Spring 5-15-2015

Understanding Escherichia coli Urinary Tract Infection: A Niche Perspective

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A dissertation presented to the
Graduate School of Arts and Sciences
Of Washington University in
Partial fulfillment of the
Requirements for the degree
Of Doctor of Philosophy

May 2015

St. Louis, Missouri

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Acknowledgements

First and foremost I would like to thank Scott Hultgren, the most amazing mentor any student could have. Scott fostered my development from day one progressively entrusting me with more responsibility to ensure that my confidence in being a scientist would grow. I greatly appreciate the opportunity to travel with Scott all over the world to disseminate our science. Everything I am as a scientist and researcher, I owe to Scott, so thank you from the bottom of my heart. The first person to notice scientific potential in me was my high school chemistry teacher, Dr. Dale Epperson. I want to thank him for creating the spark of interest and kindling it with his love of science and constant support. I would also like to thank members of my thesis committee for all of their help and suggestions along the way. I could not have succeeded without the support and assistance of the Hultgren lab. Lab was always a great place with everyone willing to offer a helping hand or suggestion, and I will miss all of our time together. I would also like to thank two amazing post-doctoral fellows, Swaine Chen and Kim Kline, who were always willing to help me with anything I needed in lab, for teaching me how to design experiments properly, and for being incredible friends.

I am very grateful for the funding sources that have allowed me to pursue all of the scientific questions that I considered. Specifically I would like to thank the National Institutes of Health grants R01 DK051406, U01 AI095542, and P50 DK064540 to Scott Hultgren and F30 DK096751 to me. I would also like to thank the Medical Scientist Training Program, the Department of Molecular Microbiology, and the MSTP training grant for supporting me.

Nothing is possible without a supportive family, and my family have encouraged and supported my love of science since day one. My mom and dad have been unceasingly accommodating with my long pathway to independence. I thank them for everything, and even

that is not enough appreciation. I would also like to thank my wife Annie's parents for their interest in my work and support over the years. Visiting and traveling with my family has been one of the joys to which I have looked forward during my graduate career. Finally, I would like to thank Annie. Each day coming home to her was phenomenal, and I could never imagine a better, more caring, and supportive wife. She is the smart one in this relationship, and I am the lucky one.

ABSTRACT OF THE DISSERTATION

Understanding *Escherichia coli* Urinary Tract Infection: A Niche Perspective

By

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Doctor of Philosophy in Biology & Biomedical Sciences

(Molecular Microbiology and Microbial Pathogenesis)

Washington University in St. Louis, 2015

Professor Scott J. Hultgren, Chairperson

Urinary tract infections (UTIs) are among the most common bacterial infections worldwide, costing greater than $2 billion in healthcare costs and lost wages yearly in America alone. The lifetime risk for a woman exceeds 50% with 25-40% of these suffering recurrent infections. Two of the most important risk factors for recurrent UTI are prior UTIs and sexual intercourse. Over 80% of UTIs are caused by uropathogenic *Escherichia coli* (UPEC), which binds and invades superficial facet cells lining the bladder surface. UPEC expresses extracellular fibers called type 1 pili with a terminal mannose-binding adhesin, FimH, which interacts with mannosylated uroplakin residues on the urothelium. UPEC replicates in the cytoplasm of bladder cells into biofilm-like intracellular bacterial communities (IBCs) in a protected niche. In C3H/HeN mice, a robust, systemic, immune response at 24 hours precedes the development of persistent bacteriuria and chronic cystitis, which lasts indefinitely. A less robust immune response results in resolution of the infection.

I determined the population dynamics during UPEC infection with a set of 40 variants of a clinical isolate, UTI89, each with a unique genetic sequence detectable by multiplex PCR. I

identified a significant population bottleneck during the first 24 hours coinciding with the inflammatory response. Furthermore, I tested a panel of FimH alleles under positive selection and found several that impacted the ability of UPEC strains to form IBCs and promote chronic cystitis. These pathoadaptive alleles govern the ability of FimH to bind mannose by dynamically interconverting between a compact, low-affinity conformation and an elongated, high-affinity state. This dynamic equilibrium is crucial for virulence as alleles locked in either conformation are attenuated. I also developed a model of frequent inoculation of UPEC into the urinary tract to investigate the clinical link between frequent sexual intercourse and UTI risk. By inoculating mice twice during acute infection, I found a dramatic increase in the proportion of mice that experienced chronic cystitis. Taken together, this thesis defines bacterial and behavioral factors that increase the risk for chronic and recurrent UTI, providing rationale for the development of novel therapeutics targeting bacterial invasion to limit infection by excluding UPEC from intracellular niches.

Chapter 1: Introduction

Edited from

UPEC Virulence and Gene Regulation

Drew J. Schwartz and Scott J. Hultgren

Regulation of Bacterial Virulence, 2013. Chapter 7: pg 135-155. *eds.* Michael L. Vasil and Andrew W. Darwin. ASM Press, Washington, D.C.

Abstract

Host-pathogen interactions are often mediated by extracellular fibers known as pili. The best characterized Gram negative pili are assembled by the Chaperone Usher Pathway (CUP). CUP pili are often tipped with an adhesin that binds to receptors with stereochemical specificity allowing microbes to colonize and adapt to dynamically changing environments. Type 1 and P pili are prototype CUP pili that mediate colonization of tissues in the urinary tract during infection by uropathogenic *Escherichia coli* (UPEC). During a urinary tract infection (UTI), UPEC occupy and flux between multiple niches within the bladder and kidneys. In the acute stages of cystitis, UPEC invade superficial bladder epithelial cells, replicate within the cytoplasm, and form large biofilm aggregates termed intracellular bacterial communities (IBCs). Appropriate expression of pili and other virulence factors in the urinary tract is essential for pathogenesis. Type 1 pili, critical for bladder infection, are regulated by cis-encoded recombinases within the fim operon that reversibly invert the fim promoter, regulating expression. Recombinases and many other regulatory proteins serve to cross-regulate CUPs and fine-tune pilus expression. Understanding the structure, regulation, assembly, and function of these pili has lead to the development of novel anti-virulence compounds that prevent bacterial attachment to host tissues.

Introduction

Bacterial virulence entails an ability to persist within a host and cause disease in the face of myriad host defense mechanisms in more than one niche or under more than one immune condition. The array of virulence factors that a pathogen possesses and its ability to regulate their expression in response to environmental signals greatly contributes to the variety and specificity of niches a pathogen can colonize and impacts the spectrum of diseases that occur. Colonization factors may confer a fitness advantage in a specific niche, being required in some environments and unnecessary or deleterious in others, or they may contribute globally to persistence and disease. Thus, coordinated gene expression is critical to ensure that appropriate genes are expressed in specific environments and turned off in others. For example, when a pathogen encounters a susceptible host, the ability to attach to a specific tissue or niche in a timely manner is vitally important to establishing infection in a dynamically changing local environment. A host-pathogen interaction is frequently the consequence of an adhesin expressed by the pathogen recognizing a receptor on host cells with exquisite stereochemical specificity [1, 2]. Several families of bacterial adhesins exist to mediate specific interactions to various hosts, tissues, organisms, or surfaces: outer membrane proteins, secreted soluble, extracellular proteins that create an adhesive matrix, type 4 pili, auto-transporters and two-partner secreted adhesins, and chaperone-usher pathway (CUP) assembled pili (see [1, 3] for review). Myriad gram-negative and gram-positive organisms utilize adhesins to attach to epithelial surfaces to persist within a host. Gram-negative organisms such as *Porphyromonas gingivalis*, *Salmonella enterica*, *Yersinia enterocolitica*, and *Neisseria gonorrhoeae* utilize surface exposed adhesins to colonize host mucosal surfaces (reviewed in [4]). The gram-positive organisms *Enterococcus faecalis* and *Clostridium perfringens* also encode pili or pilus-like adhesins that mediate attachment to surface

structures. In this chapter I will describe the virulence and regulation of CUP pili focusing on type 1 in uropathogenic *Escherichia coli* (UPEC), the most common cause of urinary tract infections (UTIs).

Urinary Tract Infections

E. coli are normally commensal colonizers of the gastrointestinal (GI) tract. Several pathotypes of *E. coli* exist, however, that can cause disease in a variety of organ systems in both immuno-competent and immuno-compromised individuals dependent on the arsenal of virulence factors and adhesins they express. *E. coli* are generally classified into 3 major groups: commensals, intestinal pathogens, and extra-intestinal pathogens (ExPEC) [5]. Commensal strains peaceably coexist with numerous other bacterial species in the GI tracts of healthy humans and many other animals. The majority of these strains cause no disease in healthy individuals, but may colonize foreign bodies if present. Additionally, these commensals lack many of the virulence genes that other *E. coli* possess. Intestinal pathotypes of *E. coli* are rarely found in the asymptomatic host, but instead are obligate pathogens of the gastrointestinal tract. Among the intestinal *E. coli* pathogens, there are several classifications based on virulence genes expressed: enterotoxigenic (ETEC), enteropathogenic (EPEC), Shiga toxin producing/enterohemmorhagic (STEC/EHEC), enteroinvasive (EIEC), and diffusely adherent (DAEC) (reviewed in [6]). ETEC adhere via fimbriae to the small intestine and produce one or both of labile toxin and stable toxin, which lead to secretory diarrhea. EPEC cause attaching-and-effacing lesions via adherence with bundle-forming pili and export of type III secretion effectors resulting in loss of absorptive microvilli and diarrhea. EHEC produce shiga toxin leading to bloody diarrhea, hemolytic uremic syndrome, and possibly death. Attachment of EHEC to the

tissue has been speculated to occur via multiple different adhesins, and many of these may contribute to colonization of more than one pathotype, but no clear adherence profile exists [7]. EIEC invade colonocytes and secrete enterotoxins, very similar to the pathogenesis of *shigellae*. DAEC diffusely adhere to the epithelial surface and auto-aggregate, yet do not produce either of the toxins common to ETEC. Infection with DAEC results in edematous villi and mucoid diarrhea. Extraintestinal infections caused by *E. coli* (ExPEC) include meningitis, pneumonia, soft-tissue infections, and UTI. ExPEC are characterized by their lack of gastrointestinal pathogenesis, however they can stably colonize the GI tract [5]. In order to cause disease, these organisms must access the site of infection: brain, lungs, medical device, or urinary tract. ExPEC generally acquire stretches of chromosomal DNA called pathogenicity islands (PAI) conferring the virulence factors often including operons encoding adhesins necessary to colonize a particular niche. Uropathogenic *E. coli* (UPEC) are one such member of ExPEC, primarily responsible for UTI by virtue of the adhesins and other virulence genes they express. The array of virulence factors and adhesins expressed by the various *E. coli* pathotypes defines their pathogenic niche and virulence profile.

UPEC causes >80% of community-acquired UTI and ~50% of nosocomial UTI [8, 9] and has evolved elaborate mechanisms that allow it to survive within and exploit multiple niches in the urinary tract [10, 11]. Although UTIs afflict both men and women, females are disproportionately affected, a trend that begins as early as 8 years old [12]. Approximately 50% of women will have at least one symptomatic UTI in their lifetime, with 20-40% of these experiencing a recurrence within six months of the first infection [13, 14]. Clinically UTIs are divided into categories based on symptoms. One category is infections of the bladder (cystitis), which are generally low mortality infections involving bacteriuria, pain or burning during

urination, urgency, and frequency. The other category includes infections of the kidneys (pyelonephritis), which in addition to the symptoms of cystitis include chills, fever, flank pain and tenderness. Antibiotics are generally successful at clearing bacteriuria and many other symptoms; however, many women suffer frequent recurrent infections with increased morbidity, necessitating long-term, daily, prophylactic antibiotics. Furthermore, studies have shown that frequently, the recurrent infection is caused by the same strain that caused the original, symptomatic UTI [15], emphasizing the presence of a bacterial reservoir within the host that cannot be effectively eradicated by current treatments. Additionally, certain patients experience asymptomatic bacteriuria or chronic cystitis marked by persistent bacteriuria with accompanying cystitis symptoms [16, 17].

Risk factors for urinary tract infection development

The balance between bacterial virulence factors, host genetics, and behavior dictates symptoms and disease state. Certain polymorphisms and reduced expression of the Toll-like receptor 4 (TLR4) gene are associated with less severe UTI and asymptomatic bacteriuria (ASB/ABU) [18, 19]. Conversely, reduced expression of the Interleukin (IL) -8 receptor, CXCR-1, is correlated with severe pyelonephritis [19]. Other human genetic factors that impact acute, chronic, and recurrent UTI (rUTI) have not yet been elucidated, but studies to determine polymorphisms to address these common syndromes are currently being conducted. Several of the most important risk factors for the development of a rUTI are behavioral. For the majority of women, the onset of urinary tract infection coincides with becoming sexually active [20]. One of the most dominant risk factors is the frequency with which a woman engages in sexual intercourse. Having sexual intercourse greater than nine times in a month was associated with a

ten-fold increase in the likelihood of developing a rUTI compared to having intercourse zero to three times [20]. In combination with sexual intercourse, bacterial colonization of the periurethral area increases in the days preceding a UTI [21]. Thus, depending on the host factors and behaviors, susceptibility to a UPEC UTI differs.

The increasingly accelerated rate of global trafficking of antibiotic resistant uropathogens is necessitating the development of better therapeutic strategies. In addition to the rising resistance to first line therapies like trimethoprim-sulfamethoxazole (TMP-SMX), UPEC are becoming increasingly resistant to floroquinolones, which are being used more as a result of TMP-SMX resistance [22-24]. Furthermore, strains of the clonal group ST131 that are resistant to the majority of antibiotics have globally spread, causing UTI and bloodstream infections in North America, Europe, Asia, and Australia [25]. The spread of these multi-drug resistant strains combined with the increased resistance among UPEC strains is poised to create a public health crisis. Thus, it is essential to thoroughly reexamine the molecular basis of UPEC infection, determining the role and regulation of essential virulence factors to create novel therapeutics that target these virulence factors while reducing the negative side-effects accompanying antibiotic treatment.

Chaperone usher pathway overview

UPEC and *E. coli* in general, heavily rely on CUP pili to mediate attachment to biotic and abiotic surfaces [26-29]. The chaperone-usher pathway (CUP) is a nearly ubiquitous system among *enterobacteriaceae* [30] used for the assembly of surface exposed pili (see [1] or [31] for thorough reviews). CUPs are genetically organized as operons encoding a pilus fiber, a periplasmic chaperone, and an outer membrane usher. Whole genome sequencing has revealed

the presence of many canonical or hypothetical CUP operons in UPEC genomes [32-34] (Table 1). These operons are *fim* (type 1), *pap* (P), F17-like, *sfa* (S), *yad*, *auf*, *yfc*, *ygi*, *yeh*, *fml*, and *foc* (F1C), *yde* (F9), *dra/afa* (Dr family), *fso* (F7), *fst*, *pix* (reviewed in [35]) (Table 1). In fact, it was recently shown that UPEC possess on average twice as many fimbrial types as commensal *E. coli* [36], presumably because of the increased array of environments these organisms colonize. I will focus on type 1 and P pili, given that they have been the most thoroughly characterized CUP pilus systems via mechanistic studies that have revealed their biochemical, structural, and regulatory properties as well as their contribution to UPEC pathogenesis.

Type 1 pili are encoded by the *fim* operon. They are composite fibers with a ~2nm wide fibrillar tip joined to a 0.3-1.5 μm length rod consisting of repeating subunits arranged in a 7nm wide right-handed helical rod [37-39]. Upon translation, pilus subunits are translocated across the inner membrane via the Sec translocation machinery (Fig. 1) [40]. Once in the periplasm, they are bound by the chaperone FimC, which is critical for subunit stability and serves to facilitate folding and cap interactive surfaces to prevent nonproductive aggregation [41]. Pilus subunits have an incomplete immunoglobulin (Ig)-like fold, missing the C-terminal 7^{th} β-strand [42, 43]. The periplasmic chaperones attain a 'boomerang' shape with two Ig-like domains connected by a linker ([44, 45]. Upon binding to the chaperone, the incomplete subunit folds are templated to fold by a process termed donor-strand complementation (DSC), in which four residues from the chaperone's G1 strand provide the steric information to complete the fold of the bound subunit [42, 43]. Chaperone subunit complexes are targeted to the outer membrane usher, the protein through which the growing pilus is extruded across the outer membrane (Fig. 1). The outer membrane usher is a large β-barrel protein consisting of a translocation domain, a ~120 residue N-terminal domain (NTD) and a ~170 residue C-terminal domain (CTD) that binds

chaperone-subunit complexes [46]. The current model suggests that incoming chaperone-subunit complexes are targeted to the usher NTD and transferred to the CTD where donor strand exchange (DSE) occurs (Fig. 1B) [46-48]. In DSE, the chaperone is displaced by a 10-20 residue N-terminal extension (Nte) present on the next subunit to be incorporated into the growing pilus. This process completes the subunit Ig fold in a canonical fashion resulting in the addition of a single subunit to the growing pilus rod. The order of pilus assembly corresponds to the affinity of the chaperone-subunit complex for the usher and the efficacy and specificity each Nte has for DSE [49]. Subunits are added to the base of the growing pilus, with the FimH adhesin first incorporated. Accordingly, the usher, FimD, has the highest affinity for FimC-FimH complexes [50]. After incorporation of FimH, a single subunit each of FimG and FimF are added to complete the tip fibrillum. FimC-FimA complexes are then added sequentially to yield a fully formed pilus rod containing ~1000 FimA subunits arranged in a right-handed helical cylinder with approximately 3.2 subunits per turn (Fig. 1) [51, 52]. The pilus is constructed from tip to base through targeting of chaperone-subunit complexes to the usher and DSE to complete the incomplete Ig-like fold of each subunit.

DSE occurs via a concerted ‘zip-in zip-out’ mechanism at a groove of the previously added subunit containing five crucial residues called P1-P5 [53]. The Nte of an incoming subunit binds to the open P5 pocket of the previous subunit. The incoming subunit then zips in, replacing the hydrophobic interactions between the previous subunit and the chaperone from P5 to P1, displacing the chaperone. The P pilus rod is terminated by the addition of a single PapH subunit. PapH contains a loop partially occluding this P5 pocket, therefore the chaperone bound to PapH cannot be displaced and pilus rod formation is terminated [54]. How type 1 pili are terminated is

unclear, as no PapH equivalent has been discovered, though some studies suggest that FimI is the anchoring subunit [55].

Based on sequence identity and genomic organization, it is very likely that other CUP systems are assembled in a manner that is similar to type 1 and P pilus biogenesis [1]. The presence of multiple CUP pili within *E. coli* genomes is thought to be required for tuning adhesive properties specific for different environmental niches [10, 56-59]. For example, the type 1 pilus adhesin, FimH, and the P pilus adhesin, PapG, bind mannosylated and digalactoside receptors respectively, thereby mediating UPEC colonization of bladders and kidneys [10, 58, 60, 61]. Transcriptional profiling and genetic studies have revealed that other UPEC CUP systems are expressed and may contribute to virulence [26, 36, 62]. However, how expression of multiple CUP operons is coordinated in response to environmental signals in different niches during infection is essentially unknown.

UPEC virulence

The pathogenesis of community-acquired UPEC UTI is thought to begin with UPEC colonization of the peri-urethral area from the fecal flora. Transmission of UPEC into the bladder can then occur via urethral manipulation [63, 64], sexual intercourse [65], or possibly direct ascension, although peri-urethral presence of UPEC does not necessarily lead to infection [66]. Infection is likely multifactorial with colonization in nearby "staging" areas combined with mechanical disruption such as sexual intercourse [21]. During infection UPEC is capable of colonizing the urine, the bladder epithelium (both extracellularly and intracellularly), and the kidneys [67]. Depending on the specific niche UPEC inhabits, it encounters different elements of

the immune response as well as different environmental pressures, necessitating precise gene regulation and immune avoidance or manipulation strategies.

Although multiple studies have correlated particular genes with virulence [68-71], to date there is no common virulence profile among cystitis isolates. However, the vast majority (>95%) encode type 1 pili, and expression of type 1 pili is highly correlated with cystitis [33, 72, 73]. Indeed the most consistent virulence factor expressed among cystitis isolates is type 1 pili [73]. UPEC isolated from women suffering rUTI expressed type 1 pili when grown in broth cultures [74]. Additional studies demonstrated that 75% of UPEC isolated from the urine of 41 adult patients had type 1 pili on their surface and were often found in association with or attached to exfoliated epithelial cells and leukocytes [75]. Several studies have indicated that planktonic cells within the urine are usually not expressing type 1 pili, implying genetic regulation that is niche specific [76, 77]. However, other studies have shown that expression patterns of planktonic UPEC in the urine are not necessarily indicative of tissue-associated bacteria [76-78]. Planktonic UPEC inhabiting the bladder lumen have limited ability to maintain residence in the urinary tract without the ability to adhere to the epithelial surface due to the flushing action of micturition except in conditions that lead to incomplete voiding or reflux.

Because of the myriad outcomes that result in human UTI, it is imperative to utilize an interdisciplinary approach in multiple model systems to completely capture the complexities of UTI pathogenesis that may be differentially exhibited spatially and temporally. Several murine models of UPEC infection have been developed [79, 80]. Each model takes advantage of different available, genetically defined, inbred mouse strains that exhibit varied immunological responses to UPEC infection to dissect several aspects of UTI progression. Studies performed in C3H/HeN, C3H/HeJ and C57Bl/6J backgrounds revealed stages of acute, sub-acute and chronic

infection that bear striking parallels to human UTI [80-83], indicating that outcomes from these mouse models strongly parallel human disease. These and other models, utilizing CBA/J, C3H/HeOUJ, and BalB/c mouse strains provided insights into immune checkpoints that may be predisposing humans to chronic UTI [80, 82, 83]. Using these mouse models, significant strides have been made in understanding the onset and progression of UPEC through the urinary tract.

Type 1 pilus-dependent bladder invasion

The FimH adhesin located at the tip of the type 1 pilus is instrumental in mediating UPEC interactions with the bladder epithelium (Fig. 2). FimH has been shown to bind mannose and its derivatives, thereby interacting with mannosylated moieties on abiotic and biotic surfaces. UPEC expressing type 1 pili were shown *in vitro* to bind to human and mouse uroplakins (UP) Ia and Ib, integral membrane glycoproteins that create a hexagonal array on the surface of the superficial facet cells of the bladder that create an impermeable barrier to toxic compounds in urine [84]. UPs are highly conserved across mammals both structurally and via sequence homology [85]. UPIa and UPIb are members of the tetraspanin family of molecules that modulate immune signaling via leukocyte differentiation. Using high-resolution, freeze-fracture, deep-etch electron microscopy, Mulvey *et al.* showed that the type 1 pilus tip fibrillum interacts with the hexagonal array of uroplakins on the terminally differentiated superficial facet cell layer of C57BL6 mice (Fig. 2A) [10], validating previous *in vitro* data [84]. This interaction, combined with the high tensile strength of the pilus rod, enables UPEC to withstand the strong shear forces applied by urine flow and persist in the bladder [86, 87]. The crystal structure of FimH with α-D-mannose bound in its recognition pocket, combined with mutational analysis, revealed the structural details of the precise specificity of FimH for mannose [60]. Subsequently,

analysis of over 300 sequenced UPEC strains revealed that the FimH binding pocket is invariant, further demonstrating the importance of this domain for pathogenesis [88]. FimH binding to UPIa results in global conformational changes of the uroplakin plaques, which lead to cytoskeletal rearrangement and the alignment of the cytoplasmic tails of UPIa, UPIb, UPII, and UPIIIa, initiating second messenger signaling [89, 90]. The resultant increase in intracellular Ca^{2+} elevation mediates host cell apoptosis and Interleukin-6 (IL-6) secretion, in an effort to control the infection and alert the innate immune system to the bacterial presence in the urinary tract [90, 91]. Through the precise interaction between FimH on type 1 pili and uroplakins on the bladder epithelial surface, UPEC bind to the tissue, initiating downstream signaling events within the host cell.

UPEC binding triggers host and bacterial responses

The binding of type 1 pili to a surface enacts bacterial transcriptional changes in addition to host responses. Binding of UPEC to mannosylated yeast enacts transcriptional change in an *E. coli* strain (CSH50) [92]. In addition to upregulation of genes involved in general metabolism, several genes classified as being involved in removal of reactive oxygen species and hydrophobic compounds were also upregulated. These latter genes are likely involved in defense against antimicrobial peptides in the urine and potentially antibiotics as well as attack from neutrophils and other inflammatory cells. Once bound, UPEC are capable of invading the superficial layer of the transitional epithelial surface in a FimH-dependent fashion (Fig. 2B) [10]. Interaction of UPEC with α3 and β1 integrins in cell culture leads to actin rearrangement and bacterial engulfment in an active process requiring Rho-family GTPases [93-95]. The invasion process occurs via a cholesterol- and dynamin-dependent process modulated by calcium levels

and clathrin [96]. By an unknown mechanism, UPEC escape the endocytic vesicle, where they replicate in the cytoplasm to form biofilm-like intracellular bacterial communities (IBCs) of 10^4-10^5 bacteria that protrude into the luminal surface of the bladder (Fig. 2D) [29]. While type 1 piliated UPEC binding and invasion into the superficial facet cells provides a niche for rapid replication, studies in C57BL/6J mice demonstrated that it also leads to a robust apoptotic response and exfoliation of the facet cells within a few hpi [10], jettisoning infected cells (Fig. 2C). In addition to facet cell exfoliation, host signaling mechanisms lead to a massive influx of immune cells, mainly PMNs, and upregulation of other immune effectors. In spite of this robust response designed to thwart infection, UPEC evolved mechanisms to subvert and exploit elements of the innate immune response and persist within the urinary tract. UPEC utilize the pore-forming toxin hemolysin (HlyA) to induce the degradation of cytoskeletal scaffold protein paxillin, leading to host cell exfoliation [97]. Exfoliation must be properly tuned to ensure the appropriate balance between IBC maturation and exposure of the underlying transitional epithelium. The two-component signal transduction system, CpxRA, regulates the expression of hemolysin (Nagamatsu *et al*. In Preparation). Strains lacking CpxR increase hemolysin expression and are attenuated during infection [98]. Similarly, overexpression strains of HlyA are also attenuated, suggesting that hemolysin deficiency explains the deficit of the CpxR mutant. A hemolysin knockout does not activate the inflammasome, stimulates a lesser degree of host cell exfoliation, but is not attenuated, implying other pathways that induce exfoliation (Nagamatsu *et al.* In Preparation). The precise degree of exfoliation governs the eventual outcome of exfoliation by modulating bacterial niche-specific replication.

Upon IBC maturation, UPEC at the outer edges of an IBC become motile and disperse into the extracellular compartment of the lumen. Concomitant with this fluxing event, some

UPEC adopt a filamentous morphology [99] (Fig. 2E), a finding that along with IBCs has been observed in urine from women suffering rUTI [100]. Once extracellular, the immune cell infiltrate attacks luminal UPEC. Filamentous UPEC are resistant to phagocyte killing, providing a mechanism whereby UPEC emerging from an IBC into an inflamed environment can survive and reinitiate the intracellular cascade [101]. Deletion of the cell division inhibitor-encoding gene *sulA*, prevented filamentation and attenuated virulence at 24 hpi and onward in C3H/HeN mice [102]. Thus, the Δ*sulA* strain formed first generation IBCs, but was incapable of filamentation and second generation IBCs in C3H/HeN mice. Bacterial filamentation was not observed during acute infection in C3H/HeJ mice that lack the ability to signal through TLR4 [99]. Infection of C3H/HeJ mice with UTI89Δ*sulA* restored virulence of this bacterial strain [102]. Thus, filamentation and possibly second round IBC formation are dependent, in part, on TLR4 responses in the bladder, leading to regulatory responses by UPEC. TLR4, in addition to recognizing bacterial LPS to stimulate innate immune cells, may play a direct role in controlling UPEC access to an intracellular niche. TLR4 mediated expulsion of UPEC may play a role in limiting the numbers of UPEC that successfully form IBCs [103]. Thus, multiple bacterial and host factors regulate the ability of UPEC to mature within an IBC.

Genetic regulation within IBCs

As a result of the rapid and dramatic shift in local environment, robust transcriptional changes occur in UPEC inhabiting an IBC. At 6 hpi each C3H/HeN mouse bladder contains between 1 and 700 IBCs (Fig. 2D) [104, 105]. In the 2 hours subsequent to invasion, WT bacteria coalesce into loose collections 2-4 μM beneath the surface of superficial facet cells [99]. UPEC replicate every 30 to 35 minutes in this environment and retain bacillary morphology.

Invasion alone; however is not sufficient to lead to IBC formation. The commensal strain MG1655 invades the urothelium, but does not form IBCs, and is thus rapidly cleared [106]. Using a tet inducible copy of FimH, it was shown that the development of IBCs requires the expression of FimH and type 1 pili [105]. Additionally, deletion of the gene encoding the outer membrane protein OmpA or the capsule synthesis genes disrupted IBC formation despite an equivalent invasion efficiency relative to WT UPEC [107, 108]. Host and bacterial transcriptional changes that accompany intracellular replication within IBCs in C3H/HeJ mice have been deduced [78]. UPEC within IBCs upregulate the siderophores enterobactin and salmochelin to scavenge available iron. Concomitantly, host upregulation of the transferrin receptor and lipocalin-2 within the IBC may restrict iron availability [78]. The battle for iron within the host is an important battleground during the host-pathogen interaction for many pathogens. Indeed, in women suffering from rUTI, urinary isolates were more likely to express the siderophores salmochelin and yersiniabactin compared to rectal isolates from the same patients [109]. Thus, regulatory changes including the upregulation of siderophores and continued expression of type 1 pili accompany the transition from the luminal niche of the bladder to the intracellular niche within the bladder epithelium.

In addition to epithelial mechanisms that restrict IBC formation, the specific allele of FimH also impacts the ability of UPEC to form IBCs. The mannose-binding pocket of FimH is comprised of invariant residues among all clinical UPEC strains [88]. Variation outside of this pocket occurs frequently among UPEC strains. Sokurenko *et al.* have used a variety of methods to examine the natural variation and shown that FimH is under positive selection in UPEC [110-112]. We performed *in silico* analysis of FimH gene sequences from 279 diverse *E. coli* isolates, which identified several amino acid (aa) residues outside of the mannose-binding pocket under

positive selection [88]. Our structure/ function analyses of these positively selected residues indicated that although they are not part of the mannose-binding pocket, they can impact mannose binding, IBC formation, and virulence [88]. It was previously shown that the majority of *E. coli* isolates including the pyelonephritis isolate CFT073 and the commensal K12 strain MG1655 have a serine at position 62 (S62) while cystitis isolates NU14 and UTI89 have an alanine [88, 113]. An alanine at position 62 (A62) was associated with increased collagen and monomannose binding [114], and increased virulence in the cystitis isolate NU14 [113]. We found that an A62S *fimH* mutation in the chromosome of UTI89 significantly attenuated invasion, IBC formation and virulence and affected phase variable type 1 pilus regulation. In addition, we found that a double mutation of the positively selected residues at positions 27 and 163 (UTI89 *fimH*::A27V/V163A) had no effect on pilus assembly or mannose binding *in vitro*, but exhibited a 10,000-fold reduction in mouse bladder colonization at 24 hpi and was unable to form IBCs even though it bound normally to mannosylated receptors in the urothelium [88]. Thus, the A27V/V163A double mutation identified a function of FimH that is required, in addition to mannose binding, for IBC formation and *in vivo* fitness, suggesting that IBC formation is critical for successful UTI. Thus, many factors contribute to the ability of invaded UPEC to form IBCs including TLR4-mediated expulsion, bladder cell exfoliation, and FimH allele, which may alter regulation or the ability of UPEC to aggregate intracellularly.

Reservoirs form in the underlying bladder tissue

The intracellular pathogenic cascade that UPEC undergoes has been shown to be critical for several of the outcomes of infection. If the immune response effectively eradicates luminal bacteria as evidenced by sterile urine, mice may still have bacterial CFU in the bladder [80] (Fig.

2G). As mentioned earlier, during the acute infection cascade, infected and uninfected cells of the superficial epithelium are exfoliated in a caspase-dependent manner [10, 115, 116] (Fig. 2C). While this defense mechanism jettisons bacteria attached to and replicating within the epithelium, it exposes cells of the underlying, transitional cell layer, which UPEC can invade. UPEC establishes reservoirs consisting of 8-12 bacteria within Lamp1+ rosettes in the bladder tissue called Quiescent Intracellular Reservoirs (QIRs) (Fig. 2G) [82]. The mechanism of QIR formation is unknown. UPEC does not actively replicate within these reservoirs, but instead persists in a dormant state. Bacteria in the QIR can reactivate (by an unknown mechanism) to release bacteria into the lumen to begin the IBC cascade anew. Reactivation of QIRs has been triggered pharmacologically resulting in a recurrent infection with high titers of bacteria in the urine of mice [81, 82]. Reservoirs can thus form in the mouse bladder that can reactivate leading to a rUTI.

An exuberant immune response during acute infection predisposes to chronic cystitis

Different murine models of UTI recapitulate the range of clinical outcomes of UTI [80]. WT inbred C57BL/6J mice resolve bacteriuria rapidly, but are susceptible to rUTI [81], possibly as a result of the large amount of QIRs formed in the bladder tissue [82] (Fig. 2G). C3H/HeOUJ mice are exquisitely susceptible to persistent bacterial replication of an inflamed bladder throughout the lifetime of a mouse, a phenomenon referred to as chronic cystitis [83] (Fig. 2F). Elevated levels of the cytokines IL-5, IL-6, Keratinocyte Cytokine (KC), and Granulocyte Colony-Stimulating Factor (G-CSF) in the serum of mice at 24 hpi predict the development of persistent bacteriuria and chronic cystitis at 4 weeks post infection (wpi) [83]. C3H/HeN mice exhibited a bimodal distribution of these two outcomes with persistent bacteriuria and chronic

cystitis occurring in 20% when infected with 10^7 CFU UTI89, a prototypical virulent UPEC isolate. Infection of mice with a dose of 10^8 CFU increased the proportion of C3H/HeN mice that developed chronic cystitis to 50% [83]. In the bladders of mice that experienced chronic cystitis, the superficial facet cells become completely denuded, and IBC formation ceases to occur after 24 - 48 hpi. Thus, during chronic cystitis, bacteria proliferate in association with the underlying tissue, but do not invade to an appreciable degree (Fig. 2F). Furthermore, treatment of these mice with an antibiotic was shown to completely sterilize the bladder [83] in contrast to the tolerance observed for bacteria in QIRs [81]. Thus, these outcomes are likely mutually exclusive. An overexuberant immune response during acute infection to invasive UPEC leads to cytokine secretion and rampant bacterial replication.

Dissemination to the kidneys is accompanied by regulatory changes

The dynamics of UTI are complex, especially when bacteria seed the kidneys. The mouse models of UTI differ in their propensity for kidney infection [80]. C3H/HeJ mice that lack the ability to respond to LPS via TLR4 sustain persistent kidney infection [117]. Kidney infection in C3H/HeN mice generally mirrors the outcome in the bladder; however, bacterial titers in the kidney are generally lower than in the bladders of the same animals [83]. The ability to ascend from the bladder to the kidneys depends on many factors including vesicoureteral reflux (VUR) and bacterial motility (Fig. 2). Bacterial inoculation into the bladder leads to reflux into the kidneys to varying degrees depending on the volume and speed with which the inoculum is introduced as well as the mouse strain [118, 119]. A minor, but significant role for flagella has been documented contributing to ascension to the upper urinary tract. Expression of flagella coincided with ascension to the upper urinary tract [120], and lacking flagella decreased the

ability of UPEC to colonize murine kidneys [120, 121]. Expression levels of the flagella promoter *flhDC* varied within IBCs, perhaps corresponding to their maturity and likelihood of releasing bacteria into the bladder lumen. Robust and rapid gene expression including activation of motility occurs prior to a transition from a bladder niche allowing transition and colonization of the upper urinary tract.

Bacterial strains that colonize the upper urinary tract are more likely to contain the *pap* operon than those strains isolated from patients suffering lower UTI only [122]. It has been shown that several UPEC CUP pili can bind receptors in the human kidney [123]. Type 1 pili were shown to bind tissue from the proximal tubules and the vessel walls, whereas P and S fimbriae bound Bowman's capsule, the glomerulus, the renal tubules, and the vessels. F1C pili bound to endothelial cells and the collecting duct and distal tubules. The role of P pili has been clearly demonstrated in cynomolgus monkeys, where the receptor specificity more closely matches that of humans [124]. The PapG containing strain DS17 caused significantly more pathology in the monkey kidneys as assessed histologically as well as resulted in a greater loss of renal function as compared to an isogenic knockout [125]. Furthermore, immunization against PapG effectively reduced renal histopathology in monkeys [126]. The role of P pili has been difficult to assess in mouse models because the receptor for PapG is not present in the mouse kidney [124, 127]. However, Pap-expressing UPEC were isolated in higher numbers from the kidneys of CBA/J mice, a commonly used murine UTI model strain, than those lacking P pili in co-infection experiments [128]. Knockout analysis of the Pap genes in CFT073 was utilized to determine that P pili play only a minor role in kidney colonization in the CBA/J model [129]. It was recently shown that Ygi fimbriae bound kidney epithelial cells *in vitro* [36]. Therefore, P pili

appear to be an essential virulence determinant in causing human UTI, however other CUP pili may also contribute to kidney tropism in humans and animal models.

UPEC may also occupy disparate niches in the kidneys, as illustrated by the ability of different adhesins to mediate attachment to different receptors within the kidney [123]. The infection dynamics of UPEC infecting the kidneys was recently demonstrated utilizing intravital multi-photon microscopy in rats [130]. Direct microinfusion of bacteria into the nephron revealed a synergistic role of P and type 1 pili. Interestingly, knockouts of FimH and P pili in distinct UPEC strains colonized the rat renal tubule, but the kinetics and dynamics of the infection differed. In the absence of P pili, attachment was temporally retarded, but spatially indistinguishable from the WT. Inversely, a FimH knockout rapidly colonized the rat renal tubules, but only areas adjacent to the epithelium. Likely, in the absence of FimH, UPEC was incapable of coalescing into a biofilm occupying the lumen of the renal tubule [130]. FimH-mediated bacterial aggregation may play a role in biofilm formation in the kidney and IBC formation in the bladder [88]. Because of the variety of niches UPEC can occupy in the bladder and kidneys, the dynamics of UPEC UTI are complex with genetic regulatory networks likely governing the appropriate confluence of virulence factor expression for each given niche.

Regulation of type 1 pili

The most well associated virulence factor with UPEC strains causing cystitis is type 1 pili, present in greater than 95% of cystitis isolates [73, 122]. Type 1 piliated UPEC are more effective at colonizing the mouse bladder than non-piliated UPEC [131-133]. Although type 1 pili are essential for bladder colonization, their expression is dispensable and perhaps disadvantageous in other niches such as the kidneys, colon, or environment. In fact, loss of type

1 piliation does not decrease intestinal colonization of individual animals [134]; however, it does significantly reduce communicability [135], further supporting the argument that *E. coli* expressing type 1 pili is better suited for life in the urinary tract compared to the GI tract [88]. However, reports to the contrary have shown that type 1 pili are essential for GI tract colonization [136]. Interestingly, clinical data may be shifting this paradigm of a source-sink relationship in this complex disease. In a study of women experiencing multiple rUTIs, it was shown that at the time of infection, the dominant *E. coli* strain in the bladder and the GI tract was the same [137]. The role of type 1 pili in the gut niche has not been fully characterized. It has been demonstrated; however, that in transitioning niches out of the bladder, gene expression changes regulate pilus expression. Accordingly, type 1 pilus expression is reduced in kidney infections of mice [118]. Within the bladder niche where type 1 pilus expression governs pathogenesis, the *fim* genes are more likely to be expressed. Bacteria attached to epithelial cells sloughed in mouse urine were significantly more likely to express type 1 pili than planktonic bacteria in the same urine sample [138]. This niche specific difference in type 1 pilus expression provides strong evidence for the spatial and temporal regulation of this CUP operon and its necessity in causing cystitis. Accordingly, a vaccine against FimH was shown to dramatically decrease infection in both infected mice and monkeys [139, 140].

Type 1 pili are phase variable, transcriptionally regulated by the inversion of the 314bp *fim* promoter, *fimS,* that is located between flanking invertible repeats which are recognized by the recombinases, FimB and FimE (Fig. 3A). FimB and FimE, which are 48% identical are encoded by two regulatory genes, *fimB* and *fimE*, 5' of the *fimS* region [141-143]. FimB recombines the invertible repeats showing no preference in promoter orientation, while FimE inverts the promoter to the OFF orientation only [142, 144]. FimB appears to show no bias in

turning the switch ON or OFF; however, the rate with which FimE turns the switch OFF is an order of magnitude greater than FimB [145]. Thus, the abundance and activity of FimE and FimB appear to be the determining factors of the orientation of the phase switch.

A number of factors have been identified that in turn control expression or activation of FimB and FimE, thus contributing to modulation of type 1 pili expression in response to environmental changes. For example, in broth cultures at 37^{o}C, FimB is produced in excess leading to the population distribution skewed to more piliated bacteria [146, 147]. Recently, *in silico* analysis has demonstrated stringent control of the type 1 fimbriation switch, such that at temperatures lower than body temperature, FimE dominates, inverting the promoter to the OFF orientation [145]. At physiological or elevated host temperatures, during febrile episodes for example, the ON-to-OFF switching rates are lowered dramatically via increasing activity of FimB, locking UPEC into the more fimbriate state. Additionally, mutation of one of the invertible repeats of the *fim* promoter such that the promoter was irreversibly "locked" in the OFF orientation, resulted in significantly fewer CFUs retrieved from urine and bladders at 24hpi, compared to the WT strain, CFT073 [148]. As expected, when the promoter was locked in the ON orientation, the resulting strain slightly out-competed WT CFT073 in the bladder at 4 and 24 hpi. In a recently conducted study with globally spreading, multi-drug resistant UPEC strains of the clonal group ST131, Totsika *et al.* found that growth in static conditions, known to enrich for *fim* expression [149], increased bladder colonization without affecting urine titers at 18 hpi [133]. In contrast strains from the same collection that lacked the ability to turn *fim* ON were unable to colonize the bladder [133]. Affecting the orientation of the promoter via genetic manipulation or through natural environmental changes alters the degree of piliation and fitness in the bladder.

Multiple studies have demonstrated that UPEC lacking both FimB and FimE rapidly inverted the *fim* promoter to the ON orientation when inoculated into mice [150, 151]. These experiments revealed that additional recombinase enzymes influence phase variation. Genome analysis of the pyelonephritis isolate CFT073 revealed 3 such recombinases *ipuA*, *ipuB*, and *ibpA* with 65-70% sequence similarity to FimE and FimB (Fig. 3A) [151]. *ipuA* and *ibpA* were able to invert the *fim* switch in the absence of FimE and FimB *in vitro* and when inoculated into the urinary tract of mice. Further analysis revealed that IpuA acts similarly to FimB, inverting the switch bidirectionally, whereas IpbA can only invert the promoter from OFF to ON. The cystitis isolate UTI89 contains FimX, an IpbA homolog, with 49.1% amino acid similarity to FimB [150] but does not contain IpuA or IpuB. A phase OFF triple *fimBEX* deletion mutant was completely deficient in bladder colonization at 6 hpi, whereas a phase OFF *fimBE* mutant colonized at WT levels, indicating that *fim* phase inversion was due to the activity of FimX. Indeed, complementation of the *fimBEX* mutant with *fimX* alone was sufficient to restore wild type levels of bladder colonization at 6 hpi, suggesting that UPEC has several functionally redundant recombinases that enable colonization of the bladder by mediating *fim* promoter inversion to the ON orientation [150]. In addition, these functionally redundant recombinases may serve to fine-tune the phase status of the population in certain niches or they may represent redundant regulators in the event of a *fimB* or *fimE* mutation, which occurs in certain UPEC strains, such as the multi-drug resistant clones of the ST131 lineage [133]. The majority (59 to 71%) of strains from this clonal group were shown to have an insertional mutation in *fimB*, yet 87% of tested isolates were still able to express type 1 pili as assessed by yeast agglutination, and adherence to and invasion into bladder epithelial cells [133]. One representative member of this group, EC958 contained *ipbA*, which may account for the observed *fim* promoter recombination.

By virtue of its importance in bladder colonization, type 1 pili are regulated by many functionally redundant recombinases.

DNA binding proteins that impact structure of the chromosome and global regulators also influence the directionality of the *fim* promoter. Integration host factor (IHF) plays a dual role in governing phase status of UPEC [152]. Mutations in IHF locked the phase switch in the OFF or ON orientation, thereby preventing inversion of the promoter [153]. Additionally, when the *fim* switch was locked in the ON orientation, mutation of IHF led to a sevenfold reduction in LacZ expression of a FimA-LacZ fusion [152]. Mutations in the histone-like protein H-NS were shown to dramatically increase the rate of *fim* promoter inversion [154]. Conversely, mutations in the master regulator Leucine-responsive regulatory protein (Lrp) decreased the rate of *fim* promoter inversion. Lrp increased the transcription of *fimB* and decreased *fimE* expression [155]. Additionally, Lrp binds directly to the *fim* invertible repeat region, thereby sterically hindering FimB/FimE binding or RNA polymerase complex recruitment [156]. Binding of the catabolite repression system through cAMP-CRP (cAMP receptor protein or Catabolite Activator Protein, CAP) also influenced the *fim* promoter (Fig. 3A) [157]. Deletion of the *crp* gene led to higher expression of *fimA* transcript while decreasing P pili and flagella. The effects of cAMP-CRP on the *fim* promoter are complex likely because this regulator imparts pleiotropic effects in response to the nutritional status of the cell and growth phase. Additionally in log phase of growth, CRP-cAMP repressed fimbriation whereas in stationary phase, it appeared to have little effect. The contribution of multiple regulatory networks and proteins like the ones we discussed here are likely to be critical in ensuring appropriate expression of type 1 pili during transitions between niches with varying temperatures and nutritional availability.

FimH allele regulates type 1 pili

In addition to the aforementioned external regulators of type 1 pili, the allele of *fimH* can also regulate type 1 pilus expression. A mutation of the FimH binding pocket from a glutamine to a lysine at position 133 resulted in 50% of the bacterial population to turn type 1 pili off relative to 90% in WT UTI89 [88]. Similarly an A62S mutation had the same effect. This regulation may be due in part to the structural conformations that FimH adopts during its translocation. The pilus initiation complex for type 1 pili has recently been crystallized, demonstrating that FimH adopts an elongated conformation when being assembled [158]. Immediately therafter, FimH adopts a bent, compressed conformation when it is extruded out of the FimD usher [159]. These conformational differences are similar to those observed when FimH is bound by the chaperone FimC versus when it is assembled into a tip [160]. When FimH binds mannose or when it is bound by its chaperone, it adopts an elongated, high affinity conformation [43, 161]. When mannose is absent, FimH is in a low affinity, compressed state [160]. FimH allelic differences may govern both mannose binding as well as the regulation of the expression of this virulence factor.

Differential methylation regulates P pili

Unlike the DNA-inversion based phase variation of type 1 pili, phase variation of the *pap* operon is mediated by differential methylation of two DNA adenine methyltransferase (DAM) sites in the promoter (Fig. 3B) [162], while additional regulation is accomplished by the cis-encoded regulators PapB [163] and PapI [164] as well as several DNA binding proteins including H-NS, Lrp, and CRP-cAMP. PapB and PapI, are encoded upstream of the *pap* operon.

PapI is transcribed from its own promoter in the opposite direction to the *papBA* transcript [164]. Two GATC methylation regions are present upstream of the *papBA* promoter [162]. Methylation of $GATC_{1130}$ ($GATC^{prox}$) results in phase ON cells that can be actively transcribed, whereas methylation of $GATC_{1028}$ ($GATC^{dist}$) results in phase OFF bacteria (Fig. 3B). Because methylation is the main mechanism for P pilus phase variation, P piliation is linked to cell division during which these epigenetic changes can be enacted [165]. Furthermore, *pap* genes were not expressed when Dam was present in too high or too low quantities within the bacterial cell, implying that appropriate methylation is essential for proper regulation. The regulator PapI binds to DNA upstream of the *papBA* promoter at distal sites. With Dam absent, the proper methylation of $GATC_{1130}$ does not occur, and transcription is abrogated. Conversely, overexpression of Dam prevented phase OFF bacteria from turning ON, presumably as a result of aberrant methylation of GATC sites [162]. As for type 1 pili, Lrp also affects P piliation. Methylation of $GATC_{1028}$ (distal) and cooperative Lrp binding at proximal sites prevented RNA polymerase from transcription at the *papBA* promoter [165].

Autoregulation of the *pap* operon by PapB is exquisitely tuned to the amount of the regulator present. PapB binds to three locations within the operon: 200 bp upstream of the *papI* promoter adjacent to the binding site of CRP, adjacent to the -10 area of the *papBA* promoter, and within the PapB coding sequence (Fig. 3B) [166, 167]. PapB binds upstream and activates the transcription of *papI*, perpetuating a positive feedback loop, maintaining bacteria in the P piliated state [168]. However, overexpression of PapB led to repression of P piliation, by blocking the binding of RNA polymerase at the -10 region of the *papBA* promoter [167, 168]. CRP-cAMP is essential for the transcription of the *pap* operon [169]. Binding of CRP-cAMP 215 bp upstream of the *papBA* promoter led to increased levels of transcript. Binding of Lrp 140

bp upstream of the promoter was essential for CRP-mediated *papBA* transcription [170]. Addition of PapI in trans dramatically increased OFF to ON switching rates and subsequent *papBA* transcription. The precise control of P pilus regulation by metabolite binding proteins and cis-encoded regulators serves to assess local environmental conditions and respond accordingly with the timely expression of the appropriate pilus system.

Pilus cross-regulation

In order to accomplish accurate and rapid exchange of expressed CUPs, regulators of the P, type 1, and S pilus systems interact with other operons, regulating their expression (Fig. 3C). These regulatory networks are likely responsible for ensuring UPEC expresses the appropriate pili in a timely manner to colonize a specific niche. Most *E. coli* only express one pilus type at a time [171] with cross-regulatory networks likely responsible for switching between expressed pilus systems [172].

Deletion of a pathogenicity operon encoding P-related fimbriae in the UPEC strain 536 diminished expression of S pili encoded by the *sfa* operon [173, 174]. The regulators PrfB and PrfI encoded by the P-related fimbriae operon are 76% and 87% homologous to the S pili regulators, SfaB and SfaI, respectively. PrfB and PrfI act in trans on S fimbriae promoting their expression and complemented *sfaB* and *sfaI* knockouts [174]. Coordinate expression of these two pilus operons and other interactions not yet known may serve to enhance the probability of attaching to a host surface. It is possible that attachment feedback through master regulators selects for the expression of appropriate adhesin systems for that environment. This theory is consistent with data suggesting that attachment altered complex genetic regulatory networks [92, 175, 176]. Further, negative cross-regulatory interactions between pili may serve to divert

resources to the conditions for most effective persistence or transit throughout hosts. Transcription of both the P and S pilus operons repressed type 1 pili expression (Fig. 3C) [177, 178]. PapB binds to several regions of the *fim* operon and enhances the expression of *fimE* and also prevents the ability of FimB to invert *fimS* [178] (Fig. 3). SfaB represses FimB-mediated recombination without affecting FimE [177] (Fig. 3C). When expressed from its native promoter in *E. coli* K12 and CFT073, PapB was shown to inhibit type 1 pili. Furthermore, the expression of P pili in clinical isolates from patients with varying clinical UTI syndromes repressed type 1 pili [179]. These data present a model of coordinate regulation of virulence factors in which during cystitis, type 1 pilus expression is dominant whereas other CUPs such as P or S pilus expression are expressed in the kidneys during pyelonephritis, repressing type 1 pilus expression.

Altering the balance of the two-component regulatory system QseBC that is present in many pathogenic bacteria [180], affects many UPEC virulence factors, CUP systems chief among them [62, 181]. Aberrant and uncontrolled phosphorylation of QseB via deletion of the sensor kinase QseC in UTI89 lead to decreased type 1 pilus expression, decreased flagella expression, and increased expression of the *sfa* operon encoding S pili, the F-17 operon, and the *fml* operon. The expression of the CUP operons *yqi*, *yeh*, and *auf* were decreased. The alteration of conserved metabolic processes likely accounts in part for these pleiotropic effects [62]. It is at present unclear whether these changes are direct effects of QseB phosphorylation or whether the decrease in *fim* expression leads to the concomitant changes in other CUP expression.

Anti-virulence compounds to treat UTI

UPEC colonization of the diverse urinary tract niches largely depends on its ability to adhere to different receptors that are niche-specific. Therefore, inhibiting the adhesive organelles

that mediate attachment poses an attractive strategy for treating or preventing UTIs. The mannose-binding pocket of FimH is invariant among sequenced UPEC, and a mutation in this region rendered FimH defective in mannose binding, as well as bladder cell invasion and IBC formation [60, 88]. FimH is known to bind human uroplakins that coat the luminal surface of the bladder [84]. Recently small molecular weight orally available compounds called mannosides that bind the FimH binding pocket with low nanomolar affinities were developed using rational structure-directed design [182-185]. The addition of mannosides to an oral regimen of the antibiotics trimethoprim-sulfamethoxazole (TMP-SMX) was additive in reducing bacterial burden by the sensitive strain UTI89 [185]. By utilizing a strain resistant to TMP-SMX, PBC-1, it was shown that mannoside addition potentiated the effects of this antibiotic combination. Mannoside prevented PBC-1 from accessing the intracellular niche of the bladder, thus partitioning organisms to the urine and bladder lumen, where antibiotic levels were above the minimum inhibitory concentration for the clinically resistant PBC-1 strain. Mannoside was also effective at reducing bacterial titers by greater than 4 logs in as little as 6 hours after its oral administration to mice experiencing chronic cystitis at 2 wpi [185]. Because mannosides do not need to access the bacterial cytosol, they are not subject to efflux pumps, degradation, or changes in outer membrane permeability, implying that resistance development is unlikely.

While type 1 pilus inhibition would likely be effective for preventing and treating cystitis, a more general approach targeting multiple CUPs could incapacitate multiple pilus systems limiting colonization of multiple niches. Accordingly, compounds have been designed to block pilus biogenesis by taking advantage of the structural similarity between CUPs [27]. These bicyclic 2-pyridinones known as pilicides reduced type 1 and P pilus based hemagglutination and biofilm formation. Structural analysis revealed that these compounds bind to the surface of

the chaperone that interacts with the usher [27]. Therefore, pilicides function by entering the bacterial periplasm and binding to the chaperone to prevent pilus assembly through the usher. Anti-virulence compounds such as pilicides and mannosides represent novel strategies to translate basic knowledge from the investigation of pilus structure and function into new therapeutics that may have efficacy in treating UTIs by affecting CUP expression and function.

Conclusions and Perspectives

Surface expressed organelles with terminal adhesins mediate the first interaction between host and pathogen. In colonizing a host or the environment, bacteria encounter many niches with disparate surfaces on which to attach. Accordingly, many pathogens have the capacity to express different adhesins assembled into different surface organelles to mediate attachment to the various niches and surfaces they encounter. Understanding the regulatory interplay between pilus function and regulation and the cross-talk between operons has led to the development of novel anti-virulence therapeutics such as mannosides and pilicides, which target the FimH adhesin expressed at the distal tip of type 1 pili or multiple chaperone usher pili, respectively [27, 182, 185, 186]. Furthermore, understanding the complex population dynamics, niche occupation, and bacterial fluxing between urinary tract niches in time and space is crucial to dissecting the importance of virulence factors. Virulence gene expression is precisely regulated over time and space by the local environmental conditions including the receptors for attachment, immunological response, and other bacterial populations nearby.

Overview

This thesis describes the ability of UPEC to occupy intracellular and extracellular bladder niches throughout infection in multiple models. I hypothesized that occupation of an intracellular niche would precede and lead to the development of chronic cystitis. To this end, I determined the population dynamics and niche distribution throughout infection using a panel of isogenic, signature-tagged, UPEC strains. This work revealed a dramatic population bottleneck that accompanies acute infection. UPEC transit this bottleneck by occupying intracellular niches in the form of IBCs. I also determined the role of several residues of FimH that are evolving under positive selection. UTI89 and CFT073 harboring FimH::A62/V163 were more fit throughout infection, especially in the ability to cause and persist during chronic cystitis. Thus, these positively selected alleles conferred a colonization advantage to both intracellular and extracellular niches. By conducting biolayer interferometry, I determined that these residues impacted the ability of FimH to bind mannose. Finally, I developed a model of repeat infection to mimic the clinical situation of frequent sexual intercourse. I found that this process induced higher rates of chronic cystitis in susceptible and resistant mouse strains. This increase in chronic cystitis depended on invasion, IBC formation, and regulation of host cell exfoliation. The information herein will help to identify certain factors, both bacterial and behavioral, that might portend worse prognosis for women suffering an acute UTI, necessitating more aggressive and novel treatments.

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Figures

Table 1. Distribution of chaperone usher pathway pili in sequenced uropathogenic *E. coli* genomes.

CUP	**K12**	**UPEC**		
Operons	MG1655	UTI89	CFT073	536
fim	+	+	+	+
pap	-	+	+*	+
F17-like	-	+	-	+
sfa	-	+	-	+
yad	+	+	+	+
auf	-	±	+	+
yfc	+	+	+	+
ygi	+	+	+	-
yeh	+	+	+	+
fml	±	±	+	-
foc	-	-	+	-
yde	+	-	+	-
Dr family[1]	-	-	-	-
fso[1]	-	-	-	-
fst[1]	-	-	-	-
pix	-	-	-	+

(+) indicates presence, (-) indicates absence, and (±) indicates that an operon is present, but likely nonfunctional due to mutation or deletion of genes.

(*) indicates that two copies of the operon are present.

[1] indicates that these operons are not present in these UPEC, but are present in many clinical isolates.

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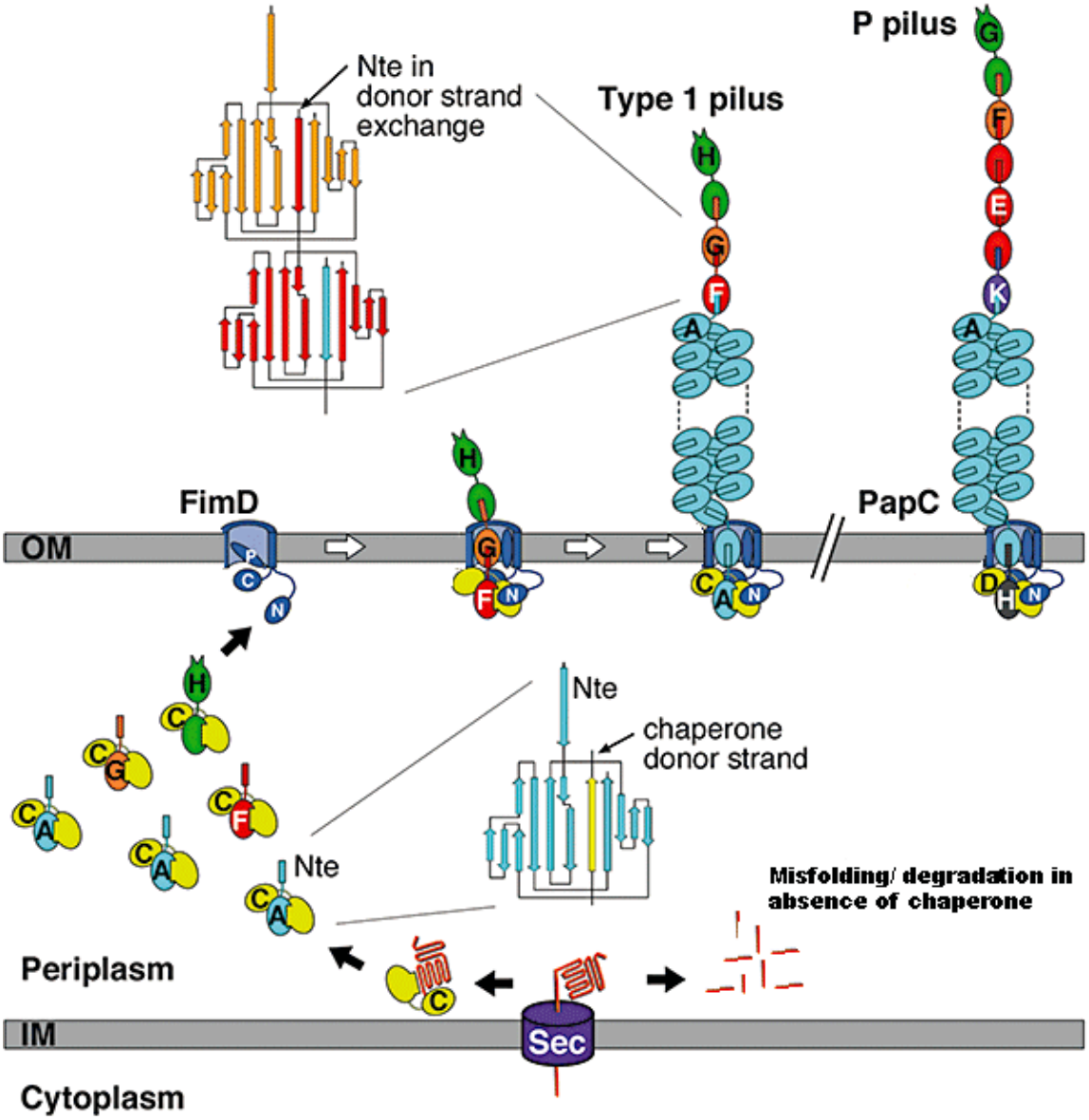

Figure 1. Model of type 1 pilus biogenesis.

A) Subunits are secreted across the Sec apparatus and immediately bound by the cognate periplasmic chaperone, the absence of which results in subunit misfolding and degradation. The chaperone donates its G1 beta strand to the subunit to complete its incomplete Ig-like fold in donor strand complementation. B) The chaperone then delivers the subunit to the NTD of the membrane usher, FimD. The subunit is then transferred to the CTD, where donor strand

exchange occurs with the previously added subunit. P pilus biogenesis occurs in a similar fashion. (A) modified with permission from John Wiley and Sons. Henderson *et al*. 2011. *Molecular Microbiology*.

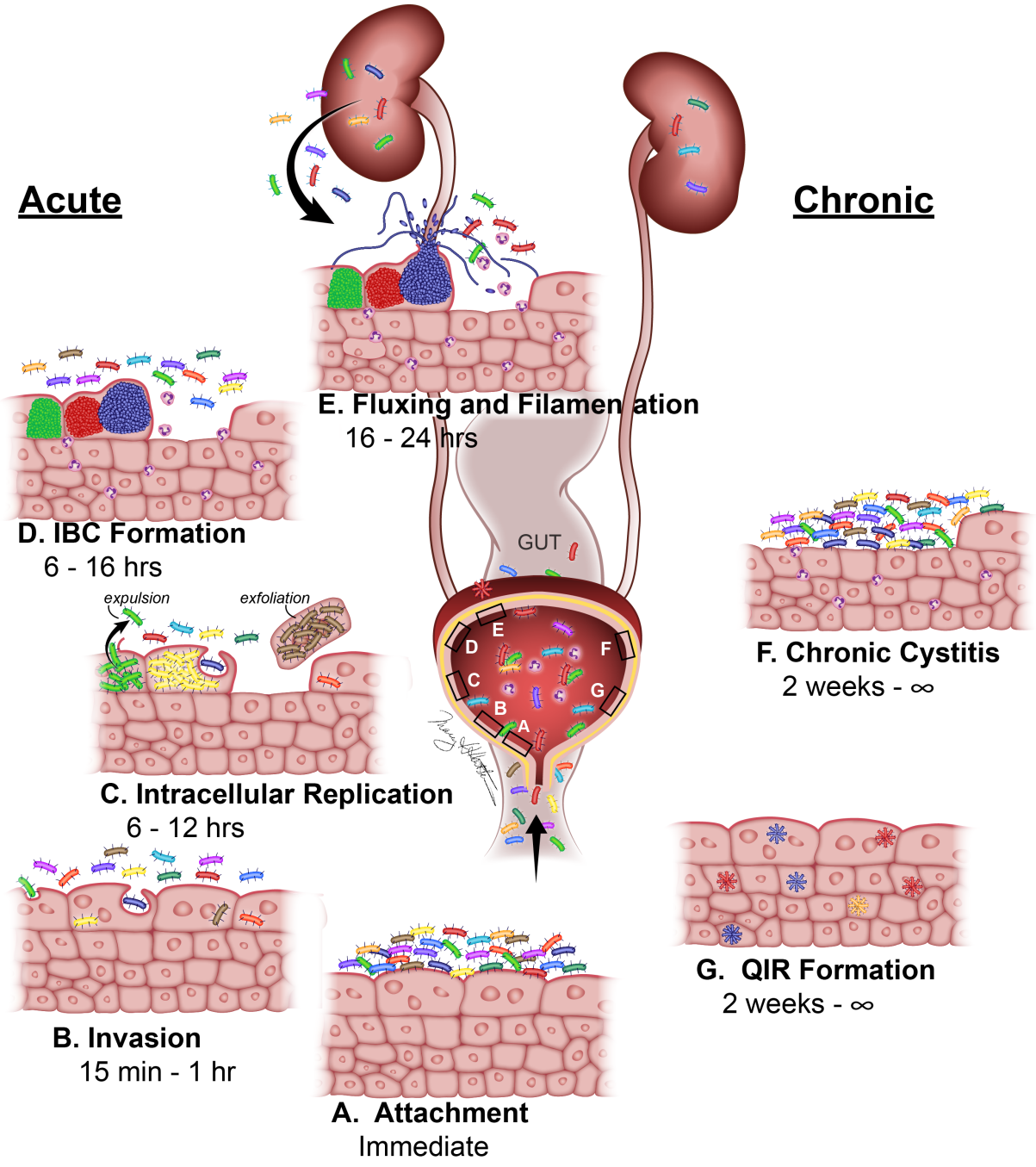

Figure 2. Pathogenesis of UPEC UTI.

A) A population of UPEC from the GI tract is introduced into the bladder where the bacteria attach to the epithelial surface with the FimH adhesin at the tip of type 1 pili. B) UPEC invades

the superficial facet cells of the bladder within the first hour of infection. C) UPEC replicates within the facet cells in a type 1 pili-dependent manner unless they are expelled via a TLR4-dependent process or infected epithelial cells are jettisoned by an apoptotic, exfoliation mechanism. D) UPEC form intracellular bacterial communities (IBCs) within the cytoplasm of superficial facet cells of the bladder. E) Between 16-24 hpi, UPEC flux out of the IBC with some bacteria becoming filamentous to become the dominant population of the bladder. Additional UPEC clones may descend from the infected kidneys. F) chronic cystitis is marked by bacterial replication in the lumen of the bladder and adherence to the tissue via type 1 pili. If the infection resolves, the formation of Quiescent Intracellular Reservoirs (QIRs) may result, as shown in (G).

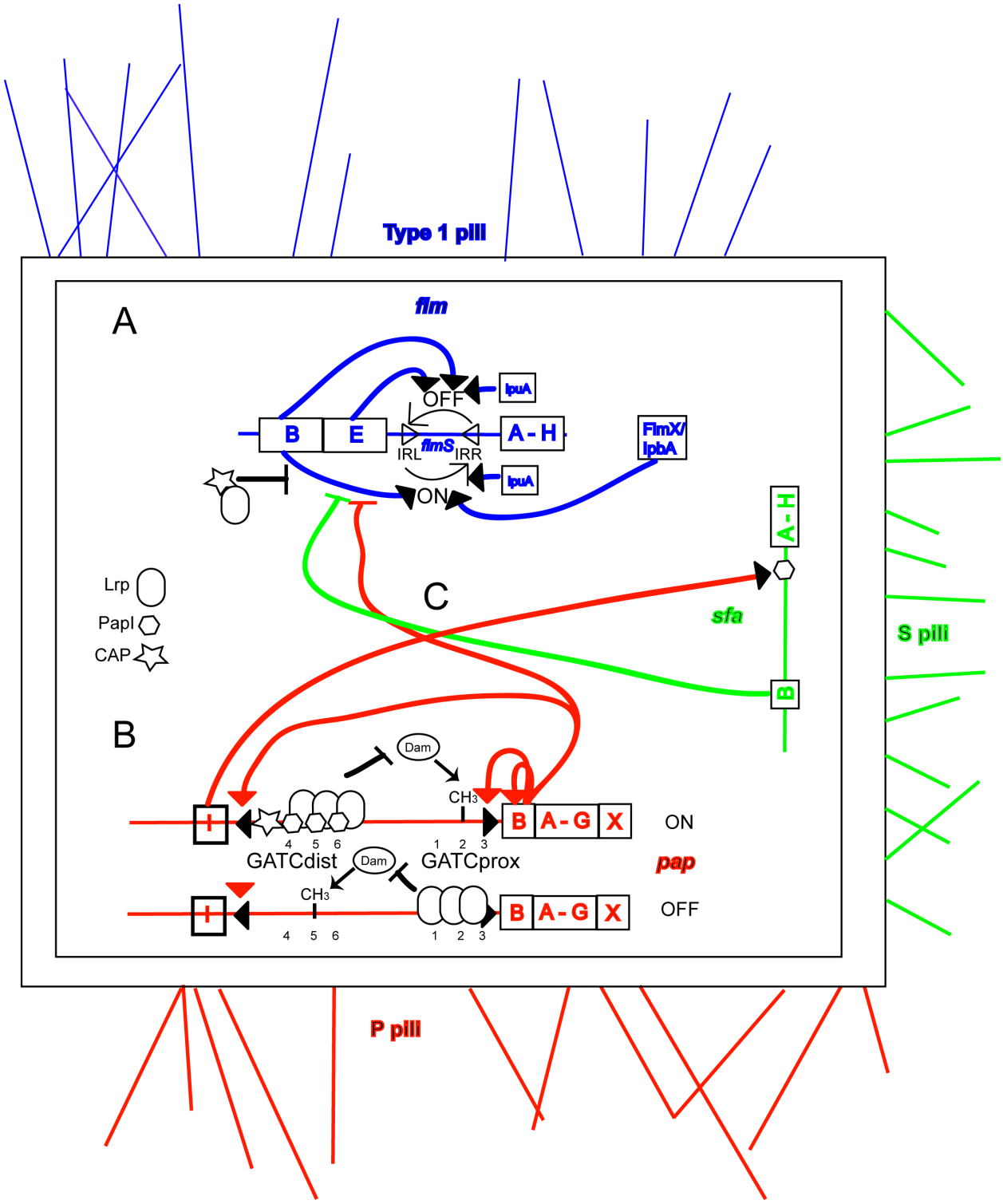
Type 1 pili
A
fim
OFF
IpuA
B
E
A - H
FimX/
IpbA
IRL
fimS
IRR
IpuA
ON
Lrp
PapI
CAP
C
A-H
sfa
S pili
B
B
Dam
CH3
B
A - G
X
ON
4 5 6
1 2 3
GATCdist
Dam
GATCprox
pap
CH3
B
A - G
X
OFF
4 5 6
1 2 3
P pili

Figure 3. Type 1, P, and S pilus regulation.

A) Type 1 pili are transcriptionally regulated by recombinases that invert the promoter, *fimS*. FimE binds the inverted repeats, turning the promoter OFF. FimB can invert the promoter bidirectionally; however, its major function is turning the promoter ON. The functionally redundant recombinases FimX/IpbA can also turn the promoter ON, whereas ipuA functions similarly to FimB. Lrp and CAP inhibit FimB-mediated OFF-to-ON inversion. B) The *pap* operon is regulated via differential Dam methylation. In the ON orientation, PapI-Lrp complexes bind the distal methylation sites, allowing RNA polymerase to transcribe the *pap* genes. Binding of Lrp to proximal GATC sites prevents Dam methylation and RNA polymerase binding, shutting down P pilus transcription. C) The cross-regulation between pilus operons serves to ensure appropriate adhesin expression for *in vivo* niche. PapI binds the *sfa* operon and enhances its transcription. PapB and SfaB inhibit FimB-mediated OFF-to-ON inversion of the *fim* promoter, preventing type 1 pilus transcription.

Chapter 2: Population Dynamics and Niche Distribution of Uropathogenic *Escherichia coli* during Acute and Chronic Urinary Tract Infection

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Infection and Immunity 79 (10), 4250-4259. doi:10.1128/IAI.05339-11. PMID: 21807904.

Abstract

Urinary tract infections (UTIs) have complex dynamics with uropathogenic *E. coli* (UPEC), the major causative agent, capable of colonization from the urethra to the kidneys in both extracellular and intracellular niches while also producing chronic persistent infections and frequent recurrent disease. In mouse and human bladders, UPEC invades the superficial epithelium, and some enter the cytoplasm to rapidly replicate into intracellular bacterial communities (IBCs) comprised of ~10^4 bacteria each. Through IBC formation, UPEC expands in numbers while subverting aspects of the innate immune response. Within 12 hr of murine bladder infection, half of the bacteria are intracellular, with 3-700 IBCs formed. Using mixed infections with GFP and WT UPEC, we discovered that each IBC is clonally derived from a single bacterium. Genetically tagged UPEC and a multiplex PCR assay were employed to investigate the distribution of UPEC throughout urinary tract niches over time. In the first 24 hpi, the fraction of tags dramatically decreased in the bladder and kidney while CFUs increased. The percentage of tags detected at 6 hpi correlated to the number of IBCs produced, which closely matched a calculated multinomial distribution based on IBC clonality. The fraction of tags remaining thereafter depended on UTI outcome, which ranged from resolution of infection with or without quiescent intracellular reservoirs (QIR) to the development of chronic cystitis as defined by persistent bacteriuria. Significantly more tags remained in mice that developed chronic cystitis arguing that a higher number of IBCs formed during the acute stages of infection precedes chronic cystitis, while fewer precede QIR formation.

Introduction

Population bottlenecks exist for many infections and are particularly well-documented during transmission between hosts for RNA viruses and parasites [1-6]. Localizing bottlenecks in time and space during an infection can identify steps in pathogenesis where an organism encounters the strongest barriers to establishing a foothold within a host. Bottlenecks may also represent important steps in host colonization and pathogenesis to target with therapeutics. Similar studies have been undertaken to identify genes important for tissue colonization and transit between tissues for bacterial pathogens ([7-9]. Several potential bottlenecks limiting the progression of uropathogenic *E. coli* (UPEC) to later stages of infection exist in the pathogenic cascade of UTI: i) invasion of the superficial bladder epithelium, ii) avoidance of Toll-like receptor 4 (TLR4)-mediated expulsion [10], iii) persistence in the face of superficial facet cell exfoliation, iv) the maturation process of IBCs, v) ascension from bladder to the kidneys, and vi) possible descent from kidneys to the bladder. These population dynamics all occur in the face of clearance mechanisms, including micturition and the innate immune system [11]. Understanding these bottlenecks in the setting of mucosal infection of the urinary tract will provide insight into the pathogenesis of this complex infection with the goal to develop better treatments.

UTIs are painful, expensive to the individual and society, and will affect 50% of women during their lifetime [12]. The vast majority of community-acquired UTIs are caused by UPEC. The clinical diagnosis of UTI hinges upon the ability to culture bacteria from clean-catch urine samples. When the uropathogen is sensitive to the chosen agent, oral antibiotics typically produce a rapid improvement in symptoms and sterilization of the urine [13, 14]. Despite appropriate treatment of a primary UTI, 25-40% of adult women will have at least one recurrence (rUTI) within 6 months of her initial infection [15, 16]. Additionally, up to 20% of

women may experience the symptoms of cystitis, or infection of the bladder, with accompanying urine cultures from clean catch specimens below the diagnostic cutoff of 10^5 CFU/mL [17]. This implies that bacterial occupation of urinary tract niches even in the absence of clinical diagnosis can contribute to symptoms of UTI. The exact location of bacteria within the urinary tract in these syndromes is, at present, unknown. The high prevalence of UTI, frequent repeated antibiotic therapy for rUTI, and the failure to use stringent diagnoses of UTI may drive rising antibiotic resistance [13, 14, 18]. Through a thorough examination of the molecular basis for rUTI and the identification of the major persistent reservoirs for UPEC within the urinary tract, new therapeutic strategies may be designed to eliminate UPEC from the urinary tract and thus, better guide appropriate antibiotic usage.

Current knowledge of the pathogenesis of UPEC UTI is incomplete, but accumulated molecular studies demonstrate tremendous complexity in the pathogenesis of the disease. In most primary UTIs, UPEC is thought to ascend the urethra from the perineum to colonize the bladder lumen. Additionally, UPEC can ascend the ureters and colonize the kidneys. Increased vesicoureteral reflux (VUR) enhances the likelihood of bacterial ascension into the kidneys and subsequent renal scarring in children and individuals with neurogenic bladder [19]. Through studies in a murine model of UTI, several novel intracellular pathways within the bladder important for UPEC pathogenesis have been elucidated. After experimental transurethral inoculation of UPEC into the urinary tract, UPEC invades the superficial facet cells of the bladder in a type 1-pilus dependent manner [11, 20, 21]. In order to evade expulsion from these cells via a TLR4-dependent mechanism [10], UPEC must escape into the cell cytoplasm, where they rapidly replicate and aggregate into cytosolic clusters of bacteria called intracellular bacterial communities (IBCs), a process that occurs independent of specific host genotypes [22,

23]. After full maturation of the IBCs, UPEC become filamentous and flux out of the superficial facet cell in response to a TLR4-dependent host signal [24]. The luminal bacteria may then invade other superficial facet cells, renewing the invasion and intracellular replication cascade [24, 25]. Thus, UPEC undergo at least two rounds of IBC formation over the first 24 hours post infection (hpi). Occupation of an intracellular niche by UPEC is not unique to the mouse model, as IBCs and filamentous bacteria have been frequently identified in urine from women with acute cystitis [26]. Replication within epithelial cells may protect UPEC from being cleared by neutrophils, antimicrobial peptides, micturition, and antibiotic administration [24, 27]. In the C3H/HeN mouse model of UTI, the IBC stage is most active during the first 24 hpi [25]. Thus, during UTI many niches within the urinary tract are colonized including the bladder lumen, within cells of the bladder epithelium, and the kidneys.

IBCs are not typically observed in C3H/HeN mice after 48 hpi. However, outcomes of the infection are dependent on the immune response to acute events [28]. The C3H/HeN mouse model of UTI recapitulates several of the outcomes present in humans. The outcome of bladder infection in these mice is bimodal with twenty to forty percent of infected C3H/HeN mice developing persistent bacteriuria and chronic cystitis [28]. Placebo-controlled trials have demonstrated persistent bacteriuria in women with or without resolution of symptoms in the absence of antibiotic treatment [15, 29]. High levels of interleukin (IL) -5, IL-6, keratinocyte cytokine (KC), and granulocyte colony-stimulating factor (G-CSF) in the serum of C3H/HeN mice at 24 hpi are nearly 100% predictive of ensuing chronic cystitis [28]. The remaining mice have low levels of these cytokines and resolve the acute infection, evidenced by sterile urine. However, even upon resolution of bacteriuria, UPEC may occupy niches in the underlying epithelium in quiescent intracellular reservoirs (QIRs) [30]. Thus, UTI pathogenesis has

complexity in both space (tissue niche) and time. During the acute stages of pathogenesis, IBC formation facilitates the expansion of bacterial numbers in the face of innate defenses. A multitude of consequences to the acute events exist ranging from resolution of infection with or without accompanying QIRs, or ensuing chronic cystitis or pyelonephritis. The relationship between IBC formation during acute infection and the subsequent outcome of infection is not known. The dynamics of acute infection and the subsequent outcomes may also be altered if the kidneys are infected concurrently with the bladder. Underlying conditions such as vesicoureteral reflux (VUR) increase the likelihood of co-infection of the kidneys and bladders. These complicated population dynamics may be modeled in specific mouse strains such as C3H/HEN. These mice are also susceptible to kidney infection due to a high rate of VUR [31-33].

With all of the transitions UPEC makes between anatomic and cellular spaces, it is likely that only a fraction of the total bacterial population transitions from one niche to another, in which case, population bottlenecks likely occur. Based on the complexity of UTI and the various niches UPEC inhabits during infection, we sought to determine transit between relevant niches and barriers within the urinary tract over time and whether the penetration of infection barriers resulted in an expansion of those descendents. A prior study indicated that intracellular UPEC make up a significant proportion of bacteria in the bladder during the first 48 hours post infection (hpi), as determined by *ex vivo* gentamicin protection assays [11]. Early in infection, micturition and neutrophil influx may disproportionately reduce or eliminate luminal bacteria, while intracellular UPEC are largely protected from these clearance mechanisms.

We addressed the role of bottlenecks in UPEC population dynamics during UTI using a panel of 40 unique genetically-tagged isogenic UPEC that were tracked using a multiplex PCR assay. We modeled the dynamics of population flux using a calculated multinomial distribution

and compared theoretical values to a complicated model of infection using the C3H/HEN mouse, which is susceptible to VUR, thus resulting in UPEC being distributed during acute infection in the bladder and kidneys. In the bladder, we demonstrated that each IBC arises from replication of a single invasive bacterium and that the number of IBCs present at 6 hpi strongly correlates with the number of unique tags present. In mice that would likely resolve bacteriuria and/or develop QIRs, the fraction of uniquely tagged UPEC decreased to 10-20% of the tags present in the initial inoculum during the first 24 hpi in whole bladder specimens. In contrast, in mice with persistent bacteriuria, a hallmark of chronic cystitis, nearly 60% of unique tags remained. Thus, a high number of IBCs formed at 6 hpi and/or an inability to clear bacteria from the bladder lumen precedes chronic cystitis. Utilizing *ex vivo* gentamicin protection assays to separate intracellular and extracellular bladder populations, we found that during acute UTI the majority of remaining clones occupied all niches within the urinary tract while later in infection disparate clonal populations existed independently in the bladders or kidneys.

Results

IBCs arise from a single invasive bacterium.

UPEC invade the superficial facet cells of the bladder 15 minutes – 1 hour post inoculation into the urinary tract as determined by *ex vivo* gentamicin protection assays [34]. Over the next 8-12 hours, IBC formation ensues for bacteria that have successfully invaded and escaped into the cytosol of the superficial facet cells [25]. To address whether IBCs contain a clonal population expanded from a single bacterium or an aggregate of multiple distinct founders, we performed co-infections of mice with 1:1 and 50:1 ratios of unlabeled and GFP-marked isogenic UPEC [35]. Six hours post infection, mice were euthanized, and the bladders were splayed, fixed, and stained with TOPRO-3 and examined by confocal microscopy. As a

result the unlabeled UPEC appeared red and the GFP-marked UPEC appeared yellow-green. Of the approximately 500 IBCs examined, each was exclusively red or yellow-green, regardless of the inoculation ratio (Figure 1). When superficial facet cells contained more than one IBC derived from a different inoculating strain, a clear demarcation existed such that no bacterial mixing occurred (Figure 1 C-D), indicating that IBCs are clonal formations arising from a single invasive bacterium.

Formation of IBCs strongly correlates to tag diversity.

Given that each IBC is derived from a single bacterial founder and IBC formation is rare, with only 0.01-0.001% of the initial inoculum successfully undergoing IBC formation, we anticipated that various bottlenecks restricting invasion and subsequent IBC formation may result in a significant founder effect in later stages of infection. At 6 hpi following an initial inoculation of 1-5 X 10^7 CFU UPEC, each mouse bladder contained between 3 and 700 IBCs (Figure 2; median 49.5; geometric mean 40), consistent with previously reported data for C3H/HeN mice [36-38]. Combining prior knowledge that the bacterial population is primarily intracellular at 12 hpi [11, 39] with the finding that IBCs are clonal, we hypothesized that the invasion and IBC formation cycle acts as a strong population bottleneck that restricts overall bacterial diversity and may subsequently contribute a significant proportion of bacteria populating later stages of infection. In order to test this hypothesis, we designed 40 isogenic strains of UTI89 each with a unique 100-300 bp genetic sequence inserted into the λ phage region of the genome (See Materials and Methods). Bacteria containing these unique tags were identified using 8 multiplex PCR reactions. We reasoned that the small number of clonal IBCs

relative to the total inoculum, which was at primarily intracellular by 12 hpi [11], may contribute to a founder effect in the number of unique genotypes arising from this early bottleneck event.

We infected mice with a pool of the tagged strains in equal proportions. At 6 hpi, the mice were sacrificed and the infected bladders were splayed and stained with X-gal to detect bacterial beta-galactosidase and thus localize UPEC in mature IBC formations, which stained punctate purple. Following enumeration of IBCs, the bladders were homogenized, and genomic DNA was extracted from the bacteria present in the bladder. We found a broad range of unique bacterial signatures corresponding to the range of IBCs formed per bladder at 6 hpi (Figure 2, black squares). Based on the hypothesis that the bacteria contained in IBCs account for the majority of bacteria in the bladder at 6 hpi, we calculated the fraction of tags that most likely would be remaining relative to the number of IBCs present using a multinomial distribution (Figure 2, line). Superimposition of the theoretical multinomial distribution onto the experimental data revealed a close fit (see Discussion and Supplemental Material). The five data points marked with asterisks represent bladders that contained more tags than IBCs. The increased fraction of tags in these bladders likely represented invasion events of bacteria that had not yet replicated into a mature IBC and thus not yet discerned in the X-Gal-stained bladders but were detected by the more sensitive multiplex PCR assay. For mice whose bladders contained >100 IBCs at 6 hr, all 40 tags were present. These data suggest that early in infection, the clonal IBC populations, arising from a small number of bacterial founders, may contribute the majority of bacterial diversity to later stages of infection.

Bacterial diversity decreased dramatically in the bladders and kidneys over time.

During the most acute stages of isolated cystitis, the formation of IBCs each from a single invasive bacterium, coincident with luminal clearance of bacteria, would be anticipated to constitute a stringent bottleneck, limiting the genetic diversity of organisms progressing to later stages of infection. We first determined the overall dynamics of infection using our set of 40 uniquely tagged UPEC as a proxy for bacterial diversity to understand the occupation of the urinary tract over time. We infected C3H/HeN mice with 1-5 X 10^7 total CFU (2.5 X 10^5 – 1.25 X 10^6 CFU/unique strain) and tracked them over the course of 4 wks. At the designated times after infection, the bladders and kidneys were homogenized, and 6% of the sample was plated to enumerate CFU (Figure 3 A-B). Through this approach, rare abundance tags are likely amplified to above the limit of detection in the multiplex PCR assay. A tag comprising <20 CFU in the sample could potentially be lost with this method, but such rare abundance tags are not likely to persist within the urinary tract for an extended period of time.

Total bacterial diversity in the bladder decreased significantly over the first 24 hpi ($p = 0.003$, 1 hpi vs. 24 hpi) to a median plateau of 25-40% of initial tags remaining (Figure 3C) while CFUs increased over this same time period to 10^5 CFU (Figure 3A). The simultaneous reduction in tag diversity with an increase in CFUs occurred during the time in which IBCs formed, suggesting a relationship between the IBC bottleneck and a founder effect in populating the bladder. Bacterial diversity in the kidneys also decreased over this same timespan ($p = 0.003$, 1 hpi vs. 24 hpi) with approximately the same fraction of tags present in the bladders and kidneys by 1 week post infection (Figure 3D).

IBC formation correlates with outcome of infection.

In C3H/HeN mice, the fate of disease is determined within the first 24 hours of infection [28]. The two disease outcomes of resolution of bacteriuria concomitant with the establishment of QIRs and the development of persistent bacteriuria indicative of chronic cystitis, result in a bimodal distribution of bacterial CFU within the bladder that occurs after 1 week [40]. Thus, we investigated the relationship between the number of IBCs formed early in infection and the impact on the fraction of tags remaining later in infection and disease outcome [28, 30].

We infected 20 mice with equal proportions of the 40 unique, tagged UTI89 and determined the bacterial CFU and the number of unique tags that remained by 2 weeks post infection (wpi). Mice were stratified based on their urine titers over time and their 2 wpi bladder titer. Chronic cystitis was categorized by persistent bacteriuria of $>10^4$ CFU/mL at 1,3, 7,10, and 14 days post infection (dpi) and a 2 wpi bladder titer of $>10^4$ CFU [28]. The remaining mice were classified based on bladder titers $<10^4$ CFU and at least one urine collection over the 2 week infection that contained $<10^4$ CFU/mL UPEC (Figure 4 A, B). Mice that developed persistent bacteriuria and chronic cystitis had significantly more unique UPEC signatures present in the bladder by 2 wpi than mice that resolved infection (Figure 4C). Based on the founder effect theory, this would argue that development of chronic cystitis is correlated with increased IBC formation during the acute stage of infection, which is consistent with the strong correlation between IBC formation and the fraction of tags present throughout the bladder during the IBC cycle (Figure 2). Conversely, decreased IBC formation is more likely correlated with resolution of bacteriuria with reservoir bladder titers $<10^4$ CFU.

Population dynamics in acute vs. chronic infection.

UTI is a dynamic infection sometimes entailing concomitant kidney infection. Populations of UPEC within the bladder and kidneys of C3H/HeN mice may thus be shaped by different independent environmental forces, including unique bottlenecks. These physically separated populations may subsequently intermix to generate some intermediate, combined population. Abundant influx of neutrophils and immune mediators into the bladder occurs very early during UTI in both mice and humans [31, 41, 42]. Invasion of the bladder epithelium may represent a mechanism for UPEC to increase its population during UTI while remaining separated from much of the innate immune response. Gentamicin protection assays on *ex vivo* infected bladders support this concept, demonstrating that by 12 hpi in murine UTI, the majority of UPEC are intracellular [11]. On the basis of these prior studies, we sought to determine the location of UPEC within urinary tract niches throughout infection. In order to obtain a more complete understanding of how bacterial subpopulations are distributed within the urinary tract at a given time, sampling of different niches within the urinary tract was performed using mice infected with equal numbers of each of the 40 genetically tagged UTI89 strains at different times post infection. We obtained clean-catch urine from mice by gentle suprapubic pressure and plated it to enumerate CFU (Figure 5A). Afterwards, the mice were sacrificed, and the bladder was bisected twice and washed with sterile PBS to liberate loosely bound and planktonic UPEC, heretofore referred as "luminal" or "extracellular" fraction (Figure 5B). The intact, quadrisected bladder was incubated with the antibiotic gentamicin, which does not penetrate the bladder epithelium, thus selectively eliminating extracellular organisms. After the gentamicin was washed away, the bladder tissue was homogenized to liberate the intracellular bacteria, designated as the "gentamicin-protected bladder" population (Figure 5C). During the first 24 hpi,

this fraction represented bacteria that invaded superficial facet cells and were, in some cases, within IBCs. After 48 hpi when IBC formation no longer occurs, the gentamicin-protected fraction consisted of bacteria within QIRs or another niche protected from gentamicin. Kidneys were also homogenized and plated to enumerate CFU (Figure 5D).

All of the 40 uniquely tagged UPEC strains were present in both the extracellular and intracellular populations at 1 hpi (Figure 5F-G). This amount of tag diversity was not statistically different than that observed in the whole bladder at 1 hpi (Figure 3C; $p > 0.05$, Wilcoxon signed rank, hypothetical median = 1.0). At 6 hpi, the proportion of genetic tags approached 1 in the kidneys, likely due to VUR of the inoculum in the C3H/HEN mice at the initiation of the infection. However, the proportion of genetic tags was decreased in the urine and all compartments of the bladder (Figure 5F-G) during the time when host innate defenses have been shown to be induced and engaged [42]. The data re-confirmed that only 0.1-1% of invasive events lead to the formation of an IBC, perhaps as a result of TLR4 mediated bacterial expulsion [10]. Furthermore, the wide range of unique tags present in the intracellular compartment at 6 hpi (Figure 5G) corresponded with the natural variation in IBC number between mice, correlating with our earlier results (Figure 2).

Between 6 and 24 hpi, the bacterial tags detected in the kidneys decreased (Figure 5H). However, the tags detected in the bladder lumen and intracellular bladder fractions increased (Figure 5F-G), suggesting that the overall bacterial diversity of the bladder was increasing due to shedding of bacteria carrying independent, unique tags from the kidney. In order to understand the overlap of bacteria in different niches, the identities of each tag were determined in each niche at each time point. We then determined on a per-mouse basis the fraction of unique and shared tags present within and between the following niches: urine, bladder lumen, within the

bladder epithelium, and kidneys. For each time point, we determined the fraction and identity of each tagged strain present in each of the 15 possible overlapping and distinct niche combinations out of the total number of tags present within the urinary tract (Figure 5I-M). At 6 hpi, the kidneys and urine contained 34% of bacterial diversity not present in a bladder niche, likely reflecting the population that gained access to the kidney earlier in the infection as a result of VUR. At 24 hpi, 38% of the total tagged strains still present within the urinary tract were shared between all of the compartments (Figure 5J). These data show that at 24 hpi the majority of individual clones of bacteria are populating every niche within the urinary tract likely as a result of emergence from IBCs earlier in infection and descent from the kidneys to seed other niches of the urinary tract.

By 48hpi, the CFU in the bladder stratified into a bimodal distribution reflecting the development of persistent bacteriuria and bladder titer of $>10^4$ CFU or resolution of bacteriuria and establishment of QIR with UPEC titer of $<10^4$ CFU [28, 30]. The bimodal distribution was evident in the bladder lumen and gentamicin-protected fractions at 1 wpi (Figure 5C). At 2 wpi in this experimental set, bacteria were no longer present in the lumen and urine in 4/5 of the mice, and instead exclusively occupied gentamicin-protected reservoirs within the bladder epithelium or kidney (Figure 5L). Among the mice 1 wpi with high bladder bacterial counts indicative of chronic cystitis, 21% of the tags were shared in all niches. In contrast, 15% of the tags remaining in mice that resolved bacteriuria and formed QIRs, were located exclusively in a gentamicin-protected niche, which increased to 28% of all urinary tract diversity by 4 wpi (Figure 5K,M). The majority of tags at 4 weeks post infection (60%) were present only in the urine without a bladder or kidney niche colonized, potentially suggesting that these bacteria colonized the ureter, urethra or peri-urethral area, explaining why they were not found in another

niche (Figure 5M). The lumen in these mice was not colonized with bacteria, while the gentamicin-protected fraction had 10^3 CFU, suggesting that these mice were resolved with QIR formation. Thus at different times during the infection, the urine fraction contains bacteria shed from the kidneys, planktonic bacteria from the bladder lumen, bacteria strongly adhered to or within exfoliated epithelial cells, or bacteria otherwise lost from bladder and kidney niches (Figure 5I-M).

Discussion

We created 40 isogenic uniquely tagged UPEC strains and assessed the presence of these bacteria in niches within the urinary tract over time with a multiplex PCR assay. This approach allowed us to provide an accurate accounting of the strains distributed throughout different compartments in the urinary tract over the course of UTI. By recovering bacteria from the samples on agar overnight to amplify even rare genetic tags, our approach resulted in a highly sensitive assay that provided a conservative estimate of the strains present in each compartment and their distribution throughout the urinary tract at the time of sampling. Using this molecular approach to better understand the obstacles in UPEC pathogenesis, we have revealed a series of population bottlenecks and interactions between distinct populations of the bladder and kidneys that impact on the dynamics of the UPEC population during infection as a whole.

When C3H/HeN mice are inoculated with 10^7 CFU UTI89, 10^3-10^4 CFU invade the bladder epithelium at 1 hpi, and all of bacterial tags can be accounted for in the bladder intracellular population (Figure 5G). Subsequently, 3-700 IBCs are formed per bladder by 6 hpi, and as demonstrated herein, each IBC is derived from a single bacterium that enters into the cytoplasm of a superficial facet cell (Figure 1, 2). Together, 0.1% of the inoculum invades the epithelium, and <2% of the invasive bacteria successfully form IBCs. The most stringent population bottleneck observed is between 1 and 24 hpi corresponding to the time frame when a limited number of invasive bacteria have entered into the IBC cycle while innate immunity is engaged to clear luminal organisms. These dynamics produce a founder effect. The variance of the fraction of tags present within the bladder epithelium at 6 hpi and in the whole bladder at 24 hpi (Figures 2, 3C, 5G) reflects the large bladder to bladder variance in IBC number and begins to reflect the bimodal distribution in infection outcome. The cause of the variance in IBC number

is unknown, but presumably reflects factors necessary to penetrate the various bottlenecks described here.

Our experimental data demonstrating the clonality of IBCs suggest that IBC formation with a concurrent reduction of the luminal UPEC population places a significant constraint on the number of organisms progressing through to later stages of infection. Indeed, modeling clonal IBC formation as the only pathway by which organisms can persist in the urinary tract fits the observed data very well and supports this hypothesis (Supplemental Figure 1). Given that IBCs are clonal, a single IBC can only contribute to the detection of a single tag. So long as a tag is detected in our PCR assay, we cannot distinguish whether it was present in one or many IBCs. Therefore, assuming that tags are equivalent and detection by PCR is efficient, this is akin to an experiment where for every IBC, a die with 40 different numbers (each corresponding to a tag) is cast, and we count how many different numbers were thrown as a result of all casts of the die. With these assumptions, the probability distribution of the number of tags detected (different numbers of the die) given a number of IBCs formed (number of casts of the die) can be calculated exactly using a multinomial distribution (Supplemental Figure 1). If there are other pathways to persistence that do not involve IBC formation (such as persistence exclusively in the lumen of the bladder), the number of tags detected can be modeled as additional throws of the die, 1 per each additional extracellular clone (Supplemental Figure 1A). Given the close fit between the multinomial expectation (based solely on IBC number) and the observed number of tags (Figure 2), if there is another pathway to persistence, its contributions in terms of diversity are very small and effectively not measurable in our data. Small exclusively luminal populations (not necessarily clonally derived from a single founder) would be predicted to drastically increase the expected fraction of tags persisting when low numbers of IBCs are present, though

this is not experimentally observed (see Fig. S1A in the supplemental material and Fig. 2). Although it is formally possible that clonal, extracellular populations contribute to bacterial diversity within the bladder at 6 hpi, the most likely interpretation of our data is that by 24 hpi, the extracellular population is largely a subset of bacteria arising from bacterial subpopulations that have emerged from other niches with IBCs being a major source (Figure 5I, J).

The outcomes of acute infection are: 1) resolution of bacteriuria which can be accompanied by formation of a QIR, which can lead to recurrent infection or 2) chronic active cystitis marked by persistent bacteriuria for >7 months, which presumably reflects extracellular replication of bacteria [28, 30]. Our data described above indicate that the strongest population bottlenecks exist within the first 24 hpi. Prior studies of UPEC mutants with defects in IBC development suggest that the first generation IBCs are important for the establishment of acute cystitis [24, 34].

The studies presented herein have several key implications. First, in species such as humans, where isolated cystitis is common, the bottlenecks due to invasion and the IBC cycle may be even more stringent than observed in our studies in C3H/HeN mice because there may be no mixing of bacteria being shed from the kidneys into the bladder. Inhibitors at the point of the major bottlenecks within the first 24 hours of infection may significantly attenuate infection, both acute and chronic. For instance, inhibition of the IBC pathway, either by blocking IBC formation, development, or dispersal, may alter outcomes by reducing the QIR and limiting chronic active cystitis. Combined with a FimH vaccine, invasion inhibitors may be particularly potent in preventing and treating UTI [43]. A recent study showed that in a urological cohort of women, UPEC isolated from urine from 6/8 patients were not actively expressing type 1 pili [44]. Our results from figure 5M showing that 60% of strains detected were present only in the

urine argue that these UPEC may indeed reflect strains lost from the bladder as they were not detectable in any other urinary tract niche. Thus, although it is possible that these strains are not expressing type 1 pili as suggested by the results of Hagan *et al* 2010 [44], their existence presumably depended on earlier colonization events involving type 1 pili and other virulence factors. Finally, the development and maintenance of genomic changes by UPEC during UTI may be contingent on the timing of acquisition of mutations, rearrangements, or deletions in the genome due to the bottleneck constraints present in the system. For instance, a mutant subpopulation obtaining antimicrobial resistance to trimethoprim through a mutation in dihydrofolate reductase (DHFR) may be extinguished by a stringent stochastic bottleneck even under circumstances when the antibiotic is being administered and an antibiotic resistance mutation provides a selective advantage. This would be expected to occur if the proportion of the population with that advantage is sufficiently too small to overcome the inherent stochastic barriers that eliminate the vast majority of the population and produce a founder effect, likely in this case to arise from majority members that do not have the advantageous mutation. Alternatively, hosts in which bacteria more effectively penetrate the early bottlenecks such as those developing chronic cystitis where diversity is maintained, may provide productive environments for the selection and perpetuation of adaptive mutations. Thus, diversity and selective evolution may be constrained by these stochastic mechanisms during cystitis and/or dramatically favor mutations that facilitate invasion and IBC formation. Indeed, several genes important for invasion, IBC formation and living within host cells were shown to be under positive selection in UPEC [37, 45].

The findings herein present the hypothesis that an increased number of IBCs activates a host immune response that predisposes to persistent bacteriuria and chronic cystitis. In C3H/HeN

mice, increasing the inoculum to 10^8 CFU/mL increases the average number of IBCs formed and increases the percentage of mice experiencing persistent bacteriuria [28]. Currently, it is not possible to evaluate these metrics of infection within the same mouse. Longitudinal monitoring of bacterial populations *in vivo* using techniques in development such as intravital multi-photon microscopy to enumerate acute IBC formation at 6 hpi and determine bacteriuria and tissue titers at 2 wpi will provide further resolution to the consequences of early infection events on subacute and chronic outcomes. Our findings are especially relevant for mucosal pathogens that have to transit through potential population bottlenecks similar to those described in this work [5, 6, 46]. Finally, the methodology described herein would be ideal to analyze population bottlenecks, niche occupation, and clonality by other bacterial pathogens of mucosal sites.

Materials and Methods

Construction of 40 isogenic tagged UPEC strains.

Unique tags were inserted into the chromosome following the *ybhC* gene in the region of the lambda phage attachment site into the clinical isolate UTI89 [39]. This site was chosen since prior insertions in this region (*gfp* reporter fusions) did not adversely affect pathogenesis [35]. The tags were designed using unique sequences as reported by Lehoux *et al* [47, 48]. Primer sequences are shown in Supplemental Table 1. For the construction of the genomic insertion cassettes, the chloramphenicol or kanamycin cassette from pKD3 or pKD4 [49] was amplified using primers BP-1 in combinations with BP-2A, BP-2B, BP-2C. The respective products were digested with EcoRI and column purified (Qiagen). Each of the BP-00F primers was phosphorylated in a reaction that included 1 x T4 ligase buffer (NEB), 10 mM dATP (Promega) and 1 U of T4 polynucleotide kinase (Invitrogen) for 30 min at 37^{o}C. The appropriate BP 00F

and BP 00R primers were annealed by mixing an equal molar ratio in dH_2O, heating to 95°C for 5 min, and cooling slowly to RT. In a similar method, primers BP-5 and BP-6 were annealed to each other. A tripartite ligation was performed using the BP-1/BP-2 digested PCR products with the annealed products of BP-00F/R and BP-5/BP-6 in 1 x T4 ligase buffer and 10 U of T4 ligase for 1 hr at RT. To a PCR reaction containing 1 x Pfx buffer (Invitrogen), 2 mM $MgSO_4$, 0.2 mM dNTP, 2.5 U of Pfx polymerase (Invitrogen), and 200 pmol each of primers BP-1 and BP-6, 0.5 μl of each ligation was added. The reactions were cycled at 94 °C for 3 min followed by 30 cycles of 94 °C for 15 sec, 55 °C for 45 sec, and 68 °C for 1.5 min. A final extension at 68 °C for 7 min was performed. The ~1.6 kb products were verified by gel electrophoresis and column purified (Qiagen). Chromosomal insertion of the tag constructs was performed using the Red Recombinase method [49]. The tags were verified by colony PCR in a reaction mixture containing 1 x PCR buffer (Invitrogen) with 0.2 mM dNTP, 2.5 mM $MgCl_2$, 2.5 U of Taq polymerase (Invitrogen) and 200 pmol each of the appropriate primers pairs BP-8K (kanamycin template) or BP-8C (chloramphenicol template) and BP-00F. Growth curves of each individual strain were performed to ensure no gross growth defects in broth culture (data not shown).

Evaluation of isogenic UPEC strains in a murine cystitis model.

In preparation for inoculation into mice, each of the 40 isogenic UTI89 derivatives was grown individually in LB statically at 37°C overnight and then sub-cultured 1:1000 into 2 ml of fresh LB with static growth at 37°C for 18-24 hr. Cell density was measured using an OD_{600} value, and each clone was added in approximately equal cell numbers to a central pool. The pooled bacteria were centrifuged for 10 min at 6,500 x g at 4°C. The pellet was resuspended in 20-25 ml of sterile PBS to yield a final cell suspension where 50 μl contained ~1-2 x 10^7 CFU. Fifty μL was introduced over 10 seconds into 7-8 week old C3H/HeN mice under 2.5%

isofluorane inhalation via a transurethral route. The infections were allowed to proceed for 1 hr up to 4 wks at which time the mice were sacrificed under anesthesia, and the bladders and kidneys were removed and homogenized in 1 mL or 0.800 mL sterile PBS, respectively. Homogenates were serially diluted and plated to enumerate the bacteria in the tissues. Genomic DNA was obtained from the bacterial lawns by the addition of two milliliters of sterile water in each Petri dish and scraped using a bacterial cell scraper (BD Falcon). The bacteria were pelleted by centrifugation and washed with PBS. The final pellet was processed using the Promega Genomic DNA Isolation Kit per the manufacturer's instructions. Genomic DNA was also prepared from a bacterial pellet of the pooled input inoculum.

BAR-PATH multiplex PCR was performed using 50 ng of genomic DNA in a PCR reaction mix including 1 x Taq buffer (Invitrogen), 2.5 mM MgCl2, 0.2 mM dNTP, 100 pmol each of BP-8C and BP-8K, and 2.5 U Taq DNA polymerase (Invitrogen). The BP 00F primers (see Table S1 in the supplemental material) were mixed in sets of 3 primers for inclusion in PCR reactions: [BP 01F, BP 02F, BP 03F], [BP 04F, BP 05F, BP 06F], etc. and added to the reaction mixture at final concentrations of ~66.6 pmol/primer. Reactions were cycled with a hot start then 94 C x 3 min followed by touchdown PCR with 10 cycles of 94 ^{o}C for 30 sec, 62 ^{o}C for 30 sec with a 1 degree decrease per cycle, and 72^{o}C for 30 sec. Next, 30 cycles were performed at 94^{o}C for 15 sec, 55^{o}C for 15 sec, 72^{o}C for 30 sec. A final 7 min extension at 72^{o}C was performed. Ten microliters of the 25 μl reaction was run a 2.5 % TBE-agarose gel. Presence or absence of an individual strain present in the multiplex PCR was determined by eye in comparison to the intensity of its cognate band in the inoculum. If a strain was not detected in the inoculum pool, it was not included in the analysis for that experiment. Thirty-seven of the forty tags were routinely detected in the inoculum. Three tags were statistically underrepresented in the PCR of the

inoculum pool and were thus excluded in the subsequent analyses of the endpoint samples. Ambiguous bands were analyzed in an independent multiplex PCR replicate. All mouse infection studies were approved by the Animal Studies Committee of Washington University in St. Louis.

IBC enumeration.

To quantify the number of IBCs per bladder, bladders were bisected and splayed onto sterile silica plates and fixed with 3% paraformaldehyde (Sigma). The bladders were washed 3X with 2mM $MgCl_2$ (Sigma), 0.01% Na deoxycholate (Sigma), 0.02% Nonidet-P40 (Roche) in sterile PBS, pH 7.4. Bladders were then stained in 0.4 mL 25 mg/mL X-gal (Sigma) and a solution containing 1 mM potassium ferrocyanide and 1mM potassium ferricyanide (Sigma). After incubating at 30^{o}C for 16 hours, bladders were visualized under a dissecting microscope where IBCs appeared as bright blue punctate circles.

Confocal scanning laser microscopy.

Female 6-7 week old C3H/HeN mice were infected with a 1:1 or 1:50 mixture of UTI89 and UTI89::HKGFP and sacrificed at 6 or 12 hpi [35]. Bladders were extracted, splayed, and counterstained with Topro-3 and imaged with a Zeiss LSM410 confocal scanning laser microscope.

Ex vivo gentamicin protection assay.

At the indicated times post-infection, bladders were removed aseptically and bisected twice. The bladders were washed three times with 500 μL sterile PBS. The washes were pooled, spun at 500 rpm for 5 min and dilution plated as described earlier. This wash was termed "luminal" or "extracellular." The bladders were then incubated for 90 min. with 100 μg/mL gentamicin to kill adherent extracellular bacteria. After this incubation, the bladders were washed

twice with 1 mL sterile PBS. The bladder was then homogenized in 1 mL sterile PBS and dilution plated to enumerate intracellular CFU. Bacterial pellets were obtained and processed as described earlier.

Acknowledgements

The authors would like to thank Dr. Karen Dodson for critical review of this manuscript. This work was supported fully or in part by the National Institutes of Health R01 DK51406(SJH), P50 DK64540 (SJH and PCS) and DK07444301 (PCS).

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Figures

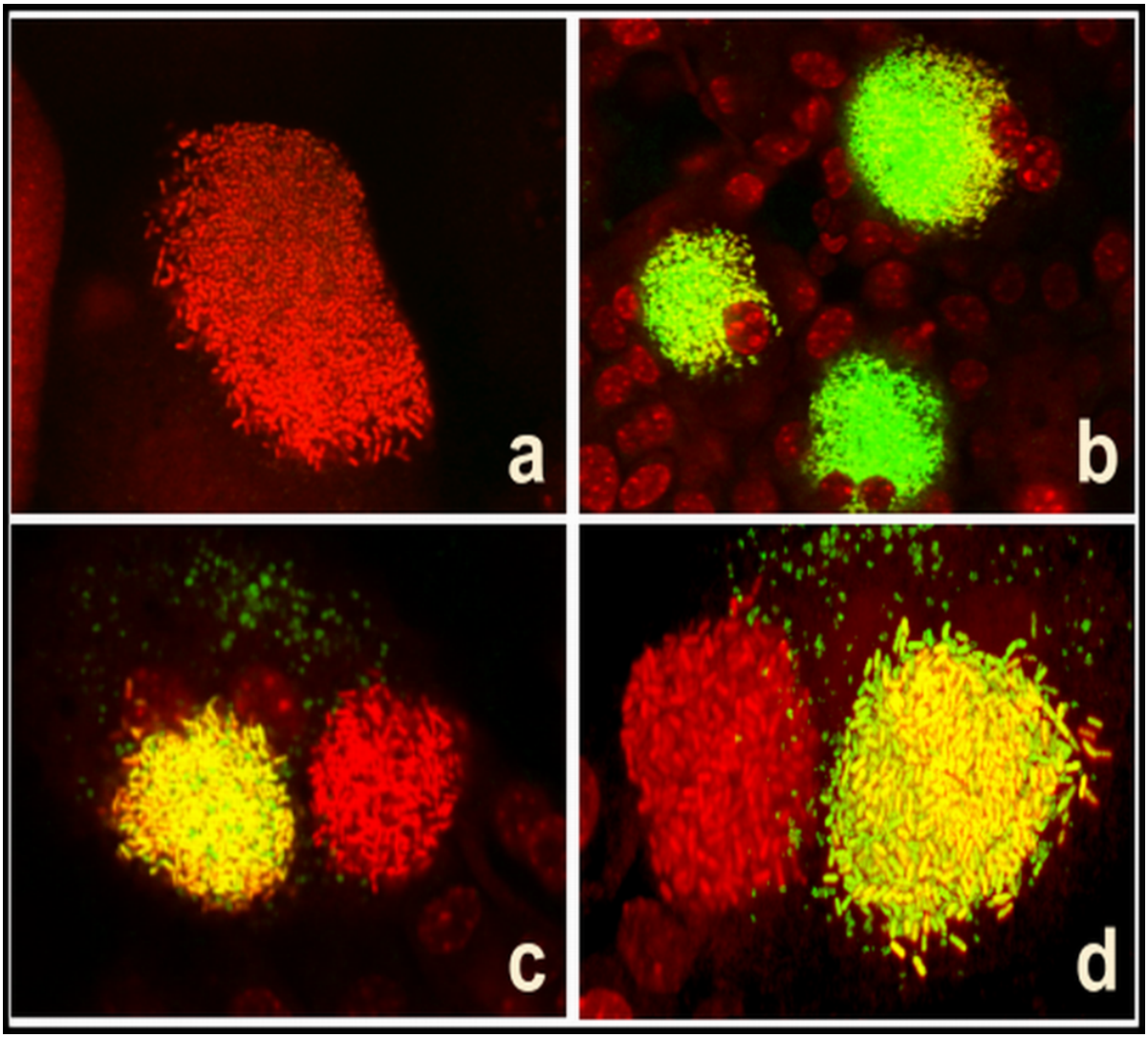

Figure 1. IBCs are clonal, derived from single invasive bacteria.

C3H/HeN mice were co-infected with UTI89 and UTI89::HKGFP and sacrificed at 6 hpi and 12 hpi. Bladders were aseptically removed and splayed and imaged with confocal microscopy. (A-B): IBCs in whole mount at 6 hpi, counterstained with TOPRO-3. Each image shows the merged red and green channel data. (C-D): Co-resident IBCs inside a single superficial facet cell at 12 hpi. Representative images of over 500 independent IBC are shown.

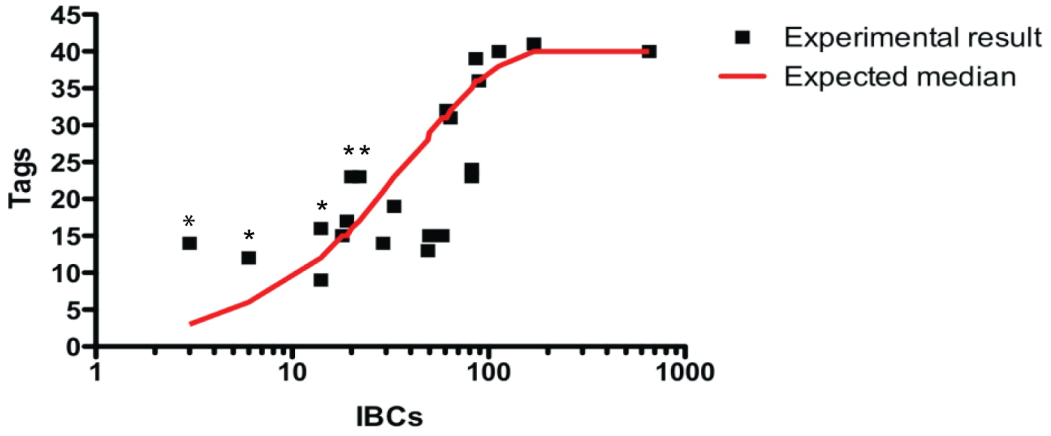

Figure 2. IBC number correlates with number of tags present.

C3H/HeN mice were infected as described and processed to enumerate IBCs and extract bacterial genomic DNA (see methods). Black squares represent the number of IBCs and the number of tagged strains present per mouse bladder. The line is the median number of tags expected based on a multinomial distribution of IBC number (see Discussion and Supplemental Material). Data represent 3 independent experiments with 5-10 mice per experiment. * represent bladders with a greater number of tags than IBCs.

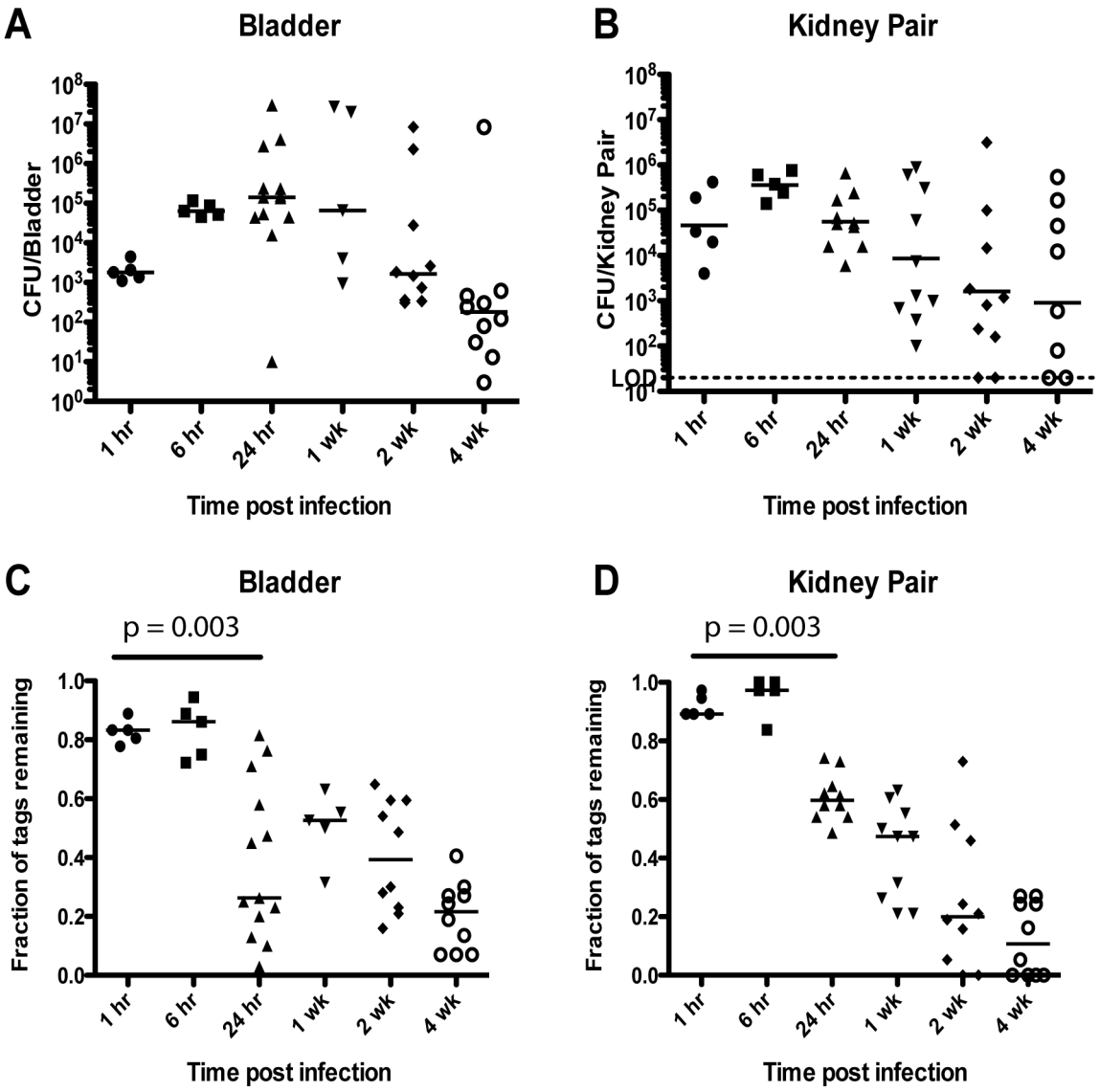

Figure 3. Bacterial diversity decreased dramatically during first 24 hpi.

At the indicated times post infection, C3H/HeN mice were sacrificed, and the bladder and kidneys were removed aseptically, homogenized in PBS, and 6% of the homogenate was plated to enumerate CFU in the bladders (A) and kidney pair (B). Genomic DNA was isolated from a lawn of UPEC isolated from the tissue homogenates, and multiplex PCR was conducted to determine the fraction of the 40 unique tags present in each bladder (C) and kidney pair (D).

CFU data are presented as CFU/whole organ. Bars are median values. N = 1-2 experiments with 5-8 mice each. P values were calculated using a two-tailed Mann Whitney non-parametric comparison. An Initial experiment conducted with 43 tags is also included in this analysis.

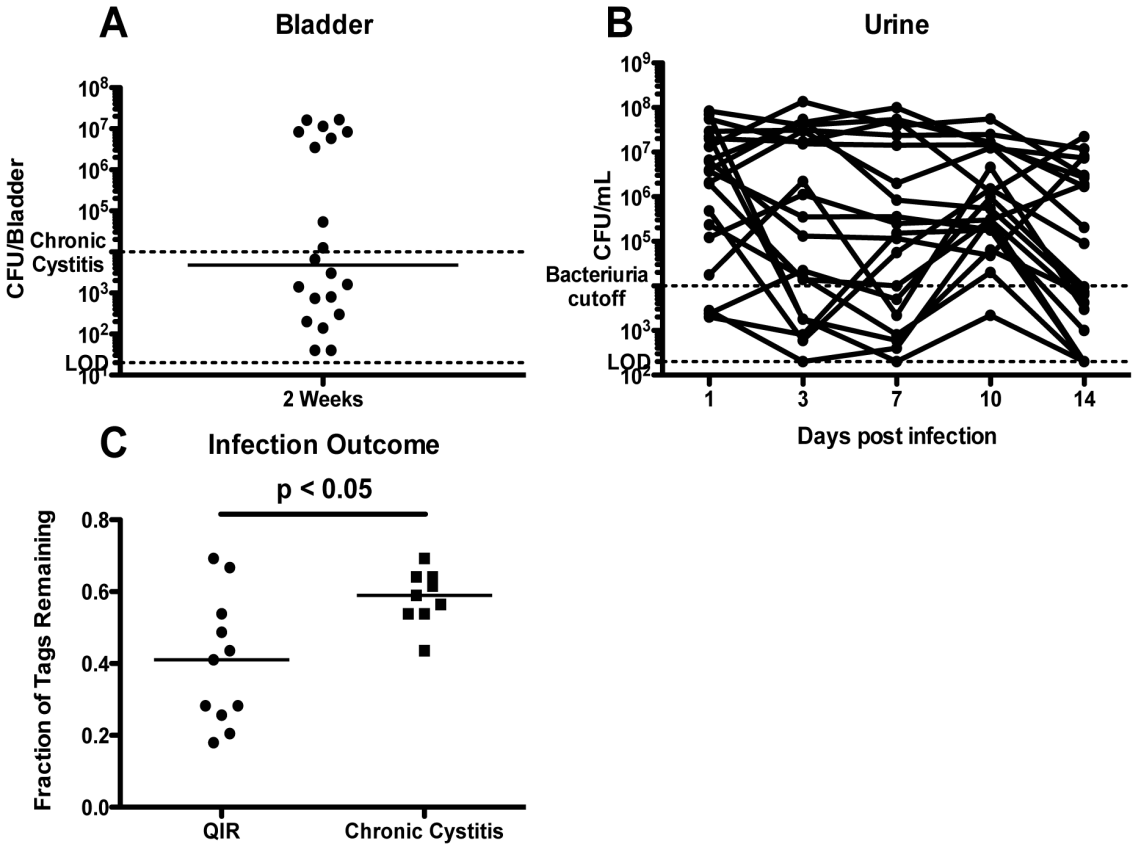

Figure 4. Higher fraction of unique bacterial signatures remain in mice experiencing chronic cystitis.

A) 2 weeks post infection, C3H/HeN mice were sacrificed, and the bladders were removed aseptically, homogenized, and plated to enumerate CFU. B) Urines were obtained by gentle suprapubic pressure and plated to enumerate CFU. The dashed line at 10^4 CFU represents the limit for assessing the presence of UTI in clean catch urine samples (40). Urine titers above this point are considered bacteriuria indicative of a UTI, while bladder titers greater than this cutoff indicate chronic cystitis. C) Genomic DNA was then obtained from the bacteria from each bladder sample and multiplex PCR was conducted. Bars are median values. P values were calculated using a two-tailed Mann Whitney non-parametric comparison.

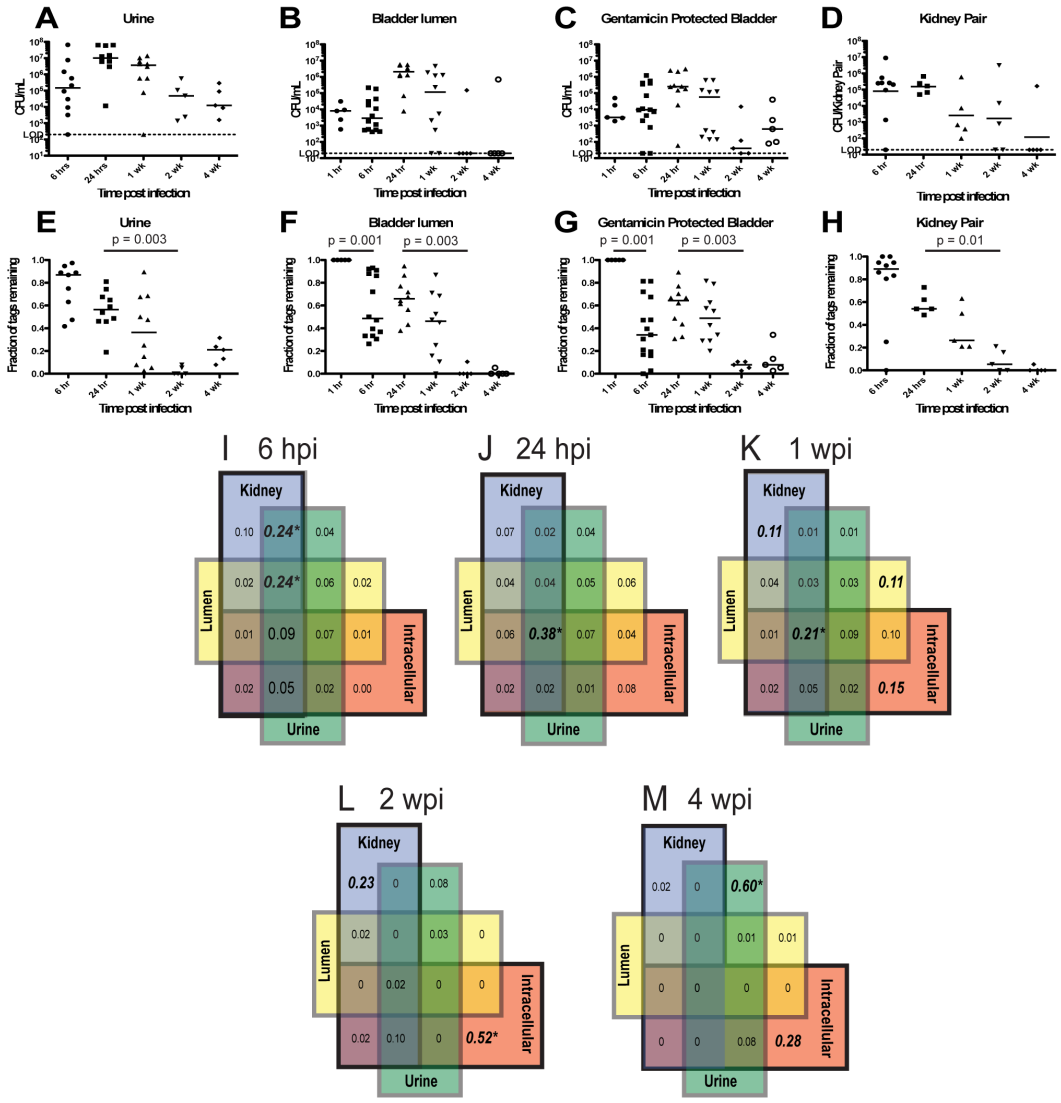

Figure 5. Niche specific diversity over time demonstrates population dynamics throughout UTI.

At the indicated times post infection, urine was obtained via gentle suprapubic pressure and plated to enumerate CFU (A) and fraction of tags remaining (E). A gentamicin protection assay was performed to enumerate luminal CFU (B) and fraction of tags remaining (F) and gentamicin protected CFU (C) and tags remaining (G). Kidneys were also plated to enumerate CFU (D) and

tags remaining (H). The niche occupation of specific tags was determined and is represented as average fraction of tags present in distinct and overlapping niches (15 total permutations) and displayed as a 4-set Venn diagram at 6 hpi (I), 24 hpi (J), 1 wpi (K), 2 wpi (L), and 4 wpi (M). Each percentage listed displays the fraction of tags present in that unique or shared niche out of the total number of tags in each murine urinary tract. Data for (A-H) represent 1-3 experiments with 5 mice per timepoint. Data for (I-M) represents experiments where kidney information was available: 2 experiments with 4-5 mice for panel I and 1 experiment with 5 mice each for panels J to M. * signifies the niche combination with the greatest unique diversity present. Bars are median values. P values were calculated using a two-tailed Mann Whitney non-parametric comparison.

Supplemental Information

2 Table 1S: Primers Used for Polymerase Chain Reaction (PCR).

Primer Name	Sequence (5'→3')	Target
kps Reg1 KO#1	GTTACAACCCATTGATTTAGCATAAATAAATTATAGTGGGTTCGGGTTTGT TGTGTAGGCTGGAGCTGCTTC	*kps* Region I, 5' / pKD4
kps Reg1 KO#2	TGGAAATGATTTTTTGGCTACTTAAAATTCAAAAGATATTGACTTGAAATAT GGGAATTAGCCATGGTCC	*kps* Region I, 3' / pKD4
kps Reg1 #1	ATGTTCCCGGTGGTCAACATGCTTCCAGCACTCCTT	*kps* Region I, 5'
kps Reg1 #2	CCTCTTTGCACGATAAAAGGATTTTCTTG	*kps* Region I, 3'
BP 1F	GTACCGCGCTTAAACGTTCAG	BAR-PATH
BP 1R	AATTctgaacgtttaagcgcggtacAGC	BAR-PATH
BP 2F	GTACCGCGCTTAAATAGCCTG	BAR-PATH
BP 2R	AATTcaggctatttaagcgcggtacAGC	BAR-PATH
BP 3F	GTACCGCGCTTAAAAGTCTCG	BAR-PATH
BP 3R	AATTcgagacttttaagcgcggtacAGC	BAR-PATH
BP 4F	GTACCGCGCTTAATAACGTGG	BAR-PATH
BP 4R	AATTccacgttattaagcgcggtacAGC	BAR-PATH
BP 5F	GTACCGCGCTTAAACTGGTAG	BAR-PATH
BP 5R	AATTctaccagtttaagcgcggtacAGC	BAR-PATH
BP 6F	GTACCGCGCTTAAGCATGTTG	BAR-PATH
BP 6R	AATTcaacatgcttaagcgcggtacAGC	BAR-PATH
BP 7F	GTACCGCGCTTAATGTAACCG	BAR-PATH
BP 7R	AATTcggttacattaagcgcggtacAGC	BAR-PATH
BP 8F	GTACCGCGCTTAAAATCTCGG	BAR-PATH
BP 8R	AATTccgagattttaagcgcggtacAGC	BAR-PATH
BP 9F	GTACCGCGCTTAATAGGCAAG	BAR-PATH
BP 9R	AATTcttgcctattaagcgcggtacAGC	BAR-PATH
BP 10F	GTACCGCGCTTAACAATCGTG	BAR-PATH
BP 10R	AATTcacgattgttaagcgcggtacAGC	BAR-PATH
BP 11F	GTACCGCGCTTAATCAAGACG	BAR-PATH

BP 11R	AATTcgtcttgattaagcgcggtacAGC	BAR-PATH
BP 12F	GTACCGCGCTTAACTAGTAGG	BAR-PATH
BP 12R	AATTcctactagttaagcgcggtacAGC	BAR-PATH
BP 13F	CTTGCGGCGTATTACGTTCAG	BAR-PATH
BP 13R	AATTctgaacgtaatacgccgcaagAGC	BAR-PATH
BP 14F	CTTGCGGCGTATTATAGCCTG	BAR-PATH
BP 14R	AATTcaggctataatacgccgcaagAGC	BAR-PATH
BP 15F	CTTGCGGCGTATTAAGTCTCG	BAR-PATH
BP 15R	AATTcgagacttaatacgccgcaagAGC	BAR-PATH
BP 16F	CTTGCGGCGTATTTAACGTGG	BAR-PATH
BP 16R	AATTccacgttaaatacgccgcaagAGC	BAR-PATH
BP 17F	CTTGCGGCGTATTACTGGTAG	BAR-PATH
BP 17R	AATTCTACCAGTAATACGCCGCAAGAGC	BAR-PATH
BP 18F	CTTGCGGCGTATTGCATGTTG	BAR-PATH
BP 18R	AATTCAACATGCAATACGCCGCAAGAGC	BAR-PATH

BP 19F	CTTGCGGCGTATTTGTAACCG	BAR-PATH
BP 19R	AATTCGGTTACAAATACGCCGCAAGAGC	BAR-PATH
BP 20F	CTTGCGGCGTATTAATCTCGG	BAR-PATH
BP 20R	AATTCCGAGATTAATACGCCGCAAGAGC	BAR-PATH
BP 21F	CTTGCGGCGTATTTAGGCAAG	BAR-PATH
BP 21R	AATTCTTGCGGCGTATTTAGGCAAGAGC	BAR-PATH
BP 22F	CTTGCGGCGTATTCAATCGTG	BAR-PATH
BP 22R	AATTCACGATTGAATACGCCGCAAGAGC	BAR-PATH
BP 23F	CTTGCGGCGTATTTCAAGACG	BAR-PATH
BP 23R	AATTCGTCTTGAAATACGCCGCAAGAGC	BAR-PATH
BP 24F	CTTGCGGCGTATTCTAGTAGG	BAR-PATH
BP 24R	AATTCCTACTAGAATACGCCGCAAGAGC	BAR-PATH
BP-2A	Tgattaagatgaattcatgggaattagccatggtcc	BAR-PATH
BP-2B	Tgattaagatgaattcgtgacacaggaacacttaacggctgac	BAR-PATH
BP-2C	tgattaagatgaattccgcactgagaagcccttagagcctc	BAR-PATH

BP-8K	gcttcaaaagcgctctgaagttcctatac	BAR-PATH
BP-8C	Cgtgccgatcaacgtctcattttcg	BAR-PATH
BP-1	gaaccgtaggccggataaggcgtttacgccgcatccggcacatagttaacagctcgtgtaggctggagctgcttc	BAR-PATH
BP-5	/5Phos/ctacttcttcgcctctgcaaccactttgctacccacgccgcggttattgtattcc	BAR-PATH
BP-6	ggaatacaataaccgcggcgtgggtagcaaagtggttgcagaggcgaagaagtaggct	BAR-PATH

Mathematical model of tag diversity subsequent to a population bottleneck

As discussed in the main text, IBC formation is clonal and hypothesized to be the main contribution to bacterial persistence in the bladder. Assuming that bacteria carrying different tags are equivalent both in terms of IBC formation and in detection efficiency, the number of tags expected to be detected due solely to IBCs can be modeled as a multinomial distribution. Because tag detection does not differentiate between whether one or multiple IBCs contained that tag, we are interested simply in the number of different tags (multinomial outcomes) detected at all, and not in their relative abundance. In short, the problem of how many tags remain following a population bottleneck is identical to asking how many distinct bar codes are chosen when they are randomly sampled with replacement, the number of samples taken representing the population bottleneck. From the point of view of tags detected, we can calculate other populations from other bacterial niches by including additional samplings (multinomial trials), with one additional sample per bacterial clone.

This problem has been previously examined in (Ma, 2001), where formulas for the number of "live" terms in a complete multinomial expansion were presented - this corresponds precisely to our problem of calculating how many tags are expected to be detected in a given experiment. In the notation of (Ma, 2001), n is the number tags, representing mutually exclusive categories that result from a sampling (i.e. multinomial choices); k is the number of bacterial clones (either IBCs or clones included in other niches), or the number of multinomial trials; m is the number of tags detected, or those that have been sampled at least once in an experiment (live terms). A closed formula for the complete multinomial distribution is well known: $a_{k,p}^{i_1,i_2,\ldots,i_n} = n!/k!(n-k_1)!(n-k_2)!\ldots i_1^{k_1} i_2^{k_2} \ldots i_n^{k_n}$ However, as noted by (Ma, 2001), this is computationally intractable for values of n and k that we are interested in. (Ma, 2001) developed a closed formula for calculating individual terms of the multinomial expansion, but use of this requires iterating over all combinations of live terms, which is equivalent to finding all combinations of a set of k integers that sum to n. To translate from this into the distribution of total number of live terms is also computationally intractable for reasonable n.

Therefore, we have extended the reasoning used by (Ma, 2001) to directly calculate the number of multinomial samplings that have a given number of live terms. Our calculation has polynomial complexity because it does not explicitly calculate each term of the multinomial; instead, it calculates sums of distinct sets of the multinomial expansion, which are precisely the values that we are interested in.

We do a direct calculation of the number of ways that k samples from a multinomial with n possibilities, all with equal probability, results in exactly m outcomes occuring at least once. We let T_m be the number of possibilities that have exactly m live terms. For $m = 1$, this is trivial; for every sample, there is only 1 possibility, and thus there is $1^k = 1$ combination. However, there are n choices for which outcome is the only successful one; thus, there are $n \times 1 = n$ total ways that exactly one outcome is successful in all k samples: $T_1 = \binom{n}{1}1^k = n$.

For $m = 2$, each of the k samples has two possibilities, resulting in 2^k total combinations. However, 2^k counts all the possibilities of no more than 2 successful outcomes; this includes outcomes where only one of the two is successful (all k choices result in the same outcome) and thus these must be subtracted. There are $\binom{2}{1} = 2$ possibilities for the single successful outcome, thus we have $2^k - \binom{2}{1}1^k$ total outcomes where exactly two possibilities are successful. There are $\binom{n}{2}$ ways to pick which two outcomes are successful, giving us a total of $T_2 = \binom{n}{2}[2^k - \binom{2}{1}1^k]$ ways to have exactly two successful outcomes after k trials.

A similar reasoning is used for $m = 3$. Given the 3 outcomes, there are 3^k possibilities that have no more than those 3 outcomes; we must now subtract the possibilities that only 2 or 1 of the 3 desired outcomes is successful. When subtracting the possibilities that only 2 of the desired 3 outcomes is successful, we must only count those outcomes where exactly 2 of the outcomes is successful and not include the subset where only 1 of the 2 outcomes is successful. As above, the possibilities for exactly 2 successful outcomes is $2^k - \binom{2}{1}1^k$, and there are $\binom{3}{2}$ combinations of two outcomes, giving $\binom{3}{2}[2^k - \binom{2}{1}1^k]$. The correction for outcomes with only one successful outcome is $\binom{3}{1}[1^k]$. Again, there are $\binom{n}{3}$ ways of choosing a subset of three successful outcomes, giving a total of $T_3 = \binom{n}{3}[3^k - \binom{3}{2}[2^k - \binom{2}{1}1^k] - \binom{3}{1}[1^k]]$.

We now introduce a recursive definition for the term $c_{m,k} = m^k - \Sigma_{i=1}^{m-1}\binom{m}{i}c_{i,k}$ for $m > 1$, and $c_{1,k} = 1^k = 1$. The term $c_{m,k}$ represents the number of ways to get exactly m successful outcomes in k trials. Note that $c_{m,k}$

does not depend on the total number of possible outcomes. Then we can simplify the above expressions to:

$T_1 = \binom{n}{1} c_{1,k}$

$T_2 = \binom{n}{2} c_{2,k}$

$T_3 = \binom{n}{3} c_{3,k}$

In general, we have

$T_m = \binom{n}{m} c_{m,k}$

which is now, as expected, dependent on the total number of possible outcomes.

References

Ma, N. 2001. "Complete multinomial expansions". Applied Mathematics and Computation 124(3):365-70.

Supplementary Figure 1

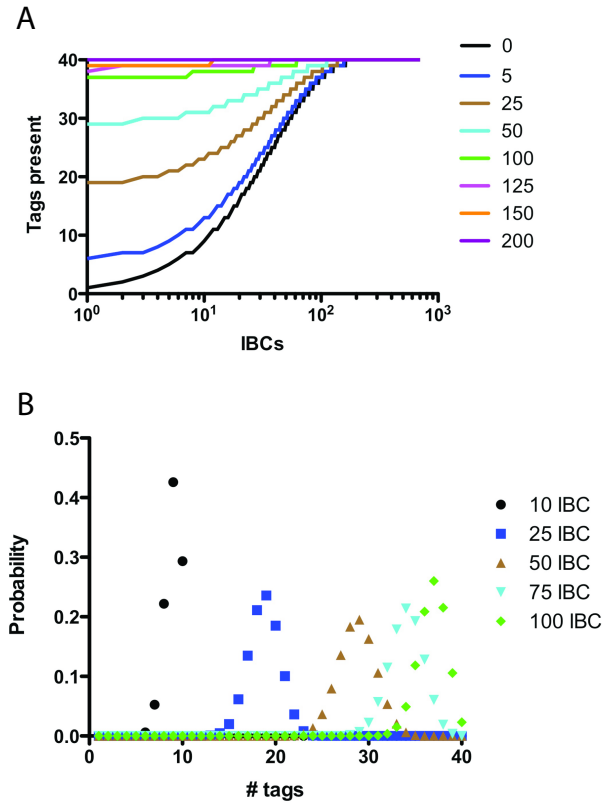

Figure 1. Predictive stochastic selection models of tag diversity in relationship to clonal intra- and extra-cellular communities. (A): Median number of tags (total = 40) expected based on a multinomial expansion with increasing contributions of tags from theoretical, clonal extracellular populations (see supplemental material). (B): The probability distributions for the likelihood of detecting a specific number of tags based on the amount of IBCs formed.

Chapter 3: Positively selected FimH residues enhance virulence during urinary tract infection by altering FimH conformation.

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Proceedings of the National Academy of Sciences 110 (39), 15530-15537.

doi:10.1073/pnas.1315203110. PMID: 24003161.

ABSTRACT

Chaperone-usher pathway (CUP) pili are a widespread family of extracellular, Gram-negative bacterial fibers with important roles in bacterial pathogenesis. Type 1 pili are important virulence factors in uropathogenic *Escherichia coli* (UPEC), which cause the majority of urinary tract infections (UTI). FimH, the type 1 adhesin, binds mannosylated glycoproteins on the surface of human and murine bladder cells, facilitating bacterial colonization, invasion, and formation of biofilm-like intracellular bacterial communities (IBCs). The mannose-binding pocket of FimH is invariant among UPEC. We discovered that pathoadaptive alleles of FimH with variant residues outside the binding pocket affect FimH-mediated acute and chronic pathogenesis of two commonly studied UPEC strains, UTI89 and CFT073. *In vitro* binding studies revealed that while all pathoadaptive variants tested displayed the same high affinity for mannose when bound by the chaperone FimC, affinities varied when FimH was incorporated into pilus tip-like, FimCGH, complexes. Structural studies have shown that FimH adopts an elongated conformation when complexed with FimC, but when incorporated into the pilus tip, FimH can adopt a compact conformation. We hypothesize that the propensity of FimH to adopt the elongated conformation in the tip corresponds to its mannose binding affinity. Interestingly, FimH variants, which maintain a high-affinity conformation in the FimCGH tip-like structure were attenuated during chronic bladder infection arguing that FimH's ability to switch between conformations is important in pathogenesis. Our studies argue that positively-selected residues modulate fitness during UTI by affecting FimH conformation and function, providing an example of evolutionary tuning of structural dynamics impacting *in vivo* survival.

Significance

Evolution of multidrug-resistance in pathogenic bacteria, including uropathogenic E. coli (UPEC) that cause most urinary tract infections (UTIs), is becoming a worldwide crisis. UPEC utilize a variety of virulence factors and adhesins, including the mannose-binding FimH adhesin, to colonize and invade bladder tissue, often forming intracellular biofilms and quiescent reservoirs that can contribute to recurrent infections recalcitrant to treatment. Using two prototypical UPEC strains, we discovered that positively-selected residues outside of the FimH mannose-binding pocket, affect transitions between low and high-affinity FimH conformations which extraordinarily impacts FimH function during pathogenesis. Thus, this work elucidates mechanistic and functional insights into pathoadaptation and evolutionary fine-tuning of critical virulence interactions.

Introduction

Urinary tract infections (UTI) are common infections causing serious morbidity and significant expenditures in healthcare dollars and lost wages. Women are disproportionately affected, with over half of women experiencing at least one UTI during their lifetime [1]. In the absence of treatment, 50-80% of women will resolve a UTI within 2 months, but up to 60% of women may remain bacteriuric with or without symptoms for at least 5-7 weeks after the initial infection [2]. Furthermore, even when effective therapy is given and bacteriuria and symptoms of the acute UTI resolve, 25-40% of women experience a recurrent UTI (rUTI) [2, 3]. rUTI can occur by recolonization of the urinary tract from the gastrointestinal (GI) tract or from another environmental source by the same or different strain or may be due to reactivation of the original UTI strain from a bladder reservoir [4-6]. Uropathogenic *Escherichia coli* (UPEC) cause 80-90% of community acquired UTI and 50% of nosocomial UTI [7]. The increasing prevalence of multidrug-resistant organisms can prolong the infection [8]. Thus, chronic and recurrent UTI represents a major health concern worldwide, necessitating molecular understanding of disease pathogenesis and investigations into novel diagnostics and therapies.

UTI is a highly complex disease involving colonization of multiple niches, each of which presents a unique set of evolutionary pressures shaping host-microbe and microbe-microbe interactions involving a multitude of virulence factors that determine disease onset, progression, and outcome. Adhesive pili assembled by the chaperone-usher pathway (CUP), such as type 1 pili, are well-characterized UPEC UTI virulence determinants. Type 1 pili, like other CUP pili, contain an adhesin (FimH) at their tip that plays an important role in host-pathogen interactions and biofilm formation. Type 1 pili are nearly ubiquitous among clinical UPEC isolates [9, 10] as well as commensal *E. coli* and other *Enterobacteriaceae*. Expression of type 1 pili is essential for

colonization of the murine urinary tract [11]; however, expressing type 1 pili is not sufficient for long-term colonization, as commensal *E. coli* are rapidly cleared [12].

Upon UPEC entrance into the bladder, FimH binds mannosylated glycoproteins, including uroplakins expressed throughout human and murine bladders [13]. Subsequent to attachment, UPEC invade superficial facet cells in a FimH-dependent manner [12, 14] and replicate in the cytoplasm, forming large biofilm-like structures called intracellular bacterial communities (IBCs) [15]. The formation of IBCs has been observed for numerous clinical UPEC isolates in multiple mouse models and in exfoliated uroepithelial cells in urines of patients with acute UTI, but not from healthy controls [16, 17]. The process of invasion and IBC formation provides UPEC an ability to survive stringent bottlenecks during pathogenesis in the urinary tract [18, 19]. Outcomes of infection range from resolution with or without accompanying quiescent intracellular reservoirs (QIRs) in the bladder tissue [4] to persistent bacteriuria and chronic cystitis [20]. In C3H/HeN mice, the formation of a high number of IBCs at 6 hours post infection (hpi) and an exuberant systemic innate immune response at 24 hpi, measurable in both urine and serum, correlate with the development of chronic cystitis marked by persistent urine and bladder titers $>10^4$ CFU/mL and severe bladder immunopathology [18, 20]. In addition to colonizing the bladder, UPEC can ascend the ureters and infect the kidneys, leading to pyelonephritis. The connection between acute and chronic UTI is just now beginning to be characterized [21-23]. Type 1 pili and the tip adhesin, FimH, are encoded by the *fim* operon [24, 25]. Mature FimH is a 279 amino acid (aa) two-domain protein containing a mannose-binding lectin domain (residues 1 – 150) and a pilin domain (residues 159 to 279) with an 8 amino acid (aa) linker connecting the domains (Fig. 1) [26-28]. The mannose-binding pocket of FimH is invariant among sequenced UPEC [29, 30]; however, several residues outside the mannose-binding pocket (positions 27, 62,

66 and 163) were found to be evolving under positive selection in clinical UPEC isolates compared to fecal strains (Fig. 1) [29, 31, 32]. Among four fully sequenced UPEC isolates (UTI89, CFT073, 536, and J96), differences exist in positively selected residues 27, 62, and 163 (Table 1). Clinical isolates expressing different *fimH* alleles have observable differences in the degree of pathogenicity as measured by IBC formation [16] and the development of chronic cystitis [20]. We found that UTI89, a cystitis isolate, formed more IBCs and had higher bladder titers at 6 hpi than CFT073, a pyelonephritis isolate, in single and co-infections. Because of the demonstrated importance of type 1 pilus function in pathogenesis, we conducted *fimH* allele swap experiments to determine whether the differences in *fimH* between UPEC strains were responsible for the phenotypic differences. We generated CFT073 and UTI89 strains containing different *fimH* alleles inserted into the normal chromosomal position. We found that presence of a *fimH* sequence encoding FimH from UTI89 (denoted FimH::A62/V163) resulted in significant increases in IBC development and the propensity to cause chronic cystitis compared to expression of CFT073 FimH (FimH::S62/A163). In co-infections, strains expressing FimH::A62/V163 significantly outcompeted otherwise isogenic strains harboring FimH::S62/A163 in both CFT073 and UTI89. FimH complexed with its chaperone FimC adopts an elongated conformation (Fig. 1A), which binds mannose with high affinity [26, 33]. When complexed with the FimG adaptor, FimH can adopt a compact conformation which binds mannose with low affinity (Fig. 1B) [33]. The identity of residues at positively selected positions outside the binding pocket dramatically impacts the mannose binding affinity of FimH when in the FimCGH tip-like complex but not in the FimCH complex. Thus, we argue that the combination of residues at positively selected positions affects the propensity of FimH to adopt an elongated conformation in the tip and thus its relative mannose binding affinity. FimH alleles

that retained the high affinity binding conformation in the tip were significantly attenuated in a mouse model of UTI suggesting that equilibrium between FimH conformations, which is modulated by positively-selected residues, is critical in pathogenesis.

Results

Prevalence of positively selected FimH alleles in humans

Comparing UPEC-infected urine isolates to fecal *E. coli* from healthy humans and animals has previously identified genes and residues within genes under positive selection in UPEC [29, 34, 35]. We analyzed a collection of *fimH* sequences obtained from 33 fecal *E. coli* from healthy, uninfected humans as well as 232 urine and periurethral isolates from women suffering acute or rUTI, asymptomatic bacteriuria, or pyelonephritis [16, 17, 29, 36-39]. We were particularly interested in cataloguing naturally occurring residue combinations at positions 27, 62, and 163, since in previous studies these residues showed evidence of positive selection, and mutations in these residues impacted fitness of the cystitis isolate UTI89 [29]. In sequences of fecal and *E. coli* from infected urine, we found one of two residues at each of these positions. An alanine occurred at position 27 in 85% of fecal isolates and 81% of urine isolates, while a valine occurred at position 27 in the other sequenced isolates. A serine occurred at position 62 in all of the fecal isolates and in 93% of urine isolates, while an alanine occurred in 7% of urine isolates. At position 163, a valine occurred in all fecal isolates and in 93% of urine isolates, with alanine occurring in the other 7% of urine isolates. Of the eight possible combinations of these three residues, we found only the four combinations shown in Table 1. Most *E. coli* isolates encode FimH::A27/S62/V163, with this allele being present in a higher percentage of fecal strains than urine strains. The prototypical UPEC isolates, UTI89 and CFT073 encode two different pathoadaptive FimH variants, A27/A62/V163 and A27/S62/A163, respectively. Neither *fimH* allele was observed in healthy feces (Table 1). All other residues in the mature FimH of UTI89 and CFT073 are identical. UTI89 was isolated from the urine of a patient experiencing an uncomplicated acute UTI [12]. CFT073 was isolated from the blood and urine of a woman

suffering acute pyelonephritis and sepsis [40]. Because of these differences in occurrence of different *fimH* alleles in different types of clinical samples, we examined their impact on pathogenesis in the urinary tract.

FimH sequence modulates IBC number

We first performed a systematic analysis of the differences in infection between UTI89 and CFT073. We transurethrally inoculated female C3H/HeN mice with 10^7 or 10^8 CFU of UTI89 or CFT073 in 50 μL of PBS (Fig. 2). The median number of IBCs formed at 6 hpi was significantly higher for UTI89 than the number of IBCs formed by CFT073 at both inoculum concentrations (Fig. 2A). Similar, statistically significant results were observed at 6 hpi in whole bladder titers (Fig. 2B). Bacterial titers at 6 hpi in the kidneys (Fig. 2C) did not significantly differ between strains, and we observed only a slight increase in kidney titers with an increased UTI89 inoculum.

To assess the impact of different *fimH* alleles in isogenic UTI89 and CFT073 backgrounds, we conducted *fimH* allele swaps. Hereafter, we will refer to these FimH alleles by the residue expressed at the relevant, positively selected position. We examined the effect on pathogenesis of three *fimH* alleles in each background: A62/V163, S62/V163 and S62/A163. Except for the previously published *in vitro* defects of FimH::S62/V163 [29], we saw no effect of FimH variants on growth in LB media in single and co-culture (Fig. S1A, Fig. S2), status of the *fim* promoter (Fig. S1B), total surface piliation (Fig. S1C), or 1 hpi invasion into 5637 cultured bladder cells (Fig. S1D) in either the UTI89 or CFT073 backgrounds. We found a moderate, but statistically significant, 2-fold increase in guinea pig red blood cell hemagglutination (HA) for FimH::A62/V163 in both strain backgrounds (Fig. S1E) and 48 hr

biofilm formation in LB in PVC plates in CFT073 (Fig. S1F). A reproducible HA titer difference of 2-fold has previously been shown to affect IBC formation [29]. Accordingly we saw that CFT073 encoding FimH::A62/V163 (UTI89 FimH) resulted in a greater number of IBCs (Fig. 3A) than WT CFT073 in an independent challenge experiment. No statistically significant difference in 6 hpi IBC number was obtained between UTI89 FimH::A62/V163 and UTI89 FimH::S62/A163, suggesting possible strain-dependent effects. Additionally, it is possible that subtle *fim* regulation defects exist *in vivo* even though we observed no differences *in vitro* with these strains.

FimH sequence modifies ability to persist during chronic cystitis

We then assessed the effect of FimH variation on development of chronic cystitis. Sixty-eight percent of mice developed persistent bacteriuria and chronic cystitis with bladder titers greater than 10^4 CFU at sacrifice 4 weeks post infection (wpi) when infected with 10^7 CFU UTI89 expressing its cognate FimH::A62/V163 compared to 40% of mice infected with UTI89 expressing the CFT073 cognate allele, FimH::S62/A163 ($p<0.05$; Fig. 3B). CFT073, expressing its cognate FimH::S62/A163 caused chronic cystitis in only 23% of mice, statistically significantly different from both UTI89 FimH::A62/V163 (**68%)** ($p<0.0005$) and CFT073 FimH::A62/V163 (**47%)** ($p<0.05$; Fig. 3B). Kidney titers were also increased for both UTI89 and CFT073 strains expressing FimH::A62/V163 (Fig. 3C), which likely reflects the higher bladder titers and the noted vesicoureteral reflux and/or ability of UPEC to ascend the ureters in this model [18, 41].

Strains expressing FimH::A62/V163 displace strains expressing FimH::S62/A163 during chronic co-infection

We found that in both backgrounds, strains expressing FimH::A62/V163 had subtle, yet significant, increases in tissue occupation versus strains expressing FimH::S62/A163 after co-infections (Fig. S3). At three hpi, 1.7-1.8-fold more bacteria encoding FimH::A62/V163 were found in the gentamicin-protected fraction in both strain backgrounds (Fig. S3). At 6 hpi, 2.2-fold more CFU of CFT073 FimH::A62/V163 was obtained (Fig. S3B). In addition to this acute advantage, strains expressing FimH::A62/V163 outcompeted strains expressing FimH::S62/A163 in the bladders and kidneys of mice experiencing chronic cystitis at four wpi based on $\log_{10}$ competitive index in both strain backgrounds (Fig. 4; $p<0.005$, Wilcoxon Signed Rank). With the average chronic cystitis bladder containing 10^7 CFU at 4 weeks, a $\log_{10}$CI of > 5 indicates that strains with FimH::S62/A163 are near the limit of detection (20 CFU). The median $\log_{10}$ CI for co-infecting UTI89 FimH::A62/V163 $Spect^R$ and UTI89 FimH::S62/A163 Kan^R was also significantly higher than a control competition between UTI89 Kan^R and UTI89 $Spect^R$ with the same *fimH*, confirming that the difference in antibiotic resistance marker could not account for the infection phenotypes (Fig. S4). Thus, in co-infections, independent of strain background, expressing FimH::A62/V163 was advantageous over expressing FimH::S62/A163 in the urine as well as in chronic organs at four wpi (Fig. 4).

UTI89 outcompetes CFT073 in co-infection experiments even when expressing the same FimH

To further clarify the relative contribution of FimH differences versus other factors in UTI89 and CFT073 for different pathogenic phenotypes, we conducted additional co-infection experiments. We first, co-inoculated CFT073 and UTI89 each expressing their natural *fimH*

allele. UTI89 had higher titers than CFT073 in the bladder lumen as well as the intracellular compartment (Fig. S5A – B, D). We observed no difference between UTI89 and CFT073 in the kidneys (Fig. S5C). We then co-inoculated UTI89 and CFT073, both of which were expressing FimH::A62/V163 and found that UTI89 outcompeted CFT073 during chronic cystitis even when both expressed the same *fimH* allele (Fig. 4C), suggesting UTI89 expresses other factors, which in addition to its *fimH* allele contribute to chronic cystitis.

Positively selected FimH residues impact affinity, but only when FimH is incorporated into a tip-like complex

We hypothesized that the fitness advantage of expressing FimH::A62/V163 over FimH::S62/A163 throughout infection was due to differences in the ability to bind mannosylated epitopes on the bladder surface. Therefore, we used BioLayer Interferometry to assess mannose binding of different FimH variants. FimH is known to adopt two different conformations, which vary in their affinity for mannose [33]: an elongated conformation with high mannose affinity and a compressed conformation with low mannose affinity (Fig. 1). In the first set of binding experiments, FimCH complexes were used in which FimC traps FimH in the elongated, high mannose binding conformation [33, 42]. We immobilized biotinylated BSA-mannose on SuperStreptavidin pins and tested for binding of FimCH complexes in solution. For these assays we utilized FimH with variations at positions 62 and 163 as seen in UTI89 and CFT073. We also varied position 27 since this position was previously found to be under positive selection [29]; this residue is variable in both uropathogens and fecal strains (Table 1). All FimCH complexes had very similar affinity to BSA-mannose regardless of the variant with the exception of FimH::Q133K, which has a mutation within the mannose binding pocket resulting in an inability

to bind to mannose (Fig. 5A) [26]. The FimCH fit curves had very high R^2 values and K_D values between 1.5 and 4.2 μM (Table 2), consistent with previously reported affinity calculations for the isolated FimH lectin domain [42].

Each variant was then reconstituted into a tip-like $FimC_{his}GH$ complex *in vitro* by combining $FimC_{his}G$ and FimCH (Fig. 5B). In addition to $FimC_{his}GH$ complexes, small quantities of higher order structures such as $FimC_{his}GGH$ and $FimC_{his}GGGH$ were detected, but the distributions of these complexes were equal among FimH alleles. FimH within the $FimC_{his}GH$ complex is able to adopt the compact conformation seen in tip structures, as the lectin domain is no longer restrained from bending by FimC [33, 43, 44]. In the tip-like $FimC_{his}GH$ complexes, different FimH alleles elicited dramatic differences in BSA-mannose binding affinity (Fig. 5C), with three general affinity patterns: high, intermediate, and low. $FimC_{his}GH$::V27/A62/A163 and $FimC_{his}GH$::A27/A62/A163 maintained the same high affinity as their FimCH counterparts (Table 2, Fig. 5), suggesting that these variants may not adopt the compressed, low-affinity conformation in the pilus or that their propensity to transition to the elongated state is increased accounting for higher relative affinities. The UTI89 natural variant (FimH::A27/A62/V163) and CFT073 natural variant (FimH::A27/S62/A163) both showed intermediate-affinity when in the $FimC_{his}GH$ complexes. However, $FimC_{his}GH$::A27/A62/V163 had a significant ($p = 0.0087$, Mann-Whitney U Test), two-fold higher affinity than $FimC_{his}GH$::A27/S62/A163 (1.2 vs. 2.5 x 10^{-4} M; Table 2). A two-fold difference in FimH K_D would likely translate to a very large difference in adherence in piliated UPEC containing 20-200 pili per bacterium (Fig. S1C). $FimC_{his}GH$::A27/S62/V163 and $FimC_{his}GH$::Q133K both had low affinity with K_D values in the 3 mM range (Table 2). This suggests that FimH::A27/S62/V163 may not be able to adopt the elongated high-affinity conformation as efficiently.

To assess whether higher binding correlated with increased *in vivo* infection, we tested virulence at 4 wpi mice were co-infected with UTI89 expressing either of the high affinity FimH variants, FimH::A27/A62/A163 or FimH::V27/A62/A163 and WT UTI89 (Fig. 5D). Both FimH::A27/A62/A163 and FimH::V27/A62/A163, but not the WT control were attenuated in the bladders of mice 4 wpi compared to WT UTI89 (Table 2). This result suggests that mannose-binding affinity does not directly correlate with pathogenicity. Instead, an intermediate binding affinity is the best predictor of virulence, implying that FimH may dynamically interconvert between conformations depending on the local environment.

Discussion

The looming worldwide crisis of globally spreading, multi-drug resistant microorganisms [45], the growing body of work delineating the important benefits of the host's normal microbiota [46], and the deleterious effects of broad-spectrum anti-microbial treatments on these symbiotic/commensal relationships [47], argue that we need to develop new approaches to treat and prevent common infectious diseases such as UTI. With the delineation of Koch's postulates and Falkow's molecular postulates [48, 49], infectious disease experts have learned to associate a particular disease with the presence or absence of a particular pathogen or "virulence factor", respectively, in a diseased host. In considering UTI pathogenesis, a critical factor is that virulence is not simply dichotomous (presence/absence of a microorganism in the host or virulence factor within a bacterium). Outcomes of infectious disease in general, and UTIs specifically, are determined by a complex, yet seemingly subtle, interplay between differing host genetics and immune states [50, 51], host experiences/exposures [52, 53], bacterial gene carriage [9, 10, 16], co-infecting species [54], and many other factors. In this study, we detail an instance that goes beyond presence or absence of virulence factors, showing that possession of particular allelic variants of a critical adhesin of UPEC modulates the pathogenic process. We have combined an evolutionary analysis of *fimH*, multiple clinical UPEC isolates, representative *in vivo* models of infection, and biochemical structure-function correlation to explain how the identity of individual amino acid residues, far from the ligand binding "active site" (Fig. 1), alter FimH binding affinity likely through modulation of conformational dynamics, governing virulence of two pathogenic UPEC strains.

Adhesive pili assembled by the chaperone-usher pathway (CUP), such as P and type 1 pili, contain adhesins at their tips that play important roles in host-pathogen interactions. Each

sequenced UPEC strain has been found to encode numerous CUP operons [55]. Some CUP adhesins are known to recognize specific receptors with stereochemical specificity, such as FimH for mannosylated glycoproteins. Type 1 pili, like other CUP pili, are encoded in a gene cluster comprised of regulatory genes (FimB, FimE), a major pilin subunit (FimA), a periplasmic chaperone (FimC), an outer membrane usher (FimD), minor tip pilins (FimF, FimG) and the adhesin (FimH). Type 1 pili, like P pili and others, are composite structures, consisting of long, rigid, helical rods made up of FimA, joined at the distal end to a short, linear fiber consisting of FimF, FimG and the FimH adhesin [56, 57]. Tip adhesins consist of two domains: a lectin domain and a pilin domain (Fig. 1). Pilin domains and subunits are incomplete immunoglobulin (Ig)-like folds, encoding just six of the needed seven strands and thus pilin subunits require the action of dedicated periplasmic chaperones for folding and stability. We discovered that the molecular basis of chaperone-assisted folding was a reaction that we named donor-strand complementation (DSC) [58-60] in which the chaperone transiently completes the Ig-like fold of each subunit, providing the missing 7^{th} beta strand needed for pilin subunit folding [61]. The DSC subunit-chaperone complex holds the subunit in a primed high-energy state with connecting loops disordered and the subunit hydrophobic core incompletely collapsed [60-62]. These complexes are then differentially targeted to the outer membrane usher [63, 64] (Fig. 6A), which is a five domain gated channel that catalyzes pilus assembly by driving subunit polymerization in a reaction we termed donor strand exchange (DSE). Each of the non-adhesin subunits contains a short N-terminal extension (Nte). DSE occurs in a zip-in-zip-out mechanism where an incoming subunit's Nte zips into the chaperone-bound groove of a nascently incorporated subunit at the growing terminus of the pilus resulting in chaperone dissociation [65]. This allows the final folding of the subunit with the collapse of the hydrophobic core and the ordering of loop regions,

such that every subunit in the pilus completes the Ig fold of its neighbor. The usher converts subunit binding and folding energy into work, acting by sequential allosteric interactions, promoting sequential DSE and incorporation of subunits into the growing pilus while stably maintaining contact with the growing pilus and ensuring the integrity of the outer membrane barrier in the absence of ATP [66, 67].

FimH has a distal mannose binding lectin domain ($FimH_L$) and a proximal pilin domain ($FimH_p$). The FimG pilin adapts FimH to the tip of the pilus (Fig. 1). Le Trong *et al* recently compared $FimH_L$ and Fim tip crystal structures, and found that FimH adopts at least two conformations with $FimH_L$ elongated or compact (Fig. 1, 6A) [33, 68]. Functional biochemical assays indicated that mannose binds tightly to the elongated $FimH_L$ but weakly, if at all, to the compact $FimH_L$ [33, 42]. Recent structural "snapshots" of pilus assembly captured these same FimH conformations during its assembly across the usher: elongated while still bound to FimC prior to DSE and compact after DSE with FimG, which incorporates FimH into the pilus tip and its extrusion through the usher pore (Fig. 6A insets) [43, 67].

The advent of high-throughput sequencing has allowed the examination of hundreds of FimH alleles, providing insight into the evolutionary process of this virulence factor [29, 35]. FimH is highly conserved both in clinical and commensal *E. coli* isolates, with all alleles encoding identical amino acids at 90% of the positions [29]. The mannose-binding pocket of FimH is invariant among sequenced UPEC, but residues 27, 62, and 163 outside of this binding pocket showed evidence of positive selection (Fig. 1), suggesting the identity of residues at these positions can confer increased fitness in the urinary tract [29, 35]. All of the positively selected residues in FimH are in locations previously identified to displace significantly between

elongated and compressed conformations, suggesting that residues may impart steric clashes in one conformation or the other (Fig. 1) [33, 44, 69].

The FimH of the two prototypical UPEC isolates, UTI89 (a cystitis isolate) and CFT073 (a pyelonephritic/sepsis isolate) are identical at residues 27 (both having A), but differ at 62 and 163; UTI89 expresses A62/V163 and CFT073 expresses S62/A163 and are otherwise identical. Here, we demonstrate that both of these alleles show high affinity for mannose when complexed with FimC and an intermediate affinity when complexed with FimG in a tip-like $FimC_{his}GH$ complex. Strains harboring a FimH sequence coding for A27/A62/V163, present in the cystitis isolates NU14 and UTI89, demonstrated increased fitness in the bladder compared to strains expressing A27/S62/A163, present in UPEC 536 and CFT073, as determined in single and co-infections at 4 weeks post infection (Table 2; Fig. 6B). Because strains with FimH::A27/S62/A163 were essentially absent in 4 week bladders (Fig. 4, 6B), it is likely that bottlenecks enforced by innate defenses were more easily transcended by strains harboring FimH::A27/A62/V163 likely because of differences in binding and invasion efficiencies (Fig. 6B) leading to higher IBC numbers during early infection [18]. In addition, the UTI89 *fimH* allele demonstrated enhanced fitness relative to all of the other tested wild type and mutant variations of these alleles during chronic cystitis (Table 2), when the superficial facet cells are completely denuded [20] suggesting continued selection based on *fimH* allele during chronic infection.

All of the FimH alleles tested, except Q133K, exhibited high affinity for mannose when complexed with FimC and held in the elongated conformation, and all naturally occurring FimH alleles exhibited a low or intermediate affinity for mannose when incorporated into the tip structure. Interestingly, while UTI89 FimH functions with an A62 and CFT073 functions with an

A163, in a survey of FimH sequences from naturally occurring *E. coli* strains the combination of A62/A163 has never been observed. We show that A62/A163 variants (both FimH::V27/A62/A163 and FimH::A27/A62/A163) retain their high affinity for mannose even when incorporated into tip-like complexes suggesting that FimH::A62/A163 does not adopt the low-affinity compact conformation when in the pilus tip or that it's transition to the high affinity elongated form is greatly enhanced in the presence of mannose. Despite their increased relative affinity for mannose, strains harboring these *fimH* alleles were severely attenuated, and it was previously shown that FimH::V27/A62/A163 does not form IBCs at 6 hpi [29]. This may reflect a critical role in pathogenesis for the compact state in pathogenesis and/or for the ability to convert between the compact and elongated conformation states. The elongated conformation may prevent detachment from the uroplakin receptor, making UPEC more vulnerable to TLR4-mediated expulsion from the urothelium [70]. Alternatively, the compact or low-affinity state may be needed within the IBC or to resist shear stress in the urinary tract. Sokurenko *et. al.* discovered that FimH binds via a "catch-bond" mechanism in which shear force can influence the strength of binding [71, 72]. We posit that a dynamic equilibrium of FimH between the two conformations may allow UPEC to resist shear forces within host niches and that residues at positions 27, 62 and 163 may impact the transitioning between the two conformations. It is possible that FimH::A62/A163 has dramatically higher mannose binding affinity in its compact state thus potentiating transitioning to the elongated state, but the binding pocket of the compact state of FimH is partially occluded, and would have to adapt to accommodate mannose to an appreciable degree [33].

Possession of an alanine at position 62 (instead of serine) has been associated with increased virulence, mannose binding, and an increased ability to bind to collagen [29, 73,

74]. Further, it was previously determined that mutation of position 62 from a serine to a leucine or glutamic acid enhanced bacterial binding to surface mannose [69], consistent with our findings. Our work would suggest that mutation of this residue from alanine to serine, in the context of V163 (FimH::A27/S62/V163) may decrease the ability of FimH to transition from the compressed, low-affinity conformation seen in the tip in the absence of mannose to the high affinity, elongated conformation. Thus, the combination of amino acids present at positions 62 and 163 modulate FimH function. We propose that evolutionary pressure on UPEC isolates has led to the selection of FimH residue combinations, which alter the stability or affinity of one conformation or the other, affecting the equilibrium between the different conformations and thus mannose affinity and pathogenesis. The FimH alleles with intermediate binding affinity, A27/A62/V163 and A27/S62/A163, expressed by UTI89 and CFT073, respectively, may be able to dynamically switch between conformations allowing them to bind to and invade superficial facet cells, and then detach and enter the cytoplasm to replicate into IBCs (Fig. 5, 6B).

We have recently characterized the population dynamics between gastrointestinal (GI) and bladder niches in women suffering UTI (Fig. 6C). At the time of UTI in four patients, we found that the UPEC strain that occupied the urinary tract was the same as the dominant *E. coli* found in the GI tract [6]. Interestingly, in one patient from this study, when a recurrent UTI was caused by a different strain of UPEC, this new strain also replaced the previous strain in the GI tract, implying that increased fitness in the urinary tract was not accompanied by a decrease in fitness in the gut, which was corroborated by a fitness analysis in both niches in animal models (Fig. 6C) [6]. The most common combination of positively-selected residues we found in both healthy human feces as well as the urinary tracts of women suffering UTI was A27/S62/V163, perhaps suggesting a generalist FimH, capable of colonizing both niches. The low affinity for

mannose of a UTI89 FimH mutated to carry this combination of residues suggests that this FimH allele likely is very poor in adopting an elongated conformation perhaps signifying that the compact conformation is of greater importance throughout infection. Additionally, residues elsewhere in FimH may further modulate the conformation equilibrium of this particular combination of positively-selected residues. The two combinations we found only in diseased urine but not in healthy feces (FimH::A27/A62/V163 and FimH::A27/S62/A163) suggests that these alleles may have increased virulence in the urinary tract. It is possible that *E. coli* expressing this allele may invariably lead to UTI, which may accelerate the decision to seek treatment. Selection for *fimH* is likely quite different in the urinary and GI tracts due to differences in shear stress, which has been shown to influence FimH binding, due to urine flow and peristalsis [71, 72, 75]. Thus, it will be interesting to assess the effect of FimH variants in the GI tract. These types of studies and investigations similar to those performed by Chen *et al* in which multiple niches are sampled longitudinally may further help refine the source-sink hypothesis of virulence and pathoadaptation of UPEC in multiple niches [6, 76].

Increasing rates of antibiotic resistance in bacteria, including uropathogens [77], the spread of multi-drug resistant strains [45], and the dearth of new antibiotic candidates in pharmaceutical development pipelines make a compelling argument and opportunity for novel virulence-based therapeutics (Fig. 6D-E) [78]. Multiple groups are investigating methods to inhibit the ability of UPEC to bind and invade bladder tissue as well as to block catheter colonization via anti-adhesive small molecule inhibitors and vaccines (Fig. 6D-E) [79-82]. High affinity mannosides that bind in the FimH binding pocket have been shown to block invasion and IBC formation as well as biofilm formation on abiotic surfaces [79, 80]. Additionally, mannosides delivered orally were shown to dramatically reduce bacterial burden during chronic

cystitis (Fig. 6D) [80]. Pilicides have been designed aimed at disrupting assembly of multiple CUP pili in order to incapacitate bacterial adhesion in various tissue niches and body habitats (Fig. 6E) [83]. These molecules work, in part, by blocking the interaction of the usher with chaperone-subunit complexes, thus blocking pilus assembly [83]. These anti-virulence therapeutics hold promise for targeting recalcitrant UPEC infections.

Conclusion

Our current understanding of the pathogenic lifestyles of UPEC is in part an extension of the molecular and structural characterization of type 1 pili. This has led to a new understanding of clinical UTIs and its complicating factors. Combining this with genomic and pathoadaption studies, we have elucidated a connection between evolutionary pressures, protein structural dynamics, and *in vivo* disease pathogenesis. UTIs are one of the greatest contributors to antibiotic consumption and thus likely an important driver of the development of antibiotic resistance. Our analysis here would argue that, in addition to the therapeutic strategies addressed above, therapeutics that block the interconversion of the FimH adhesin between conformations; locking it in either conformation would impede UPEC pathogenesis [69, 84]. Analyses of other pathogens and virulence genes by combining positive selection analyses, *in vivo* models of pathogenesis, and biochemical and structural characterization could lead to identification of similar targets for the development of novel therapeutics that will be needed to fend off the looming crisis of multi-drug resistant bacterial pathogens.

Materials and Methods

Bacterial Strains.

UTI89 strains with chromosomally integrated *fimH* variants were generated using the λ Red recombinase system [85] and are the same as previously published [29]. The same method was used to create similar *fimH* variants in CFT073. Creation of HK marked antibiotic resistant strains was conducted similarly.

Mouse infections.

Infections, LacZ staining of bladders, and *ex vivo* gentamicin protection assays were conducted as previously described [18, 41, 86]. All mouse infection studies were approved by the Animal Studies Committee of Washington University in St. Louis, MO.

Protein Purification.

See Supplemental Materials for purification details.

Biolayer Interferometry (BLI).

OCTET was used to attain BLI progress curves, which allowed us to extract kinetic parameters (k_{on}, k_{dis}, and K_D). See Supplemental Materials for more details. Analysis was conducted on ForteBio Data Analysis 6.4. Processed data was fit globally for each variant at 3 concentrations between 1.25 and 12 μM for FimCH and 1.8 to 200 μM for $FimC_{his}GH$ per experiment in a 1:1 kinetic binding model.

Statistical analysis.

Statistical analysis was performed in GraphPad Prism 5.0. CFU values of zero were set to the limit of detection of the assay. Competitive Index (CI) = (CFU_{output} strain A/ CFU_{output} strain B)/(CFU_{input} strain A/ CFU_{input} strain B). Wilcoxon Signed Rank was used on log transformed CI

to determine if the median value was different than 0. Statistical analysis to compare median values between groups was determined by Mann-Whitney U test. To compare proportions of mice experiencing persistent bacteriuria and chronic cystitis, we used Fisher's Exact Test. $p < 0.05$ was considered significant.

Acknowledgements

The authors would like to thank members of the Hultgren laboratory for helpful suggestions. We would also like to thank Will Planer for help with strain creation, Dan Liu for urine collection, Patrick Olson for technical assistance with mouse infections, and Wandy Beatty for TEM studies. Thanks to Stanley Falkow, Staffan Normark, Tom Silhavy, Carl Frieden, and Jeffrey Gordon for guidance and inspiration. S.J.H. was supported by NIH grants NIAID R01 AI029549, NIAID R01 AI048689, ORWH NIDDK P50 DK064540, NIDDK R01 DK051406, NIAID U01 AI095542, and NIAID R01 AI099099. DJS was supported by NIH F30 DK096751, and SLC was supported by K01 DK081620 and administrative supplement.

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Figures and Tables

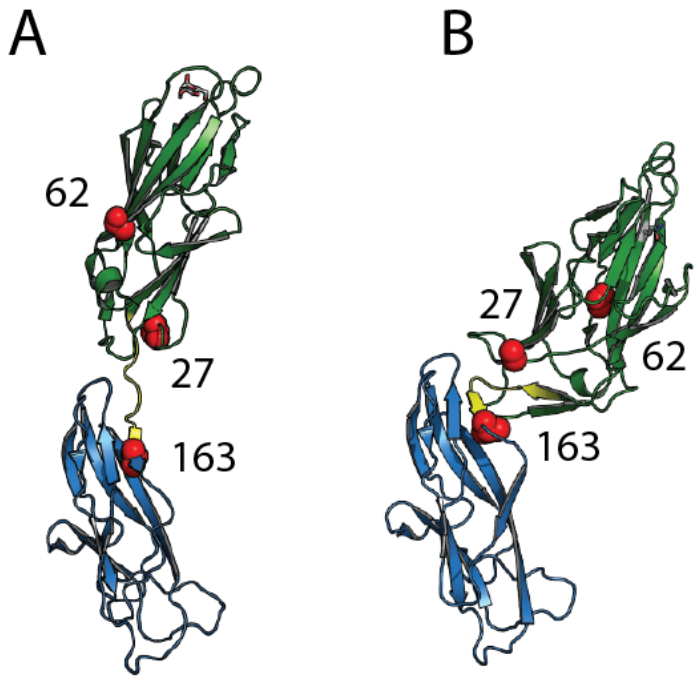


Figure 1. FimH positively selected residues

FimH is a two-domain adhesin comprised of a lectin domain of residues 1-150 (green) and a pilin domain with residues 159-279 (blue), and a linker loop (yellow) connecting them. Positively selected residues are mapped onto the structures of FimH as red spheres. A) In the elongated FimH (V27/S62/V163) structure, mannose is observed at the distal binding pocket in white sticks (J96 FimH; PDB:1KLF; FimC removed for clarity). B) In the compressed FimH (A27/S62/A163) structure in the absence of mannose, position 133 of the binding pocket is colored white (F18 FimH; PDB:3JWN). Note the distance of these positively selected residues from the mannose binding pocket.

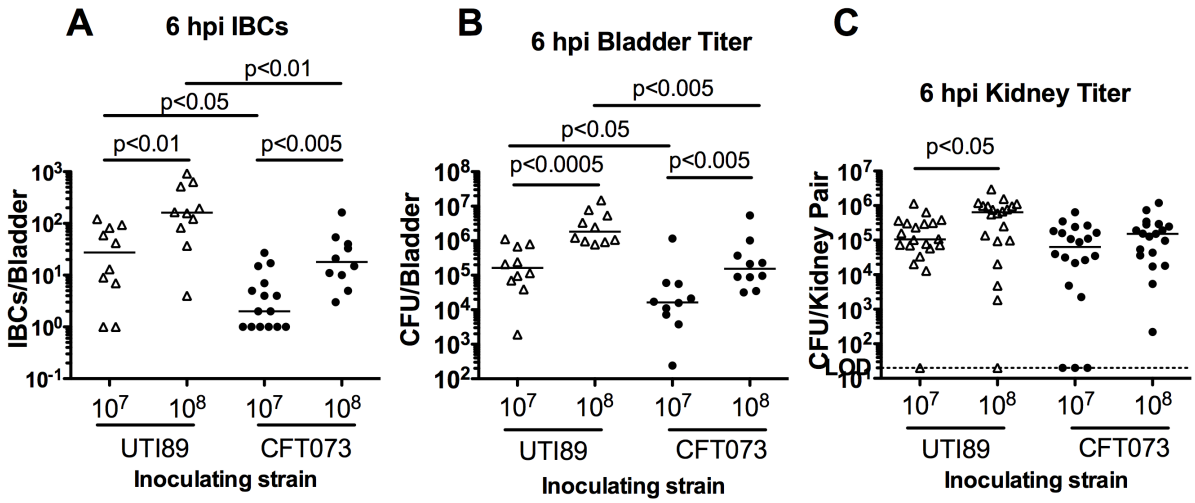


Figure 2

CFT073 and UTI89 acutely infect mouse urinary tracts. A) IBCs per bladder were enumerated with LacZ stain. B) Total bladder bacterial counts were determined after homogenization. C) Total bacteria present in both kidneys was enumerated. N = 2 experiments with 5 mice per group. Panel C includes kidney titers of mice from panels A and B.

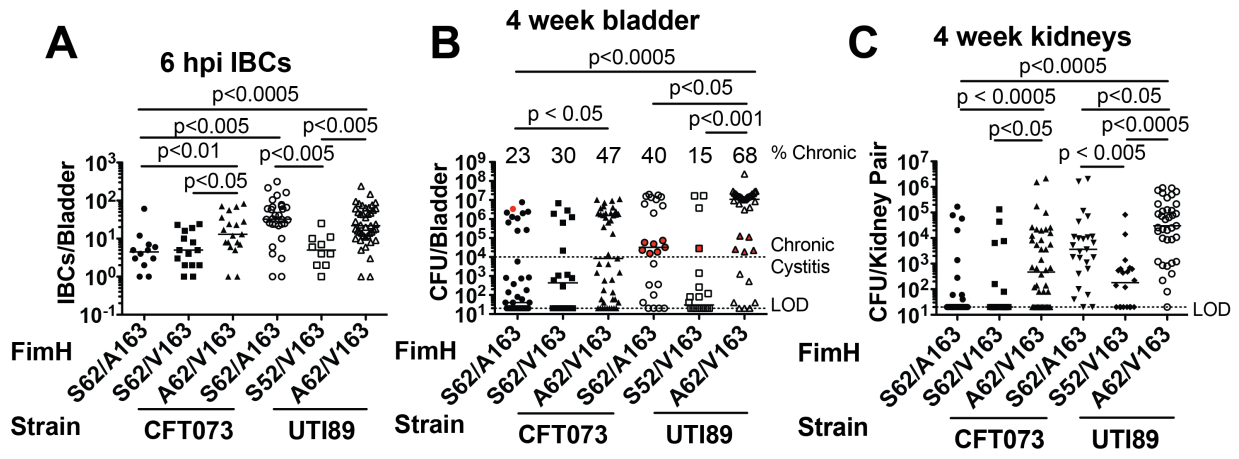


Figure 3

FimH allele modulates acute and chronic pathogenesis. Mice were infected with 10^7 CFU (range 3.4 x 10^6 – 1.8 x 10^7; median 1.02 x 10^7) of the indicated strains. A) IBCs were enumerated after LacZ staining. B) Urine was collected at days 1, 7, 14, and 21, and the number of bacteria present in bladders was determined at 4 wpi. Data points above 10^4 CFU reflect mice that had persistent bacteriuria and are considered to have chronic cystitis. Red symbols denote mice that resolved bacteriuria and either had a recurrence or high levels of reservoir titers, and were thus included in the resolved category because they did not experience persistent bacteriuria and chronic cystitis. The percentage of mice per group experiencing chronic cystitis is displayed at the top of each column. C) Kidney titers of the mice from panel B. A) N = 2 – 11 experiments with 2 – 8 mice per group. B-C) N = 2 – 8 experiments with 5 – 10 mice each.

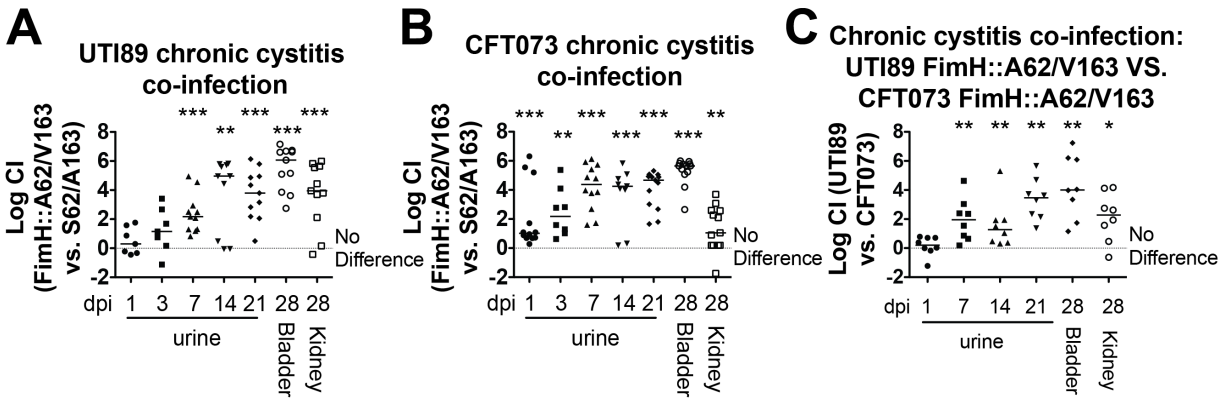


Figure 4

FimH::A62/V163 displaces FimH::S62/A163 during chronic co-infection. Co-infections were conducted A) between UTI89 with FimH::A62/V163 and FimH::S62/A163, B) between CFT073 with FimH::A62/V163 and FimH::S62/A163, and C) between UTI89 FimH::A62/V163 and CFT073 FimH::A62/V163. Urine was collected at days 1, 3, 7, 14, and 21 days post infection (dpi), and bladder and kidney titers were determined at 28 dpi. Log_{10} of competitive indices of the mice experiencing chronic cystitis is plotted as determined via plating on selective antibiotics. N = 2-3 experiments with 5-10 total mice per group. Wilcoxon signed rank test was conducted to evaluate whether the median value was significantly different from 0; **, $p<0.01$, ***, $p<0.005$.

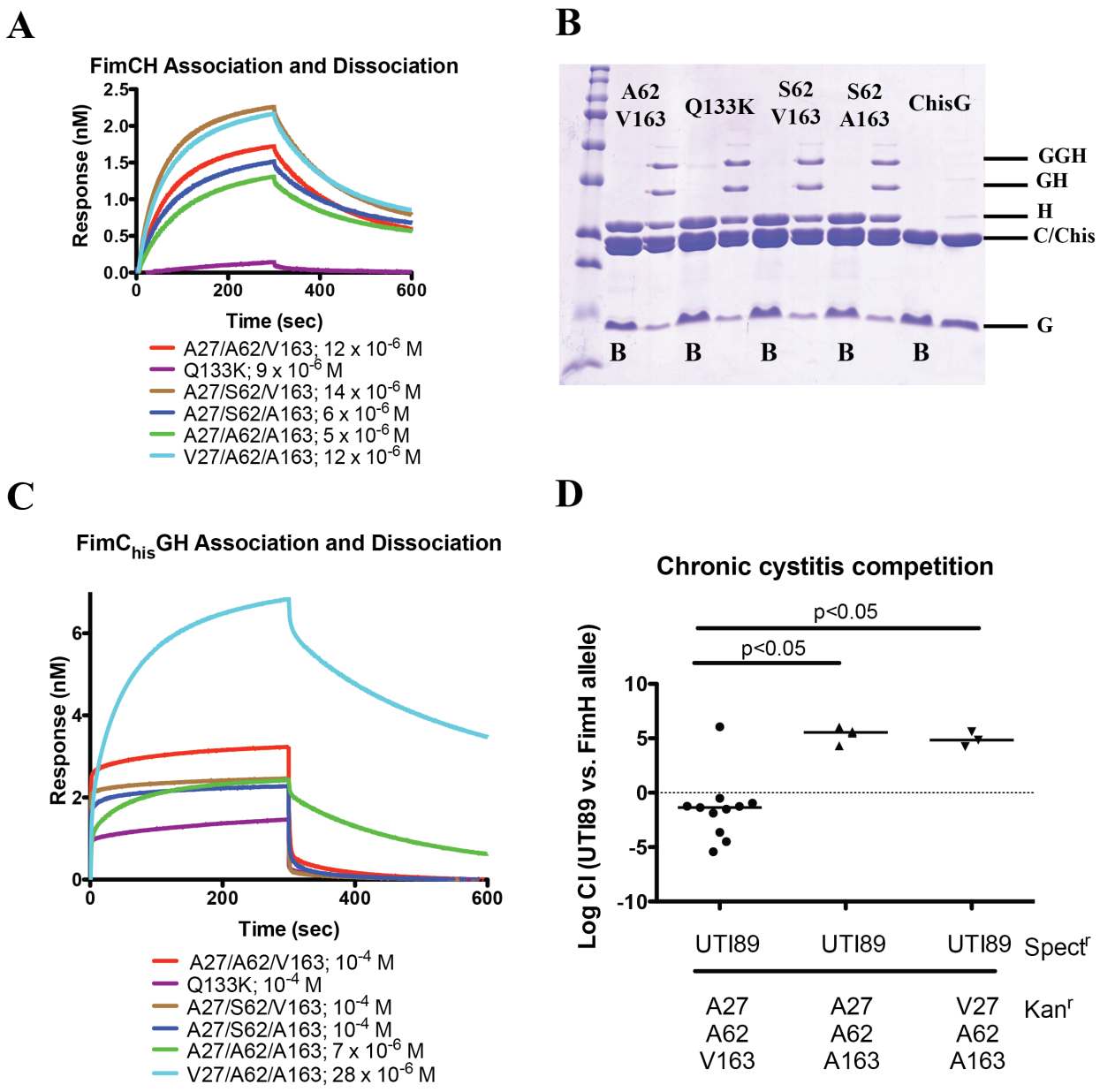
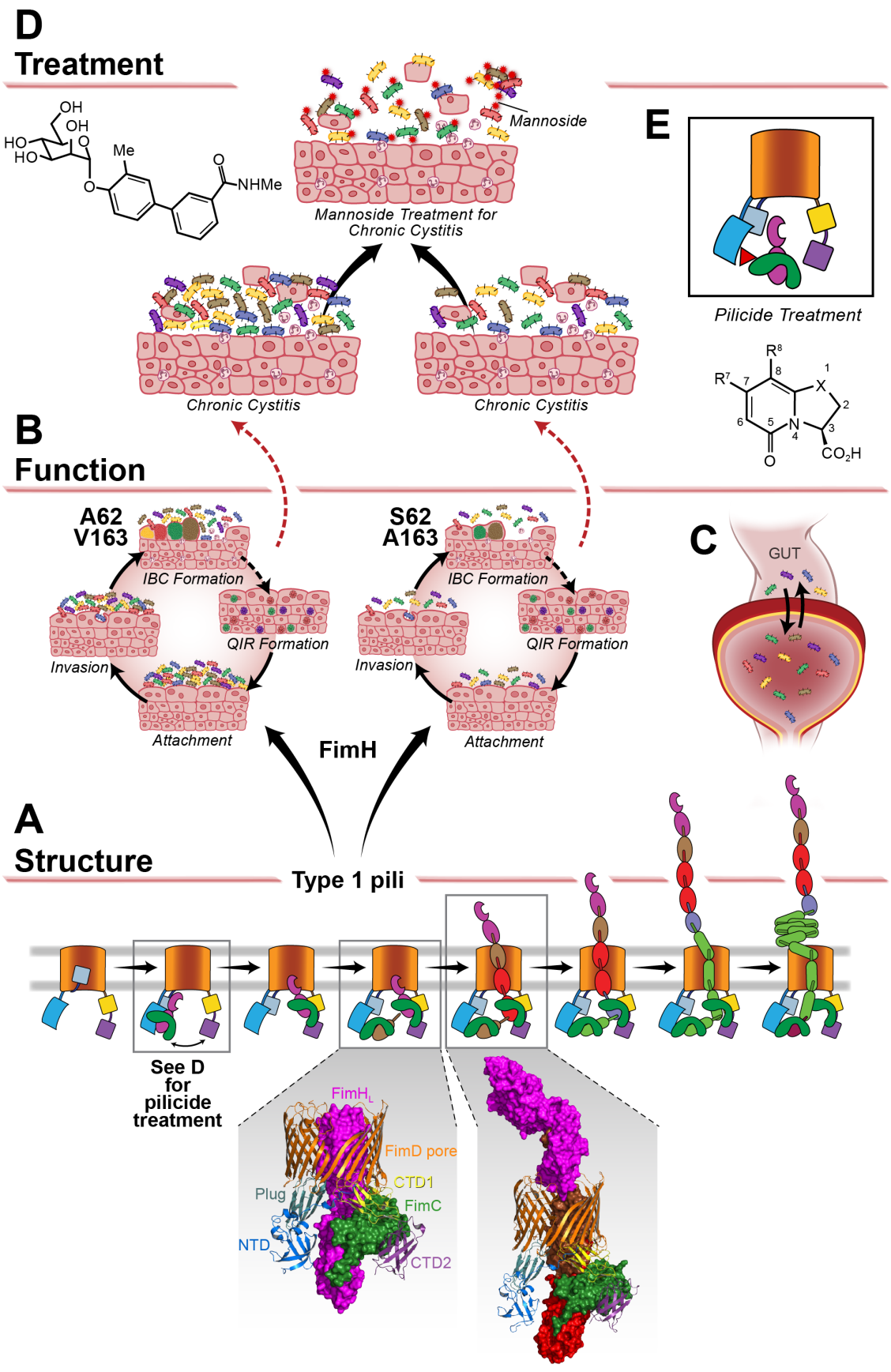


Figure 5

Effect of FimH on mannose binding and chronic fitness. A) Representative curves showing FimCH association to and dissociation from immobilized BSA-mannose at indicated concentrations. Two biological replicates conducted. B) Representative 14% SDS-PAGE gel indicating select variants in $FimC_{His}GH$ samples. "B" marks the boiled lanes. C) Representative curves showing $FimC_{His}GH$ association to and dissociation from immobilized BSA-mannose at

the indicated concentrations. Representative of three biological replicates with two technical replicates each with combined K_D data shown in Table 2. D) Four week bladder titer is shown for mice experiencing chronic cystitis with co-infections of the listed strains. N = 1-3 experiments with 5-10 mice each.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Mannoside Treatment for Chronic Cystitis
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R8
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7
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4
3
CO2H
O
B
Function
A62
V163
IBC Formation
QIR Formation
Invasion
Attachment
S62
A163
IBC Formation
QIR Formation
Invasion
Attachment
C
GUT
FimH
A
Structure
Type 1 pili
See D for pilicide treatment
FimHL
FimD pore
CTD1
Plug
FimC
NTD
CTD2

Figure 6

Structure-function-treatment model of UPEC pathogenesis. A) Model of pilus biogenesis including delivery of chaperone-subunit complexes to the NTD (blue) of the usher (orange) with transfer to the CTDs (yellow and pink). The next subunit reacts with the previous by DSE polymerizing the pilus rod. Insets represent recent crystal structures demonstrating the orientation of FimH as it binds to and exits the usher [43, 67] (PDB: 3RFZ and PDB: 4J30). B) UPEC pathogenic cascade showing type 1 piliated UPEC attaching to superficial cells of the bladder, invasion into these cells, and replication in the cytoplasm to form IBCs. Mice can then either resolve the infection with potential recurrences thereafter or develop persistent bacteriuria and chronic cystitis. C) The dynamics of transmission from the gut to the bladder and vice versa is an intriguing concept that is just now beginning to be studied [6]. D) Mannoside treatment can detach bacteria from the bladder epithelium during chronic cystitis leading to clearance. E) Pilicides block the ability of the pilus to polymerize.

Table 1. Prevalence of positively selected FimH residues.

Residue[a]	Frequency in healthy feces[b] (%)	Frequency in urine/ periurethra[b] (%)	Sequenced Analog[c]
V27/S62/V163	5/33 (15)	49/254 (19)	MG1655/J96
A27/A62/V163	0	19/254 (7)	UTI89/NU14
A27/S62/A163	0	17/254 (7)	CFT073/536
A27/S62/V163	28/33 (85)	169/254 (67)	None

[a] All other possibilities not observed.

[b] 287 strains utilized from human ECOR and clinical *E. coli* isolates with 33 fecal samples from uninfected women and 254 infected urine/periurethral isolates.

[c] Published strain for which *fimH* sequence is available [34, 73, 87-90]. No fully sequenced analog of A27/S62/V163 is published.

Table 2. Affinity values and fits for Fim allele complexes

FimH allele[a]	FimCH Mean K_D (μM)	Mean Fit (R^2)	$FimC_{his}GH$ Mean K_D (μM)	Mean Fit (R^2)	Pathogenesis[b]	
					Acute	Chronic
Q133K	0	0.46	7193 ± 2957	0.94	-	--
A27/S62/V163	3.6 ± 0.2	0.99	3198 ± 1917	0.99	+	-
A27/S62/A163 (CFT073)	1.5 ± 0.8	0.99	252 ± 110	0.98	+	+
A27/A62/V163 (UTI89)	4.2 ± 0.6	0.99	119 ± 33	0.98	++	++
V27/A62/A163	3.0 ± 0.0	0.99	2.4 ± 0.2	0.98	-	--
A27/A62/A163	1.7 ± 0.0	0.99	7.0 ± 0.6	0.98	?	--

[a]Relative to UTI89 FimH, where applicable

[b]from figures 2, 3, 5D, or inferred based on data from Chen *et al* 2009.

Supplementary Material

Supplemental Methods

In vitro bacterial assays.

Guinea pig hemagglutination assays (HA) were conducted as previously described [91] with minor modifications. Bacteria were grown 2 x 24 hours in 10 mL LB at 37^{o}C statically. Bacteria were centrifuged 6500 RPM for 10 min. and resuspended in PBS to an OD_{600} of 1.0. Guinea pig serum (Colorado Serum) was washed with PBS and diluted to OD_{640} of 1.9-2.0. Twenty-five μL of guinea pig blood was added to each well. The HA titer reported is the well containing the last amount of visible RBC agglutination. Invasion assays were similar to those previously described [92, 93]. Bladder 5637 cells were grown to confluence and split such that 1 – 5 x 10^5 cells were in each well in RPMI. UPEC was added to each well at a multiplicity of infection (m.o.i.) of 10. After centrifugation, we aspirated the media and replaced it with RPMI supplemented with 120 μg/mL gentamicin (Sigma) and incubated for 60 min at 37 ^{o}C. Bacteria were serially diluted and plated to LB-agar plates with the appropriate antibiotics after eukaryotic lysis with 1% Triton X-100. Biofilm assays were conducted in LB in PVC plates and quantified by Crystal Violet at 600 nm as described previously [83, 94].

Protein Purification

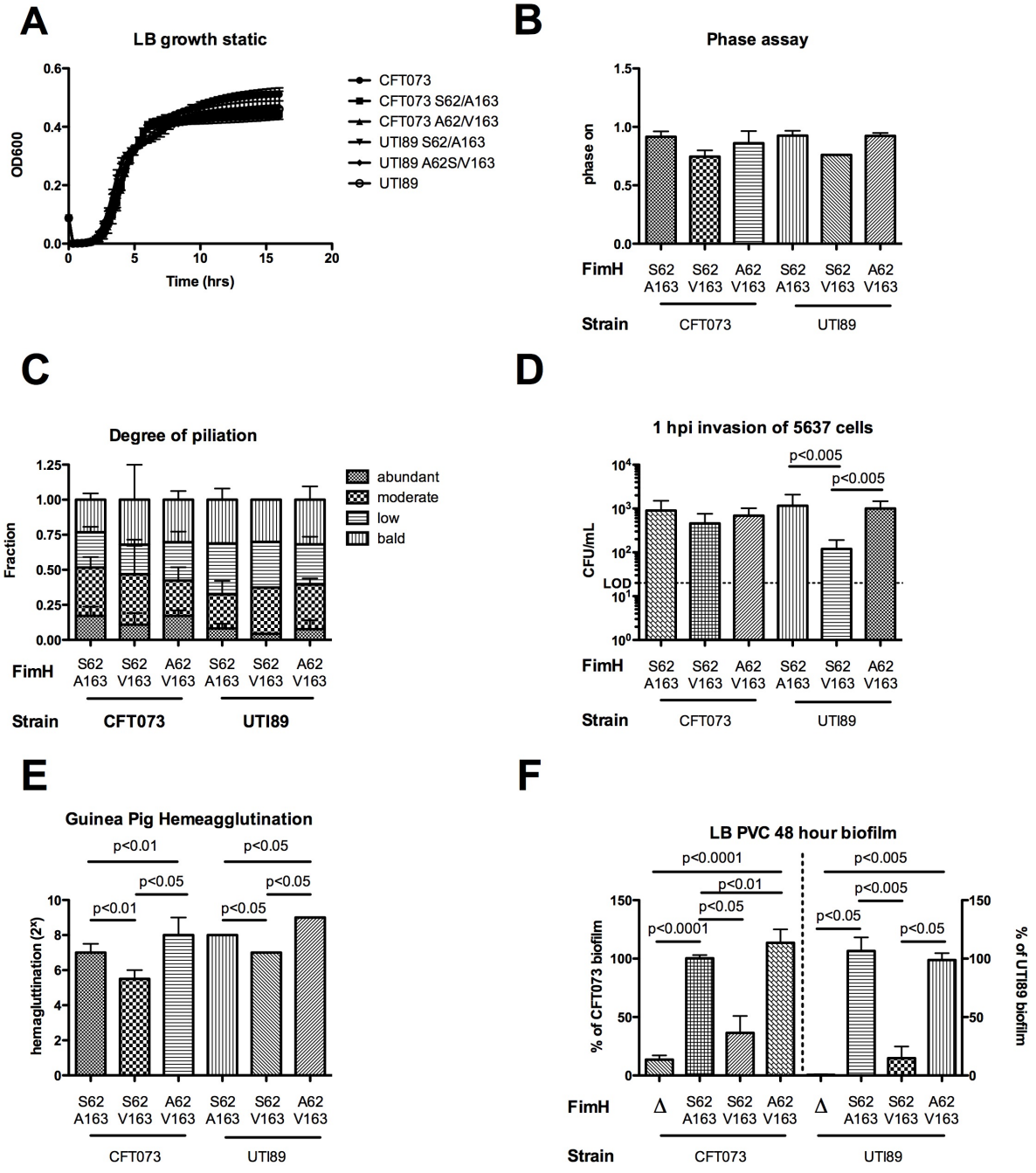
FimCH complexes were expressed in *E. coli* C600 with a pBAD33 plasmid containing the appropriate *fimH* allele and pTRC99a plasmid containing UTI89 *fimC*. The protein sequence of FimC is identical for UTI89 and CFT073 [34, 95]. Periplasm was prepared as previously described [96]. The supernatant was purified over columns as previously described [97] and

cleaned on an additional Source 15S column in 15mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.8 in the absence of mannose. His-tagged FimC was co-expressed on pETS1000 with FimG expressed on pETS2A and $FimC_{his}G$ purified essentially as previously described [64]. Periplasm was dialyzed with PBS and 250 mM NaCl overnight. A Talon column (Clontech) was run with a gradient of PBS with 300 mM imidazole. Pooled fractions were dialyzed against 15 mM MES at pH 5.8 overnight. A 2nd Source column (15S GE) was run with a gradient of 15 mM MES pH 5.8 with and without 400 mM NaCl. For generation of $FimC_{his}GH$ variant complexes, FimCH variant complexes were mixed with $FimC_{his}G$ complexes in equimolar ratios and incubated at 4 ^{o}C for 18-20 hrs.

Biolayer Interferometry

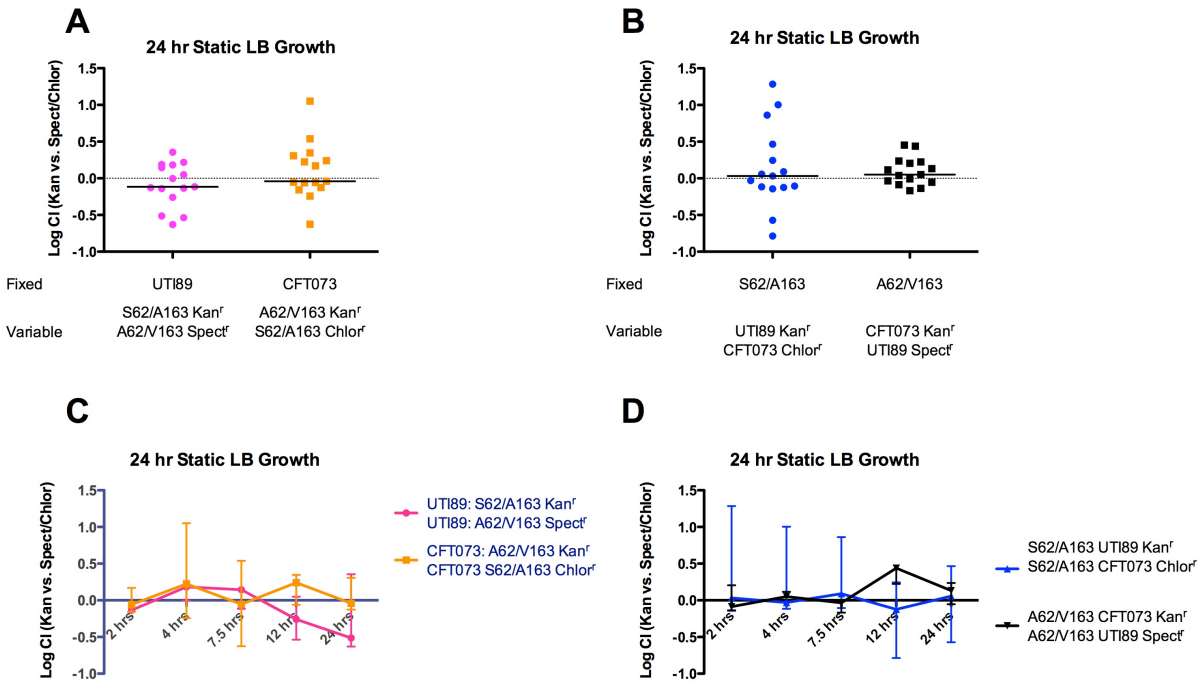
For BLI experiments, SuperStreptavidin (SSA) pins were first dipped in a baseline in HEPES-Buffered Saline (HBS) pH 7.5 for 120 seconds, followed by loading of 10 μg/ml biotinylated BSA-mannose (DEXTRA) in HBS for 240-300 seconds, quenching by 10 μg/ml biocytin in HBS for 180 seconds, and another baseline step in HBS for 120 seconds. Thereafter, pins were dipped in HBS + 1% BSA + 0.05% TWEEN-20 to block non-specific interactions for 300 seconds, transferred to protein samples (either FimCH or $FimC_{his}GH$ variants) for association for 300-600 seconds, and moved to HBS for dissociation for 300 seconds. K_D values for pilot $FimC_{his}GH$ experiments were not included for FimH::A27/A62/V163, FimH::A27/S62/A163, FimH::A27/S62/V163, and FimH::Q133K because determined K_D values were greater than 10-fold above the highest tested concentration, below the limit of detection of the instrument. R^2 values greater than 0.98 and 0.90 were included in the analysis for FimCH and $FimC_{his}GH$ complexes, respectively.

Supplementary Figures



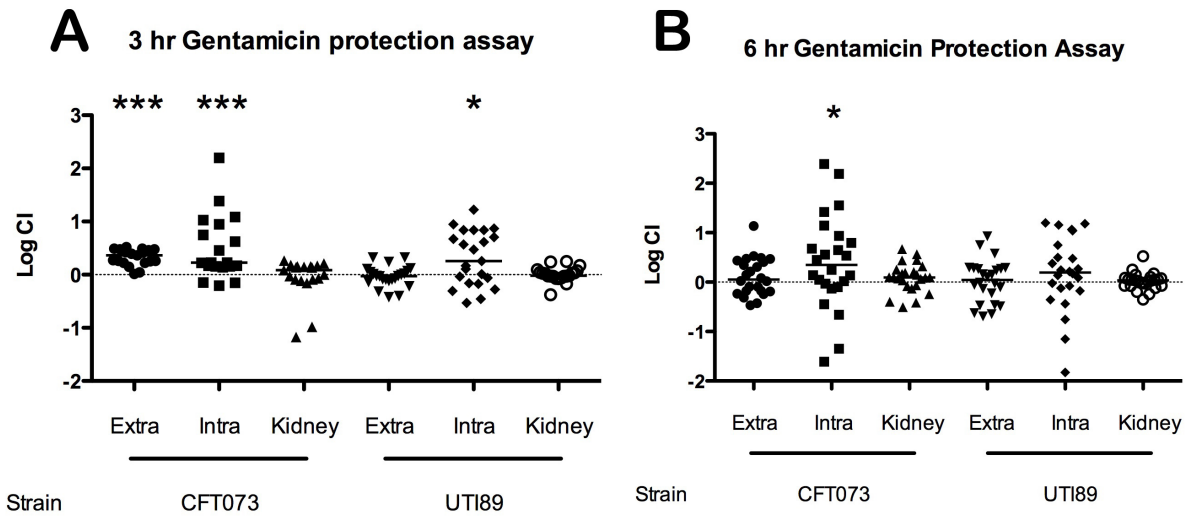
Supplementary figure 1: in vitro effects of fimH allele

A) Growth was determined for 16 hrs in LB in 96 well plates. B) PCR was conducted on 1 μL of bacteria grown 2 x 24 hrs in static culture at 37 ^{o}C and graphed as the fraction of the bacterial population with the *fim* promoter in the ON orientation. C) Degree of piliation was assessed by TEM for each strain. Fraction of cells expressing no (bald), 1-20 (low), 20-100 (moderate) or >100 (abundant) pili per cell. Represents 2 pooled experiments. D) Titers of bacterial strains after 1 hr invasion into cultured 5637 cells is shown. Data are pooled from 3 biological replicates with 3 technical replicates each. E) Guinea pig RBC HA in the absence of mannose was conducted for the strains indicated. Titer is listed as the last well showing visible agglutination. Pooled data from 2-9 biological replicates with 2 technical replicates each. F) Strains were grown in LB statically at room temperature in a 96-well PVC plate for 42-45 hrs to evaluate biofilm formation. Percent of biofilm relative to UTI89 is displayed. 3 biological replicates with 6-8 technical replicates each are pooled.



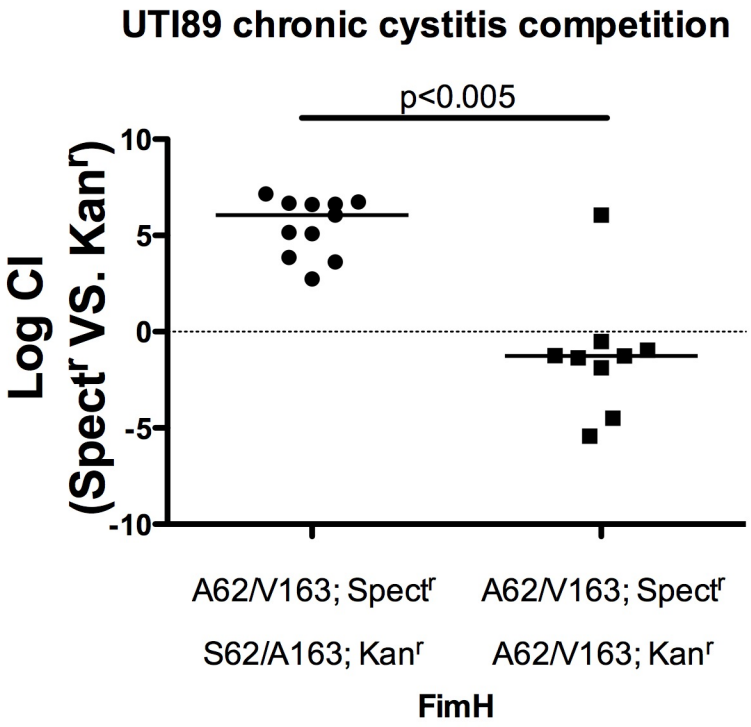
Supplementary Figure 2: in vitro *LB co-infections*

One μL of a 2 x 24 LB culture was inoculuated after dilution into 10 mL LB and allowed to grow statically for 24 hrs. Two hundred microliters was taken 2, 4, 7.5, 12, and 24 hrs after inoculation and plated to selective media. A) All Log_{10} CIs are graphed for a sample co-inoculated with UTI89 FimH::S62/A163 Kan^r and UTI89 FimH::A62/V163 $Spect^r$ in magenta. Orange is a co-inoculation of CFT073 FimH::A62/V163 Kan^r and CFT073 FimH::S62/A163 $Chlor^r$. B) All Log_{10} CIs are graphed for a sample co-inoculated with UTI89 FimH::S62/A163 Kan^r and CFT073 FimH::S62/A163 $chlor^r$ (blue). Black squares represent all timepoint Log_{10}CIs of a co-inoculation of CFT073 FimH::A62/V163 Kan^r and UTI89 FimH::A62/V163 $Spect^r$. C-D) Log_{10}CI for each strain comparison over time for panels A and B, respectively.



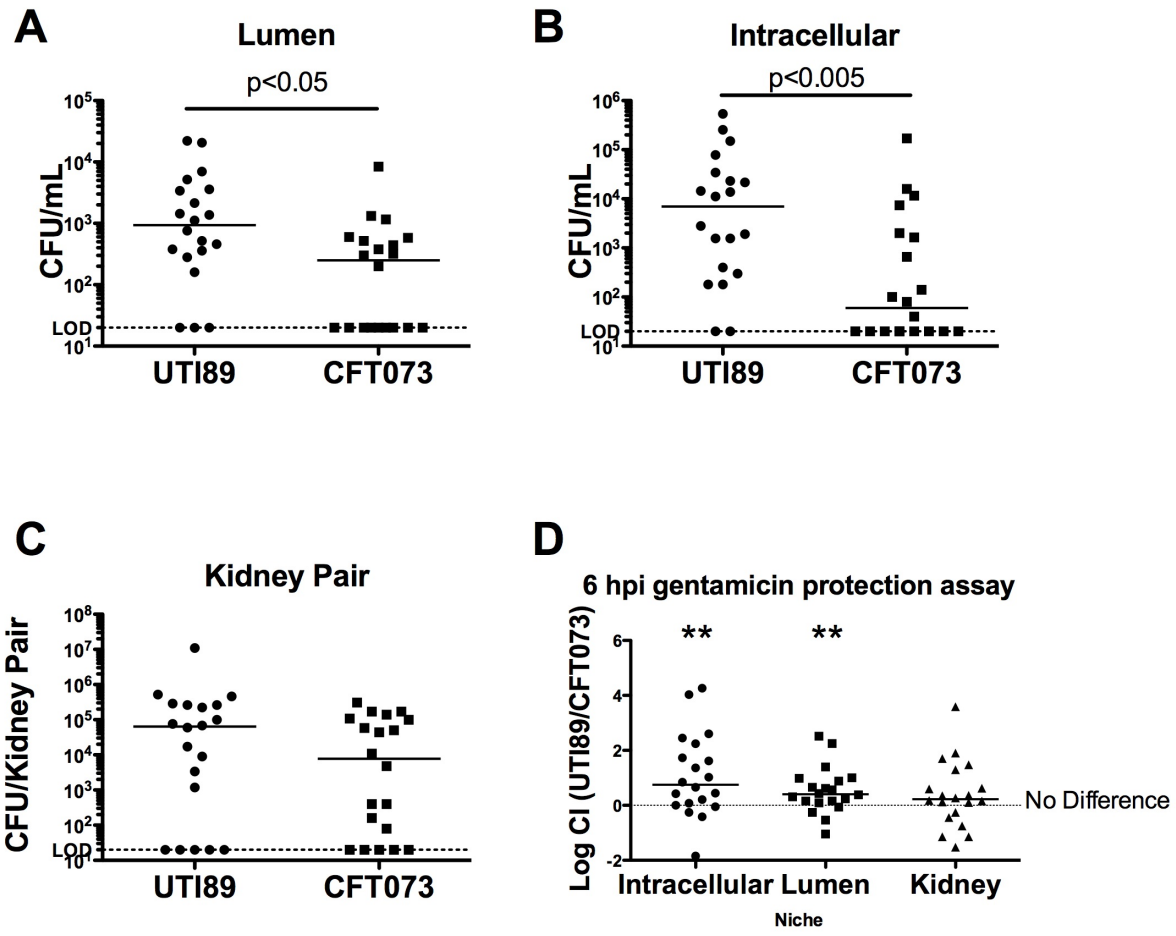
Supplementary Figure 3: FimH allele modulates acute niche occupation

Ex vivo gentamicin protection assay was conducted on bladders extracted at 3 and 6 hpi. $Log_{10}CI$ of FimH::A62/V163 relative to FimH::S62/A163 is shown for 3 (A) and 6 (B) hpi for competitions in CFT073 and UTI89 backgrounds. Wilcoxon Signed Rank, *, $p<0.05$; **, $p< 0.01$; ***, $p<0.005$.



Supplementary Figure 4.

Log_{10}CI of chronic bladder titers graphed for a co-infection between isogenic strains differing by antibiotic marker. Data in the left column reproduced from Figure 3A representing the effect of FimH on chronic fitness. Data in the right column is a control co-infection of isogenic strains differing only by antibiotic resistant marker.



Supplementary Figure 5: UTI89 outcompetes CFT073 during acute infection

7-8 week old female C3H/HeN were infected with a 50 μL inoculum containing CFT073 and UTI89 totaling 2 x 10^7 CFU (range 1.46 x 10^7 – 2.2 x 10^7). At 6 hpi, mice were sacrificed, and an *ex vivo* gentamicin protection assay was conducted on the bladder. A) Bacterial titers present in the lumen were enumerated by plating onto antibiotic selective agar plates. B) Intracellular UPEC were enumerated after gentamicin treatment. C) Kidney pairs were homogenized and plated. D) Log_{10} CI were computed for UTI89 titers relative to CFT073 to control for varying inoculum concentrations. Data is pooled from 2 experiments with 2 competitions of 5 mice each

where UTI89 Kan^R and CFT073 chlor^R were co-infected as well as UTI89 chlor^R and CFT073 Kan^R. A-C) statistical analysis was conducted using Mann-Whitney U-test. D) Wilcoxon Signed rank relative to zero was determined. **, $p<0.01$.

Chapter 4: Uropathogenic *Escherichia coli* Superinfection Enhances the Severity of Mouse Bladder Infection

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PLoS Pathogens 2015 11(1). doi:10.1371/journal.ppat.1004599. PMID25569799

Abstract

Urinary tract infections (UTIs) afflict over 9 million women in America every year, often necessitating long-term prophylactic antibiotics. One risk factor for UTI is frequent sexual intercourse, which dramatically increases the risk of UTI. The mechanism behind this increased risk is unknown; however, bacteriuria increases immediately after sexual intercourse episodes, suggesting that physical manipulation introduces periurethral flora into the urinary tract. In this paper, we investigated whether superinfection (repeat introduction of bacteria) resulted in increased risk of severe UTI, manifesting as persistent bacteriuria, high titer bladder bacterial burdens and chronic inflammation, an outcome referred to as chronic cystitis. Chronic cystitis represents unchecked luminal bacterial replication and is defined histologically by urothelial hyperplasia and submucosal lymphoid aggregates, a histological pattern similar to that seen in humans suffering chronic UTI. C57BL/6J mice are resistant to chronic cystitis after a single infection; however, they developed persistent bacteriuria and chronic cystitis when superinfected 24 hours apart. Elevated levels of interleukin-6 (IL-6), keratinocyte cytokine (KC/CXCL1), and granulocyte colony-stimulating factor (G-CSF) in the serum of C57BL/6J mice prior to the second infection predicted the development of chronic cystitis. These same cytokines have been found to precede chronic cystitis in singly infected C3H/HeN mice. Furthermore, inoculating C3H/HeN mice twice within a six-hour period doubled the proportion of mice that developed chronic cystitis. Intracellular bacterial replication, regulated hemolysin (HlyA) expression, and caspase 1/11 activation were essential for this increase. Microarrays conducted at four weeks post inoculation in both mouse strains revealed upregulation of IL-1 and antimicrobial peptides during chronic cystitis. These data suggest a mechanism by which caspase-1/11 activation and

IL-1 secretion could predispose certain women to recurrent UTI after frequent intercourse, a predisposition predictable by several serum biomarkers in two murine models.

Author Summary

Urinary tract infections (UTIs) affect millions of women each year resulting in substantial morbidity and lost wages. Approximately 1.5 million women are referred to urology clinics suffering from chronic recurrent UTI on a yearly basis necessitating the use of prophylactic antibiotics. Frequent and recent sexual intercourse correlates with the development of UTI, a phenomenon referred to clinically as "honeymoon cystitis." Here, using superinfection mouse models, we identified bacterial and host factors that influence the likelihood of developing chronic UTI. We discovered that superinfection leads to a higher rate of chronic UTI, which depended on bacterial replication within bladder cells combined with an immune response including inflammasome activation and cytokine release. These data suggest that bacterial inoculation into an acutely inflamed urinary tract is more likely to lead to severe UTI than bacterial presence in the absence of inflammation. Modification of these risk factors could lead to new therapeutics that prevent the development of recurrent UTI.

Introduction

Nearly nine million people present each year to primary care physicians with a urinary tract infection (UTI), costing nearly $2 billion yearly [1, 2]. Women suffer the majority of these infections, with the lifetime risk approaching 50% [3]. Furthermore, 25-40% of these women will suffer recurrent UTI (rUTI), with 1.5 million women referred to urology clinics and often requiring prophylactic antibiotics to prevent recurrence [4-6]. Uropathogenic *E. coli* (UPEC) are responsible for >80% of community acquired UTI and 50% of nosocomial UTI [7, 8]. In the absence of antibiotic therapy, up to 60% of women experience symptoms and/or bacteriuria lasting months after initial infection [9-12], implying that cystitis is not always self-limiting. Furthermore, if the infection persists without adequate treatment, the organisms have the capacity to ascend the ureters, causing pyelonephritis and sepsis [13]. Antibiotic resistant organisms further complicate infection and threaten to increase the likelihood of chronic UTI, pyelonephritis and potentially bacteremia [14, 15]. UTIs are increasingly being treated with fluoroquinolones, which in turn has led to a rise in resistance and the spread of multi-drug resistant microorganisms globally, which is a looming worldwide crisis [16, 17]. It is therefore imperative to understand the molecular mechanisms that underlie this problematic disease in order to develop novel therapies.

Sexual intercourse is one of the most significant risk factors predisposing otherwise healthy women to UTI. Early studies demonstrated that sexual intercourse led to a 10-fold increase in bacteria/ml of urine and a subsequently increased predisposition to developing a UTI within 24 hours thereafter [5, 18-21]. More recent studies have shown that the frequency with which a woman has sexual intercourse dramatically impacts the likelihood of developing both acute and rUTI [4, 22, 23]. Scholes *et. al* found a direct association between the number of

episodes of sexual intercourse in a given month and the risk of developing rUTI. However the significance of the timing between these episodes of sexual intercourse is unknown. Are evenly spaced episodes associated with an equal risk or, instead, does an episode prime the bladder for rUTI if another insult follows within a sensitive period? To address this question, we developed a model of sequential infection in mice to explore the hypothesis that a sensitive period exists after an initial bacterial insult to the bladder in which the likelihood of developing severe, chronic infection is dramatically increased.

Murine models of UTI have been used to decipher complexities of this disease in naïve individuals. UPEC are capable of colonizing multiple body habitats and niches, including both intracellular and extracellular locations within the bladder, as well as in the gastrointestinal (GI) tract and the kidneys. Selective pressure and bacterial population bottlenecks during colonization impact the ultimate fate of disease [24-27]. Adhesive pili assembled by the chaperone/usher pathway (CUP), such as type 1 pili, contain adhesins at their tips that function in adherence and invasion of host tissues and in biofilm formation on medical devices. Upon introduction of UPEC into the bladder, bacteria bind to either mannosylated uroplakin plaques or b1-a3 integrin receptors on the epithelial surface of the bladder via the type 1 pilus FimH adhesin [28-30]. Upon internalization, UPEC can be exocytosed as part of a TLR4 dependent innate defense process [31]. In addition to expulsion of individual bacteria, the host can exfoliate superficial facet cells to shed attached and invaded bacteria into the urine for clearance [29]. A small fraction of invaded bacteria escape into the host cell cytoplasm, where they are able to subvert expulsion and innate defenses by replicating into biofilm-like intracellular bacterial communities (IBCs) [24, 32]. UPEC eventually flux out of these communities with a substantial proportion existing as neutrophil resistant filaments [33, 34]. Importantly, evidence of IBCs and bacterial

filaments have been observed in women suffering acute UTI, one to two days post self-reported sexual intercourse, but not in healthy controls or infections caused by Gram-positive organisms, which do not form IBCs [21]. IBCs have also been observed in urine from children with an acute UTI [35]. Additionally, IBC formation and the innate immune response of cytokine secretion and exfoliation have been observed in all tested mouse strains, but the long-term outcome of infection differs [36-38].

There are two main, mutually exclusive, outcomes to acute infection in C3H/HeN mice: either chronic bacterial cystitis (chronic cystitis), which is characterized by persistent high titer bacteriuria (>10^4 CFU/ml) and high titer bacterial bladder burdens (>10^4 CFU) two or more weeks after inoculation, accompanied by chronic inflammation [37, 39], or resolution of bacteriuria [37]. Mice that resolve infection may harbor small populations of dormant UPEC called Quiescent Intracellular Reservoirs (QIRs) [40]. Other mouse strains exhibit varied proportions of these two outcomes. C57BL/6J mice resolve bacteriuria within days and thus are resistant to chronic cystitis, but are susceptible to QIR formation [40, 41]. In contrast, other TLR4-responsive C3H background sub-strains and closely related CBA/J and DBA/2J mice experience persistent high-titer bacteriuria and bladder colonization by UPEC in the presence of chronic inflammation lasting at least four weeks post-infection (wpi). During chronic cystitis, persistent lymphoid aggregates and urothelial hyperplasia with lack of superficial facet cell terminal differentiation accompany luminal bacterial replication [37]. These same histological findings of submucosal lymphoid aggregates and urothelial hyperplasia have been observed in humans suffering persistent bacteriuria and chronic cystitis [42]. Since murine chronic cystitis predisposes to recurrent chronic UTI after antibiotic-mediated bacterial clearance, this is also a relevant model to interrogate the mechanism of recurrent cystitis [37]. In mouse models of UTI,

mice initially experience urinary frequency and dysuria as determined by reaction to noxious stimuli and nerve responses during acute infection [43, 44]; however, during chronic cystitis bacterial replication may exist in an asymptomatic carrier state as studies have not been conducted to determine whether dysuria persists. Interestingly, higher serum levels of interleukins (IL) 5 and 6, keratinocyte cytokine (KC/CXCL1), and granulocyte colony-stimulating factor (G-CSF) in C3H/HeN mice at 24 hours post infection (hpi) predicted the development of persistent bacteriuria and chronic cystitis thereafter, suggestive of a host-pathogen checkpoint during acute infection that predicts long term outcome [26, 37]. In women with an acute UTI, increased amounts of serum CXCL1, M-CSF, and IL-8 correlated with subsequent rUTI, suggesting a similar checkpoint [45].

In this manuscript, we developed a superinfection model to mimic the clinical scenario of frequent sexual intercourse whereby sequential inocula are introduced within a brief period of time. C57BL/6J mice are resistant to chronic cystitis when singly infected; however, 30% of C57BL/6J mice developed chronic cystitis when superinfected 24 hours after the initial infection. Serum elevations of IL-6, KC, and G-CSF prior to superinfection predicted the development of persistent bacteriuria in C57BL/6J mice similar to singly infected C3H/HeN mice. Superinfecting C3H/HeN mice 1-6 hours after the initial inoculation increased the proportion of mice experiencing chronic cystitis. In order for this elevation to occur, we found that the initial UPEC inoculum (the “priming” inoculation) must be alive, invasive, capable of intracellular replication, and able to regulate hemolysin expression. Inhibition of the caspase 1/11 inflammasome prior to priming reduced bacterial CFU at four wpi relative to DMSO-treated mice. Microarray analysis of mouse bladders four wpi revealed that both C57BL/6J and C3H/HeN mice secreted antimicrobial peptides and IL-1 during chronic infection. In contrast to

C3H/HeN mice, immunoglobulin expression was upregulated in C57BL/6J mice experiencing chronic cystitis. This immunoglobulin expression was absent in C57BL/6J mice that resolved infection and in C3H/HeN mice. Our data suggest mechanisms whereby certain women may be susceptible to rUTI after frequent sexual intercourse dependent on intracellular bacterial replication and the host immune response.

Results

Time-sensitive enhancement of infection

Studies suggest that a host-pathogen checkpoint within the first 24 hpi determines UTI outcome in C3H/HeN mice [26, 37]. In addition, the chronic inflammation observed in mice experiencing chronic cystitis was found to predispose to rUTI after re-infection [37]. Thus, we hypothesized that superinfecting mice during this period of acute inflammation would increase the proportion of mice experiencing chronic cystitis. We transurethrally infected 7-8 week old female C3H/HeN mice with 10^7 CFU UTI89 or PBS as the priming inoculation and superinfected them 1-2, 6, or 24 hours thereafter. Enumeration of bacterial CFU at one wpi as an initial screen revealed a dramatic increase in the proportion of mice experiencing chronic cystitis in mice superinfected 1-6 hours after priming compared to singly infected or PBS treated mice (Fig. 1A). We used a cutoff of 10^6 CFU to demarcate mice experiencing high-titer bacterial infection at one week. Importantly, we did not observe a significant increase in CFU when a single inoculum was doubled (2×10^7 CFU). Superinfection at 24 hpi had no effect on bacterial titers at one week, suggesting that the factors predisposing to increased susceptibility to chronic cystitis upon superinfection wane over time [26]. However, inoculation with PBS followed by UTI89 24 hpi did lead to high titers in 60% of mice. While this result is perplexing, it possibly

reflects that sacrifice six days post infection was not sufficient to delineate the typical bimodal distribution of outcomes [37]. The process of catheterization also induces inflammation, which may not have resolved by 6 dpi [46]. We conducted all subsequent C3H/HeN superinfections one hour after priming.

Since early severe inflammatory responses predispose to chronic cystitis [37], we hypothesized that the initial inoculum primed the bladder by initiating an innate immune response to intracellular bacteria that predisposed to a higher proportion of mice experiencing chronic cystitis upon superinfection. We utilized a panel of UTI89 mutants in *fimH*, *ompA*, and *kps* that have been shown to differ in their ability to: i) invade and form IBCs and ii) persist during chronic cystitis in co-infection experiments [47, 48]. Mature IBCs caused by WT bacteria are clonally derived from a single invasive event [24]. The mannose-binding pocket of FimH is invariant among sequenced UPEC [47], and the binding pocket mutant, FimH::Q133K, is defective in mannose-binding and can neither invade the bladder epithelium nor form IBCs. FimH undergoes compact and elongated conformational changes wherein the receptor binding domain bends approximately 37° with respect to the pilin domain. The mannose-binding pocket is deformed in the compact conformation whereas the elongated conformation is mannose binding proficient [49, 50]. Several residues outside the mannose-binding pocket (positions 27, 62, 66 and 163) are under positive selection in clinical UPEC isolates compared to fecal strains [47] and have been shown to function in modulating the conformational changes between the elongated and compact states [48]. FimH::A27V/V163A predominantly adopts a high-mannose binding, elongated conformation. Its expression results in: i) a 10-fold reduction in intracellular CFU one hpi and ii) a defect in the ability to form IBCs at six hpi. FimH::A62S shifts the equilibrium towards the compact conformation. Expression of this allele results in: i) a 10-fold

reduction in intracellular CFU one hpi and ii) a 10-fold reduction in IBC formation compared to WT UTI89 [47, 48]. UTI89Δ*ompA* forms half the number of IBCs as UTI89 [51], and UTI89Δ*kps* is defective in IBC formation. UTI89Δ*kps* can replicate intracellularly and the IBC defect can be rescued by co-inoculation with WT UTI89, which results in mixed strain, non-clonal, IBCs [52].

We primed mice with these strains and superinfected one hpi with WT UTI89 and assessed bacteriuria at days 1, 7, 14, and 21 and enumerated bladder titers at 28 dpi. Mice were designated as having chronic cystitis if they had urine bacterial titers greater than 10^4 CFU/ml at each time point *and* bladder titers greater than 10^4 CFU at sacrifice [37]. We found that the FimH::A27V/V163A allele was incapable of priming the bladder for the development of chronic cystitis ($p<0.05$ relative to WT superinfection). In contrast, FimH::A62S did not significantly differ from PBS or WT superinfection; therefore, it may be capable of priming, though to a lesser degree. UTI89Δ*ompA* and UTI89Δ*kps* were both able to prime the bladder for enhanced chronic cystitis relative to PBS when superinfected one hpi with WT UTI89 ($p<0.05$ and $p<0.01$ respectively; Fig. 1C). We also primed with heat-killed UTI89 and found that live, but not heat killed, UTI89 were capable of priming the bladder indicating that bacterial products such as LPS were insufficient (Fig. 1B). These data indicate that live and invasive UTI89 capable of at least some degree of intracellular replication are required for the priming to enhance the incidence of chronic cystitis upon superinfection of UTI89. Taken together these data suggest that priming begins during invasion and early IBC formation.

UPEC Hemolysin and Caspase 1/11 activation are essential

One of the most potent host defenses to eliminate adherent and invaded UPEC is superficial facet cell exfoliation [29]. The process of exfoliation is activated in part by the bacterial expression of hemolysin (HlyA) [53](Nagamatsu *et al.* in review). UTI89Δ*cpxR* overexpresses HlyA, leading to exfoliation and attenuation in our murine model of cystitis (Nagamatsu *et al.* in review). The UTI89Δ*cpxR*Δ*hlyA* double mutant was not attenuated, suggesting that the *in vivo* defect was due to increased hemolysin expression (Nagamatsu *et al*. in review). The ability of UPEC to rapidly build up in numbers in the form of IBCs and then disperse to neighboring cells may be part of a mechanism to subvert an exfoliation response. Thus, fine-tuning the expression of HlyA during acute bladder infection may serve to maximize UPEC persistence and give UPEC a fitness edge against the host innate inflammatory response. Interestingly, in C3H/HeN mice, UTI89 Δ*hlyA* is not attenuated throughout infection and causes chronic cystitis comparable to UTI89; however, other reports suggest deletion of HlyA in UPEC CFT073 decreases virulence [54]. We investigated the role of hemolysin in priming the bladder for chronic cystitis upon superinfection by utilizing UTI89Δ*hlyA* or UTI89Δ*cpxR* as the initial inoculation followed by WT UTI89 one hpi. Both of these strains were statistically significantly different when compared to WT UTI89 as the priming inoculum. Therefore, we conclude that neither was capable of priming the bladder for enhanced chronic cystitis (Fig. 1D). Thus, too high or low expression of hemolysin abolished the ability of UTI89 to prime for enhanced chronic cystitis implying that an optimal level of hemolysin expression is critical for priming the bladder for enhanced chronic cystitis.

HlyA-mediated exfoliation is in part due to its ability to trigger degradation of paxillin, a scaffold protein that modulates the dynamics of cytoskeletal rearrangements [55]. HlyA can also

trigger cell death in human bladder epithelial cells and release of IL-1α via caspase-4 (the murine ortholog is caspase-11) activation and caspase-1-dependent IL-1β secretion via activation of the NLRP3 inflammasome pathway, which orchestrates additional cell death (Nagamatsu *et al.* in review). We hypothesized that inflammasome and caspase 1/11 activation were essential for superinfection. Thus, mice were treated intravesically with a dose of caspase 1/11 inhibitor or DMSO one hour prior to priming and a second dose with the priming inoculum to test this hypothesis (Fig. 2A). Providing two doses of the inhibitor was previously shown to be effective in dampening *in vivo* inflammatory responses. *In vitro*, the inhibitor dramatically reduced downstream elements of inflammasome activation, IL-1α and IL-1β secretion, when bladder cells were infected with UTI89 (Nagamatsu *et al.* in review). Caspase 1/11 inhibition significantly reduced median bladder titers at four weeks after superinfection relative to the DMSO control group (Fig. 2B). We also saw a trend of caspase 1/11 inhibition in reducing the proportion of WT superinfected mice experiencing chronic cystitis to single infection levels (Fig. 2B). DMSO also reduced the proportion of mice experiencing persistent bacteriuria and chronic cystitis, but to a lesser degree than caspase 1/11 inhibition (Fig. 2B vs. Fig. 1B-D), suggesting an anti-inflammatory role of DMSO alone. Intriguingly, DMSO was recently found to inhibit the NLRP3 inflammasome [56]. Taken together, these data implicate hemolysin and the NLRP3 inflammasome in the priming response to enhanced chronic cystitis.

We further investigated whether chemical exfoliation could enhance the proportion of mice experiencing chronic cystitis prior to a single infection. We utilized the cationic protein, protamine sulfate, which has previously been used to exfoliate the superficial facet cell layer of the urothelium [40, 57]. A 10 mg/mL dose delivered intravesically in 50 μL PBS was shown to exfoliate 65% of the facet cell layer 12 hours after treatment while an additional booster dose of

50 mg/mL led to 95% exfoliation [40]. We utilized these concentrations to initiate, but likely not complete, the process of exfoliation one hour prior to infection with UTI89. We did not observe a significant increase in the proportion of mice experiencing chronic cystitis over PBS pretreatment (Fig. 2C). Thus, these data suggest that at least partial IBC formation in conjunction with caspase 1/11 activation primes the bladder for enhanced chronic cystitis, but chemical initiation of exfoliation is not sufficient. Taken together, these data suggest that exfoliation per se might not play a significant role in impacting the likelihood of enhanced chronic cystitis but instead may reflect a downstream marker of the priming event.

Superinfection leads to chronic cystitis in a resistant mouse strain

C57BL/6J mice typically rapidly resolve bacteriuria and are resistant to chronic cystitis upon single inoculation with UPEC [37, 38]. Five to ten percent of the time after inoculation with UTI89, C57BL/6J mice experience persistent bacteriuria, but this is generally due to kidney infection without concomitant high titer bladder infection [37, 41]. This degree of kidney infection is not infectious dose dependent and therefore likely due to ureteric reflux of the bacteria during experimental inoculation [37]. We investigated whether superinfecting C57BL/6J mice during acute infection would stimulate an immune response leading to chronic cystitis. We inoculated bladders with PBS or 10^7 CFU of UTI89 followed by superinfection with UTI89 1, 6, 24, 48 hours or one week after initial infection and collected urine at days 1, 7, 14, and 21 dpi followed by enumeration of bladder and kidney titers at 28 dpi (Fig. 3). A 24 hpi superinfection resulted in 35% of mice sustaining persistent bacteriuria with bladder titers > 10^4 CFU at four weeks compared to 0% in the singly infected group (Fig. 3A). Kidney titers were also increased in the mice with persistent bacteriuria, but we did not observe a significant increase in the

proportion of mice with kidney infection greater than 10^4 CFU (Fig. 3B). These data suggest that at 24 hours after infection the bladders of C57BL/6J mice were primed to develop chronic cystitis upon superinfection. We investigated whether an ascending kidney infection plays a role in predisposing these mice to chronic cystitis by inoculating PBS into the bladder, either 24 hours before or after infection with UTI89, to stimulate a bladder and ureter stretch response or potentially increase reflux of bacteria into the kidneys, respectively. We determined the percentage of mice with persistent bacteriuria and those with bladder and kidney titers greater than 10^4 CFU at sacrifice (Table 1). We found in all conditions that persistent bacteriuria was a 100% predictor of kidney titers $>10^4$ CFU at four wpi. Persistent bacteriuria also predicted bladder titers greater than 10^4 CFU at four wpi in C57BL/6J mice superinfected 24 hpi with UTI89. For the group of mice inoculated with PBS before the initial UTI89 infection, persistent bacteriuria did not correlate with high bladder titers suggesting these bacteria were only replicating in the kidneys. Serially infecting with two inocula of UTI89 trended towards increased persistent bacteriuria and chronic cystitis compared to the group inoculated with UTI89 followed by PBS at 24 hpi ($P = 0.066$; Table 1 and Fig. 4A). Kidney titers of UTI89 superinfected mice were significantly higher than when PBS was used to prime or superinfect perhaps suggesting that repeat infection may also increase susceptibility to pyelonephritis (Fig. 4B). Thus, a 24 hpi superinfection of WT UTI89 led to increased rates of persistent bacteriuria and chronic cystitis; however, bladder/ureter stretch or kidney ascension at 24 hpi may contribute to this increase.

C3H/HeN mice that progress to chronic cystitis upon single inoculation can be predicted by elevated serum levels of IL-5, IL-6, KC, and G-CSF at 24 hpi [37]. We hypothesized that similar elevations would predict sensitization to chronic cystitis in C57BL6/J mice if they were

subsequently superinfected. Thus, we determined levels of 23 serum cytokines from C57BL/6J mice 24 hrs after initial inoculation with PBS or UTI89 prior to superinfection. We then superinfected a subset of the mice initially infected with UTI89 (superinfection in Figure 5) leaving the other mice untouched (UTI89 group). All mice were evaluated with urine titers over 28d and sacrificed to enumerate bladder titers. We stratified the superinfected mice based on outcome four weeks later as determined by persistent bacteriuria and chronic cystitis. We found elevations of serum KC (Fig. 5A), IL-6 (Fig. 5B), and G-CSF (Fig. 5C) in mice that progressed to chronic cystitis relative to those that resolved infection or were mock-infected with PBS. Therefore, higher levels of these cytokines correlate with chronic cystitis that develops later if mice are superinfected. At the time we obtained serum, the single infection and superinfection groups were identical, and no statistical differences existed among them. These data demonstrate that a subset of C57BL/6J mice respond to an initial infection in a way that results in higher specific serum cytokine levels and primes them to develop chronic cystitis if an additional insult is delivered 24 hpi.

Response to infection differs between C3H/HeN and C57BL/6J

During chronic cystitis of singly-infected C3H/HeN mice, the bladder epithelium is hyperplastic and normal terminal differentiation of the superficial facet cell layer, including the expression of surface uroplakins, does not occur [37]. In this environment, the bacteria are able to persist extracellularly by an unknown mechanism. To assess this, we conducted scanning electron microscopy analysis on bladder tissue harvested at four wpi and found that bacteria replicate in the presence of ongoing epithelial exfoliation and neutrophil influx in chronic cystitis of both C3H/HeN and C57BL/6J mice (Fig. S1A-D). This analysis supports previous

experiments that have shown that during chronic cystitis the majority of bacteria are extracellular, replicating in the urine or adherent to underlying transitional epithelial cells [24, 37]. The mechanism by which bacteria adhere in the absence of uroplakins has not been demonstrated *in vivo*, but *in vitro* studies have shown that FimH binds integrins and other host proteins such as TLR4 [30, 58, 59]. Alternatively additional adhesive factors such as other CUP pili may play a role. Interestingly, during chronic cystitis, neutrophils, which we observed to be actively engulfing bacteria, are insufficient for clearing infection; however, the reason for this is unclear. Mature superficial facet cells could not be discerned at this time point, but were present in mock-infected mice (Fig. S1E). Patients with persistent bacteriuria or rUTI have been reported to have similar histopathology [42]. In order to identify the bladder micro-environment in which UPEC replicate during chronic cystitis, we conducted microarray analysis on RNA extracted from bladders four wpi. C3H/HeN mice were singly-infected and C57BL/6J mice were superinfected to develop chronic cystitis. Mice from each strain inoculated with PBS were used as controls. Depicted in Figure 6 are the expression profiles relative to the global average with green indicating increased expression and red denoting decreased. C3H/HeN mice experiencing chronic cystitis had a dramatically different expression profile from resolved and mock-infected mice (Fig. 6A). Uroplakins were among the most downregulated genes during chronic cystitis in both mouse models, consistent with the lack of terminally differentiated superficial facet cells (Fig. S1). Eleven of the 20 (55%) most upregulated genes during chronic cystitis were the same in both mouse strains (Table S1). The functional categorization revealed that most of the up-regulated genes function in inflammatory response, cytokine release, and ion binding [60-62]. Of interest among these genes in both of these mouse models is the inflammasome-related cytokines IL-1. We have shown that UPEC activate the caspase 4 murine homologue, caspase 11, during

acute infection in a hemolysin-dependent fashion (Nagamatsu *et. al.* in review). Despite these similarities, interesting differences existed in the ongoing inflammatory response in mice experiencing chronic cystitis (Table S1). In C57BL/6J mice, the inflammatory response is immunoglobulin- and cytokine-mediated whereas in C3H/HeN mice, we noted a remarkable absence of upregulated immunoglobulin genes. The increased expression of antimicrobial peptides such as RegIIIγ and the calgranulins (s100a8 and s100a9) is interesting because this increased expression is not sufficient to eliminate bacterial replication during chronic cystitis. Interestingly, C3H/HeN mice that were mock infected exhibited a very similar profile to mice that resolve infection (Fig. 6A). Contrary to C3H/HeN mice, C57BL/6J mice that resolved infection differed significantly from either chronic cystitis or mock infected mice, suggesting an element of altered physiology and immunological memory of the infection (Fig. 6B). This information supports research that serially infecting mice that resolve infection makes them less susceptible to recurrent infection [37, 63]. What is interesting here is that the mechanisms by which this occurs may differ between mouse strains, and possibly by extension, women.

Discussion

We have developed models of bacterial superinfection of the urinary tract, which may provide insight into the connection between recent and frequent sexual intercourse and the susceptibility to the development of chronic UTI [5, 22]. Our results demonstrate that superinfection resulted in increased susceptibility to chronic cystitis in both susceptible and resistant mouse genetic backgrounds, but the time window for priming differed between strains. We have previously shown that chronic cystitis predisposes to severe rUTI upon a subsequent infection weeks to months after clearance of the first infection with antibiotics [37]. Clinically, millions of women take post-coital and prophylactic antibiotics so as not to develop rUTI [64]. Therefore, if clinically applicable, our results detailed here may partially explain why frequent sexual intercourse is such a strong risk factor for UTI. The necessity of prophylactic antibiotics could be obviated if the risk factors and bacterial traits identified here can be altered in the clinical population of women suffering chronic rUTIs.

Frequent sexual intercourse is among the most important risk factors for rUTI in young women [22]. Peri-urethral carriage of the causal strain and sexual intercourse immediately precede the development of a rUTI [5]. Sexual intercourse likely introduces mixed populations of bacteria into the urinary tract, with *E. coli* being the most common [18]. In this environment, UPEC invade bladder tissue and replicate, forming IBCs and bacterial filaments, which have been observed in human urine in 40% of patients suffering acute UTI, 24-48 hours after reported sexual intercourse [21]. These data may provide mechanistic insight as to the frequent clinical observation that recent and frequent sexual intercourse over a brief period of time leads to increased rates of rUTI [23]. Furthermore, elevated levels of serum CSF1, CXCL-1, and CXCL-8 in women with acute UTI were associated with a higher rate of rUTI [45]. Using C3H/HeN and

C57BL/6J mice, we have shown that superinfection during the period of acute infection dramatically increases the proportion of mice that experience chronic cystitis with inoculations of 10^7 UPEC (Fig. 1A and 3A). The bacterial characteristics responsible for frequent recurrences are beginning to be assessed [65]. Hemolysin is expressed by 50% of UPEC isolates, but is more likely to be associated with symptomatic UTI [66]. It is possible that hemolysin-mediated exfoliation and caspase 1/11 activation leads to UTI-associated symptoms. In our studies, we found that an increase in priming for chronic cystitis correlated with the bacterial ability to invade and replicate within the bladder tissue (Fig. 1B-C), and through hemolysin to activate caspase 1/11 leading to IL-1 secretion and bacterial replication (Fig. 1D and 2B). Activation of caspase 1/11 has been shown to contribute to epithelial cell death *in vitro* and exfoliation *in vivo* in C3H/HeN mice, suggesting that caspase-mediated exfoliation may expose the underlying epithelium upon which UPEC replicates during chronic cystitis (Nagamatsu *et. al.* in review). Inhibition of caspase 1/11 protected superinfected mice from chronic cystitis (Fig. 2), suggesting a role for cytokines downstream of caspase activation including IL-1α and IL-1β, identified in our microarray of four-week bladders (Fig. 6; Table S1). A microarray analysis revealed that in C3H/HeN and C57BL/6J mice, 11/20 of the most upregulated genes during chronic cystitis were the same. Differences between the responses to infection in these mouse strains may result from the dramatic increase in kidney infection or QIR presence in C57BL/6J relative to C3H/HeN mice [37, 40]. Further, this data supports the hypothesis that a muted inflammatory response to UPEC infection is more likely to lead to resolution [26]. Also, our studies suggest that serum biomarkers such as IL-6, KC, and G-CSF may predict a predisposition to rUTI (Fig. 5) [37]. Recently, it was demonstrated that cytokines involved in immune cell chemotaxis and maturation

(the human homolog of KC included) during acute UTI enhanced the likelihood of developing rUTI [45].

We have created mouse models that have identified both bacterial and host immune factors that may predispose women to rUTI. Inhibiting caspase-mediated inflammation or downstream effectors may serve to prevent a UTI from becoming a chronic or recurrent UTI. Further work to identify bacterial and host factors that influence the balance between resolution and chronic infection is required to lead to better treatments clinically. The ability of UPEC to invade bladder tissue allows it to transcend stringent bottlenecks during infection [24, 25, 27]. The ability to replicate intracellularly also impacts the ability of a second invading strain to proliferate in the bladder environment (Fig. 1B-C). The molecular basis of bacterial colonization of the bladder during chronic cystitis is an area of active investigation. Previously, it has been shown that mannosides are effective in treating chronic cystitis arguing that FimH-mediated binding plays an important role [67]. It has recently been demonstrated that FimH variation outside of the binding pocket affects protein conformation and pathogenicity of UPEC [48]. This variation may impact bacterial adherence and replication during chronic cystitis. Furthermore, because invasion and intracellular replication appear to influence the likelihood to develop chronic cystitis, treatments with soluble compounds such as mannosides that block the ability of UPEC to invade the tissue or compounds that might alter FimH conformation hold promise as effective means to prevent or treat rUTI [67-70]. These analyses may allow us to identify high-risk patients for more aggressive therapy and/or anti-virulence compounds to limit this troubling disease.

Materials and Methods

Bacterial strains

All WT bacterial strains utilized were derivatives of UTI89, including tagged, isogenic UTI89 isolates, kanamycin resistant UTI89 att_{HK022}::COM-GFP, kanamycin resistant UTI89 with re-integrated UTI89 FimH, spectinomycin resistant UTI89 att_{λ}::*PSSH10-1*, and chloramphenicol resistant UTI89 [24, 47, 71]. FimH mutant strains, *ΔompA*, *Δkps*, *ΔhlyA*, *ΔcpxR* were all previously published [47, 51, 52](Nagamatsu *et al*. in review).

Mouse infections

Bacteria for infection were prepared as previously described [72]. Six to seven week old female C3H/HeN (Harlan) or C57BL/6J (Jackson) were transurethrally infected with a 50 μL suspension containing 5 x 10^6 – 2 x 10^7 CFU of UTI89 or relevant mutant in PBS under 3% isofluorane. Protamine Sulfate (Sigma) was dissolved in PBS and caspase 1/11 inhibitor Ac-YVAD-CMK (BACHEM) was dissolved in DMSO and transurethrally inoculated into the bladder. At indicated timepoints after infection, mice were anesthetized and infected again. Venous blood was obtained at 24 hpi, just prior to re-infection, by submandibular puncture and centrifuged at max speed at 4 ^{o}C in Microtainer serum separation tubes (BD) and stored at -20 ^{o}C until use. Cytokine expression was measured using the Bio-Plex multiplex cytokine Group I bead kit array (Bio-Rad), which measures 23 cytokines. Urine was obtained by gentle suprapubic pressure and serially diluted and plated on appropriate antibiotic plates. Mice were sacrificed by cervical dislocation under isofluorane anesthesia, and their organs were aseptically removed. Chronic cystitis was determined if animals had urine titers > 10^4 CFU/mL at 1, 7, 14, 21 dpi and

bladder titers > 10^4 CFU at sacrifice at 28 dpi [37]. Animals that resolved infection and had a recurrence or had resolved the infection with reservoir titers >10^4 CFU were marked in red and considered to have resolved the chronic infection. Organ titers shown are the total bacterial burden.

Ethics statement

The Washington University Animal Studies Committee approved all mouse infections and procedures as part of protocol number 20120216, which was approved 01/11/2013 and expires 01/11/2016. Overall care of the animals was consistent with *The guide for the Care and Use of Laboratory Animals* from the National Research Council and the USDA *Animal Care Resource Guide*. Euthanasia procedures are consistent with the "AVMA guidelines for the Euthanasia of Animals 2013 edition."

Microarray experiments

C3H/HeN or C57BL/6J mice were infected as discussed above. After 28 days, animals that had developed chronic cystitis, resolved the infection, or aged matched PBS controls were sacrificed for RNA isolation. Upon sacrifice, 5 bladders from each condition were immediately pooled and homogenized in Trizol for RNA isolation according to the manufacture's suggested protocol. DNase treatment was performed to remove any contaminating DNA before submission to the Genome Technology Access Center for sample processing and hybridization on Affymetrix Mouse Gene 1.0 chips in triplicate. Data was analyzed using the Partek Genomics Suite. Gene lists were compiled using fdr-ANOVA analysis with a significance cut off of $p<0.001$. Experiments were repeated twice with a representative analysis shown. Microarray data

are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-2930.

Scanning electron microscopy

Mice were infected as described above. Bladders were aseptically harvested, bisected, and splayed. Bladders were fixed in 2.0% glutaraldehyde in 0.1M sodium phosphate buffer overnight. Bladders were then washed three times with 0.1M sodium phosphate buffer and de-ionized water before being fixed in 1.0% osmium tetroxide. Bladders were washed and then critical point drying was performed with absolute ethanol and liquid carbon dioxide. Sputter coating was performed with gold-palladium using a Tousimis Samsputter-2a. Images were obtained on a Hitachi S-2600H operated at 20kV accelerating voltage.

Statistical analysis

Datapoints below the limit of detection (LOD) were set to the LOD for graphical representation and statistical analysis. For cytokine data, values out of the range of the instrument were not included for analysis. Fisher's exact test was utilized to determine differences between groups for rates of chronic cystitis. One-way ANOVA was utilized to determine whether any cytokine differences were apparent and pairwise assessment of median values was determined by Mann-Whitney test. Unless otherwise indicated, $p<0.05$ was considered significant. Analyses were performed in Graphpad Prism 5.0.

Acknowledgements

We would like to thank Dan Liu and Aaron Lynch for help with urine collection and mouse infections. We would like to thank David Hunstad, Karen Dodson, and Kanna Nagamatsu for valuable ideas.

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Funding: We would like to thank the NIH for funding through grants F30 DK096751 to DJS, F32 DK101156 to MSC, K08 AI083746 to TJH, and SCOR P50 DK064540, R01 DK051406, and U01 AI095542 to SJH. The Scanning Electron Microscopy core facility is supported by NIH NIDCD grant P30DC04665. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Author contributions: Conceived and designed the experiments: DJS TJH SJH. Performed the experiments: DJS MSC TJH. Analyzed the data: DJS MSC TJH SJH. Contributed reagents/materials/analysis tools: MSC TJH. Wrote the paper: DJS SJH. Edited the manuscript: MSC TJH.

Figures and Tables

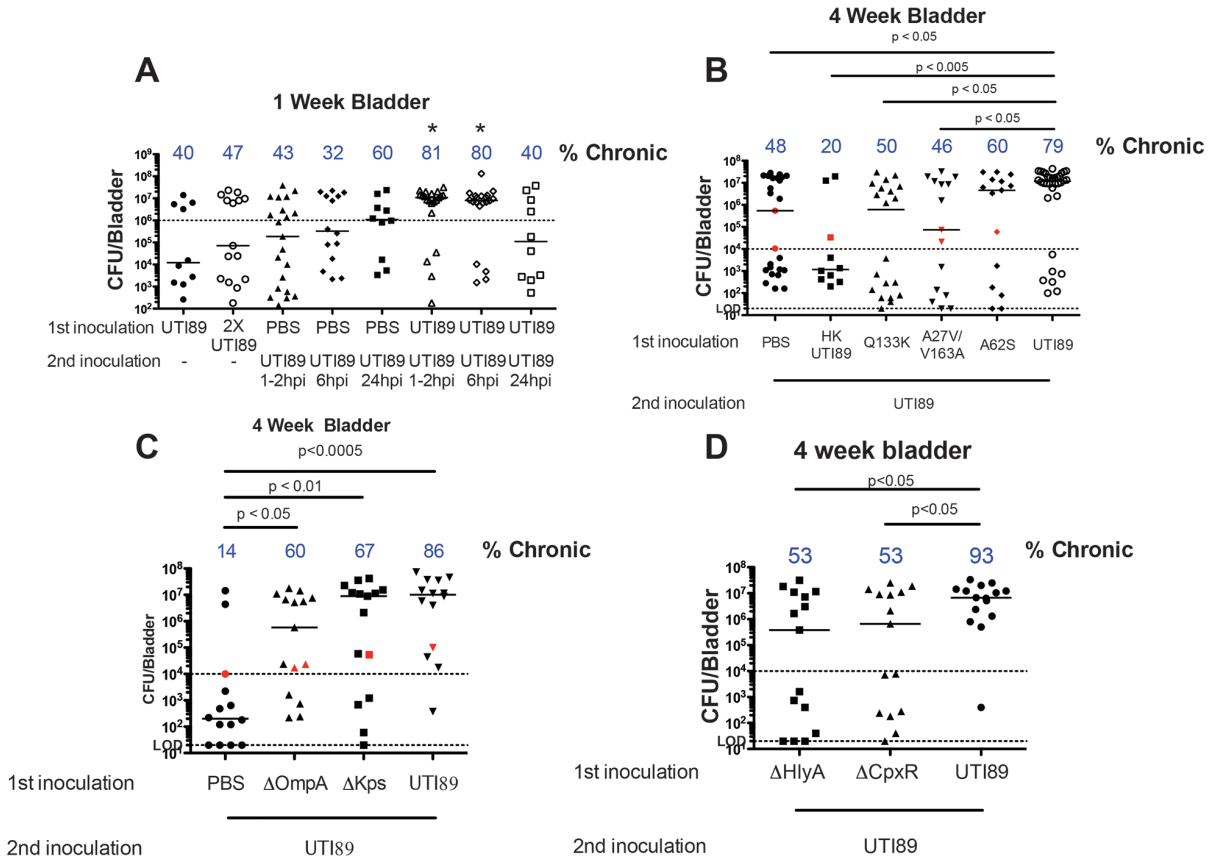

Figure 1. Superinfections of C3H/HeN mice.

A) Mice were infected with 10^7 CFU UTI89, 2 x 10^7 CFU UTI89, or PBS and re-infected with UTI89 at the indicated time points. One week total bladder titers are shown. Percentage of mice likely to develop chronic cystitis is displayed at the top of the column based on a CFU cutoff of 10^6. Asterisks indicate $p<0.05$ from the PBS control and singly infected mice. B-D) Mice were infected with the indicated strain or PBS and re-infected one hour later. Urine titers were determined over time and four-week bladder titers are displayed. The fraction of mice with chronic cystitis is displayed at the top of each column. Red data points indicate resolved infection. Horizontal bars indicate median values. The dashed line at 20 CFU represents the

LOD, and the dashed line at 10^6 (A) or 10^4 CFU (B-D) represents the chronic cystitis cutoff for urine and bladder titers. Panel A reflects 2-4 experiments with 5-9 mice per group. Panel B is 2-7 experiments with 4-5 mice per group. Panel C-D are 3 experiments with 4-5 mice per group. Statistical comparisons were determined using Fisher's exact test based on the fraction of mice experiencing chronic cystitis.

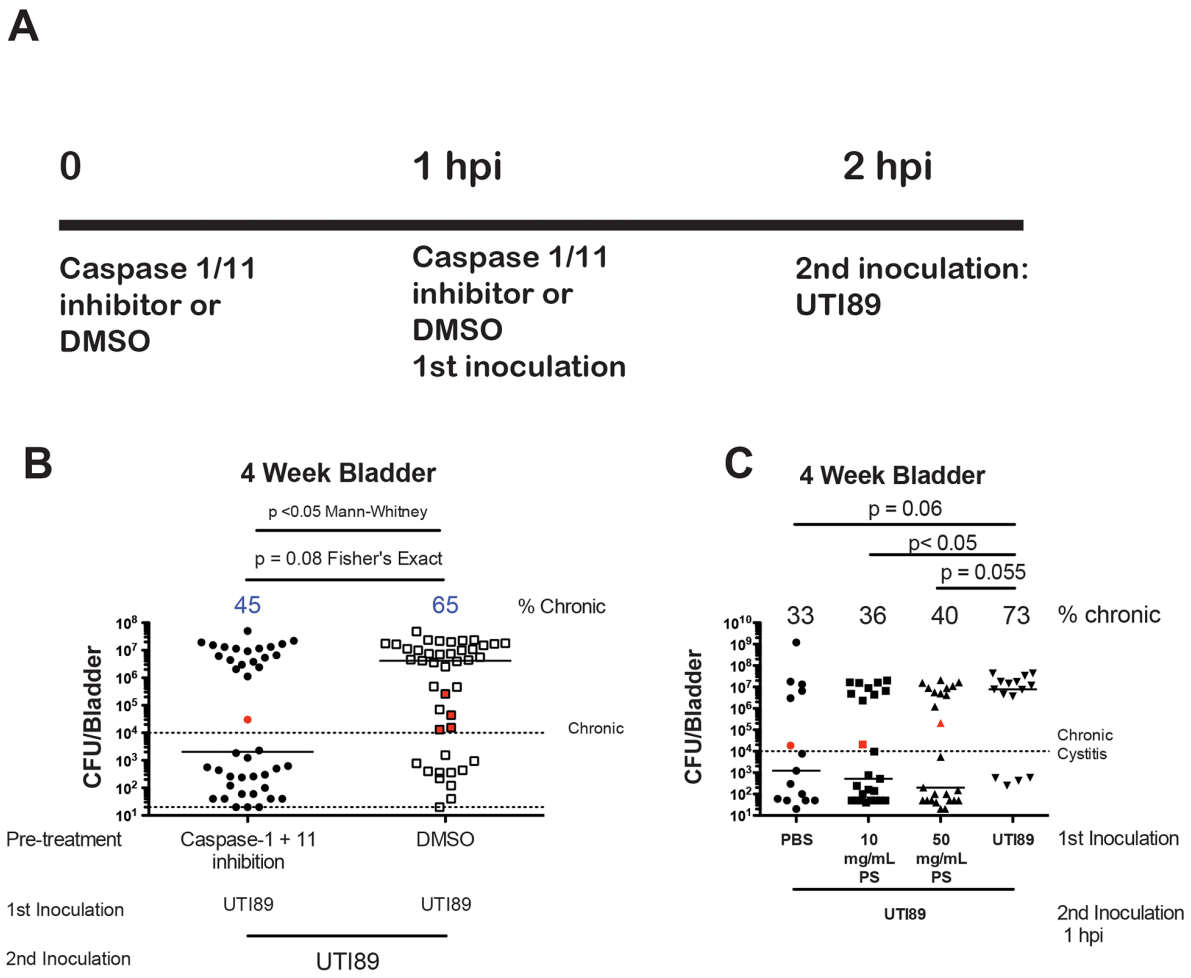

Figure 2. Role of caspase 1/11 and exfoliation in C3H/HeN superinfections.

A) Inoculation protocol shown for caspase inhibition studies of panel B. B) Four-week total bladder titer based on inhibitor or vehicle. C) Mice were inoculated with PBS, UTI89 or the indicated dose of protamine sulfate in 50 μL PBS and inoculated one hour later with UTI89. Urine was collected weekly and overall bladder titers are shown at four weeks. Panel B represents 5 experiments with n = 5-10 mice per group. Panel C represents 2 experiments with n = 5-10 mice per group. B-C) Observations in red indicate resolved infection. Percent of mice with persistent bacteriuria and chronic cystitis is shown at the top of each column. For Panel B

and C, a Fisher's Exact Test was used to determine significance between proportions of mice experiencing chronic cystitis. Mann-Whitney U Test was used to compare median CFU values in Panel B.

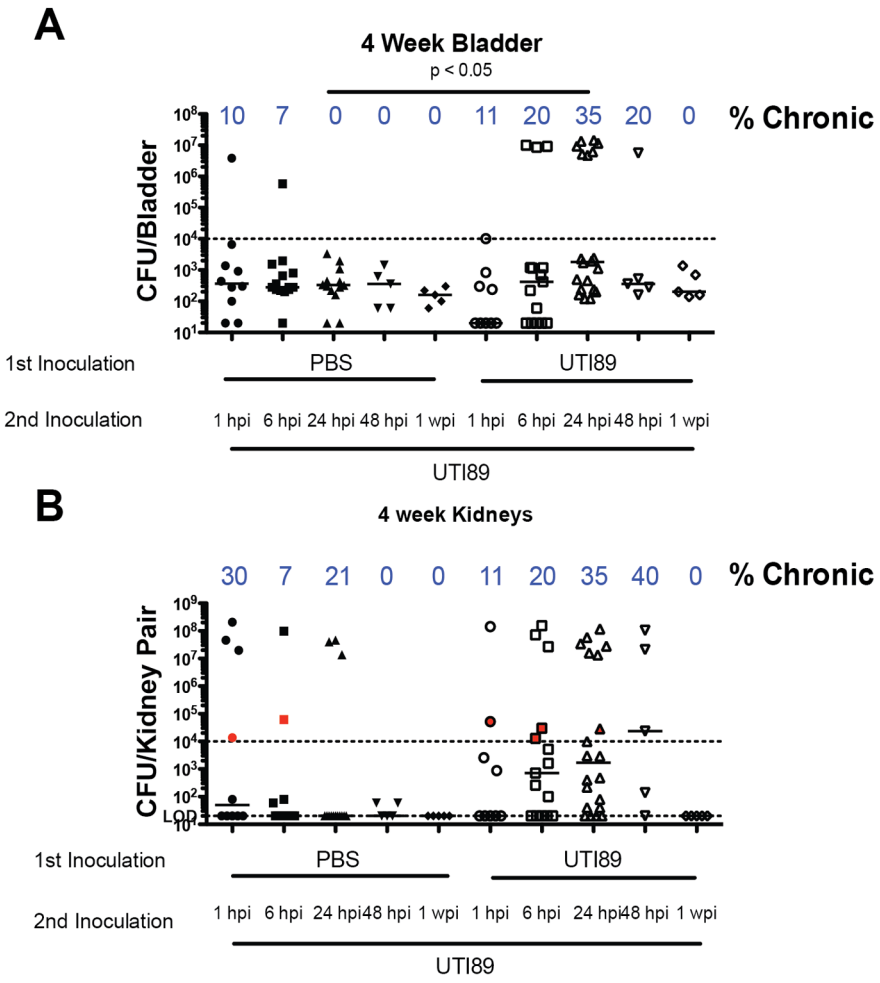

Figure 3. C57BL/6J mice are susceptible to chronic cystitis when superinfected 24 hpi.

A-B) Mice were transurethrally infected with PBS or UTI89 and re-infected at the indicated timepoints with UTI89. Urine was tracked weekly and four-week total bladder (**A**) and kidney pair (**B**) titer is displayed. N = 2-4 experiments with 4-5 mice per group. Statistical differences determined by Fisher's Exact test.

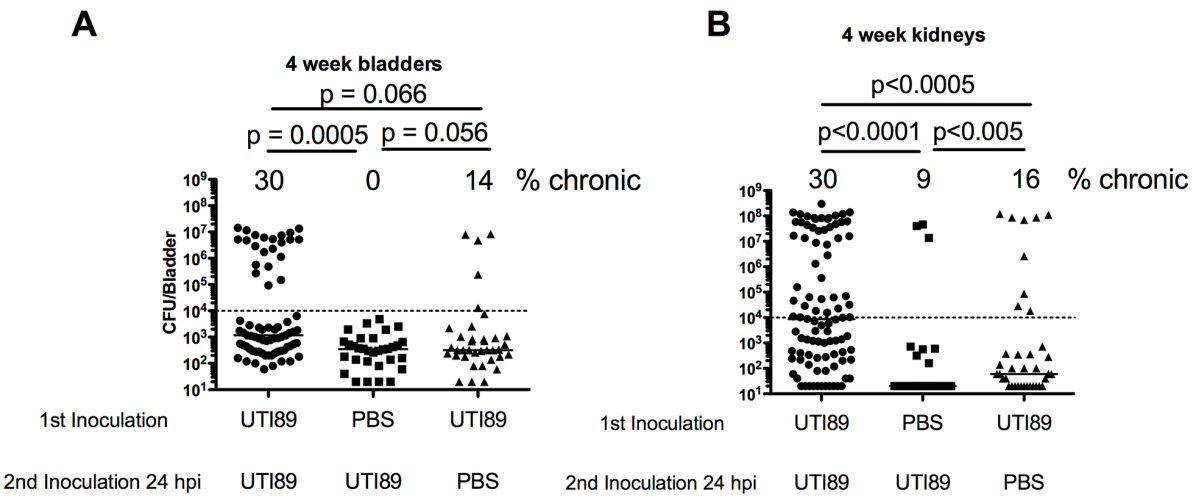

Figure 4. UTI89 Superinfection of C57BL/6J mice increases bladder and kidney infection.

A-B) mice were infected with PBS or UTI89 Kan^r and re-infected 24 hrs later with PBS or UTI89 Spect^r. Urine was tracked over four weeks and total bladder (**A**) and kidney pair titer (**B**) is displayed. Number above columns indicates number of mice with persistent bacteriuria with bladder (**A**) or kidney (**B**) titer > 10^4 CFU. Data represents 3-8 experiments with n = 4 – 29 mice per group. Panels also include data reproduced from Figure 3.Statistical differences determined by Fisher's Exact test.

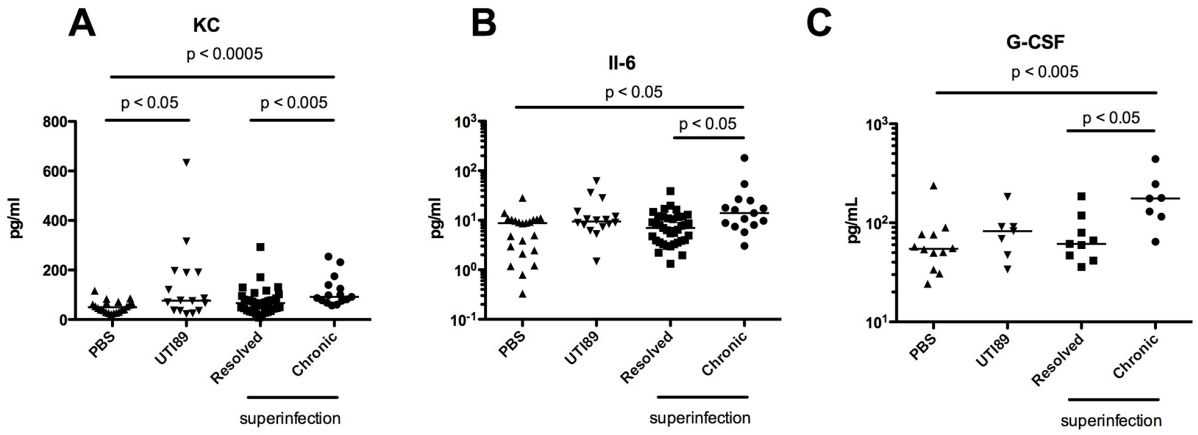

Figure 5. Serum cytokine signature of C57BL/6J mice with persistent bacteriuria.

Serum was obtained 24 hrs after initial inoculation prior to superinfection. Levels of 23 cytokines were determined and cytokines showing significant differences between resolved and chronic superinfected mice are shown. Levels of KC (**A**), IL-6 (**B**), and G-CSF (**C**) shown in pg/mL. Data represent 4- 6 experiments with n = 4 – 29 mice per group. Statistical differences determined by One-Way ANOVA overall and Mann-Whitney U test for pairwise comparisons.

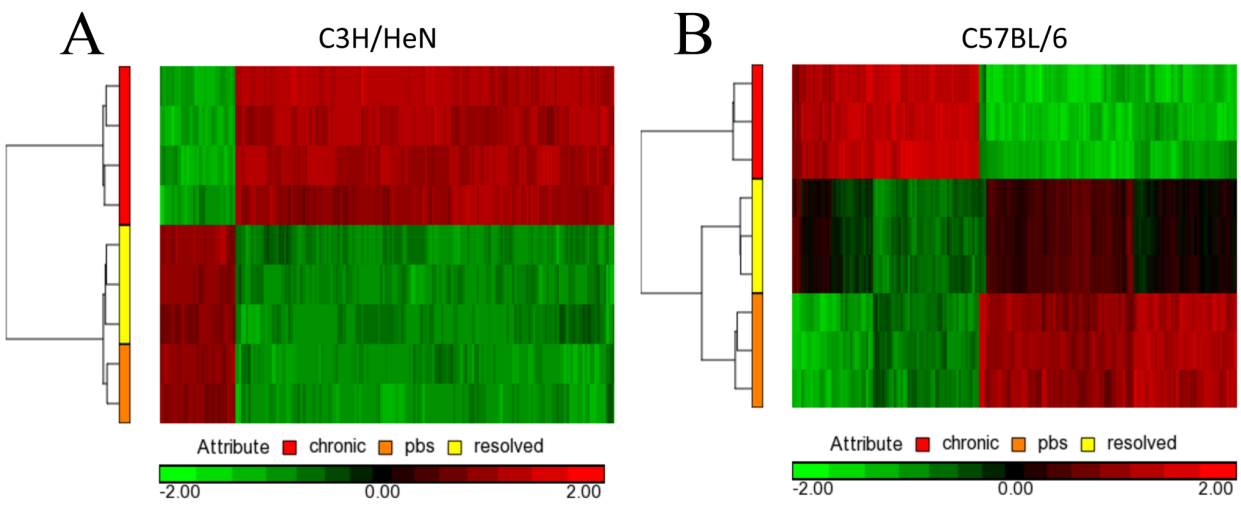

Figure 6. Microarray gene changes for C3H/HeN and C57BL/6J bladders.

A) C3H/HeN heatmap analysis for mice that resolved infection, experienced chronic cystitis, or were mock infected with PBS. **B**) C57BL/6J heatmap analysis for mice mice that resolved infection, experienced chronic cystitis, or were mock infected with PBS. Depicted is a representative analysis of two biological and three technical replicates.

Table 1. **Characteristics of C57BL/6J superinfections**

Condition[a]	Incidence % (n)	Persistent Bacteriuria[b] Bladder Titers[c,d]	Persistent Bacteriuria[b] PPV[e]	Resolved Bacteriuria Bladder Titers	Resolved Bacteriuria NPV[e]	Persistent Bacteriuria Kidney Titers	Persistent Bacteriuria PPV[e]	Resolved Bacteriuria Kidney Titers	Resolved Bacteriuria NPV
UTI89→ UTI89	30 (28/94)	$4.4x10^6$	92 (22/24)	$5.5x10^2$	100 (50/50)	$3.6x10^7$	100 (28/28)	$1.2x10^3$	73 (49/6
UTI89→ PBS	16 (6/37)	$2.5x10^6$	83 (5/6)	$3.2x10^2$	100 (31/31)	$8.6x10^7$	100 (6/6)	$6.0x10^1$	90 (28/3
PBS→ UTI89	9 (3/32)	$1.9x10^3$	0 (0/3)	$3.4x10^2$	100 (29/29)	$4.0x10^7$	100 (3/3)	$2.0x10^1$	100 (29/2

a. All mice infected 24 hours after initial infection

b. Defined as $>10^4$ CFU/mL bacteria in clean catch urine throughout four-week infection

c. Median values listed

d. Bladders of 20 mice were used for imaging purposes so no titers were available.

e. PPV is the positive predictive value of persistent bacteriuria predicting bladder/kidney titer greater than 10^4 CFU. NPV is the negative predictive value of resolved bacteriuria predicting bladder/kidney titer less than 10^4 CFU.

Supporting information

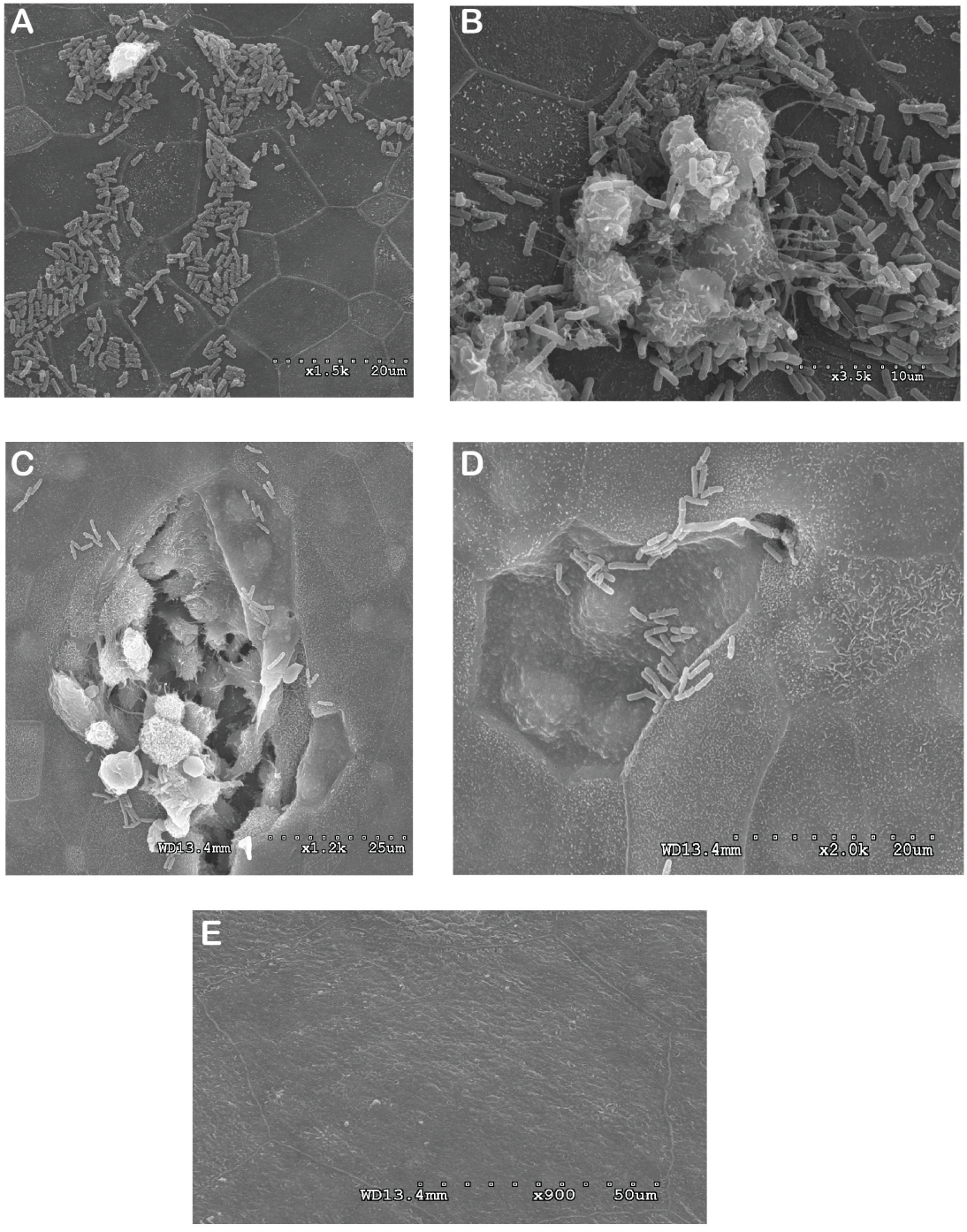

Figure S1. Bacteria replicate on the bladder surface during chronic cystitis.

Bladders of C3H/HeN and C57BL6 mice were splayed four wpi and fixed in glutaraldehyde. **A-B**) Chronic C3H/HeN bladders. **C-D**) Chronic C57BL/6J bladders. **E**) Mock infected C57BL/6J bladder shown for comparison.

Table S1. Genes with highest fold change of chronic cystitis versus resolved.

C57BL6			C3H/HeN		
Gene name	Fold change (chronic v resolved)	Functional Annotation	Gene name	Fold change (chronic v resolved)	Functional Annotation
Ubd	**119.405**	**Proteosome**	BC100530	144.401	Unknown
Saa3	**112.203**	**Inflammatory Response**	**S100a8**	**88.7327**	**Cytokine/Ion Binding**
S100a8	**85.4193**	**Cytokine/ Ion Binding**	**Mmp7**	**87.0008**	**Ion Binding**
Mmp7	**77.6183**	**Ion Binding**	**Ubd**	**77.6292**	**Proteosome**
RegIIIγ	**66.2146**	**Inflammatory Response**	**S100a9**	**74.4965**	**Cytokine/ Ion Binding**
S100a9	**44.9876**	**Cytokine/ Ion Binding**	**RegIIIγ**	**62.1077**	**Inflammatory Response**
Igk-V19-14	31.1745	Immunoglobulin	**Cxcl2**	**39.3707**	**Cytokine**
Igk-V28	27.7003	Immunoglobulin	Il1b	38.9361	Cytokine
Fam3b	**25.7301**	**Cytokine**	**Sprr2f**	**38.7662**	**Tissue Remodeling**
Plekhs1	24.3332	Tissue Remodeling	**Saa3**	**36.8235**	**Inflammatory Response**
Duox2	23.492	Ion Binding	Cxcl5	34.2451	Cytokine
Gm5571	22.2598	Immunoglobulin	**Il1a**	**33.0689**	**Cytokine**
Sprr2f	**22.0386**	**Tissue Remodeling**	Gabrp	31.6717	Ion Binding
AA467197	21.9697		Sprr2d	31.4934	Tissue Remodeling
Cxcl9	21.46	Cytokine	**Fam3b**	**29.3965**	**Cytokine**
Cxcl2	**17.698**	**Cytokine**	Cxcr2	27.6748	Cytokine
Pigr	17.1008	Immunoglobulin	**Tmprss11g**	**26.263**	**Tissue Remodeling**
Tmprss11g	**15.7865**	**Tissue Remodeling**	Lcn2	25.9349	Ion Binding
Nos2	15.644	Inflammatory Response	Il1f9	24.0818	Cytokine
Il1a	**15.0219**	**Cytokine**	Sprr2g	23.4676	Tissue Remodeling

Chapter 5: Discussion, Future Directions, and Concluding Remarks

The pathogenesis of UPEC UTI is complex because UPEC invades tissue and colonizes multiple niches during infection, each niche presenting different advantages and challenges to persistence. Many of these niches allow UPEC to resist immune defense and antibiotic treatment [1-3]. Furthermore, the high rate of same strain recurrence and the rising incidence of infections with antiobiotic resistant pathogens necessitates a thorough examination into the pathogenesis of this complex disease [4-7]. It is imperative to investigate novel therapeutics targeting unique elements of the UPEC pathogenic cascade to specifically target the disease and avoid unwanted side effects [8-10]. Prior to this work, the role of the intracellular bladder niche during acute infection was believed to be important; however, the degree to which intracellular replication impacted infection outcome was unknown. Additionally, a host-pathogen checkpoint at 24 hpi was predictive of infection outcome, but the specific mechanisms feeding into this checkpoint had not yet been characterized [11]. The work presented in this thesis identifies key elements of the UPEC pathogenic cascade as well as implicates human behavioral factors that predispose to chronic and recurrent urinary tract infections. I have discovered and detailed the stringent population bottleneck that accompanies acute infection [12]. Additionally, I elucidated the molecular mechanism whereby pathoadaptive changes in the adhesive protein FimH impact mannose-binding, conformation, and virulence. Moreover, this work identified a balance between mannose-binding and dynamic flexibility, which leads to maximal success within the urinary tract. This trait may be generalizable to other organisms that adhere to different surfaces. Finally, I developed a model of frequent bacterial inoculation and determined a possible mechanism whereby women are susceptible to chronic, recurrent UTI after frequent sexual intercourse. Targeting these important pathogenic elements therapeutically could dramatically reduce the morbidity and economic cost of this common disease.

This final chapter consists of three parts: Discussion (**A**), Future Directions (**B**), and Concluding Remarks (**C**). The Discussion (**A**) section will expand on the data presented herein and offer its context within the field regarding the pathogenesis of UTI. Specifically, I will discuss the 1) colonization of niches and reservoirs, 2) role of invasion and IBC formation during UTI, 3) UTI pathogenesis and symptoms, 4) factors responsible for colonization in a chronically inflamed bladder, 5) novel treatment modalities, and 6) bacterial population bottlenecks and virulence factors. In the Future Directions (**B**), I will outline the experimental design to test 1) virulence defects of high affinity pathoadaptive FimH alleles, 2) Identification of the FimH receptor during chronic cystitis, 3) mannoside efficacy against alternative FimH alleles in multiple niches, and 4) the role of IL-1 in priming the bladder during superinfection. Finally, I will offer my perspectives on the future of this disease in the Concluding Remarks (**C**).

A. Discussion

Colonization of niches and reservoirs

During UTI, UPEC is capable of colonizing multiple intracellular and extracellular niches in multiple organs. The pathogenesis is thought to begin with introduction of GI flora into periurethral or vaginal niches. The presence of UPEC in these areas increases prior to a UTI [13]. Some mechanical force such as sexual intercourse is thought to introduce bacteria into the urinary bladder [14, 15]. The bladder environment may not be sterile, as previously suspected [16]. UPEC would have to displace or interact with colonizers in order to establish productive infection of these niches. Furthermore, it is unlikely that an inoculum is monomicrobial. Mounting evidence suggests that the clinical practice of ignoring non-traditional uropathogens as contaminants may not accurately reflect the disease state [17, 18]. In fact, murine models of these polymicrobial infections suggest that the presence of other organisms may enhance UPEC pathogenesis [19]. When Group B Streptococcus and UPEC are co-inoculated, UPEC invade to a higher degree and form more IBCs than when inoculated singly [19]. Thus, the presence of other bacteria may enhance intracellular niche occupation, which would be predicted to enhance pathogenesis (**Chapter 2**) [12, 19]. Recent data suggests that other organisms may also be able to establish intracellular niche colonization [18, 20]. The ability to access this intracellular niche protected from the immune response and antibiotics may be a common component of the pathogenic cascade of multiple uropathogens.

Many recurrent UTI are caused by the same strain as the initial infection [4], suggesting that antibiotics are not effective at sterilizing niches colonized by UPEC. It was recently discovered that at the time of UTI, the dominant *E. coli* strain in the bladder was also the dominant *E. coli* species in the GI tract [21]. Antibiotic therapy may not be completely effective

in sterilizing this gut niche or Quiescent Intracellular Reservoirs (QIRs) within the bladder epithelium [22, 23]. Furthermore, it is possible that UPEC colonizes multiple niches within the GI tract as has previously been observed for other organisms [24, 25]. UPEC pili and other virulence factors may contribute to colonization of multiple niches within the GI tract in addition to the bladder [26]. Little is known about kidney colonization in humans because mice do not express the same receptor to which UPEC P pili bind in humans [27-29]. However, it has been suggested that multiple pilus systems contribute to kidney colonization in addition to P pili [30]. Type 1 and P pili may act in concert in the kidneys to facilitate attachment and biofilm formation (Figure 1) [31]. Colonization of these kidney niches may contribute to recurrent pyelonephritis [4, 32]. The ability of UPEC to ascend the ureters and colonize kidneys in an asymptomatic state may also lead to recurrent cystitis or pyelonephritis (**Chapter 2**). Data from multiple mouse models suggests that bacteria that colonize the kidney are the result of multiple cycles of ascension, colonization, and descent [12, 33]. UPEC infection is thus a highly dynamic process whereby multiple niches and reservoirs are colonized. The appropriate virulence factors are likely both niche-specific as well as general. The ability to regulate these genes to govern colonization and transition between these niches is essential for persistence [34].

Role of Invasion and IBC Formation During UTI

UPEC bind to and invade superficial facet cells of the bladder epithelium immediately upon entry into the murine urinary tract [2, 35, 36]. Rapid, intracellular bacterial replication within the cytoplasm results in biofilm-like communities known as intracellular bacterial communities (IBCs) that protrude into the lumen of the mouse bladder [37, 38]. The first round of IBC formation is well synchronized within an individual mouse and across mice for the first

six hpi [12, 36]. Each IBC contains $10^4 – 10^5$ CFU at 6 hpi derived from a single, invasive bacterium (**Chapter 2**) [12]. Thereafter, the infection is highly variable with regard to the timing of pathogenic events and the outcome [36]. That is, the variance of bladder bacterial titers at 6 hpi is very small, but at later timepoints, a bimodal distribution of bacterial titer begins to develop [39]. This bimodal distribution reflects mice that progress to chronic cystitis with high titers in the bladder, and those that resolve infection with low titers in QIRs. Thus far, the importance of the invasion and IBC cycle has only been assessed through mutations affecting this process, among other elements of pathogenesis. In this thesis, I provided evidence demonstrating the importance of this cascade during infection with WT UPEC strains.

We developed a library of 40 strains of UTI89 each with a unique DNA sequence that could be detected by eight multiplex PCR reactions (**Chapter 2**) [12]. During the first six hours of infection, bacterial CFU increased while the fraction of unique tags decreased. This increase in CFU corresponds to bacterial replication within IBCs, and the decrease in tag fraction results from innate defenses of neutrophil influx and superficial facet cell exfoliation [2, 12]. In extracted bladders, we found that higher numbers of IBCs corresponded to a higher fraction of unique tags isolated. An average C3H/HeN bladder has 50 IBCs from an inoculum of 10^7 CFU [12]. A higher fraction of tags was also present at two weeks post infection in mice experiencing chronic cystitis than mice that resolved infection [12]. Taken together, these data regarding the population bottleneck present several hypotheses regarding the transition between acute and chronic infection. These three hypothesis explain how UPEC transcend the stringent acute population bottleneck to cause chronic cystitis. The following hypotheses address how bacteria are able to cause chronic cystitis by transiting the acute population bottleneck: 1) IBC formation

and subsequent release allow clones to perpetuate infection, 2) increased luminal bacteria or increased flux out of IBCs, or 3) kidney colonization.

The first hypothesis assumes that the most stringent bottleneck occurs simultaneously with IBC formation, and that UPEC emerging from an IBC would produce a founder effect on the UPEC population. I have seen a direct relationship between IBC number and the proportion of mice experiencing chronic cystitis based on inoculum concentrations and bacterial strain (**Chapters 2-3**). Bacteria that flux out of the IBC to colonize the bladder during chronic cystitis persist luminally, adherent to exposed underlying epithelial cells (**Chapter 4**) [40]. UPEC resist the inflammatory response of this chronic condition and replicate indefinitely throughout the lifetime of the animal [40]. As a stable, extracellular infection it would seem unlikely for a continued decrease of the roughly 50% of remaining unique clones. This observation is consistent with other systems in which stable, luminal infections by WT organisms are not significantly diminished [41]. This hypothesis would predict that a larger number of IBCs precedes chronic cystitis while fewer precede resolution of the infection [11, 12, 34]. This hypothesis is consistent with the observation that an increased inoculum of either UTI89 or CFT073 leads to an increase in IBC number and an increase in the proportion of mice that experience chronic cystitis (**Chapter 3**). Furthermore, FimH mutants that form fewer IBCs, but bind the tissue equally well, are less likely to cause chronic cystitis. Based on these data, this is my favored hypothesis, but I will now describe possible alternative hypotheses.

An alternative hypothesis is that 1st generation IBC number does not significantly differ based on eventual infection outcome, but increased 2nd generation IBCs or bacterial flux precede chronic cystitis. Serum elevations of IL-5, IL-6, KC, and G-CSF at 24 hpi predict the development of chronic cystitis later [40]. Because the immune response to infection differs

between longterm outcomes, it is likely that some difference in the pathogenic cascade or niche occupation leads to this variance in immune response. Therefore, for this hypothesis, we will assume that mice that progress to chronic cystitis and mice that resolve infection have equal IBCs at 6 hpi. The difference between these conditions would result from either an increase in bacterial flux from the IBC to colonize the bladder, an increase in 2nd generation IBCs, or an increase in luminal bacteria throughout acute infection. These possibilities are not mutually exclusive. However, each of these would imply that the immune response would react to the increase in luminal bacteria. At 24 hpi, mice that progress to chronic cystitis have elevated urine titers and an increased inflammatory score relative to their littermates who resolve infection [40]. The developmental history of these bacteria is unknown, but it is unlikely that these bacteria were luminal throughout infection for several reasons. First, neutrophil and macrophage influx into the bladder would preferentially target these bacteria, eliminating them [36]. Additionally, the ability to attach to the bladder is not sufficient for longterm persistence. UTI89 FimH::A27V/V163A binds bladder tissue and invades superficial facet cells, but is not able to form IBCs nor cause chronic cystitis (**Chapter 3**) [42]. Interestingly, this mutant has *enhanced* mannose-binding ability, suggesting an increased attachment to bladder tissue (**Chapter 3**) [42]. Despite this increased binding, this strain is rapidly cleared from the urinary tract [42]. Additionally, a FimH mutant regulated by tetracycline expresses FimH in the lumen of the bladder, but once it invades the tissue, FimH expression is turned off [43]. Despite equal levels of luminal and invasive bacteria at 1 hpi, this mutant was attenuated as soon as 6 hpi. Taken together, these data suggest that IBC formation is essential for pathogenesis, and that bacteria that remain luminal throughout infection are unlikely to be the founder population later. While it is formally possible that increased bacterial flux out of initial IBCs or increased IBC formation

during the 2nd round predicts chronic cystitis, it is unlikely as these events occur between 16 and 24 hpi, when the predictive immune response has already been initiated [11, 40]. Therefore, based on these data, I do not favor this alternative hypothesis.

A third hypothesis is that bacterial ascension/replication in the kidneys results in the systemic immune response predictive of infection outcome. The C3H/HeN model of UTI is susceptible to a high rate of vesicoureteral reflux (VUR) and immediate post-inoculation kidney colonization [39, 44]. Treatment of mice with dexamethasone to dampen the inflammatory response significantly decreased the proportion of mice experiencing chronic cystitis without altering urine, bladder, or kidney titers at 24 hpi [40]. This result suggests that the host mediated inflammatory signal was blocked without dramatically affecting the pathogenic cascade. Furthermore, elevation of cytokines at 24 hpi did not correlate with kidney titers, but did correlate with urine titers suggesting a higher bladder contribution to this immune response. Increasing the inoculum to 10^8 CFU dramatically increased the proportion of mice that experienced chronic cystitis, but did not significantly increase kidney titers [40] (**Chapter 3**). While it is possible that kidneys can be colonized in a niche specific way in this model, as has been suggested (Figure 1) [30, 31], the data do not support kidney colonization differences as predictors of chronic cystitis in C3H/HeN mice. Therefore, taken together, our data best fits the hypothesis that an increase in UPEC intracellular niche presence leads to the systemic immune response predictive of chronic cystitis. Bacteria that colonize IBCs and flux out are therefore the most likely to colonize the bladder after the IBC cycle has concluded.

UTI pathogenesis and symptoms

Much of the work in this thesis attempts to explain UTI pathogenesis based on virulence factors and bacterial niche occupation in the urinary tract. Understanding the pathogenesis of a disease can also explain elements of symptomatology. Recent work is beginning to shed light on how UPEC and other inflammatory responses lead to UTI symptoms [45-47]. Interestingly, many of these studies have shown that bacterial presence is not essential, as LPS introduction into the bladder can lead to pain [45]. However, many of these symptoms are likely the result of inflammation and bacterial replication in preventing adequate bladder stretch or activating and repressing neuronal communication [48]. I believe bacterial replication in the urinary tract in the context of virulence factors and niche occupation may influence immediate and recurrent symptoms.

Although *E. coli* LPS has been shown to lead to a bladder pain response [45], the degree to which it affects the host is likely multifactorial. The particular LPS O-antigen structure and LPS shed frequency affect this bladder response [45]. Furthermore, the composition of the bacterial inoculum can likely influence the bladder response. As mentioned above, it is unlikely that a typical UTI-causing inoculum is monomicrobial [14, 15]. It is possible that other organisms, such as the gram positive, *Streptococcus agalactiae,* that are often co-inoculated, can dampen the pain response in addition to the inflammatory response [19, 20]. Additionally, the degree to which UPEC invades the epithelium will likely regulate the ability of the bladder to stretch during filling and voiding [47]. The confluence of bacterial virulence factors including the specific allele of FimH governs the pathogenesis of this organism. These virulence factors and the attributes they confer likely also impact how the host responds to the infection, resulting in symptoms. Ongoing bacterial replication during chronic cystitis would likely lead to persistent

and common UTI symptoms of dysuria, urgency, and frequency. If the infection resolves and QIRs form in the tissue [22], ongoing host responses may lead to symptoms in the absence of bacteriuria [4, 49]. Clinical investigations into these issues may begin to shed light on confusing diseases such as interstitial cystitis [48, 50].

Factors responsible for colonization in a chronically inflamed bladder

During the transition from acute to chronic infection, bladder physiology changes depending on outcome (**Chapter 4**) [11, 40]. In mice that resolve infection, the superficial facet cell layer regenerates with basal stem cell proliferation [51, 52]. Alternatively, during chronic cystitis, the entire superficial facet cell layer is denuded, no mature uroplakins are found on the bladder surface, and uroplakin genes are among the most downregulated murine genes in this state (**Chapter 4**). Several mutants in bacterial genes exhibit subtle defects during acute pathogenesis, but are dramatically outcompeted once chronic cystitis develops (**Chapter 3**) [11]. Therefore, different virulence factors may be required for bacteria to maintain bladder presence in this chronic inflammatory environment. During chronic cystitis, invasion does not occur to an appreciable degree [40]. Despite this observation, roughly 20% of the bacteria are present in a gentamicin-protected niche, which may exist at tight junctions (**Chapter 4**). What UPEC are binding in this niche and in the entire bladder during this period remains to be characterized because of the absence of mannosylated uroplakins.

Several alleles of the FimH adhesin are under positive selection among UPEC compared to fecal *E. coli* [42] FimH alleles such as FimH::S62/A163 have a slight competitive defect during the first 6 hpi (**Chapter 3**), which is dramatically accelerated thereafter. I have shown that FimH is able to adopt at least two different conformations, which alter mannose-binding affinity.

It is unclear as yet whether FimH is able to dynamically interconvert between these conformations or whether binding affinity differs in the elongated, mannose-bound, state between FimH alleles. FimH alleles with the highest mannose-binding affinity are attenuated *in vivo*, as are strains with the lowest affinity (**Chapter 3**). Furthermore, a gradient exists whereby subtle differences in mannose binding between FimH::A62/V163 and FimH::S62/A163 lead to large differences in pathogenesis. Thus, FimH::A62/V163 may bind even tighter to a different epitope that is exposed during chronic cystitis. UTI89 FimH has been shown to bind to α3 and β1 integrins in a cell culture system resembling undifferentiated, transitional bladder epithelial cells such as those exposed during chronic cystitis [53]. Additionally, strains possessing FimH::A62 were shown to have enhanced binding to type I and type IV collagen *in vitro* [54]. Type IV collagen is present in the basement membrane, which is unlikely to be exposed during chronic cystitis, but an epitope with a similar terminal sugar may be present. UTI89 FimH is more likely to adopt the compressed conformation (**Chapter 3**). In this state, shifts in loop positions cause a partial occlusion of the mannose-binding pocket, but an adjacent, deep groove may accommodate a different epitope.

Other non-FimH colonization factors may also be important during chronic cystitis, including other CUP pili [34]. As mentioned above, due to superficial facet cell exfoliation and ongoing neutrophil influx and inflammation, additional binding epitopes and nutrients may be exposed. UPEC has the ability to downregulate neutrophil influx and function [55], perhaps implicating immune-downregulatory factors during chronic infection. Similarly, nutritional acquisition or other virulence factors may confer benefit in this context. UTI89 outcompetes CFT073 expressing UTI89 FimH, but only after 7 dpi (**Chapter 3**). Therefore, UTI89 expresses

other factors allowing for longterm survival including transcendence of possible extracellular bottlenecks that CFT073 is incapable of transiting [11].

Novel treatment modalities

The rising rates of antibiotic resistant uropathogens and their worldwide spread necessitates the need for developing alternative therapies for UTI. Anti-virulence compounds represent one promising target for enhanced target specificity without increased side effects and off target effects of antibiotics [8]. Mannosides are mannose analogs that bind with high specificity to the FimH binding pocket of UPEC, preventing attachment to the bladder epithelium [56-58]. These molecules have enhanced affinity over the natural substrate, are readily available in mouse urine after one oral dose, and are effective against multi-drug resistant strains [57, 59]. The mannose-binding pocket of FimH is invariant among UPEC, likely because of its essential role in uropathogenesis [42]. Thus, complete resistance to mannosides would be unlikely because it would dramatically reduce affinity for the natural substrate. Furthermore, based on recent data demonstrating the presence of uropathogens in the GI tract during the time of infection, mannosides may help to eliminate a gut reservoir of bacteria [21]. The data presented in this thesis provides a strong rationale for the use of these compounds.

UPEC transit the stringent bottleneck during acute infection by invading bladder tissue and forming IBCs (**Chapter 2**). Blocking this crucial step would help eliminate infection. Furthermore, animals are more likely to develop chronic cystitis and recurrent, chronic cystitis, when infected with two invasive UPEC strains (**Chapter 4**). Inhibiting a fraction of the invasive organisms would also likely lead to clearance or a less severe infection. Alternatively, if the drug is admistered after the initiation of chronic cystitis, it effectively reduces bacterial burden

10,000-fold after three doses [57]. The FimH allele varies among UPEC, but mannosides have been shown effective for at least three different alleles encompassing over 70% of natural sequence variation [42, 57, 59, 60].

I can envision several potential problems with mannoside treatment. First, if the drug is administered during acute infection, but after the bacteria have established IBCs, it would not be effective [57]. Because of the possibilities of QIR formation leading to recurrences or the development of chronic cystitis, the invasion process may already establish negative downstream sequelae. Second, downregulation of type 1 pili because of mannoside administration might lead to the expression of other pilus systems, increasing the risk for pyelonephritis [12, 33, 61]. Nevertheless, these molecules can be utilized instead of antibiotics or in synergistic combination [57]. In addition to inhibiting type 1 pili by blocking its interaction with the mannosylated receptors *in vivo*, compounds known as pilicides can block the synthesis of multiple types of extracellular fibers [62, 63]. Pilicides could obviate some of the negative side effects including possible ascension and pyelonephritis. These molecules may have more side effects because of the near ubiquitous presence of CUP pili among enterobacteriaceae, including gut commensals [64, 65]. Further evaluation of mannosides and pilicides is necessary, but these treatments hold promise as alternative or complementary therapies for UTI. The work described in this thesis describes the key elements of UPEC pathogenesis, providing rationale for aggressive and novel treatments aimed at limiting this disease.

Bacterial population bottlenecks and virulence factors

UPEC must overcome several bottlenecks and barriers to establish colonization of the urinary tract [12, 33]. The bottlenecks that organisms transit to cause disease are numerous and

likely similar between mucosal infections [11, 66-70]. These host and environmental filters attempt to reduce the numbers of pathogenic organisms that reach protected sites. For UPEC, the increased immune response corresponds with the ability to access deeper tissues [11, 40]. Organisms of the genus *Vibrio* have several virulence factors allowing them to carve out niches within the host. *Vibrios* express Type VI secretion systems allowing them to target other species to eliminate niche competition [71]. Once certain *Vibrio* species have attached to the appropriate niche, they can also invade the epithelium, likely presenting another bottleneck to pathogenesis [72]. Within these genes, positive selection has likely occurred favoring certain allelic variants. Thus, many of the findings presented for UPEC in this thesis can and should apply to the consideration of other infectious diseases.

One very important consideration that should be taken from this thesis is that presence or absence of a virulence factor is not sufficient to mediate disease phenotype. Furthermore, allelic variation among virulence factors may serve to modulate that particular event in the host-pathogen interface. For example, positively selected residues in the FimH adhesin modulate the ability of UPEC to attach to bladder tissue, invade, form IBCs, and bind mannose (**Chapter 3**). A spectrum of disease severity exists partially regulated by the specific FimH variant an *E. coli* expresses. Similar investigations into other mucosal pathogens would likely yield similar findings [72]. Therefore, I propose that it is not what specific virulence factors an organism expresses, but rather *which* allele of each virulence factor that it expresses that confers specific niche pathogenicity.

B. Future Directions

Virulence defects of high affinity pathoadaptive FimH alleles

UTI89 expressing FimH::A27V/V163A and FimH::V163A are attenuated during chronic infection, and UTI89 FimH::A27V/V163A invades the urothelium but does not form IBCs (**Chapter 3**) [42]. Purified Fim tips containing these mutations have high mannose affinity and presumably only exist in the elongated conformation. The experiments outlined below will determine the reason for the inability of these strains to form IBCs and whether these high-affinity strains can colonize the bladder after the onset of chronic cystitis.

Is high mannose affinity inhibiting IBC formation a general phenomenon?

I hypothesize that the high affinity state is mutually exclusive with the ability to form IBCs *in vivo*. UTI89 FimH::A27V/V163A does not form IBCs at 6 hpi and is rapidly cleared by 24 hpi [42]. UTI89 FimH::V163A was not dramatically different from UTI89 at 24 hpi in single infection; however, this result was accompanied by a large titer variance and was not explored at other timepoints [42]. Both FimH mutants exhibit high mannose affinity and an increased ability to form biofilm *ex vivo* (**Chapter 3**) [42]. Both strains were also defective in co-infection with WT UTI89 during chronic cystitis. In order to test my hypothesis, I would inoculate C3H/HeN mice with UTI89 or UTI89FimH::V163A and determine IBC number at 6 hpi using whole mount LacZ staining [73]. I expect UTI89 FimH::V163A will not form IBCs.

Why are strains expressing high mannose affinity FimH incapable of forming IBCs?

I hypothesize that FimH cannot detach from its uroplakin receptor on the bladder cell surface if it is locked in the elongated, high-affinity conformation. To test this hypothesis mice could be infected UTI89 with FimH::A27V/V163A and FimH::V163A as well as strains artificially locked in the elongated or compressed conformation via cysteine bonds [74]. Invasive UPEC co-stained with uroplakin III and Rab27b, indicating UPEC internalization into fusiform vesicles with its receptor [75]. A TLR4-dependent process can expel bacteria within these vesicles into the lumen of the bladder [76]. Hours later, bacteria within IBCs are no longer coated with Rab27b, and appear to replicate in the cytoplasm of the epithelial cell [38]. How bacteria burst out of this vesicle and replicate within the cytoplasm is unknown. The following experiments will determine the role of FimH in this mechanism:

A) I would determine whether UTI89 FimH::A27V/V163A cannot form IBCs because it is trapped within fusiform vesicles by utilizing a GFP expressing strain and staining bladders at 6 hpi for uroplakin III, Rab27b, and host cell nuclei with DAPI. By examination using confocal microscopy, I would expect to find UTI89 FimH::A27V/V163A and other strains with highest mannose affinity to remain in vesicles, whereas UTI89 will be free within the cytoplasm.

B) UPEC can be expelled from within the epithelial cell by elevations of cAMP and fusion of fusiform vesicles to the bladder surface [75]. I would inhibit this elevation of cAMP by introduction of compound H89, which has been previously shown to decrease UPEC expulsion [75]. I predict that administration of this molecule will lessen the defect of strains expressing high mannose affinity. It is also possible that the ability of these high affinity strains to escape the vacuole is reduced temporally, in which case H89 may restore IBC formation. Alternatively, these strains may be incapable of detaching from the uroplakin receptor, which would result in

increased bacterial titers without increasing IBC formation. This hypothesis can be tested using a uroplakin transgenic mouse and intravital multiphoton microscopy [77]. C57BL/6J mice expressing RFP from the uroplakin 1 locus (*UpkIb*) are commercially available through Jackson Laboratories. These mice can be infected with GFP-expressing UTI89 strains expressing various FimH alleles. As a pilot experiment, confocal microscopy can be conducted on bladders extracted at 1, 3, 6, and 12 hpi to determine bacteria-uroplakin colocalization. If this staining is successful, I would image, in real time in a live mouse, the engagement and detachment of the uroplakin receptor and the impact of FimH allele on this process. I predict that strains with high mannose affinity will be unable to detach from the receptor resulting in a high degree of bacteria-uroplakin colocalization, whereas FimH that can adopt the compact conformation will separate from the receptor by 6 hpi to form IBCs.

3) Can fitness defects of high affinity FimH alleles be rescued after the onset of chronic cystitis?

When co-inoculated with WT UTI89, UTI89 with FimH::V163A and FimH::A27V/V163A are attenuated in the urine and in the bladders at four weeks. UTI89 FimH::A27V/V163A cannot form IBCs, likely explaining this virulence defect [42]. I have shown that both of these mutations *increase* mannose-binding affinity (**Chapter 3**). During chronic cystitis, uroplakins are not present, implicating another receptor for FimH (see Discussion). If binding affinity is the sole determinant of success for otherwise isogenic strains, I would predict that FimH::A27V/V163A would outcompete UTI89 in this environment.

To test whether enhanced binding increases pathogenesis during chronic infection, I would infect mice with UTI89 Spect^r and re-infect at four weeks with UTI89 FimH::A27V/V163A or FimH::V163A; both kanamycin resistant. WT UTI89 Kan^r will serve as

the control in a separate set of mice. In a pilot of a small number of mice, a WT re-infection was not able to colonize a mouse experiencing chronic cystitis. If this result replicates, it suggests that adaptation to the inflamed environment has occurred, excluding the incoming inoculum. In that event, I would treat mice with antibiotics to sterilize the urinary tract, and challenge them with FimH::A27V/V163A after the antibiotics have cleared. Preliminary data suggests that IBC formation does not occur at 6 hpi after a re-infection when mice are sensitized to develop chronic cystitis by the above protocol (Figure 2). I predict that in mice that have developed chronic cystitis, exfoliation is so rapid, that there is no invasive bacterial population. This hypothesis can be tested by conducting *ex vivo* gentamicin protection assays and confocal microscopy 1, 6, and 24 hours after infection of sensitized mice with UTI89 expressing GFP. The degree of exfoliation can be assessed using scanning electron microscopy [2, 78]. Whole bladders from naïve mice can be examined as well as mice that resolved infection or developed chronic cystitis on the first infection. If sensitized mice exfoliate more completely and more rapidly than mice that resolved infection, I would predict that UTI89 FimH::A27V/V163A can cause chronic cystitis. If this strain is still attenuated and IBC formation is not essential in this condition, it would suggests that the compact state of FimH is favored during chronic cystitis.

If the introduced inoculum is capable of colonizing to an equal degree, I would predict that introduction of UTI89 FimH::A27V/V163A or UTI89 FimH::V163A would outcompete the WT strain because of its enhanced mannose binding. If it is not able to colonize, this also suggests that the compact state that FimH adopts in WT UTI89 is important for colonizing the chronic bladder. These experiments can also be conducted with strains locked in the elongated and compact states [74].

Identification of the FimH receptor during chronic cystitis

As mentioned above and in the Discussion (**A**), the superficial epithelial layer is denuded during chronic cystitis, and mature uroplakins are not expressed [40]. I will outline a biased and unbiased approach to identify the receptor for FimH in a chronic bladder.

Biased approach

Several epitopes have been reported to bind FimH *in vitro* including α3 and β1 integrins, TLR4, and collagen [53, 54, 79]. I would infect mice with GFP-expressing UTI89 and stain bladders at 4 weeks post infection with antibodies to α3 integrin, β1 integrin, TLR4, and collagen IV. I would determine the percentage of these receptors that co-localize with green bacteria and vice versa. In chronic mice, if one of these is the potential receptor, bacteria and the receptor will colocalize to a high degree. I would additionally extract bladders from chronically infected mice, wash them with α-methyl-D-mannopyranoside to detach bacteria, and incubate with fluorescein-labeled UTI89 or FimH mutants, and stain with antibodies for the prospective receptors and DAPI. The ability to bind purified FimCH and FimCGH could then be analyzed using biolayer interferometry with OCTET.

Unbiased approach

I would use a similar biolayer interferometry setup with biotinylated FimCH and FimCGH bound to OCTET pins and add the following homogenized bladder preparations: uninfected, protamine sulfate-exfoliated urine and bladder tissue, and bladder tissue from mice experiencing chronic cystitis. If the biotinylation reaction prevents binding, I would immobilize FimCH and FimCGH on eliza plates, and conduct the following analyses. I would run these samples in separate wells to bind mouse bladder proteins to immobilized WT or binding-null FimCH or FimCGH. I would then wash the wells to remove unbound samples, add mannose to elute the

receptor, and run these samples on a gel to identify bands specific to chronic mouse bladders. The band corresponding to uroplakins in naïve mouse bladders should be present in uninfected as well as protamine-treated bladders and absent from chronic cystitis bladders. This internal control ensures the assay is functional. Mice that are pretreated with protamine sulfate and infected 1 hpi later do not develop chronic cystitis at a higher rate (**Chapter 4**), suggesting either that the receptor is not exposed without immune activation or that IBC formation is required prior to exfoliation. Comparing bladders of protamine-treated mice and mice experiencing chronic cystitis will help to identify the receptor as well as answer the previous question. Protein bands satisfying these conditions will be sent for mass spectrometry analysis to identify the receptor that FimH binds during chronic cystitis.

Mannoside efficacy against multiple FimH alleles in multiple niches

Oral administration of mannosides has proven effective in dramatically reducing bacterial colonization of the mouse bladder [57, 59, 60]. The infecting strain in these experiments has been UTI89, EC958, and PBC1, all of which express different FimH alleles. In order to test whether mannosides are equally effective against common FimH alleles of UPEC strains, I would infect mice with UTI89 differing only by FimH allele. For strains less effective than UTI89 at causing chronic cystitis, an inoculum of 10^8 CFU can be introduced to initiate a greater proportion of chronic cystitis. I would administer a single oral dose of a high affinity mannoside and evaluate bladder and kidney bacterial burden 6 hrs after mannoside or PBS administration.

An interesting experiment would be to colonize the GI tracts of mice with UTI89 expressing FimH alleles after a four-day course of streptomycin [80]. Oral gavage of bacteria with or without mannoside will test whether FimH is necessary for gut colonization. Previous reports regarding the role of type 1 pili in gut colonization are conflicting [80, 81]. Inocula

containing multiple strains differing only by FimH can be introduced to answer the question of whether these differences impact gut fitness. These competitive experiments may help us understand why the most common alleles observed in the population are less fit in the urinary tract (**Chapter 3**). These gut colonization experiments will answer multiple questions. First, is FimH necessary for gut colonization? Second, if FimH is necessary, does the FimH allele impact GI colonization as has been shown previously for one allele in gnotobiotic mice [42]? Third, can mannoside effectively eliminate UPEC from this niche as well as from the bladder? This last question is important given the recent data showing gut and bladder colonization of UPEC in humans at the time of UTI [21]. Mannoside could be used bifunctionally to eliminate the immediate cause of UTI as well as reduce bladder and GI reservoirs that could serve as sources of recurrent UTI [22, 23].

The role of IL-1 in priming the bladder for superinfection

The precise molecular mechanism underlying the proportional increase in the development of chronic cystitis for superinfected mice is unknown (**Chapter 4**). This increase does not occur when the initial inoculum has mutations in *hlyA* or *cpxR*, nor does it occur when the bladder is pretreated with a caspase1/11 inhibitor prior to infection. Taken together, these results suggest that activation of the inflammasome without an exaggerated response is essential to increase the proportion of mice suffering chronic cystitis.

The inflammasome is a mutli-protein assembly complex resulting in the activation and release of caspase-1/11, IL-18, IL-1β, and Il-1α, dependent on whether caspase-11 is also activated [82]. Inflammasome activation depends on the type of stress, and can be named based on a particular scaffolding protein that aids in stimulus recognition. UPEC activates the non-

canonical bladder inflammasome via NLRP3 in conjunction with caspase-11 elaboration by the sensing of hemolysin (Nagamatsu et al. in preparation). Strains overexpressing hemolysin (ΔCpxR) lead to increased activation, while strains lacking hemolysin do not activate the inflammasome. mRNA levels of caspase-1, caspase-11, nlrp3, and IL-1β are increased in the serum of infected mice in a dose-dependent manner. Although systemic levels of IL-1α and IL-1β were not predictive of ensuing chronic cystitis [40] (**Chapter 4**), the local bladder environment may be enriched for these cytokines. Indeed, infected bladders had increased levels of IL-1β in a hemolysin-dependent manner (Nagamatsu *et. al*. In Preparation). UTI89ΔHlyA is not attenuated for the development of chronic cystitis, but does not prime the bladder for enhanced chronic cystitis in superinfected C3H/HeN mice. This result suggests that an alternative pathway may also lead to chronic cystitis, but that elevations in Hemolysin-mediated exfoliation and inflammasome activation increase this response.

In order to test the role of the inflammasome during superinfection further, I would superinfect mice 1-6 hours after the initial infection and determine serum and bladder levels of IL-1α, IL-1β, IL-5, IL-6, KC, and G-CSF using ELISA or Bioplex at 24 hpi. Bladder levels of IL-1α and IL-1β will be correlated to serum levels of IL-5, IL-6, KC, and G-CSF to predict which mice had triggered the checkpoint to develop chronic cystitis [11, 40]. I would predict that superinfected mice have increased bladder IL-1α and/or IL-1β, and that this would correspond to mice with elevated serum IL-5, IL-6, KC, and G-CSF. If this result is seen, I would treat mice with Anakinra to block the IL-1 receptor, which I predict would block the enhancement of chronic cystitis in superinfected mice. If Anakinra prevents increased chronic cystitis, it could be used instead of, or in combination with, antibiotics for extreme cases of UTI. For additional proof of the role of the inflammasome in superinfected mice, I would backcross C3H/HeN mice

with caspase-1/11$^{-/-}$ C57BL/6 mice for > 10 generations to develop C3H/HeN mice that lack caspase 1/11. I would predict these mice do not develop chronic cystitis.

C. Concluding Remarks

This dissertation expounds upon the growing complexity of UPEC UTI that is just now being appreciated. I detailed the complex population dynamics and niche distribution of UPEC throughout UTI, identifying a key bottleneck that bacteria need to transit in order to establish long-term colonization. This work additionally showed that the degree of IBC formation correlates with disease outcome. Further, positively selected FimH residues conferred enhanced fitness during acute and chronic UTI. These studies brought up a mannose-binding paradox, whereby affinity extremes were less fit than FimH exhibiting moderate affinity. This affinity relates to the ability of FimH to adopt at least two conformations, both of which are essential during bladder colonization. Finally, I established a model to mimic frequent sexual intercourse, showing that caspase 1/11 activation leads to enhanced chronic cystitis in a susceptible mouse strain. I also showed that this model lead to chronic cystitis in a strain resistant when infected singly. This thesis provides added rationale for treating the invasive stage of the UPEC pathogenic cascade aggressively before long-term, negative sequelae develop. Accordingly, the ability of UPEC to occupy specific niches in a time and space dependent manner is crucial for successful colonization. By targeting UPEC within these niches at the appropriate times during the complex pathogenic cascade, we can eradicate this troublesome disease.

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Figures

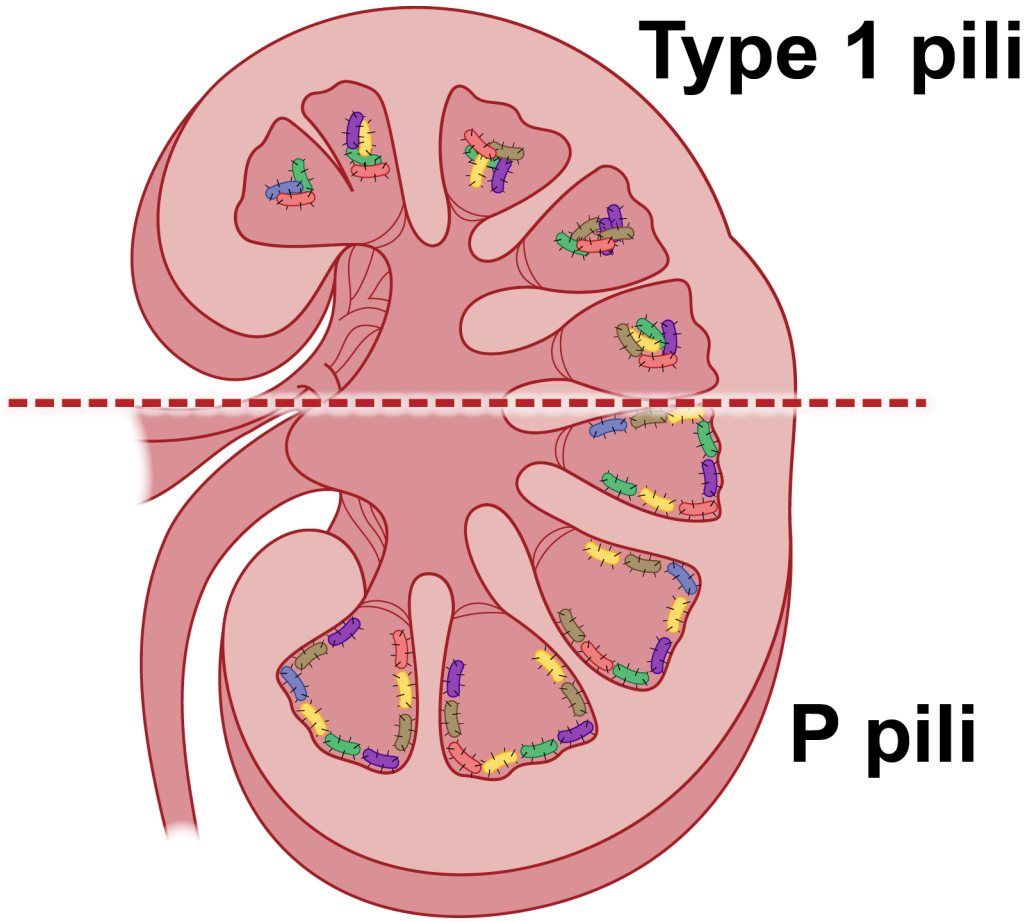

Figure 1. Potential role of type 1 and P pili during pyelonephritis.

P pili bind to receptors on the kidney epithelium when UPEC enters the kidney. Type 1 pili may serve to coalesce bacteria within biofilms in this environment [31]. Possible intracellular niches or antibiotic resistant niches may also exist.

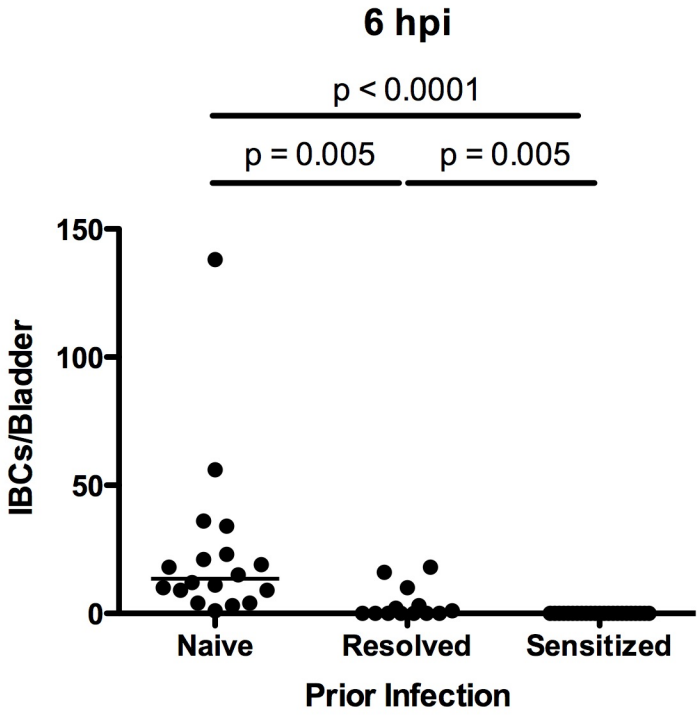

Figure 2. IBC formation in sensitized mice.

Mice were infected with 10^8 CFU UTI89 or PBS and urine was tracked for 28 days. Mice were then treated with trimethoprim-sulfamethoxazole for 7 days in drinking water. After 21 days free from antibiotics, mice were infected with 10^7 CFU UTI89 and bladders were extracted for whole mount LacZ analysis 6 hpi.