay (Fig. 1C). Similarly, subinhibitory antibiotics increased sheep erythrocyte hemagglutination with *S. saprophyticus*, suggesting increased exposure or the presence of adhesin uro-adherence factor A (UafA) (Fig. 1D). Since capsular polysaccharide can interfere with UafA-ligand interactions, hemagglutination was also evaluated in an acapsular strain to discern whether our observations were due to increased UafA expression or decreased capsular polysaccharide abundance. We found that subinhibitory antibiotics also increased hemagglutination in the acapsular strain, indicative of increased UafA surface exposure (Fig. 1D).

**Antibiotic-induced adhesin expression is SOS dependent in UPEC.** RNA sequencing analysis confirmed significant induction of both a *fimH*-like adhesin, *fmlD* (4.84-fold), and the suspected uropathogenic adhesin/invasin *hek* (2.47-fold) (Table 1). Interestingly, changes in type 1 fimbrial transcripts were not observed via RNA sequencing, nor were any changes observed in the proportion of the population that had the invertible *fim* promoter in the ON orientation (data not shown). As the SOS-responsive elements *lexA* and *recA* were also highly induced with treatment (4.08- and 3.37-fold, respectively), we assessed their contributions to adhesin expression and biofilm formation by using SOS-deficient *E. coli* UTI89 strains. These strains demonstrated a modest suppression in biofilm formation (Fig. 2A) and type 1 fimbria-dependent hemagglutination (Fig. 2B) and did not respond to subinhibitory ciprofloxacin or gentamicin treatment (Fig. 2C and D). Expression analysis using quantitative PCR (qPCR) confirmed *fmiD* and *hek* induction with ciprofloxacin challenge in wild-type UPEC; however, transcription was not altered in the *recA*-deficient strain (Fig. 2E). Ampicillin increased biofilm mass in all strains tested, but only at concentrations near the MIC (Fig. 2C and D).

**Subinhibitory antibiotics prime uropathogens for urothelial colonization.** Ciprofloxacin priming prior to infection resulted in higher overall bladder and kidney titers for both *S. saprophyticus* 15305 and *E. coli* UTI89 at 24 h postinfection (p.i.) (Fig. 3A). Almost all murine kidneys inoculated with primed uropathogens lacked signs of bacterial clearance. Ciprofloxacin priming also resulted in increased risk of severe UTI, manifesting as persistent bacteriuria, high bacterial bladder titer, and chronic inflammation, an outcome referred to as chronic cystitis, at 14 days p.i. (Fig. 3B). Chronic cystitis represents unchecked luminal bacterial replication and is defined histologically by urothelial hyperplasia
and submucosal lymphoid aggregates, a histological pattern similar to that seen in humans experiencing chronic UTI (33, 42, 43). The bladder colonization threshold for assessing the risk of chronic cystitis is the presence of *E. coli* UTI89 loads of $\geq 10^4$ CFU/ml at 14 days p.i. or later (such data do not exist for *S. saprophyticus*) (44). Considering this threshold, priming resulted in 100% of mice presenting with a high risk of chronic *E. coli* UTI89 cystitis. Although there are no established infection thresh-

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### TABLE 1 Subinhibitory ciprofloxacin induces urothelium adhesin gene expression* 

| Gene  | Fold change (log$_2$) | Median effect size | Functional annotation                             |
|-------|----------------------|-------------------|--------------------------------------------------|
| fmlD  | 2.275                | 1.891             | Fimbrial, FimH-like protein, mannose binding      |
| Hek   | 1.304                | 3.475             | Hek adhesin/virulence factor                     |
| lexA  | 2.03                 | 5.635             | Repressor of LexA                                 |
| recA  | 1.751                | 3.75              | DNA repair, SOS induction                         |

* Significant changes in differentially expressed transcripts of interest in *E. coli* UTI89 after 4-h exposure to 1/4 MIC ciprofloxacin.
olds for *S. saprophyticus* 15305, it is noteworthy that 100% of mice inoculated with untreated pathogen completely resolved their infection, while 50% maintained low, but measurable, bladder titers with ciprofloxacin priming. Among primed *S. saprophyticus* 15305-treated mice, 100% maintained at least some level of infectious kidney titer, while in control groups only 60% exhibited the same result.

**Antibiotic-driven colonization promotes tissue invasion and IBC formation.** Changes in IBC formation during the early stages of infection were assessed for *E. coli* UTI89 by staining for *lacZ* expression using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). This technique indicated significantly more IBCs in ciprofloxacin-primed groups, indicative of more numerous urothelial invasion events (Fig. 4A). IBC morphology was further explored using a green fluorescent protein (GFP)-expressing *E. coli* UTI89 strain and confocal microscopy. Appearance of untreated *E. coli* UTI89 was unremarkable, as IBCs demonstrated characteristic tight, globular clustering (Fig. 4B). However, examination of ciprofloxacin-primed *E. coli* UTI89 revealed larger, diffuse IBCs with atypical morphology (Fig. 4C). Early filamentation was also noted in several of the IBCs belonging to the ciprofloxacin-treated *E. coli* UTI89 group (Fig. 4D). Volumetric analysis of the IBCs confirmed that ciprofloxacin priming significantly increased overall size 2.0-fold (Fig. 4E). The ability for ciprofloxacin to induce filamentation was investigated further, as this process might contribute to observed early IBC evacuation, which has been shown to lead to spreading of the infection to neighboring cells (45). Cultures of *E. coli* UTI89 were treated with subinhibitory ciprofloxacin and imaged using transmission electron microscopy (TEM). Control *E. coli* UTI89 cells were unremarkable, appearing to be ~1 to 2 μm in length (see Fig. S2A in the supplemental material). Most cells in the ciprofloxacin treatment
A group appeared similar in nature; however, ~10% of the overall population presented with the observed filamenting phenotype, sometimes increasing to ~10 μm in length (see Fig. S2B).

**Subtherapeutic ciprofloxacin dosing induces recurrence and severe infection in mice.** The effects of oral subinhibitory ciprofloxacin therapy were considered in two groups of mice that had been previously inoculated with *E. coli* UTI89 and had either (i) naturally resolved their infection (described as a <10^4 CFU/ml bacterial urine load >30 days p.i.) or (ii) maintained chronic urine titers (described as >10^4 CFU/ml for >30 days p.i.). Prior to the initiation of oral antibiotic therapy, the urine titers for all mice were measured over 3 days in the absence of antibiotics to determine the baseline bacterial burden. Supplementation of ciprofloxacin into drinking water at 1/50 the empirical therapeutic concentration caused 80% of previously resolved mice to develop significant infection (>10^4 CFU/ml) after 3 to 6 days (Fig. 5A). Although 1/25 the empirical therapeutic dose had no effect, 1/10 the empirical therapeutic dose resulted in retraction of bacterial urine loads to the limit of detection, suggesting that the observed antibiotic-dependent increases were not due to the appearance of resistant mutants (data not shown). In chronically infected mice, supplementation with 1/50 the empirical therapeutic dose for 3 days significantly increased mean UPEC urine titers by 26-fold (Fig. 5B). Again, treatment with 1/10 the therapeutic dose was sufficient to decrease bacteriuria to the limit of detection (data not shown).

**Ciprofloxacin modulates human and murine mucosal immune responses.** Luminex profiling demonstrated induction of the cytokines interleukin-6 (IL-6) and IL-8 in T24 bladder cells infected with *S. saprophyticus* 15305, but this induction was suppressed to below baseline levels with the application of subinhibitory ciprofloxacin (Fig. 6A). IL-8 secretion in human 5637 bladder cells treated with *S. saprophyticus* 15305 or lipopolysaccharide (LPS) was also reduced by ciprofloxacin, slightly by gentamicin, but not affected by ampicillin, as determined in an enzyme-linked immunosorbent assay (ELISA) (Fig. 6B). Thus, we hypothesized that these observed immunomodulatory effects suppress the secretion of proinflammatory mediators and decrease immune cell infiltrate *in vivo*. Urine sediment polymorphonuclear leukocytes (PMNs) taken 3.5 h after UPEC inoculation was reduced in mice receiving subtherapeutic ciprofloxacin (Fig. 6C). Ciprofloxacin-dependent modulation of cytokine secretion was assessed using a Luminex bead-based multiplex assay of extracted bladder tissue homogenates. A total of seven urinary-relevant cytokines were assessed and included IL-1β, IL-6, keratinocyte chemoattractant (KC, also called IL-8), granulocyte colony-stimulating factor (G-CSF), IL-17, IL-10, and tumor necrosis factor alpha (TNF-α) (46). All produced reliable profiles with the exception of TNF-α (data not shown). Changes in IL-17 secretion were unremarkable in either the control or ciprofloxacin treatment groups (Fig. 6D). Similarly, KC was not significantly impacted by ciprofloxacin, although there was a trend of reduced secretion when antibiotic was
present (Fig. 6D). The proinflammatory mediators IL-1β and IL-6 were suppressed, while secretion of the anti-inflammatory cytokine IL-10 was increased (Fig. 6D). In contrast, the proinflammatory cytokine G-CSF was significantly increased in the presence of ciprofloxacin and *E. coli* UTI89 infection (Fig. 6D).

**Prophylactic intervention is ineffective in curtailing recurrence and increases intracellular UPEC burden.** The possibility that low-dose prophylaxis promotes virulence and enhances pathogenesis was investigated in the C57BL/6J model of rUTI, where frequent recurrent infections originate from QIRs (33, 39, 47). Following inoculation with nonprimed UPEC, changes in bacterial urine load were assessed at least every 2 days to track the course of infection using a novel prophylaxis therapy model. Establishment of infection after 24 h and response to trimethoprim-sulfamethoxazole (“co-trimoxazole”) intervention were observed (see Fig. S3 in the supplemental material). The following 7-day antibiotic-free period revealed dynamic changes in *E. coli* UTI89 urine titers, with frequent recurrences observed. Initiation of prophylactic therapy did not appear to improve the frequency of infection resolution over the 7-day treatment period, as 2/20 mice in this group presented with clinically significant bacteriuria while 3/20 in the untreated group did so at the study’s end (Fig. 7A). Both bladder and kidneys demonstrated greater bacterial loads in the prophylaxis group, although this difference was not significant (Fig. 7B). However, comparison of bladder titers revealed prophylaxis significantly increased the presence of low-level bladder CFU

![Image](https://example.com/image.png)

**FIG 4** Antibiotic priming increases the invasive capacity of UPEC. (A) *E. coli* UTI89 IBC quantity at 6 h p.i. of C3H/HeN bladders. (B and C) Representative confocal images of control (B) and antibiotic-pretreated (C) *E. coli* UTI89 IBCs in C3H/HeN mouse bladders 6 h p.i. (red, GFP/UTI89; blue, Syto9/nuclei; green, WGA/cell). (D) Early pathogen evacuation from an IBC via filamentation (arrows) in a ciprofloxacin-pretreated sample. (E) Volumetric analysis of confocal images is depicted for both control and antibiotic-pretreated IBCs. Means from at least two independent experiments are shown. Significance was determined using a one-way ANOVA, Dunnett’s multiple comparison test, or the Mann-Whitney test. *, *P* < 0.05; **, *P* < 0.01.

![Image](https://example.com/image.png)

**FIG 5** Subtherapeutic ciprofloxacin increases bacterial burden and recurrence frequency. (A) The fraction of resolved C3H/HeN mice presenting with clinically significant (>10⁴ CFU/ml) bacterial urine titers following subtherapeutic ciprofloxacin dosing over time (n = 10 mice). The antibiotic dosing period is indicated on the upper x axis as fractions of the therapeutic dose. (B) Urine titers of chronically infected mice receiving either no antibiotic or subtherapeutic ciprofloxacin for 3 days. Means from at least two independent experiments are shown. Significance was determined using a log rank test (panel A; *P* = 0.0131) or Mann-Whitney test (panel B; *P* = 0.0142).
as determined via ex vivo gentamicin protection; this was likely indicative of more numerous QIRs (Fig. 7C).

**DISCUSSION**

**Subinhibitory antibiotics promote SOS-dependent virulence factor expression.** We have demonstrated that subinhibitory antibiotics enhance adherence and biofilm production in uropathogens. However, unlike previous studies, which focused on single pathogen-antibiotic combinations, we found that several agents targeting distinct cellular processes were capable of similar effects in unrelated pathogens and that these changes translated to enhanced virulence in vivo. Thus, in addition to increasing the risk of resistant infection, subinhibitory antibiotic therapy may also pose an additional risk of promoting the expression of virulence factors. We further characterized the nature of adherence and determined that adhesins critical to uropathogenesis were induced in both *S. saprophyticus* 15305 and *E. coli* UTI89 by subinhibitory concentrations of antibiotics. RNA sequencing of subinhibitory ciprofloxacin-treated UPEC revealed that *fmlD* and *hek* induction coincided with SOS activation. Further phenotypic and qPCR investigations of strains deficient in SOS revealed the importance of this stress response system to adhesin gene expression and enhanced adherence, as deficient strains no longer responded to treatment. This finding provides a general mechanism by which diverse classes of antibiotics might enhance virulence via a system essentially ubiquitous across bacterial species. Investigations further characterizing the poorly established role of the SOS in virulence regulation are warranted (48).

**Subinhibitory ciprofloxacin priming enhances pathogenesis in a murine model of UTI.** Previous results have demonstrated that antibiotics are capable of priming organisms for downstream colonization, primarily through the upregulation of adhesins (49–51). In mice, ciprofloxacin treatment prior to infection shifted the equilibrium toward infection persistence in bladders and kidneys. This effect was most dramatic in the kidneys, where both uropathogens showed no signs of clearance. The results imply that infection severity could be worsened by the application of ciprofloxacin prophylactically, a practice that is widely conducted (52–54).
107 CFU of accounts for this (55–57). C3H/HeN mice inoculated with kidneys. The absence of UafA ligand in the mouse bladder likely the C3H/HeN model of chronic cystitis. Ciprofloxacin also altered but showed a similar infective capacity as 
primed enhanced IBC-forming capacity observed in ciprofloxacin-
are associated with an increased risk of chronic cystitis (36). The
induced hemagglutination (61, 62). Increased numbers of IBCs
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ification or capsule downregulation and enhanced FimH expo-
ment is likely paramount. Although changes in type 1 fimbria
sponse to ciprofloxacin and subsequent chronic infection devel-
urothelial cells. Type 1 fimbriae are known to be critical in inter-
flections is likely dependent on its ability to induce invasion of
host tissues before exfoliation can occur, promoting the establish-
morphology of IBCs, resulting in a dispersed phenotype com-
pared to that of untreated controls. The characteristics of these
IBCs, such as increased numbers of bacterial filaments, are remi-
niscent of those observed during later stages of infection (63),
suggesting that ciprofloxacin priming alters IBC development, re-
sulting in an earlier dispersal and fluxing of the bacteria from the
IBC biomass and their spread to neighboring cells. Ciprofloxacin-
triggered filamentation could thus hasten the spread to distal tis-
ze before exfoliation can occur, promoting the establishment of
scient intracellular reservoirs in the bladder that can serve as
seeds for recurrent UTI (39). Thus, ciprofloxacin-induced changes in 
E. coli UTI89 might contribute to increased urothelial
adherence, immune evasion, and invasion potential (45, 64).
These processes might further help uropathogens subvert aspects
of early host immunity by rapidly gaining access to the intracellu-
lar environment, and at a higher frequency. Combined, altera-
tions in these pathogenic mechanisms are likely responsible for
driving the dynamics of the host-pathogen equilibrium in favor of
bacterial persistence.

**Subtherapeutic ciprofloxacin augments infection severity and recurrence risk in chronically infected and resolved mice.**

Two experimental groups were evaluated for the influence of sub-
therapeutic ciprofloxacin treatment on (i) the worsening of prog-
osis in chronically infected mice and (ii) the predisposition of
previously inoculated but resolved mice for clinically significant
recurrences. In both cases, subtherapeutic ciprofloxacin was asso-
associated with increased E. coli UTI89 urine titers. The effects of sub-
therapeutic ciprofloxacin dosing on modulating cytokine expres-
sion could, in part, account for increased pathogen urine load in
chronically infected mice (discussed below). However, the ability
of ciprofloxacin to trigger clinically significant recurrences in re-
solved mice was unexpected and might occur through reemergence of E. coli from QIRs. As fluoroquinolones inhibit phosho-
diesterase activity in mammalian cells (65, 66), a resulting
accumulation of cyclic AMP has been proposed to trigger exocy-
tosis of intracellular UPEC (67). Although chronically infected
mice were housed in separate cages from resolved mice, cross-
infection between mice experiencing recurrences within the same
cage cannot be ruled out. Interestingly, there were mice that never
experienced recurrences with ciprofloxacin therapy, indicating

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**FIG 7** Low-dose ciprofloxacin therapy increases intracellular UPEC reservoirs. (A and B) E. coli UTI89 titers in C57BL/6 mouse urine (A) or bladders and kidneys (B) following a 1-week prophylactic treatment regimen with either ciprofloxacin or vehicle. (C) Gentamicin protection assays indicated the presence of intracellular E. coli reservoirs. The dotted line indicates the limit of detection. Means from at least two independent experiments are shown. Significance was determined using an unpaired, two-tailed t test. ns, not significant; *, P = 0.0148.

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54). *S. saprophyticus* 15305 poorly colonized the bladders of mice but showed a similar infective capacity as *E. coli* UTI89 in the kidneys. The absence of UafA ligand in the mouse bladder likely accounts for this (55–57). C3H/HeN mice inoculated with 10⁷ CFU of *E. coli* UTI89 and then presenting with >10⁴ CFU/ml at 14 days p.i. are known to be at very high risk of chronic UTI (44). The increased titers observed following ciprofloxacin priming suggested that these organisms were better equipped to colonize the host than were the untreated counterparts. The host-pathogen interactions during the first 24 h p.i. are thought to play a significant role in infection establishment or resolution, suggesting that increased titers at this time have some predictive value in determining the severity of UTI (33). The data obtained at 14 days p.i. with *E. coli* UTI89 validated this finding, as ciprofloxacin priming prior to inoculation was sufficient to increase the frequency of chronic cystitis to 100%, compared to 60% in untreated groups. While no such data exist that describe the infection thresholds of *S. saprophyticus* 15305, it is noteworthy that 100% of mice resolved infection when inoculated with untreated *S. saprophyticus* 15305, while 50% of ciprofloxacin-primed inoculated mice maintained low-grade bladder colonization. Although lifetime infection carriage needs to be evaluated in this model, the data are highly suggestive that organism virulence is enhanced by the presence of subinhibitory antibiotic levels, such that there is a direct impact on persistence within host tissues.

The capacity for ciprofloxacin to promote chronic UPEC infec-
tions is likely dependent on its ability to induce invasion of
urothelial cells. Type 1 fimbriae are known to be critical in inter-
inalization and IBC formation (58–60); their expression in re-
sponse to ciprofloxacin and subsequent chronic infection develop-
ment is likely paramount. Although changes in type 1 fimbria
transcript abundance was not observed, posttranscriptional mod-
ification or capsule downregulation and enhanced FimH expo-
sure might contribute to the observed changes in antibiotic-
induced hemagglutination (61, 62). Increased numbers of IBCs
are associated with an increased risk of chronic cystitis (36). The
enhanced IBC-forming capacity observed in ciprofloxacin-
primed *E. coli* UTI89 indicated that establishment of an intracel-
ular niche is an important step in developing chronic infection in
the C3H/HeN model of chronic cystitis. Ciprofloxacin also altered

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urinary clearance and providing an argument against cross-infection. These findings are the first to directly associate sub-therapeutic antibiotic therapy with increased infection risk in vivo. If translatable to the clinic, noncompliance with antibiotic prescriptions, or failure to follow strict intraoperative redosing guidelines, could contribute to infection complications (19, 20).

Ciprofloxacin modulates aspects of host immunity. Ciprofloxacin was found to have an immunomodulatory effect in urothelial tissues, which might account for the changes in bacterial urine titers observed in mice during subtherapeutic treatment. The effect was noted in both human bladder epithelial cell lines in addition to murine bladder tissues extracted following infection. Low doses of ciprofloxacin were sufficient to depress the release of IL-6 and IL-8 in T24 and 5637 bladder cells, suggesting that residual levels left over following therapy might predispose to infection later on. IL-6 and IL-8 are both important proinflammatory mediators for host cell immunity during UTIs, and suppression of either cytokine has important implications on neutrophil chemotaxis to sites of infection (68–70). This was confirmed in mice, where there were significantly depressed PMN infiltrates in urine sediments when animals were treated with subtherapeutic ciprofloxacin. In addition to IL-6 and IL-8 suppression in vivo, the proinflammatory mediator IL-1β, produced by activated macrophages, is an important early response element to UPEC infection, and this cytokine was suppressed with ciprofloxacin treatment. Alternatively, the anti-inflammatory cytokine IL-10 was upregulated with infection and ciprofloxacin presence. IL-10 is important in urothelial protection during UTI as it limits macrophage activation (71–73). Interestingly, ciprofloxacin dramatically increased the expression of G-CSF. This cytokine increases neutrophil migration from the bone marrow, and UPEC is known to trigger its upregulation during UTIs (46). G-CSF presence could facilitate its upregulation during UTIs (46). G-CSF presence could increase neutrophil chemotaxis to sites of infection (68–70). This was confirmed in mice, where there were significantly depressed PMN infiltrates in urine sediments when animals were treated with subtherapeutic ciprofloxacin. In addition to IL-6 and IL-8 suppression in vivo, the proinflammatory mediator IL-1β, produced by activated macrophages, is an important early response element to UPEC infection, and this cytokine was suppressed with ciprofloxacin treatment. Alternatively, the anti-inflammatory cytokine IL-10 was upregulated with infection and ciprofloxacin presence. IL-10 is important in urothelial protection during UTI as it limits macrophage activation (71–73). Interestingly, ciprofloxacin dramatically increased the expression of G-CSF. This cytokine increases neutrophil migration from the bone marrow, and UPEC is known to trigger its upregulation during UTIs (46). G-CSF presence could be effective in increasing levels of circulating neutrophils; however, the local suppression of chemotactic cytokines in the bladder would diminish the effect at sites of infection. As serum was not collected from these mice, ciprofloxacin augmentation of systemic responses was not assessed. G-CSF also has immunomodulatory effects on macrophages and attenuates IL-1 production, leading to less efficient bacterial clearance, which further corroborates our findings (66, 74, 75). The capacity for ciprofloxacin to induce one cytokine and suppress another while having no effect on others might depend on the augmentation of immune populations and their activity within the bladder during infection. Further characterization of cytological profiles of immune population changes in response to ciprofloxacin would corroborate this hypothesis.

Ciprofloxacin prophylaxis is not associated with improved outcome and increases the intracellular bladder reservoir in mice. We were able to demonstrate therapeutically relevant effects of ciprofloxacin treatment on UPEC pathogenesis using a murine model of prophylaxis. The results indicated that the effect of prophylaxis on bacterial urine loads was negligible and not effective in preventing recurrence risk. Further assessment of sacrificed mice for augmentation in bladder and kidney E. coli UTI89 loads revealed a trend toward higher titers when ciprofloxacin was provided. The most important finding from these studies was in revealing the propensity for prophylaxis to enhance UPEC tissue invasion in bladders. These results mirror clinical observations, in that while prophylaxis might assist in decreasing UTI symptoms, they inexorably do not alter the long-term risk of recurrence and may in fact encourage future episodes by promoting the persistence or accumulation of intracellular reservoirs (28, 52, 76). The presence of intracellular UPEC has been associated with recurrence risk in both humans (77) and mice (36) in the past. Further studies are warranted to determine the longevity of these antibiotic-induced bacterial reservoirs and whether they result in future recurrence episodes.

Clearly, there is a need to reevaluate the effectiveness of prophylactic strategies in patients with highly recurrent UTIs, especially when treatment length is short or when individuals are non-compliant. The findings presented herein likely extend beyond recurrent UTI management, raising important questions regarding the appropriateness of even well-defined antibiotic therapeutic approaches in other disease milieux. For example, empirical therapy for emergent bacteremia may compromise some patients when pathogens are resistant and thus exposed to only subtherapeutic antibiotic levels. The issues to overcome may include ensuring appropriate Gram-positive and -negative coverage, but also only using agents which are known to not induce the release of potentially deadly toxins. This study highlights the need to validate and optimize current regimens and ensure their use is based upon empirical evidence of efficacy rather than anecdotal observation, theory, or instinct on what is thought to work.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** Organisms were grown in lysogenic broth (LB) supplemented with antibiotics when appropriate. MIC determinations were performed via the broth microdilution method as per CLSI protocols. Enumeration of bacterial CFU was performed using LB agar (Bacto agar; BD) plates prepared as per the manufacturer’s instructions and supplemented with antibiotics when required. Cultures were grown statically unless stated otherwise. If shaking was implemented, it was done so at 200 rpm. The UPEC strain used in this study was a kanamycin-resistant derivative of the human cystitis isolate UTI89: \(^{atT}_{16k0222}:comGFP\). E. coli UTI89 (78), S. saprophyticus 15305 was purchased from the American Type Culture Collection (Manassas, VA). SOS-deficient strains E. coli PAS0209 (ΔrecA) and PAS0211 (lexA\(^{T55G}\); also referred to as lexA\(^{T55G}\) or the Ind\(^{−}\) mutant) derived from the UTI89 background were provided by Sheryl Justice (The Research Institute at Nationwide Children’s Hospital, Columbus, OH). The acapsular S. saprophyticus C1 strain derived from the 15305 background was provided by Toshiko Ohta (Graduate School of Comprehensive Human Science, University of Tsukuba, Tsukuba, Japan).

**Biofilm formation assays.** Overnight (24-h) cultures of S. saprophyticus 15305 or E. coli strains UTI89, ΔrecA, and lexA\(^{T55G}\) grown in LB were subcultured 1,000-fold in fresh medium. The wells of a 96-well plate were prepared by loading various subinhibitory concentrations of either ciprofloxacin, ampicillin, gentamicin, or vehicle in 100 μl of LB. Organisms were added at 10 μl per well, and plates were sealed and then incubated for either 24 or 48 h at 37°C. If incubation periods continued for longer than 24 h, wells were washed and replaced with fresh LB containing the appropriate concentration of antibiotic. Following these incubation periods, wells were washed, stained with crystal violet, and decolorized with 70% ethanol. Biofilm abundance was determined by measuring the optical density at 600 nm (OD\(_{600}\)).

**In vitro human cell line assays.** Please refer to the further description of our methods provided in the supplemental material for details used regarding our in vitro human cell line assays.

**Hemagglutination titers.** Alsever guinea pig red blood cells (RBCs; Colorado Serum Company, Denver, CO), for E. coli hemagglutination, or sheep RBCs (Fisher Scientific), for S. saprophyticus hemagglutination, were prepared as previously described (79, 80). E. coli UTI89, ΔrecA, or lexA\(^{T55G}\) and S. saprophyticus 15305 or C1 were subcultured 1,000-fold.
from overnight (24-h) cultures into fresh LB medium containing 1/4 MIC of relevant antibiotics or vehicle for 4 h. Bacteria were then pelleted at 6,500 rpm and resuspended in phosphate-buffered saline (PBS) to an OD$_{600}$ of 1.0. One milliliter of the resulting suspension was transferred to a 1.5-ml microcentrifuge tube and centrifuged at 6,500 rpm for 2 min using a Sorvall Legend Microfuge 21 centrifuge (Thermo Scientific) to pellet. Supernatants were then aspirated, and pellets were resuspended in 100 μl of PBS. V-bottom, 96-well plates were prepared by transferring 25 μl of PBS into each well. Additionally, 4% (wt/vol) α-methyl-o- + -mannopyranoside (Sigma) was prepared in a second plate in a similar manner and served as a measure of mannose-resistant hemagglutination. In duplicate, 25 μl of each suspension was transferred to the first column of the plate and serially diluted 2-fold to each subsequent well to create a dilution gradient of organisms. Twenty-five microliters of the prepared blood suspension was added per well and mixed by gently tapping the plate. Plates were then sealed, covered, and placed at 4°C for 2 to 3 h prior to analysis. Titers were read by determining the last well to display RBC agglutination.

Soft agar motility assay. Soft agar plates (0.3% agar) were prepared by loading with either 1/4 MIC levels of antibiotic or vehicle as previously described (81). E. coli UTI89, ΔlexA, or lexA$_{ppss}$ was subcultured 1,000-fold in fresh LB without antibiotic, incubated at 30°C with shaking (200 rpm) for 4 h, and adjusted to an OD$_{600}$ of 1.0 before being spot plated onto the surface of antibiotic-loaded or unloaded soft agar plates. Organisms were also pretreated with 1/4 MIC levels of relevant antibiotics for 4 h prior to plating on unloaded soft agar plates. The radius of the resulting swimming zone (in millimeters) was measured 24 h postincubation.

Mouse infection protocols. Murine infections models (82) routinely made use of 6- to 8-week-old female C3H/HeN or C57BL/6 mice obtained from Harlan (Harlan Sprague Dawley Inc., Indianapolis, IN) or Jackson (Jackson Laboratory, Bar Harbor, ME), respectively. All animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals under Animal Welfare Assurance A3381-01 at the Washington University School of Medicine, St. Louis, MO. The Washington University School of Medicine Animal Study Committee approved animal protocol 20120216 (expiration, 01/11/2016). E. coli UTI89 and S. saprophyticus 15305 used for infections were inoculated into 10 ml LB directly from 80°C freezer stocks, grown statically overnight at 37°C, subcultured (100-fold) in fresh LB medium with or without 1/4 MIC ciprofloxacin, and grown for another 4 h at 37°C (83). These cultures were then centrifuged for 10 min at 3,000 × g, resuspended in 10 ml of PBS, and diluted to an OD$_{600}$ of 0.35 (~2.0 × 10$^8$ CFU/ml). Fifty microliters of this suspension (~1 × 10$^7$ to 2 × 10$^8$ CFU) was used to inoculate the bladders of mice via transurethral catheterization as previously described (82). Urine bacterial load (titer) was determined via serial dilution in PBS and spot plating (10 μl per drop, 50 μl total per dilution, 1 plate per sample) during the course of infection where indicated. At the indicated times, mice were sacrificed, and bladders and kidneys were aseptically removed and processed for microscopy or mechanically homogenized for CFU titration.

The effect of subtherapeutic ciprofloxacin dosing on the murine response to UTI was investigated in previously infected mice. The animals were initially infected with a dose (~10$^5$ CFU) of E. coli UTI89 and were left for at least 30 days to either spontaneously resolve infection or develop persistent high-titer bacteriuria (>10$^5$ CFU/ml for ≥ 28 days p.i.), which is an accurate indicator of the presence of chronic cystitis (44). The urine titers of mice were additionally monitored for 3 days prior to initiation of subtherapeutic ciprofloxacin supplementation. The empirical therapeutic ciprofloxacin dose in mice was estimated based on human data (40 μg/ml ciprofloxacin administered daily). Ciprofloxacin was supplemented into the drinking water for ad libitum consumption, and water intake was monitored and did not differ between antibiotic-supplemented and control groups, indicating ciprofloxacin was well tolerated and did not alter palatability (data not shown). Dosing ranges of <1/25 the empirical therapeutic dose were found to not significantly decrease bacterial urine titers over a 3-day period (data not shown). Following optimization of dosing parameters, such that UPEC was not negatively influenced by the level of ciprofloxacin present, 1/50 the empirical ciprofloxacin therapeutic dose was supplemented into the drinking water and replaced each day for 3 to 6 days. The urine titer of each mouse was monitored daily for changes as described elsewhere. At either 3 or 6 days, the dose was further increased to 1/25 the empirical therapeutic dose for another 3- to 6-day period, with urine titers again determined daily. Lastly, the dose was increased further to 1/10 the empirical therapeutic dose for 3 days to clear infection and ensure that spontaneous development of ciprofloxacin-resistant mutants did not occur.

Evaluation of intracellular bacterial populations within the bladder. E. coli UTI89 IBC enumeration, visualization, and volumetrics were performed using lacZ staining and confocal microscopy as previously described (36, 84). For IBC enumeration, animals were sacrificed 6 h p.i., and bladders were excised, bisected longitudinally, and placed onto a silicone bladder pinning pad. They were fixed with 3% paraformaldehyde (Sigma), washed thrice with 2 mM MgCl$_2$ (Sigma), 0.01% sodium deoxycholate (Sigma), and 0.02% Nonidet P-40 (Roche, Mississauga, ON, Canada) in PBS. Staining was performed using 25 mg/ml X-Gal (Sigma) and a solution containing 1 mM potassium ferrocyanide and 1 mM potassium ferricyanide (Sigma). After an incubation period of 16 h at 30°C, bladders were visualized under an Olympus SZX12 dissecting microscope (Olympus, America, Center Valley, PA).

For IBC visualization, infections and bladder fixation were conducted as described elsewhere, with the exception that E. coli UTI89 carrying the GFP-expressing plasmid pANT4 was utilized. Fixed bladders were washed and counterstained with nuclear ToPro3 (Molecular Probes) and recombinant wheat germ agglutinin (r-WGA) to outline superficial umbrella cells (1:700 dilution for each). Bladders were imaged using a Zeiss LSM 510 Meta laser scanning inverted confocal microscope (Thornwood, NY). IBCs were rendered in three-dimensions (3D) via reconstructive z-stacking of images, and volumes were determined by using Volume 4 image analysis software (PerkinElmer, Waltham, MA).

Murine and human tissue cytokine profiling and urine sediment analysis. Mice were supplemented with 1/50 the empirical ciprofloxacin therapeutic dose or vehicle for 3 days, then inoculated with 10$^7$ non-treated E. coli UTI89 and sacrificed at 3.5 h p.i. Bacterial urine load and immune cell infiltrate were determined at the time of sacrifice, and bladders and kidneys were excised and snap-frozen in liquid nitrogen for future cytokine profiling. Murine urine immune cell sediment analysis was carried out using the CytoPro 7620 cytocentrifuge (Wescor, Logan, UT) as per the manufacturer’s instructions. Stained urine sediments were examined by light microscopy on an Olympus BX51 light microscope, and the average number of PMN per 400× magnification field (high-powered field) was calculated by counting at least five fields and using a semiquantitative scoring system as previously described (44).

The cytokine expression profiles for murine bladders were determined using an xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokines were liberated from tissues by using homogenization in extraction buffer containing 20 mM Tris-HCl (pH 7.5; Sigma), 150 mM NaCl (Sigma), 1-mM phenylmethylsulfonyl fluoride (PMSF; Sigma), 0.05% Tween 20 (Sigma), and a protease inhibitor cocktail (100-fold dilution; Roche). Conversely, supernatants from previous in vitro human T24 tissue cell line experiments were also extracted and analyzed. Total protein levels were assessed using a bicinchoninic acid (BCA) kit (Thermo Scientific) as per the manufacturer’s instructions and diluted when required. Levels of cytokines IL-1β, IL-6, IL-12, KC, G-CSF, IL-17, IL-10, and TNF-α were measured using multiplexed immunoassay kits according to the manufacturer’s instructions (Bio-Rad Laboratories, Inc., Hercules, CA). Cytokine levels (in picograms per milliliter) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1; Bio-Rad). ELISAs were used to determine IL-8 expression levels in human 5637 bladder cells. Infections were carried out as previously described, with the
exceptions that LPS (Sigma) was added at 5 µg/ml in place of S. saprophyticus 15305 and ampicillin and gentamicin were also tested at 1/4 MIC levels in addition to ciprofloxacin. Four hours post-5637 cell infection, supernatants were extracted and cytokines were quantified using the human CXCL8/IL-8 Quantikine ELISA kit (R&D Systems, Minneapolis, MN) as per the manufacturer’s instructions.

**Model of ciprofloxacin prophylaxis.** C57BL/6 mice were inoculated as previously described with ~10^7 CFU of nonprimed E. coli UTI89. Infections were allowed to develop for 24 h prior to initiation of a 3-day regimen of normal co-trimoxazole therapy (270 µg/ml replaced daily; Qualitest Pharmaceuticals, Huntsville, AL) (40). Following therapy cessation, mice were provided a 7-day rest period prior to initiation of ciprofloxacin prophylaxis. They then received either a typical prophyllactic dose of ciprofloxacin (1/4 the empirical therapeutic dose; 100 µl of a 1-µg/ml solution) or vehicle (distilled H2O) daily via oral gavage for 7 days. Following prophyllactic therapy, animals were sacrificed, and kidneys and bladders were excised for determinations of bacterial titers. In some cases, ex vivo gentamicin protection assays were conducted on bladders for enumeration of the intracellular population. Urine was taken at least every 2 days throughout the course of the experiment to allow tracking of in-fection.

**Gene expression analyses.** For details on the methods used for the gene expression analyses, please refer to the supplemental material.

**Statistical analyses.** Statistical analyses were conducted using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Significance was determined using one-way analysis of variance (ANOVA) and Dunnett’s multiple comparison test or Bonferroni’s multiple comparison test, a Mann-Whitney test with Gaussian approximation, a log-rank test, or a two-tailed or unpaired t test. Each test used for the various experiments is described in detail in the corresponding figure legend. Statistical analyses for the differential abundance of mapped mRNA reads from RNA sequencing were conducted by the ALDEx2 R package version 2.0.6 (85, 86). A log, median effect size (calculated by using ALDEx, as the mean ratio of the difference between groups versus the maximum difference within groups) of at least 1.5 (representing at least a 2.5-fold-greater difference between groups versus within groups) was required for genes to be considered differentially expressed. In addition, a >1% overlap in the distributions between the two conditions was permitted for inclusion (85, 87). This approach ensures that the within-group variance is consistently and substantially smaller than the between-group difference when small sample sizes are used.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article can be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00356-15/-/DCSupplemental.

Figure S1, TIF file, 8.1 MB.
Figure S2, TIF file, 9.1 MB.
Figure S3, TIF file, 28.2 MB.
Text S1, DOCX file, 0.02 MB.

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