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Conservation Of The Intracellular Bacterial Community Pathogenic Pathway In Urinary Tract Infection

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CONSERVATION OF THE INTRACELLULAR BACTERIAL COMMUNITY
PATHOGENIC PATHWAY IN URINARY TRACT INFECTION

by

David Aaron Rosen

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the Miller lab offered tremendous guidance with initial *K. pneumoniae* cloning and methods.

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DEDICATION

To my inspiration, best friend, and wife.

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ABSTRACT OF THE DISSERTATION

Conservation of the Intracellular Bacterial Community Pathogenic Pathway
in Urinary Tract Infection

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David Aaron Rosen

Doctor of Philosophy in Biology and Biomedical Sciences
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Professor Scott J. Hultgren, Chairperson

Urinary tract infections (UTIs) affect 13 million women annually in the United States. Uropathogenic *Escherichia coli* (UPEC) is the predominant etiologic agent of UTI; however, several other uropathogens, including *Klebsiella pneumoniae,* are also significant causative agents. In a murine cystitis model, UPEC utilize a multistep pathogenic pathway in which they invade and form intracellular bacterial communities (IBCs) within bladder facet cells. Type 1 pili, adhesive fimbriae, are necessary for UPEC binding and invasion of urothelial cells and formation of IBCs. UPEC ultimately disperse from the IBC, many with filamentous morphology, and proceed to infect other host urothelial cells. This work evaluates the conservation of the IBC pathogenic pathway across both host and uropathogen.

To determine if the IBC pathway occurs in human UTI, urine samples from women with acute, uncomplicated cystitis and from asymptomatic women were blindly analyzed. We found evidence of IBCs in 18% and filamentous bacteria in 41% of urines

from women with UTI. None of the urines from the asymptomatic comparative group or from Gram-positive UTIs showed evidence of IBCs or filaments. These findings suggest that the IBC pathogenic pathway characterized in the mice also occurs in humans.

Numerous non-UPEC, Gram-negative uropathogens were found competent for IBC formation in the murine cystitis model. The uropathogenesis of one of these IBC formers, *K. pneumoniae*, was compared to UPEC. *K. pneumoniae* was able to colonize the murine bladder and form IBCs, but to a lesser extent than UPEC early in infection. Much of this disparity can be attributed to differences in expression and function of type 1 pili. Specifically, *K. pneumoniae* encodes an extra *fim* operon gene, *fimK*, which inhibits expression of type 1 pili. Additionally, *K. pneumoniae* has a defect in the mannose-sensitive hemagglutination phenotype of type 1 pili specific to its FimH adhesin domain. These differences in expression and function of *K. pneumoniae* type 1 pili explain, in part, why *K. pneumoniae* is a less prevalent etiologic agent of UTI than UPEC. To further analyze host factors involved in UTI pathogenesis of *K. pneumoniae* and other uropathogens, we developed a streptozocin-induced diabetic model of UTI. Diabetic mice were found to be more susceptible to UTI, especially by non-UPEC uropathogens, compared to healthy mice.

This work revealed that the IBC pathogenic pathway occurs in human UTI and is common to several uropathogens, albeit to varying degrees of efficiency. Further insight into this conserved pathway may lead to enhanced UTI treatments and prevention of recurrence.

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Molecular Variations in *Klebsiella pneumoniae* and *Escherichia coli* FimH Affect Function and
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Abstracts

Rosen DA, Hooton TM, Stamm WE, Humphrey PA, Hultgren SJ, Evidence of Intracellular
Bacterial Communities in Humans with Acute Urinary Tract Infections. Presented at the
American Society of Microbiology General Meeting, Toronto, Ontario, May, 2007.

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Bacterial Community Pathway during Urinary Tract Infection in Mice and Humans, in BIRCWH
and SCOR Presentation Abstracts, Journal of Women's Health 16(8): 1108-1113, 2007.

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Chapter 1

Introduction

Overview

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting greater than half of all women in their lifetimes. While these infections can often be treated with short-course antibiotic therapy, recurrent infection is a serious and menacing problem. Interestingly, a high proportion of recurrent UTIs are caused by the same strain of bacteria as the initial infection. Uropathogenic *Escherichia coli* (UPEC) is the predominant etiologic agent of UTI, however, many other uropathogens are also capable of causing disease. A murine model of cystitis provides a valuable tool for studying host-pathogen interactions during the infection process. In this model, it has been shown that UPEC utilize adhesive structures known as type 1 pili to bind and invade the bladder urothelium. Within the superficial facet cells of the bladder, UPEC form biofilm-like intracellular bacterial communities (IBCs). This intracellular niche allows for bacterial replication in an environment largely protected from host innate immunity and possibly antibiotic treatment. UPEC disperse from IBCs, many in filamentous morphology, and progress to infect other urothelial cells. This IBC pathogenic pathway occurs in the murine bladder with multiple UPEC isolates, but has not been studied with other uropathogens or in the human host. Additionally, it has been shown that UPEC can form quiescent intracellular reservoirs (QIRs) within the urothelium that may ultimately seed recurrent infection. These findings change the paradigm of UTI pathogenesis and provide an additional explanation for recurrent infection. UTIs are not as simple as once thought and further insight into this pathogenic pathway and its conservation may lead to better therapies and treatments for this disease.

Urinary Tract Infections

Epidemiology

Urinary tract infections (UTIs) are the second most common infection and a significant cause of morbidity in the United States (35). They are responsible for 13 million office visits annually in the United States and an economic burden of greater than 2 billion dollars (19, 25). It has been estimated that there are 175 million cases of UTI worldwide each year (77). UTIs are more common in women than men, largely because of anatomical differences, and it is estimated that half of all women will experience at least one UTI in their lifetime (76).

Risk factors for developing a UTI in sexually active young women include recent sexual intercourse, recent use of a diaphragm with spermicide, and a history of recurrent urinary tract infections (34). It is widely known that certain subpopulations, such as diabetic or pregnant women, are more prone to UTIs which often result in serious sequelae (3, 74). In addition to community-acquired UTIs, nosocomial infection is extremely common, especially amongst elderly or catheterized patients, and can result in sepsis with significant morbidity and mortality (15).

Clinical Presentation, Diagnosis and Treatment

UTIs are often subdivided into multiple categories including uncomplicated and complicated UTIs. Complicated infections often involve anatomic abnormalities or medical instrumentation (97). Furthermore, infections can be categorized based on site of infection. These classifications include cystitis (infection of the bladder) and

pyelonephritis (infection of the kidney). Pyelonephritis is several-fold less common than cystitis but can result in more serious sequelae (19, 21). Patients with UTI can present with a wide range of symptoms including dysuria, frequency, hematuria, cloudy urine, suprapubic pain, low back pain, fever, chills, nausea and vomiting (35).

Diagnosis of UTI is confirmed by urine culture, however, patients are often treated empirically based on history, pyuria or urine reagent test strip analysis. A positive diagnosis requires greater than 10^2 bacteria/ml of a uropathogen in a symptomatic patient or greater than 10^5 bacteria/ml under any conditions (33, 47). Occasionally patients are found to have asymptomatic bacteriuria which may ultimately progress to symptomatic UTI.

Community-acquired UTIs are generally treated with oral antibiotics. Only short courses (usually 3 or 7 days) of antibiotics are generally required for acute, uncomplicated cystitis (32). A variety of antibiotics are used including antimetabolites, fluoroquinolones, cephalosporins and nitrofurantoin. In the majority of cases, antibiotics lead to quick resolution of symptoms, however, in rare cases UTI can progress to sepsis and even death (15, 83). Additionally, many uropathogens are developing resistance to multiple classes of antibiotics (26-28) warranting the need for new and effective UTI treatments.

Etiologic Agents

Uropathogenic *Escherichia coli* (UPEC) is the leading causative agent of UTI, responsible for up to 85% of community-acquired UTIs and 25% of nosocomial UTIs

(75). *Staphylococcus saprophyticus*, *Klebsiella pneumoniae* and other

Enterobacteriaceae are also significant outpatient uropathogens. Less frequent causes of

uncomplicated UTI are enterococcus and group B streptococcus. Complicated UTIs have

a more diverse etiology; organisms that rarely cause disease in healthy individuals can

cause significant disease in patients with underlying anatomic, immunologic, or

metabolic disorders (73). Diabetics, for example, have a much higher rate of UTI caused

by *Klebsiella pneumoniae* (30, 51).

Paradigm of Pathogenesis and Recurrence

The majority of UTIs are thought to arise when uropathogens present in the fecal

flora are able to colonize the vaginal introitus, enter the urethra and ascend into the

normally sterile urinary bladder (46). It has been assumed that upon entering the bladder,

uropathogens adhere to the host epithelium and initiate disease from this extracellular

niche. UPEC and other uropathogens have historically been thought to initiate the host

response from within the bladder lumen manifested by secretion of cytokines, pyuria and

the development of physical symptoms.

Recurrence after an initial episode of UTI is a serious and menacing problem. It

has been estimated that within six months of an index UTI, 25% of women experience a

recurrent episode and 3% have a third (20). Another study found that within one year of

an index UTI, 44% of women will have a recurrence (38). These recurrent episodes

occur despite appropriate therapy of the initial infection and negative follow-up urine

cultures. In addition to the frustration and morbidity associated with these recurrent

infections, a history of recurrent cystitis is associated with an increased risk of developing pyelonephritis and antibiotic resistant infection (69, 76).

The most apparent risk factor for developing recurrent UTI is having had a recent initial UTI (38). An event, or series of events, may occur during the primary UTI that predisposes the patient to a subsequent infection. Interestingly, a high proportion of recurrent UTIs are caused by the same strain of bacteria as the initial infection. One study that followed 58 women with an index UTI episode, found that 68% of recurrences were caused by the same strain of *E. coli* as determined by restriction fragment length polymorphism (RFLP) analysis (78). Another long-term prospective study demonstrated that the same strain of UPEC can cause a recurrent UTI up to 3 years later (9).   A third study involving female college students demonstrated that after first-time UTIs, 50% of recurrent strains appeared genotypically identical to the original bacterial strain (22). In a subset of patients from these studies, the causative strain was found in the vaginal and/or fecal cultures of these women despite negative urine cultures at the time. Based on these data, researchers have proposed that recurrences occur through reascension and reinoculation of the bladder lumen by a uropathogenic strain of bacteria that has persisted in the periurethral or fecal flora. The notion that these strains may be persisting in the bladder epithelium and seeding recurrence has not been studied.

Women who get frequent recurrent UTIs have been offered a variety of prevention strategies. Often these women are given antibiotics to take upon first presentation of symptoms, after sexual intercourse, or daily as UTI prophylaxis (62, 85, 86). These courses, however, promote uropathogen antibiotic resistance. Additional

studies have looked at cranberry products (5), *Lactobacillus* probiotic therapy (90), and vaccination against bacterial adhesins (49, 50). While many of these strategies are promising, they have yet to be validated in large-scale clinical trials.

Bladder Histology and Physiology

The anatomy of the urinary bladder is important for proper investigation of bacterial pathogenesis during cystitis. The bladder is a sac-like structure designed for the storage of urine prior to micturition. It is typically a sterile organ and the presence of microbes indicates an infectious process. The bladder is composed of four layers: the adventitia, muscularis, lamina propria, and urothelium (31) (Figure 1). The adventitia, or serosa, surrounds the bladder and consists of connective tissue and fat (not shown). The muscularis is composed of three layers of smooth muscle which contribute to bladder tone. The lamina propria is made of connective tissue and contains blood vessels, fibroblasts, collagen fibers and elastic fibers. The urothelium is a pseudostratified epithelium consisting of intermediate and basal transitional cells and superficial facet cells. This pseudostratified architecture has evolved in order to maintain impermeability while allowing for the constant expansion and contraction of the organ. Superficial facet cells are large, highly differentiated, typically binucleate cells composed of a thicker luminal leaflet of the apical plasma membrane termed the asymmetric unit membrane (AUM). The AUM is composed of plaques of hexagonal arrays of uroplakin complexes important in allowing the bladder to stretch while maintaining the permeability barrier (87). Uroplakin proteins (Ia, Ib, II, and III) are incorporated into fusiform vesicles that
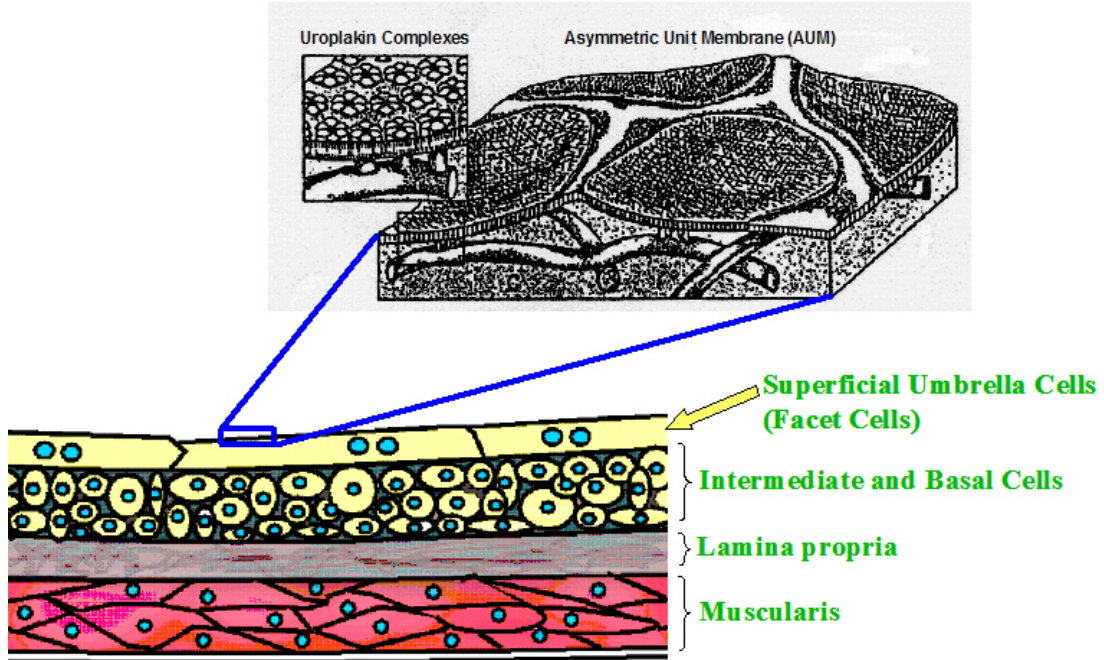
Figure 1. Structure of the Urinary Bladder
The bladder consists of four distinct layers: the serosa (not shown), muscularis, lamina propria and urothelium. The tunica muscularis is responsible for the contraction of the bladder during micturition. The lamina propria, or submucosal layer, consists largely of connective tissue and contains blood vessels and nerves. The mucosal layer, or urothelium, is a pseudostratified epithelium responsible for maintaining the impermeable barrier of the bladder preventing the leakage of toxins back into the body. The most superficial umbrella cells of the bladder, or facet cells, are often binucleate, very large, and form the asymmetric unit membrane containing hexameric uroplakin plaques.

are retracted from and fused into the facet cell membrane allowing for changes in surface area during bladder distention (57, 95). The bladder also produces a mucinous glycosaminoglycan (GAG) layer. GAGs are long anionic polysaccharides and include hyaluronic acid, chondroitin, and heparin. Their negative charge creates a stable water layer creating a shielding effect which also aids in prevention of toxin and solute leakage (68).

The transitional epithelium creates an impermeable barrier and prevents the leakage of toxins, solutes and other waste materials into tissue. The urothelium responsible for maintaining this barrier has a very slow rate of turnover. Under normal circumstances, the bladder epithelium has a slow but continuous turnover rate; a year or more is required in humans and approximately 40 weeks in mice for the bladder to fully turnover (40, 41).   While epithelial regeneration signaling pathways are generally under tight inhibition, sloughing of the terminally-differentiated superficial umbrella cells, or facet cells, leads to activation of bladder progenitor cells and fusion of bladder intermediate cells to form new facet cells (60). The bladder has evolved numerous mechanisms to maintain impermeability and disruption of this barrier likely leads to host symptoms associated with UTI and other bladder diseases.

Type 1 Pili

UPEC employ a wide array of virulence factors to successfully colonize and survive within the urinary tract, including adhesive organelles such as P, F1C, and S pili (4, 39, 65, 66), iron acquisition/transport systems (84), hemolysin (63), and flagella (48,

94). Among them, the best characterized and arguably most important virulence factor is type 1 pili. Type 1 pili are adhesive hair-like fibers that enable bacteria to bind numerous moieties including the bladder surface (36), Tamm-Horsfall protein (67), collagens (71), and laminin (45). They are critical in causing cystitis in several models of UTI (49, 50, 64). Specifically, type 1 pili are required for the binding to and the invasion of bladder urothelial cells. The proteins involved in biogenesis and composition of type 1 pili are encoded by *fimA-H* of the *fim* operon (Figure 2), the expression of which is regulated by an invertible promoter switch element (often termed *fimS*). This phase-varying switch is controlled by neighboring recombinases *fimB* and *fimE*, along with other recombinases in the genome including *fimX* (10, 29).

Type 1 pili consist of a thicker pilus rod, composed of FimA, and a thinner tip fibrillum, composed of FimF, FimG and the adhesin, FimH. Type 1 pili are assembled via the chaperone-usher pathway (37) (Figure 3). Subunits, such as FimA, the major subunit, contain an incomplete immunoglobulin fold and an N-terminal extension. Donor-strand complementation takes place in which the N-terminal extension of an adjacent pilus subunit complements and completes the immunoglobulin fold of its neighbor (8, 81). FimC is a periplasmic chaperone required to stabilize pilus subunits and escort them to FimD, the outer membrane usher (80). The type 1 pilus adhesin, FimH, contains a pilin domain and an adhesin domain which confers receptor specificity and tissue tropism (36). Atomic force microscopy studies have demonstrated that, once assembled, type 1 pili are able to reversibly unravel. This unraveling may be an essential mechanism for absorbing physiological shear forces in the urinary tract (56).
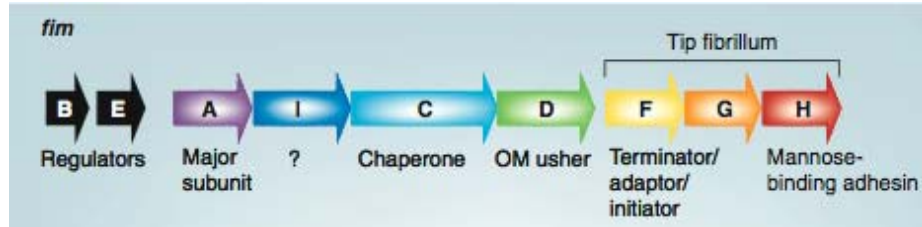
Figure 2. UPEC Type 1 Pili Encoded by the *fim* Operon
This schematic representation shows the UPEC *fim* operon. FimB and FimE are
regulatory recombinases controlling the expression of *fimA-H*. FimA is the major subunit
of type 1 pili and forms the helical rod. FimC acts as a chaperone, stabilizing subunits
prior to secretion through FimD, the outer membrane usher. FimF, FimG, and FimH
form the distal tip fibrillum. The adhesin FimH is composed of a pilin domain and an
adhesin or receptor binding domain. Figure adapted from (92).
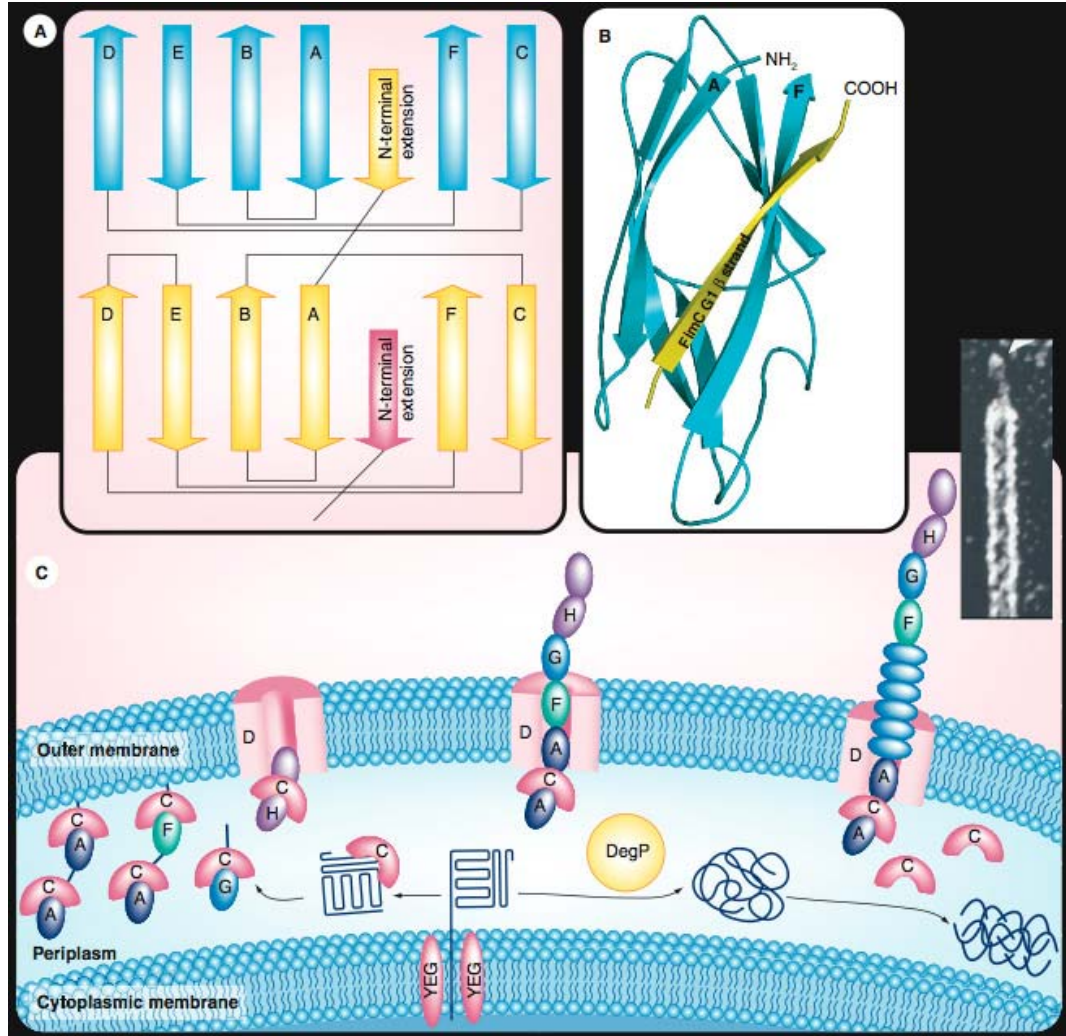
Figure 3. Donor Strand Exchange and Biogenesis of Type 1 Pili
(A) Schematic diagram showing donor strand complementation in which the N-terminal extension of one pilus subunit completes the immunoglobulin fold of its neighbor. (B) Ribbon diagram of FimH pilin domain stabilized by the chaperone, FimC, G1 strand. (C) Chaperone-Usher pathway of pilus biogenesis. FimC stabilizes subunits prior to incorporation into the pilus at the FimD usher. Excess or misfolded proteins are degraded by proteases such as DegP. (Inset) High resolution electron micrograph of type 1 pilus. Figure adapted from (92).

Intracellular Bacterial Community Pathogenic Pathway

A murine cystitis model has been established to better study the interplay between host and pathogen during a UTI. In this system, bacteria are inoculated directly into the bladder of the mouse via a small transurethral catheter. Using this murine model, it was demonstrated that UPEC utilize a multi-step pathogenic cascade during infection in which they invade bladder facet cells and replicate intracellularly (Figure 4) (2, 42, 58). Virtually all UPEC strains express type 1 pili (11). The FimH adhesin of type 1 pili recognizes mannosylated uroplakins on the luminal surface of facet cells (96). Recently, it was shown that UPEC type 1 pili are also able to bind β-1 and α-3 integrins on host urothelial cells (16).

Binding of UPEC to host urothelial cells induces a cascade of signaling events that ultimately leads to bacterial internalization (53). Within the superficial umbrella cells of the bladder, UPEC replicate to form early intracellular bacterial communities (IBCs) of loosely-packed bacteria (42). The bacteria then progress to form large, mid-stage IBCs of morphologically coccoid bacteria. This large IBC has biofilm-like characteristics including positive periodic acid-Schiff (PAS) staining (2). Type 1 pili also have an intracellular role and are required for UPEC to form tightly packed biofilms (93). As the IBC matures, the bacteria begin to flux out of the epithelial cell, some in filamentous morphology. These long filaments subvert engulfment by neutrophils, permitting survival of the bacteria and allowing them to re-enter the IBC developmental cascade. The transient SulA-mediated inhibition of cell division leading to bacterial filamentation is essential for UPEC persistence in the murine model of cystitis (43).
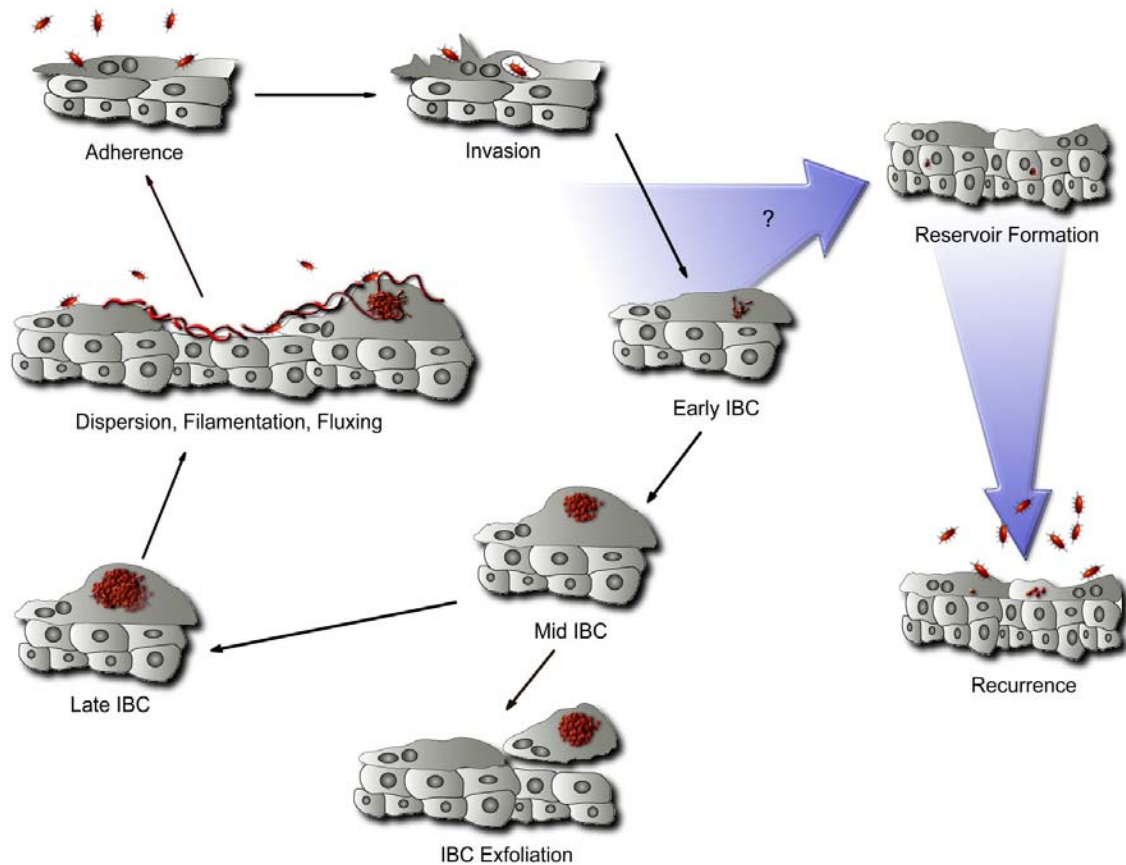
Figure 4. UPEC Intracellular Bacterial Community Pathogenic Pathway
UPEC utilize a multi-step pathogenic pathway in a murine cystitis model. Upon introduction into the bladder, UPEC bind to the host urothelium via type 1 pili and an invasion event ensues. UPEC escape into the facet cell cytoplasm and replicate to form loosely-packed early IBCs and ultimately tightly packed mid and late IBCs. Bacteria disperse from the IBC, many in filamentous morphology, bind to another host cell and can ultimately reenter this developmental pathway. In addition, UPEC are able to form intracellular reservoirs within different layers of the urothelium that may lead to recurrent bacteriuria.

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UPEC also form quiescent intracellular reservoirs (QIRs). QIRs are small membrane bound collections of bacteria within host urothelial cells. This reservoir can persist for several weeks in a quiescent state, protected from antibiotics and seemingly undetected by the host immune system (58, 82). External stresses and other cues may trigger bacterial replication and the reemergence of the reservoir, which may reside as bacterial rosettes within Lamp-1 positive vesicles, leading to recurrent bacteriuria (59). The presence of QIRs, if confirmed in humans, may explain recurrent UTI in a subset of women.

The host has evolved a variety of defense mechanisms in the event of bacterial colonization of the bladder. The utilization of the IBC pathogenic pathway by UPEC largely subverts many of these host responses allowing for bacterial persistence. Micturition represents a powerful force that bacteria must withstand in order to colonize the bladder. UPEC are able to do so via their strong interactions with facet cells using type 1 pili. The host recruits neutrophils to the bladder in the event of infection. UPEC are able to resist phagocytosis while hiding within urothelial cells or while in the filamentous morphology. Finally, infected host facet cells undergo apoptosis and are exfoliated and expelled in the urine. UPEC are able to form QIRs deep in the bladder tissue and remain dormant for weeks prior to causing recurrent bacteriuria.

The IBC pathogenic pathway has been characterized using the cystitis isolate, UTI89, in primarily C3H/HeN or C57BL/6 mice. Recently, however, numerous UPEC isolates from various clinical syndromes of UTI were shown to be competent for IBC formation in the murine cystitis model (23). Additionally, UPEC are able to form IBCs

in the urothelium of a variety of murine backgrounds (23). It would seem advantageous for other uropathogenic bacteria to progress through a similar pathogenic cascade in order to efficiently infect the host. Many other Gram-negative uropathogens also produce type 1 pili highly homologous to that of UPEC. However, with few exceptions, it is unknown whether other uropathogens are able to progress through an IBC-like pathway and/or establish an intracellular reservoir in the murine cystitis model.

K. pneumoniae and Other Non-UPEC Uropathogens

UTIs Caused by Non-UPEC Uropathogens.

While UPEC are the most common causative agent of UTI, there are several other important Gram-negative and Gram-positive uropathogens (73). Little is known about the pathogenesis of these organisms *in vivo*, however, many share common features with UPEC. For example, *K. pneumoniae and Proteus mirabilis* both express type 1 pili highly homologous to that of UPEC. *Enterococcus faecalis*, a Gram-positive bacterium responsible for many nosocomial UTIs, has been tested in the mouse model and no evidence of progression through the IBC pathogenic cascade similar to that of UPEC has been found (44). Instead, *E. faecalis* displayed a kidney tropism in the murine model. Additional studies need to be done to evaluate other non-UPEC uropathogens including *K. pneumoniae, P. mirabilis, S. saprophyticus,* Group B streptococci, *Enterobacter aerogenes, Citrobacter diversus* and *Pseudomonas aeruginosa* in the murine model of UTI.

K. pneumoniae Epidemiology

*K. pneumonia*e is a Gram-negative, non-motile, encapsulated, rod-shaped bacterium of the family *Enterobacteriaceae*. *Klebsiella* species are ubiquitous in both the environment and on mammalian mucosal surfaces. The detection rate for these bacteria in normal human stool samples ranges from 5 to 38% although they are identified in 77% of stool samples of hospitalized patients (89). The urinary tract is the most common site of *Klebsiella* infection, however it also causes pneumonia in compromised hosts (12). *Klebsiella* accounts for up to 17-29% of all nosocomial UTI and even shows higher incidence among specific groups of patients (51, 70). Among diabetic women, asymptomatic bacteriuria is several-fold more prevalent and acute pyelonephritis is ten times more common (61). A significant proportion of UTIs in diabetics are caused by *K. pneumoniae*. In one study, *Klebsiella* caused 26.7% of community-acquired diabetic UTI compared to 10.1% of non-diabetic community-acquired UTI (51). *Klebsiella* also caused greater than double nosocomial diabetic UTIs as compared to *E. coli* (37.7% versus 18.3%, respectively). Over the past twenty years, there has been a substantial increase in the spread of drug resistant strains of *Klebsiella*, particularly those producing extended-spectrum β-lactamases (55).

Fimbrial Expression in K. pneumoniae

K. pneumoniae encodes type 1 pili highly homologous to that of *E. coli* with greater than 80% amino acid sequence identity between both the adhesin, FimH, and the major pilus subunit, FimA, of the two species (24). However, in addition to the common

components, *Klebsiella* appears to contain an additional gene, termed *fimK* (formerly

fimI), downstream of the adhesin gene *fimH* (24). The function of the *fimK* gene, or

whether it is co-transcribed as part of the *Klebsiella fim* operon, is unknown. Like the *E.*

coli system, expression of *Klebsiella* type 1 pili appears to be controlled by phase

variation in which an invertible promoter element is flipped. However, the details of this

switch in *Klebsiella* are still unclear. It is known that *Klebsiella* encodes the regulatory

genes *fimB* and *fimE*, the recombinases involved in turning this promoter switch on and

off. It has been difficult to stimulate natural expression of these pili in *Klebsiella* species

in the laboratory setting. While some researchers have used serial passages through

various broth media over the course of weeks to stimulate the expression of type 1 pili,

most have utilized inducible plasmid expression systems or even recovered piliated

bacteria directly from the urine of patients (17, 52). *K. pneumoniae's* type 1 pili have

been shown to mediate attachment to rat bladder epithelial cells *in vitro* and the urothelial

surface of the rat bladder *in vivo* (17, 18).

   K. pneumoniae also possesses a type 3 pilus system that, unlike type 1 pili,

mediate a mannose-resistant, *Klebsiella*-like hemagglutination (MR/K-HA) of

erythrocytes that have been treated with tannin. These pili mediate attachment to the

basolateral surface of tracheal epithelial cells and basement membrane components (88).

Type 3 pili are encoded by the *mrk* operon with *mrkA* encoding the major subunit and

mrkD encoding the adhesin (1). Additionally, MrkF appears to be an anchoring protein

responsible for attachment of the pilus to the bacterial membrane and MrkE appears to be

a regulatory protein encoded upstream of *mrkA*. The regulation of type 3 pili is not clear

and subclones lacking the *mrkE* gene have no evident change in the level of pilus expression (1).

Additional Adhesins and Virulence Factors Expressed by K. pneumoniae

In addition to type 1 and type 3 pili, *K. pneumoniae* encodes at least two other non-pilus adhesins. The CF29K adhesin mediates attachment to Intestine-407 and CaCo-2 cell lines and is thought to be important in mediating attachment within the mammalian intestine (13). Another adhesin, KPF-28, has been observed in the majority of *Klebsiella* strains producing extended spectrum β-lactamases (14). In addition, it has recently been discovered that *K. pneumoniae* encodes a *luxS* homologue that produces functional type 2 quorum sensing signaling molecules (7). Analysis of a *luxS* mutant strain revealed that the LuxS-dependent signal plays a role in the early stages of *K. pneumoniae* biofilm formation *in vitro*.

K. pneumoniae generates massive layers of complex acid polysaccharides covering the bacterial surface. This capsule, which can be classified into 77 serological types, is essential for *Klebsiella* virulence (6). It is thought to aid in pathogenesis by protecting the bacterium from phagocytosis by polymorphonuclear leukocytes and prevent killing of the bacterium by bactericidal serum factors (91). Strains expressing the capsule antigens K1 and K2 are the most ubiquitous serotypes isolated from UTI and pneumonia patients and are thought to be especially virulent (72). Interestingly, this thick capsule has been shown to impede the *in vitro* adhesion to and invasion of ileocecal and bladder epithelial cells by *K. pneumoniae* (79). A separate study looking at *K.*

pneumoniae clinical isolates found that all 22 blood isolates were encapsulated and did not express type 1 pili while 10 of 11 UTI isolates expressed type 1 fimbriae but were unencapsulated (54). Studies of the contributions of these and other *Klebsiella* virulence factors to pathogenesis in the urinary tract are still needed.

Summary

UTIs are one of the most common forms of bacterial infection and new aspects of pathogenesis are being elucidated using a murine model of infection. The discovery of an IBC pathogenic pathway challenges the current dogma of UPEC pathogenesis. Moreover, the ability of UPEC to utilize an intracellular niche also has dramatic implications for UTI recurrence. The research presented in this dissertation examines the conservation of this IBC pathogenic pathway across both host and uropathogen.

While it is clear that UPEC progresses through an IBC pathway in the murine bladder, this pathogenic pathway has yet to be investigated in humans. It is imperative to determine whether UPEC possesses this ability in human infection. This finding would change the way the scientific and medical community think about UTI. Extrapolation to the human host is absolutely required before new treatment and therapeutic modalities can be employed to conquer this disease or prevent recurrence.

The IBC pathway has been discovered and characterized exclusively with the most prominent UTI etiologic agent, *E. coli*. Perhaps this pathway represents a virulence property that sets UPEC apart from other uropathogens. It is unclear whether other non-UPEC uropathogens are able to utilize an intracellular niche during infection. If so,

elucidating differences in aspects of the pathogenic cycle may help explain differences observed in the clinical course of non-UPEC UTIs.

K. pneumoniae is a prime uropathogen with which to initiate these studies because it is closely related to UPEC and may employ many of the same virulence factors in uropathogenesis. Despite its similarities to UPEC, *K. pneumoniae* causes significantly fewer UTIs in healthy individuals. By studying this uropathogen in the murine cystitis model, not only can we learn about *K. pneumoniae* uropathogenesis, but we can also begin to distinguish the factors and characteristics that make UPEC the predominant uropathogen.

Type 1 pili are extremely important virulence factors in UPEC infection, as they are required for multiple steps of the IBC pathogenic pathway. *K. pneumoniae* also encodes type 1 pili similar to those of UPEC. However, small differences in expression or structure of type 1 pili can have significant effects on uropathogenesis. Examining the extra gene, *fimK*, of *K. pneumoniae* and small differences in the FimH adhesin compared to UPEC may provide insights into pathogenesis and the role of type 1 pili.

Finally, while UTI has been extensively studied in healthy mouse models, UTI has seldom been studied in the diabetic setting. Diabetics are significantly more susceptible to UTI and are infected with a wider range of uropathogens. Development and characterization of a murine model of diabetic UTI could aid in our understanding of why diabetics are more prone to UTI and why specific uropathogens, such as *K. pneumoniae*, are more apt to infect diabetic individuals.

In summary, UTIs are one of the most common bacterial infections in the United States and the entire world. These infections are not simple extracellular infections as once thought. UPEC are able to invade the bladder and form IBCs during acute UTI. This research examines the IBC pathogenic pathway in different host settings and investigates the ability of different uropathogens to undergo these events. This work has revealed that the IBC pathogenic pathway occurs during human UTI and is common to several uropathogens, albeit to varying degrees of efficiency. Further insight into this conserved pathway may lead to enhanced UTI treatments and prevention of recurrent infections.

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Chapter 2

Detection of Intracellular Bacterial Communities

in Human Urinary Tract Infection

Modified from

Rosen et al. (2007) *PLoS Medicine*.

Abstract

Background.

Urinary tract infections (UTIs) are one of the most common bacterial infections and are

predominantly caused by uropathogenic *Escherichia coli* (UPEC). While UTIs are

typically considered extracellular infections, it has been recently demonstrated that UPEC

bind to, invade, and replicate within the murine bladder urothelium to form intracellular

bacterial communities (IBCs). These IBCs dissociate and bacteria flux out of bladder

facet cells, some with filamentous morphology, and ultimately establish quiescent

intracellular reservoirs that can seed recurrent infection. This IBC pathogenic cycle has

not yet been investigated in humans.

Methods and Findings.

We collected midstream, clean-catch urine specimens from 80 young healthy women

with acute uncomplicated cystitis and 20 asymptomatic women with a history of UTI.

Investigators were blinded to culture results and clinical history. Samples were analyzed

by light microscopy, immunofluorescence and electron microscopy for evidence of

exfoliated IBCs and filamentous bacteria. Evidence of IBCs was found in 14 of 80 (18%)

urines from women with UTI. Filamentous bacteria were found in 33 of 80 (41%) urines

from women with UTI. None of the twenty urines from the asymptomatic comparative

group showed evidence of IBCs or filaments. Filamentous bacteria were present in all 14

urines with IBCs compared to 29% (19/66) of samples with no evidence of IBCs

($P<0.001$). 14 of 65 (22%) urines from patients with *E. coli* infections had evidence of

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IBCs and 29 of 65 (45%) had filamentous bacteria, while none of the Gram-positive

infections had IBCs or filamentous bacteria.

Conclusions.

The presence of exfoliated IBCs and filamentous bacteria in the urines of women with

acute cystitis suggests that the IBC pathogenic pathway characterized in the murine

model may occur in humans. The findings support the occurrence of an intracellular

bacterial niche in some women with cystitis that may have important implications for

UTI recurrence and treatment.

Introduction

Urinary tract infections (UTIs) affect nearly 13 million women annually in the

United States alone and can result in significant costs and morbidity (7, 11, 13, 14, 25).

Uropathogenic *Escherichia coli* (UPEC) is the predominant causative agent, responsible

for up to 85% of community-acquired infections (23, 24). The majority of UTIs are

thought to arise when uropathogens present in the fecal flora colonize the vaginal

introitus, ascend into the bladder, and initiate a host response manifested by secretion of

cytokines, pyuria and the onset of symptoms (18).

Women have a 25% chance of experiencing a recurrent UTI within six months (8)

of an index episode and a 44% chance of recurrence within one year (15) despite

appropriate therapy of the initial infection and negative follow-up urine cultures. Over

one-half of all recurrent episodes of acute uncomplicated cystitis are caused by the same

bacterial strain as the initial infection (9, 26). As with initial UTIs, it is widely thought that recurrences occur through re-ascension and re-inoculation of the bladder lumen by a UPEC strain that has persisted in the periurethral or fecal flora following the previous UTI.

Recently, it has been demonstrated in a murine model of cystitis that UPEC utilize a multi-step pathogenic cycle during infection in which they progress through an intracellular niche within the bladder (Figure 1) (1, 16, 21). UPEC express adhesive fibers known as type 1 pili that mediate binding and invasion into luminal facet cells of the bladder (19, 20, 30). This intracellular niche is conducive for UPEC replication and formation of intracellular bacterial communities (IBCs) with biofilm-like properties (1). IBCs exist only transiently before the bacteria dissociate and flux out of the facet cell, many adopting a filamentous morphology (16). The filamentous UPEC avoid engulfment by neutrophils, thus allowing them to reinvade the urothelium (16). Upon infection, the host exfoliates and expels bladder epithelial cells into the urine. Ultimately, UPEC are able to form quiescent intracellular reservoirs comprised of small rosettes of bacteria within Lamp-1 positive endocytic vesicles that can persist for several weeks protected from antibiotics and presumably undetected by the host immune system (21, 22, 27). Epithelial turnover may cause the quiescent bacteria to revert into an actively replicative form leading to recurrent bacteriuria (22).

To date, this phenomenon has not been investigated in humans. In this study, with the knowledge that superficial bladder cells exfoliate in response to infection, we analyzed urine samples from women with acute, uncomplicated cystitis and from

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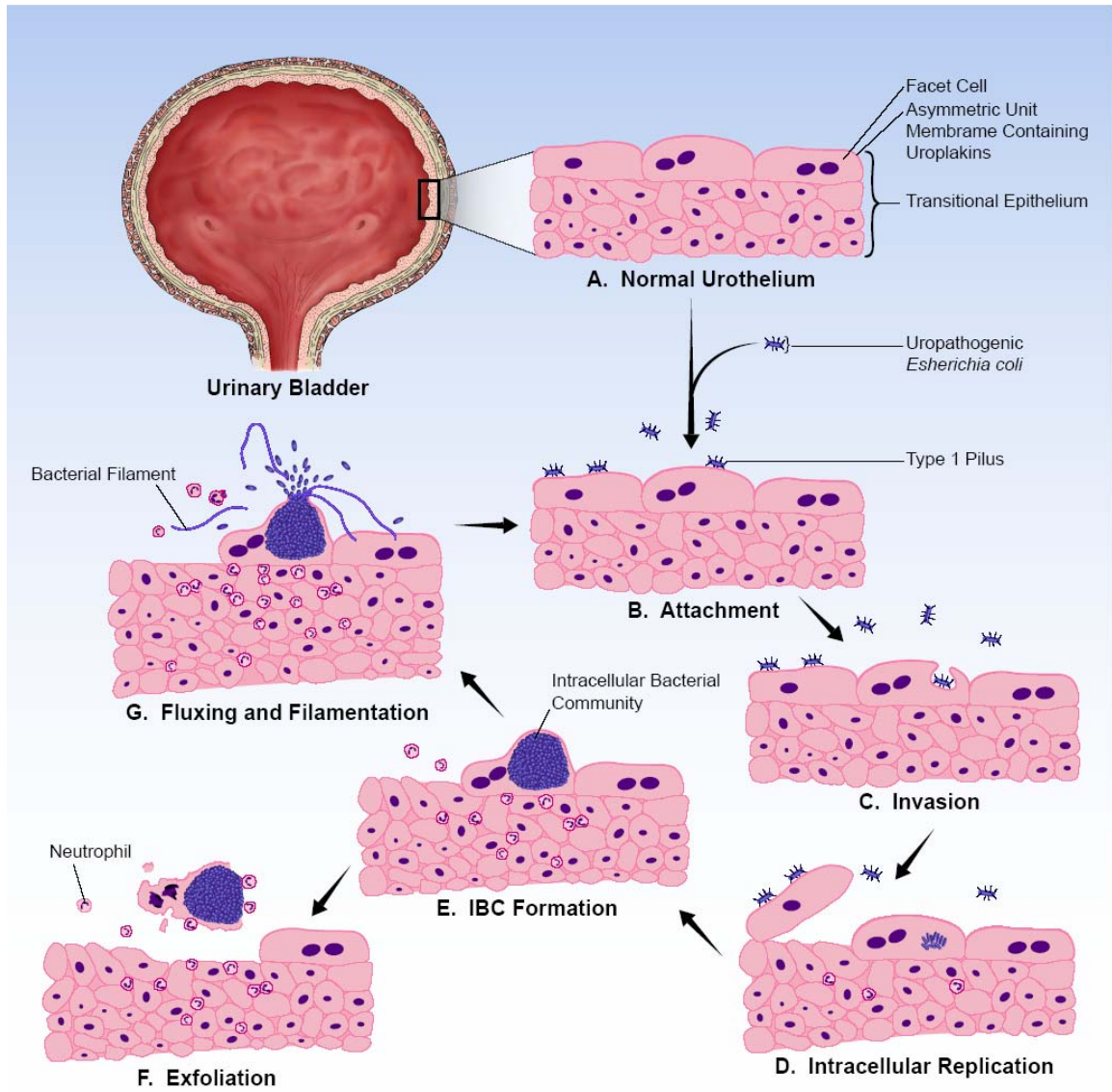
Figure 1. UPEC IBC Pathogenic Pathway Observed in the Murine Cystitis Model
The bladder urothelium (A) is a pseudostratified transitional epithelium lined by large facet cells. These cells have an apical asymmetric unit membrane containing uroplakins that help form the impermeable bladder barrier and also serve as receptors for UPEC. Bacteria introduced into the bladder adhere to the bladder surface via type 1 pili (B). Upon attachment, bacteria are able to invade (C) and replicate (D) within the facet cell cytoplasm. UPEC form large biofilm-like IBCs within these cells (E). Ultimately the bacteria flux out of their intracellular niche (G), some adopting a filamentous morphology, adhere to other host cells and re-enter the infectious cycle. During this process, infected urothelial cells are sloughed into the urine (F) and neutrophils are recruited to the site of infection.

asymptomatic women to investigate whether the IBC and/or filamentous *ex vivo* hallmarks of the IBC cycle were present.

Materials and Methods

Specimen Collection.

Specimens were collected from women between the ages of 18 and 49 who were enrolled in various studies of acute cystitis at the Student Health Center of the University of Washington in Seattle, Washington between January 2005 and January 2007. Urine samples were collected from 80 women with acute cystitis prior to treatment and a comparative group of 20 different asymptomatic women seen for follow-up at least one week after successful cystitis treatment. Exclusion criteria for both groups included antibiotic use in the prior week, phenazopyridine use in the prior two days, symptoms or signs of pyelonephritis, pregnancy, chronic illness such as diabetes, recent catheterization, or known anatomic or functional abnormalities of the urinary tract. Subjects provided midstream, clean-catch urine specimens for culture and analysis and questionnaire data which included information about their general health, current UTI episode, and UTI and sexual histories. All studies were approved by the Human Subjects Review Committee at the University of Washington and all participants provided written informed consent. A small number of urine samples from women with acute cystitis at the Medical Campus Student Health Clinic at Washington University in St. Louis, Missouri were also collected for optimization of microscopy and staining methods. The

collection and analysis of all specimens was approved by the Washington University Human Research Protection Office.

Urines were cultured by standard methods and acute cystitis isolates were banked at the University of Washington. A woman was considered to have a UTI if she had urinary symptoms and a urine culture of $\geq 10^2$ CFU/ml of a uropathogen(29). Asymptomatic bacteriuria was defined as the presence of $\geq 10^5$ CFU/ml of a uropathogen in a woman with no urinary symptoms. Urine hemocytometer white blood cell (WBC) counts were determined using Kova Glasstic Slide 10s with Grids (Hycor Biomedical Inc.). Within one hour of micturition, urines were separated into three aliquots that were fixed with 1% final concentration of formalin, 2.5% final concentration of glutaraldehyde or left unfixed and sent overnight on ice to Washington University in St. Louis, Missouri. To reduce potential bias, no clinical information was sent to investigators at Washington University prior to examination of the urine specimens for IBCs and filamentous bacteria.

Light microscopy.

Fixed and unfixed urine samples were cytocentrifuged for 6 min at 1,000 rpm onto poly-L-lysine-coated glass slides using a CytoPro 7620 cytocentrifuge (Wescor). Samples were then briefly heat fixed and stained with filter sterilized Protocol Hema 3 stains (Wright-Giemsa method, Fisher Scientific). Between 800 µl and 5 ml of each sample were screened by light microscopy using an Olympus BX51 light microscope (Olympus America).

Immunofluorescence.

Slides of unfixed and formalin-fixed urine specimens were prepared as described above, washed in filter-sterilized PBS, blocked in 1% BSA/0.3% Triton X-100 for 1 hr at room temperature, and subsequently incubated for 1 hr with rabbit anti-*E. coli* (1:1000, cross reacts with other *Enterobacteriaceae*, U.S. Biological) 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-, 594- or 546-conjugated donkey secondary antibodies (1:1000, Molecular Probes) for 30 min, slides were washed, stained with Hoescht or TOPRO-3, coverslipped with Fluoromount G (Southern Biotechnology Assoc.) and examined using an epifluorescent Zeiss Axioskop or Zeiss LSM410 confocal laser scanning microscope (Carl Zeiss).

Electron microscopy.

Electron microscopy was utilized to gain higher resolution images of IBCs in human urines. For transmission electron microscopy (TEM) and immunostaining, glutaraldehyde-fixed urine specimens were gently pelleted and embedded in 1% agarose. Samples were processed and immunostained as previously described using rabbit anti-*E. coli* antibody (1:200) followed by 18nm colloidal gold-conjugated anti-rabbit IgG (1:30, Jackson ImmunoResearch Laboratories) (19). All labeling experiments were conducted in parallel with controls in which primary antibodies were omitted. Sections were viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA) at 80 kV accelerating voltage.

For scanning electron microscopy (SEM), glutaraldehyde-fixed urine specimens were cytocentrifuged as described above onto 1% polyethylenimine-coated, 12 mm, round, glass cover slips. Processing of samples and SEM was done as previously described(19) and viewed with a Hitachi S-450 scanning electron microscope (Hitachi) at 20 kV accelerating voltage.

IBC and filament criteria.

The presence of IBCs and filamentous bacteria in urine was screened for by light microscopy and, if detected, verified by immunofluorescence. Specimens were considered positive for IBCs if large, dark-staining epithelial cells were observed containing collections of what appeared to be intracellular bacteria. Positive staining with anti-uroplakin antibodies detected by immunofluorescence and fluorescent intensity profiles were used as confirmation that bacteria were located within facet cells. Filamentation was confirmed using immunofluorescent staining and measurements were made to determine whether bacteria greater than 20 μm in length were present.

Mouse infection studies.

An *E. coli* isolate from a cystitis patient with findings of urine IBCs and filamentous bacteria was inoculated by transurethral catheterization into the bladders of eight-week-old female C3H/HeN mice (Harlan-Sprague Dawley) as previously described (20) in order to compare human and mouse urine cytology. Mouse urine samples were collected 30 hours post inoculation by bladder massage over a sterile 1.5 ml Eppendorf tube,

cytocentrifuged and stained as described above. Bladders were aseptically removed, fixed in neutral buffer formalin, paraffin embedded and sections were stained with hematoxylin and eosin (H&E).

Statistical analysis.

Categorical variables were compared using Pearson chi square and Fisher's exact tests as appropriate. Continuous variables were compared using the Mann-Whitney U test since these variables were not normally distributed. All tests were two-tailed and a P value less than 0.05 was considered significant. Analyses were performed using SPSS (version 14.0) and SAS (version 9.0).

Results

Study population.

The urines of 100 women at the University of Washington were analyzed in this study, 80 with acute uncomplicated cystitis and 20 asymptomatic women with a history of recent UTI. The median age of these 100 women was 22 (range 18-41) and the majority were white (75%) and never married (77%). This population reported a median of 3 (range 0-30) previous UTIs and 53% reported having had sexual intercourse in the 24-48 hours prior to sample collection.

Urine cultures.

The pathogens cultured from the 80 urines obtained from women with UTI included 65 (81%) *E. coli*, 4 (5%) *Staphylococcus saprophyticus*, 3 (4%) *Enterococcus*, 2 (3%) each of *Enterobacter aerogenes*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, and 1 (1%) *Citrobacter diversus*.   There was also 1 (1%) UTI with high levels of both *E. coli* and *S. saprophyticus*. None of the 20 comparative asymptomatic women had significant bacteriuria.

Light microscopy, immunofluorescence, and electron microscopy.

Light microscopic analysis of some urine samples revealed large biofilm-like collections of morphologically coccoid bacteria, often in association with cell nuclei or debris (Figure 2A). Analysis also revealed large, dark-staining cells containing what appeared to be intracellular bacteria in several samples (Figure 2B-D). Bacteria were sometimes observed seemingly protruding or exiting from these often binucleate cells – a common characteristic of facet cells. Additionally, long filamentous bacteria were seen in many of the urine samples (Figure 2E-H). Neutrophils, epithelial cells and morphologically normal bacteria were also observed in the majority of specimens.

Immunofluorescence verified the presence of urine IBCs and filaments (Figure 3). Cells were stained with antibodies to *E. coli* (Figure 3A) and uroplakin III, a marker that stains facet cell membrane and cytoplasmic vesicles (Figure 3B). Merged images (Figure 3C) revealed communities of bacteria within exfoliated urine facet cells. The distribution of fluorescent staining was defined quantitatively by generating profiles of fluorescent
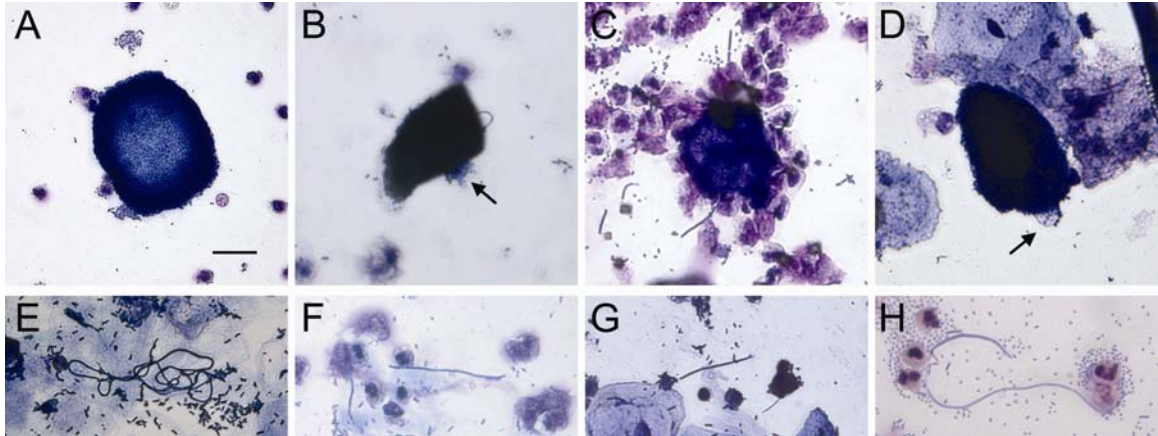
Figure 2. Light Microscopy Findings of Potential IBCs and Filaments in Urines from Women with Cystitis
Light microscopy screening of urines from cystitis patients revealed biofilm-like collections of bacteria (A), potential intracellular bacterial communities (B-D) and filamentous bacteria (E-H). Many large biofilm-like collections of small, morphologically coccoid bacteria were found in cystitis urine samples. Dark-staining epithelial cells with potential IBCs were observed often with bacteria that appear to be protruding from within (arrows). Tangled collections and individual long filamentous bacteria were also found in several of the samples. Scale bar, 20 μm, applies to all panels.
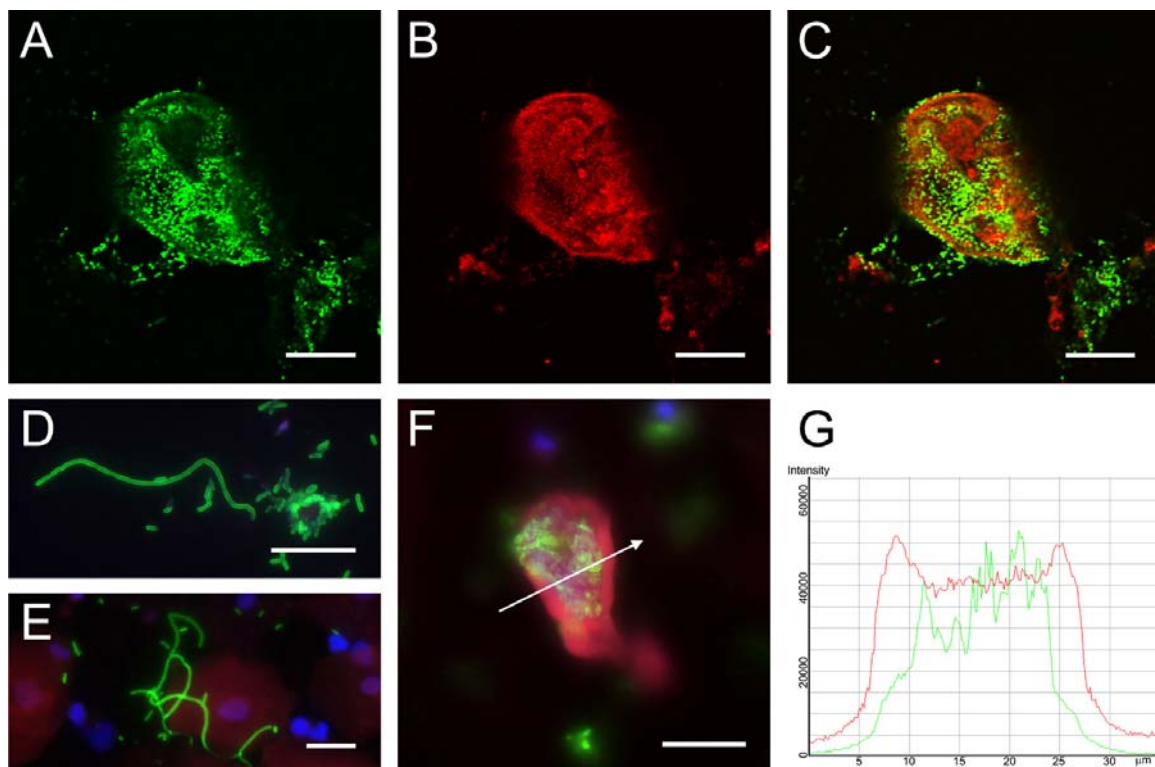
Figure 3. Immunofluorescence Confirmation of IBCs and Filaments in Urines from Women with Cystitis

Urines from women with cystitis were stained with antibodies against *E. coli* (green) and uroplakin III (red). Confocal microscopy analysis revealed large collections of bacteria (A) and cells with partial membrane and cytoplasmic uroplakin staining (B). Merged images (C) show these bacteria to be intracellular. Filaments of the uropathogen greater than 20 μm in length were also observed in many of the urine samples (D-E). To quantify fluorescence, a slice was taken through the middle of an IBC (F) and fluorescent intensity was analyzed along a traversing line (arrow). A representative fluorescent intensity distribution profile (G) shows peaks of uroplakin (red) staining corresponding to the facet membrane and *E. coli* (green) staining localized intracellularly. Scale bars, 20 μm.

intensity along lines traversing the middle of IBCs (Figure 3F-G). IBCs generally had higher peaks of uroplakin staining (red) for the cellular membrane while the uropathogen staining (green) was primary localized within the cell itself between the membrane peaks. In addition, filamentous bacteria were stained (Figure 3D-E) and measured to verify multiple bacterial filaments greater than 20 μm in a given positive sample.

SEM and TEM were used to generate higher resolution images of IBCs and filaments (Figure 4). Large collections of bacteria were often observed associated with nuclei, lipid membrane or other cellular debris (Figure 4A). The size, morphology and spacing of the bacteria in these large collections in human urines (Figure 4B) were similar to what has been observed in murine urine samples (Figure 4C). Intracellular bacterial filaments were also found within an exfoliated epithelial cell from an *E. coli* urine specimen (Figure 4D). By SEM, large spherical biofilm-like collections of bacteria (Figure 4E-F) were observed in fixed urine positive for IBCs and filaments. Higher magnification revealed that these bacteria adopted a smaller, more coccoid morphology as typically seen in mouse IBCs. Long filamentous bacteria were also found in these samples (Figure 4G). Immunoelectron microscopy of these *E. coli* urine specimens with anti-*E. coli* antibody demonstrated positive staining of the large collections of bacteria and filaments.

Comparison of cystitis and asymptomatic subjects.

Women with cystitis had higher urine WBC counts, more frequently reported recent sexual intercourse, and were slightly older than comparative subjects. The two

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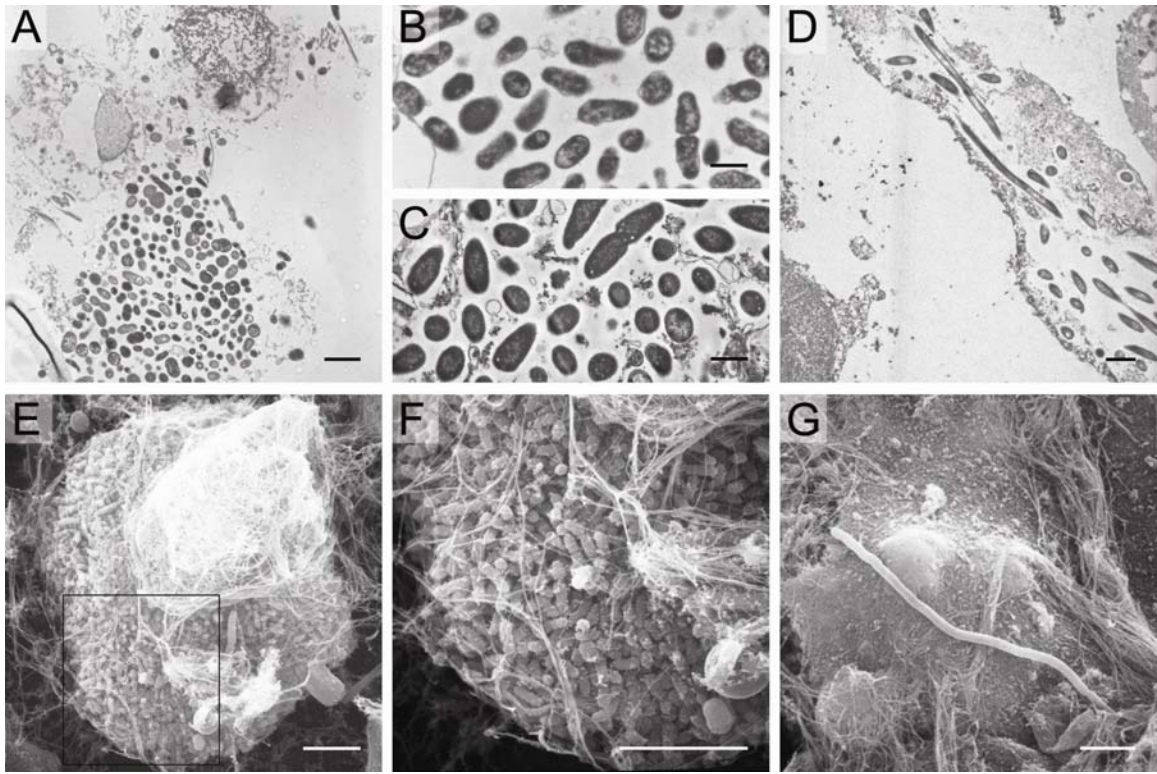
Figure 4. Electron Microscopy Findings in Urines from Women with Cystitis
TEM analysis of human cystitis urine specimens (A) revealed large collections of
bacteria associated with nuclei and other cellular debris. These collections of bacteria
from human urines (B) have similar morphology and organization as those recovered
from intact murine intracellular bacterial communities (C). Bacteria and filaments were
also observed intracellularly within exfoliated epithelial cells in a urine sample quickly
fixed and analyzed from an *E. coli* cystitis patient (D). SEM analysis of cystitis urines
deemed positive for IBCs and filaments captured large bacterial biofilm-like collections
(E-F) composed of bacteria with a smaller, more coccoid morphology than typical *E. coli*.
Long filaments were also captured by SEM (G).   Scale bars, 2 μm (A, D), 1 μm (B, C),
and 5 μm (E-G).

groups were similar in race, education, marital status, and number of previous UTIs.

IBCs were detected in 14 (18%) and filamentous bacteria in 33 (41%) of the 80 urine

specimens from women with acute cystitis. None of the 20 urines from the comparative

group showed evidence of IBCs or filaments (Table 1).

Characteristics of urines from women with cystitis.

All 14 (100%) of the urines with IBCs had filamentous bacteria compared with 19

of 66 (29%) urines without IBCs (P<0.001). IBCs and filaments were observed only in

urines with Gram-negative uropathogens, with none of the Gram-positive urines showing

such evidence (P=0.038). Every specimen that contained IBCs and the majority of

specimens positive for filamentous bacteria (30 of 33, 88%) were from UTIs caused by *E.*

coli. Filamentous bacteria were also observed in urine samples from cases of cystitis

caused by *E. aerogenes*, *K. pneumoniae*, and *P. mirabilis*.

Comparison of cystitis patients with and without urine IBCs and filaments.

To determine if evidence of IBCs and filaments was associated with any patient

or UTI characteristics, cystitis patients with and without IBCs or filaments were

compared. IBCs and filaments were associated with higher bacterial burdens (P=0.014)

and a longer duration of symptoms (P=0.007) (Table 2). In addition, patients with urine

IBCs or filaments were significantly younger than those without these findings (P=0.032)

(Table 2). There were no statistically significant differences in other demographic or

behavioral factors (Table 2).

Table 1. Comparisons of Women with Acute Cystitis versus Asymptomatic Women				
Characteristic	**Units**	**Cystitis Subjects** **(N=80)**	**Comparative Subjects** **(N=20)**	**P Value**
Demographics				
Age – median (min-max)	years	23 (18-41)	20.5 (18-37)	0.031
Race – no. (%) *	white	62 (77.5)	13 (65.0)	0.309
Education – no. (%) *	13-15 years	38 (47.5)	16 (80.0)	0.079
Marital Status – no. (%) *	never married	59 (73.8)	18 (90.0)	0.557
Previous UTIs – median (min-max)	no. episodes	3 (0-30)	2 (1-20)	0.574
Recent Intercourse – no. (%) [†]	yes	49 (61.3)	4 (20.0)	<0.001
Urine Findings				
IBCs – no. (%)	positive	14 (17.5)	0 (0.0)	0.066
Filaments – no. (%)	positive	33 (41.3)	0 (0.0)	<0.001
WBCs – median (min-max) [‡]	WBCs/μl	137.5 (0-2400)	0 (0-10)	<0.001

* P values refer to grouped categorical data, not all groups shown. Unknowns are excluded from analysis.

[†] Reported having had sexual intercourse 24-48 hours prior to sample collection.

[‡] WBC values too numerous to count ($\geq$2400/μl) were considered 2400 WBC/μl in analyses.

Table 2. Comparisons of Cystitis Subjects With and Without Urine IBCs or Filaments				
Characteristic	**Units**	**IBC or Filament Positive**	**IBC and Filament Negative**	**P Value**
		(N=33)	**(N=47)**	
Demographics				
Age – median (min-max)	years	21 (18-33)	23 (19-41)	0.032
Race – no. (%) *	white	25 (75.8)	37 (78.7)	0.915
Education – no. (%) *	13-15 years	19 (57.6)	19 (40.4)	0.483
Marital status – no. (%) *	never married	24 (72.7)	35 (74.5)	0.963
Previous UTIs – median (min-max)	no. episodes	2 (0-30)	3 (0-20)	0.664
Recent Intercourse – no. (%) *[†]	yes	18 (54.5)	31 (66.0)	0.333
Characteristics of Cystitis Episode				
First or Recurrent UTI – no. (%) *	recurrent	26 (78.8)	39 (83.0)	0.491
Uropathogen – no. (%)	Gram-negative$^{\alpha}$	33$^{\beta}$ (100.0)	40 (85.1)	0.038
	Gram-positive	0 (0.0)	7 (14.9)	
Quantity of Uropathogen – no. (%)	$<10^5$ CFU/ml	6 (18.2)	21 (44.7)	0.014
	$\geq 10^5$ CFU/ml	27 (81.8)	26 (55.3)	
Symptom Duration – med. (min-max)‡	days	3 (1-9)	2 (1-7)	0.007
WBCs – median (min-max) §	WBCs/µl	200 (10-2400)	75 (0-2400)	0.856

* P values refer to grouped categorical data, not all groups shown. Unknowns are excluded from analysis.

[†] Reported having had sexual intercourse 24-48 hours prior to sample collection.

[‡] Number of symptomatic days prior to and including the day of sample collection.

[§] WBC values too numerous to count ($\geq$2400/µl) were considered 2400 WBC/µl in analyses.

[α] Sample containing both *E. coli* and *S. saprophyticus* was considered Gram-negative in analysis.

[β] *E. coli* accounted for 30/33 of these Gram-negative infections.

Comparison of urine cytology in human and murine UTIs.

Histologic examination of mouse bladders infected with an *E. coli* strain isolated from a woman with acute cystitis revealed IBCs in superficial facet cells present in the tissue (Figure 5A), and sloughed into the lumen (Figure 5B). Filamentous bacteria and a robust inflammatory response were also observed. The urines of these mice contained several large, dark-staining IBCs (Figure 5C) that appeared morphologically indistinguishable from those of the original human urine specimen (Figure 5D).

Discussion

Our study provides evidence that episodes of *E. coli* cystitis in humans may involve an IBC pathogenic pathway similar to that observed in mice. Evidence of filamentous bacteria and exfoliated bladder facet cells containing large collections of *E. coli* were observed by light microscopy and confirmed by immunofluorescence. High resolution electron microscopy showed large biofilm-like IBCs and long filamentous bacteria. The expected variety of uropathogens (23) was cultured from infected women in the study, yet IBCs and filaments were not observed in urines from women infected with Gram-positive pathogens or in urines from asymptomatic women. Additionally, the urine cytologies observed in human and murine UTIs were indistinguishable.

Previous studies support our findings of an intracellular bacterial niche during UTI. In one study, human bladder biopsies from 33 women with recurrent urinary tract symptoms were analyzed after antibiotic therapy (5). Sixteen of these patients had sterile

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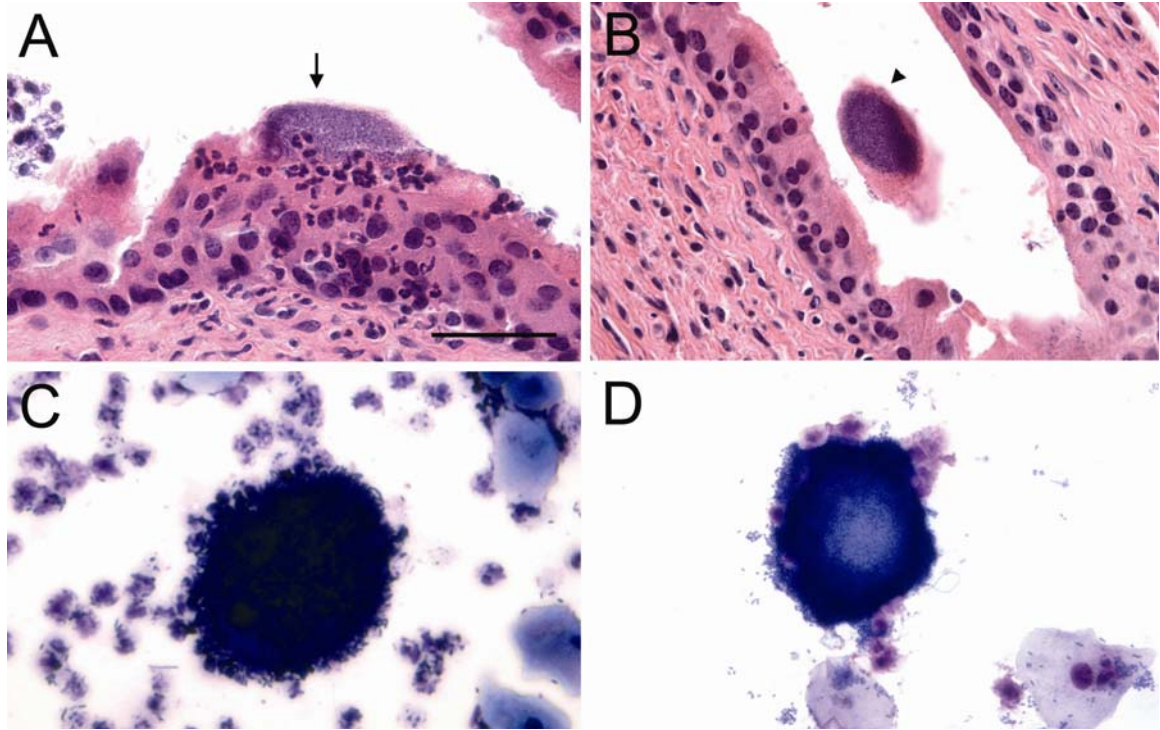
Figure 5. Mouse Trial and Comparison of Human and Mouse Urine
The *E. coli* isolate from a urine specimen positive for IBCs and filaments was inoculated
into mice where it progressed through the IBC pathogenic cycle. Several IBCs were
observed by H&E in the mouse bladder at 30 hours post infection (A, arrow). IBCs could
also be seen exfoliated into the bladder lumen (B, arrowhead). Urine collected from mice
at this time point were positive for IBCs (C). These IBCs were similar in morphology
and size to those formed by the same *E. coli* isolate in the original human urine specimen
(D). Scale bar, 50 μm, applies to all panels.

urine cultures; however, bacteria were cultured from eight patients' biopsies. These findings suggest that urine culture results may not necessarily reflect the bacteriologic status of the bladder epithelium and that there may be a persistent niche for uropathogens associated with bladder tissue. In addition, multiple studies have shown that UPEC strains are able to invade and replicate within human urothelial cell lines (6, 19, 21). More recently, it has been demonstrated that the majority of UPEC isolates from patients with various clinical syndromes of UTI are competent for IBC formation in the murine cystitis model (10).

The ability to form filaments is an important virulence property that facilitates persistence in the murine cystitis model (17). Filamentation can be the result of the Gram-negative bacterial SOS response which is induced by a variety of stressful stimuli, including antibiotics (28). In this study, however, patients had no recent history of antibiotic usage. Also, filamentation did not correlate with WBC counts in the urines of these patients as one might expect if this were a non-specific stress response. Thus, the filaments likely represented bacteria that had emerged from an intracellular niche as seen in the murine model. The filamentation event has been shown to be triggered by TLR-4-dependent inflammatory responses in the murine model of cystitis (17), however, whether this is also true in the human remains to be determined. We were able to capture electron microscope images of these filamentous bacteria within the intracellular niche of sloughed urothelial cells. In addition, the urine finding of filamentous bacteria significantly correlated with the presence of IBCs in these samples. This correlation could be explained by the association of these two entities in the same pathogenic

pathway or may be due to the higher bacterial burden in these samples and an enhanced ability to detect these endpoints by microscopy.

Interestingly, the presence of IBCs or filaments was associated with patients who had significantly longer self-reported durations of symptoms. This may relate to the kinetics of the IBC cycle and the time point at which IBCs are exfoliated into the urine, or it may be related to the higher burden of bacteria in the urines at these time points. Each urine analyzed represents a single point in what may be a temporally regulated pathogenic pathway. IBCs and filaments are likely transient (16) and, thus, could be missed if the sampling interval is not appropriate. In addition, the volume of urine analyzed represents a small proportion of the total sample micturated. Thus, the findings in this study may underestimate the prevalence of the IBC pathway.

Exfoliated cells found in the urine, while a useful reflection of the bladder tissue, have generally lost considerable structural integrity. Bacterial invasion into these damaged cells after they have been shed from the urothelium, while possible, seems unlikely. Mistaking such rare events for IBCs is implausible because samples were fixed upon micturition and bacteria would have needed significant time to multiply into large biofilm-like communities. While bladder biopsies are usually contraindicated in actively infected patients, future studies could assess biopsies from selected women with a history of recurrent UTI for the presence of an intracellular reservoir. As is observed in the murine model (21, 22, 27), a quiescent intracellular bacterial reservoir forms within the transitional epithelium. In this model, epithelial turnover and differentiation induce the bacteria within this reservoir to emerge and initiate the formation of new IBCs and

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recurrent bacteriuria. Reservoir formation, not explored in this study, could possibly

serve as a seed for recurrence in same-strain UTIs in some women.

This study involved a large number of well-characterized young healthy women

with acute cystitis and provides strong evidence that IBCs and filamentous bacteria can

be found in this group of women. However, there are also several limitations to this

study. We cannot extrapolate our results to women with different demographic

characteristics or with different clinical syndromes, such as asymptomatic bacteriuria,

pyelonephritis, or catheter-associated infections. We were not able, because of treatment

considerations, to collect serial specimens from these women with acute cystitis, and thus

we could not optimize collection time points when IBCs and filaments might be most

abundant. The patients were not followed prospectively and thus we cannot evaluate

important temporal associations between presence of IBCs or filaments and response to

treatment and patterns of recurrence. It is possible that the sensitivity of our assays might

have been better if we had been able to analyze urines quickly without the agitation of

shipping. The low number of non-*E.coli* infections makes it difficult to assess the ability

of other uropathogens to form filaments and IBCs during human infection. Lastly, it was

outside the scope of this study to perform genetic analyses of bacteria collected as has

been done in previous studies (4, 10). Thus, potential genetic differences and other

characteristics of bacteria collected in this study are not known at this time.

Despite these limitations, our data provide compelling evidence that there is an

association between IBCs, filaments and acute uncomplicated cystitis in young women

and are suggestive of an IBC pathway in a subset of these women. In this pathway in

mice, bacteria are able to invade and replicate within the urothelium where they are largely protected from host innate immunity, which may explain how the relatively few bacteria introduced into the bladder with urethral milking or sexual intercourse (2, 3, 12) are able to survive and multiply to numbers high enough to elicit symptoms in the host. The implications of these observations with regard to clinical management remain unclear. Urine IBCs and filaments may be prognostic indicators of specific clinical outcomes or women with these findings may benefit from different management or prevention strategies such as longer treatment or use of antimicrobials with better intracellular penetration. It is imperative that additional studies with appropriate patient follow-up be conducted to address these specific questions. Further understanding of the IBC pathogenic pathway in human infection may provide new potential targets and approaches to the treatment and prevention of UTI.

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Chapter 3

Utilization of an IBC Pathway in *Klebsiella pneumoniae* Urinary Tract Infection and the Effects of FimK on Type 1 Pilus Expression

Abstract

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 1721, was compared to the uropathogenic *Escherichia coli* (UPEC) isolate, UTI89, in a murine cystitis model. Bladder and kidney titers of TOP52 1721 were lower than UTI89 at early timepoints, but similar at later timepoints. TOP52 1721, like UTI89, formed biofilm-like intracellular bacterial communities (IBCs) within the murine bladder, albeit at significantly lower levels than UTI89. Additionally, filamentation of TOP52 1721 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 1721'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* encodes 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 of *fimK* was constructed and TOP52Δ*fimK* had higher titers and formed more IBCs in the murine cystitis model than wildtype. The loss of *fimK* or expression of *E. coli fimX* from a plasmid in TOP52 1721 resulted in a larger phase ON population, higher expression of type 1 pili, and gave TOP52 1721 the ability to form type 1-dependent biofilms. Complementation with p*fimK* decreased type 1 pilus expression and biofilm formation of TOP52Δ*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.

Introduction

Nearly 13 million women get urinary tract infections (UTIs) per year in the United States alone and greater than half of all women will experience a UTI during their lifetime (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 80-85% 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 UTI 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 twenty five 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). Like E. coli, expression of the *fim* operon is phase variable controlled by an invertible promoter element, *fimS*. *FimE* and *fimB*, are recombinases encoded in the type 1 pilus gene cluster that mediate 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* display superior *in vitro* binding to ciliated hamster tracheal epithelial cells and rat bladder epithelial cells

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(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 utilize a multi-step pathogenic pathway during human and murine infection in which they invade bladder facet cells and replicate intracellularly (1, 19, 30, 44, 53). Almost all UPEC strains express type 1 pili which consist of a thin tip fibrillum attached to a thicker pilus rod (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 beta1 and alpha3 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 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 are also able to 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 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, a *K. pneumoniae* cystitis isolate. TOP52 1721 (abbreviated TOP52) was isolated from the urine of a 26 year old woman with acute cystitis in 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.

TOP52 1721 and UTI89 mutant construction and complementation.

Targeted deletions of *fimK* and *fimA-H* in the *K. pneumoniae* isolate TOP52 were constructed with the pKOV vector as described previously (35). Flanking sequences of approximately 1000 bp on each side of the targeted gene were amplified with the indicated primers (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 wildtype.

UTI89Δ*fimA-H* was constructed using the red recombinase method as previously described (9, 45), with pKD4 as a template and the primers indicated (Table 2). PCR using flanking primers was used to confirm the deletion.

Table 1. Bacterial Strains and Plasmids		
Strain	**Description**	**Reference**
UTI89	UPEC cystitis isolate	(44)
UTI89Δ*fimA-H*	Knockout of *fimA-H* in UTI89	This study
TOP52 1721	*Klebsiella pneumoniae* cystitis isolate	(53) and this study
TOP52Δ*fimK*	Knockout of *fimK* in TOP52	This study
TOP52Δ*fimA-H*	Knockout of *fimA-H* in TOP52	This study
Plasmid	**Description**	**Reference**
pBAD33	Empty expression vector, P_{ara}, Cm^R	(24)
p*fimK*	*fimK*$_{TOP52}$ expression vector, P_{ara}, Cm^R	This study
p*fimX*	*fimX*$_{UTI89}$ expression vector, P_{ara}, Cm^R	(25)

Table 2. Primer Sequences

Primer Name	Primer Use	Primer Sequence
NotIFimKF1	Amplify 1kb *fimK* 5' Flanking Region	5'-CGGTAACGCGGCCGCGACTATCC GGAAACGATCACC-3'
HindIIIFimKR2	Amplify 1kb *fimK* 5' Flanking Region	5'-AACAAGCTTAGACGATCCGGATG ACTCAC-3'
HindIIIFimKF3	Amplify 1kb *fimK* 3' Flanking Region	5'-CATAAGCTTAGAAAAGCGCACCG GTTA-3'
SalIFimKR4	Amplify 1kb *fimK* 3' Flanking Region	5'-AACGTCGACAAAACAGAAACCAC AGCAACG-3'
3FimKCheckF	Check TOP52Δ*fimK*	5'-GTCGATTATCGGCATCACCT-3'
3FimKCheckR	Check TOP52Δ*fimK*	5'-GTGGCGAAGGTAGTGGAAAA-3'
NotIFimF1	Amplify 1kb *fimA* 5' Flanking Region	5'-CGGTAACGCGGCCGCGCATTAGC GAACTGCTGGAT-3'
BamHIFimR2	Amplify 1kb *fimA* 5' Flanking Region	5'-AACGGATCCCAGGGCTGACACAA CAATCA-3'
BamHIFimF3	Amplify 1kb *fimH* 3' Flanking Region	5'-TACGGATCCGTCAATCTCGGCCTG ACG-3'
SalIFimR4	Amplify 1kb *fimH* 3' Flanking Region	5'-AACGTCGACGGAGGGTATGTTGAC CGAAA-3'
3FimCheckF	Check TOP52Δ*fimA-H*	5'-CCAACGCCATCGCTATTC-3'
3FimCheckR	Check TOP52Δ*fimA-H*	5'-GATACACCACGATCCGCTTC-3'
3FimKcompF	Amplify TOP52 *fimK* for cloning into pBAD	5'-AATTCCATGGGGCATCACCTTTGT CTATCAATGATG-3'
3FimKcompR	Amplify TOP52 *fimK* for cloning into pBAD	5'-AATTGGTACCCGGTGCGCTTTTCT CGCACCCTCAACG-3'
pBADF	Check pBAD33 Clones	5'-TATCGCAACTCTCTACTGTTTCTC CA-3'
pBADR	Check pBAD33 Clones	5'-CTGTATCAGGCTGAAAATCTTCTC TCA-3'
UTI89fimA-H KO F	Knockout *fimA-H* in UTI89	5'-ATGAAAATTAAAACTCTGGCAATTG TTGTTCTGTCGGCTCTGTCCCTGAGCAT ATGAATATCCTCCTTAG-3'
UTI89fimA-H KO R	Knockout *fimA-H* in UTI89	5'-TTATTGATAAACAAAAGTCACGCCAA TAATCGATTGCACATTCCCTGCAGGTGT AGGCTGGAGCTGCTTC-3'
UTI89fimA-HcheckF	Check UTI89Δ*fimA-H*	5'-GTTTGTGCGCGATGCTTTCC-3'
UTI89fimA-HcheckR	Check UTI89Δ*fimA-H*	5'-GAGCACCTGAGCCTGCCATT-3'
2KlebphaseF	Amplify *fimS* Region for Phase Assay	5'-GGGACAGATACGCGTTTGAT-3'
2KlebphaseR	Amplify *fimS* Region for Phase Assay	5'-GGGACAGATACGCGTTTGAT-3'

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. This *fimK* nucleotide sequence has been deposited in the GenBank database under the accession number EU315065. 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 eight-week-old female C3H/HeN mice (National Cancer Institute) by transurethral catheterization as previously described (43). 25 ml static cultures were started from freezer stocks and grown at 37°C for 18 h and then subcultured 1:250 into 25 ml fresh media. These cultures were then grown statically at 37°C for 18 h, centrifuged for 5 min at 5800 rpm, and the resultant pellet was resuspended in PBS and diluted to approximately 2×10^8 CFU/ml. 50 μl of this suspension was used to infect each mouse with an inoculum of $1\text{-}2 \times 10^7$ 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 post inoculation 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).

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 post infection, 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 indicated times, 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, paraffin embedded and sections were stained with hematoxylin and eosin (H&E). For immunohistochemistry, unstained slides were deparaffinized, washed in filter-sterilized PBS, blocked in 1% BSA/0.3% Triton X-100 for 1 h at room temperature, and

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subsequently incubated for 1 h with rabbit anti-*E. coli* (1:1000, 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 (1:1000, Molecular Probes) for 30 min, slides were washed, stained with Hoescht, coverslipped with Fluoromount G (Southern Biotechnology Assoc.) and examined using an epifluorescent Zeiss Axioskop (Carl Zeiss).

FimA immunoblots, 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 1:2000 rabbit anti-type 1 pili primary antibody (48). Bacteria were normalized by OD_{600} and Coomassie staining was performed to verify similar levels of protein in each lane. 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) was 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. Serological titers using anti-type 1 pili monospecific serum or anti-type 3 pili monospecific serum (20, 21) were determined as previously described (10).

Negative stain electron microscopy.

Strains were cultured as described above for mouse infections and were prepared for electron microscopy (EM) as follows. Bacteria were absorbed onto Formvar-carboncoated 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 electron microscope (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, stained with crystal violet, and biofilms were quantified as previously described (47). Resulting graphs compare means +/- SEM of two independent experiments, each with duplicate plates.

Statistical analysis.

Continuous variables were compared using the 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 (GraphPad Software, version 4.03).

Results

TOP52 1721 compared to UTI89 in a murine cystitis model.

To contrast urinary tract infection of *K. pneumoniae* and *E. coli*, C3H/HeN female mice were inoculated by transurethral catheterization with 10^7 TOP52 1721 (abbreviated TOP52), a *K. pneumoniae* cystitis isolate, or 10^7 UTI89, an *E. coli* cystitis isolate. Inoculations with less than 10^7 TOP52 did not result in consistent infection in the mouse model (data not shown). Bladders and kidneys were harvested at various timepoints post inoculation and bacterial titers were determined. In the bladder (Figure 1A), TOP52 had significantly lower titers than UTI89 at both 6 h (P<0.0001) and 24 h (P=0.0487) timepoints. At later timepoints, TOP52 and UTI89 had similar bladder titers. At 2 weeks post inoculation, TOP52 and UTI89 had similar distributions of bladder titers. In the kidney (Figure 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 timepoints tested.

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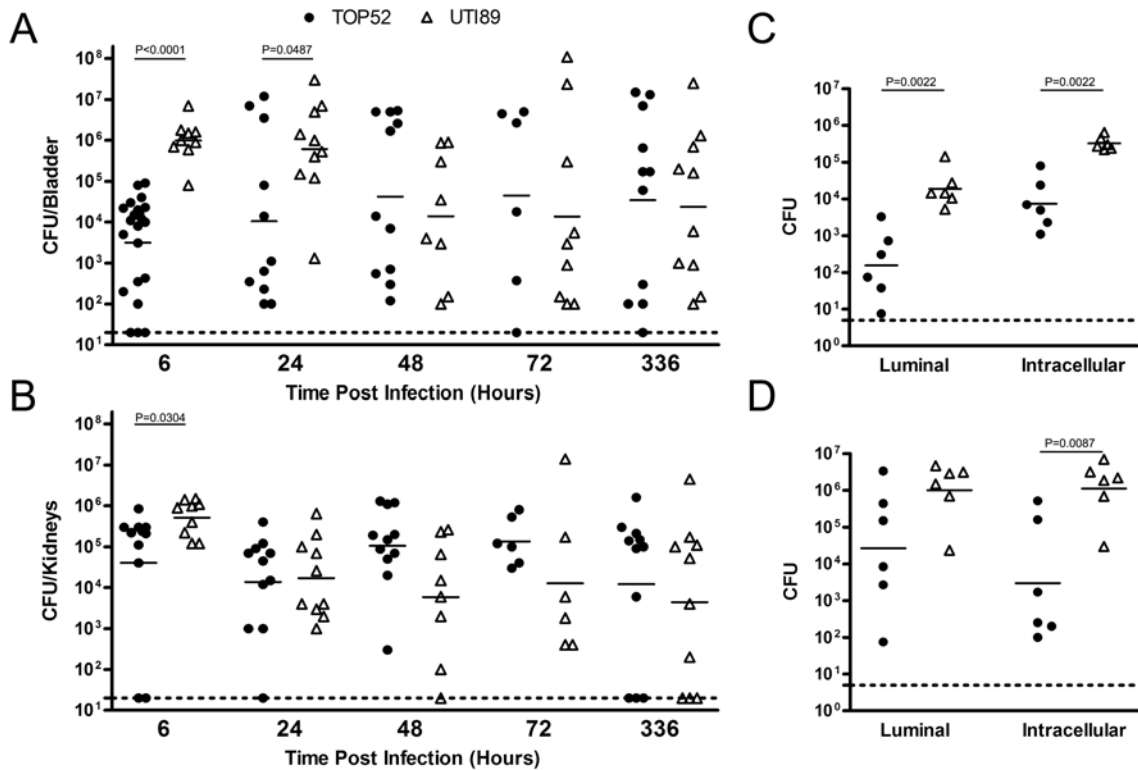
Figure 1. Timecourse of TOP52 and UTI89 bladder and kidney infections and gentamicin protection assays. Female C3H/HeN mice were inoculated with 10^7 TOP52 (●) or UTI89 (Δ) by transurethral inoculation. For organ titers, bladders (A) and kidneys (B) were harvested at various timepoints post infection and CFU were calculated. Titer data are combined from N=3 independent experiments. For *ex vivo* gentamicin protection assays, bladders were harvested at 6 h (C) and 24 h (D) post infection and luminal and intracellular populations of bacteria were quantified. Short bars represent geometric means of each group and full dotted lines represent limits of detection. Significant P-values, as calculated using the Mann-Whitney *U* test, are displayed.

It has been shown that UPEC invade into bladder epithelial cells during infection. The ability of *K. pneumoniae* to invade into bladder epithelial cells during UTI was investigated using gentamicin protection assays of the bladder performed at 6 h and 24 h post inoculation. At each timepoint, 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 post inoculation. At 6 h (Figure 1C), TOP52 had significantly more intracellular bacteria than luminal bacteria (P=0.0087) as did UTI89 (P=0.0022). By 24 h (Figure 1D), the number of intracellular bacteria was similar to the number of extracellular bacteria for both TOP52 and UTI89. However, the number of both luminal and intracellular TOP52 bacteria was significantly less than 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 post inoculation and numerous sections were analyzed in order to further characterize the intracellular

population of TOP52. Large intracellular bacterial communities (IBCs) were found in

TOP52-infected bladders (Figure 2A). These biofilm-like communities, found within

superficial bladder facet cells, appeared morphologically identical to those found in

UTI89-infected bladders (Figure 2B). Immunohistochemistry staining with *Klesbsiella*-

specific antibodies confirmed that these IBCs were composed of *K. pneumoniae* (data not

shown). Staining with antibodies against type 1 pili (green) confirmed that type 1 pili are

expressed within TOP52 IBCs (Figure 2C) and UTI89 IBCs (Figure 2D), similar to what

has been reported previously for UTI89 (62). At 24 h post inoculation, filamentous

bacteria, another hallmark of the IBC pathway, were found in urines from both TOP52-

infected (Figure 2E) and UTI89-infected (Figure 2F) mice.

IBCs were visualized and quantified by *lacZ* staining of whole mounted, fixed

bladders at 6 and 24 h post inoculation of TOP52 or UTI89 (Figure 2G). TOP52 had

significantly fewer IBCs than UTI89 at both 6 h (P=0.0012) and 24 h (P=0.0221)

timepoints. 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 TOP52ΔfimK has higher bacterial titers in

vivo.

We investigated whether the differences in bacterial burden and IBC formation

between TOP52 and UTI89 observed at early timepoints in infection may, in part, relate
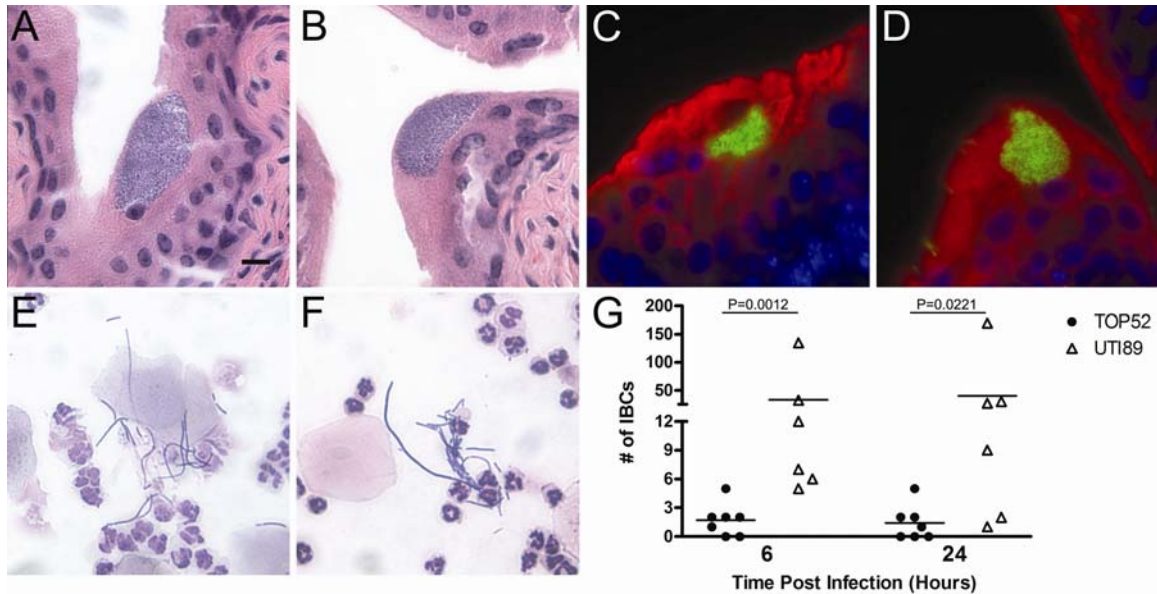
Figure 2. TOP52 progresses through an IBC pathogenic pathway. Histologic analysis of murine bladders after 6 h infection with TOP52 (A) or UTI89 (B) revealed morphologically identical IBCs within superficial urothelial cells. Immunohistochemistry demonstrated that bacteria express type 1 pili within IBCs of both TOP52 (C) and UTI89 (D) infections. Staining was done with antibodies against type 1 pili (green), the facet cell marker, uroplakin III (red), and nuclei were visualized with Hoescht staining (blue). Urine samples from mice at 24 h post infection with TOP52 (E) or UTI89 (F) contained long bacterial filaments. IBCs were quantified (G) after visualization by LacZ staining and revealed that TOP52 forms significantly fewer IBCs than UTI89 at both 6 and 24 h post inoculation. Short bars represent geometric means and significant P-values, as calculated using the Mann-Whitney U test, are displayed. Scale bar, 10 μm, applies to panels A-F.

80

to 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* (Figure 3A). *FimK* was also found to be present

immediately downstream of *fimH* in both other strains of *K. pneumoniae* in which

sequence of this region was available (22, 41). Sequence of the 1410 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 of *fimK* was constructed and the ability of TOP52Δ*fimK* to colonize

and form IBCs was investigated in the murine cystitis model. At 6 h post inoculation,

TOP52Δ*fimK* had significantly higher bladder (Figure 3B) and kidney (Figure 3C) titers

than wildtype 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. TOP52Δ*fimK*/p*fimK* had significantly lower bladder and kidney titers than the

empty vector control, TOP52Δ*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 (Figure 3D). While

wildtype TOP52 formed a mean of 1.8 IBCs per bladder, TOP52Δ*fimK* formed 4.6

(P=0.0312). TOP52Δ*fimK*/pBAD vector control formed a mean of 5.7 IBCs per bladder

while TOP52Δ*fimK*/p*fimK* formed 3.3 IBCs per bladder (P=0.0939). These data suggest
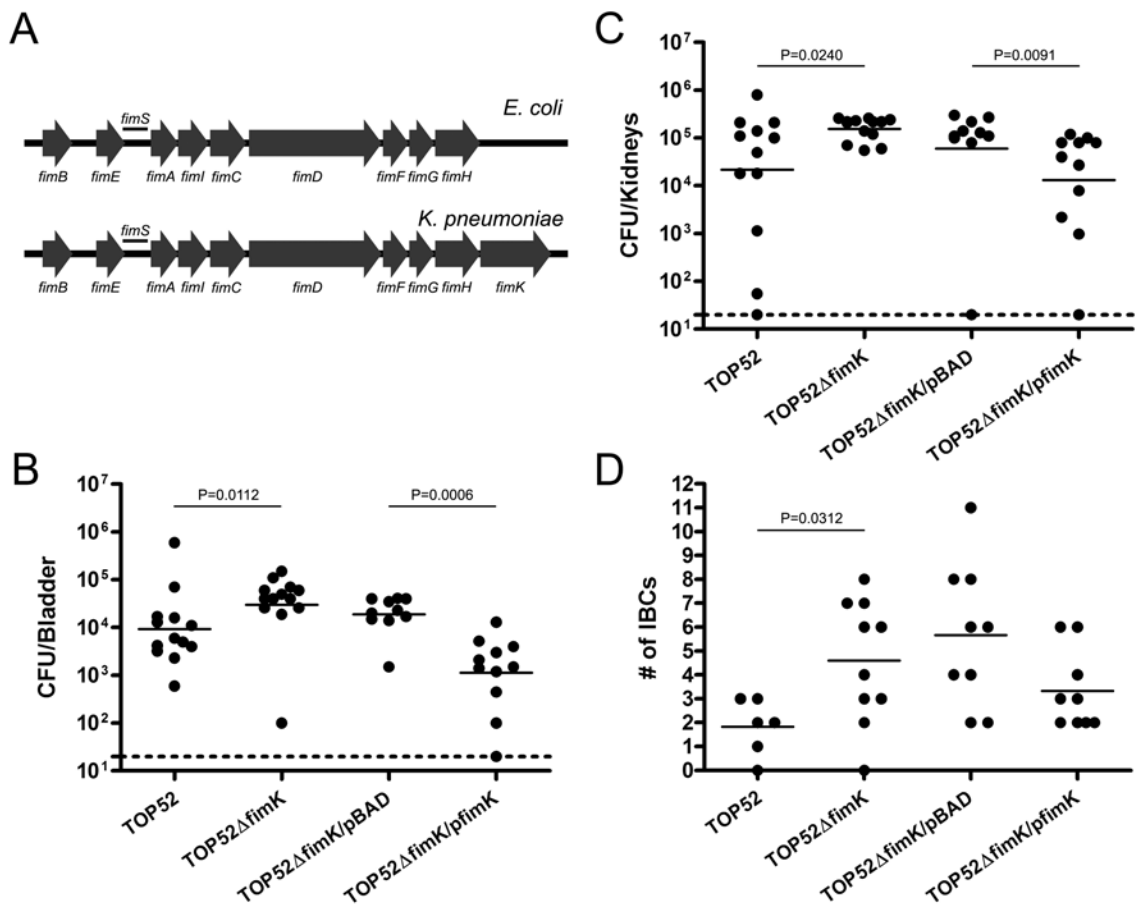
Figure 3. TOP52Δ*fimK* has higher organ titers and forms more IBCs in the murine cystitis model than wildtype. (A) The *fim* operons of *E. coli* and *K. pneumoniae*, which encode type 1 pili, have similar composition and organization, but *K. pneumoniae* contains an extra gene of unknown function, *fimK*. (B) TOP52Δ*fimK* had higher 6 h titers in the bladder than wildtype and this phenotype was complemented with p*fimK*. (C) TOP52Δ*fimK*, compared to wildtype, also had consistently higher kidney titers at 6 h and this difference could be complemented back to wildtype levels. (D) Enumeration of IBCs at 6 h post infection revealed that TOP52Δ*fimK* forms more IBCs than TOP52. Titer and IBC data are combined from N=3 independent experiments. Short bars represent geometric means of each group and full dotted lines represent limits of detection. Significant P-values, as calculated using the Mann-Whitney *U* test, are displayed.

that loss of *fimK* results in higher bacterial titers, greater number of IBCs, and overall more efficient bladder infection.

TOP52ΔfimK and TOP52/pfimX have higher expression of type 1 pili.

We hypothesized that the lower bladder colonization and IBC formation 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 bladder titers and increased abundance of IBCs observed with the loss of *fimK* could potentially be explained by higher expression of type 1 pili. 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 immunoblots 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 (Figure 4). TOP52Δ*fimK* produced a larger FimA band compared to TOP52 as determined by immunoblot. Complementation of TOP52Δ*fimK* with p*fimK* reduced the size of the FimA band close to wildtype levels. Moreover, the addition of *fimX* in TOP52 also led to the production of a larger FimA band compared to vector control. As a control, *fimA-H* was deleted in TOP52. This negative control strain, TOP52Δ*fimA-H,* did not produce a FimA band.
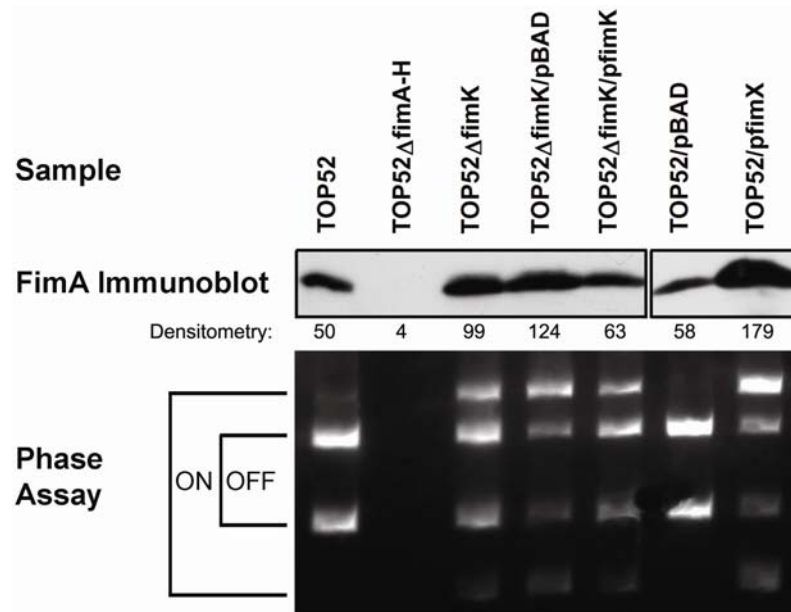
Figure 4. TOP52Δ*fimK* and TOP52/p*fimX* have higher expression of type 1 pili than wildtype and vector controls. FimA immunoblots showed that TOP52Δ*fimK* makes more type 1 pili than wildtype TOP52. Expression of FimA was complemented down to wildtype levels with p*fimK*. TOP52/p*fimX* also produced a much larger FimA band than vector control. Bacteria were normalized by OD_{600} and Coomassie 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 wildtype TOP52 and TOP52/pBAD vector control were largely phase OFF while TOP52Δ*fimK* and TOP52/p*fimX* had significant phase ON populations.

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. TOP52Δ*fimK* had a larger proportion of phase ON cells compared to TOP52 which was primarily phase OFF. This phenotype could be partially complemented using pfimK. TOP52Δ*fimK*/p*fimK* had a slightly larger phase OFF population than TOP52Δ*fimK*/pBAD vector control. Expression of *fimX* in TOP52 resulted in a significant shift in the population to the phase ON position compared to TOP52/pBAD vector control which was primarily phase OFF. The negative control, TOP52Δ*fimA-H,* did not yield a PCR product because a portion of the corresponding DNA had been deleted.

For semi-quantitative 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 pili monospecific antisera to agglutinate bacteria. For wildtype TOP52, only dilutions of antibody up to 1:160 caused agglutination, while TOP52Δ*fimK* agglutinated at levels up to 1:2560, indicating much higher expression of type 1 pili in TOP52Δ*fimK*. While vector control resulted in similar antibody titers, complementation of TOP52Δ*fimK* with p*fimK* resulted in a titer of 1:80 with arabinose induction, thus fully decreasing type 1 piliation down to levels similar to wildtype. The negative control TOP52Δ*fimA-H*, as expected, did not agglutinate even with the highest concentration of type 1 antisera. It should also be noted that all strains had similar titers using antisera to type 3 pili (data not shown) indicating that *fimK* does not affect expression of type 3 pili.

Table 3. Serological titers of *K. pneumoniae* strains using anti-type 1 pili monospecific serum.		
Strain	**Arabinose**	**Titer[ab]**
TOP52	-	160
TOP52Δ*fimK*	-	2560
TOP52Δ*fimK*/pBAD	-	1280
TOP52Δ*fimK*/pBAD	+	2560
TOP52Δ*fimK*/p*fimK*	-	1280
TOP52Δ*fimK*/p*fimK*	+	80
TOP52Δ*fimA-H*	-	<20[c]

[a]Titers are the reciprocal of the highest dilution of serum causing visable agglutination after 5 minutes.

[b]Titers represent the median of three independent experiments.

[c]The lowest dilution of antibody used in this assay was 1:20.

Additionally, negative stain EM was performed to visualize type 1 pili in the different strains (Figure 5). Wildtype TOP52 showed low to moderate levels of type 1 piliation while TOP52Δ*fimA-H* was exclusively bald. TOP52Δ*fimK* and TOP52Δ*fimK*/pBAD had a dramatic shift in the proportion of heavily type 1 piliated bacteria. This hyperpiliation phenotype was reduced in TOP52Δ*fimK*/p*fimK*. TOP52/p*fimX* also displayed higher levels of piliation compared to TOP52/pBAD vector control.

These data suggest that *fimK* inhibits type 1 piliation in TOP52 and addition of *fimX* can increase piliation. This was demonstrated at the transcriptional level in which loss of *fimK* or addition of *fimX* resulted in an increased population of phase ON bacteria and increased amounts of FimA. Wildtype 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 UTI89.

fimK and fimX affect in vitro biofilm formation.

To determine if the loss of *fimK* or the gain of *fimX* have effects on type 1-dependent biofilm formation which may be important *in vivo*, TOP52 biofilm assays were performed (Figure 6A). Crystal violet was used to stain biofilms and the amount of biofilm was quantified by absorbance at OD_{600}. Wildtype TOP52 failed to make biofilm
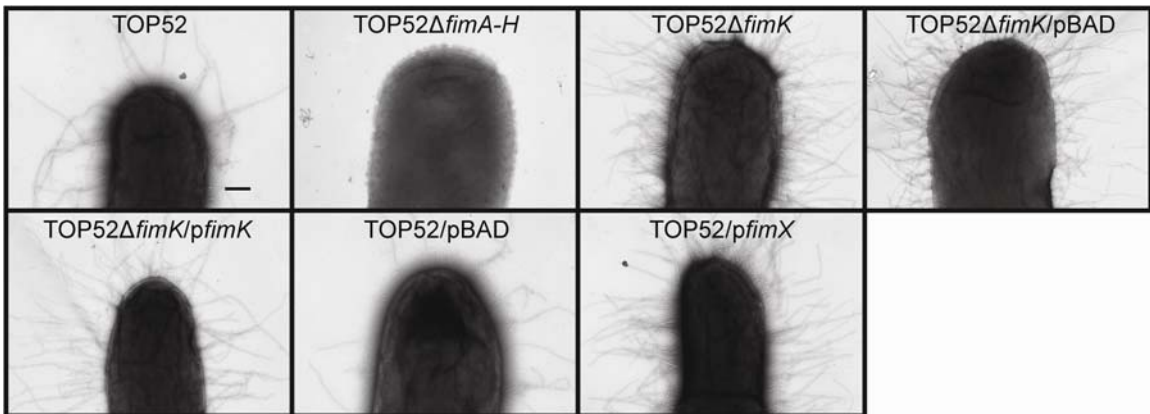
Figure 5. TOP52Δ*fimK* and TOP52/p*fimX* appear hyperpiliated by negative stain electron microscopy. Negative stain EM was performed on TOP52 cultures are representative images are displayed. TOP52 had low to moderate piliation and the negative control TOP52Δ*fimA-H* appeared bald. TOP52Δ*fimK* was hyperpiliated compared to wildtype and this phenotype could be partially complemented with p*fimK*. TOP52/p*fimX* also displayed higher levels of piliation compared to vector control.
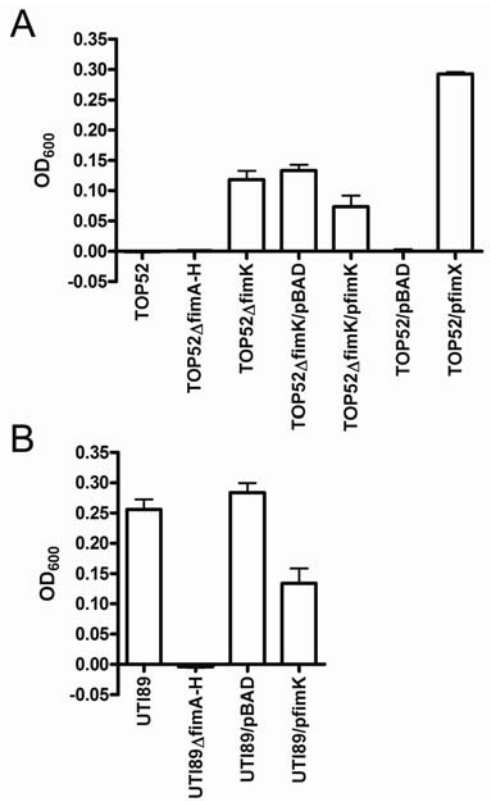
Figure 6. The presence of *fimK* **and** *fimX* **affect biofilm formation of TOP52 and UTI89.** A 48 h biofilm assay was used to quantify biofilms produced by TOP52 (A) strains and UTI89 strains (B). TOP52 and the negative control TOP52Δ*fimA-H* failed to form biofilms. However, TOP52Δ*fimK* and TOP52/p*fimX* were able to form biofilm. TOP52Δ*fimK*/p*fimK* formed significantly less biofilm than vector control. Wildtype UTI89 formed robust biofilm and the negative control UTI89Δ*fimA-H* was unable to form biofilm. Complementation of UTI89 with TOP52 *fimK* resulted in significantly less biofilm formation than UTI89/pBAD vector control. Data combine means of 2 independent experiments, each with duplicate plates. Error bars represent standard error of the mean.

in this assay after 48 hours as did the negative control, TOP52Δ*fimA-H*. TOP52Δ*fimK*,

however, formed biofilm under the same conditions. TOP52Δ*fimK*/p*fimK* made

significantly less biofilm than TOP52Δ*fimK*/pBAD vector control (P=0.0128). Thus,

complementation of TOP52Δ*fimK* with p*fimK* 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/p*fimX* made significantly more biofilm than

TOP52/pBAD vector control which was unable to form biofilm (P=0.0002).

TOP52Δ*fimA-H*/p*fimX* did not form biofilm (data not shown) verifying that biofilm

formation required type 1 pilus expression.

UTI89 was transformed with p*fimK* in order to determine if *fimK* expression

would also reduce UTI89 biofilm formation (Figure 6B). Wildtype UTI89 formed a

robust biofilm after 48 hours. The negative control, UTI89Δ*fimA-H,* was unable to form

biofilm. Expression of p*fimK* in UTI89 decreased biofilm formation compared to

UTI89/pBAD vector control (P=0.0022).

While wildtype TOP52 is unable to form type 1 pili-dependent biofilms, loss of

fimK or addition of *fimX* transforms a non-biofilm former into a biofilm producer.

Interestingly, these data suggest that *fimK* acts as an inhibitory factor for biofilm

formation in both TOP52 and UTI89, likely through affecting changes in expression of

type 1 pili. Thus, *fimK* is likely affecting type 1 pili or biofilm regulatory pathways that

are conserved in both *K. pneumoniae* and *E. coli*.

Discussion

Despite being 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 1721, a *K. pneumoniae* cystitis isolate, and found that, early in infection, it had lower bladder and kidney titers than UTI89, a UPEC strain. The reason for this early deficiency is likely related, in part, to the type 1 piliation state of TOP52.

TOP52 encodes the gene *fimK* which acts to reduce type 1 piliation. Additionally, TOP52 lacks *fimX*, a recombinase able to increase type 1 piliation. These factors may predispose *K. pneumoniae* to be a less effective uropathogen compared to 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 those of *E. coli*. More work needs to be done to determine if there are structural or functional differences of *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 compared to UTI89. Interestingly, despite this defect, TOP52 titers are similar to those of UTI89 at later timepoints in infection. TOP52 may be able to persist at a relatively constant level throughout the course of infection, while UTI89

infection levels decrease to that 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 fashion in the murine cystitis model with a 10^7 inoculum. In humans, relatively few bacteria are introduced into the bladder and if *K. pneumoniae* has a lower probability of forming IBCs relative to UPEC, than *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 quiescent intracellular reservoirs (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 exclusively acting on expression of type 1 pili or FimK may also affect the expression of other adhesins or virulence factors in *K. pneumoniae* which may play a role in UTI. The ability of FimK to decrease type 1-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 towards 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 bladder titers 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* UTI and emphasizes the importance of type 1 pili 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.

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Chapter 4

Molecular Variations in *Klebsiella pneumoniae* and *Escherichia coli*

FimH Affect Function and Pathogenesis in the Urinary Tract

Abstract

Type 1 pili mediate binding, invasion and biofilm formation of uropathogenic

Escherichia coli (UPEC) in the host urothelium during urinary tract infection (UTI) via

the adhesin FimH. In this study, we characterized the molecular basis of functional

differences between FimH of the UPEC isolate, UTI89, and the *Klebsiella pneumoniae*

cystitis isolate, TOP52 1721. Type 1 pili characteristically mediate mannose-sensitive

hemagglutination (MSHA) of guinea pig erythrocytes. Although the adhesin domain of

K. pneumoniae TOP52 1721 FimH (FimH$_{52}$) is highly homologous to that of *E. coli,* with

an identical mannose binding pocket and surrounding hydrophobic ridge, it lacks the

ability to mediate MSHA. In addition, FimH-dependent biofilm formation in *K.*

pneumoniae is inhibited by heptyl mannose, but not methyl mannose, suggesting the need

for contacts outside of the mannose binding pocket. The binding specificity differences

observed for FimH$_{52}$ resulted in significant functional differences seen in *K. pneumoniae*

UTI pathogenesis compared to *E. coli*. Infections in a murine model of UTI

demonstrated that although the *K. pneumoniae* strain TOP52 1721 required FimH$_{52}$ for

invasion and IBC formation in the bladder, FimH$_{52}$ was not essential for early

colonization. This work reveals that a limited amount of sequence variation between the

FimH of *E. coli* and *K. pneumoniae* results in significant differences in function and

ability to colonize the urinary tract.

Introduction

Bacterial adherence to host mucosal surfaces is often an important first step in the infection process. This is especially true in the case of urinary tract infections (UTIs) (59). It is estimated that half of all women will experience at least one UTI in their lifetime (49), the vast majority of which are caused by uropathogenic *Escherichia coli* (UPEC) and other *Enterobacteriaceae* (48). An essential step in UPEC infection of the bladder is adherence to the host urothelial surface via type 1 pili (2, 27, 29). Type 1 pili are assembled via the chaperone/usher pathway (3, 30, 53). They are adhesive hair-like fibers consisting of cylindrical pilus rods composed of FimA pilin subunits and small tip fibrillae composed of FimF, FimG and the adhesin, FimH (6, 31). The FimH adhesin recognizes mannosylated uroplakins and β-1 and α-3 integrin receptors on the luminal surface of bladder urothelial cells (17, 29, 63). Binding of UPEC to host cells induces a cascade of signaling events that ultimately leads to bacterial internalization and the formation of biofilm-like intracellular bacterial communities (IBCs) (1, 17, 22, 32, 39, 43, 51). IBC formation is also dependent on type 1 pili (62). Ultimately bacteria disperse from this intracellular niche and progress to infect other urothelial cells.

Type 1 piliated bacteria have historically been characterized by their ability to agglutinate guinea pig red blood cells (RBCs) in a mannose-sensitive manner (14, 15, 52). This mannose-sensitive hemagglutination (MSHA) phenotype of *E.coli* expressing type 1 pili requires the FimH adhesin. FimH consists of two domains: an amino-terminal adhesin domain (receptor binding domain) and a carboxy-terminal pilin domain (8, 29, 31). FimH recognizes mannosylated glycoproteins including those present on the host urothelium through its adhesin domain. FimH-mediated adhesion can be inhibited by D-mannose or oligosaccharides containing terminal mannose residues (5, 19-21).

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Additionally, it has been demonstrated that the FimH adhesin domain binds more tightly to α-D-mannosides with longer alkyl chains. Heptyl mannose was found to have the highest affinity for FimH (5). In animal models, neutralization of the adhesin by FimH-specific antibodies protects from UPEC cystitis (35, 36). X-ray crystal structures of FimH reveal a highly-conserved mannose-binding pocket at the tip of the FimH adhesin domain surrounded by a distal hydrophobic ridge (8, 29). Minor sequence differences in *E. coli* FimH, many of which are not located in close proximity to the mannose binding pocket, have been found to correlate with differential binding phenotypes (54-56).

Klebsiella pneumoniae is the second leading cause of Gram-negative UTI and encodes numerous chaperone/usher pili including type 1 pili and type 3 pili (23). While type 1 pili have historically been defined by their MSHA phenotype, type 3 pili display a mannose-resistant hemagglutination (MRHA) with tannin-treated RBCs (47). Type 1 pili of *K. pneumoniae* are highly homologous to those of UPEC (23) and have been previously implicated in UTI pathogenesis (18, 40). The *fim* operon of *K. pneumoniae*, encoding type 1 pili, contains a terminal *fimK* gene, not present in UPEC, which plays a role in suppressing the expression of type 1 pili (50). *K. pneumoniae* binds, invades and forms IBCs within host urothelial cells, albeit less efficiently than UPEC in the murine cystitis model. Similar to UPEC, *K. pneumoniae* also expresses type 1 pili within these IBCs (50). In this study, we discovered that type 1 piliated *K. pneumoniae* are unable to mediate MSHA despite the presence of wild type FimH containing an identical mannose binding pocket as *E. coli* FimH. We analyzed functional and structural differences in FimH of the *K. pneumoniae* strain, TOP52 1721, and the effects of these differences on UTI pathogenesis.

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 (43), and TOP52 1721 (abbreviated TOP52), a *K. pneumoniae* cystitis isolate (50). Bacteria were cultured at 37°C in Luria-Bertani (LB) broth containing, as appropriate, 20 μg/ml chloramphenicol and 0.4% arabinose as indicated.

TOP52 and UTI89 mutant construction and complementation.

A targeted deletion of *fimH* in the *K. pneumoniae* isolate, TOP52, was constructed with the pKOV vector as described previously (37). Flanking sequences of approximately 1000 bp on each side of the targeted gene were amplified with the indicated primers (Table 2) and cloned into pKOV. Potential knockouts were screened by PCR and the knockout region was sequenced. Growth curves were performed for mutant strains and showed no differences in growth compared to wildtype.

UTI89Δ*fimH* was constructed using the red recombinase method as previously described (10, 44), with pKD4 as a template and the primers indicated (Table 2) followed by expression of the FLP recombinase to eliminate the kanamycin cassette. PCR using flanking primers was used to confirm the deletion.

For complementation studies, the adhesin domains (ADs) and pilin domains (PDs) of both UTI89 *fimH* and TOP52 *fimH* were amplified using the primers indicated (Table 2). Subscripts 89 and 52 were used to indicate a given domain was from UTI89 or

Table 1. Bacterial Strains and Plasmids		
Strain	**Description**	**Reference**
UTI89	UPEC cystitis isolate	(43)
UTI89Δ*fimH*	Knockout of *fimH* in UTI89	This study
TOP52 1721	*Klebsiella pneumoniae* cystitis isolate	(50)
TOP52Δ*fimH*	Knockout of *fimH* in TOP52	This study
TOP52Δ*fimK*	Knockout of *fimK* in TOP52	(50)
Plasmid	**Description**	**Reference**
pBAD33	Empty expression vector, P_{ara}, Cm^R	(24)
p*fimX*	$fimX_{UTI89}$ expression vector, P_{ara}, Cm^R	(25)
p*fimH*$_{89}$ (p*fimH*-AD$_{89}$PD$_{89}$)	$fimH_{UTI89}$ expression vector, P_{ara}, Cm^R	This study
p*fimH*-AD$_{89}$PD$_{52}$	*fimH* chimera expression vector, P_{ara}, Cm^R	This study
p*fimH*-AD$_{52}$PD$_{89}$	*fimH* chimera expression vector, P_{ara}, Cm^R	This study
p*fimH*$_{52}$ (p*fimH*-AD$_{52}$PD$_{52}$)	$fimH_{TOP52}$ expression vector, P_{ara}, Cm^R	This study

Table 2. Primer Sequences

Primer Name	Primer Use	Primer Sequence
NotIFimHF1	Amplify 1kb *fimH* 5' Flanking Region	5'-CGGTAACGCGGCCGCGGCGAATC GAAGGATTTTACT-3'
BamHIFimHR2	Amplify 1kb *fimH* 5' Flanking Region	5'-AACGGATCCAAAGGACCAGGCGT TCATC-3'
BamHIFimHF3	Amplify 1kb *fimH* 3' Flanking Region	5'-AACGGATCCACGCGATCTTCACCA ATACC-3'
SalIFimHR4	Amplify 1kb *fimH* 3' Flanking Region	5'-AACGTCGACGTCTGGGGGTGAAGT ACCTG-3'
FimHCheckF1	Check TOP52Δ*fimH*	5'-GGACAGCACCGGCTATTACA-3'
FimHCheckR2	Check TOP52Δ*fimH*	5'-GGGATCGTCAGGGAGATACA-3'
UTI89fimH KO F	Knockout *fimH* in UTI89	5'-ATGAAACGAGTTATTACCCTGTTTGCT GTACTGCTGATGGGCTGGTCGGTAGTGTA GGCTGGAGCTGCTTC-3'
UTI89fimH KO R	Knockout *fimH* in UTI89	5'-TTATTGATAAACAAAAGTCACGCCAA TAATCGATTGCACATTCCCTGCAGTCATAT GAATATCCTCCTTAG -3'
UTI89fimHcheckF	Check UTI89Δ*fimH*	5'-CAATCAGCGCACTTCCCGTTACAGG-3'
UTI89fimHcheckR	Check UTI89Δ*fimH*	5'-CTCGAATTATAAACAACCCGCGGCG -3'
2KlebphaseF	Amplify *fimS* Region for Phase Assay	5'-GGGACAGATACGCGTTTGAT-3'
2KlebphaseR	Amplify *fimS* Region for Phase Assay	5'-GGGACAGATACGCGTTTGAT-3'
ADecoliF	Amplify *E. coli fimH* Adhesin Domain	5'-AATTCCATGGGATGAAACGAGTTATT ACCCTGTTTGCTG-3'
ADecoliR	Amplify *E. coli fimH* Adhesin Domain	5'-TCGCAGCCGCCAGTGGGCACCACCAC-3'
PDecoliF	Amplify *E. coli fimH* Pilin Domain	5'-GTGGTGGTGCCCACTGGCGGCTGCGA-3'
PDecoliR	Amplify *E. coli fimH* Pilin Domain	5'-AATTGGTACCATTGATAAACAAAAGTC ACGCCAATAATCG-3'
ADklebF	Amplify *Kleb. fimH* Adhesin Domain	5'-AATTCCATGGGATGATGAAAAAAATAA TCCCCCTGTTCACC-3'
ADklebR	Amplify *Kleb. fimH* Adhesin Domain	5'-TCGCAGCCGCCGGTGGGGACCACC AC-3'
PDklebF	Amplify *Klebsiella fimH* Pilin Domain	5'-GTGGTGGTCCCCACCGGCGGCTGCGA-3'
PDklebR	Amplify *Klebsiella fimH* Pilin Domain	5'-AATTGGTACCCATTGATAGACAAAGGT GATGCCGATG-3'
pBADF	Check pBAD33 Clones	5'-TATCGCAACTCTCTACTGTTTCTCCA-3'
pBADR	Check pBAD33 Clones	5'-CTGTATCAGGCTGAAAATCTTCTCT CA-3'

TOP52, respectively. Single ADs and PDs were added together as templates in a PCR

reaction to create a full length *fimH* gene that was subsequently cloned into the arabinose-

inducible pBAD33 vector (abbreviated pBAD). The four permutations of the ADs and

PDs yielded *fimH* complementation vectors $pAD_{89}PD_{89}$ ($pfimH_{89}$), $pAD_{89}PD_{52}$,

$pAD_{52}PD_{89}$, and $pAD_{52}PD_{52}$ ($pfimH_{52}$). All constructs were verified and sequenced using

pBAD plasmid primers. The TOP52 *fimH* nucleotide sequence has been deposited in the

GenBank database under the accession number EU327536.

Hemagglutination assays.

Hemagglutination assays (HAs) were performed with guinea pig red blood cells (OD_{640} =

2.0, Colorado Serum Company) as previously described using serial dilutions in

microtiter plates with and without the addition of 100 mM methyl α-D-mannopyranoside

(28).

Biofilm assays.

Bacteria were grown in LB broth in wells of microtiter plates in the presence of 0.01%

arabinose and either no mannose, 1 mM methyl mannose, 100 mM methyl mannose or 1

mM heptyl mannose. After 48 hours of growth at room temperature, wells were rinsed,

stained with crystal violet, and biofilms were quantified as previously described (46).

Modeling of K. pneumoniae FimH.

K. pneumoniae TOP52 FimH was modeled onto the X-ray crystal structure of *E.coli* FimH from the J96 isolate FimC-H complex structure (PDB ID: 1KLF) (29) using the protein structure threading program Phyre (4). The resulting model was compared to the J96 structure and UTI89 amino acid sequence (a structure for UTI89 FimH has not been solved to date and J96 FimH only differs by 4 amino acids from UTI89 FimH). Figures were rendered in the molecular modeling program Pymol (11).

Mouse infections.

Bacterial strains were used to inoculate eight-week-old female C3H/HeN mice (National Cancer Institute) by transurethral catheterization as previously described (42). 25 ml static cultures were inoculated from freezer stocks and grown at 37°C for 18 hours and then subcultured 1:250 into 25 ml fresh media. These cultures were then grown statically at 37°C for 18 hours, centrifuged for 5 min at 5800 rpm, and the resultant pellet was resuspended in PBS and diluted to approximately 2×10^8 CFU/ml. 50 μl of this suspension was used to infect each mouse with an inoculum of $1\text{-}2 \times 10^7$ CFU. All studies were approved by the Animal Studies Committee at Washington University School of Medicine.

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 post infection, 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 (33). For *ex vivo*

enumeration of IBCs, infected bladders were harvested at 1 hour post infection, bisected,

splayed, washed with PBS, fixed with 3% paraformaldehyde for 1 hour at room

temperature and *lacZ* stained as previously described (33). IBCs were visualized and

counted using an Olympus SZX12 dissecting microscope (Olympus America).

Fim operon phase assay.

To determine the orientation of the type 1 pili phase-variable promoter switch (*fimS*) in

UTI89Δ*fimH*, a phase assay was performed as previously described (58). Briefly, PCR

primers were used to amplify a 589 bp DNA region including *fimS*. The PCR product

was then digested with the restriction endonuclease Hinf1 (New England Biolabs) and

was separated on a 2.5% agarose gel. A phase ON switch results in products of 489 and

70 bp and a phase OFF switch results in products of 359 and 200 bp.

Immunoelectron microscopy.

Bacteria were prepared as described above for mouse infection, fixed with 1%

paraformaldehyde for 10 min, and absorbed onto formvar/carbon-coated copper grids for

2 min. Grids were washed two times with PBS, blocked with 1% FBS for 5 min, and

incubated with rabbit anti-FimH antibody (1:100) for 30 min at room temperature. The

rabbit anti-FimH antibody was raised against the FimH adhesin domain (1-159, T2) of *E.

coli* J96 (26) (SigmaGenesis). Grids were subsequently washed two times with PBS,

blocked with 1% FBS for 5 min, and incubated with 18nm colloidal gold-conjugated anti-

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rabbit IgG (Jackson ImmunoResearch Laboratories) for 30 min at room temperature.

Following two PBS washes, grids were rinsed in dH$_2$O, and stained with 1% aqueous

uranyl acetate (Ted Pella Inc.) for 1 min. Excess liquid was gently wicked off and grids

were air dried. Samples were viewed on a JEOL 1200EX transmission electron

microscope (JEOL USA) at 80 kV accelerating voltage.

Statistical analysis.

Continuous variables were compared using the Mann-Whitney *U* test since these

variables were not normally distributed. 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) and SAS (version 9.0).

Results

Type 1 piliated TOP52 is MSHA negative.

In contrast to UPEC, statically passaged *K. pneumoniae* TOP52 1721 (abbreviated

TOP52) produced no detectable hemagglutination of guinea pig RBCs despite expression

of type 1 pili (Table 3). The MSHA titer of the UPEC strain UTI89 was 1:512. Deletion

of *fimH* abolished the ability of UTI89Δ*fimH* to produce MSHA. UTI89Δ*fimH* produced

a low MRHA titer of 1:4, unlike UTI89. Wildtype TOP52 was MSHA negative.

Deletion of *fimH* to create TOP52Δ*fimH* thus had no effect and this strain was also

MSHA negative. Recently, we discovered that deletion of *fimK*, a gene

Table 3. FimH$_{52}$ is Unable to Produce a Mannose-Sensitive Hemagglutination of Guinea Pig Red Blood Cells.		
Strain	Guinea Pig RBC HA Titer $(1:2^x)^*$	Guinea Pig RBC + Mann. HA Titer $(1:2^x)^*$
UTI89	9	2
UTI89Δ*fimH*	2	2
TOP52	0	0
TOP52Δ*fimH*	0	0
TOP52Δ*fimK*	0	0
TOP52/pBAD	0	0
TOP52/pfimX	0	0
TOP52Δ*fimH*/pBAD	0	0
TOP52Δ*fimH*/p*fimH*$_{52}$	0	0
TOP52Δ*fimH*/p*fimH*$_{89}$	4	0

*HA titer data are representative of 3 independent experiments.

unique to *Klebsiella fim* gene clusters, resulted in a hyper-type 1 piliated phenotype (50). The hyperpiliated TOP52Δ*fimK* was also MSHA negative. The *fimX* recombinase has been shown to have *fimB*-like properties (7, 25) and it's overexpression results in increased expression of type 1 pili in both *E. coli* (25) and *K. pneumoniae* (50). The hyperpiliated TOP52/p*fimX* was also MSHA negative. Thus, the type 1 piliated *Klebsiella* TOP52 strain is unable to mediate MSHA.

FimH$_{52}$ and FimH$_{89}$ are highly similar in amino acid sequence and structure.

To further investigate the functional differences of TOP52 type 1 pili, we sequenced *fimH$_{52}$* (GenBank accession number EU327536) and compared it to other known *K. pneumoniae* FimH sequences and the sequence of *E. coli* UTI89 FimH (FimH$_{89}$). The FimH$_{52}$ amino acid (aa) sequence shares 100% identity to the FimH adhesin domain of *K. pneumoniae* strain IA565 (23), 99.6% aa identity with the FimH of *K. pneumoniae* ATCC 700721 strain (41), 85.3% aa identity with *K. pneumoniae* strain IA551 (16). FimH$_{52}$ has 86.4% aa identity to FimH of *E. coli* UTI89 (FimH$_{89}$) (Fig. 1A) and maintains the general bi-domain composition of *E. coli* FimH with an amino-terminal adhesin domain (aa 1-157) and a carboxy-terminal pilin domain (aa 161-279) separated by a short linker region.

We threaded the *K. pneumoniae* FimH onto the X-ray crystal structure of FimH from the complex structure of FimC-H from the J96 *E. coli* isolate (29). We then
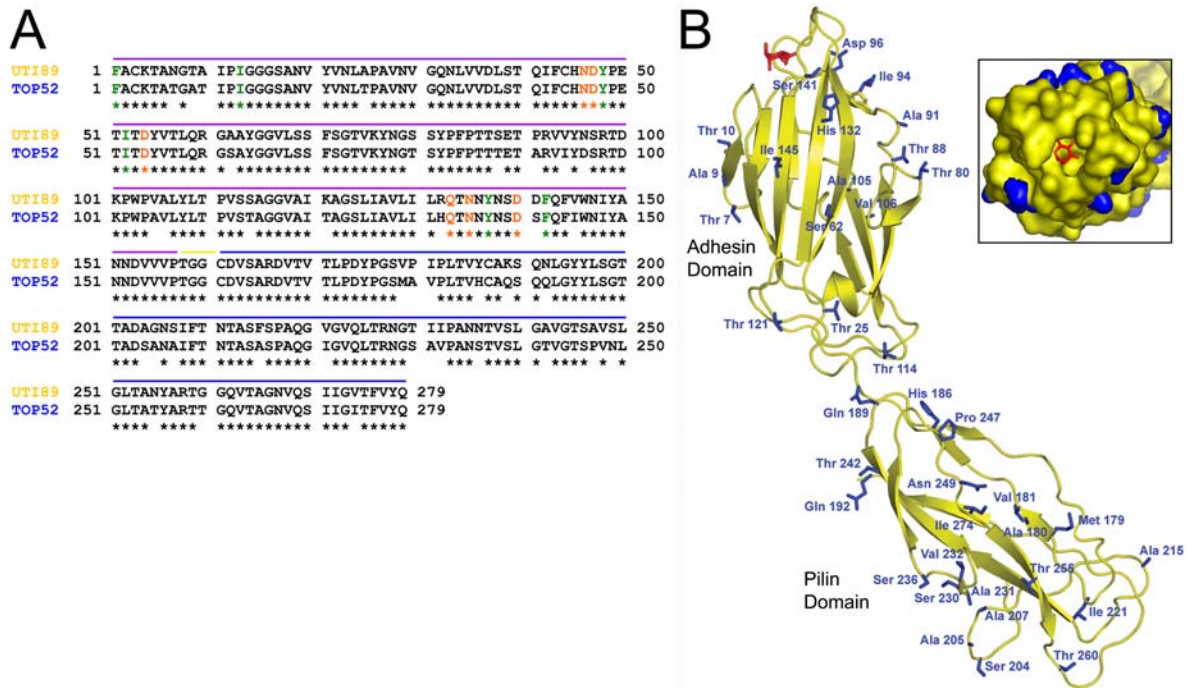
Figure 1. FimH₅₂ and FimH₈₉ are highly conserved in sequence and structure.
(A) The FimH amino acid sequences of UTI89 and TOP52 are shown. Residues known to interact with mannose (orange) and form the hydrophobic ridge (green) are fully conserved. The purple line denotes sequence of the adhesin domain, the green line denotes sequence of the pilin domain, and the yellow line denotes the short linker region. Amino acid numbers refer to the mature protein without signal sequence. (B) Structure of *E. coli* FimH (yellow) (from the J96 strain FimC-H complex, PDB ID: 1KLF) bound to mannose (red) overlayed with a threaded model of *K. pneumoniae* TOP52 FimH. Side chains of TOP52 amino acids that vary from the UTI89 sequence are shown as blue sticks. (Inset) Space filling model of a view into the mannose-binding pocket (same colors as above). This shows that all residues in direct contact with the mannose moiety and those that form the hydrophobic ridge are fully conserved between UTI89 and TOP52.

overlayed J96 FimH and TOP52 FimH and compared the positions and identities of

amino acid (aa) differences in $FimH_{89}$ and $FimH_{52}$ (Fig. 1B). This comparison assumes

that residues conserved between J96 FimH and UTI89 FimH have the same conformation

as shown in the three-dimensional J96 FimH structure. There are only 4 amino acid

differences between FimH of these two strains. 17 adhesin domain aa differences and 21

pilin domain aa differences exist between $FimH_{89}$ and $FimH_{52}$. Interestingly, despite its

MSHA deficit, $FimH_{52}$ displays full conservation of the residues known to interact with

mannose in the mannose binding pocket (orange) and those that form the surrounding

hydrophobic ridge (green) (Fig 1). Residue differences exist in areas adjacent to the

receptor binding site and the hydrophobic ridge and in other more distal part of the

molecule and may together slightly alter the molecular details of the interaction with

mannose; these differences may help to explain the lack of MSHA in the case of TOP52

FimH.

The loss of MSHA in TOP52 is specific to the adhesin domain of $FimH_{52}$.

Although all residues involved in direct interactions with the mannose moiety and

all those in the surrounding hydrophobic ridge are identical between $FimH_{52}$ and $FimH_{89}$,

nearly 14% of amino acids differ between the two proteins. We hypothesized that if this

variation in FimH sequence accounts for the lack of the MSHA phenotype, then

complementation of TOP52Δ*fimH* with *fimH* cloned from UTI89 (*fimH*$_{89}$), should restore

the MSHA phenotype. Thus, TOP52Δ*fimH* was complemented with the *fimH* gene of

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TOP52 ($fimH_{52}$) or the $fimH$ gene of UTI89 ($fimH_{89}$) on inducible plasmids. While TOP52$\Delta fimH$/pBAD vector control and TOP52$\Delta fimH$/p$fimH_{52}$ did not produce a positive MSHA titer, TOP52$\Delta fimH$/p$fimH_{89}$ had an MSHA titer of 1:16 (Table 3). Thus, the complementation indicated that FimH$_{89}$ is able to participate in type 1 pilus biogenesis with the Fim proteins of TOP52 and confers a gain of MSHA function to the TOP52 *K. pneumoniae* strain.

To test the expression of exogenous *fimH* in the UTI89$\Delta fimH$ background, phase assays were conducted analyzing the phase-variable promoter switch of type 1 pili (Fig. 2). The *fim* operon of wildtype UTI89 was primarily phase ON after static growth, however, loss of *fimH* in UTI89$\Delta fimH$ and UTI89$\Delta fimH$/pBAD vector control resulted in bacterial populations that were primarily in the phase OFF orientation. Complementation with either p$fimH_{89}$ or p$fimH_{52}$ did not result in a robust OFF to ON switch as the populations remained primarily phase OFF. However, enough ON phase switching occurred to detect MSHA in bacteria complemented with p$fimH_{89}$ (Table 3).

FimH$_{52}$ and FimH$_{89}$ function was investigated further by constructing FimH chimeras. We used the chimeras to complement *fimH* knockout strains and then examined the final pilus assembly on each strain by immunoelectron microscopy. The adhesin domains (ADs) and pilin domains (PDs) of each strain were amplified and expressed in different combinations on the arabinose inducible pBAD33 vector. This resulted in 4 different *fimH* construct-expressing plasmids: pAD$_{89}$PD$_{89}$ (p$fimH_{89}$), pAD$_{89}$PD$_{52}$, pAD$_{52}$PD$_{89}$, and pAD$_{52}$PD$_{52}$ (p$fimH_{52}$).
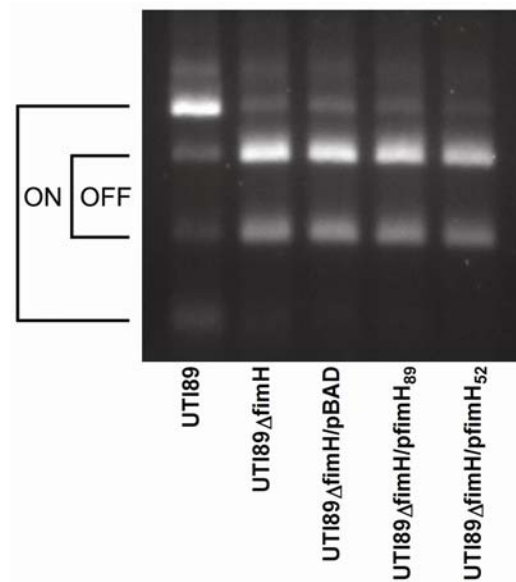
Figure 2. The *fim* operon of UTI89ΔfimH is primarily in the phase OFF orientation.
Phase assays of the *fimS* invertible promoter region of the fim operon were done for
UTI89, UTI89Δ*fimH*, UTI89Δ*fimH*/pBAD, UTI89Δ*fimH*/p*fimH*$_{89}$ and
UTI89Δ*fimH*/p*fimH*$_{52}$. UTI89 was largely phase ON while the UTI89Δ*fimH* strains were
all primarily phase OFF despite complementation.

The incorporation of p$fimH_{89}$, p$AD_{89}PD_{52}$, p$AD_{52}PD_{89}$, and p$fimH_{52}$, into pili was confirmed by immunoelectron microscopy using anti-FimH antibodies. Consistent with the phase switch being primarily OFF in these complementations, the majority of bacteria were bald without noticeable pili. However, a subpopulation of bacteria existed in each sample that were moderately piliated and immunolabeling at the tips of pili was observed for UTI89Δ$fimH$ complemented with each construct (Fig. 3). The low level of type 1 pilus expression explains the inability to fully complement UTI89Δ$fimH$ to wildtype UTI89 levels of MSHA.

TOP52Δ$fimH$ was also complemented with each FimH chimera and the MSHA titers for all strains were analyzed (Table 4). Wildtype UTI89 produced an MSHA titer of 1:512 while wildtype TOP52 did not have a MSHA titer. UTI89Δ$fimH$, UTI89Δ$fimH$/pBAD, TOP52Δ$fimH$, and TOP52Δ$fimH$/pBAD all lacked MSHA titers. UTI89Δ$fimH$/p$fimH_{89}$ and UTI89Δ$fimH$/p$AD_{89}PD_{52}$ both produced an MSHA titer of 1:32 while UTI89Δ$fimH$/p$AD_{52}PD_{89}$ and UTI89Δ$fimH$/p$fimH_{52}$ did not produce a MSHA titer. This trend was recapitulated in the TOP52Δ$fimH$ background. While TOP52Δ$fimH$/p$fimH_{52}$ and TOP52Δ$fimH$/p$AD_{52}PD_{89}$ both lacked MSHA titers, TOP52Δ$fimH$/pfimH$_{89}$ and TOP52Δ$fimH$/p$AD_{89}PD_{52}$ both produced MSHA titers of 1:16.

These results demonstrate that the *K. pneumoniae* TOP52 FimH MSHA defect is specific to its adhesin domain. The adhesin domain of UTI89 FimH is capable of agglutinating guinea pig RBCs with the native UTI89 pilin domain or with the pilin
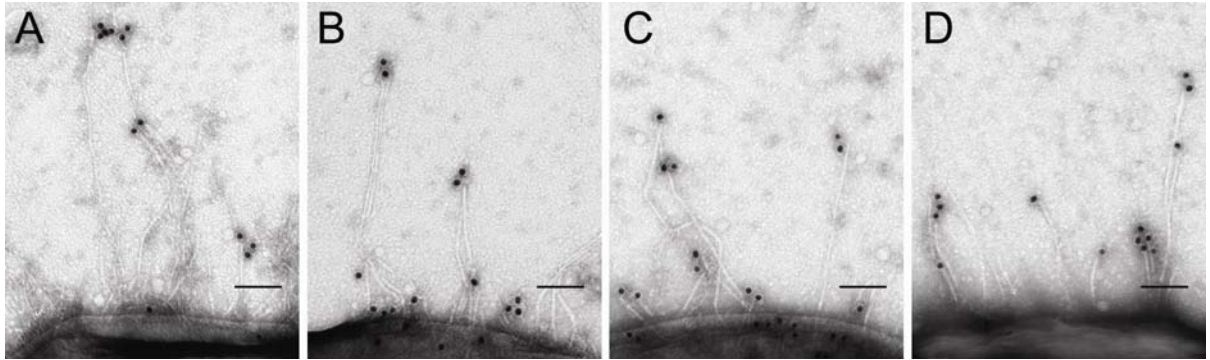
Figure 3. *FimH* constructs in UTI89Δ*fimH* are expressed in some bacteria and localized at the tips of pili.
Immunoelectron microscopy using an anti-FimH antibody was performed against A) UTI89Δ*fimH*/p*fimH*$_{89}$, B) UTI89Δ*fimH*/pAD$_{89}$PD$_{52}$, C) UTI89Δ*fimH*/pAD$_{52}$PD$_{89}$ and D) UTI89Δ*fimH*/p*fimH*$_{52}$. While the majority of bacteria did not appear to be expressing type 1 pili, piliated bacteria could be found in all four samples. Piliated bacteria displayed FimH immmunostaining at the distal tips of pili.

Table 4. Mannose-Sensitive Hemagglutination Deficiency is Specific to the Adhesin Domain of FimH$_{52}$.		
Strain	Guinea Pig RBC HA Titer (1:2^x)*	Guinea Pig RBC + Mann. HA Titer (1:2^x)*
UTI89	9	3
UTI89Δ*fimH*	2	2
UTI89Δ*fimH*/pBAD	0	0
UTI89Δ*fimH*/pfimH$_{89}$	5	0
UTI89Δ*fimH*/pAD$_{89}$PD$_{52}$	5	0
UTI89Δ*fimH*/pAD$_{52}$PD$_{89}$	0	0
UTI89Δ*fimH*/pfimH$_{52}$	0	0
TOP52	0	0
TOP52Δ*fimH*	0	0
TOP52Δ*fimH*/pBAD	0	0
TOP52Δ*fimH*/pfimH$_{89}$	4	0
TOP52Δ*fimH*/pAD$_{89}$PD$_{52}$	4	0
TOP52Δ*fimH*/pAD$_{52}$PD$_{89}$	0	0
TOP52Δ*fimH*/p*fimH*$_{52}$	0	0

*HA titer data are representative of 3 independent experiments.

domain of TOP52. Thus, variation between the FimH adhesin domains of UTI89 and

TOP52 are likely responsible for their differences in function.

TOP52 FimH-dependent biofilms are inhibited by heptyl mannose but not methyl

mannose.

TOP52 is able to form biofilm at room temperature when the production of type 1

pili is induced via the *E. coli* recombinase, *fimX* (50). Thus, we investigated whether this

biofilm was FimH-dependent. Biofilms were stained with crystal violet and quantified

after 48 h of incubation. Wildtype TOP52 and TOP52/pBAD vector control did not form

biofilm, however TOP52/p*fimX* formed biofilm. TOP52Δ*fimH*, TOP52Δ*fimH*/pBAD,

and TOP52Δ*fimH*/p*fimX* did not form biofilm suggesting that the TOP52/p*fimX* biofilm

is FimH-dependent. Thus, although $FimH_{52}$ is unable to mediate MSHA, it is capable of

mediating biofilm formation. UTI89 formed a robust biofilm while UTI89Δ*fimH* did not.

The formation of UTI89 biofilm was fully inhibited by 100 mM methyl mannose or 1

mM heptyl mannose (Fig 4). In contrast, TOP52/p*fimX* biofilm formation was not

affected by the presence of 100 mM methyl mannose, but was fully inhibited by 1 mM

heptyl mannose.

Therefore, TOP52/p*fimX* forms a FimH-dependent biofilm that is inhibited by

heptyl mannose but not methyl mannose. This phenotype is distinct from UTI89 FimH-
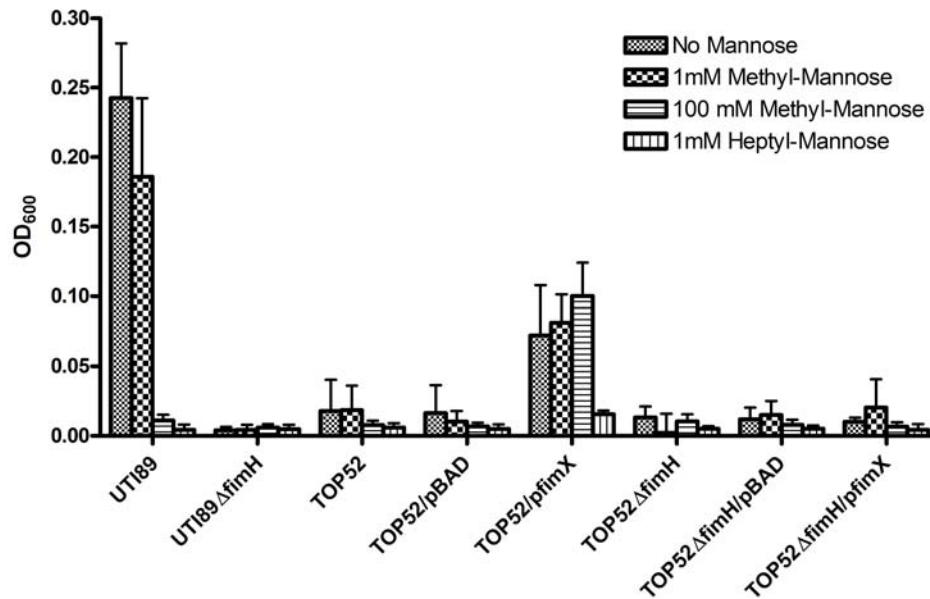
Figure 4. Heptyl mannose, but not methyl mannose, inhibits FimH-dependent biofilm formation of TOP52/p*fimX*.
A 48 h biofilm assay was used to quantify biofilms produced by UTI89 and TOP52 strains in the presence of no mannose, 1mM methyl mannose, 100 mM methyl mannose or 1 mM heptyl mannose. TOP52 forms a FimH-dependent biofilm with induced expression of type 1 pili via the *E. coli* recombinase, *fimX*. This biofilm formation is inhibited by heptyl mannose but not high concentrations of methyl mannose. UTI89 forms robust biofilm without mannose but is inhibited by heptyl mannose or high concentrations of methyl mannose. Error bars represent standard deviations.

dependent biofilms, which are fully inhibited by the presence of 100 mM methyl mannose.

UTI89 and TOP52 both require fimH for effective persistence in the urinary tract.

To analyze the respective roles of $FimH_{52}$ and $FimH_{89}$ in urinary tract infection, 10^7 CFU of UTI89, UTI89Δ*fimH*, TOP52 or TOP52Δ*fimH* were inoculated into the bladders of C3H/HeN mice by transurethral catheterization. Bladders and kidneys were harvested at various timepoints post inoculation and bacterial titers were determined. In the bladder (Fig. 5A), UTI89 had significantly higher titers than UTI89Δ*fimH* at 6 ($P<0.0001$), 24 ($P<0.0001$) and 336 hours ($P=0.0007$) post infection. UTI89Δ*fimH* was cleared from the bladder as time progressed. TOP52 had slightly but significantly higher titers ($P=0.0244$) than TOP52Δ*fimH* in the bladders of mice at 6 hours post inoculation. By 24 hours there was no significant difference between TOP52 and TOP52Δ*fimH* bladder titers. However, by 336 hours post infection, TOP52Δ*fimH* had significantly lower titers than wildtype TOP52 ($P=0.0012$). In the kidney (Fig. 5B), UTI89 had significantly higher titers than UTI89Δ*fimH* at 6 hours post infection ($P<0.0001$), however the two strains had similar titers at both 24 and 336 hours post infection. TOP52 and TOP52Δ*fimH* had similar levels of bacterial burden in the kidney at all timepoints tested. Thus, FimH-containing type 1 pili in the *K. pneumoniae* isolate, TOP52, does not play a critical role early in bladder infection as is the case with UTI89; however, it is required for effective persistence in the bladder in both strains.
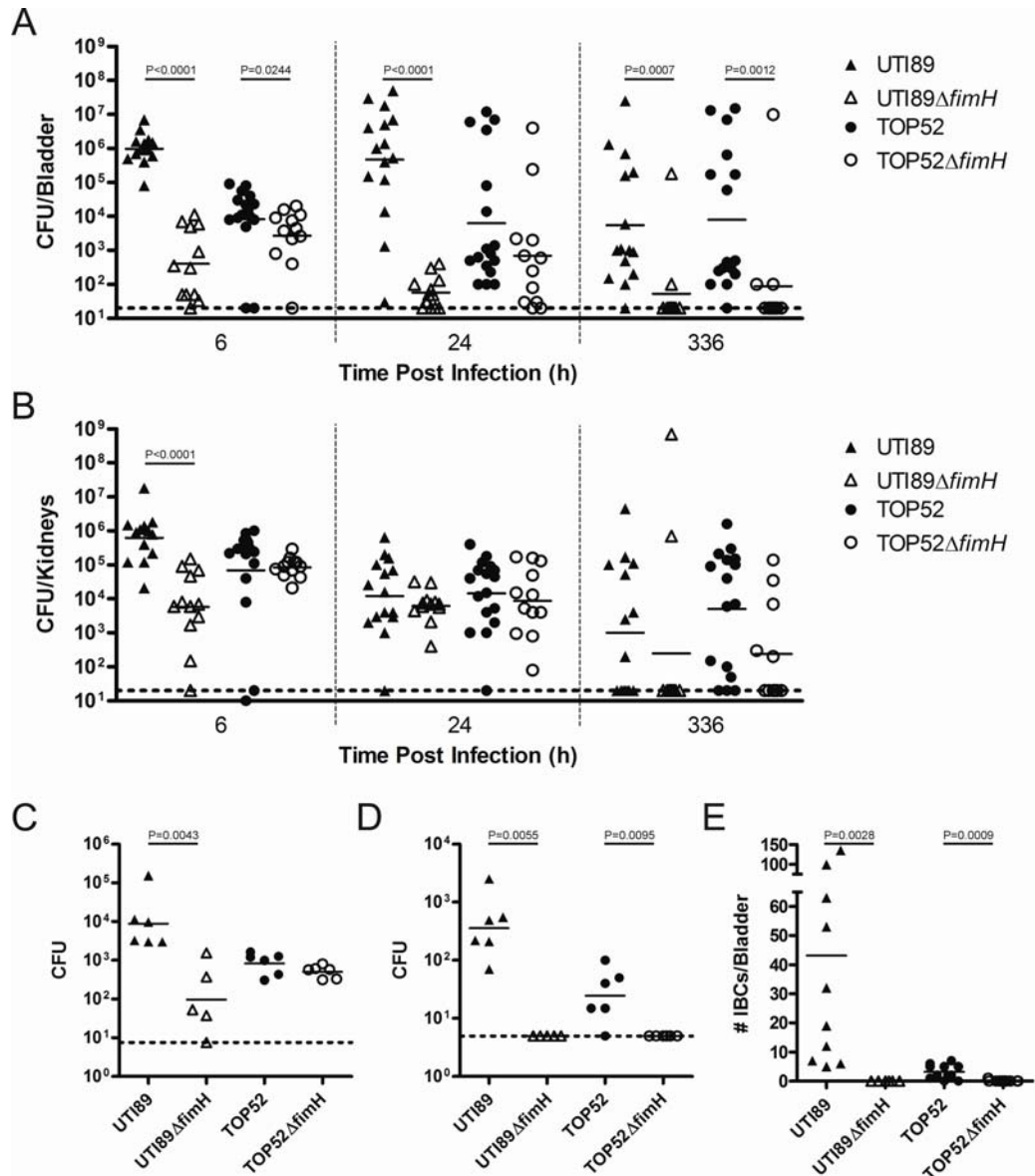
Figure 5. FimH of TOP52 is required for invasion, IBC formation, and persistence but not colonization in the murine model of UTI.

Female C3H/HeN mice were inoculated with 10^7 UTI89 (▲), UTI89Δ*fimH* (Δ), TOP52 (●), or TOP52Δ*fimH* (○) by transurethral inoculation. For organ titers, bladders (A) and kidneys (B) were harvested at various timepoints post infection and CFU were calculated. Titer data are combined from N=3 independent experiments. For *ex vivo* gentamicin protection assays, bladders were harvested at 1 h post infection and luminal (C) and intracellular (D) populations of bacteria were quantified. IBCs were quantified (E) after visualization by LacZ staining at 6 h post inoculation. Short bars represent geometric means of each group and horizontal dotted lines represent limits of detection. Significant P-values, as calculated using the Mann-Whitney *U* test, are displayed.

FimH$_{52}$ is required for TOP52 bladder invasion and IBC formation.

In order to further assess the role of FimH$_{52}$ in acute TOP52 cystitis, bladder invasion assays were performed at 1 hour post infection with UTI89, UTI89Δ*fimH*, TOP52 or TOP52Δ*fimH*. In these assays, luminal bacteria were collected by successive bladder washes (Fig. 5C), prior to gentamicin treatment of the bladder to kill extracellular bacteria, as previously described (33). After 1.5 hour incubation in gentamicin, bladders were homogenized and titered to reveal the intracellular bacterial burden (Fig. 5D). UTI89 had 100-fold higher luminal bacterial counts compared to UTI89Δ*fimH* at 1 hour post infection (P=0.0043). However, TOP52 and TOP52Δ*fimH* had similar levels of luminal colonization. At this 1 hour timepoint, UTI89 had significantly higher levels of intracellular bacteria than UTI89Δ*fimH* (P=0.0055), which did not have any intracellular titers above the limit of detection (5 CFU). TOP52 also invaded into the bladder tissue and had intracellular bacterial titers that were significantly higher than TOP52Δ*fimH* (P=0.0095), which did not have titers above the limit of detection.

To determine if the presence of FimH$_{52}$ affects the ability of TOP52 to form IBCs, we visualized and quantified IBCs by *lacZ* staining of whole mounted, fixed bladders as described previously (33) at 6 hours post inoculation of UTI89, UTI89Δ*fimH*, TOP52 or TOP52Δ*fimH* (Fig. 5E). UTI89 formed a wide range of IBCs with a median of 25.5 per bladder while UTI89Δ*fimH* formed no detectable IBCs. TOP52 had a median of 2.0 IBCs per bladder, while TOP52Δ*fimH* was unable to produce detectable IBCs (P=0.0009).

These data suggest that *K. pneumoniae* TOP52 FimH$_{52}$, in contrast to *E. coli* UTI89 FimH$_{89}$, does not play a significant role in early bladder colonization. However, FimH$_{52}$ is required for TOP52 invasion and IBC formation in the murine bladder as is the case for UTI89.

FimH$_{52}$ does not restore the ability of UTI89ΔfimH to effectively infect the bladder.

UTI89 relies on FimH to successfully cause UTI in the murine model. The ability of *fimH$_{52}$* to restore the ability of UTI89Δ*fimH* to bind, invade, and infect murine bladders, was investigated by using UTI89Δ*fimH* complemented with pBAD vector control, p*fimH$_{89}$*, and p*fimH$_{52}$*. In 1 hour gentamicin protection assays (Fig. 6A-B) UTI89Δ*fimH* complemented with p*fimH$_{89}$* had significantly higher luminal titers than the same strain complemented with vector control (Fig. 6A, P=0.0001) despite the known expression deficiencies observed above. Additionally, UTI89Δ*fimH*/p*fimH$_{89}$* had significantly higher 1 hour luminal titers than UTI89Δ*fimH*/p*fimH$_{52}$*, which had colonization levels similar to vector control. Examination of the intracellular population at 1 hour (Fig. 6B) revealed that UTI89Δ*fimH*/pBAD vector control did not have titers above the limit of detection, whereas, UTI89Δ*fimH*/p*fimH$_{89}$* did produce significantly higher burdens of intracellular bacteria (P=0.0028). UTI89Δ*fimH*/p*fimH$_{52}$* was able to invade into the bladder tissue, but at significantly lower levels compared to UTI89Δ*fimH*/p*fimH$_{89}$* (P=0.0106). At 6 hours post inoculation, UTI89Δ*fimH*/p*fimH$_{89}$* had significantly higher burdens of bacteria in the bladder than both UTI89Δ*fimH*/pBad
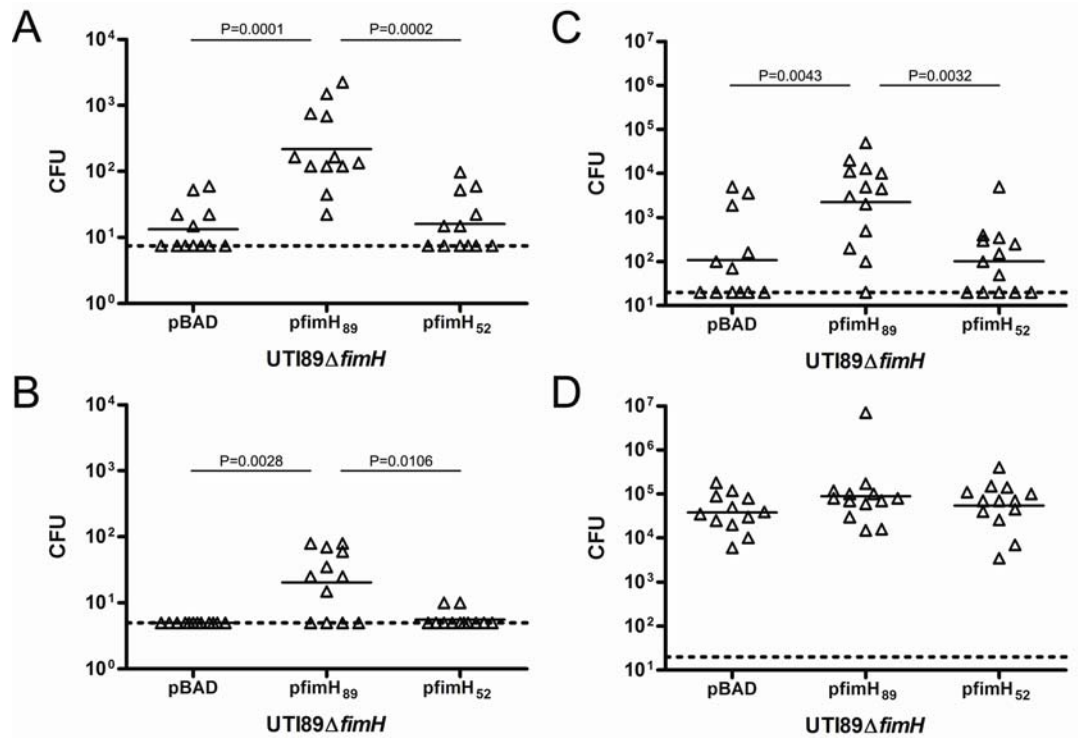
Figure 6. Complementation of UTI89Δ*fimH* with p*fimH*₈₉ but not p*fimH*₅₂ leads to increased bacterial burden in the murine model of UTI.

Female C3H/HeN mice were inoculated with 10^7 UTI89Δ*fimH*/pBAD vector control, UTI89Δ*fimH*/p*fimH*₈₉ or UTI89Δ*fimH*/p*fimH*₅₂ by transurethral inoculation. *Ex vivo* gentamicin protection assays were performed in which bladders were harvested at 1 h post infection and luminal (A) and intracellular (B) populations of bacteria were quantified. For organ titers, bladders (C) and kidneys (D) were harvested at 6 h post infection and CFU were enumerated. Short bars represent geometric means of each group and horizontal dotted lines represent limits of detection. Significant P-values, as calculated using the Mann-Whitney *U* test, are displayed.

(P=0.0043) and UTI89Δ*fimH*/p*fimH*$_{52}$ (P=0.0032). Complementation of UTI89Δ*fimH* with *fimH* from either UTI89 or TOP52 did not significantly affect 6 h kidney titers compared to vector control.

The *fimH*$_{52}$ gene was not able to restore UTI89Δ*fimH* to levels above vector control while complementation with *fimH*$_{89}$ yielded higher bacterial burdens at 1 and 6 hours. This suggests a potential defect in the function of FimH$_{52}$ in the bladder compared to FimH$_{89}$.

Discussion

FimH of the *K. pneumoniae* strain TOP52 1721 (FimH$_{52}$) has an amino acid sequence highly homologous to the sequence encoded by dozens of *fimH* genes that have been sequenced from *E. coli* (34, 54, 56). The residues that form the mannose-binding pocket (Asn46, Asp47, Asp54, Gln133, Asn135, Asp140) and hydrophobic ridge (Phe1, Ile13, Try48, Ile52, Tyr137, Phe142) surrounding the pocket are completely identical between FimH$_{52}$ and all known FimH adhesins of *E. coli*.   Despite this identity, FimH$_{52}$ has a receptor specificity unique from that of UPEC FimH. FimH$_{52}$ is unable to mediate MSHA of guinea pig erythrocytes whereas all known UPEC FimH's are defined by their ability to mediate MSHA. Different *E. coli* FimH variants have been classified as high

affinity monomannose binders or lower affinity trimannose binders (45, 54). However, both trimannose and monomannose variants display MSHA of guinea pig erythrocytes.

E. coli FimH recognizes mannose and has been shown to be able to interact with Manα1,3Manβ1,4GlcNAcβ1,4GlcNAc in an extended binding site (61). These additional interactions between FimH and extended oligomannose moieties are mimicked by butyl α-D mannose (61). Extended alkyl-α-mannosides have higher affinities for *E. coli* FimH compared to methyl-α-D-mannopyranoside (methyl mannose), with heptyl α-D-mannopyranoside (heptyl mannose) having the lowest K_d of 5 nM (5). *K. pneumoniae* FimH-dependent biofilms could only be inhibited by heptyl mannose and not methyl mannose arguing that *K. pneumoniae* FimH requires additional contacts of the alkyl chain outside of the mannose binding pocket.

$FimH_{52}$ differs at 17 positions from *E. coli* FimH and was threaded onto the three dimensional structure of *E. coli* FimH. In the DE loop, adjacent to the hydrophobic ridge, Val94 and Asn96 of UTI89 FimH ($FimH_{89}$) are changed to Ile and Asp, respectively, in $FimH_{52}$. In the G strand, immediately C-terminal to key residues in the hydrophobic ridge, Val145 in $FimH_{89}$ is changed to Ile in $FimH_{52}$. Combined, these differences may alter the structural stability of the hydrophobic ridge of $FimH_{52}$ through changes in hydrophobic and hydrogen bond contacts. Thus, although $FimH_{52}$ is unable to bind methyl mannose, these amino acid changes may facilitate interactions with longer oligomannose substrates (61).

The inability of $FimH_{52}$ to mediate MSHA may be due to amino acid changes in proximity to the mannose binding pocket. Gln133 and Asp140 *E. coli* FimH residues are required for HA titers and mannose binding (29). Two differences in TOP52 primary sequence exist in residues adjacent to these mannose-binding residues (His132 and Ser141, changed from Arg and Asp, respectively, in $FimH_{89}$). The threaded model $FimH_{52}$ suggests that these residues would lie ~8.5 Å away from the bound mannose moiety (Fig 1B). Arg132 and Asp141 form a salt bridge in J96 FimH that helps stabilize the structure of the FG loop that contains mannose binding residues Gln133, Asn135, and Asp140 and forms part of the hydrophobic ridge. Arg132 NH1 also makes two hydrogen bonds to Gln59 OE1 and Glu 89 OE1. In $FimH_{52}$, His132 is only able to make a hydrogen bond with Glu89 OE2. Thus, in $FimH_{52}$ this salt bridge as well as the hydrogen bond to Gln59 would be lost. Thus, at least two hydrogen bonds are lost in the combined differences in residues 132 and 141, which may have a destabilizing effect on interactions at the mannose site around the Asp140 and Gln133 mannose binding residues.

Sequence variation in regions of FimH not in close proximity to the mannose-binding pocket can also significantly affect FimH function (56, 57). Differences in areas both adjacent to the receptor binding site and hydrophobic ridge or other more distal differences may together alter the molecular details of the interaction with mannose and help explain the loss of affinity for methyl mannose and the differences in MSHA.

Studies have suggested that fimbrial shafts can influence binding specificities of type 1 pili (16, 38). These effects do not account for the binding specificity differences

observed for $FimH_{52}$. $FimH_{52}$ assembled into UTI89 type 1 pili was also MSHA negative and $FimH_{89}$ assembled into TOP52 type 1 pili was MSHA positive. Thus, the major functional disparities between *E. coli* and *K. pneumoniae* type 1 pili were specific to the adhesin domain of FimH, not the strain background or fimbrial shaft. However, fimbrial shafts may influence FimH binding in more subtle ways that could have been missed in this study due to the lower expression of type 1 pili in *fimH* knockout backgrounds.

The binding specificity differences observed for $FimH_{52}$ result in dramatic functional differences seen in *K. pneumoniae* UTI pathogenesis compared to *E. coli*. Although the *K. pneumoniae* strain TOP52 requires FimH for invasion and IBC formation in the murine bladder, FimH is not essential for early colonization. TOP52 and TOP52Δ*fimH* have similar 1 hour luminal bladder titers, 24 hour whole bladder titers and only modest titer differences at 6 hours post infection. The small but significant differences at 6 hours likely represent the intracellular population of bacteria in IBCs within TOP52-infected bladders that are absent in TOP52Δ*fimH*-infected bladders. *K. pneumoniae* may use a different, non-type 1 pilus adhesin for initial binding to the bladder surface that *E. coli* lacks. This would explain why TOP52Δ*fimH* had higher 1 hour luminal titers and 6 hour whole bladder titers compared to UTI89Δ*fimH*. *K. pneumoniae* encodes type 3 pili, however, these pili have not been implicated in binding to the bladder surface and are thought to be to mediate attachment to the basolateral surface of tracheal epithelial cells and basement membrane components (60). In addition to type 1 and type 3 pili, *K. pneumoniae* encodes at least two other non-pilus adhesins. The CF29K and KPF-28 adhesins may play important roles in mediating attachment

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within the mammalian intestine, but a role for them in UTI has not been investigated (9, 12).

For many years, the glycoprotein uroplakin Ia has been considered the main receptor mediating FimH-dependent adhesion in the bladder (42, 63). Recently, it has been shown that host cell integrins also can mediate type 1 pilus-dependent invasion of urothelial cells (17). We currently do not know if *K. pneumoniae* FimH$_{52}$ is capable of binding these receptors. It is possible that FimH$_{52}$ may only be capable of binding integrin receptors (and not uroplakin Ia) for invasion of urothelial cells but not necessarily mediating significant adhesion to the uroplakin-coated bladder surface.

This work focused on a single uropathogenic isolate of *K. pneumoniae* and it is important to extend this work to other strains. The sequence of TOP52 FimH was almost identical to other sequenced *K. pneumoniae* FimH proteins and thus may be representative. The inability of TOP52 to produce an MSHA titer is not an isolated finding. The ATCC 700721 strain also lacks an MSHA titer. The first studies of fimbriae and adhesive properties of 154 *K. pneumoniae* isolates found that 57.6% of strains produced little or no MSHA titer (13). Many considered this to be due to poor type 1 expression in *K. pneumoniae*. However, *K. pneumoniae* remained MSHA negative when expression of type 1 pili was increased by deletion of *fimK* or overexpression of *fimX*. And when *E. coli* type 1 pili were expressed at similar levels as TOP52 type 1 pili, it resulted in a positive MSHA.

This study suggests that limited sequence variation between the FimH of *E. coli* and *K. pneumoniae* results in differences in function and ability to colonize the urinary

tract. Despite its poor adhesive properties in the urinary tract, FimH of *K. pneumoniae* remains an important virulence factor despite its poor adhesive properties in the urinary tract. It enables *K. pneumoniae* to progress through an IBC pathway during UTI and ultimately persist in the host. *K. pneumoniae* FimH likely requires ligand/receptor contacts outside of the mannose binding pocket for efficient binding. Further insight into these structural determinants will aid in our understanding of the altered host-pathogen interactions of *K. pneumoniae* UTI.

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Chapter 5

A Streptozocin-Induced Diabetic Mouse Model

of Urinary Tract Infection

Abstract

Diabetics have a higher incidence of urinary tract infection (UTI), are infected with a broader range of uropathogens, and more commonly develop serious UTI sequelae. To better study UTI in the diabetic host, we created and characterized a murine model of diabetic UTI using the pancreatic islet β-cell toxin streptozocin in C3H/HeN, C3H/HeJ and C57BL/6 mouse backgrounds. Intraperitoneal injections of streptozocin were used to initiate diabetes in healthy mouse backgrounds as defined by consecutive blood glucose levels >250 mg/dl. UTIs caused by uropathogenic *Escherichia coli* (UTI89), *Klebsiella pneumoniae* (TOP52 1721), and *Enterococcus faecalis* (0852) were studied and diabetic mice were found to be considerably more susceptible to infection. All three uropathogens produced significantly higher bladder and kidney titers than buffer-treated controls. Muted effects were seen in the TLR-4 defective C3H/HeJ diabetic mouse arguing that the dramatic increase in colonization seen in C3H/HeN diabetic mice may be partially due to diabetic-induced defects in innate immunity. Competition experiments demonstrated that *E. coli* had a significant advantage over *K. pneumoniae* in the bladders of healthy mice and less of an advantage in diabetic bladders. In the kidneys, *K. pneumoniae* outcompeted *E. coli* in healthy mice but in diabetic mice *E. coli* outcompeted *K. pneumoniae* and caused a severe pyelonephritis. Diabetic kidneys contained renal tubules laden with matrix-embedded extracellular *E. coli* UTI89 bacterial communities. Diabetic mice also had glucosuria, which may enhance bacterial replication in the urinary tract. These data support that this murine diabetic UTI model is consistent with known characteristics of human diabetic UTI and can provide a powerful tool for dissecting this infection in the multi-factorial setting of diabetes.

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Introduction

Urinary tract infection (UTIs), which include infections of the bladder (cystitis) and kidney (pyelonephritis), primarily affect women and are responsible for nearly 13 million annual office visits in the United States (16). One third of women will experience a recurrent infection within 3-6 months of the initial episode (19) and 44% will experience a recurrence within one year (21). These infections are most commonly caused by the Gram-negative bacterium *Escherichia coli,* which is responsible for 80-85% of community acquired UTIs, however there are numerous other pathogens capable of infecting the urinary tract (20, 46). Uropathogenic *E. coli* (UPEC) employs a wide array of virulence factors to successfully colonize and survive within the urinary tract including adhesive organelles such as type 1, P, F1C, and S pili (2, 22, 40, 41), iron acquisition/transport systems (49), hemolysin (39), and flagella (27, 51). It has recently been found that UPEC has the ability to invade bladder urothelial cells and replicate to form intracellular bacterial communities (IBCs) largely protected from host innate immunity (1, 9, 23, 47). Bacteria disperse from these IBCs, some in filamentous morphology, which subverts elimination by polymorphonuclear leukocytes (PMN) and allows for further dissemination throughout the urinary tract (24, 47).

Diabetes mellitus is the most common endocrine disease and worldwide incidence of this ailment is increasing (38). Type 1 diabetes is an autoimmune disorder by which insulin-producing beta-islet cells are destroyed by one's own immune system (15). These patients, even with proper management and glycemic control, can develop a variety of diabetic sequelae including retinopathy, neuropathy, nephropathy and numerous

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cardiovascular complications. Additionally, diabetics are more prone to infection and these infections are more severe than in non-diabetics (6, 43).

The urinary tract is the most common site of infection in the diabetic host (30). Diabetics are more likely to have asymptomatic and symptomatic bacteriuria (14, 42). Acute pyelonephritis is approximately ten times more common in the diabetic population (37). In addition to higher risk of developing UTI, diabetic women have increased risk of developing complications of UTI such as emphysematous cystitis, abscess formation and renal papillary necrosis (42). Although UPEC remains the predominant etiologic agent of UTI in diabetic individuals, infections by *Klebsiella* species (17, 29), enterococci (5, 29), *Acinetobacter* species (36), group B streptococci (35), fungi (26) and other less common uropathogens are more prevalent in diabetic women. Many hypotheses have been attributed to the increase of UTI in diabetic women, such as glucosuria, impaired immune cell function or functional abnormalities of the urinary tract, however, these theories have not been fully tested or confirmed in an animal model of UTI (42). While there are multiple murine models of diabetes, including non-obese diabetic (NOD) mice (31), few have been used to effectively investigate diabetic UTI. This study presents a streptozocin-induced murine model of diabetic UTI. This model is consistent with epidemiologic observations of diabetic UTI and thus will greatly assist in understanding the physiological and molecular mechanisms underlying uropathogenesis in the diabetic setting. Using this model, we discovered that diabetic mice are more prone to UTI and differences in virulence observed in the kidney and bladder are dependent on the bacterial strain and host background.

Materials and Methods

Bacterial strains and growth conditions.

Clinical strains used in this study were UTI89, a UPEC cystitis isolate (34), UTI89 hk::comGFP, a kanamycin resistant and GFP expressing strain of UTI89 (51), TOP52 1721, a *K. pneumoniae* cystitis isolate, and 0852, an *Enterococcus faecalis* UTI isolate (25). Bacteria were cultured at 37°C in Luria-Bertani (LB) broth (UTI89 and TOP52) or brain heart infusion (BHI) broth (0852). UTI89 hk::comGFP growth media also contained 50 μg/ml kanamycin.

Induction of diabetes in mice.

To develop a diabetic mouse model of UTI, we gave 4-5-week-old female C3H/HeN (National Cancer Institute), C3H/HeJ (Jackson Laboratories) or C57BL/6 (Jackson Laboratories) mice 2-3 intraperitoneal (IP) injections of streptozocin (STZ, Sigma-Aldrich) to induce pancreatic islet β-cell death. Mice were weighed prior to injections and STZ was freshly dissolved in dilution buffer (0.1M Sodium citrate, pH 4.5 with HCl, stored at 4°C) and filter sterilized. To induce diabetes, mice were given 0.1 ml IP injections of 200 mg/kg STZ using Precision Sure Dose ½ CC syringe with 30 x 3/8" gauge needle. Dilution buffer-injected mice were used as healthy controls. Blood glucose levels were measured daily beginning 5 days after the second IP injection. STZ-injected mice with glucose levels <250 were given a third STZ IP injection. This method

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consistently resulted in 80-85% penetrance of diabetes in STZ-injected mice. Mice were considered diabetic after two consecutive readings of >250 mg/dl blood glucose.

Mouse infections, competitions and organ titers.

Eight-week-old female diabetic or healthy control mice were infected by transurethral catheterization as previously described (33). Static bacterial cultures were started from freezer stocks and grown at 37°C for 18 h and then subcultured 1:250 (for UTI89 and TOP52) or 1:100 (for 0852) into fresh media. These subcultures were then grown statically at 37°C for 18 h (for UTI89 and TOP52) or 2-3 h (for 0852), pelleted, resuspended in PBS and diluted appropriately to yield 50 μl inocula (for UTI89 and TOP52) or 200 μl inocula (for 0852) of 1-2 x 10^7 CFU unless otherwise indicated. For competition experiments, 10^7 CFU each of *K. pneumoniae* TOP52 and *E. coli* UTI89 hk::comGFP were inoculated together in a total volume of 50 μl. To quantify bacteria present in mouse organs, bladders and kidneys were aseptically harvested at the indicated times post infection, homogenized in PBS, serially diluted, and plated onto LB plates, BHI plates or LB-kanamycin (50 μg/ml) and BBL CHROMagar Orientation media (BD-Diagnostics) for competition experiments. All studies were approved by the Animal Studies Committee at Washington University School of Medicine.

Mouse urine collection and analysis.

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Urine samples were collected from diabetic and healthy mice by bladder massage over a

sterile 1.5 ml Eppendorf tube prior to bacterial inoculation. Urine samples were analyzed

using Multistix Pro 10 LS urine reagent test strips (Bayer) according to manufacturer's

instructions.

Histology and electron microscopy.

Infected mouse bladders and kidneys were aseptically removed, fixed in neutral buffer

formalin, and paraffin embedded. Sections were stained with hematoxylin and eosin

(H&E) and examined using an Olympus BX51 light microscope (Olympus America).

For transmission electron microscopy (TEM), glutaraldehyde-fixed kidneys were

harvested and processed as previously described (32). Sections were viewed on a JEOL

1200 EX transmission electron microscope (JEOL USA) at 80 kV accelerating voltage.

Urine growth curves.

Overnight UTI89 and TOP52 shaking LB cultures were subcultured 1:100 into filter-

sterilized urine specimens from healthy volunteers with and without 2% glucose

supplementation. Samples were grown shaking at 37°C and OD_{600} readings were taken

at various timepoints. Doubling times were calculated as $t_d = \ln 2\,(t_2 - t_1)/(\ln OD_2 - \ln OD_1)$.

Statistical analysis.

Competitive indices were calculated as (UTI89 hk::comGFP $_{out}$/ UTI89 hk::comGFP $_{In}$)/ (TOP52$_{out}$/TOP52$_{In}$). The Wilcoxon signed rank test was used to compare log of competitive indices to a theoretical mean of zero. Continuous variables were compared using the Mann-Whitney *U* test since these variables were not normally distributed. All tests were two-tailed and a P value less than 0.05 was considered significant. These analyses were performed using GraphPad Prism (GraphPad Software, version 4.03). To calculate ID$_{50}$ values, the Reed and Muench mathematical technique (45) was used and infection was defined as organs with bacterial titers above the limit of detection (20 CFU) at 72 h post infection.

Results

Diabetic mice have increased susceptibility to UPEC infection and higher bacterial burden.

It is widely known that diabetics are more susceptible to UTI than non-diabetics. To determine if STZ-induced diabetic C3H/HeN mice are more susceptible to UTI compared to buffer-treated control mice, various doses of the UPEC cystitis isolate, UTI89, were inoculated by transurethral catheterization. After 72 h of infection, mice were sacrificed and bladder and kidney bacterial titers were determined (Fig. 1A).

Inocula of 10^7 (P=0.0006), 10^6 (P=0.0079), 10^5 (P=0.0173), 10^4 (P=0.0080) and 10^3 (P=0.0317) CFU of *E. coli* UTI89 all resulted in significantly higher bladder titers in diabetic mice compared to buffer-treated controls. The majority of healthy buffer-treated mice inoculated with 10^3 or 10^4 CFU of UTI89 were able to efficiently clear infection after 72 h; however, almost all diabetic mice had significant infection even with these relatively low inocula. Defining infection as a titer at 72 h greater than 20 CFU (minimum level of detection), the ID_{50} of *E. coli* UTI89 was 1.97 x 10^4 CFU in healthy murine bladders and was less than 100 CFU (approximately 68.1) in diabetic bladders. Kidney titers were also significantly higher in diabetic mice compared to buffer-treated control mice after inoculation with 10^7 (P=0.0006), 10^6 (P=0.0079), 10^5 (P=0.0087), 10^4 (P=0.0200) and 10^3 (P=0.0159) CFU of *E. coli* UTI89 (Fig. 1B). By 72 h after inoculation with 10^3 CFU of UTI89, all diabetic mice had significant kidney titers while only half of the healthy mice remained infected. The ID_{50} of UTI89 was 2.08 x 10^3 CFU in healthy murine kidneys and was less than 100 CFU (approximately 46.4) in diabetic kidneys.

The kinetics of urinary tract colonization by *E. coli* UTI89 were compared between diabetic mice and healthy controls (Fig. 2A-B). UTI89 efficiently colonized mouse bladders as early as 6 h post infection in both healthy and diabetic mice (Fig. 2A). The bacterial load in infected bladders of healthy mice decreased to a geometric mean of
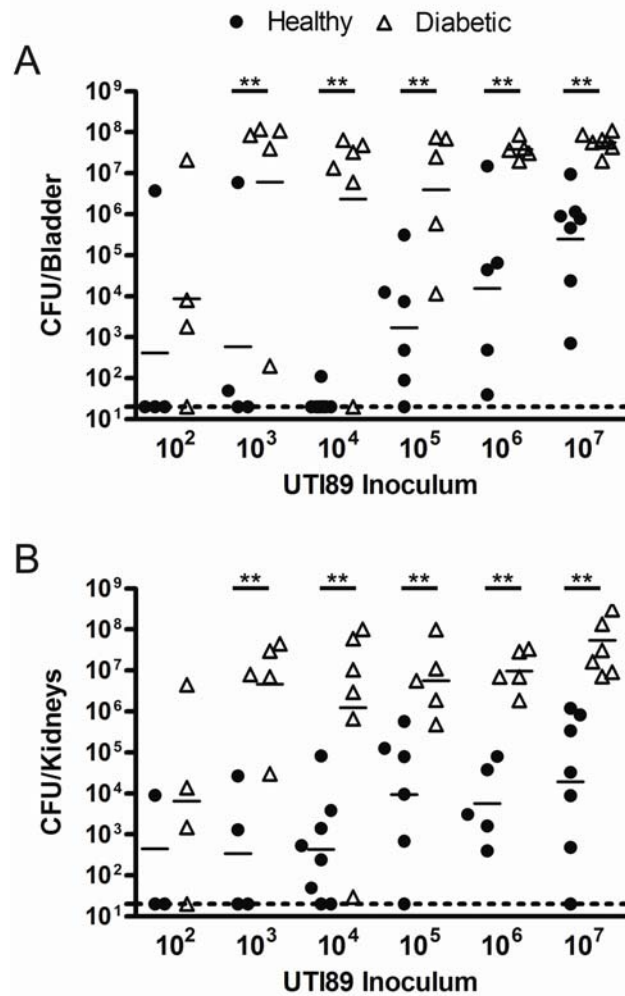
Figure 1. Diabetic Mice have Increased Susceptibility to *E. coli* UTI89 UTI compared to Healthy Mice

Female C3H/HeN diabetic mice (Δ) and buffer-treated control mice (●) were infected with varying inocula of UTI89, a UPEC cystitis isolate. At 72 h post infection, bladders (A) and kidneys (B) were harvested, homogenized and CFU were determined. Titer data are combined from 3 independent experiments. Short bars represent geometric means of each group and dotted lines represent limits of detection. The symbol (**) indicates significant P values less than 0.05.
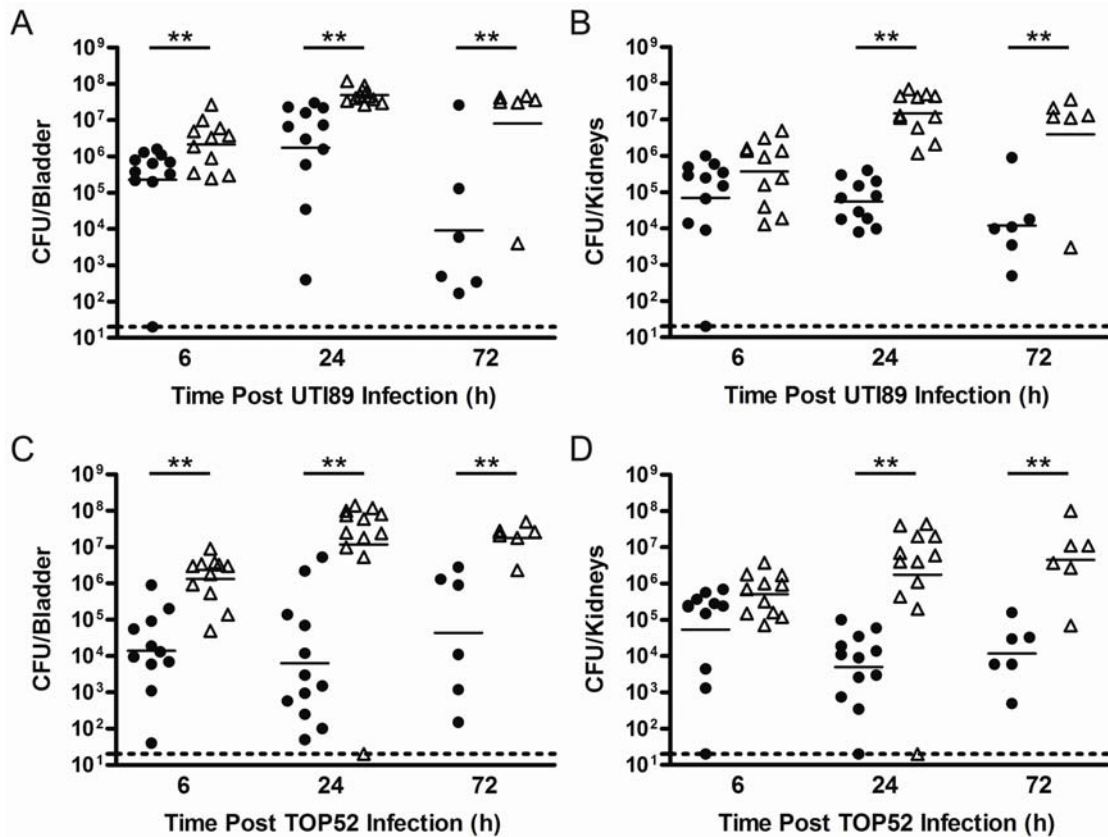
Figure 2. Timecourse of *E. coli* UTI89 and *K. pneumoniae* TOP52 Bladder and Kidney Infections in Healthy and Diabetic mice

Female C3H/HeN diabetic mice (Δ) and buffer-treated control mice ($\bullet$) were inoculated with 10^7 CFU of the UPEC isolate, UTI89 (A-B), or with 10^7 CFU of the *K. pneumoniae* isolate, TOP52 (C-D) by transurethral catheterization. For organ titers, bladders (A, C) and kidneys (B, D) were harvested at various timepoints post infection and CFU were calculated. Graphs of bacterial burden of *E. coli* UTI89 in the bladder (A) and kidneys (B), and *K. pneumoniae* TOP52 in the bladder (C) and kidneys (D) are shown. Titer data are combined from 3 independent experiments. Short bars represent geometric means of each group and dotted lines represent limits of detection. The symbol (**) indicates significant P values less than 0.05.

9.2×10^3 CFU per bladder by 72 h post infection. Diabetic mouse bladders, on the other hand, retained high levels of bacterial colonization at 72 h post infection. Similar patterns of persistent high-level colonization through out the course of infection were seen in infected kidneys of diabetic mice (Fig. 2B).

These data show that diabetic mice are more susceptible to infection by the UPEC isolate, UTI89. UTI89 had a considerably lower ID_{50} in the diabetic background than in healthy control mice. UTI89 also had significantly higher bladder titers at 6, 24, and 72 h post infection in diabetic mice than in healthy mice. Additionally, diabetic mice had greater UTI89 kidney infections compared to healthy mice, especially at later timepoints in infection.

Diabetic mice infected with K. pneumoniae or E. faecalis have higher burdens of infection.

Urinary tract infections caused by non-UPEC uropathogens are more prevalent in diabetic individuals. To determine how non-UPEC uropathogens perform in the diabetic mouse model over time, we inoculated diabetic and buffer-treated control mice with either the *K. pneumoniae* isolate, TOP52 1721 (abbreviated TOP52, Fig. 2C-D), or the *E. faecalis* isolate, 0852 (Fig. 3), in their respective murine models of UTI. As early as 6 h post infection, the bacterial load of *K. pneumoniae* TOP52 was 100-fold higher in the bladders of diabetic C3H/HeN mice than in healthy C3H/HeN mice (Fig. 2C, P=0.0005).

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This difference between healthy and diabetic bladder TOP52 bacterial burdens was further exaggerated to greater than 1000-fold by 24 h post infection (P=0.0007). *K. pneumoniae* TOP52 showed similar levels of infection in the kidneys of diabetic and healthy mice at 6 h but diabetic bacterial burdens were significantly higher by 24 h (P=0.0007) and 72 h (P=0.0043) post infection.

In the C57BL/6 UTI model, the *E. faecalis* isolate, 0852, showed no difference in bacterial titer at 2 d post infection in the bladders (Fig. 3A) and kidneys (Fig. 3B) of diabetic and control mice. However, diabetic mouse kidneys had significantly higher bacterial burdens than buffer-treated control mouse kidneys at both 7 d (P=0.0229) and 14 d (P=0.0135) post infection. Significantly higher *E. faecalis* 0852 titers were also observed in diabetic mouse bladders compared to control mouse bladders at 7 d (P=0.0119) and 14 d (P=0.0420) post infection.

These data demonstrate that diabetic mice have greater burdens of UTI caused by the *K. pneumoniae* isolate, TOP52, or the *E. faecalis* isolate, 0852, compared to healthy mice. The experiments involving *E. faecalis* in the C57BL/6 background also illustrate the versatility of STZ-induction of diabetes and the ability to adapt this method to preexisting models of UTI characterized for specific uropathogens. Interestingly, the *K. pneumoniae* strain, TOP52, has even larger differences between bladder bacterial loads of diabetic and control mice than does the *E. coli* strain, UTI89, at 6 and 24 h post infection. This greater advantage afforded to TOP52 in the diabetic background is consistent with the higher rates of *K. pneumoniae* cystitis observed in diabetic patients.
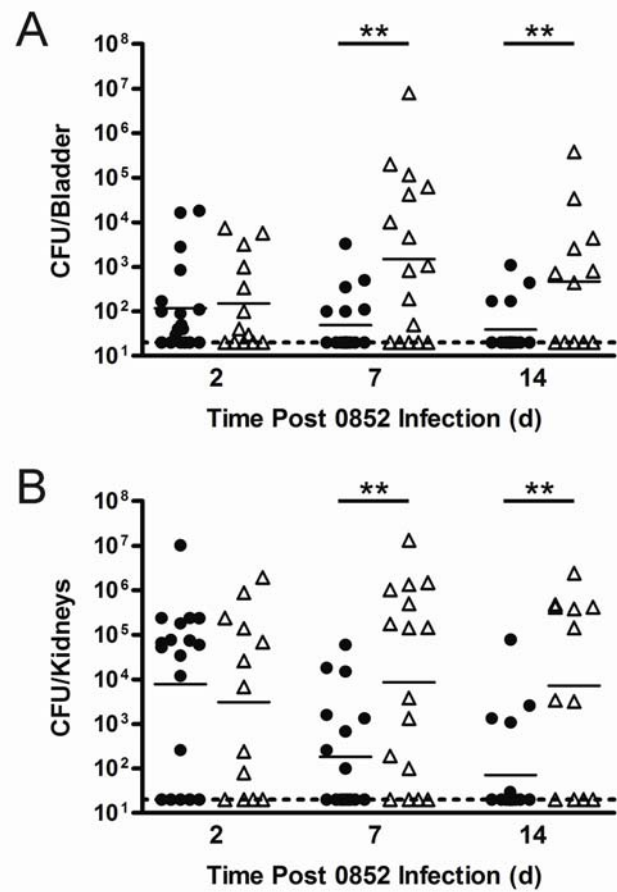
Figure 3. Timecourse of *E. faecalis* 0852 Bladder and Kidney Infections in Healthy and Diabetic Mice

Female C57BL/6 diabetic mice (Δ) and buffer-treated control mice (●) were inoculated with 10^7 CFU of the *Enterococcus faecalis* isolate, 0852, by transurethral catheterization. Bladders (A) and kidneys (B) were harvested at various timepoints post infection and CFU were calculated. Graphs of bacterial burden of *E. faecalis* 0852 in the bladder (A) and in the kidneys (B) are shown. Titer data are combined from 2 independent experiments. Short bars represent geometric means of each group and dotted lines represent limits of detection. The symbol (**) indicates significant P values less than 0.05.

Advantages between UPEC and non-UPEC uropathogens shift in the diabetic host.

To directly compare UPEC to Gram negative non-UPEC uropathogens in the diabetic model, we conducted competition experiments in which both 10^7 *E. coli* UTI89 hk::comGFP and 10^7 *K. pneumoniae* TOP52 were co-inoculated into diabetic and buffer-treated C3H/HeN mice. UTI89 hk::comGFP has a kanamycin resistance cassette allowing for selection on antibiotic media. After 24 h, bladders and kidneys were harvested, titers of each pathogen were enumerated, and competitive indices were calculated. We then compared the log competitive index of healthy and diabetic mice in the bladder (Fig. 4A) and kidney (Fig. 4B). A value of greater than 0 indicates UTI89 hk::comGFP outcompetes TOP52 while a value of less than 0 indicates TOP52 outcompetes UTI89 hk::comGFP. In the bladders of buffer-treated mice, the log of the competitive indices was significantly greater than 0 (P=0.0020), indicating *E. coli* UTI89 hk::comGFP has a significant advantage over *K. pneumoniae* TOP52. In diabetic bladders, the log of competitive indices was also significantly greater than 0 (P=0.0371), albeit to a lesser degree than in healthy bladders. Thus the competitive advantage of UTI89 hk::comGFP over TOP52 seems more pronounced in healthy bladders than in diabetic bladders. In the kidney, while *K. pneumoniae* TOP52 had a significant advantage over *E. coli* UTI89 hk::comGFP in the healthy background (P=0.0137), UTI89 hk::comGFP substantially outcompeted TOP52 in the diabetic host (P=0.0200).

An increased prevalence of non-UPEC strains cause cystitis in diabetic patients. In our model, the diabetic condition gave *E. coli* a lesser advantage over *K. pneumoniae*
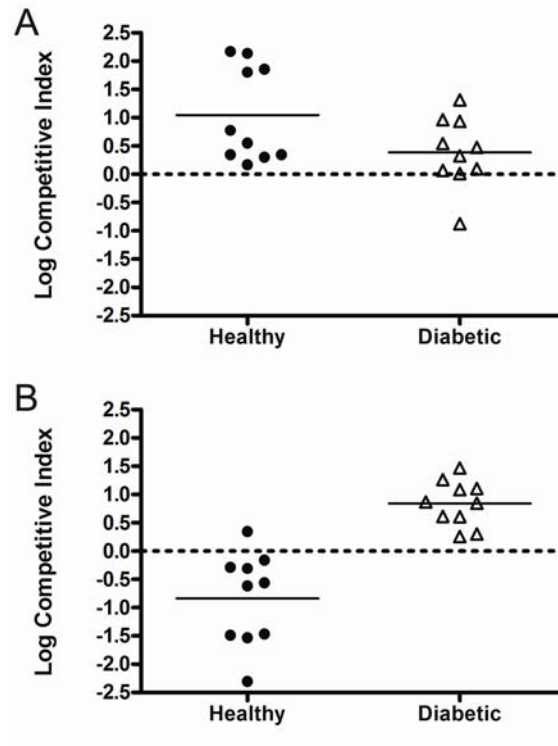
Figure 4. Competition of *E. coli* UTI89 and *K. pneumoniae* TOP52 in the Bladder and Kidneys of Healthy and Diabetic Mice

Female C3H/HeN diabetic mice (Δ) and buffer-treated control mice (●) were infected with 10^7 CFU each of *E. coli* UTI89 hk::comGFP and *K. pneumoniae* TOP52. After 24 h, bladders (A) and kidneys (B) were harvested, CFU of each pathogen were enumerated, and competitive indices equal to (UTI89 hk::comGFP $_{out}$/ UTI89 hk::comGFP $_{In}$)/ (TOP52$_{out}$/TOP52$_{In}$) were calculated. A value greater than zero indicates an *E. coli* UTI89 hk::comGFP advantage, while a value less than zero indicates a *K. pneumoniae* TOP52 advantage. The log of the competitive indices were significantly different than zero in all cases. Data are combined from 2 independent experiments. Bars represent means of each group and dotted lines represents value at which each uropathogen competes equally.

in the bladder, consistent with the increased prevalence of *K. pneumoniae* cystitis in diabetics. In the kidney, the situation was different. We found a dramatic shift in the kidney from an environment favoring *K. pneumoniae* in control mice to an environment favoring UPEC colonization in the diabetic setting.

UPEC causes marked interstitial pyelonephritis in diabetic mice.

To further investigate the shift favoring an *E. coli* UTI89 competitive advantage in the diabetic kidney, we examined histologic H&E-stained sections of single 72 h infections of *E. coli* UTI89 or *K. pneumoniae* TOP52. The renal pelvises (Fig. 5A) of buffer-treated control mice inoculated with UTI89 or TOP52 both showed low levels of inflammation. The pelvises of infected diabetic mice infected with UTI89 or TOP52 were dilated and significantly inflamed, often with large sheets of PMN. The urothelium lining the pelvis was hyperplastic with intraurothelial neutrophilia and collections of bacteria were observed within the pelvic space. The kidney parenchyma (Fig. 5B) of healthy control mice infected with TOP52 or UTI89 and of diabetic TOP52-infected mice were largely unremarkable with patent tubules and normal-appearing glomeruli. In contrast, the diabetic *E. coli* UTI89-infected kidney parenchyma displayed a marked acute interstitial pyelonephritis with cortical and medullary involvement. There were multiple areas of abcessation with wide-spread destruction of renal architecture. High power views of these regions (Fig. 5C-D) showed marked inflammation that was largely neutrophilic in nature with a small component of lymphocytes and plasma cells. Vast
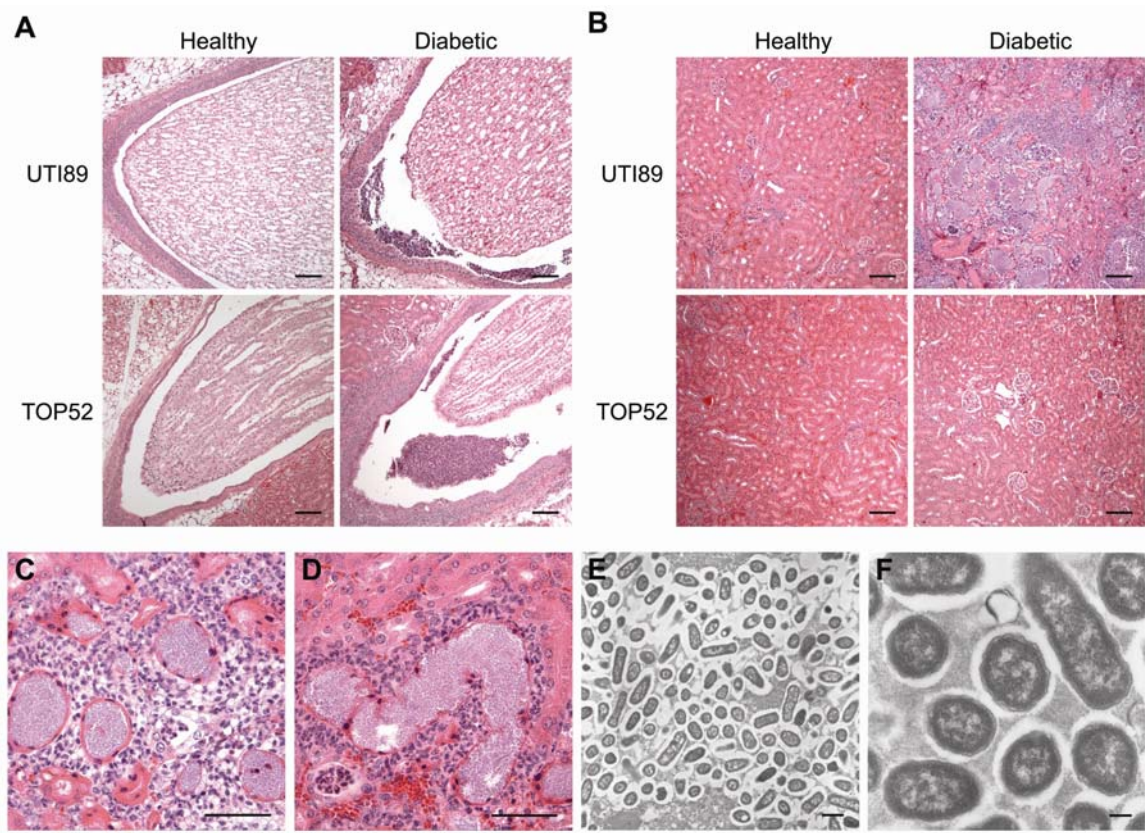
Figure 5. *E. coli* **UTI89 Causes an Acute Interstitial Pyelonephritis in the Kidneys of Diabetic Mice**

Kidney sections from 72 h post infection with *E. coli* UTI89 or *K. pneumoniae* TOP52 were analyzed by light microscopy. (A) Renal pelvises from diabetic mice infected with either UTI89 or TOP52 revealed increased inflammatory cells, primarily PMN. (B) Kidney parenchyma appeared largely normal in healthy mice and in diabetic mice infected with TOP52, however, UTI89-infected kidneys showed marked histopathology with loss of tissue architecture and a significant inflammatory infiltrate. (C-D) High power magnification of UTI89-infected kidneys revealed large collections of bacteria filling renal tubule lumen and collections of intratubular and peritubular PMN. (E-F) Electron microscopy of these renal tubules showed tight collection of bacteria embedded in an extracellular matrix material. Scale bars, 100 μm (A, B), 50 μm (C, D), 1 μm (E) and 0.2 μm (F).

collections of extracellular bacteria filled the lumen of renal tubules. Intratubular and peritubular PMN were also observed.

To further characterize the collections of bacteria observed within the kidney tubules, TEM was performed (Fig. 5E-F). Bacteria were tightly packed between simple tubular epithelial cells of the kidney. An extracellular matrix material was observed between bacteria. These UPEC collections had similar morphology and spacing to that of the intracellular bacterial communities formed in the bladder during cystitis.

Bladder histology was similar between *E. coli* UTI89-infected and *K. pneumoniae* TOP52-infected bladders. In the healthy mouse bladders at 72 h, moderate acute inflammation and epithelial hyperplasia were observed. Diabetic mouse bladders had increased luminal bacteria and PMN compared to buffer-treated controls (data not shown).

These findings demonstrate a significant pyelonephritic phenotype in the kidneys of UPEC-infected diabetic mice. This phenotype is specific to *E. coli* UTI89, as it was not observed in *K. pneumoniae* TOP52-infected diabetic kidneys, and is consistent with the significant advantage of UTI89 over TOP52 in the diabetic kidney.

Diabetes in TLR-4 deficient mice.

Many studies have argued that diabetics have a defect in host inflammatory cell function which may contribute to their increased infection rate. To further investigate

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whether innate host immunity or other factors irrespective of innate immunity play roles in the differences observed between diabetic and healthy infections, we inoculated diabetic and healthy C3H/HeJ and C3H/HeN female mice with *E. coli* UTI89 or *K. pneumoniae* TOP52 (Fig. 6). C3H/HeJ mice contain a mutation in the Toll-Like Receptor 4 (TLR-4) signaling domain and thus fail to transmit signal. It has been shown that UPEC colonize the bladders and kidneys of C3H/HeJ mice to significantly higher levels than C3H/HeN mice without inducing a significant neutrophil response early in infection (18, 48). Diabetic C3H/HeJ mice were more prone to infection by *E. coli* UTI89 at 72 h post infection compared to healthy controls (Fig. 6A, P=0.0221). The presence of a diabetic advantage in C3H/HeJ mice suggests that additional TLR-4-independant factors may be important in diabetic UTI. However, the difference in UTI89 titers between healthy and diabetic bladders was greater in C3H/HeN mice (10,000 fold increase) than in C3H/HeJ mice (100 fold increase) suggesting that innate immune factors related to TLR-4 regulated processes may also be important. UTI89 had less than a ten fold advantage in diabetic kidneys of C3H/HeJ mice (Fig. 6B, P=0.0023) at 72 h post infection compared to healthy controls compared to the 10,000 fold advantage seen in C3H/HeN kidneys. Thus, TLR-4 regulated factors may account for much of the increase in bacterial burdens in diabetic mouse kidneys compared to healthy controls.

K. pneumoniae TOP52 had significantly higher titers in the bladders (Fig. 6C) of both diabetic C3H/HeN (P=0.0043) and C3H/HeJ (P=0.0043) mice than in buffer-treated controls. Interestingly, the differences in geometric means of the 72 h titer data were
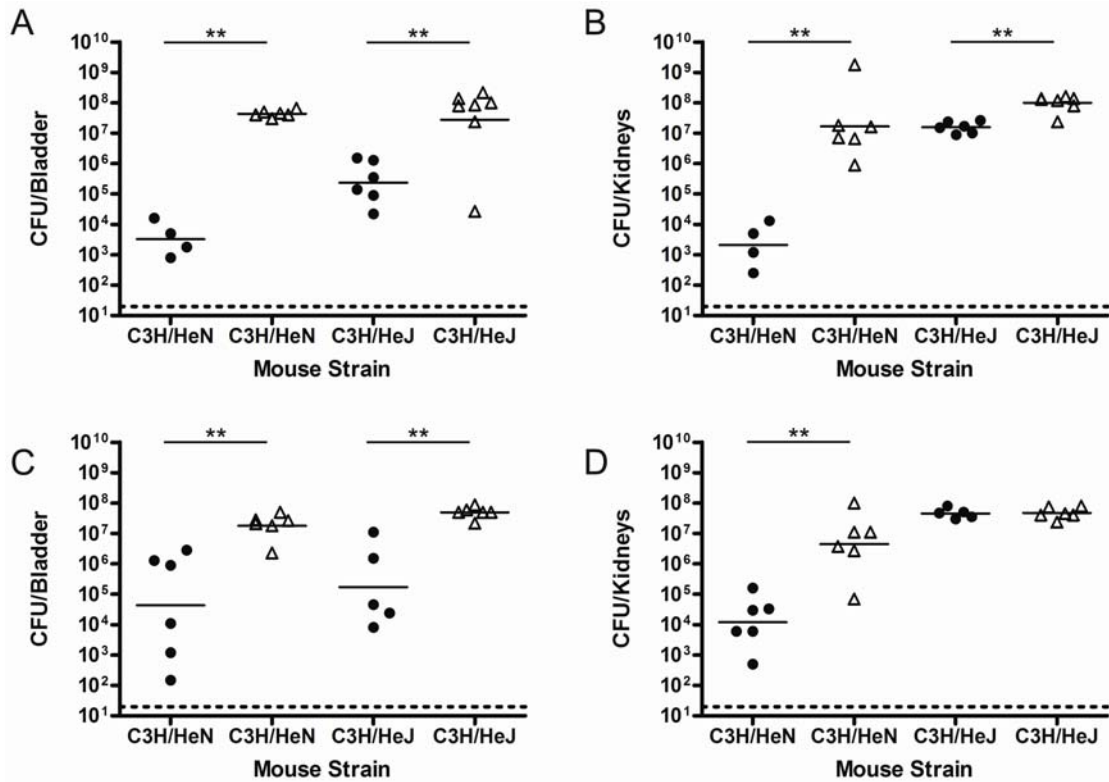
Figure 6. Differences in Diabetic UTI in C3H/HeN and C3H/HeJ Mice
Female C3H/HeN and C3H/HeJ diabetic mice (Δ) and buffer-treated control mice (●)
were inoculated with 10^7 CFU of *E. coli* UTI89 (A-B), or with 10^7 CFU of *K.*
pneumoniae TOP52 (C-D). For organ titers, bladders (A, C) and kidneys (B, D) were
harvested at 72 h post infection and CFU were calculated. Graphs of bacterial burden of
UTI89 in the bladders (A), and kidneys (B) and TOP52 in the bladders (C) and kidneys
(D) of both mouse backgrounds are shown. Titer data are combined from 2 independent
experiments. Short bars represent geometric means of each group and dotted lines
represent limits of detection. The symbol (**) indicates significant P values less than
0.05.

roughly equivalent in the C3H/HeN and C3H/HeJ backgrounds. Similar to UTI89, while

TOP52 displayed a significant advantage in the kidneys (Fig. 6D) of diabetic C3H/HeN

mice compared to healthy controls (P=0.0043), TOP52 had no advantage in diabetic

kidneys of C3H/HeJ mice compared to healthy C3H/HeJ controls (P=0.9307). Thus,

TLR-4 regulated factors seemingly account for much of the increased advantage of

TOP52 in the C3H/HeN kidney. Inducing diabetes in the C3H/HeJ mice produced no

additional observable effects. Thus, much of the increase in C3H/HeN kidney

colonization conferred upon inducing diabetes may be due to certain defects in TLR-4

regulated innate immune factors, although non-TLR-4 related factors may also be

important.

Taken together, these experiments suggest that the diabetic phenotypes observed

for both UPEC and non-UPEC organisms are likely multi-factorial. A defect in TLR-4

regulated factors may be playing a role in diabetic mice. Nevertheless, other diabetic

effects, irrespective of TLR-4, are also important in the ability of uropathogens to cause

high burdens of infection in the urinary tract.

Urine Growth Curves.

Poorly regulated diabetic patients often spill glucose into their urine. To

determine if STZ-treated diabetic mice have glucosuria, we collected urine samples from

diabetic and buffer-treated control mice. Urine test reagent strips were used to determine

the glucose status and specific gravity of the mouse urines prior to inoculation with a uropathogen. Diabetic mouse urine consistently had ≥ 200 mg/dl (2%) glucose and a low specific gravity of 1.010. Healthy control mouse urine was negative for glucose and had a specific gravity of 1.030. Only similar trace amounts of protein and ketones were found in diabetic and healthy mouse urines.

E. coli UTI89 and *K. pneumoniae* TOP52 grow equally well in LB with doubling times of 34 minutes (Fig. 7A). To determine if the glucosuria of the diabetic mice affects uropathogen growth, *E. coli* UTI89 and *K. pneumoniae* TOP52 were grown in filter-sterilized urine from healthy human subjects with and without 2% glucose supplementation (Fig. 7B). Optical density readings of both UTI89 and TOP52 correlated with CFU during logarithmic growth. The doubling times of both UTI89 (1.36 h with glucose, 2.24 without) and TOP52 (1.05 h with glucose and 1.87 without) were significantly shorter in glucose supplemented urine. TOP52 displayed higher yields than UTI89 in urine with and without glucose supplementation.

These data show that STZ-induced diabetic mice have glucosuria similar to that of poorly controlled diabetics. This urine glucose may contribute to the increased bacterial burden within the urinary tract of diabetic mice.
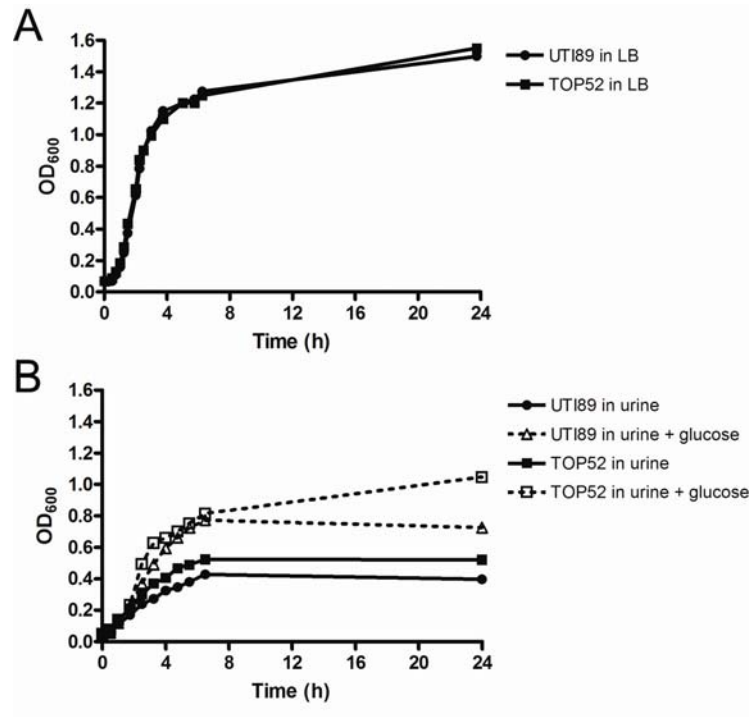
Figure 7. The Addition of Glucose Enhances *E. coli* UTI89 and *K. pneumoniae* TOP52 Growth in Urine

Growth of *E. coli* UTI89 and *K. pneumoniae* TOP52 over time was measured in LB (A) and filter sterilized urine with and without the supplementation of 2% glucose (B). Growth curves shown are representative of 3 independent experiments.

Discussion

Models of STZ-induced diabetes have been used for decades (8, 28, 31), however little has been done to study diabetic urinary tract infection. UTIs are more prevalent and more severe in the diabetic population and murine models of infection that accurately mirror human infection are required to better understand disease (42). STZ-induced diabetic mice were more susceptible to UPEC UTI and had higher burdens of infection than buffer-treated controls. Remarkably low inocula of the UPEC strain, UTI89, were able to effectively infect diabetic mice but were largely cleared from healthy mice. It is also known that diabetic patients are more likely to be infected with non-UPEC uropathogens (29). In STZ-induced diabetic mice, the *K. pneumoniae* strain, TOP52, and the *E. faecalis* strain, 0852, had significantly higher bacterial titers in the bladders and kidneys compared to healthy mice. *E. coli* UTI89 outcompeted *K. pneumoniae* TOP52 1721 in both healthy and diabetic bladders. However, the advantage of UTI89 over TOP52 in the bladders of diabetic mice was reduced. This result reflects the clinical diabetic situation in which UPEC remains the predominant uropathogen with increased frequency of *K. pneumoniae* infection. In contrast, the advantage of UTI89 in the kidneys of diabetic mice over TOP52 was dramatic, since TOP52 outcompeted UTI89 in healthy kidneys. Finally, experiments in C3H/HeJ mice suggested that defects in the diabetic urinary tracts to manage and clear bacterial infection are likely multi-factorial, possibly involving TLR-4 regulated factors, glucosuria and other unknown factors. All of these findings are consistent with human diabetic UTI epidemiologic data and suggest

that this STZ-induced model of diabetic UTI will provide a valuable tool for studying this disease.

Histologic analysis of mouse kidneys revealed a severe pyelonephritic phenotype specific to *E. coli* UTI89 in the diabetic setting. Large extracellular bacterial biofilm-like communities were observed filling renal tubules. Bacteria within these communities were tightly packed within an extracellular matrix. Morphologically these bacterial collections appeared similar to the UPEC biofilm-like intracellular bacterial communities observed within facet cells of the bladder (1, 47). Further studies are required to determine what enables UTI89 to form these communities within the renal tubules of the diabetic host.

There are numerous hypotheses for the enhanced ability of uropathogens to infect the urinary tract of diabetic individuals (12). Poorly controlled diabetes often results in the spilling of glucose into the urine. Glucosuria increased the growth rates of UTI89 and TOP52 and has been shown to increase growth of numerous other uropathogenic isolates (10). Multiple studies have suggested that neutrophil dysfunction and differences in cytokine secretion may play important roles in diabetic UTI (11, 42, 50), however, data contradicting these claims have also been reported (3). We discovered that diabetes in C3H/HeJ mice resulted in significantly increased colonization of the bladders and kidneys by *E. coli,* albeit the effect was decreased compared to C3H/HeN mice. Additionally, induction of diabetes resulted in no advantage for *K. pneumoniae* in the diabetic kidneys of C3H/HeJ mice. These results argue that both TLR-4 related and

unrelated factors may be defective in the setting of diabetes. TLR-4 regulated factors affected by diabetes may include the inability of neutrophils to effectively clear bacteria as was seemingly the case in the diabetic kidneys infected by *E. coli* UTI89. UTI89 was able to establish massive extracellular biofilm-like collections even in the presence of a robust neutrophilic inflammatory infiltrate. Finally, studies have suggested that bacteria have increased adherence abilities in the urinary tract of diabetics, possibly due to lower levels of urine Tamm-Horsfall protein in diabetics (4, 7, 44) or potential changes of the uroepithelial cells themselves (13). This murine model of diabetic UTI provides a powerful tool for dissecting these and other factors involved in diabetic uropathogenesis.

The STZ-induced diabetic UTI mouse model is extremely versatile. A type 1 diabetic syndrome can be induced in different murine backgrounds allowing for the analysis of specific host factors in knockout or mutant mouse backgrounds. Additionally, uropathogens have been studied in various mouse strains and this method of STZ-induction can be applied to the mouse strain best characterized for a given uropathogen. For example, we were able to induce diabetes and analyze infection using the previously established mouse model of *E. faecalis* UTI in the C57BL/6 murine background (25). The STZ method of diabetic induction is extremely practical with high levels of penetrance (80-85%) in a relatively short period of time (2-3 weeks) and can be well-controlled with buffer IP injections in the identical mouse strain. While STZ produces a type 1-like, irreversible insult to pancreatic β-cells leading to a severe diabetic phenotype in these mice (8), it may be possible to treat these mice with insulin or other agents to contrast UTI in the settings of controlled and uncontrolled diabetes. It is currently

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unclear whether the diabetic UTI phenotypes observed will be present in a euglycemic diabetic host. Additionally, this model could be used to test various UTI preventative measures, including vaccinations, which may be especially beneficial for this predisposed population.

This STZ-induced murine model of diabetic UTI is consistent with many known characteristics of human diabetic UTI including increased susceptibility to infection and more severe infection. Many of these traits of diabetic UTI are poorly understood and require an infection model to test hypotheses of diabetic uropathogenesis. Using this model, we discovered that diabetes affects *E. coli, K. pneumoniae and E. faecalis* pathogenesis in diverse ways. Defects in TLR-4 dependent and independent pathways appear to provide differential advantages to these pathogens. A dramatic decrease in the ID_{50} may explain the increased susceptibility of diabetics in general and the ability of *E. coli* to establish biofilms in the diabetic kidney provides insights into the increased virulence seen in diabetics. This model of diabetic UTI is versatile, practical and can provide a powerful tool for dissecting UTI in the setting of diabetes.

Acknowledgements

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Chapter 6

Conclusions and Future Directions

Conservation of the IBC Pathogenic Pathway in Human UTI

Analysis of human urine specimens suggests an association between intracellular bacterial communities (IBCs), filamentous bacteria and acute uncomplicated cystitis in young women (27). Episodes of cystitis caused by uropathogenic *Escherichia coli* (UPEC) in humans likely involve an IBC pathogenic pathway similar to that observed in mice. The ability to utilize an intracellular bladder niche in both murine and human infection may represent an imperative virulence determinant. Intracellular bacteria are largely protected from host innate immunity. While in the intracellular niche, bacteria are not subject to the shear forces of urine flow and are not subject to phagocytosis by host neutrophils or macrophages. Additionally, the formation of intracellular biofilms may allow bacteria to withstand many antimicrobial therapies, as is the case for bacteria in extracellular biofilms (8). IBC formation may also explain how the relatively few bacteria introduced into the bladder with sexual intercourse (4, 5, 16) are able to survive and replicate to numbers high enough to elicit symptoms in the host. The introduction of low numbers of bacteria into the bladder is likely a relatively common occurrence, but it may be that only in the event of invasion and IBC formation can bacteria replicate and persist to sufficiently initiate symptomatic cystitis.

The knowledge that the IBC pathway takes place during human cystitis raises several more questions that require investigation. To begin with, it is unclear whether the presence of urine IBCs or filamentous bacteria may be prognostic indicators of clinical outcome. Studies with proper follow-up can determine whether these patients are more likely to get recurrent cystitis, pyelonephritis or other UTI sequelae. Also, it is unknown

whether IBC formation in human hosts occurs in cycles as has been demonstrated in mice; furthermore, it is unknown whether IBC numbers increase with successive cycles. To investigate this, patients should be studied in a temporal fashion to analyze the timing of this IBC pathway. It is possible that only a few IBCs are formed upon introduction of bacteria to the bladder and bacteria dispersing from these IBCs progress to form greater numbers of IBCs with successive cycles. Filamentation may also show a temporal pattern. Perhaps filaments are less likely to be observed just as a patient becomes bacteriuric prior to a strong host inflammatory response or fluxing from IBCs. Finally, the prevalence of this IBC pathway in humans has not been examined in a broad population. Work described here has only analyzed a relatively homogenous population of young, healthy, single, well-educated women. Studies should be extended to additional populations including elderly patients, catheterized patients or patients with neurogenic bladders. It is also important to carefully assess the response of patients to antibiotic therapy; bacterial strains from patients with urine findings of IBCs and filaments may be more likely to develop antibiotic resistance. Utilizing antibiotics with better cell penetration or longer durations of therapy may more efficiently clear the bladder of bacteria.

Many of the above studies would require verification of IBCs and filaments in urine specimens. This process, which currently requires multiple microscopy modalities, can be extremely laborious, especially for large-scale trials. A urine molecular marker or signature of the IBC pathway would be invaluable in further assessing this process in human UTI. For instance, a mass spectrometry profile could be established that would be

both sensitive and specific to bacteria in IBCs. An automated analysis of urine

specimens could also provide a beneficial lab tool that clinicians may be able to use to

stratify patients based on likelihood of recurrent infection or efficacy of treatment

regimen.

Further compelling evidence of this pathway occurring in human bladders would

be biopsy evidence of IBCs. Biopsy samples from hundreds of surgical pathology cases

have identified no IBCs in the studied bladder sections. However, it was not known

whether sampled patients had a history of UTI and, in the vast majority of cases, bladder

facet cells could not be found. This may be due to sampling bias; urologists are more

likely to biopsy grossly erythematous or inflamed areas of the bladder where an intact

epithelium is rarely present. Additionally, it is contraindicated to knowingly take

biopsies from actively infected patients due to sepsis or other potential complications.

Still these findings would be valuable and studies of cystectomy specimens or autopsy

bladder specimens are warranted.

While the herein described urine studies revealed the presence of IBCs in

exfoliated facet cells, they have not assessed the presence of a quiescent intracellular

reservoir (QIR) in human bladders. In the murine model, small numbers of UPEC have

been shown to persist within endocytic vesicles of the urothelium. QIRs consist of small

foci of bacteria dormant within LAMP1-positive endocytic vesicles that can ultimately

emerge to seed recurrent bacteriuria (25, 29). QIRs were discovered and have been

characterized primarily in C57BL/6 mice, but have also been found in C3H/HeN mice.

Upon epithelial maturation and differentiation, these foci of bacteria can escape their

vesicular compartment and form IBCs in the host urothelium seeding recurrent UTI. Chemical compounds such as protamine sulfate, or the introduction of bacteria, can induce epithelial turnover resulting in a recurrent UTI caused by the dormant reservoir strain. It is imperative to determine whether QIRs are formed in bladders of human hosts because they could have considerable implications on UTI recurrence. This could be done through biopsy studies of female patients with a known recent history of UPEC cystitis. Given the small size of QIRs, identification may prove challenging, thus a human study should include a large number of patients with several biopsies per patient to provide the power necessary to detect these small bacterial foci.

If QIRs are found in humans, perhaps many recurrent UTIs can be prevented by promoting urotheial turnover, as they can be in mice. The drug protamine sulfate, commonly known to counteract heparin, could potentially be used to induce the turnover of the bladder urothelium and clear the organ of QIRs. A trial could be performed in which patients with a history of frequent, recurrent UTI could receive bladder lavages of protamine sulfate or phosphate buffered saline (placebo) with an adjuvant antibiotic. If protamine sulfate can successfully induce the emergence of intracellular bacteria, the presence of antimicrobials will eliminate the uropathogen. The main outcome measure would be recurrent cystitis rates among patients in one year of follow-up following treatment. Ideally oral drugs could be developed that are excreted in the urine to induce urothelial turnover to be taken as an adjuvant to antibiotic therapy.

Studies are currently being conducted in which women with a history of UTI are followed temporally with urine and periurethral cultures taken daily. These studies are

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providing new data regarding the periurethral ascension and the bladder reservoir models of recurrence. Many patients have periurethral colonization with the recurrent uropathogen for days or weeks prior to bacteriuria, possibly supporting an ascension model of recurrence. Other patients display periurethral cultures devoid of uropathogens prior to bacteriuria, supporting a bladder reservoir model. However, one thing apparent from these studies is that there is constant seeding of faecal, periurethral and bladder sites with bacteria from the other sites. The development of treatment modalities for UTI and UTI prevention in addition to antibiotics is necessary. Vaccines or probiotic therapies have shown promise in the past (23, 24, 33) and may help reduce uropathogen colonization and seeding of these anatomic sites, especially in extremely susceptible populations.

The IBC pathogenic pathway is conserved in human UTI. The extent of this conservation and the prevalence of this pathway in patients with cystitis remains obscure, however, the presence of IBCs in humans is a remarkable discovery. This finding changes the historical paradigm that UTI is an extracellular infection and validates the already established murine cystitis model. UTI pathogenesis is clearly more complex than once considered and these newly elucidated steps in the infectious process raise several novel questions and may provide additional therapeutic targets for this common disease.

Conservation of the IBC Pathogenic Pathway Among Uropathogens

The IBC pathogenic pathway was discovered and characterized with the UPEC strain, UTI89 (2, 19). Recently, work by Garofalo and colleagues has demonstrated that the majority of UPEC isolates from various clinical syndromes of UTI are capable of forming IBCs within the murine bladder (11). However, the ability of non-UPEC uropathogens to progress through an intracellular niche had not been assessed until now. The ability to form intracellular biofilms during infection that are largely protected from host innate immunity is likely a valuable virulence property for bacteria. Perhaps this ability is what sets UPEC apart from other pathogens; IBC formation may be the reason UPEC is by far the most predominant uropathogen. Alternatively, other uropathogens may have also evolved the ability to progress through an intracellular niche during infection of the bladder. If this is the case, one must also investigate mechanistic conservation of the pathway. Are there differences between the IBC pathways of UPEC and other uropathogens? Do non-UPEC uropathogens utilize the same set of virulence factors as UPEC in causing infection and just how similar are these factors? These are some of the questions that should be addressed in analyzing the conservation of the IBC pathogenic pathway among uropathogens.
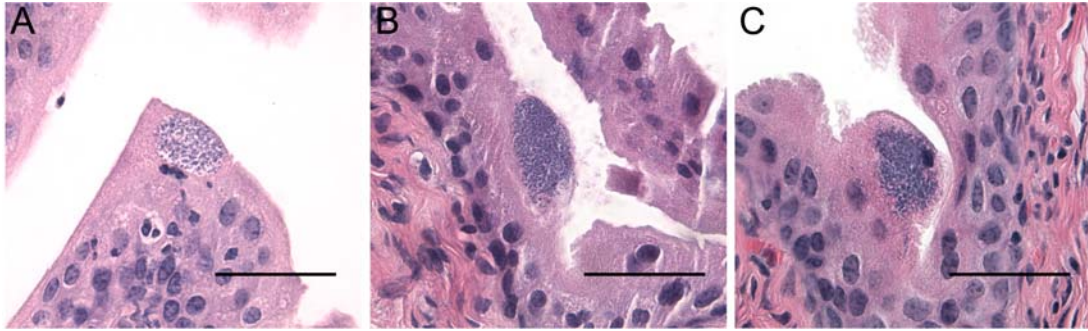
Several non-UPEC uropathogens form IBCs

Cystitis isolates were tested in the C3H/HeN murine model of UTI for the ability to form IBCs. IBCs were observed in several of the clinical isolate infections including *Citrobacter diversus*, *Enterobacter aerogenes*, and two strains of *Klebsiella pneumoniae*

(Figure 1). A summary of the findings for each species of uropathogen tested can be found in Table 1. It is important to note that in this study, only two timepoints were assessed, 6 hours and 24 hours, previously shown to be ideal for UPEC IBC detection; it is possible that some of these strains may produce IBCs at timepoints not analyzed. Moreover, the method utilized here for IBC detection was histological bladder section analysis. Although this is sufficient for IBC detection with robust IBC formers such as UPEC, it may not be sensitive enough for locating IBCs formed by strains producing few IBCs. For infections with isolates in which IBCs could not be found, further testing is warranted. Gentamicin protection assays could be used at various timepoints to determine whether populations of intracellular bacteria exist in these infections.

Despite these limitations, it is clear that the ability to form IBCs is conserved among some, but not all, non-UPEC uropathogens. All strains that were found to form IBCs were Gram-negative uropathogens that are thought to encode type 1 pili. All of these strains, with the exception of the *K. pneumoniae* isolates, had mannose-sensitive hemagglutination (MSHA) of guinea pig erythrocytes indicative of type 1 piliation. This suggests that uropathogens that progress through an IBC pathway likely all utilize type 1 pili or other adhesins during pathogenesis, however, further testing is required to confirm this hypothesis.

For those uropathogens that do form IBCs, their respective pathogenic pathways in the urinary tract should be characterized and dissected with respect to UPEC. Small differences in the progression of the IBC pathway may have profound differences on the virulence of that organism or the ability to persist. These comparisons may also further

Figure 1. Intracellular Bacterial Communities are Formed by Several Non-UPEC Uropathogens.
Intracellular bacterial communities were found in histologic sections (hematoxylin and eosin) of mouse bladders after infection with A) *K. pneumoniae* TOP67 2444, B) *Enterobacter aerogenes* FOSF6600 26-1445, and C) *Citrobacter diversus* TOP68 2384. Scale bars, 50 μm.

Table 1. Ability of Uropathogens to Form IBCs in Murine Model		
Uropathogen	**6 h IBCs***	**24 h IBCs***
Escherichia coli	Yes	Yes
Klebsiella pneumoniae	Yes	Yes
Enterobacter aerogenes	Yes	Yes
Citrobacter diversus	Yes	ND
Pseudomonas aeruginosa	No	No
Staphylococcus saprophyticus	No	No
Enterococcus faecalis	No	No

*"ND" means not determined. "No" means IBCs were not observed in multiple experiments, however they cannot be entirely ruled out.

elucidate virulence properties of UPEC that are important in pathogenesis and provide explanations for its predominance as a uropathogen. However, differences in host response to bacterial pathogens likely have significant effects on UTI outcome. Therefore, the host response to these uropathogens should also be assessed. The level of host urothelial exfoliation, influx of inflammatory cells, and cytokine release could all be important elements in clearing the uropathogen.

Extensive experiments investigating the ability of a *Proteus mirabilis* isolate to form IBCs were not done, however, a small trial was performed with two mice. No IBCs were observed in histologic sections, although this is clearly not a sufficiently large population to draw any conclusions. Given that *Proteus mirabilis* is the third leading cause of Gram-negative UTI, more detailed studies are warranted. Also, *P. mirabilis* has the ability to swarm, a property that may be interesting to further study in the setting of UTI (1). Finally, another intriguing pathogen to study in the murine cystitis model is *Salmonella,* the close relative of *E. coli*. *Salmonellae* are not commonly isolated from the urinary tract, however, recent studies have shown that *Salmonella* serotype Tennessee is an emerging uropathogen (14).

Not all Gram-negative strains tested in this study formed IBCs. *Pseudomonas aeruginosa* did not form detectable IBCs and had extremely low bladder titers at both 6 and 24 hours post infection. This result was not surprising considering *P. aeruginosa* does not encode type 1 pili. It would be interesting to see if expression of type 1 pili rendered *P. aeruginosa* competent for IBC formation in the murine bladder. This could be done by cloning the *fim* operon in a vector that can replicate in *P. aeruginosa*. This

has been successively done previously in the Enterohemorrhagic *E. coli* (EHEC) strain O157:H7, which gained the ability to form IBCs with expression of type 1 pili (11).

There is no evidence of Gram-positive uropathogens utilizing an intracellular niche during infection. *Staphylococcus saprophyticus* and *Enterococcus* isolates did not form detectable IBCs and may have greater kidney tropism in the murine model of UTI (21). In C3H/HeN mice, these Gram-positive uropathogens had extremely low bladder titers compared to the other uropathogens tested. The C3H/HeN murine cystitis model may not be a useful model for studying Gram-positive infections. Further efforts should be made to better understand the pathogenesis of Gram-positive uropathogens, especially *S. saprophyticus*, the second leading cause of UTI among sexually-active young women.

Quiescent intracellular reservoir formation

While various uropathogens were tested for their ability to form IBCs, their ability to form QIRs was not evaluated. Gentamicin protection assays on bladders of abacteriuric mice 2 weeks post infection would be helpful in determining which uropathogens maintain an intracellular niche. Follow-up immunohistochemistry studies could validate the ability of a uropathogen to form QIRs. Whether a bacterium is able to form a QIR may be important in that pathogen's ability to cause non-reascension recurrent infection. One may expect higher same-strain recurrence rates for uropathogens with the ability to form QIRs. An assessment of same-strain recurrence rates for women in the SCOR study at the University of Washington are shown based on index strain in Table 2. This study, like many in the literature, can provide accurate estimates of *E. coli*

Table 2. SCOR Study Recurrence Rates with 3 Months Follow-Up			
Index Uropathogen	**Enrollment UTIs No. (%)**	**Same Strain[*] Recurrent UTIs No. (%)**	**Same Strain[*] Recurrent UTIs x 2[#] No. (%)**
Escherichia coli	85	19 (22.4)	5 (5.9)
Staph. saprophyticus	7	0 (0.0)	0 (0.0)
Klebsiella pneumoniae	2	1 (50.0)	0 (0.0)
Coag.-negative *Staph.*	2	1 (50.0)	0 (0.0)
Group B Streptococci	2	0 (0.0)	0 (0.0)
Proteus mirabilis	1	0 (0.0)	0 (0.0)
Enterococcus	1	0 (0.0)	0 (0.0)
Enterobacter aerogenes	1	0 (0.0)	0 (0.0)

[*]Determination of "same strain" was done by PFGE typing for Gram-negative strains and antibiotic profiling for Gram-positive strains.

[#]This group of patients had multiple recurrent UTIs in the 3 month follow-up period.

cystitis recurrence rates in the three month follow-up period, however, the population tested is not sufficiently large enough for estimating recurrence rates of non-UPEC uropathogens. Interestingly, the second most predominant uropathogen, *S. saprophyticus*, did not cause recurrences in the seven cases followed in this study. Further work needs to be done following women with non-UPEC UTIs, in order to assess outcome and recurrence rates specific to each non-UPEC uropathogen.

Enterobacter aerogenes uropathogenesis

The *Enterobacter aerogenes* cystitis isolate FOSF6600 was also assessed for its ability to progress through an IBC cycle. *Enterobacter aerogenes* is a member of the *Enterobacteriaceae* and is part of the normal flora of the large intestine. *Enterobacter* species cause 1% of community-acquired, 2.4% of pediatric, 7% of nosocomial UTI (18). The incidence of *Enterobacter* UTI is increasing and drug resistance is an escalating problem in these strains (15, 28). FOSF6600 produced an MSHA titer similar to that of UTI89 after static growth. This isolate was then inoculated into the C3H/HeN murine cystitis model and was monitored for IBC formation. Although many IBCs were found at both 6 and 24 hour timepoints, interestingly, no filamentous bacteria were observed in these bladder sections. Notably, bladder bacterial titers were similar to UTI89 at early timepoints of infection, yet FOSF6600, in contrast to UTI89, was cleared from the bladder by two weeks post infection.

To further investigate the lack of filamentation by FOSF6600 *in vivo*, mitomycin C studies were performed. Mitomycin C induces the SOS response ultimately leading to

inhibition of cell division and the onset of filamentation. The pathway has been well characterized: the LexA protein binds to conserved SOS boxes in the promoter region of genes such as SulA, an FtsZ inhibitor. Upon induction of stress, RecA stimulates the autoproteolysis of LexA leading to transcription of SulA and other SOS response genes. SulA inhibits FtsZ ring formation preventing septum formation and leading to bacterial filamentation (7). In our investigation, while UTI89 formed long filaments in response to mitomycin C SOS induction, FOSF6600 did not (Figure 2). To further investigate this finding, an *in vitro* timecourse of mitomycin C SOS-induction was performed on UTI89, UTI89Δ*sulA*, *Enterobacter aerogones* FOSF6600, and the *Klebsiella pneumoniae* strains TOP52 and TOP67 and percent filamentation was measured on a semi-quantitative scale (Figure 3). UTI89Δ*sulA* and FOSF6600 had severely delayed and deficient filamentation compared to UTI89, TOP52 and TOP67.

The phenotypes observed with FOSF6600 are extremely similar to those observed with UTI89Δ*sulA*. UTI89Δ*sulA* does not filament in the murine cystitis model and is unable to persist in the bladder (20). These findings suggest the importance of bacterial filamentation for persistence *in vivo*. Additionally, it was found that UTI89Δ*sulA* was able to persist in the C3H/HeJ murine background with deficient TLR-4 signaling. Thus, filamentation may be less important in infections of murine bladders lacking a robust innate immune response. To investigate this further, C3H/HeJ mice were infected with *Enterobacter aerogenes* FOSF6600. FOSF6600 was able to persist and produced higher bladder and kidney titers in C3H/HeJ mice than in C3H/HeN mice (Figure 4). In C3H/HeJ mice, FOSF6600 had bladder and kidney titers similar to those of UTI89.
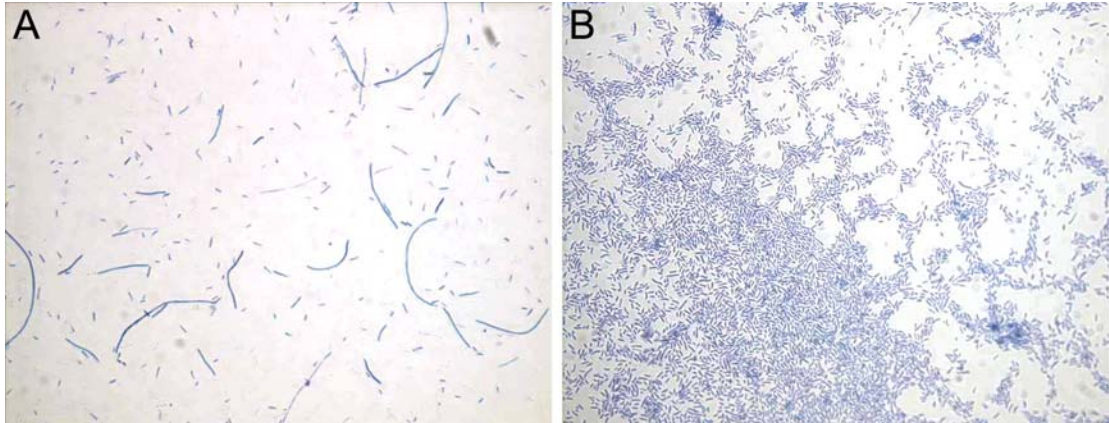
Figure 2. Mitomycin C Induces Filamentation of UTI89 But Not FOSF6600.
Cultures of A) *E. coli* UTI89 and B) *E. aerogenes* FOSF6600 were induced with 150
ng/ml mitomycin C in early log phase. Bacteria were cytocentrifuged onto poly-lysine
coated slides and stained with Hema 3. Bacteria were observed by light microscopy
using a 40X objective. Many long filamentous organisms were found in the UTI89
cultures but not the FOSF6600 cultures.
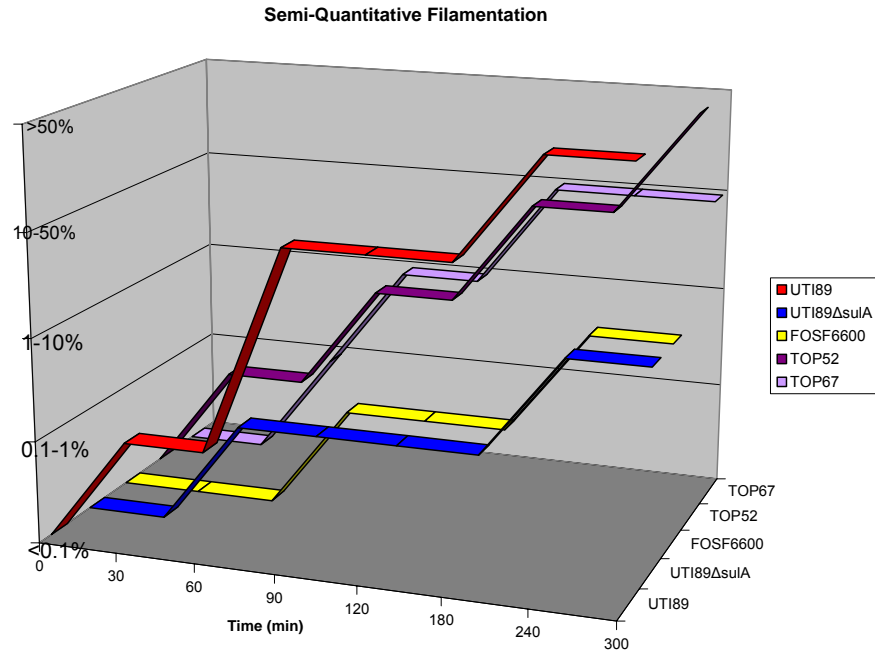
Figure 3. Semi-quantitative Timecourse of Bacterial Filamentation after Mitomycin C Induction.
Cultures of *E. coli* UTI89, UTI89ΔsulA, *E. aerogenes* FOSF6600, and *K. pneumoniae* strains TOP52 and TOP67 were induced with 150 ng/ml mitomycin C in early log phase and analyzed at given timepoints post induction. Filamentation was measured on a semi-quantitative scale based on percent of all bacteria in filamentous morphology. FOSF6600 displayed poor filamentation similar to UTI89Δ*sulA*.
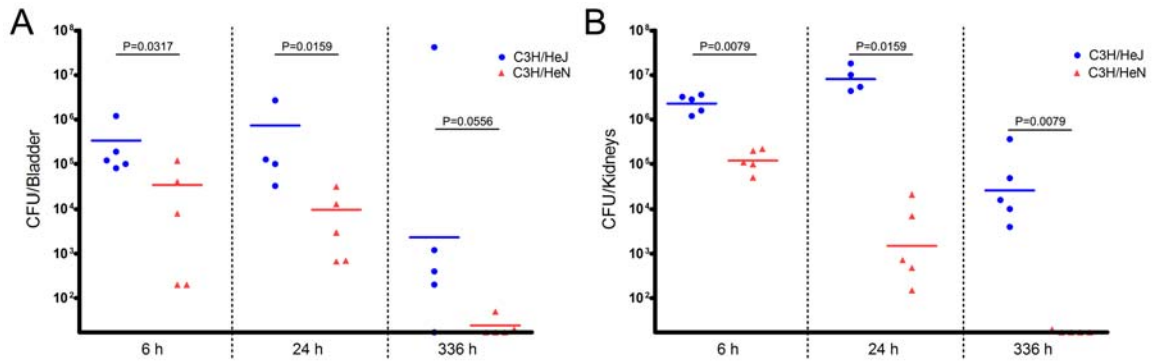
Figure 4. *E. aerogenes* FOSF6600 Bladder and Kidney Titers in C3H/HeN and C3H/HeJ Murine Backgrounds.

Female C3H/HeN mice (▲) and C3H/HeJ mice (●) were inoculated with 10^7 CFU of the *E. aerogenes* isolate, FOSF6600, by transurethral catheterization. For organ titers, bladders (A) and kidneys (B) were harvested at various timepoints post infection and CFU were calculated. Short bars represent geometric means of each group. All significant P values, as calculated by Mann-Whitney U tests, are displayed.

To determine if *E. aerogenes* encoded *sulA*, polymerase chain reactions were performed and verified the presence of a *sulA* locus in FOSF6600 and two other *E. aerogenes* isolates. The genes had a high percent identical to each other and the SOS box was highly conserved with that of *E. coli*. There may be differences in SulA, LexA, or even RecA that could affect the SOS response of *E. aerogenes* compared to *E. coli*. *E. aerogenes* may have a higher SOS threshold than *E. coli*, thus not filamenting *in vivo* at early timepoints and making it susceptible to early clearance in the murine cystitis model.

To further investigate these possibilities, LexA production and cleavage can be monitored by western blot analysis, after SOS induction with varying levels of mitomycin C. Additionally, qRT-PCR can be performed on *sulA* and other SOS response genes in FOSF6600 to determine relative levels of upregulation with SOS induction. Finally, SOS response genes including *sulA* can be swapped between UTI89 and FOSF6600 to see if FOSF6600 will gain the ability to filament or UTI89 will lose its ability to filament. To this point, *in vivo* and *in vitro* studies have been done with the *E. aerogenes* strain FOSF6600. It is important to see if these lack of filamentation and inability to persist phenotypes occur with other *E. aerogenes* strains. If they do, this may be a more general phenomenon that helps explain why *E. aerogenes* is not a more prevalent uropathogen.

Klebsiella pneumoniae Uropathogenesis

K. pneumoniae is the second leading cause of Gram-negative UTI and thus represents an ideal pathogen to study in comparison to the predominant uropathogen, *E.*

coli. Only a single *K. pneumoniae* isolate has been fully sequenced and this ATCC 700721 strain is a pneumonia isolate. One 26 year old woman in the SCOR study at the University of Washington was enrolled with an index *K. pneumoniae* UTI. This patient was treated with trimethoprim-sulfamethoxazole and subsequently reported back to the clinic with recurrent UTI nine days later (Figure 5). This recurrent infection was caused by the same *K. pneumoniae* strain as the index UTI. Periurethral and urine cultures demonstrated that in the days leading up to recurrent UTI, this patient developed *K. pneumoniae* bacteriuria prior to detectable periurethral cultures. These findings may suggest a bladder reservoir model of UTI recurrence, as opposed to the common paradigm of re-ascension. This patient's index strain, TOP52 1721, was used for subsequent studies on *K. pneumoniae* uropathogenesis.

Infections in the murine cystitis model have demonstrated that *K. pneumoniae* is able to form bacterial filaments and IBCs which are morphologically identical to those formed by UPEC. While TOP52 had lower bacterial titers early in urinary tract infection, it had similar titers to UPEC at later timepoints. At 2 weeks post infection, TOP52 displayed similar bimodal bladder titers as those observed for UTI89. In UPEC, some mice have high titers at 2 weeks representing chronic active infection that has not been cleared by the host or, in a few cases, recurrent bacterial infection following an abacteriuric period. Other mice have lower titers at 2 weeks, likely represent bacteria in QIRs. In TOP52-infected mice, the nature of this bimodal titer distribution has not been assessed, but may represent syndromes similar to those in UTI89-infected mice. To determine whether TOP52 is able to form QIRs, gentamicin protection assays and
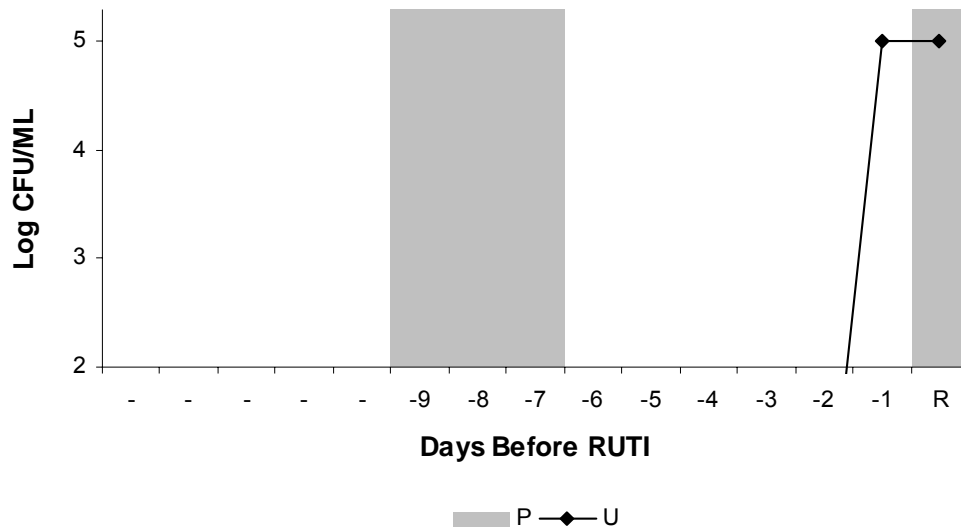
**Figure 5. Patient with *Klebsiella pneumoniae* Recurrent UTI.**
Selected 14-day window patterns prior to *K. pneumoniae* recurrent UTI. Days with positive periurethral (P) cultures for K. pneumoniae are shaded and urine culture (U) results are graphed. In this case, urine cultures were positive prior to periurethral colonization possibly suggesting a potential bladder reservoir model of recurrence.

subsequent immunofluorescent microscopy need to be performed on bladders 2 weeks post inoculation. For these studies, the C57BL/6 murine background may be more appropriate since UPEC QIRs are more prevalent after 2 week infection in this mouse strain.

Differences in UPEC and *K. pneumoniae* acute cystitis are likely related to type 1 pilus expression and function. The *K. pneumoniae fim* operon contains an additional gene, *fimK*, which has an effect on the *K. pneumoniae* phase switch, primarily keeping it in the OFF orientation. The stop codon of *fimH* overlaps the start codon for *fimK*. RT-PCR analysis verified that *fimK* is co-transcribed as part of the *fim* operon. RNA purified from TOP52 and TOP52/pfimX cultures was DNAse treated twice and was verified to be DNA free by PCR using generic 16S RNA primers. cDNA was then synthesized and used as a template for PCR reactions using primers to amplify *fimA*, *fimH*, and a region of DNA spanning *fimH* and *fimK*. RT-PCR showed bands for TOP52 and TOP52/p*fimX* with all three primers sets confirming that *fimK* is co-transcribed with the *fim* operon (Figure 6). As expected, induction of the TOP52 type 1 operon with the recombinase *fimX* resulted in greater PCR product compared to TOP52 alone.

While it is clear that the presence of FimK inhibits type 1 piliation in *K. pneumoniae*, the mechanism of this inhibition requires further study. Also, FimK may be exclusively acting on expression of type 1 pili or it may affect the expression of other adhesins or virulence factors in *K. pneumoniae*. The C-terminal domain of FimK contains an EAL domain. EAL domains have been implicated in the cleavage of the ubiquitous second messenger molecule, cyclic di-GMP (6, 30, 31). Loss of FimK may
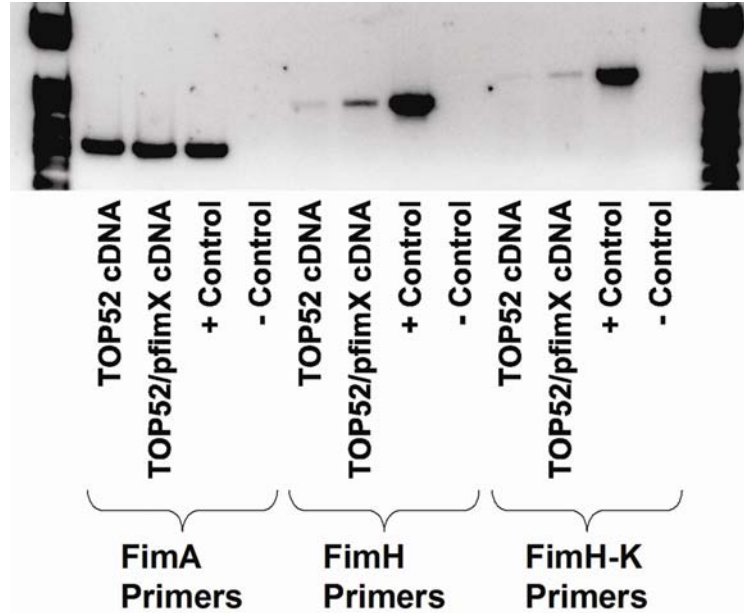
Figure 6. RT-PCR Confirmation that *fimK* **is Co-transcribed with the** *K. pneumoniae* **TOP52 1721** *fim* **Operon.**
RNA was isolated from TOP52 and TOP52/pfimX cultures and DNAse treated twice. cDNA was synthesized from the RNA and PCR reactions were run using cDNA as a template with primers to *fimA*, *fimH* and primers spanning *fimH-fimK*. The presence of a PCR product with the primers spanning *fimH-fimK* indicates that these are polycistronic in mRNA. Complementation with *fimX* increases expression of the *fim* operon and thus the intensity of these bands.

lead to elevated levels of cyclic di-GMP which have been implicated in biofilm formation, production of adhesive surface structures, and inhibition of motility (10, 22, 32). To determine if FimK is an active phosphodiesterase with the ability to cleave cyclic di-GMP, *in vitro* experiments can be performed. FimK can be purified, used to react with synthetic cyclic di-GMP and products can be analyzed by mass spectrometry.

In addition to the expression differences between UPEC and *K. pneumoniae* type 1 pili, sequence variation of their FimH adhesins leads to significant functional differences both *in vitro* and *in vivo*. In the murine cystitis model, FimH of TOP52 ($FimH_{52}$) was not essential for early colonization, but was required for invasion and IBC formation in the murine bladder. *In vitro*, the adhesin domain of $FimH_{52}$ lacks the mannose-sensitive hemagglutination (MSHA) phenotype typically characteristic of type 1 pili. TOP52 FimH-dependent biofilms were inhibited by heptyl mannose, but not methyl mannose, suggesting the need for additional contacts outside of the mannose binding pocket.

FimH residues of TOP52 and UTI89 known to interact with mannose or form the hydrophobic ridge are fully conserved. There are only 17 amino acid changes between the adhesin domains of the FimH proteins. Two of these changes are located adjacent to important mannose-binding residues Gln133 and Asp140 (17). Arg132 and Asp141 of *E. coli* FimH form a salt bridge helping stabilize and orienting important mannose-binding residues. These amino acids are changed to His132 and Ser140 in $FimH_{52}$ and may affect mannose binding (Figure 7). These two residues were mutated, $FimH_{52}$ residues to $FimH_{89}$ residues, and vice versa, and were expressed in UTI89Δ*fimH*. Hemagglutination
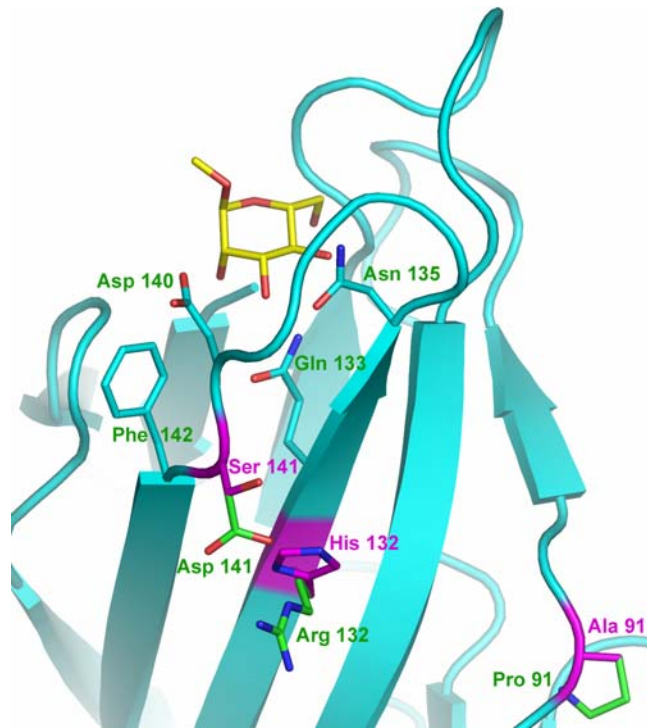
Figure 7. Model of the Mannose Binding Pocket of *K. pneumoniae* FimH.
The TOP52 FimH sequence was threaded onto the crystal structure of FimH from the *E. coli* strain J96 (17). This view shows the tip of the adhesin domain binding to mannose (yellow). Arg132 and Asp141 of *E. coli* FimH (green) are changed to His and Ser, respectively, in FimH of TOP52 (pink). These residues form a salt bridge in *E. coli* FimH which helps to stabilize the FG loop and mannose binding residues Gln133 and Asp140. These changes in *K. pneumoniae* FimH may affect mannose binding.

assays (HAs) with guinea pig red blood cells were performed with these site-directed mutants (Table 3). $FimH_{52}$ did not display a gain of function with mutations in these residues. However, $FimH_{89}$ did exhibit lower HA titers. Single changes, either R132H or D141S, resulted in loss of HA titer. The double mutant R132H-D141S resulted in a $FimH_{89}$ with an HA titer of 1:4 compared to the 1:16 titer of wildtype $FimH_{89}$. It should be noted that the stability and expression of all of these mutants are being confirmed. These amino acids may be playing a role in the altered function of $FimH_{52}$ but they alone likely do not account for the entire phenotype.

A number of approaches can be taken to further investigate the structural differences in the *E. coli* and *K. pneumoniae* FimH proteins. While expression of *fimH* on a plasmid *in trans* to the *fim* operon is poor, *fimH* site-directed mutants can be inserted into the native *fim* operon site in UTI89Δ*fimH* by homologous recombination. This will allow for wildtype levels of pilus expression utilizing the native promoter dynamics. With relatively little variation in the FimH adhesin domains of TOP52 and UTI89, these methods are feasible to test many of these changes and their effects on FimH function. Additionally, while structural modeling provides insight into $FimH_{52}$, a crystal structure of $FimH_{52}$, possibly bound to heptyl mannose, would greatly aid in our understanding these structural considerations.

While many questions remain, much has been discovered regarding *K. pneumoniae* pathogenesis in the urinary tract in this work. Despite being able to progress through an IBC pathway, *K. pneumoniae* forms fewer IBCs and has lower bladder titers than UPEC early in infection. These differences are related, in part, to its extra gene,

Table 3. Hemagglutination Assays with FimH Mutants		
Strain	Guinea Pig RBC HA Titer $(1:2^x)$	Guinea Pig RBC + Mann. HA Titer $(1:2^x)$
UTI89	9	3
UTI89Δ$fimH$	3	3
UTI89Δ$fimH$/pBAD	0	0
UTI89Δ$fimH$/pfimH$_{89}$	4	0
UTI89Δ$fimH$/pfimH$_{89}$R132H	0	0
UTI89Δ$fimH$/pfimH$_{89}$D141S	0	0
UTI89Δ$fimH$/pfimH$_{89}$RHDS	2	0
UTI89Δ$fimH$/pfimH$_{52}$	0	0
UTI89Δ$fimH$/pfimH$_{52}$H132R	0	0
UTI89Δ$fimH$/pfimH$_{52}$S141D	0	0
UTI89Δ$fimH$/pfimH$_{52}$HRDS	0	0

fimK, and its effects on type 1 pilus expression. Additionally, small sequence variations between the FimH of *E. coli* and *K. pneumoniae* result in differences in function and ability to colonize the urinary tract. Despite its poor adhesive properties in the urinary tract, *K. pneumoniae* FimH remains an important virulence factor allowing *K. pneumoniae* to progress through an IBC pathway during UTI and ultimately persist in the host. It remains unclear why *K. pneumoniae* has evolved altered FimH and and active suppression of type 1 pili. *K. pneumoniae*'s primary niche may be outside of the urinary tract and expression of type 1 pili may be disadvantageous in this setting. This work begins to explain the difference in prevalence of *E. coli* and *K. pneumoniae* UTI and emphasizes the importance of type 1 pili in the ability of uropathogens to infect the urinary tract.

Uropathogenesis in the Diabetic Setting

Diabetics have a higher incidence of urinary tract infection (UTI), are infected with a broader range of uropathogens, and more commonly develop serious UTI sequelae. Very low inocula, less than 100 CFU, of UTI89 can lead to robust infection in diabetic mice. Also, non-UPEC uropathogens are more capable of causing robust infection in the diabetic urinary tract. The development of a diabetic mouse model provides a powerful and versatile tool for dissecting this infection in the multi-factorial setting of diabetes.

IBCs were observed in bladders of diabetic mice infected with UTI89 or TOP52, however, it is not clear how important the IBC pathway is to pathogenesis in these

compromised hosts. Are uropathogens not capable of causing robust acute infection (i.e. UTI89Δ*fimH*) or persisting in healthy hosts (i.e. UTI89Δ*sulA*) able to do so in diabetic mice? Do uropathogens filament in diabetic bladders and, if so, is this filamentation required for persistence? One may expect that an impaired inflammatory response in diabetic mice may not induce the filamentation of uropathogens during infection. Trial studies with a UTI89 mutant deficient in capsule formation have shown successful infection in diabetic mice but poor infection in the healthy host. The diabetic setting may be compromised enough to allow persistence of uropathogens that lack virulence factors otherwise important in the healthy host.

Numerous hypotheses can be raised to explain the enhanced ability of uropathogens to infect the urinary tract of diabetic individuals including glucosuria, neutrophil dysfunction, decreased cytokine secretion and enhanced adherence properties of urothelial cells (12). These hypotheses can be tested in the murine model. Neutrophil dysfunction can be assayed via neutrophil killing assays assessing the potency of neutrophils isolated from diabetic and non-diabetic mice against various uropathogens. Cytokine secretion can be compared by carrying out bladder, kidney and urine bioplex assays. Studies have suggested that bacteria have increased adherence abilities in the urinary tract of diabetics, possibly due to lower levels of urine Tamm-Horsfall protein (3, 9, 26) or potential changes of the uroepithelial cells themselves (13). Levels of Tamm-Horsfall protein can be assayed in the urine using immunoblot or antigenic titration analyses. To probe for changes in the urothelium, overlay binding studies can be performed in which bacteria are placed onto diabetic and healthy bladder sections and

bound bacteria are quantified after several washes. It would be interesting to find that

diabetic bladders have higher levels of integrins, uroplakins or a yet undiscovered

bacterial receptor expressed on their surface.

We do not yet know how the effects of insulin therapy affect diabetic UTI.

Presumably, euglycemic diabetic mice will not be as susceptible to UTI as uncontrolled,

hyperglycemic diabetic mice. However, it is also possible that after a given amount of

time in an uncontrolled diabetic state, elicited damages will render mice particularly

susceptible to UTI despite glycemic control. Further understanding of UTI in controlled

diabetic mice is especially applicable to the millions of individuals receiving treatment

for diabetes. Additionally, this predisposed population may gain the most benefit from

prophylactic UTI vaccinations. The streptozocin-induced diabetic model of UTI is the

ideal setting for testing the efficacies of these treatments.

Overall Conclusions

In summary, this thesis work set out to better understand the pathogenesis of

uropathogens in the urinary tract. Prior studies have revealed that UPEC employs a

complex developmental process in mice, initiated by the intimate contacts between the

pathogen adhesive organelles and the host epithelium, to successfully colonize and persist

within the harsh environment of the urinary tract. The IBC pathogenic pathway is critical

in the ability of a uropathogen to cause UTI and interferences at any step in this

pathogenic cycle could alter the infection course. This thesis extends our knowledge of

the IBC pathogenic pathway in two important ways. First, this study has demonstrated the presence of IBCs and bacterial filaments in human samples suggesting an IBC pathogenic pathway in human cystitis. Second, this work has confirmed the ability of a number of non-UPEC uropathogens to progress through an IBC pathogenic pathway in a murine model of cystitis. These findings indicate the importance of thoroughly understanding the mechanisms that underlie this process and how these differ among uropathogens. These discoveries alter the current paradigm of bacterial pathogenesis in the urinary tract and could ultimately lead to better treatment modalities for patients suffering UTIs.

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