# Utilization of an Intracellular Bacterial Community Pathway in *Klebsiella pneumoniae* Urinary Tract Infection and the Effects of FimK on Type 1 Pilus Expression[▽]

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*Klebsiella pneumoniae* is an important cause of urinary tract infection (UTI), but little is known about its pathogenesis in vivo. The pathogenesis of the *K. pneumoniae* cystitis isolate TOP52 was compared to that of the uropathogenic *Escherichia coli* (UPEC) isolate UTI89 in a murine cystitis model. Bladder and kidney titers of TOP52 were lower than those of UTI89 at early time points but similar at later time points. TOP52, like UTI89, formed biofilm-like intracellular bacterial communities (IBCs) within the murine bladder, albeit at significantly lower levels than UTI89. Additionally, filamentation of TOP52 was observed, a process critical for UTI89 evasion of neutrophil phagocytosis and persistence in the bladder. Thus, the IBC pathway is not specific to UPEC alone. We investigated if differences in type 1 pilus expression may explain TOP52's early defect in vivo. The type 1 pilus operon is controlled by recombinase-mediated (*fimE*, *fimB*, and *fimX*) phase variation of an invertible promoter element. We found that *K. pneumoniae* carries an extra gene of unknown function at the 3′ end of its type 1 operon, *fimK*, and the genome lacks the recombinase *fimX*. A deletion mutant of *fimK* was constructed, and TOP52 Δ*fimK* had higher titers and formed more IBCs in the murine cystitis model than wild type. The loss of *fimK* or expression of *E. coli fimX* from a plasmid in TOP52 resulted in a larger phase-ON population and higher expression levels of type 1 pili and gave TOP52 the ability to form type 1-dependent biofilms. Complementation with p*fimK* decreased type 1 pilus expression and biofilm formation of TOP52 Δ*fimK* and decreased UTI89 biofilm formation. Thus, *K. pneumoniae* appears programmed for minimal expression of type 1 pili, which may explain, in part, why *K. pneumoniae* is a less prevalent etiologic agent of UTI than UPEC.

Nearly 13 million women get urinary tract infections (UTIs) per year in the United States alone, and more than half of all women will experience a UTI during their lifetimes (16, 23, 28, 29, 52). These infections often recur, and over half of all recurrent episodes are caused by the same bacterial strain as the initial infection (17, 54). Uropathogenic *Escherichia coli* (UPEC) is the most common etiologic agent, responsible for 80 to 85% of community-acquired UTIs (51). However, there are several other significant uropathogens; including *Staphylococcus saprophyticus*, *Klebsiella pneumoniae*, and *Proteus mirabilis* (49).

*K. pneumoniae* causes up to 5% of community-acquired UTIs and is significantly more common in diabetic patients and in the nosocomial setting (26, 36, 50). The urinary tract is the most common site of *Klebsiella* infection, although it may be better recognized as a cause of pneumonia in compromised hosts (7). Over the past 25 years, there has been a substantial increase in the spread of drug-resistant strains of *Klebsiella*, particularly those producing extended-spectrum β-lactamases (42). *K. pneumoniae* encodes type 1 pili, and its corresponding *fim* operon is highly homologous to that of *E. coli* (14, 22). As with *E. coli*, expression of the *fim* operon is phase variable, controlled by an invertible promoter element, *fimS*. *fimE* and *fimB* are recombinase genes in the type 1 pilus gene cluster that mediates the phase switching of *fimS* (33). *fimX* is not part of the *fim* gene cluster but encodes a recombinase that is also important in *fimS* phase switching in vivo (4, 25). ON-phase *K. pneumoniae* displays superior in vitro binding to ciliated hamster tracheal epithelial cells and rat bladder epithelial cells (12, 15) and in vivo infection of the rat urinary tract (13) compared to OFF-phase organisms. It has also been shown that infection of the mouse urinary tract leads to a population shift favoring organisms expressing type 1 pili (37).

UPEC strains utilize a multistep pathogenic pathway during human and murine infection in which the bacteria invade bladder facet cells and replicate intracellularly (1, 19, 30, 44, 53). Almost all UPEC strains express type 1 pili, which consist of thin-tipped fibrillae attached to thicker pilus rods (5). Genes essential for UPEC type 1 pilus biogenesis are organized in the *fim* operon, with *fimH* encoding the adhesin that recognizes mannosylated uroplakins on the luminal surface of facet cells (63). Binding of UPEC to the host cell, possibly through β1 and α3 integrins, induces a cascade of signaling events that leads to bacterial internalization (11, 38, 39). Within the superficial facet cells of the bladder, piliated UPEC bacteria replicate to form intracellular bacterial communities (IBCs) with biofilm-like properties (1, 62). IBCs are transient, and the bacteria ultimately flux out of epithelial cells, some adopting a filamentous morphology. These long filaments evade engulfment by neutrophils and are necessary for persistence in the murine cystitis model (31). UPEC is also able to

TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Description | Reference or source |
|---|---|---|
| **Strains** | | |
| UTI89 | UPEC cystitis isolate | 44 |
| UTI89 Δ*fimA-fimH* | Knockout of *fimA-fimH* in UTI89 | This study |
| TOP52 1721 | *K. pneumoniae* cystitis isolate | 53; also this study |
| TOP52 Δ*fimK* | Knockout of *fimK* in TOP52 | This study |
| TOP52 Δ*fimA-fimH* | Knockout of *fimA-fimH* in TOP52 | This study |
| **Plasmids** | | |
| pBAD33 | Empty expression vector; arabinose inducible; Cm$^r$ | 24 |
| p*fimK* | TOP52 *fimK* expression vector; arabinose inducible; Cm$^r$ | This study |
| p*fimX* | UTI89 *fimX* expression vector; arabinose inducible; Cm$^r$ | 25 |

form quiescent intracellular reservoirs (QIRs) that can persist for several weeks protected from antibiotics and seemingly undetected by the host immune system (44, 46, 57). It has not yet been determined whether non-UPEC uropathogens, such as *K. pneumoniae*, utilize an intracellular bladder niche during infection. In this study we investigated the pathogenesis of *K. pneumoniae* in the murine cystitis model compared to UPEC. We discovered interesting differences in the pathogenic pathway of *K. pneumoniae* that were related to the expression of type 1 pili.

## MATERIALS AND METHODS

**Bacterial strains and culture conditions.** A complete list of bacterial strains and plasmids used in this study can be found in Table 1. Clinical strains used include UTI89, a UPEC cystitis isolate (44), and TOP52 1721 (abbreviated

TOP52), a *K. pneumoniae* cystitis isolate. TOP52 was isolated from the urine of a 26-year-old woman with acute cystitis from a previous UTI study (53). After successful completion of antibiotic therapy and negative follow-up urine cultures, this patient developed recurrent cystitis with the same strain of *K. pneumoniae*, as determined by restriction fragment length polymorphism analysis. Bacteria were cultured at 37°C in Luria-Bertani (LB) broth containing, as appropriate, 20 μg/ml chloramphenicol, 50 μg/ml kanamycin, and arabinose as indicated below.

**TOP52 and UTI89 mutant construction and complementation.** Targeted deletion mutants of *fimK* and *fimA-fimH* in the *K. pneumoniae* isolate TOP52 were constructed with the pKOV vector as described previously (35). Flanking sequences of approximately 1,000 bp on each side of the targeted gene were amplified with the primers indicated in Table 2 and cloned into pKOV. Potential knockouts were screened by PCR, and the knockout region was sequenced. Growth curves were done for all mutant strains and showed no differences in growth compared to the wild type.

UTI89 Δ*fimA-fimH* was constructed using the red recombinase method as previously described (9, 45), with pKD4 as a template and the primers indicated in Table 2. PCR using flanking primers was used to confirm the deletion.

For complementation studies, *fimK* was amplified and cloned into the arabinose-inducible pBAD33 vector (abbreviated pBAD) to make p*fimK*. TOP52 *fimK* was sequenced using both amplification primers and pBAD plasmid primers. Homology searches were conducted using BLASTN and BLASTP to look for homologous genes, proteins, and domains. For expression of *fimX*, the p*fimX* vector, which contains *fimX* of UTI89 cloned into pBAD, was used as previously described (25).

**Mouse infections.** Bacterial strains were used to inoculate 8-week-old female C3H/HeN mice (National Cancer Institute) by transurethral catheterization as previously described (43). Static cultures (25 ml) were started from freezer stocks and grown at 37°C for 18 h and then subcultured at 1:250 into 25 ml of fresh medium. These cultures were then grown statically at 37°C for 18 h and centrifuged for 5 min at 5,800 rpm, and the resultant pellet was resuspended in phosphate-buffered saline (PBS) and diluted to approximately $2 \times 10^8$ CFU/ml. Fifty milliliters of this suspension was used to infect each mouse with an inoculum of $1 \times 10^7$ to $2 \times 10^7$ CFU. All studies were approved by the Animal Studies Committee at Washington University School of Medicine.

**Urine collection.** For mouse urine studies, samples were collected 24 h postinoculation by bladder massage over a sterile 1.5-ml Eppendorf tube and cytocentrifuged for 6 min at 1,000 rpm onto poly-L-lysine-coated glass slides using a CytoPro 7620 cytocentrifuge (Wescor). Slides were stained with filter-sterilized Protocol Hema 3 stains (Wright-Giemsa method; Fisher Scientific) and analyzed by light microscopy using an Olympus BX51 light microscope (Olympus America).

TABLE 2. Primer sequences

| Primer name | Primer use | Primer sequence |
|---|---|---|
| NotIFimKF1 | Amplify 1-kb *fimK* 5′ flanking region | 5′-CGGTAACGCGGCCGCGACTATCCGGAAACGATCACC-3′ |
| HindIIIFimKR2 | Amplify 1-kb *fimK* 5′ flanking region | 5′-AACAAGCTTAGACGATCCGGATGACTCAC-3′ |
| HindIIIFimKF3 | Amplify 1-kb *fimK* 3′ flanking region | 5′-CATAAGCTTAGAAAAGCGCACCGGTTA-3′ |
| SalIFimKR4 | Amplify 1-kb *fimK* 3′ flanking region | 5′-AACGTCGACAAAACAGAAACCACAGCAACG-3′ |
| 3FimKCheckF | Check TOP52 Δ*fimK* | 5′-GTCGATTATCGGCATCACCT-3′ |
| 3FimKCheckR | Check TOP52 Δ*fimK* | 5′-GTGGCGAAGGTAGTGGAAAA-3′ |
| NotIFimF1 | Amplify 1-kb *fimA* 5′ flanking region | 5′-CGGTAACGCGGCCGCGCATTAGCGAACTGCTGGAT-3′ |
| BamHIFimR2 | Amplify 1-kb *fimA* 5′ flanking region | 5′-AACGGATCCCAGGGCTGACACAACAATCA-3′ |
| BamHIFimF3 | Amplify 1-kb *fimH* 3′ flanking region | 5′-TACGGATCCGTCAATCTCGGCCTGACG-3′ |
| SalIFimR4 | Amplify 1-kb *fimH* 3′ flanking region | 5′-AACGTCGACGGAGGGTATGTTGACCGAAA-3′ |
| 3FimCheckF | Check TOP52 Δ*fimA-fimH* | 5′-CCAACGCCATCGCTATTC-3′ |
| 3FimCheckR | Check TOP52 Δ*fimA-fimH* | 5′-GATACACCACGATCCGCTTC-3′ |
| 3FimKcompF | Amplify TOP52 *fimK* for cloning into pBAD | 5′-AAT TCC ATG GGGCATCACCTTTGTCTATCAATGATG-3′ |
| 3FimKcompR | Amplify TOP52 *fimK* for cloning into pBAD | 5′-AATTGGTACCCGGTGCGCTTTTCTCGCACCCTCAACG-3′ |
| pBADF | Check pBAD clones | 5′-TATCGCAACTCTCTACTGTTTCTCCA-3′ |
| pBADR | Check pBAD clones | 5′-CTGTTATCAGGCTGAAAATCTTCTCTCA-3′ |
| UTI89fimA-H KO F | Knockout *fimA-fimH* in UTI89 | 5′-ATGAAAATTAAAACTCTGGCAATTGTTGTTCTGTCGGCT CTGTCCCTGAGCATATGAATATCCTCCTTAG-3′ |
| UTI89fimA-H KO R | Knockout *fimA-fimH* in UTI89 | 5′-TTATTGATAAACAAAAGTCACGCCAATAATCGATTG CA CATTCCCTGCAGGTGTAGGCTGGAGCTGCTTC-3′ |
| UTI89fimA-HcheckF | Check UTI89 Δ*fimA-fimH* | 5′-GTTTGTGCGCGATGCTTTCC-3′ |
| UTI89fimA-HcheckR | Check UTI89 Δ*fimA-fimH* | 5′-GAGCACCTGAGCCTGCCATT-3′ |
| 2KlebphaseF | Amplify *fimS* region for phase assay | 5′-GGGACAGATACGCGTTTGAT-3′ |
| 2KlebphaseR | Amplify *fimS* region for phase assay | 5′-GGGACAGATACGCGTTTGAT-3′ |
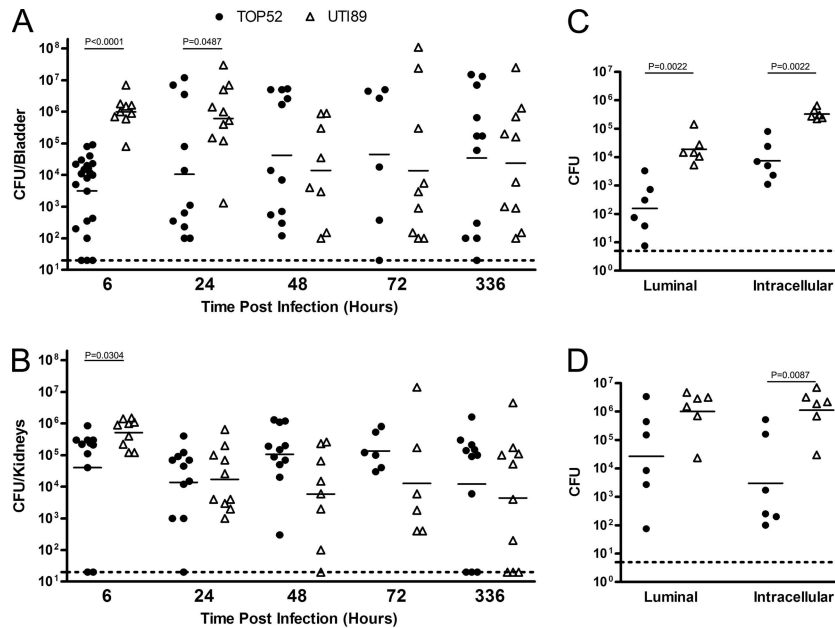
FIG. 1. Time course of TOP52 and UTI89 bladder and kidney infections and gentamicin protection assays. Female C3H/HeN mice were inoculated with $10^7$ CFU of TOP52 or UTI89 by transurethral inoculation. For organ titers, bladders (A) and kidneys (B) were harvested at various time points postinfection, and the numbers of CFU were calculated. Titer data are combined from three independent experiments. For ex vivo gentamicin protection assays, bladders were harvested at 6 h (C) and 24 h (D) postinfection, and luminal and intracellular populations of bacteria were quantified. Short bars represent geometric means of each group, and dotted lines represent limits of detection. Significant *P* values, as calculated using the Mann-Whitney U test, are shown on the figure.

**Organ titers, gentamicin protection assays, and IBC enumeration.** To quantify bacteria present in mouse organs, bladders and kidneys were aseptically harvested at the indicated times postinfection, homogenized in PBS, serially diluted, and plated onto LB agar plates. Luminal and intracellular bacteria were quantified using an ex vivo gentamicin protection assay as previously described (32). For ex vivo enumeration of IBCs, infected bladders were harvested at the times indicated in the figure legends, bisected, splayed, washed with PBS, fixed with 3% paraformaldehyde for 60 min at room temperature, and *lacZ* stained as previously described (32). IBCs were visualized and counted using an Olympus SZX12 dissecting microscope (Olympus America).

**Histology and immunohistochemistry.** Infected mouse bladders were aseptically removed, fixed in neutral buffer formalin, and paraffin embedded, and sections were stained with hematoxylin and eosin. For immunohistochemistry, unstained slides were deparaffinized, washed in filter-sterilized PBS, blocked in 1% bovine serum albumin–0.3% Triton X-100 for 1 h at room temperature, and subsequently incubated for 1 h with rabbit anti-*E. coli* (1:1,000; U.S. Biological), rabbit anti-*K. pneumoniae* (1:100; Virostat), or rabbit anti-gel-purified type 1 pili (1:500) (48) and goat anti-uroplakin III (1:100; Santa Cruz Biotechnology) primary antibodies. After three washes in PBS for 5 min and staining with Alexa Fluor 488- and 594-conjugated donkey secondary antibodies (1:1,000; Molecular Probes) for 30 min, slides were washed, stained with Hoechst, coverslipped with Fluoromount G (Southern Biotechnology Assoc.), and examined using an epifluorescent Zeiss Axioskop instrument (Carl Zeiss).

**FimA immunoblotting, phase assays of the *fim* operon switch, and antigenic titration assays.** For immunoblot analysis bacterial strains were grown statically as described above for mouse infections with 0.01% arabinose, as appropriate. Acid-treated whole-cell immunoblotting was performed as previously described (19) using 1:2,000 rabbit anti-type 1 pilus primary antibody (48). Bacteria were normalized by optical densities at 600 nm ($OD_{600}$), and Coomassie blue staining was performed to verify similar levels of protein in each lane. The intensity of each band was analyzed using ImageJ software (http://rsb.info.nih.gov/ij/).

To determine the orientation of the *fimS* phase switch in TOP52, a phase assay was adapted from the *E. coli* assay previously described (59). PCR primers were used to amplify an 815-bp DNA region including *fimS*. The PCR product was then digested with the restriction endonuclease Hinf1 (New England Biolabs) and run on a 2.5% agarose gel. A phase-ON switch results in products of approximately 495 and 320 bp, and a phase-OFF switch results in products of approximately 600 and 215 bp.

For antigenic titration assays, all strains were grown statically at 37°C in LB with appropriate antibiotics and 0.1% arabinose, as indicated in Table 3. Serological titers using monospecific antiserum to type 1 pili or type 3 pili (20, 21) required to agglutinate strains were determined as previously described (10).

**Negative-stain EM.** Strains were cultured as described above for mouse infections and were prepared for electron microscopy (EM) as follows. Bacteria were absorbed onto Formvar-carbon-coated copper grids for 1 min. Grids were washed in distilled water and stained with 1% aqueous uranyl acetate (Ted Pella Inc.) for 1 min. Excess liquid was gently wicked off, and grids were allowed to air dry. Samples were viewed on a JEOL 1200EX transmission EM (JEOL USA) at an accelerating voltage of 80 kV, and representative images are shown.

**Biofilm assays.** Bacteria were grown in LB broth in wells of microtiter plates in the presence of 0.1% arabinose (for complementation in UTI89) or 0.01% arabinose (for complementation in TOP52), as appropriate. After 48 h of growth at room temperature, wells were rinsed and stained with crystal violet, and biofilms were quantified as previously described (47). Resulting graphs compare means ± standard errors of the means of two independent experiments, each with duplicate plates.

**Statistical analysis.** Continuous variables were compared using a Mann-Whitney U test since these variables were not normally distributed. For biofilm data, means of multiple plates from two independent experiments were compared, and *t* tests were performed. All tests were two tailed, and a *P* value less than 0.05 was considered significant. Analyses were performed using GraphPad Prism (version 4.03; GraphPad Software).

**Nucleotide sequence accession number.** The TOP52 *fimK* nucleotide sequence has been deposited in the GenBank database under accession number EU315065.

## RESULTS

**TOP52 compared to UTI89 in a murine cystitis model.** To contrast urinary tract infections of *K. pneumoniae* and *E. coli*, C3H/HeN female mice were inoculated by transurethral catheterization with $10^7$ CFU of TOP52, a *K. pneumoniae* cystitis isolate, or $10^7$ CFU of UTI89, an *E. coli* cystitis isolate. Inoculations with less than $10^7$ CFU of TOP52 did not result in consistent infection in the mouse model (data not shown).
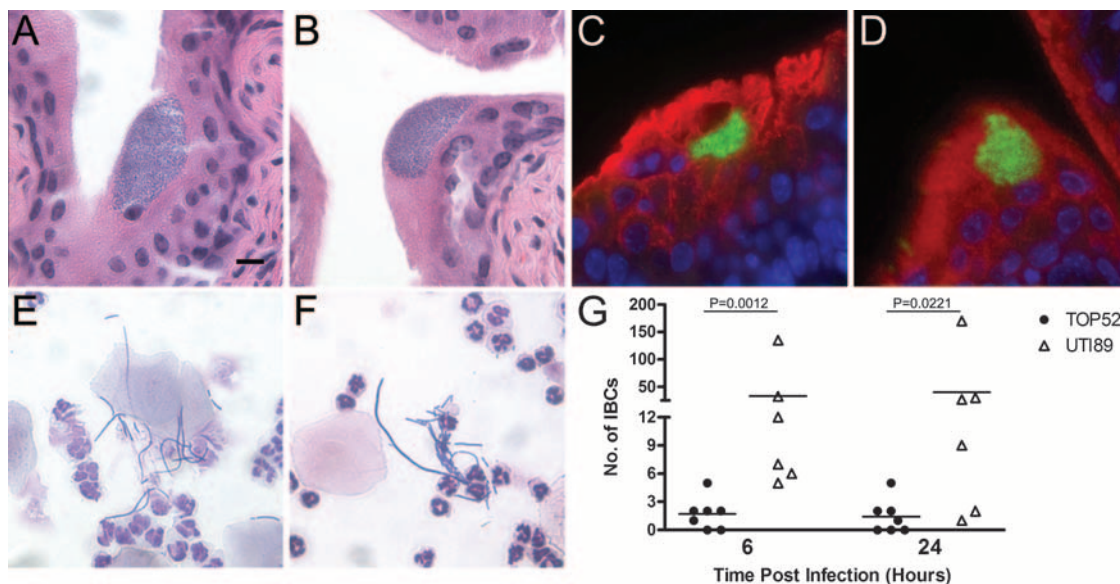
FIG. 2. TOP52 progresses through an IBC pathogenic pathway. Histologic analysis of murine bladders after a 6-h infection with TOP52 (A) or UTI89 (B) revealed morphologically identical IBCs within superficial urothelial cells. Immunohistochemistry demonstrated that bacteria express type 1 pili within IBCs of both TOP52 (C) and UTI89 (D) infections. Staining was done with antibodies against type 1 pili (green) and the facet cell marker uroplakin III (red), and nuclei were visualized with Hoescht staining (blue). Urine samples from mice at 24 h postinfection with TOP52 (E) or UTI89 (F) contained long bacterial filaments. IBCs were quantified after visualization by LacZ staining and revealed that TOP52 forms significantly fewer IBCs than UTI89 at both 6 and 24 h postinoculation (G). Short bars represent geometric means, and significant $P$ values, as calculated using the Mann-Whitney U test, are shown on the figure. Scale bar, 10 μm (A to F).

Bladders and kidneys were harvested at various time points postinoculation, and bacterial titers were determined. In the bladder (Fig. 1A), TOP52 had significantly lower titers than UTI89 at both the 6-h ($P < 0.0001$) and 24-h ($P = 0.0487$) time points. At later time points, TOP52 and UTI89 titers were similar in bladders. At 2 weeks postinoculation, the distributions of TOP52 and UTI89 titers were similar in bladders. In the kidney (Fig. 1B), TOP52 had slightly lower titers than UTI89 at 6 h ($P = 0.0304$); however, it had similar or higher titers than UTI89 in the kidney at all other time points tested.

It has been shown that UPEC bacteria invade bladder epithelial cells during infection. The ability of *K. pneumoniae* to invade bladder epithelial cells during UTI was investigated using gentamicin protection assays of the bladder performed at 6 h and 24 h postinoculation. At each time point, luminal bacteria were collected by successive bladder washes prior to gentamicin treatment. TOP52, like UTI89, had substantial intracellular populations of bacteria at both 6 and 24 h postinoculation. At 6 h (Fig. 1C), TOP52 had significantly more intracellular bacteria than luminal bacteria ($P = 0.0087$), as did UTI89 ($P = 0.0022$). By 24 h (Fig. 1D), the number of intracellular bacteria was similar to the number of extracellular bacteria for both TOP52 and UTI89. However, the numbers of both luminal and intracellular TOP52 bacteria were significantly lower than the respective UTI89 levels at 6 h ($P = 0.0022$). Thus, TOP52 may be defective in adherence and/or invasion of the bladder urothelium early in infection relative to UTI89.

These data demonstrate that the *K. pneumoniae* cystitis isolate TOP52 is able to infect the bladder and kidneys of mice in the murine cystitis model. However, TOP52 has significantly reduced titers early in infection compared to the *E. coli* cystitis isolate, UTI89. Similar to UTI89, there also appears to be a significant intracellular population of TOP52 early in infection.

**TOP52 progresses through an IBC pathway.** Histology was performed on TOP52 bladders at 6 h postinoculation, and numerous sections were analyzed in order to further characterize the intracellular population of TOP52. Large IBCs were found in TOP52-infected bladders (Fig. 2A). These biofilm-like communities, found within superficial bladder facet cells, appeared morphologically identical to those found in UTI89-infected bladders (Fig. 2B). Immunohistochemistry staining with *Klebsiella*-specific antibodies confirmed that these IBCs were composed of *K. pneumoniae* (data not shown). Staining with antibodies against type 1 pili confirmed that type 1 pili are expressed within TOP52 IBCs and UTI89 IBCs (green areas in Fig. 2C and D, respectively), similar to what has been reported previously for UTI89 (62). At 24 h postinoculation, filamentous bacteria, another hallmark of the IBC pathway, were found in urine samples from both TOP52-infected (Fig. 2E) and UTI89-infected (Fig. 2F) mice.

IBCs were visualized and quantified by *lacZ* staining of whole mounted, fixed bladders at 6 and 24 h postinoculation of TOP52 or UTI89 (Fig. 2G). TOP52 had significantly fewer IBCs than UTI89 at both the 6-h ($P = 0.0012$) and 24-h ($P = 0.0221$) time points. Taken together, these data demonstrate that TOP52 progresses through an IBC pathogenic pathway. TOP52, however, forms fewer IBCs than UTI89 early in infection.

**The *fim* operon of TOP52 contains *fimK*, and TOP52 Δ*fimK* has higher bacterial titers in vivo.** We investigated whether the differences in bacterial burden and IBC formation between TOP52 and UTI89 observed at early time points in infection may, in part, relate to the expression of type 1 pili. We found

that while *K. pneumoniae* encodes intact *fimB* and *fimE* recombinases important in mediating phase variation of the invertible promoter element, it lacks *fimX*. Compared to UTI89, the *fim* operon of TOP52 also contains an extra gene of unknown function, *fimK* (Fig. 3A). *fimK* was also found to be present immediately downstream of *fimH* in both of the other strains of *K. pneumoniae* for which the sequence of this region was available (22, 41). The sequence of the 1,410-bp open reading frame of *fimK* (GenBank accession number EU315065) showed no homology to sequenced bacterial genes. However, the carboxy-terminus contained an EAL domain, a domain which has previously been implicated in cleavage of the second messenger cyclic-di-GMP (8, 58, 60).

A deletion mutant of *fimK* was constructed, and the ability of TOP52 Δ*fimK* to colonize and form IBCs was investigated in the murine cystitis model. At 6 h postinoculation, TOP52 Δ*fimK* had significantly higher titers in bladder (Fig. 3B) and kidney (Fig. 3C) than wild-type TOP52 ($P = 0.0112$ and $P = 0.0240$, respectively). The *fimK* gene was cloned downstream of an arabinose-inducible promoter to make p*fimK* for complementation studies. TOP52 Δ*fimK*/p*fimK* had significantly lower titers in bladder and kidney than the empty vector control, TOP52 Δ*fimK*/pBAD ($P = 0.0006$ and $P = 0.0091$, respectively).

*lacZ* staining of whole mounted, fixed bladders was performed to determine if the deletion of *fimK* had a quantitative effect on IBC formation (Fig. 3D). While wild-type TOP52 formed a mean of 1.8 IBCs per bladder, TOP52 Δ*fimK* formed 4.6 ($P = 0.0312$). The TOP52 Δ*fimK*/pBAD vector control formed a mean of 5.7 IBCs per bladder while TOP52 Δ*fimK*/p*fimK* formed 3.3 IBCs per bladder ($P = 0.0939$). These data suggest that loss of *fimK* results in higher bacterial titers, greater numbers of IBCs, and overall more-efficient bladder infection.

**TOP52 Δ*fimK* and TOP52/p*fimX* have higher expression levels of type 1 pili.** We hypothesized that the low bladder colonization and IBC formation levels of TOP52 compared to UTI89 may be related to type 1 pilus expression. Possible differences in type 1 pilus expression could potentially result from the lack of the *fimX* recombinase or the presence of the *fimK* gene in TOP52. The higher titers in bladder and increased abundance of IBCs observed with the loss of *fimK* could potentially be explained by a higher level of type 1 pilus expression. Thus, the effects of *fimK* loss or *fimX* addition on type 1 pilus expression were investigated. Levels of the major type 1 pilus subunit, FimA, were monitored by whole-cell immunoblotting using anti-FimA antibodies. Cells were normalized by $OD_{600}$, and Coomassie staining was used to verify similar levels of protein in each lane. In addition, the orientation of the *fimS* promoter was analyzed in phase assays to test if the loss of *fimK* or the addition of *fimX* affects type 1 pilus expression (Fig. 4). TOP52 Δ*fimK* produced a larger FimA band than TOP52 as determined by immunoblot analysis. Complementation of TOP52 Δ*fimK* with p*fimK* reduced the size of the FimA band to close to the wild-type level. Moreover, the addition of *fimX* in TOP52 also led to the production of a larger FimA band than that produced by the vector control. As a control, *fimA-fimH* was deleted in TOP52. This negative-control strain, TOP52 Δ*fimA-fimH*, did not produce a FimA band.
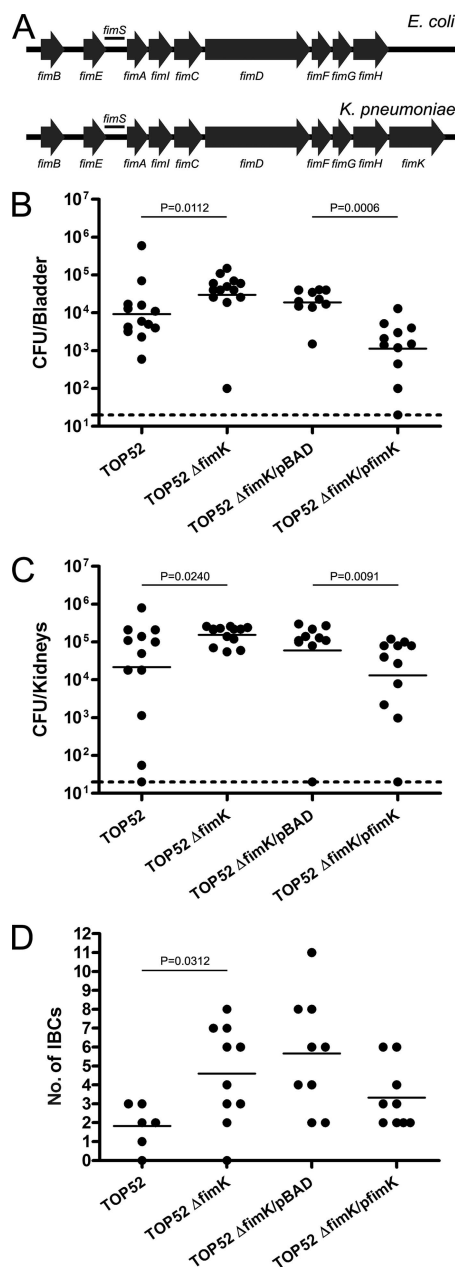


FIG. 3. TOP52 Δ*fimK* has higher titers in organs and forms more IBCs in the murine cystitis model than the wild type. (A) The *fim* operons of *E. coli* and *K. pneumoniae*, which encode type 1 pili, have similar compositions and organizations, but the *K. pneumoniae fim* operon contains an extra gene of unknown function, *fimK*. (B) TOP52 Δ*fimK* had higher 6-h titers in the bladder than the wild type, and this phenotype was complemented with p*fimK*. (C) TOP52 Δ*fimK* also had consistently higher titers in the kidney at 6 h than the wild type, and this difference could be complemented back to wild-type levels. (D) Enumeration of IBCs at 6 h postinfection revealed that TOP52 Δ*fimK* forms more IBCs than TOP52. Titer and IBC data are combined from three independent experiments. Short bars represent geometric means of each group, and dotted lines represent limits of detection. Significant *P* values, as calculated using the Mann-Whitney U test, are shown in the figure.

Phase assays were performed in which the *fimS* invertible region was amplified by PCR and digested using a restriction endonuclease to determine the orientation of the *fim* operon promoter. TOP52 Δ*fimK* had a larger proportion of phase-ON
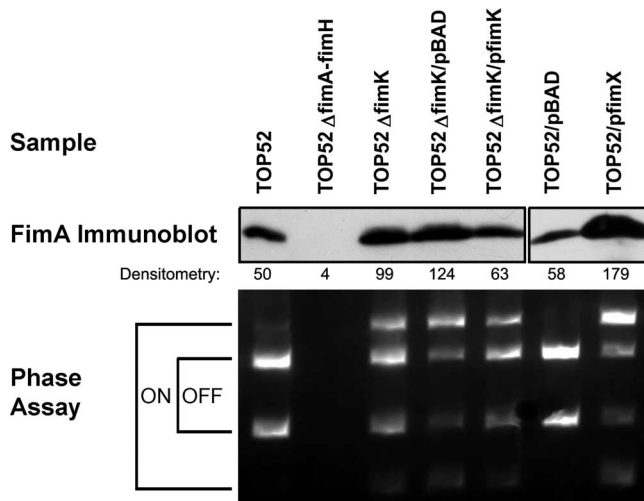
Sample

TOP52
TOP52 ΔfimA-fimH
TOP52 ΔfimK
TOP52 ΔfimK/pBAD
TOP52 ΔfimK/pfimK
TOP52/pBAD
TOP52/pfimX

FimA Immunoblot

Densitometry:    50    4    99    124    63    58    179

Phase Assay    ON | OFF

FIG. 4. TOP52 ΔfimK and TOP52/pfimX have higher expression levels of type 1 pili than wild type and vector controls. FimA immunoblot analysis showed that TOP52 ΔfimK makes more type 1 pili than wild-type TOP52. Expression of FimA was complemented down to wild-type levels with pfimK. TOP52/pfimX also produced a much larger FimA band than the vector control. Bacteria were normalized by OD$_{600}$, and Coomassie blue staining was used to verify similar levels of protein in each lane. Relative densitometry was calculated using ImageJ software. Phase assays, showing the orientation of the fimS promoter region of the fim operon, revealed that the wild-type TOP52 and TOP52/pBAD vector control were largely phase OFF while TOP52 ΔfimK and TOP52/pfimX had significant phase-ON populations.

cells than TOP52, which was primarily phase OFF. This phenotype could be partially complemented using pfimK. TOP52 ΔfimK/pfimK had a slightly larger phase-OFF population than the TOP52 ΔfimK/pBAD vector control. Expression of fimX in TOP52 resulted in a significant shift in the population to the phase-ON position compared to the TOP52/pBAD vector control, which was primarily phase OFF. The negative control, TOP52 ΔfimA-fimH, did not yield a PCR product because a portion of the corresponding DNA had been deleted.

For semiquantitative measures of type 1 pilus expression in these K. pneumoniae strains, we performed antigenic titration assays (Table 3). This assay tests the ability of various dilutions of type 1 pilus-monospecific antiserum to agglutinate bacteria. For wild-type TOP52, only dilutions of antibody up to 1:160 caused agglutination, while TOP52 ΔfimK agglutinated at levels up to 1:2,560, indicating much higher expression of type 1 pili in TOP52 ΔfimK. While the vector control resulted in similar antibody titers, complementation of TOP52 ΔfimK with pfimK resulted in a titer of 1:80 with arabinose induction, thus fully decreasing type 1 piliation down to levels similar to wild type. The negative control TOP52 ΔfimA-fimH, as expected, did not agglutinate even with the highest concentration of type 1 antiserum. It should also be noted that all strains had similar titers when antiserum to type 3 pili was used (data not shown), indicating that fimK does not affect expression of type 3 pili.

Additionally, negative-stain EM was performed to visualize type 1 pili in the different strains (Fig. 5). Wild-type TOP52 showed low to moderate levels of type 1 piliation, while TOP52 ΔfimA-fimH was exclusively bald. TOP52 ΔfimK and TOP52

ΔfimK/pBAD had a dramatic shift in the proportion of heavily type 1-piliated bacteria. This hyperpiliation phenotype was reduced in TOP52 ΔfimK/pfimK. TOP52/pfimX also displayed higher levels of piliation than the TOP52/pBAD vector control.

These data suggest that fimK inhibits type 1 piliation in TOP52 and that the addition of fimX can increase piliation. This was demonstrated at the transcriptional level in which loss of fimK or the addition of fimX resulted in an increased population of phase-ON bacteria and increased amounts of FimA. Wild-type TOP52 was primarily phase OFF, with inherently low production of type 1 pili possibly hindering its ability to cause infection in the urinary tract. The presence of fimK and lack of fimX in TOP52 may, in part, account for the significantly reduced bacterial titers early in infection compared to those in UTI89.

**fimK and fimX affect in vitro biofilm formation.** To determine if the loss of fimK or the gain of fimX has effects on type 1-dependent biofilm formation, which may be important in vivo, TOP52 biofilm assays were performed (Fig. 6A). Crystal violet was used to stain biofilms, and the amount of biofilm was quantified by measuring absorbance at OD$_{600}$. Wild-type TOP52 failed to make biofilm in this assay after 48 h as did the negative control, TOP52 ΔfimA-fimH. TOP52 ΔfimK, however, formed biofilm under the same conditions. TOP52 ΔfimK/pfimK made significantly less biofilm than the TOP52 ΔfimK/pBAD vector control ($P = 0.0128$). Thus, complementation of TOP52 ΔfimK with pfimK significantly reduced its ability to form biofilm. Expression of the recombinase fimX on a plasmid also conferred TOP52 with the ability to form biofilm. TOP52/pfimX made significantly more biofilm than the TOP52/pBAD vector control, which was unable to form biofilm ($P = 0.0002$). TOP52 ΔfimA-fimH/pfimX did not form biofilm (data not shown), verifying that biofilm formation required type 1 pilus expression.

UTI89 was transformed with pfimK in order to determine if fimK expression would also reduce UTI89 biofilm formation (Fig. 6B). Wild-type UTI89 formed a robust biofilm after 48 h. The negative control, UTI89 ΔfimA-fimH, was unable to form biofilm. Expression of pfimK in UTI89 decreased biofilm formation compared to that of the UTI89/pBAD vector control ($P = 0.0022$).

While wild-type TOP52 is unable to form type 1 pilus-dependent biofilms, loss of fimK or addition of fimX transforms a non-biofilm former into a biofilm producer. Interestingly, these

TABLE 3. Serological titers required to agglutinate K. pneumoniae strains using monospecific antiserum to type 1 pili

| Strain | Growth with arabinose | Titer[a] |
|---|---|---|
| TOP52 | − | 160 |
| TOP52 ΔfimK | − | 2,560 |
| TOP52 ΔfimK/pBAD | − | 1,280 |
| TOP52 ΔfimK/pBAD | + | 2,560 |
| TOP52 ΔfimK/pfimK | − | 1,280 |
| TOP52 ΔfimK/pfimK | + | 80 |
| TOP52 ΔfimA-fimH | − | <20[b] |

[a] Titers are the reciprocal of the highest dilution of serum causing visible agglutination after 5 min. Titers represent the median of three independent experiments.
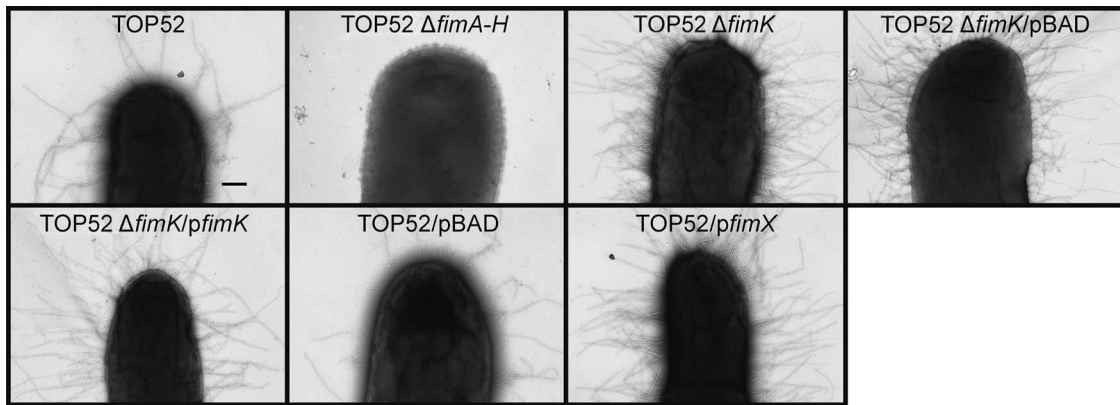[b] The lowest dilution of antibody used in this assay was 1:20.

FIG. 5. TOP52 Δ*fimK* and TOP52/p*fimX* appear hyperpiliated by negative-stain EM. Negative-stain EM was performed on TOP52 cultures, and representative images are displayed. TOP52 had low to moderate piliation, and the negative control TOP52 Δ*fimA-fimH* appeared bald. TOP52 Δ*fimK* was hyperpiliated compared to the wild type, and this phenotype could be partially complemented with p*fimK*. TOP52/p*fimX* also displayed higher levels of piliation than the vector control.
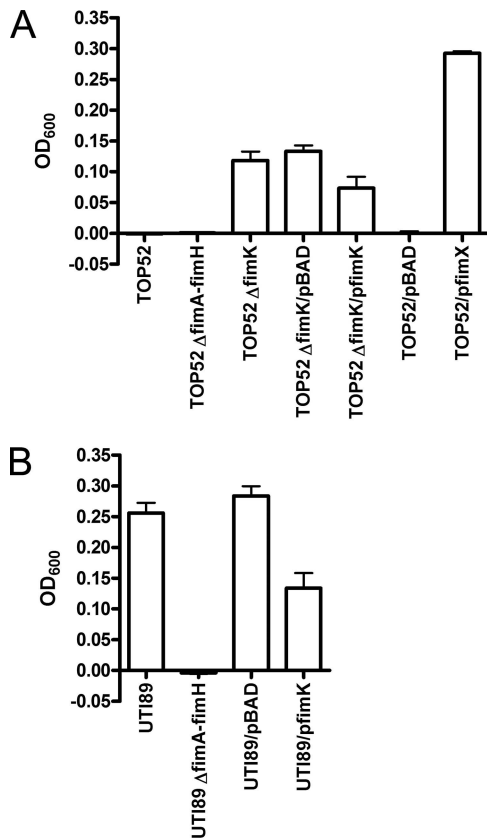


FIG. 6. The presence of *fimK* and *fimX* affects biofilm formation of TOP52 and UTI89. A 48-h biofilm assay was used to quantify biofilms produced by TOP52 strains (A) and UTI89 strains (B). TOP52 and the negative control TOP52 Δ*fimA-fimH* failed to form biofilms. However, TOP52 Δ*fimK* and TOP52/p*fimX* were able to form biofilms. TOP52 Δ*fimK*/p*fimK* formed significantly less biofilm than the vector control. Wild-type UTI89 formed a robust biofilm, and the negative control UTI89 Δ*fimA-fimH* was unable to form a biofilm. Complementation of UTI89 with TOP52 *fimK* resulted in significantly less biofilm formation than that produced by the UTI89/pBAD vector control. Data combine means of two independent experiments, each with duplicate plates. Error bars represent standard errors of the means.

data suggest that *fimK* acts as an inhibitory factor for biofilm formation in both TOP52 and UTI89, likely by affecting changes in expression of type 1 pili. Thus, *fimK* likely affects type 1 pili or biofilm regulatory pathways that are conserved in both *K. pneumoniae* and *E. coli*.

## DISCUSSION

Even though *K. pneumoniae* is the second most common cause of gram-negative UTI, few studies have analyzed the pathogenesis of *K. pneumoniae* in a UTI model. In this study, we infected mice with TOP52, a *K. pneumoniae* cystitis isolate, and found that, early in infection, it had lower titers than UTI89, a UPEC strain, in bladder and kidney. The reason for this early deficiency is likely related, in part, to the type 1 piliation state of TOP52.

TOP52 carries the gene *fimK*, which acts to reduce type 1 piliation. Additionally, TOP52 lacks *fimX*, a recombinase gene able to increase type 1 piliation. These factors may predispose *K. pneumoniae* to be a less-effective uropathogen than UPEC. The few bacteria that get introduced into the host bladder (3, 6, 27) are less likely to adhere to or invade the bladder surface because of less type 1 piliation. A less-piliated organism is presumably at a significant disadvantage upon entrance into the bladder and is less likely to survive to initiate significant infection in the host. In addition, it should be noted that while we analyzed the expression of *K. pneumoniae* type 1 pili, it is not clear that their function is the same as that of *E. coli* type 1 pili. More work needs to be done to determine if there are structural or functional differences between *K. pneumoniae* and UPEC type 1 pili that may also affect UTI pathogenesis.

TOP52 was able to form biofilm-like IBCs in the bladder; however, it made significantly fewer IBCs than UTI89. Interestingly, despite this defect, TOP52 titers are similar to those of UTI89 at later time points in infection. TOP52 may be able to persist at a relatively constant level throughout the course of infection while UTI89 infection levels decrease to the level of TOP52. This could be due to the influence of the host inflammatory responses to each uropathogen. Alternatively, there may be a lag in *K. pneumoniae* infection due to type 1 pilus

expression or other factors. Bacterial filamentation has been shown to be important for UPEC persistence in the murine cystitis model (31). It should be noted that no obvious differences in the levels of bacterial filamentation were observed in 24-h urine samples of UTI89-infected and TOP52-infected mice; however, quantitative measurements of filamentation were not performed. Ultimately, both UTI89 and TOP52 were able to effectively persist in similar ways in the murine cystitis model with an inoculum of $10^7$ CFU. In humans, relatively few bacteria are introduced into the bladder, and if *K. pneumoniae* has a lower probability of forming IBCs than UPEC, then *K. pneumoniae* may be cleared more efficiently.

This is the first account of a non-UPEC uropathogen forming IBCs in the bladder. Thus, the ability to form IBCs is not specific to UPEC. The IBC pathway may be a conserved mechanism by which many uropathogens evade the host innate immune response. Uropathogens may form these intracellular biofilms to multiply unhindered within the protected niche of the urothelial cell and even escape antibiotic penetration. Further studies need to be done to determine which uropathogens, including other *K. pneumoniae* isolates, are able to utilize an intracellular niche during acute bladder infection.

UPEC IBCs are transient in nature; however, UPEC has the ability to form long-lasting QIRs that can seed recurrent infection (46). These QIRs consist of bacterial rosettes within Lamp-1-positive endocytic vesicles. This study did not analyze long-term reservoir formation by *K. pneumoniae*. Future studies are needed to determine if *K. pneumoniae* can form QIRs and if these reservoirs can seed recurrent infection.

While it is clear that the presence of FimK inhibits type 1 piliation in *K. pneumoniae*, the mechanism of this inhibition is not known. FimK does contain an EAL domain, named after conserved residues, which has been implicated in the cleavage of the second messenger molecule, cyclic di-GMP (8, 58, 60). Loss of FimK may lead to higher levels of cyclic di-GMP, which has been implicated in biofilm formation, production of adhesive surface structures, and inhibition of motility (18, 34, 61). Further studies are needed to determine if FimK is an active phosphodiesterase with the ability to cleave cyclic di-GMP and how this second messenger may influence expression of type 1 pili in *K. pneumoniae*. FimK may be acting exclusively on the expression of type 1 pili, or FimK may also affect the expression of other adhesins or virulence factors in *K. pneumoniae* that may play a role in UTI. The ability of FimK to decrease type 1 pilus-dependent biofilm formation in UTI89 implies that it acts on a regulatory network affecting type 1 piliation that is conserved between *K. pneumoniae* and *E. coli*.

It is not clear why *K. pneumoniae* is programmed to keep type 1 pili primarily in the phase-OFF orientation. While type 1 pili have been shown to be important in cystitis, perhaps the presence of type 1 pili is detrimental for *Klebsiella* in other environments or conditions. UPEC type 1 pili have been shown to switch to a phase-OFF state in the kidney (56). *Klebsiella* may have evolved to be primarily a kidney pathogen without the need for high levels of type 1 pili in this niche. Type 1 pili may be antigenic in the lung or gut and may lead to *K. pneumoniae* clearance. Additionally, while capsule is an essential virulence factor for *K. pneumoniae* in many environments (2), it has also been shown to impede adhesion to and invasion of bladder epithelial cells (55). It has been noted that type 1 pili

and capsule may be inversely expressed or regulated in *K. pneumoniae* (40), and this may explain the predisposition toward a less-piliated state.

Our studies have demonstrated that the ability to utilize an intracellular niche during UTI is not specific to UPEC alone. Despite being able to progress through an IBC pathway, *K. pneumoniae* forms fewer IBCs and has lower titers in bladder than UPEC early in infection. These differences are related, in part, to type 1 pilus expression. This work begins to explain the difference in prevalence of *E. coli* and *K. pneumoniae* UTIs and emphasizes the importance of type 1 pilus regulatory elements in the ability of uropathogens to infect the urinary tract. Specifically, we have identified a novel regulatory gene in *K. pneumoniae*, *fimK*, capable of suppressing IBC and biofilm formation and impeding UTI pathogenesis.

## ACKNOWLEDGMENTS

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