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The Enemy Within: An Investigation of the Intracellular Bacteria in Urinary Tract Infections

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by

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ABSTRACT OF THE DISSERTATION

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Scott J. Hultgren, Chair

Urinary tract infections (UTIs) are common diseases that are associated with significant morbidities. Multiple studies have indicated that multiple species of uropathogenesis bacteria invade and persist within bladder epithelial cells as a necessary step of uropathogenesis. Interestingly, many of these species are not canonically associated with intracellular infections. Although the first study describing bacteria within the urothelium was published two decades ago, this critical step of uropathogenesis remains relatively understudied.

I established a murine model of community-acquired *A. baumannii* UTI, a previously unstudied manifestation of the disease. While immunocompetent mice resolved their infections quickly, immunocompromised mice displayed high bacterial burdens throughout their urinary tracts for several weeks. I found that mice infected using this model retained *A. baumannii* intracellular reservoirs (ABIRs) in their urothelium long after the resolution of the initial colonization event. Inserting a catheter into the bladders of these resolved mice triggered a same-strain UTI in over 50% of the mice. Stringent experimental controls suggest that these

resurgences came from bacterial reservoirs within the resolved host. Further testing implicates the ABIRs as the most likely source.

I have also characterized the intracellular phenotypes of multiple uropathogenic *E. coli* (UPEC) clinical isolates. While each isolate had unique strain-specific characteristics, all three of the phylogroup A strains proved to be incapable of properly undergoing the intracellular steps of uropathogenesis. I have also studied an important process used by intracellular UPEC, lactose metabolism. UPEC isolates with mutations in their lac operons formed significantly smaller intracellular bacterial communities, and were unable to complete IBC development. However, I found that UPEC strains with deficient lactose permeases were still capable of importing lactose-like sugars.

Overall, my dissertation contributes to field of intracellular uropathogenesis in multiple ways. With *E. coli*, I have identified a potential link between bacterial genetics, specifically when a bacterium is a member of phylogroup A, and intracellular phenotype in the bladder. I have also established that lacY-deficient UPEC isolates are capable of internalizing lactose-like sugars, which suggests the presence of a secondary mechanism. I have also developed a murine model of community-acquired UTI for *A. baumannii* and have investigated both the pathogenesis and the prevalence of this manifestation. I have also developed the first murine model of resurgent infections for *A. baumannii* and have identified a potential reservoir bringing novel strains into the hospital in the form of host reservoirs. Previous *Acinetobacter* UTI research has focused primarily on complicated UTIs. Together, my findings expand our knowledge of *Acinetobacter* uropathogenesis in the previously unstudied, community-acquired model of infection.

Chapter 1: Introduction

By Jennie Hazen

1.1 Overview

Urinary tract infections (UTIs) rank among the most common infections worldwide. Despite the prevalence of this disease, however, our knowledge of how bacteria successfully colonize the host urinary tract is incomplete. Recent work has revealed that many uropathogens invade the epithelial cells of the bladder wall and undergo intracellular steps of uropathogenesis. Relatively little is known about this step of uropathogenesis, due in part to limitations in technology. Here I discuss UTIs, their clinical manifestations and their epidemiology. Next, I describe the current body of knowledge on the intracellular steps of uropathogenesis. Finally, I discuss open questions in the field pertaining to what happens to bacteria within the cells of the bladder lining, as well as the limitations in technology that have contributed to these gaps remaining unfilled. In future chapters, I will describe my efforts to better understand the intracellular behavior of bacteria within the urothelium, focusing on uropathogenic *E. coli* and uropathogenic *A. baumannii*.

1.2 Understanding Urinary Tract Infections

1.2.1 What is a UTI? Definition, symptoms, epidemiology, and burdens

UTIs are defined as bacterial infection of the kidneys, ureters, bladder, and/or urethra[1,2]. Clinical UTIs are diagnosed based on the presence of a high bacterial burden ($\geq 10^3$ - 10^5 colony forming units (CFU)/ml urine, depending on the location) and on occurrence of symptoms[2–4] . Common UTI symptoms include dysuria, urgency, frequency, lower abdominal and/or lower back pain, fever, foul smelling and/or discolored urine, and hematuria[1,5,6] .

UTIs are extremely prevalent, ranking as the third most common infectious disease in the world, and as the most common outpatient infection in the United States[6–8]. An estimated 60.4% of all women and 5% of all men worldwide will experience at least one UTI in their lifetime[1].

UTIs can pose a significant threat to quality of life, especially in patients who experience severe pain or disruptive levels of urgency and frequency in urinating. Recent analysis of the Global Burden of Disease Study 2019 demonstrated that UTIs carry quantifiable morbidities and were associated with an estimated 520,200 disability-adjusted life years (DALY), defined as the equivalent to the loss of one full year of full health, in 2019[2,9]. In addition to morbidity, these infections carry a small but significant risk of mortality. Even though current estimates calculate the mortality rate of UTIs below 2%, the high prevalence of UTIs results in a significant number of deaths. The Burden of Disease Study 2019 analysis revealed a 2.4-times global increase in direct UTI-attributable deaths since 1990, with 236,790 deaths in 2019[10] . Furthermore, when left untreated, UTI-causing bacteria can travel from the bladder to the kidneys and finally to the bloodstream and cause bacteremia, a much deadlier disease with mortality rates that average at 40% and reach as high as 80%[11–13]. In fact, the majority of bacteremia cases are thought to originate from the urinary tract[14–17]. As the prevalence of antibiotic-resistant bacteria continues to increase worldwide, so too does the frequency of antibiotic-resistant UTIs and, in turn, the difficulty to treat these infections.

Due to their frequency, associated morbidities, and difficulty to treat, UTIs also pose a significant economic burden on patients, hospitals, and the overall economy. In 2014, the direct annual cost of UTIs in the United States was reported at $2.8 billion. The addition of indirect costs, such as lost labor, brings the total up to approximately five billion dollars each year[6,18].

1.2.2 Types of UTIs

UTIs are generally categorized as 'uncomplicated' or 'complicated' and are associated with different mechanisms of pathogenesis as well as with different bacteria. The term 'uncomplicated UTI' refers to a lower urinary tract infection in an otherwise healthy individual. Uncomplicated UTIs are primarily associated with *E. coli,* which causes up to 95% of uncomplicated UTI, and to a lesser extent with *K. pnemunoiae*[19–21]. They involve bacterial colonization of the bladder, via mechanisms discussed below in the **IBC cycle** section, with the occasional ascent into the kidneys.

Complicated UTIs involve structural, immunological, or external complications that affect the host's urinary tract, response to the infection, or both[21]. Examples of complicated UTIs include UTIs that occur in immunocompromised patients; post-organ transplant kidney infections; UTIs associated with kidney stones, bladder stones, or other obstructions to the urinary tract; and catheter-associated UTIs (CAUTI). While *E. coli* and *Klebsiella* still cause a substantial amount of complicated UTIs (~34%), there is a much wider variety of pathogens that can cause infections in these settings, including *Candida* (17.8% prevalence in CAUTI), *Enterococcus* (13.8%), *P. aeruginosa* (10.3%)[22]. Many CAUTI-causing bacteria do not substantially contribute to cases of uncomplicated UTI. For example, the bacterium *A. baumannii* is incapable of colonizing healthy c57bl/6 mice in a murine model of uncomplicated infection[23]. However, in a healthy c57bl/6 who has been implanted with a catheter, *A. baumannii* causes a robust infection[24]. The bladder itself is rarely colonized to the same extent as the implant, meaning that the bacteria preferentially bind to the device[24,25]. These differences in pathogenesis are due to the different mechanisms of complicated vs uncomplicated uropathogenesis. When a foreign body is inserted into a host, the host deposits a substance called

fibrinogen on the "non-self" object[26]. Studies from Floreles-Mireles and Walker have demonstrated that some bacteria which lack the ability to colonize a bladder have the ability to adhere to and colonize a fibrinogen-coated catheter[24–26]. Other types of complicated UTI also involve their own specific mechanisms of uropathogenesis[27]. In this way, the term UTI encompasses a constellation of manifestations, associated pathogens, and methods of colonization.

1.2.3 When a UTI won't go away

Recurrent UTIs

In addition to high prevalence UTIs can be highly recurrent, which can have severe consequences on the quality of life for patients experiencing recurrent UTIs (rUTIs). According to various estimates of UTI recurrence rates, 25% of sexually active adult women who have experienced one UTI are predicted to experience a recurrent infection within six months. A subset of these women are predicted to experience further subsequent infections. An estimated 2-3% of all women worldwide are predicted to experience six UTIs in as many months[1,28]. While it is easy to imagine the morbidities associated with rUTI, few official studies have been performed to quantify the burdens these patients face. A six-month long prospective observational study in Switzerland reported that patients who suffer from rUTIs experienced significant levels of depression and anxiety[29]. The patients reported severe decreases in their quality of life due to painful or disruptive symptoms. Severe incidences of pain, urgency, or frequency resulted in functional handicaps. Sufferers of rUTIs also faced significant social burdens[30]. In fact, Naber *et al* report in a 2022 systematic literature review that the social burdens of rUTI far outweighed the physical[31]. Direct social burdens involve judgement from peers. In the public sphere, UTIs are inaccurately attributed to poor hygiene and sexual promiscuity. These judgements are often applied to rUTI sufferers. These individuals also face the same judgements and difficulties many

patients with bathroom-related chronic illnesses face. For example, German and American participants of a 2022 UTI survey report that their frequency symptoms and resultant need to be near a restroom interrupted time that would otherwise be spent on their familial, social, work, and academic commitments[32]. Interrupted work time, the survey respondents claim, also lead to economic consequences. Other survey respondents report the negative impact rUTIs have on their romantic relationships; many individuals' partners blamed themselves because UTIs are attributed to a partner's poor hygiene just as often as they are to the individual in question. Meanwhile, indirect social burdens are related to the impact rUTIs have on a patient's mental health, and in turn the impacts that a poor mental health state has on a patient's relationships. One participant of the 2022 UTI survey spoke of the toll of her rUTIs, saying, "You're thinking, 'What are [my coworkers] thinking? They can totally smell this' … It's kind of like a social uncomfortableness … Sometimes I'll go to another floor in the building where I think I won't see anybody I know"[32]. These anxieties lead to patients feeling "irritable". These findings were supported by similar prospective studies, retrospective studies, and surveys conducted across Europe and the United States[29,30,33–36].

Why do UTIs recur?

UTIs recur through several complicated mechanisms, the majority of which relate primarily to where the bacteria reside within the host and to the host immune response.

Immune-mediated recurrence. Individuals with stronger inflammatory immune responses during an acute infection are, paradoxically, more susceptible to rUTIs. Significant work performed by *Hannan*, *O'Brien*, and *Yu* over the past several years revealed mice that experience severe COX-2 – mediated inflammatory responses during the early hours of acute infection undergo significant bladder remodeling from neutrophil transmigration, which predisposes these

mice towards chronic and recurrent UTIs[37,38]. These findings translate to humans as well. Hannan *et al* demonstrated in 2014 that humans with elevated levels of inflammatory biomarkers in their serum were more prone to experiencing rUTI, and Ebrahimzadeh demonstrated in 2021 that women who suffer from rUTI have higher levels of prostaglandin E2 (a product of COX2) in their urine[39,40]. It is important to note that not all inflammatory immune responses sensitize individuals to a more robust infection. Robust IL-17 – mediated inflammation, for example, is necessary for mice to resolve their infections and prevent chronicity[41].

The gut as a reservoir

UTI-causing bacteria can colonize and persist within the gut mucosa of infected individuals[42,43]. The bacteria in the human gut are shed in the feces, resulting in bacterial colonization of the skin around the anus. A fraction of these bacteria are mechanically transferred around the urogenital area through the course of routine activity, where they can ascend the urethra and make its way to the bladder. Thanert and Worby demonstrated in 2019 and 2022 that UTI-causing bacteria can "bloom" within the guts of affected individuals immediately preceding a recurrent UTI[44,45]. Furthermore, Worby *et al* performed a large-scale multi-omics study in 2022 to demonstrate that women who experience recurrent UTIs have dysbiostic gut microbiomes, that are overabundant in UTI-causing bacteria[46].

The 'embedded infection' model

In addition to the gut, the bladder itself is known to be a host reservoir for UTI-causing bacteria. Via mechanisms described below **[Intracellular Uropathogenesis]**, bacteria can invade the epithelial cells lining the bladder wall, and persist within bladder epithelial cells long after the initial colonization event[47]. Multiple studies have shown that disruption of the uroethelium, for

example during the insertion of a catheter, can release these intracellular bacteria back into the bladder lumen and 'reactivate' them to cause another infection[48,49].

1.3 Defining uropathogens

1.3.1 Bacterial colonizers of the urinary tract vs. uropathogenic bacteria: A strain specific phenomenon.

UTI researchers face a major hurdle in defining what makes a bacterial species uropathogenic. A uropathogen is commonly defined as any microbial species that can cause a UTI – which is, by definition, a disease where bacteria symptomatically colonize the urinary tract. It is important to note that many bacteria can, and often do, colonize the host asymptomatically, without causing damage[50–53]. By definition, these bacterial species would be considered non-uropathogenic. However, many of these same bacteria can cause damage in specific circumstances. For example, nonpregnant individuals experiencing asymptomatic bacteriuria (ASB) caused by Group B Streptococci (GBS) do not face any risk. However, pregnant individuals asymptomatically colonized GBS are at risk of experiencing adverse birth outcomes[54,55]. While these patients do not experience classic UTI symptoms, the adverse birth outcomes are representative of bacterial damage to the host. While the damage was not limited to the urinary tract, it resulted as a direct consequence of UT colonization. It is thus necessary to consider context when defining a species' uropathogenicity; even if a bacterium is not known to cause UTIs, if it can colonize the urinary tract of and cause damage to a host under the correct circumstances, then it can be uropathogneic. For the purposes of this review, we will thus define a uropathogen as a bacterium that can colonize the urinary tract and of causing damage to their host, in at least one context.

Furthermore, on a molecular level, researchers have been unable to definitively classify an entire species as 'uropathogenic' or 'non-uropathogenic'. Even within the same phylogroup of a species, individual strains can greatly differ in their ability to colonize the urinary tract or to cause harm to a host. For example, studies in UTI- versus ASB- causing *E. coli* isolates demonstrated that gene carriage is not directly linked to bacterial virulence in the urinary tract[56]. UPEC encompasses an extremely genetically and phenotypically diverse group of *E. coli*. Schreiber *et al* demonstrated that UPEC strains' individual genomes can vary greatly, with only 60% of the genome is shared amongst all strains (core), and that putative virulence factors are typically part of the variable portion of the genome[57]. For example, while it is generally accepted that UPEC strains carry at least one iron acquisition and heme utilization system, the exact identity of the specific system can vary. The prototypical urinary isolate UTI89 expresses the heme receptor ChuA at significantly higher levels in the bladder than in the cecum, and experiences attenuated urovirulence when the gene is deleted[58]. However, Clermont *et al* demonstrated that phylogroup A and phylogroup B1 *E. coli* isolates do not carry the chuA gene[59]. Many group A and B1 UPEC isolates cause many UTIs worldwide, meaning chuA itself is not necessary for uropathogenesis[57]. Thus, a specific gene carriage pattern necessary for one UPEC strain is irrelevant to another. Similarly, specific gene carriage patterns necessary for one UPEC strain may not be unique to uropathogenic *E. coli* at all; many virulence factors of uropathogenesis, including type 1 pili, are harbored by most *E. coli* strains regardless of pathogenic potential[60–62]. Similarly, individual strains of *A. baumannii* differ in their abilities to successfully colonize or cause damage to a host urinary tract[23]. It is thus necessary to consider uropathogenic bacteria on an individual, strain-by-strain basis rather than on a species or even subspecies/phylogroup level.

1.3.2 A brief aside: *A. baumannii*

While uropathogenic bacteria should be considered on a strain-by-strain basis, this review will focus on strains of two unique species: *E. coli* and *A. baumannii*. Uropathogenic *E. coli* (UPEC) is discussed in detail throughout this chapter. Thus, this specific section will serve as a primer for the two *A. baumannii*.

***Acinetobacter baumannii*: an overview**

Historically, *Acinetobacter* was regarded as an opportunistic pathogen of low virulence potential and lower clinical import. However, in recent years this bacterium has become a pathogen of emergent concern. It is currently regarded as a "high priority" and "urgent" threat by the WHO and the CDC[63,64]. *Acinetobacter baumannii* is now known for its role as a nosocomial pathogen and its ability to colonize medical devices used in the treatment of compromised patients[65]. While individual site reports vary, *A. baumannii* has been associated with 2% up to 5% of all hospital-acquired bacterial infections worldwide[66–68]. *Acinetobacter* is primarily associated with ventilator-associated pneumonia and catheter-associated bloodstream infections. However, by colonizing implanted urinary catheters, Acinetobacter spp. cause CAUTIs and act as uropathogens; ~20% of all *A. baumanii* isolates come from urinary isolates[24,69]. While some publications posit that an extra-hospital reservoir can introduce new strains of bacteria to the clinic[70,71], *A. baumannii* infections are primarily thought to be spread between patients and hospitals via reservoirs within the hospital itself. Multiple publications have ruled out the environment and the community as potential reservoirs for infection, and "infection reservoirs external to the hospital have not been identified"[72–74].

Acinetobacter, and *A. baumannii* specifically, is increasingly associated with extremely high rates of multidrug resistance. In fact, in the last few years, the frequency of multidrug resistance

(MDR) in *A. baumannii* has skyrocketed and it is currently the Gram-negative bacterium displaying one of the highest rates of multidrug resistance worldwide[66,75,76]. *Acinetobacter* is difficult to treat and carries plasmids that confer resistance to most common antibiotics such as penicillins, aminoglycosides, cephalosporins, and quinolones[77–79]. Some studies of *Acinetobacter spp*. clinical isolate panels report MDR rates of up to 99%[80]. This has led to an increased use of carbapenem-class antibiotics, which has in turn led to an increase in selective pressure and carbapenem-resistance. Carbapenem-resistant *A. baumannii* (CRAB) is highly prevalent worldwide, accounting for over 50% of all A. baumannii infections in the United states and up to 85% in other nations[81,82]. The emergence of CRAB has necessitated a shift towards use of a polymyxin-class antibiotics, usually colistin and polymyxin B, sometimes prescribed in combination with the non-polymyxin antimicrobial rifampin[83–85]. However, colistin is notably nephrotoxic, which greatly complicates the treatment of pyelonephritis and other UTIs[86]. Studies have also determined that even after treatment with colistin, patients suffering from CRAB bacteremia still suffer a mortality rate as high as 70%[87,88]. Colistin-resistant CRAB isolates have also been increasing in prevalence in recent years[89,90].

Acinetobacter baumannii genetics

A. baumannii is highly genetically diverse with a pan-genome of over 8800 genes[72]. The majority of these genes are not shared by all strains in the species and are part of the accessory genome[91,92]; depending on the number and identity of strains analyzed, only ~1455 – 2688 of those genes are part of the species' core genome[91–94]. Paradoxically, despite its genetic diversity and genomic plasticity, compelling arguments have been made asserting *A. baumannii* as a species that is much more "genetically compact" than it could be, and that the species' diversity was in fact reduced by an evolutionary bottleneck[76,95,96]. The majority of hospital outbreaks can

be traced back to one of eight lineages[76,96]. Three of these lineages are far more highly represented than the other five[93,97,98]. Diancourt *et al* performed sequence analysis of over 150 *A. baumannii* isolates in 2010 and discovered evidence of a recent and severe genetic bottleneck in the species' recent past, perhaps exposure to antibiotics[95].

A. baumannii as part of the ACB-complex

Studies on *Acinetobacter spp.* began when it was first isolated from a soil sample in 1911 , though it was only named as such in 1954 (it was originally named originally named *Micrococcus calcoaceticus*[99,100]. Even then, *Acinetobacter* was only widely accepted as a genus after *Baumann et al* published their report in 1968[100–102]. *Acinetobacter baumannii* was specifically recognized as a species following Bouvet and Grimont et al's report in 1986[103]. It has historically been difficult to differentiate between Ainetobacter varieties biochemically or phenotypically on the species level. As such, a proportion of historic *A. baumannii* clinical isolates are thought to be other species which were improperly categorized[104–106]. Species can be distinguished using molecular methods such as genome sequence or the presence or absence of specific genes[107,108]. However, molecular methods can also be difficult due to *Acinetobacter's* genetic plasticity; gene mutations and allelic variations can register as a false negative, and horizontal gene transfer can result in a false positive[109–112]. Furthermore, molecular methods are not often used over phenotypical or biochemical methods in the clinic. In fact, clinical reports indicate that diagnostic laboratories have not been utilizing the most sensitive methods to distinguish between *Acinetobacter* species as recently as 2016[106].

For these reasons, *Acinetobacter baumannii* is often grouped with other closely related *Acinetobacter* species in a taxa called the *Acinetobacter calcoaceticus–Acinetobacter baumannii* complex (ACB-complex). It consists of *Acinetobacter calcoaceticus, Acinetobacter*

baumannii, Acinetobacter pittii, Acinetobacter nosocomialis, *Acinetobacter dijkshoorniae,* and *Acinetobacter seifertii*[113–116]. The ACB-complex was first characterized in 1991 by Gerner-Smidt *et al*, after which it quickly established itself as a dynamic and expanding group. *A. pittii* and *A. nosocomialis* were defined on a species level in 2011, and the most recent genomic species included in the complex (*A. dijkshoorniae)* was defined in 2016[114,115].

The ACB-complex member species most relevant to this review, *Acinetobacter baumannii,* is overall the most frequently isolated species in the clinic and is responsible for approximately 80% of reported infections[117,118]. Further, *A. baumannii* is associated with higher rates of antibiotic resistance, with higher frequencies of morbidities, and with higher rates of mortality when compared to other *Acinetobacter* species[119,120]. However, due to the dynamic nature of ACB-complex speciation and species identification, as well as to the close similarities of said species, *A. baumannii* is sometimes referred to or studied as a proxy for all species in the complex[105].

1.4 Intracellular uropathogenesis

1.4.1 Defining intracellular uropathogenesis through the lens of UPEC

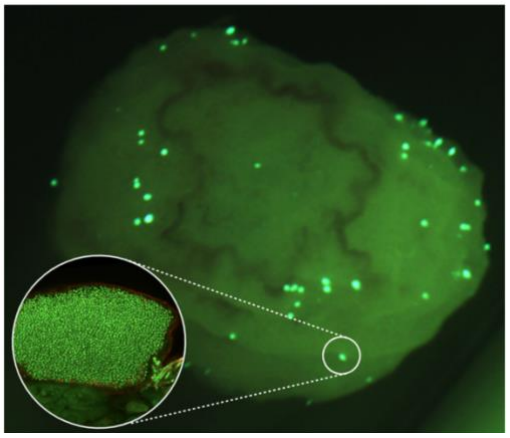

Figure 1.1 Image of IBCs. Image of an inverted 6hpi mouse bladder, decorated with GFP-fluorescing IBCs. A representative cross-section of an IBC is depicted in the bottom left corner, wherein the bright green = GFP-fluorescing *E. coli* and the red = uroplakin III. *Images were merged by Jesús Bázan Villicaña*

Despite the vast genetic diversity of UPEC strains, in murine models of acute cystitis, the majority of successful UPEC strains adhere to and invade their hosts' bladder epithelial cells and become intracellular pathogens [121,122]. This phenotype is surprising; even though the number of non-invasive strains capable of causing robust UTIs *in vitro* is low, most robust infections caused in non-UT niches by *E. coli* as a species are not intracellular. Nevertheless, during UTIs the uropathogens invade and replicate rapidly in the host umbrella cell and eventually form biofilm-like pods called Intracellular Bacterial Communities (IBCs) within the cell **[Figure 1.1]** [121,123–125]. IBC formation is a necessary step of acute UTI pathogenesis in murine models, and IBCs have also been found in shed epithelial cells in human urine from patients suffering clinical UTI[126].

As part of an IBC, the uropathogens are protected by the host cell from antibiotics, neutrophils, and other host defense responses, which cannot penetrate the bladder cell[48,127,128]. The bacteria within the IBC are able to replicate unopposed, eventually filling the bladder cell cytoplasm and fluxing out of the umbrella cells[125,129]. Once out of their host cell the bacteria adhere to and re-invade a neighboring umbrella cell, replicating (albeit at a more attenuated rate) and forming new IBCs[125]. Thus, UPEC take advantage of their intracellular behavior to gain a foothold in the bladder and perpetuate infection.

The IBC cycle

The process of IBC development, known as the IBC cycle, has been studied extensively in the murine model using the prototypical UPEC strain, UTI89, to characterize IBC development, including: **i)** invasion, the adherence of a single bacterium to a bladder umbrella cell; **ii)** IBC maturation, the process by which the replicating daughter cells of the initial intracellular bacterium form a biofilm-like community; **iii)** filamentation, wherein the bacteria within the IBC adapt an extremely long, rod-like morphology; **iv)** fluxing, the escape of the filamented bacteria in the IBC from the bladder epithelial cell, and; **v)** re-invasion, the adherence and entry of fluxed

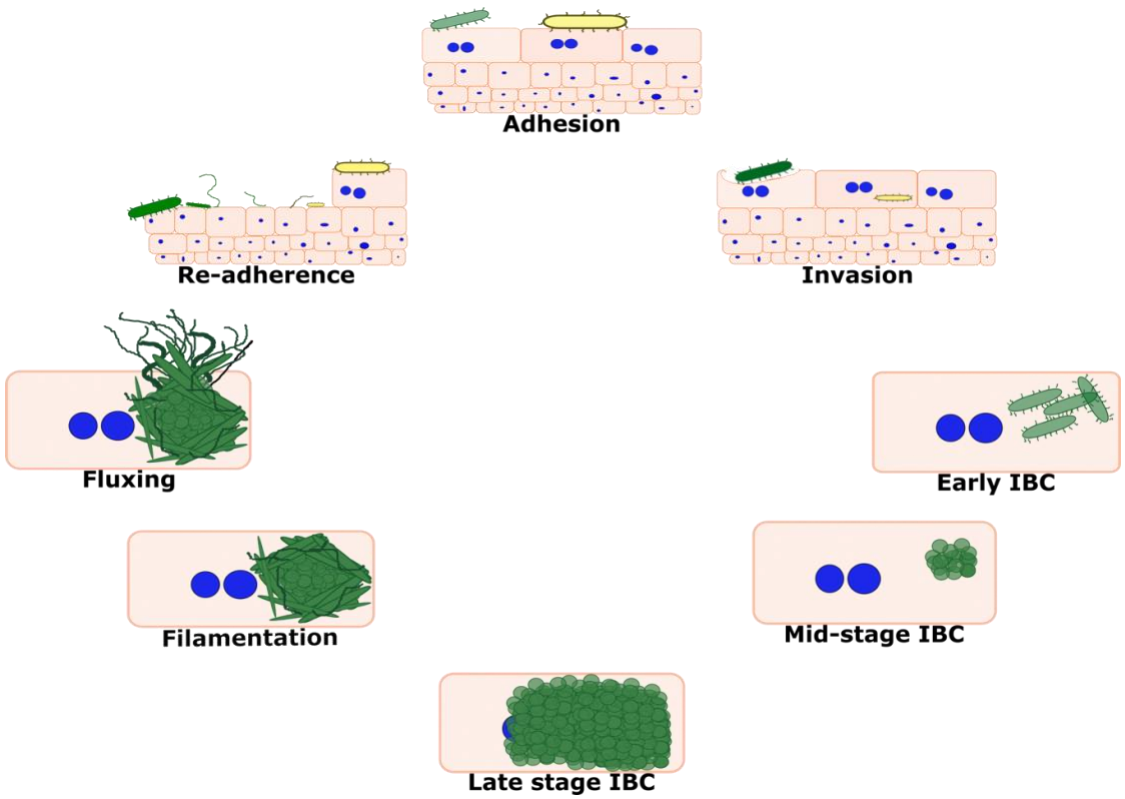

Figure 1.3 The IBC cycle. The adherence and invasion steps depict a full pseudostratified urothelium, while the steps related to IBC maturation depict individual urothelial cells.

bacteria to neighboring umbrella cells[125]. IBCs themselves undergo several stages of morphological and physiological change over the ~18-24 hours of their formation and dispersal

Invasion

The stable adhesion of UPEC via appendages known as type 1 pili to a mannose molecule decorating a bladder epithelial cell triggers a signal cascade within the host cell that results in the engulfment and internalization of the bacteria into the host cell cytosol[127,130,131]. After UPEC binds to integrins and mannosylated UPIa, there is a bacterial-induced phosphorylation of the UPIIIa uroplakin, whose cytoplasmic domain is speculated to have signal-transducing properties. UPIIIa phosphorylation results in a subsequent increase of calcium ions [Ca^{2+}] in the umbrella cell. Both of these processes play a critical role in the initial steps of the signal cascade, though the exact mechanism of UPIIIa phosphorylation is unclear[132].

Following UPIIIa phosphorylation, the focal adhesion kinase protein (FAK) within the bladder cell is phosphorylated[131]. This activation seems to be twofold; adhesion indirectly activates FAK by way of sac kinases, and adhesion also seems to directly activate FAK through an unknown, alternate mechanism[130,131]. FAK then forms complex with Phosphoinositide 3-kinase (PI3K). The PI3K signal pathway then leads to the activation of Rho GTPases[131]. Much like FAK activation, Rho GTPase activation is also two-fold; UPEC secretes a toxin called cytotoxic necrotizing factor 1 (CNF1), which is taken up via host cell endocytic pathways and activates Rho GTPases[133].

The downstream effect of these GTPases is also twofold. First, activation of the Rho-GTPases leads directly to rearrangement of the actin cytoskeleton. The host cell's membrane engulfs the bacterium in a zipper-like mechanism, and internalizes it in an acidic compartment similar to a

late endosome or early lysosome[130,134]. The bacterium then escapes the endosome through an unknown mechanism and colonizes the host cytoplasm. Meanwhile, activation of RAC1-GTP triggers an anti-apoptotic response pathway in the bladder cell. In this way, the bacteria are able to promote the bladder cell's survival in the face of invasion[133].

IBC maturation

Following invasion, the bacteria replicate within the host cell cytosol and form intracellular bacterial communities (IBCs). Over the next 18-24 hours the IBC goes through three distinct stages of maturation.

The **early phase** of the IBC cycle begins roughly between 1-3 hours post infection (hpi), and lasts until 6-8 hpi. During this time, the bacteria are loosely packed and unorganized, and lack any biofilm-like traits. These bacteria change morphologically and replicate independently of each other within their host cell. Each bacterium is large (~7 microns), non-motile and rod-shaped, with a rapid doubling time of 30-35 minutes[125].

Between 6-8 hpi, the IBC matures into the **middle stage**. This stage lasts until about 10-14 hpi. Relatively early in the middle stage, all the bacteria within the IBC appear to undergo a simultaneous "differentiation". They change from their large rod morphology into a small (~2 microns) coccoid shape and become much more closely packed as they congregate into an organized and pod-like community structure. The community begins to display biofilm-like traits, and the bacteria form an extracellular matrix between each other. From this stage forward, the bacteria change, grow, and replicate relatively in tandem with their neighbors. Various *in vivo* mouse models of infection studies have utilized immunohistochemistry, fluorescence reporter genes, and genetic deletion assays to define components of the biofilm-like substance within the IBC matrix. These components include antigen 43, polysaccharides, extracellular

DNA, and surface adhesion molecules called curli[32,123,125,135,136]. The exact signals involved in the simultaneous differentiation from early to mid-stage IBC bacteria are not fully understood, but studies have shown that deletion of IHF, a DNA-binding protein that regulates the transcription of adhesins and bacterial capsules, prevents the bacteria from adopting their coccoid morphologies[137]. It is also known that UPEC secretes antigen 43 in response to oxidative stress, and seem to form curli in response to quorum sensing-related signals[138]. The bacteria within the newly-formed biofilm continue to replicate, though their doubling time slows significantly to 1 hour, until the bacteria occupy almost the entire area of the host cell.

The IBC enters the **late stage** of maturation at about 12-16 hpi. The bacteria first revert in morphology from coccoid back to rod shape. The rods divide more quickly, and appear to have a flagellar-based motility[125]. Passage into the late stage of IBC formation is highly unsynchronized from IBC to IBC. Morphological changes of UPEC within an individual IBC are unsynchronized as well. This stage of the IBC cycle lasts until approximately 18hpi, after which the bacteria adopt a filamentous morphology. UPEC occupying the outermost layer of the spherical community that is closest to the host cell cytoplasm seem to filament much sooner than bacteria within the IBC's core.

Finally, **Filamentation** occurs when bacteria fail to divide and instead continue to grow longer until they form filaments[139,140]. Inhibiting cell division at the cytokinesis step results in a bacterium that contains twice the genetic information and is twice as long as it was before initiating the replication process[141]. Deletions of DamX, a gene whose protein product regulates FtsZ ring activity during cytokinesis, result in an inability for UPEC to form filaments in mouse bladders *in vivo*. Conversely, overexpression of DamX promotes rapid premature filamentation of UPEC[142]. The SOS response pathway, which inhibits cell division in response to DNA

damage (in this case, largely caused by oxidative stress), also plays a role in filamentation. IBCs formed during *in vivo* mouse infection assays performed using UPEC with deletions of genes involved in the pathway such as SulA, RecA, and LexA did not filament[143,144]. It is unknown exactly what stressor or combination of stressors are directly responsible for triggering the SOS response at the end of the IBC cycle[126,129,143].

Fluxing

When the filamentous bacteria grow too large to be contained from the host epithelial cell, they flux out, along with neighboring rod-shaped bacteria, from a localized area on the epithelial cell surface back into the bladder lumen[125]. After fluxing, the filamentous UPEC are protected from neutrophils in the bladder lumen, where they can re-adhere to and re-invade a neighboring epithelial cell[129,145]. Once inside a new host cell, the bacteria undergo the IBC cycle all over again.

Outcomes of the IBC cycle

After two to three rounds of invasion, IBC maturation, fluxing, and subsequent re-invasion, with the kinetics of IBC development attenuating every new cycle, the UPEC run out of intact umbrella cells to infect and thus stop forming IBCs. There are then three courses of action that the bacteria can take:

The bacteria persist in the bladder and the urinary tract infection becomes a long-lasting chronic cystitis. Bladder cystitis is specifically characterized by bacterial urine and bladder titers greater than 10^4 CFU/ml and chronic inflammation that lasts more than two weeks after initial infection[146].

The host immune system clears the infection, and the bacteria are flushed from the urinary tract.

A small number of bacteria invade underlying intermediate transitional bladder cells and become metabolically inactive. The bacterium then remains within the endocytic vesicle that it utilized to invade its host cell and becomes a quiescent intracellular reservoir (QIR)[147]. While evidence of QIRs have been identified in murine models of infection, they have not yet been found in human urine as infected bladders do not tend to shed transitional cells. QIRs are long-lasting and are capable of re-seeding same-strain infection long after clearance of the initial infection[48]. Mice that suffer from a higher burden of IBCs during the acute phase of infection tend to develop more severe outcomes of infection than mice whose bladders did not house many IBCs[146]. Thus, successful passage through the IBC cycle is an important step for UPEC to gain a foothold in the bladder.

1.4.2 Other intracellular uropathogens

It has become increasingly clear in recent years that UPEC is not the only species whose strains undergo important intracellular steps in the context of a UTI. In the laboratory setting, many bacterial species that are not canonically associated with intracellular pathogenesis have been shown to invade and persist within mouse bladder cells or *in vitro* in bladder epithelial cell lines. Rosen *et al* demonstrated that *K. pneumoniae* was capable of undergoing the full IBC cycle in the murine model of infection[124]. Molecular interrogation revealed that the *K. pneumoniae* isolates employed similar mechanisms to their UPEC counterparts to invade and persist within the urothelium, the most prominent of which was type 1 fimbriation and adhesion to D-mannose[124]. Similarly, Szabados demonstrated that *S. aureus*, which is very evolutionarily distinct from UPEC and *K. pneumoniae,* can invade and persist within the human bladder carcinoma 5637 cell line[148]. Further, the invasive abilities of *P. mirabilis* have been known since the 1980s, and is connected to the species' motility, ability to swarm, and ability to import

putrescine[149,150]. Similarly, Penaranda *et al* demonstrated in 2021 that *P. aeruginosa* can invade and persist within bladder epithelial cells in both *in vivo* cell culture models and in *in vitro* murine models of infection[151]. In March of 2022, Newman *et al* demonstrated that *P. aeruginosa* isolates obtained from *in vivo* clinical patient UTI samples were competent to invade bladder 5637 cells *in vitro*[152]. Paradoxically, the host NF-kappaB pathway, which is important in clearing *Pseudomonas* infections, is also important in allowing *Pseudomonas* to persist within the bladder epithelial cells[151].

There has recently been a "boom" in the intracellular uropathogen field, and new publications investigating intracellular uropathogens in the context of human samples are being released at an exponential rate[153–156]. Khasriya *et al* performed gentamicin protection assays on shed urothelial cells from infected patient urine samples in 2022[153]. Briefly, the antibiotic gentamicin can kill bacteria but incapable of permeating a bladder cell. In a gentamicin protection assay extracellular adherent bacteria are thus killed while intracellular bacteria are protected by the bladder cell and can be quantified[129,157]. Khasriya reports recovery of *Enterococcus faecalis, Streptococcus anginosus, E. coli*, and *Proteus mirabilis*, thus providing indirect support for the invasive capabilities of these bacteria in human UTIs[153]. Horsley *et al* supported these findings with their own direct microscopy-based observations of intracellular *E. faecalis* in shed patient urothelial cells[154]. More recently, Barrios-Villa *et al* observed intracellular *Staphylococcus aureus, Staphylococcus epidermidis, Staphylococcus simulans*, and *Streptococcus agalactiae* in a patient experiencing a recurrent polymicrobial infection[155]. The authors did not conduct any experiments to determine the mechanisms by which these bacteria invaded the bladder epithelial cells. However, they noted the presence of pyocites in the patient's urine and hypothesized that *S. aureus* utilized fibronectin-binding adhesins to invade the urothelium[155]. Interestingly, while this

patient reported UTI symptoms, the urine culture did not yield a significant bacterial burden. However, direct observation of Sternheimer-Malbin stained urine sediment revealed the presence of bacteria residing within the exfoliated bladder epithelial cells. The bacteria existed in several different forms within the bladder cell cytosol, likely reflective of the polymicrobial nature of the infection. Some intracellular bacteria seemed to resemble QIRs and existed in small, isolated groups. Others existed in bacterial aggregates that closely resembled IBCs. After processing the urine sediment to lyse exfoliated bladder cells and release any intracellular bacteria, the patients' urine culture tested positive for bacterial infection[155]. Similarly, Ognenovska *et al* published their direct observation of intracellular *Enterococcus faecalis* and Group B *Streptococcus* (GBS) within exfoliated bladder epithelial cells found in the urine of infected patients who also suffered from detrusor overactivity[156]. These observations directly support those made by Horsely *et al* and Khasriya *et al*[153,154]. The GBS closely resembled QIRs, or *A. baumannii* intracellular reservoirs seen during UTIs **(described further in Chapter 2)**; they were few in number, small and coccoid in morphology, and low in signal intensity. The intracellular *E. faecalis* appeared to form multiple small intracellular aggregates throughout the bladder cell cytosol. Molecular probing of urinary GBS and *E. faecalis* isolates indicated that, similarly to UPEC isolates, virulence factors determining invasive abilities varied on a strain-specific level. However, all the isolates had at least one of the following three abilities: D-mannose binding, haemolytic/cytolytic activity, and/or biofilm production[156].

It is becoming increasingly apparent that intracellular steps are important during uropathogenesis, even in species that are not associated with intracellular infections. Thus, further studies are necessary to learn the mechanisms of these intracellular steps.

1.5 Outstanding questions

While there are several outstanding questions in the field of intracellular uropathogenesis, this dissertation will focus on two areas of inquiry: **1.) Intracellular pathogenesis of *A. baumannii*.** Namely, is the pathogen *Acinetobacter baumannii* capable of invading and persisting within urothelial cells?; and **2.) Intracellular pathogenesis of UPEC.** Do diverse, non phylogroup-B2, isolates of uropathogenic *E. coli* exhibit canonical IBC phenotypes? What mechanisms underlie IBC development in UPEC?

1.5.1 Intracellular pathogenesis of *A. baumannii*.

Acinetobacter baumannii is known to adhere to and invade lung epithelial cells *in vivo*, and various human cell lines *in vitro*[158–162]. However, no studies have been performed to determine whether *A. baumannii* undergoes intracellular steps during uropathogenesis.

1.5.3 Intracellular pathogenesis of UPEC

Do diverse, non phylogroup-B2, isolates of uropathogenic *E. coli* exhibit canonical IBC phenotypes?

While the intracellular behavior of UPEC has been extensively characterized, the majority of these characterizations have been performed in strains from the B2 phylogroup[57,122,163–165]. The overrepresentation of B2 isolates is unsurprising, as phylogroup B2 causes the majority of UTIs in the United states, where the majority of this research was conducted[57]. However, phylogroups A, B1, and D also cause UTIs. In fact phylogroup D strains are more well-represented in other parts of the world, such as Asia, than B2 strains are[57]. Further studies are thus necessary to ensure that diverse UPEC isolates from other phylogroups follow similar IBC cycle kinetics and morphologies to the well-characterized B2 strains, and to identify any possible link between genotype and intracellular phenotype.

What mechanisms underlie IBC development in UPEC?

Until recently, experiments that probe the molecular mechanisms underlying intracellular uropathogenesis have been difficult. These difficulties can be attributed to four main complications: **i.)** The difficulties in defining a molecular signature of a uropathogen also carry over to defining the molecular signature of an intracellular uropathogen; **ii.)** the extreme genetic bottlenecks that occur between infection and intracellular persistence[148,151,152,154,156,166,167]; **iii.)** the low bacterial biomass of an IBC, QIR, or similar intracellular structure and the contaminating presence of the surrounding host bladder cell; and **iv.)** the relative lack of *in vitro* models that successfully recapitulate the urothelial environment[168–170]. As such, relatively little is known about the gene regulatory programs necessary for a bacterium to successfully invade and live within a bladder epithelial cell.

1.6 Conclusion

In this chapter, I have described the current body of knowledge on the intracellular steps of uropathogenesis, and I have demonstrated why these steps are important to study. To this end, I have conducted several studies with the goal of better understanding intracellular uropathogenesis. In the second chapter of this document, I will focus on intracellular *A. baumannii*. I investigated and characterized the intracellular capabilities and behaviors of a species not previously known to occupy the bladder cell cytosol, and uncovered a potential functional role of these intracellular bacteria in causing secondary infections. In chapter three I will describe my efforts to expand the existing body of knowledge on the general patterns of behavior of intracellular UPEC via extensive phenotypic characterizations of diverse UPEC strains. In chapter four, I interrogated the relationship of a specific molecular mechanism, lactose metabolism, to intracellular survival in the urinary tract and suggest an additional genetic

pathway used by these intracellular bacteria. Through these studies, I contribute to the growing body of knowledge about intracellular uropathogenesis in the field, and open the door to new avenues of research.

Chapter 2: Catheterization triggers resurgent *A. baumannii* infections seeded by host bacterial reservoirs

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2.1 Abstract

The highly antibiotic resistant bacterium *Acinetobacter baumannii* is a leading cause of healthcare-associated infections. Despite surveillance and infection control efforts, new *A. baumannii* strains are regularly isolated from healthcare facilities worldwide. In a mouse urinary tract infection model, we found that mice infected with *A. baumannii* displayed high bacterial burdens throughout their urinary tracts for several weeks. Strikingly, even two months after resolution of infection, the introduction of a catheter into the bladder led to the resurgence of same-strain catheter-triggered urinary tract infection in ~53% of mice in just 24 hours. The recurrent UTI triggered by insertion of a catheter suggests this strain establishes a reservoir in the host. We identified intracellular bacteria in the bladder epithelial cells of these resolved mice, which we propose act as a host reservoir. These data suggest that patients can unknowingly enter the clinic already harboring *A. baumannii* reservoirs, which can be activated upon insertion of a medical device leading to a resurgent infection. Our findings could, in the future, lead to the implementation of novel preemptive strategies to mitigate the risk for *A. baumannii* infections and subsequent hospital outbreaks.

2.2 Introduction

Healthcare-associated infections (HAI) are often associated with the use of medical devices such as catheters and ventilators, surgical procedures, transmission between patients and healthcare workers, and overuse of antibiotics. Annually, approximately two million patients suffer from HAIs and nearly 100,000 patients are estimated to die in the US[171]. According to the World Health

Organization (WHO), the direct cost of HAIs to hospitals is at least US$35.7 billion in the USA [171]. Moreover, the CDC 2020 National and State Healthcare-Associated infections Progress Report described multiple severe setbacks in the prevention of several important HAIs[172]. *Acinetobacter baumannii* is a leading global cause of HAI[173–177]. This bacterium is a known cause of medical device-associated infections like ventilator-associated pneumonia and catheter associated urinary tract infection (CAUTI), as well as nosocomial skin and soft tissue infections[178]. These infections are severe in immunocompromised or otherwise vulnerable patients, which can have high rates of morbidity and mortality[179,180]. In the last few years, the frequency of multidrug resistance (MDR) in *A. baumannii* has skyrocketed and it is currently the Gram-negative bacterium displaying one of the highest rates of multidrug resistance worldwide. Reflecting its growing impact on global health, the WHO and CDC have classified the species as an urgent, high priority threat in need of new therapeutics[63,64]. Within hospitals, infected patients act as bacterial sources for transmission, often through colonization of high-touch surfaces and equipment. Key unanswered questions are: how are new *A. baumannii* strains first introduced into hospitals to colonize these surfaces, and from where does the "patient zero" who initiates an *A. baumannii* outbreak contract their infection. Increasing reports of community-acquired infections suggest the existence of extra-hospital reservoirs[70,71], and several cases of *A. baumannii* community-acquired infections are reported annually across the globe[181–189]. Here, we present evidence indicating that patients may serve as their own reservoir carrying intracellular *A. baumannii* from a previous infection or asymptomatic colonization event. These reservoirs can be activated upon treatments such as the insertion of a medical device, triggering infections that could initiate nosocomial outbreaks.

2.3 Results

2.3.1 *A. baumannii* asymptomatic bacteriuria occurs in ~*2%* of the healthy population

Acinetobacter baumannii is able to cause both urinary tract infections (UTI) and asymptomatic bacteriuria (ASB) in both hospital and community settings[24]. Several individual center studies report *A. baumannii* as one of the most common uropathogens causing CAUTI in their facility[190,191]. Additionally, approximately one fifth of *A. baumannii* clinical strains are isolated from the urine of patients suffering from UTIs[24]. 40% of these isolates were derived from the urine of patients suffering from community-acquired UTIs and from otherwise complicated but non-catheter associated UTI (the combination of which will hereafter be referred to as non-catheter UTI, or ncUTI) [24]. Like patients with ncUTIs, most individuals with *A. baumannii* ASB (Ab ASB) are colonized from sources outside of the clinic. Upon entering the healthcare setting, such individuals would unknowingly bring *A. baumannii* into the clinic. We performed a systematic literature analysis of studies screening different populations for Ab ASB. Data was compiled on 7,060 individuals, 1,055 of whom had ASB, across 11 international publications from 1979 to 2020[52,192–201]. We focused our analysis on healthy, non-pregnant individuals who had not recently been hospitalized and thus were unlikely to have been colonized from the clinic. Four of the studies detected *A. baumannii* in their subjects' urine. Remarkably, *A. baumannii* and other *Acinetobacter* species within the ACB-complex were well represented among the total study population. Approximately 13.5% (143/1050) of reported ASB cases could be attributed to *A. baumannii*. Our analysis also indicates that approximately 2% (143/7060) of the healthy, non-pregnant population carry ASB attributable to *A. baumannii* or other related species within the ACB-complex (**Table 2.1).** This number is likely an underestimate, since several ASB studies used methods that do not

facilitate the growth of non-lactose fermenters and older bacteriological methods were not sensitive enough to detect any low-count bacteria in multi-species ASB cases[202,203]. Based on these results, we conclude that *A. baumannii* urinary tract colonization is a relevant phenomenon in both symptomatic and in asymptomatic cases, and extra-hospital acquisition of *A. baumannii* is far more common than previously estimated.

Ref	Where	When	Individuals	ASB cases	Ab-ASB cases	% of ASB cases	% of total population
Pooled population of 11 studies	**8 countries**	**1979 - 2020**	**7060**	**1055**	**143**	**14%**	**2%**
[52]	Nigeria	Pre-2006	400	64	29	45%	7.3%
[192]	Iraq	Jan 2016 – Dec 2017	862	263	32	12%	3.7%
[193]	Cameroon	Pre-2013	111	29	0	0%	0%
[194]	Nigeria	Aug 2007 – Sep 2008	200	26	0	0%	0%
[195]	South Korea	Jan 2001 – Dec 2001	30	1	0	0%	0%
[196]	Denmark	Oct 1979	2314	288	81	28%	3.5%
[197]	UK, Portugal, Spain	Jan 2010 – Dec 2011	2497	303	0	0%	0%
[198]	Nigeria	Jul 2017 – Dec 2017	116	1	1	100%	0.9%
[199]	Nigeria	Pre-2005	300	30	0	0%	0%
[200]	Nigeria	Mar 2007 – May 2007	180	27	0	0%	0%
[201]	Nigeria	Mar 2009 – May 2009	50	23	0	0%	0%

Table 2.1 *Acinetobacter* asymptomatic bacteriuria occurs in ~2% of the healthy population. Table depicts the proportions of individuals across 11 international studies in a "pooled population", who have asymptomatic bacteriuria attributed to *A. baumannii*.

2.3.2 Different host genetic backgrounds confer different severity of *A. baumannii* ncUTI

We have recently developed a mouse model to study *A. baumannii* CAUTI[24]. However, ncUTIs and asymptomatic colonization remain understudied. Bladder catheterization elicits an inflammatory response in the host that modifies both the bladder and the catheter surfaces; therefore, bacterial mechanisms of uropathogenesis differ between CAUTIs and ncUTIs[22,204–206]. We developed a murine model to study *A. baumannii*–induced ncUTIs. The severity of a UTI is highly dependent on the genetic background of the infected host[207,208]. We thus evaluated the ability of UPAB1, an MDR urinary clinical isolate obtained in 2016 from a patient suffering from ncUTI[24], to infect: **i)** immunocompetent C57BL/6, **ii)** immunocompetent C3H/HeN mice, and **iii)** TLR4-deficient, immunocompromised, C3H/HeJ mice. TLR4-deficient C3H/HeJ mice were derived from C3H/HeN mice and were chosen since TLR4 is known to play a key role in the defense of uropathogens[209]. We evaluated the outcomes of infection by performing comparative long-term urinalysis studies on the three strains of mice **(Figure 2.1A)**. Immunocompetent C3H/HeN mice were not susceptible to long-term UTIs, resolving their bacteriuria in less than two weeks **(Figure 2.1B, C)**. Approximately half of the immunocompromised C3H/HeJ, mice, however, still had urine titers higher than 10^4 CFU/ml three weeks post infection (wpi), experiencing bacteriuria for up to two months **(Figure 2.1B, C)**.

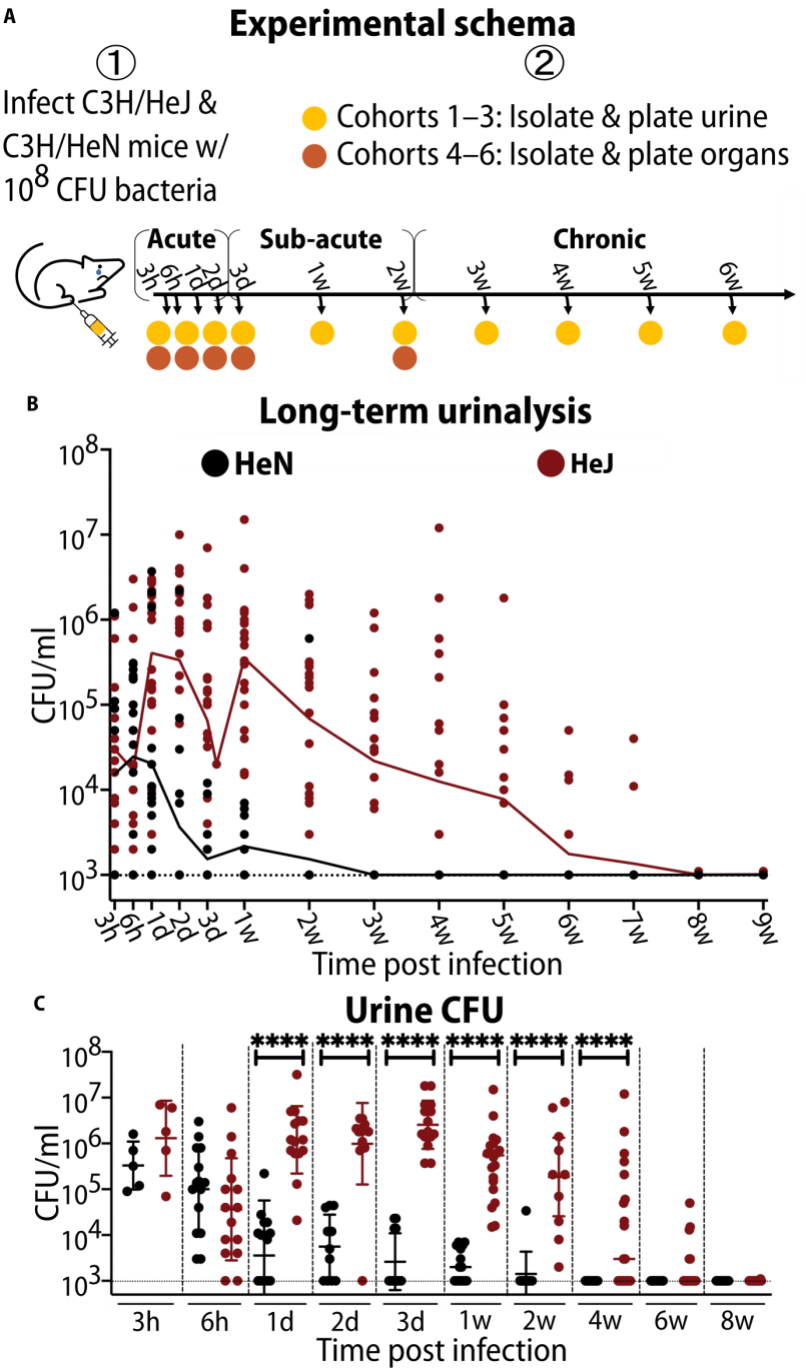

Figure 2.1 Host susceptibilities in the murine model of *A. baumannii* ncUTI mimics population dynamics seen in the clinic .**A)** Experimental design. Female mice were infected with UPAB1 and bacteriuria levels were examined at indicated time-points and tracked weekly until resolution of bacteriuria. Bladders and kidneys were harvested and bacterial burdens were enumerated at the indicated timepoints during acute and sub-acute colonization. **B)** Bacterial burdens in the urine of C3H/HeJ and C3H/HeN mice from the experiment described in (A), where the connecting line represents the geometric mean. **C)** C3H/HeJ and C3H/HeN mice were transurethrally infected with UPAB1. Urine was collected and bacterial burdens were enumerated at 3, 6, 24, 48, and 72hpi and weekly thereafter until bacteriuria levels fell to below the limit of detection (LOD). LOD = 1000 CFU/ml urine. **** = $P \leq 0.0001$

We also measured bacterial burdens in the urine, kidneys, bladders, and spleens of infected mice at 3 hours post infection (hpi), 6hpi, 24hpi, 1 wpi and 2wpi. Again, the immunocompetent C3H/HeN mice were able to clear their infections during the acute and sub-acute stages of UTI while the C3H/HeJ mice took several weeks to clear their organs of bacteria **(Figure 2.2 A, B)**. The immunocompetent C57BL/6 mice were also not susceptible to even acute colonization by *A. baumannii*. By 24hpi their urine had no detectable bacterial presence and their bladder and kidney titers had fallen below 10^4 CFU/organ (**Figure 2.2C)**. We next sought to ensure that UPAB1's ability to cause prolonged urinary tract colonization in TLR4-deficient mice depends on the strain's uropathogenic potential in addition to immunocompromised status of the host. We infected C3H/HeJ mice with ATCC 19606 ("19606"), a common lab strain isolated from urine in 1967, and a poor uropathogen in the CAUTI model[24,210]. 19606-infected mice showed significantly decreased urine, bladder, and kidney titers compared to mice infected by UPAB1 at all analyzed time points **(Figure 2.3)**. These results demonstrate both that UPAB1 is a uropathogenic strain in the ncUTI model and that the phenotypes observed were not solely due to the immunocompromised nature of the mouse. *A. baumannii* is thus capable of colonizing the urinary tract in a non-catheter associated model of infection, showing increased severity in immunocompromised mice. In this way, our model mimics patient demographics seen in the clinic.

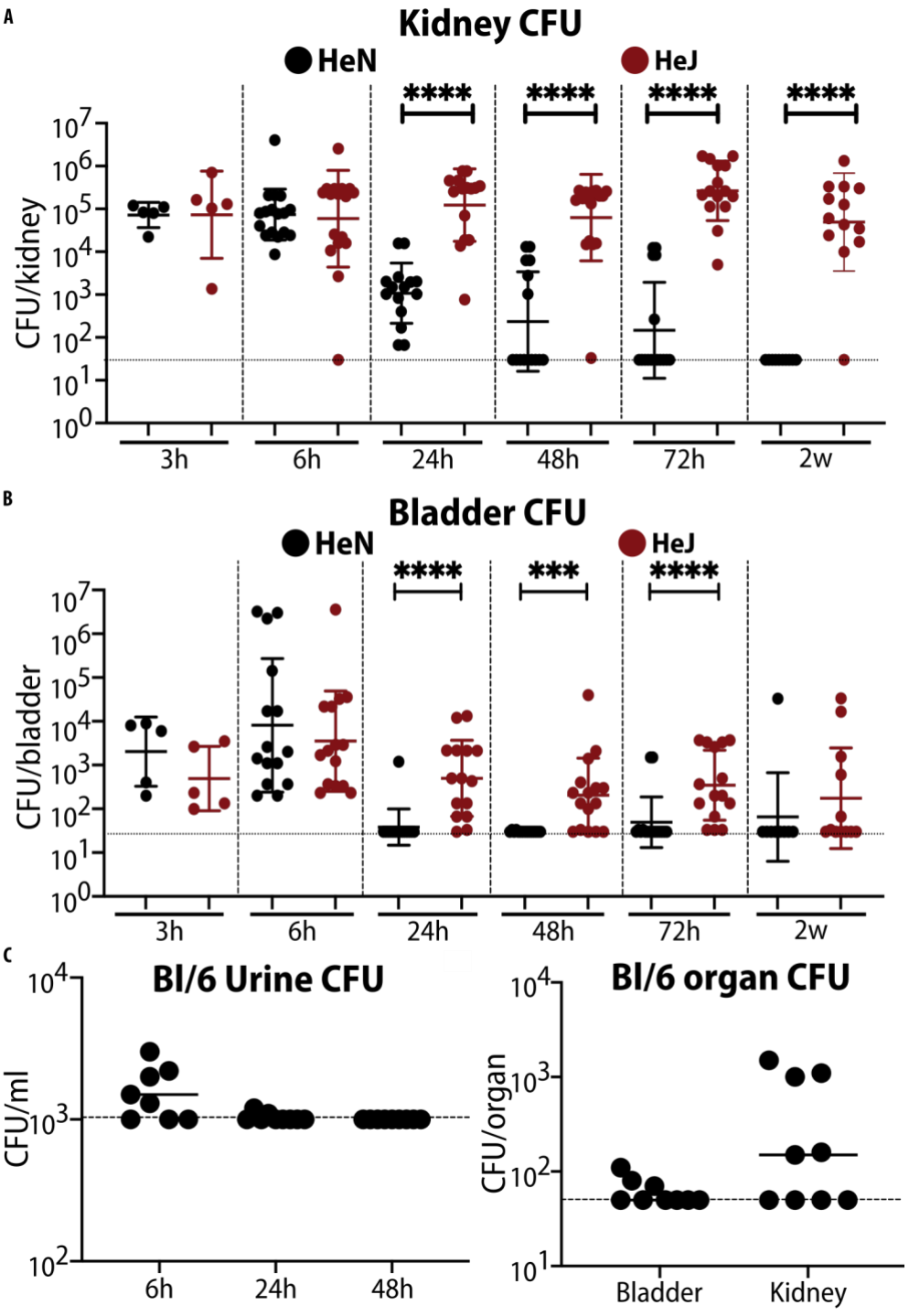

Figure 2.2 Characterizing a murine model of ncUTI A) Kidney CFU of the mice described in Fig 2.1A. **B)** Bladder CFU of the mice described in Fig 2.1A. **C)** Female C57Bl6/J mice were infected with UPAB1. Mouse urine was collected at 6, 24, and 48hpi while bladders and kidneys were collected at 24hpi and bacterial burdens were enumerated.

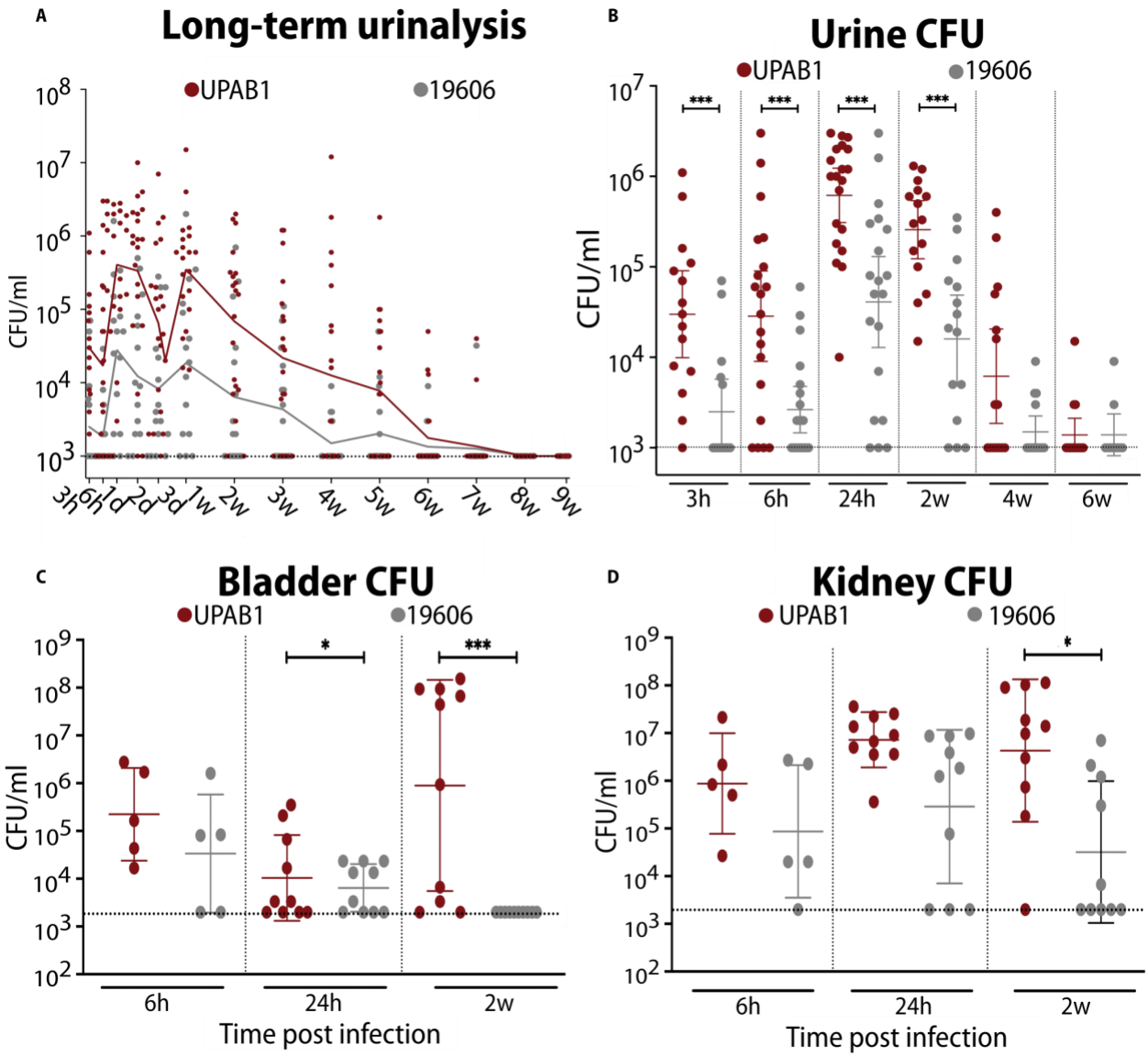

Figure 2.3 UPAB1 is a bonafide uropathogen. C3H/HeJ mice were transurethrally infected with UPAB1 or 19606. **A)** Bacteriuria levels were measured at 3, 6, 24, 48, and 72hpi, and weekly thereafter until resolution of bacteriuria below the LOD of 1000 CFU/ml urine. **B)** Selected time-points depicting bacteriuria levels of UPAB1 vs 19606 infected C3H/HeJ mice from the experiment described in (A). **C)** Bladders were harvested and bacterial burdens were enumerated at 6hpi, 24hpi, and 2wpi. LOD = 30 CFU/bladder. **D)** Kidneys were harvested from the mice described in (C) and bacterial burdens were enumerated at 6hpi,24hpi, and 2wpi. LOD = 30 CFU/kidney. *** = $P \leq 0.0005$, ** = $P \leq 0.005$, * = $P \leq 0.05$

2.3.3 Developing a model of resurgent A. baumannii UTI.

Uropathogenic *Escherichia coli* (UPEC) is the most common cause of UTI[19]. UPEC has been shown to establish quiescent intracellular reservoirs in the bladder that can seed recurrent infections upon a molecular insult to the bladder such as catheterization[49]. Therefore, we evaluated

whether an infection could reappear in animals that have resolved their *A. baumannii* primary infections. This model (activation of a host reservoir by catheterization) will be referred to as the **"resurgence model"**. Primary infections are classified as resolved when previously infected mice produce bacteriuria titers below the limit of detection in two separate urine samples taken seven days apart. Thus, resolved mice serve as a proxy to study previously colonized but outwardly healthy patients. Mice were transurethrally infected with UPAB1 and bacterial loads in the urine were tracked until their infection had been resolved. Following resolution, mice were separated from their cage-mates still experiencing active bacteriuria to reduce consumption of contaminated bedding and feces, and housed in fresh cages with at least two other resolved mice of the same litter. Following separation, the resolved mice were left to recover for one to nine weeks, depending on the specific experiment performed. Subsequently, we aseptically inserted a catheter into the bladders of resolved mice. By sanitizing the skin and fur surrounding the vagina, anus, and urethra prior to catheterization, we eliminated detectable bacteria on the perineal region of the mouse **(Figure 2.4)**.

Catheter retention was approximately 56% (38/67) over the 24 hour period. Catheter, bladder, kidney, and spleen bacterial burdens were quantified 24 hours post implantation **(Figure 2.5A)**. 53% (20/38) of mice that retained their catheters exhibited a resurgent infection (**Figure 2.5B)**. Resurgent infections in these mice manifested as CAUTI (45%, 17/38), cystitis (~42%, 16/38), pyelonephritis (~24%, 9/38), and sepsis (~8%, 3/38). Of the 29 mice who did not retain their catheters for the full 24 hours after implantation, ~38% (11/29) had bacterial burdens indicative of resurgent systemic infections **(Figure 2.5B)**. PCR analysis and antibiotic profiling confirmed that the strain recovered was UPAB1[24,211] **(Figure 2.6).**

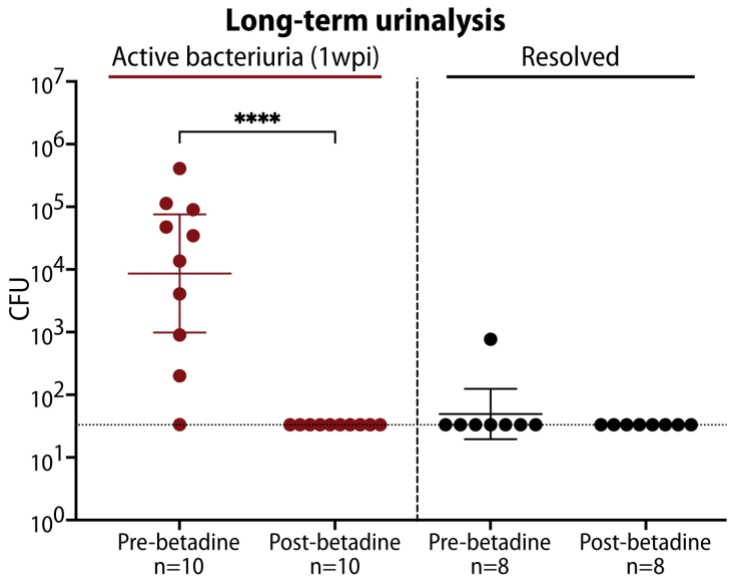

Figure 2.4 Sterilization of the perineum preceding catheter insertion.C3H/HeJ mice were transurethrally infected with UPAB1. Bacteria on the perineum (skin and fur surrounding the urethra) was quantified before and after treating the area with betadine, a step performed immediately prior to catheterizing the animal. In both cases of active bacteriuria (red, left) and after resolution (black, right), betadine treatment eliminated viable bacteria in the area surrounding the urethra.

As of now it is unknown why only some mice develop systemic infections while others only develop CAUTI. No correlation was observed between the bacterial load in the bladder or catheter, or even the retention of the catheter for the full 24 hours, with the presence of bacteria in the spleen 24 hours post implantation **(Figure 2.5B, Figure 2.7).**

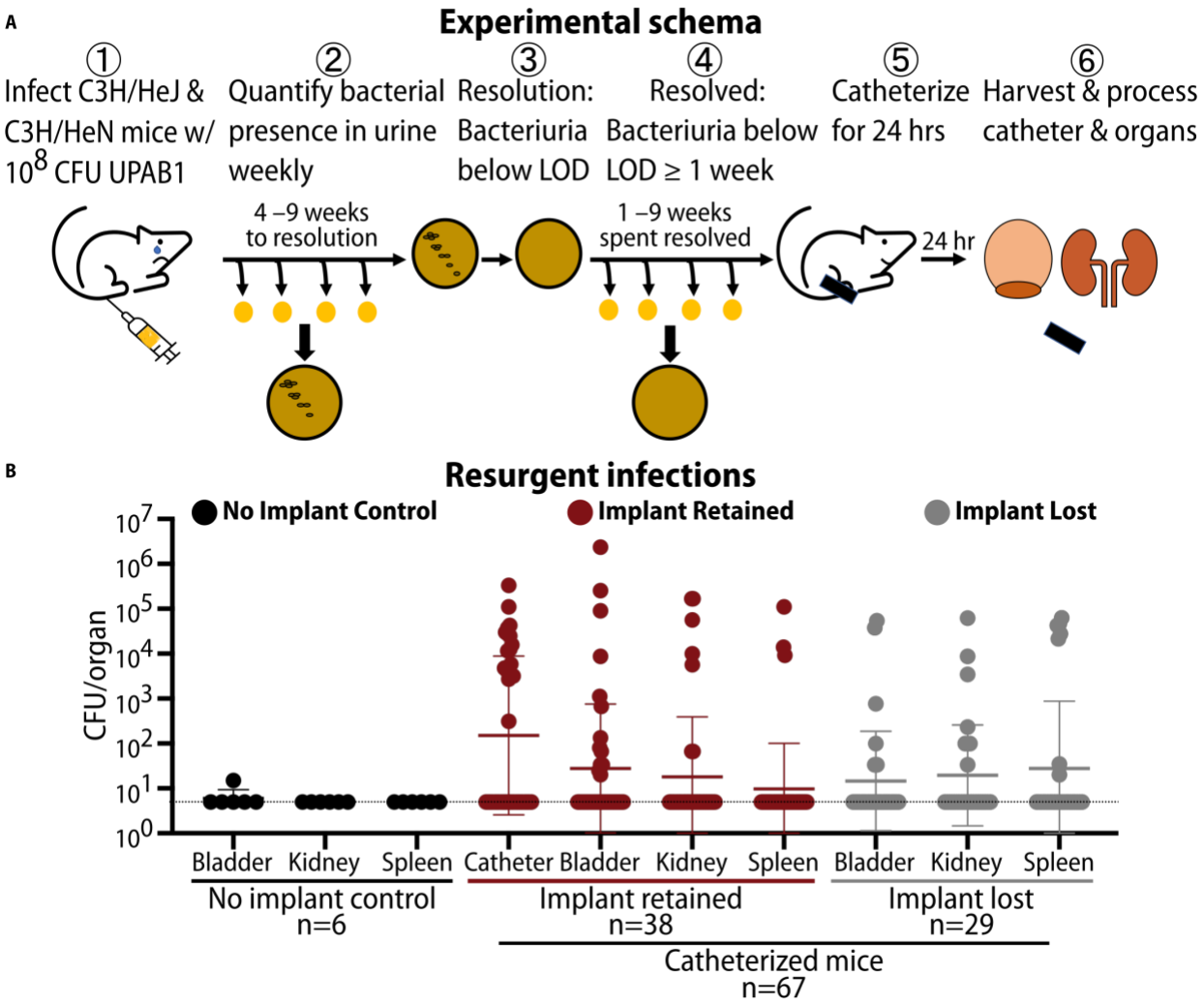

Figure 2.5 Insult to bladder epithelium after resolution of UTI leads to resurgence of secondary, catheter-triggered, UTI. A. Female mice were transurethrally infected with UPAB1 and bacteriuria was tracked weekly. Once bacterial titers in the urine fell below the LOD, the mouse was further monitored for at least one week to ensure resolved bacteriuria. After, mice were either not implanted, or a catheter was transurethrally implanted. 24h post-catheterization the bacterial burdens of recovered catheters, bladders, kidneys, and spleens were quantified. **B.** Bacterial burdens of C3H/HeJ mice after resolution, with and without a catheter. n = 67 implanted mice, and 6 non-implanted mice. Of the implanted mice, n=38 who retained their catheters and n=29 who lost their catheters. LOD = 5 CFU/structure.

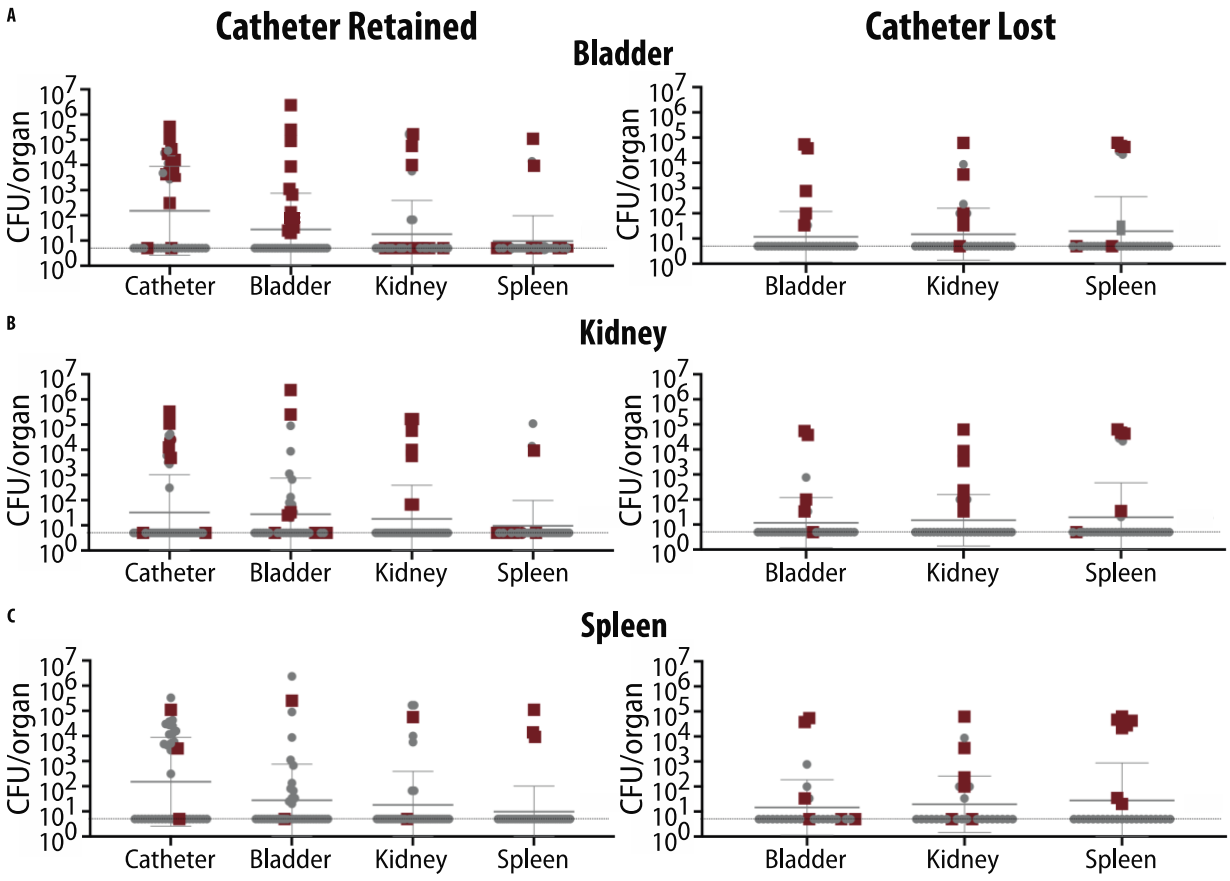

Figure 2.6 Resurgent colonization of one organ does not rely on colonization of the catheter or of other organs in the urinary tract. Data from Figure 2.5B, with individual mice highlighted in red if they experienced resurgent colonization in the specified organ. **A)** Data points in red indicate the mouse harbored detectable UPAB1 in its bladder 24 hours post catheterization. **B)** Data points in red indicate the mouse harbored detectable UPAB1 in its kidney 24 hours post catheterization. **C)** Data points in red indicate the mouse harbored detectable UPAB1 in its spleen 24 hours post catheterization.

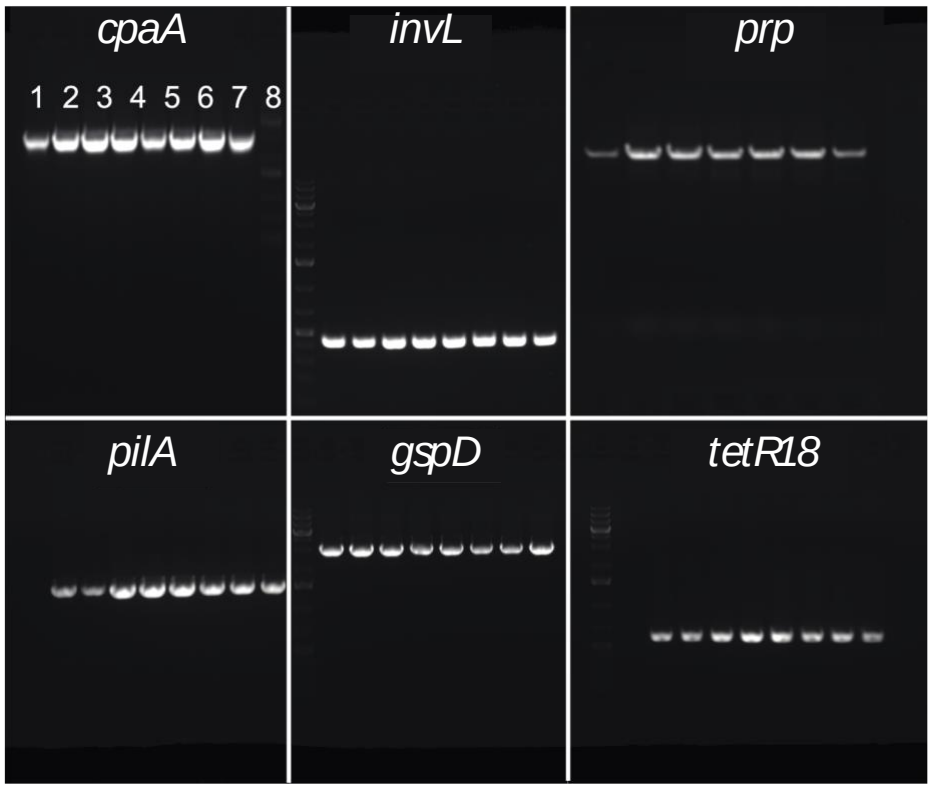

Figure 2.7 PCR confirms same-strain UPAB1. CAUTI resurgence after resolution of initial colonization event C3H/HeJ mice were transurethrally infected with UPAB1 and bacteriuria CFU were tracked weekly until resolution of bacteriuria below 1000 CFU/ml urine. Two weeks post resolution n=5 mice were catheterized. 24 hours post catheterization, genomic DNA was isolated from the following locations, corresponding to the lane number: **1)** Catheter from mouse A, **2)** Bladder from mouse A, **3)** Bladder from mouse B, **4)** Spleen from mouse C, **5)** Spleen from mouse D, **6)** Kidney from mouse D, and **7)** Kidney from mouse E. Genomic DNA was also isolated from the UPAB1 inoculum used to infect the mice **(8).** PCR amplification was performed on genes corresponding to the Prp adhesin, the type 2 secretion system (T2SS) structural component gspD, the T2SS effectors CpaA and InvL, the Type 4 pilus component pilA, and the pAB5 plasmid gene tetR18.

Immunocompetent C3H/HeN mice also experienced *A. baumannii* resurgence, although to a lower efficiency; bacteria were found on the catheters and in the urinary tracts of ~9% (2/22) of the mice (**Figure 2.8A)**. The proportion of immunocompetent mice that exhibited resurgent UTI with UPAB1 after only 24 hours of catheterization was comparable to the ones observed in similar experiments performed with UPEC after three days of catheterization[49]. *A. baumannii* resurgence did not occur in C57BL/6J mice, which were never truly colonized with UPAB1 during their primary infection **(Figure 2.8B)**. Control C3H/HeJ mice sham-infected with PBS

and housed with UPAB1-infected C3H/HeJ mice did not exhibit a resurgent infection **(Figure 2.8C).** These results indicate the existence of internal host reservoirs suitable for the long-term persistence of *A. baumannii* in mice that resolved the primary infection.

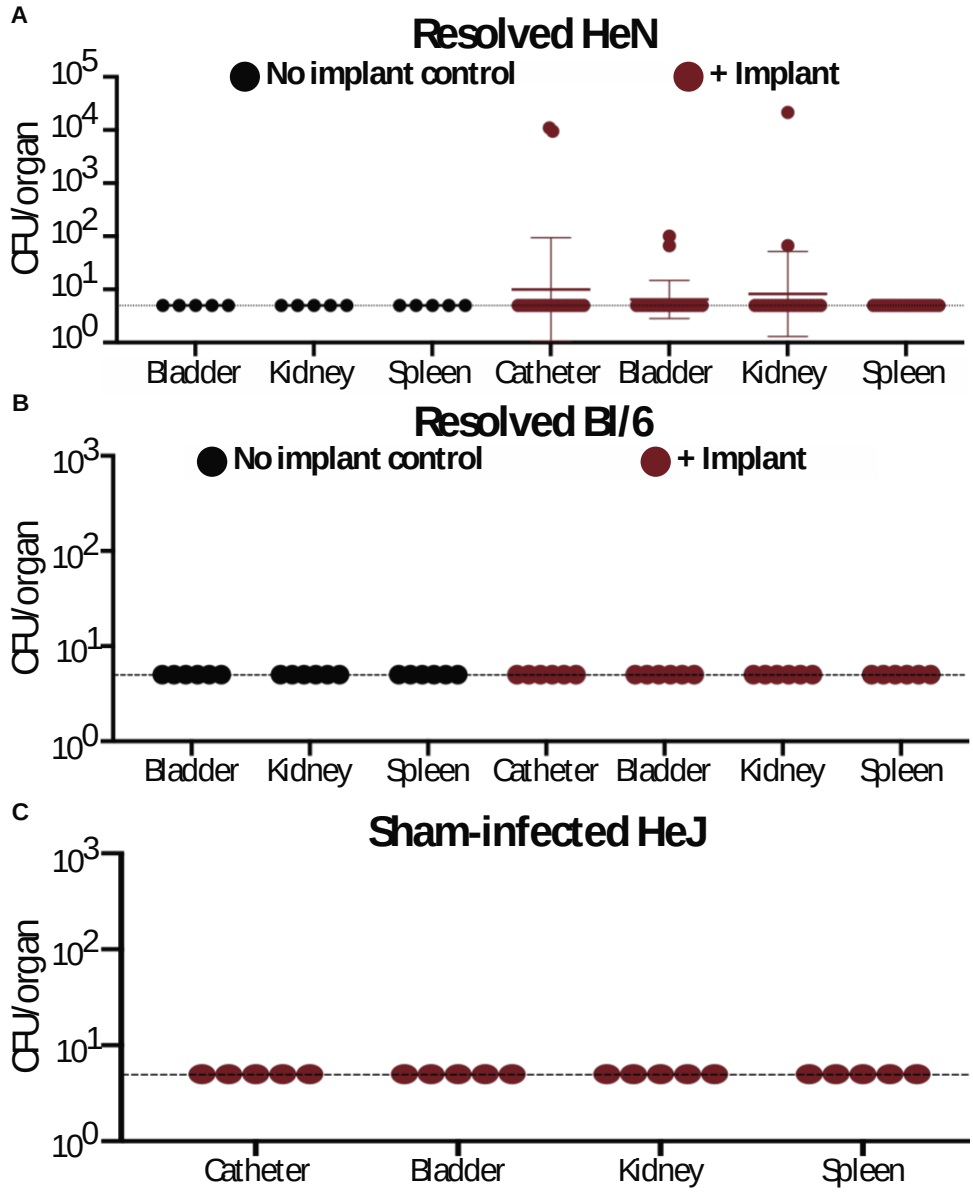

Figure 2.8 Resurgence of CAUTI is dependent on previous urinary tract colonization. A) C3H/HeN mice were transurethrally infected with UPAB1 and bacteriuria CFU were tracked weekly until resolution of bacteriuria below 1000 CFU/ml urine. One to nine weeks post resolution, a proportion of mice were catheterized. 24 hours post catheterization, the implant, bladder, kidneys, and spleen were harvested and bacterial burdens enumerated. LOD = 50 CFU/organ or implant. n= 22, five biological replicates, consisting of five mice each, were performed. **B)** n=5 C57Bl/6 mice were transurethrally infected with UPAB1 and bacteriuria CFU were tracked weekly until resolution of bacteriuria below 1000 CFU/ml

urine. Mice were catheterized, and 24 hours post catheterization, the implant, bladder, kidneys, and spleen were harvested and bacterial burdens enumerated. LOD = 50 CFU/organ or implant. **C)** n=5 C3H/HeJ mice were sham-infected transurethrally with PBS. Two weeks post 'infection', the mice were implanted with catheters for 24 hours. The catheter, bladder, kidneys, and spleen were then harvested and bacterial burdens enumerated. LOD = 50 CFU/organ or implant.

2.3.4 GI tract colonization is not linked to resurgence of resolved mice

Previous studies have ruled out the gastrointestinal (GI) tract as a potential reservoir for *A. baumannii* in healthy, uninfected hosts[74]. However, *Acinetobacter* has been found in the feces and perirectal regions of patients prior to and during the experience of same-strain infections[212–214]. Because mice practice coprophagia and often consume the corncob bedding that they are housed in, we evaluated the capacity of the GI tract to act as potential reservoir for UPAB1 for resurgence. Fecal pellets were collected from resolved mice immediately prior to catheterization and bacterial presence was enumerated. During active bacteriuria, up to 85% of mice had detectable bacteria in their feces. However, several weeks after resolution of active bacteriuria only 37% of the mice carried UPAB1 in their feces **(Figure 2.9)**.

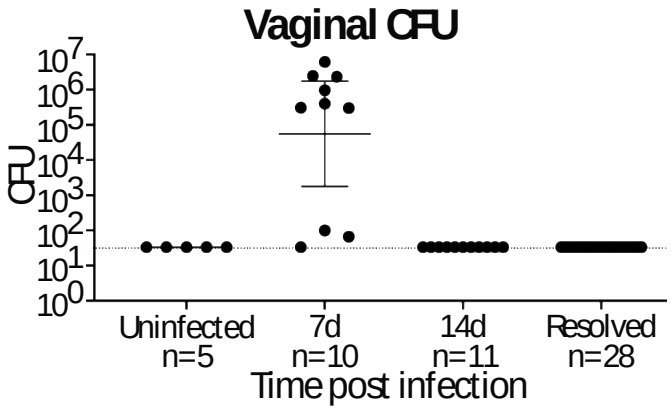

Figure 2.9 *A. baumannii* is not detected in the vaginas of resolved mice. C3H/HeJ mice were transurethrally infected with UPAB1. Vaginal bacteria were enumerated immediately prior to infection, 7dpi, 14dpi, and after resolution of bacteriuria. LOD = 50 CFU/vaginal wash. Vaginal washes were performed on randomly selected mice who were infected over the course of five biological replicate experiments.

Regardless, 70.6% (12/17) of mice that experienced resurgent UTIs did not have detectable UPAB1 in their feces. These experiments indicate that resurgence does not rely on GI colonization and suggest the presence of another host bacterial reservoir. Additionally, we performed vaginal washes that failed to detect UPAB1 in the region after the resolution of bacteriuria (**Figure 2.10)**. This ruled out the vagina as a reservoir for UPAB1.

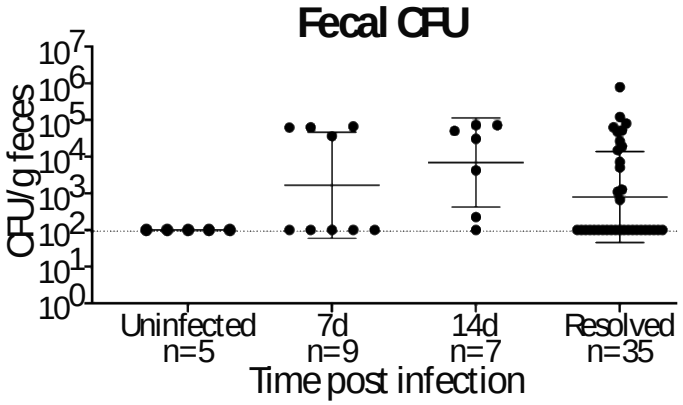

Figure 2.10. Detection of *A. baumannii* in the feces during and after resolution of ncUTI. C3H/HeJ mice were transurethrally infected with UPAB1. Fecal pellets were collected prior to infection, 7dpi, 14dpi, and after the resolution of bacteriuria. Bacterial titers were enumerated and are represented as CFU/g feces. Feces was evaluated on randomly selected mice who were infected over the course of five biological replicate experiments.

2.3.5 Detection of intracellular *Acinetobacter* in the bladders of mice

We and others have recently showed that *A. baumannii* can invade and replicate in macrophages and lung epithelial cells[159,160]. We thus hypothesized that UPAB1 could form intracellular reservoirs in the bladder upon infection and performed modified *ex vivo* antibiotic protection assays on C3H/HeJ mouse bladders excised at 24hpi and 2wpi as described by Mulvey *et al* in 2001[215] **(Figure 2.11A)**. Indeed, after two hours of antibiotic treatment to eliminate extracellular bacteria, we observed the presence of low numbers of intracellular bacteria in UPAB1-infected bladders 24hpi (approximately 10^2 CFU/organ) **(Figure 2.11B)**; intracellular bacteria were not detected in 2wpi bladders. Immunocompetent C3H/HeN mice also harbor intracellular bacteria in

their bladders at 6hpi, however, by 24hpi bladder colonization falls below the limit of detection (**Figure 2.11C**). Using confocal microscopy, we were able to visualize these intracellular bacteria in immunocompromised C3H/HeJ mice during the initial (24hpi) and chronic (2wpi) stages of colonization inside of infected bladder epithelial cells. Up to ten of these intracellular clusters of bacteria were shown to exist in groups of 1–5 small, coccoid bacteria per bladder **(Figure 2.11D)**. We named these structures *Acinetobacter baumannii* Intracellular Reservoirs (ABIRs). ABIRs were also seen in the bladders of resolved mice under the microscope for up to two months after resolution of the infection, indicating that intracellular *A. baumannii* can persist within bladder cells long after resolution of UTI symptoms **(Figure 2.11D)**. No extracellular adherent bacteria were visualized in the bladder lumens of resolved mice, nor were any bacteria visualized in their kidneys**.** The visualized ABIRs are remarkably similar to the quiescent intracellular reservoirs (QIRs) formed by UPEC during UTIs[47]. QIRs are known to act as sources for recurrence of UTI upon disruption of the bladder epithelium; for example, upon the insertion of a catheter[205]. Taken together, our data suggests that a potential host reservoir responsible for CAUTI resurgence are ABIRs within urothelial cells.

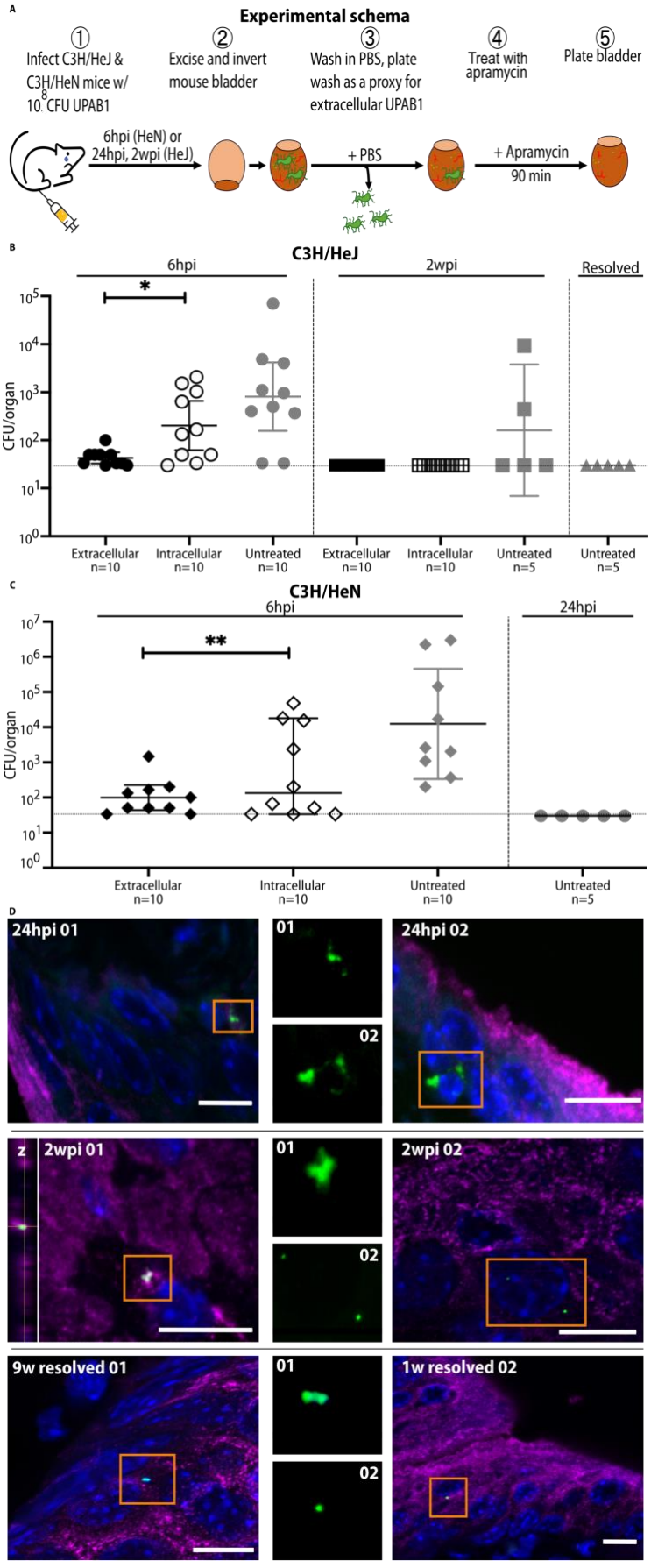
A
Experimental schema
① Infect C3H/HeJ & C3H/HeN mice w/ 10^8 CFU UPAB1
② Excise and invert mouse bladder
③ Wash in PBS, plate wash as a proxy for extracellular UPAB1
④ Treat with apramycin
⑤ Plate bladder
6hpi (HeN) or 24hpi, 2wpi (HeJ)
+ PBS
+ Apramycin 90 min
B
C3H/HeJ
6hpi
2wpi
Resolved
CFU/organ
Extracellular n=10
Intracellular n=10
Untreated n=10
Extracellular n=10
Intracellular n=10
Untreated n=5
Untreated n=5
C
C3H/HeN
6hpi
24hpi
CFU/organ
Extracellular n=10
Intracellular n=10
Untreated n=10
Untreated n=5
D
24hpi 01
01
02
24hpi 02
z
2wpi 01
01
02
2wpi 02
9w resolved 01
01
02
1w resolved 02

Figure 2.11. Detection of intracellular *A. baumannii* in bladder urothelium during and after resolution of ncUTI. **A)** Experimental design. Antibiotic protection assays were performed on infected mouse bladders as described in the Methods section. **B)** Antibiotic protection assays in the bladders of C3H/HeJ mice reveal the presence of low numbers of intracellular bacteria at 24hpi, n=10; intracellular bacteria were below the LOD at 2wpi, n=10; and total titers were below LOD post resolution of bacteriuria, n=5**.** * = $P \leq 0.05$ **C)** Antibiotic protection assays in the bladders of C3H/HeN mice reveal the presence of low numbers of intracellular bacteria at 6hpi, n=10; total bacterial titers were below the LOD at 24hpi, n=10. ** = $P \leq 0.005$ **D)** Representative images of ABIRs in C3H/HeJ mice. Sections were stained for cell nuclei (blue), UPAB1 (green), and uroplakin III (magenta). Scale bar 10um. Top panels = 24hpi. Middle panels = 2wpi. Middle-left panel includes a Z-projection to the left. Bottom left panel = 9 weeks post resolution, and bottom right panel = 1 week post resolution, right. Top insets correspond with the left panel; bottom insets correspond with the right.

2.4 Discussion

According to the CDC, on any given day, one in thirty-one hospital patients will have an HAI[216,217]. *A. baumannii* is a major cause of HAI, and it is particularly concerning due of its extensive MDR. Despite the implementation of stringent preventative interventions, new *A. baumannii* strains are continuously isolated in hospital worldwide. Although other *Acinetobacter spp* are usually found among human skin colonizers, *A. baumannii* is rarely found as a member of human microbiomes. Therefore, unlike for other pathogens, the sources of new *A. baumannii* nosocomial strains remain unknown. Here, employing a murine model of infection, we demonstrate that *A. baumannii* can survive for months in bladder reservoirs within the host, and that the insertion of a medical device, can trigger a resurgent infection. This concept is accepted for bacteria that are known to be regular components of human microbiotas, such as *E. coli* or *Klebsiella spp.*, but has not been previously shown to be applicable for opportunistic pathogens. Since about 2% of the population exhibit asymptomatic *A. baumannii* bacteriuria, we propose that patients can unknowingly enter the clinic already harboring the pathogen, long after an initial colonization event. These host reservoirs can in turn become 'activated' upon medical intervention, where they can trigger a resurgent infection as a HAI. Our experiments support bladder ABIRs as the most likely bacterial source for a resurgent infection. More work will be necessary to fully establish the role of ABIRs in *A.*

baumannii pathogenesis. Furthermore, it remains to be investigated if our findings can be extended to other opportunistic pathogens.

While ~20% of *A. baumannii* clinical isolates derive from urinary sources, ventilator-associated pneumonia is the most common type of infection caused by *A. baumannii*[178]. In this clinical manifestation, neutrophils and macrophages are quickly recruited to phagocytose and eliminate the bacteria. It has been recently established that *A. baumannii* can invade and replicate in macrophages and, lung epithelial cells[159,160]. Therefore, it is possible that *A. baumannii* strains can be asymptomatically carried by patients in ABIRs in organs such as bladders or lungs before their hospitalization. Interventions like the use of catheters and ventilators, or other events that weaken the host immune system, could trigger the resurgent infection. Our findings could, in the future, lead to the implementation of novel preemptive strategies to mitigate the risk for *A. baumannii* infections and subsequent hospital outbreaks.

2.5 Materials and Methods

2.5.1 Systematic literature analysis

We performed a literature search in PubMed and Google Scholar using combination of terms "*Acinetobacter baumannii*", "*Acinetobacter*", "asymptomatic bacteriuria", "ASB," "urine", and "asymptomatic", and an expanded search using bibliographies of identified studies. For inclusion in our analysis, an epidemiological study must have fulfilled the following criteria: (1) performed on consecutive, non-duplicate isolates obtained from a geographically and temporally associated population; (2) isolates were not exclusively obtained from a single patient population (e.g., immunocompromised) or hospital ward (e.g., only ICU patients); (3) patients were explicitly not catheterized, not pregnant, and had no known underlying conditions; (4) the study reported the

number of both positive and negative urine samples; (5) the patient population was greater than or equal to ten patients.

2.5.2 Bacterial strains and growth conditions

The two *A. baumannii* strains used were UPAB1, a MDR urinary isolate obtained in 2016, and ATCC 19606 ("19606"), a lab domesticated strain isolated from patient urine in 1967[24]. Cultures were started from freezer stocks and grown using a "2x24" system. Bacteria was first grown statically in lysogeny broth (LB) at 37°C for 24 hours. The static cultures were then subcultured at 1:1000 into 20 mL of fresh LB and grown statically for another 24 hours at 37°C.

2.5.3 Mouse infections

Eight-week-old female mice were anesthetized via inhalation of 4% isoflurane and infected transurethrally, as previously described in Mulvey *et al*, 1998[218]. Bacteria cultures were grown in 2x24 conditions, described above, pelleted, resuspended in phosphate-buffered saline (PBS), and subsequently re-concentrated or diluted in PBS to yield 50 µl innocula of 1 x 10^8 CFU. All studies were approved of and performed in accordance with the guidelines set by the Committee for Animal Studies at Washington University School of Medicine, and the mice were housed in a facility with a 12 hour light/dark cycle where they had ad libitum access to standard food and water.

2.5.4 Urine collection and organ titers

Urine samples were collected from mice by bladder massage over sterile 1.5 mL microcentrifuge tubes. Urine was serially diluted in LB and plated on LB agar plates, as well as on LB agar plates containing chloramphenicol 10 (strains lacking pAB5) or on LB agar plates containing kanamycin 30 and gentamicin 10 (strains containing pAB5). To quantify bacteria within infected mouse organs, mice were sacrificed at the appropriate time-points and their bladders, kidneys, and spleens

were aseptically harvested for enumeration. Organs were homogenized in PBS, serially diluted, and plated on LB agar plates as well as on LB agar plates antibiotics in the concentrations and conditions specified above.

2.5.5 Antibiotic protection assays

Infected mouse bladders were aseptically removed, hemisected to allow access to the bladder lumen, and washed 3x in PBS to collect adherent extracellular bacteria. The PBS washes were pooled and spun at 500 x g minutes to pellet any shed epithelial cells, after which bacterial loads of the wash were quantified. Simultaneously, the washed bladders were treated with 1 ug/ml of apramycin in serum-free RPMI at 37°C for one hour to kill any remaining adherent extracellular bacteria while leaving potential intracellular bacteria intact. Treated bladders were then homogenized in PBS, and the homogenate was serially diluted and plated on plates containing the relevant antibiotic to enumerate any intracellular bacteria.

2.5.6 Histology and Immunohistochemistry

Infected mouse bladders and kidneys were aseptically removed at relevant time-points, fixed overnight in 10% formalin, and paraffin embedded. Sections were then either stained with hematoxylin and eosin, or left unstained. Unstained slides were then processed for immunohistochemistry. Organ sections were deparaffinized in xylene (2 x 10 min), rehydrated in isopropanol (3 x 5 min), and washed in water for 5 min. Slides were then dried and antigen retrieval was subsequently performed by boiling the sections in 10mM sodium citrate buffer for 30 minutes. Slides were then washed 3 x 5 min in PBS and blocked for one hour at RT in blocking buffer (PBS containing 2% BSA and 0.2% Triton-X-100). Slides were washed in PBS 3 x 5 min and incubated in primary antibodies overnight at 4°C. Primary antibodies used on bladder sections include mouse-raised anti-*E. coli* RNA pol II at a concentration of 1:1500, which has been shown to stain

for *A. baumannii,* and goat anti-uroplakin III at a concentration of 1:1000. The slides were again washed 3 x 5 min in PBS, and then incubated for 20 min at RT with secondary antibodies: Alexa Fluor anti mouse 488 at 1:1000, and Alexa Fluor donkey anti goat 647 at 1:1000. Following a final wash step, the slides were mounted in Prolong Gold Antifade with DAPI and visualized under a confocal microscope (below). Stained bladders of PBS-mock infected mice were used as control. No bacteria were detected in these preparations.

2.5.7 Urinalysis

C57BL/6J as well as C3H/HeN and C3H/HeJ mice were anesthetized through inhalation of 4% isoflurane and transurethrally infected with either 10^7 or 10^8 CFU of UPAB1, respectively. Urine samples were collected weekly from mice by bladder massage over sterile 1.5 mL microcentrifuge tubes. Urine was serially diluted in LB and plated on LB agar plates, as well as on LB agar plates containing chloramphenicol 10. If no colonies were detected in a specific mouse's urine titer, urine samples were collected later that same week and plated directly on to LB agar plates containing chloramphenicol 10 without serial dilution. Mice whose bacteriuria titers had fallen below the limit of detection for both tests were classified as 'resolved', and were transferred to a clean cage from at least one week to approximately two months.

2.5.8 Fecal samples

Fecal samples were collected directly from each animal at indicated time-points by clean-catching one pellet into a pre-weighed microcentrifuge tube. Samples were immediately weighed, homogenized in 1mL of PBS, serially diluted by factors of 10, and plated on plates containing LB with kanamycin 30 and gentamicin 10 to enumerate *A. baumannii*.

2.5.9 Vaginal Washes

Vaginal washes were collected as described in *Gilbert et al, 2013*[219]. Briefly, mice were scruffed and their vaginas flushed with 50uL of sterile PBS using a P200 pipette. The PBS was pipetted up and down at least 5x and was then deposited into a sterile microcentrifuge tube containing 10 additional uL of PBS. The vaginal washes were serially diluted by 10s and plated on LB plates containing kanamycin 30 and gentamicin 10 to enumerate *A. baumannii.*

2.5.10 Perineal Swabbing

Mice were scruffed and a PBS-soaked cotton-tipped applicator was used to gently swab the perineal region (skin and fur from the base of the tail, around the anus & urogenital area, and slightly above the urethra). The applicator tip was deposited into 1 mL of PBS and bacteria were released by vortexing for 30 seconds, water sonicating for 7 minutes, and vortexing again for 30 seconds. The applicator tip was then removed and the bacteria were spun down for 5 minutes at 6500xg. The resultant pellet was resuspended in 100uL of PBS, serially diluted by 10s, and plated on LB plates containing kanamycin 30 and gentamicin 10.

2.5.11 Catheterizing resolved mice

Mice that had been resolved and housed in a separate cage with only resolved mice for at least one week were anesthetized by inhaling 4% isoflurane, the skin and fur surrounding their urogenital area was sterilized using a combination of betadine and ethanol, and their bladders were catheterized via the aseptic transurethral insertion of a small piece of 2-3mm silicone tubing[206]. The mice were sacrificed 24 hours post catheterization, after which approximately 50% of the implants remained in the bladder. Mouse bladders, kidneys, and spleens were aseptically removed and processed for bacterial enumeration as described above. Recovered catheters were aseptically removed and placed into Eppendorf tubes containing 1mL of sterile PBS, and sonicated in a water bath for 5 minutes to disassociate any bacterial biofilms formed on the catheter. The resulting PBS

mixtures were then serially diluted and plated on LB chloramphenicol 10 plates to enumerate bacterial presence on the catheters.

2.5.12 Conformation of bacterial identity

Bacterial identity was confirmed by antibiotic resistance profile and a combination of several PCR products that is unique to UPAB1. Genomic DNA was extracted with the Purelink genomic DNA minikit (Invitrogen) from overnight bacterial cultures recovered from a resurgent infection, following the manufactures protocol. Targeted genes present in the chromosome and the pAB5 plasmid of UPAB1 strain were amplified with the following primers:

Primer	Sequence
gspD Fw	CCATCTCAACCTACAGAGCC
gspD Rv	CAGTTTGTACTGGCTCCACC
prpD Fw	AGGTTATGTAAAGCTTTATAATTTATCG
prpD Rv	AGTAGGATGTTTGAGTAAATAGTC
pilA Fw	AAGTTATAACCCGCCACAACC
pilA Rv	CGTGGGATTAGGAATGCAAC
cpaA fw	GCTTGGCGCTGGCTTAGGTG
cpaA rv	TAAGGATGATCATGGTGTAACTCCTG
TetR18-FwdBamHI	ATATGGATCCATGACTAAAGTTATTTCAAAAAGAAAAACC
TetR18-RevPstI	ATTACTGCAGTTAGTGGTGGTGGTGGTGGTGAGCCTCAAATGTCTGAATTAGACTC
5' fdeC diag	CTAGATAAATTATCAGTTGCTGCAACG
3' fdeC diag	GCTAAAGTTGCACCATTTGC

Table 2.2. Primers used in this study.

2.5.13 Confocal microscopy

Samples were analyzed with a Zeiss LSM880 laser scanning confocal microscope with Airyscan (Carl Zeiss Inc. Thornwood, NY). The system is equipped with 405nm diode, 488nm Argon, 543nm HeNe, and 633nm HeNe lasers. A Plan-Apochromat 63X (NA 1.4) DIC objective and ZEN black 2.1 SP3 software were used for image acquisition. All microscopy was performed at the Washington University in St. Louis Molecular Microbiology Imaging Facility. Z-stacks were taken

with slices at a thickness of 0.75 - 1 um, and Z-projections were made in FIJI [220] based on maximum intensity.

2.5.14 Statistical analyses

All statistical tests were performed using GraphPad Prism[221]. Datasets from each condition (i.e. strain of *A. baumannii* used, time post infection, strain of mouse, etc.) were analyzed for Gaussian distribution using the D'Agostino-Pearson omnibus normality test. Comparisons between normally distributed datasets were statistically analyzed using the Student's *T* Test while nonparametric data were compared using the Mann-Whitney *U* test.

Chapter 3: Strain-specific intracellular behavior of diverse uropathogenic *E. coli* clinical isolates

By Jennie Hazen, Denise Dorsey, Ellie Gaylord, and Scott Hultgren

Adapted from prepared manuscript for dissertation
Hazen JE, Dorsey D, Gaylord E, Hultgren S. Strain-sepcific intracellular of diverse uropathogenic *E. coli* clinical isolates. To be submitted to Pathogens.

3.1 Abstract

Urinary tract infections (UTIs) are highly prevalent with considerable physical, psychological, and economic burdens to infected individuals particularly since they are highly recurrent with 25% of individuals with an acute infection having a recurrence within 6 months. A necessary yet understudied step of uropathogenesis in the most common uropathogenic bacteria, uropathogenic *E. coli* (UPEC) is the successful invasion of and replication and persistence within bladder epithelial cells. Within the cytosol of bladder cells UPEC undergo several stages of morphological and physiological changes as they form biofilm aggregates called intracellular bacterial communities (IBCs). This process has best been characterized for the protypical urinary isolate UTI89, but it occurs in the majority of UPEC. Here, we characterize the intracellular behaviors of nine diverse urinary clinical UPEC isolates. Each isolate displayed strain-specific behaviors in terms of IBC development kinetics, IBC morphology, and number of IBCs formed. Thus, this work highlights the phenotypic diversity of different UPEC isolate behavior within bladder cells. However, certain patterns did emerge. Interestingly, all three strains from phylogroup A were unable to successfully complete the IBC cycle. It is our hope that this work will inform future in studies probing the capacity of clade A UPEC isolates to form IBCs and in investigating the connection between gene carriage and regulation, and IBC formation.

3.2 Introduction

Urinary tract infections (UTIs), defined as symptomatic colonization ($>10^3$ - $>10^5$ CFU/mL urine, depending on the guidelines followed) of the kidneys, ureters, bladder, and/or urethra, rank among the most common infectious diseases in the world[1–5]. An estimated 60.4% of all women and 5% of all men worldwide will experience at least one UTI in their lifetime[1]. In addition,

UTIs can be highly recurrent; 25% of sexually active adult women who have experienced one UTI are predicted to experience a recurrent infection within 6 months[1,6]. The high prevalence and recurrence rates of UTIs result in a sizeable drain on the U.S. economy. In 2014, the direct annual cost of UTIs was reported at $2.8 billion. The addition of indirect costs, such as lost labor, brings the total up to approximately five billion dollars each year[1–3]. As the prevalence of antibiotic-resistant uropathogens continues to increase worldwide, so too will the symptomatic and economic burdens of this disease and risks the rise of UTI cases for which there is no effective antibiotic available. Thus, elucidating the mechanisms of UTI pathogenesis is needed to elucidate new drug targets for development of new UTI therapies.

Among the pathogens that cause UTIs, uropathogenic *E. coli* (UPEC) is the most common, accounting for over 85% of community-acquired UTIs and over 50% of nosocomial (hospital-acquired) UTIs worldwide[7–9]. UPEC as a group is extremely genetically and phenotypically diverse, consisting of many strains of *E. coli* that span multiple clades in the evolutionary tree and isolates can differ in gene carriage by up to 40%. The pangenome of *E. coli* contains upwards of 16,000 genes, of which individual strains can carry about 5,000 (~3000 of which are core genes shared by all *E. coli* and ~2000 of which are variable)[10]. Despite this genetic diversity, in murine models of acute cystitis, the majority of successful UPEC strains adhere to and invade their hosts' bladder epithelial cells and become intracellular pathogens[11,12]. This phenotype is surprising; even though the number of strains that are both non-invasive in an *in vivo* model and that are capable of causing robust UTIs *in vitro* is low, the vast majority of robust infections caused in non-UT niches by *E. coli* as a species are not intracellular. Nevertheless, during UTIs the uropathogens replicate rapidly in the host umbrella cell and eventually form biofilm-like pods called Intracellular Bacterial Communities (IBCs) within the

cell[11,13–15]. IBC formation is a necessary step of acute UTI pathogenesis in murine models, and IBCs have also been found in shed epithelial cells in the urine of women experiencing UTIs caused by UPEC[16]. Mice that suffer from a higher burden of IBCs during the acute phase of infection tend to develop more severe outcomes of infection than mice whose bladders did not house many IBCs[17]. In this way, successful passage through the IBC cycle is an important step for UPEC to gain a foothold in the bladder.

As part of an IBC, the uropathogens are protected by the host cell from antibiotics, neutrophils, and other host defense responses, which cannot penetrate the bladder cell[18–20]. The bacteria within the IBC can replicate unopposed, eventually filling the bladder cell cytoplasm and fluxing out of the umbrella cells[15,21]. Once out of their host cell the bacteria adhere to and re-invade a neighboring umbrella cell, replicating (albeit at a more attenuated rate) and forming new IBCs[15]. Thus, UPEC take advantage of their intracellular behavior to gain a foothold in the bladder and perpetuate infection.

The process of IBC development, known as the IBC cycle, has been studied extensively in the murine model using the prototypical UPEC strain, UTI89. The IBC cycle is comprised of several steps, including: **i)** invasion, the adherence of a single bacterium to a bladder umbrella cell (~15 minutes pi – 1hpi); **ii)** IBC maturation, the process by which the replicating daughter cells of the initial intracellular bacterium form a biofilm-like community; **iii)** filamentation, wherein the bacteria within the IBC adapt an extremely long, rod-like morphology; **iv)** fluxing, the escape of the filamented bacteria in the IBC from the bladder epithelial cell, and; **v)** re-invasion, the adherence and entry of fluxed bacteria to neighboring umbrella cells[15].

The bacteria within the IBC, as well as the IBC ultrastructure itself, undergo several stages of morphological and physiological change over the ~18-24 hours of their formation and

dispersal, especially during IBC maturation. In an **early-stage IBC** (~3hpi) the quickly-replicating bacteria are relatively large and rod shaped. They are loosely associated with each other and do not express hallmarks of biofilm formation. In **mid-stage IBCs** (~6hpi), which form after the bacteria undergo seemingly simultaneous morphological and physiological changes, the bacteria associate tightly with each other in aggregates that biofilm characteristics. The bacteria within a mid-stage IBC are small and coccoid in morphology, and they replicate more slowly. Once the IBC has grown to overtake the volume of the bladder cell, it is considered a **late-stage** IBC (~12hpi). From here, bacteria on the outer edge of the IBC once again adapt a rod-shaped morphology. As the rods continue to grow, the UPEC on the outer edge of the late-stage IBC form long **filaments and flux** from the uroethelium (~18hpi). Fluxed bacteria that invade neighboring urothelial cells change shape once again to smaller rods. These bacteria once again execute the IBC cycle in their new bladder cell, repeating the same process of dynamic morphological and physiological change.

While the 'gold standard' IBC cycle has been defined using UTI89, Garofalo *et al* performed significant work in 2007 to characterize the intracellular behavior of several urinary clinical isolates[12]. In their study, Garofalo found that the majority of UPEC clinical isolates can form IBCs, regardless of the type of infection they caused in their host (chronic or recurrent cystitis, acute cystitis, asymptomatic bacteriuria, pyelonephritis). However, the different UPEC strains differed wildly in their intracellular behavior. For example, of the 15 IBC-forming strains, the five isolates from acute UTIs on average formed smaller IBCs. Individual strains varied widely in IBC cycle kinetics and in the number of IBCs they formed. Schreiber *et al* also investigated the ability of eight diverse clinical UPEC isolates to form IBCs in a 2018 study that corroborated Garofalo's findings[10]. While the IBC morphologies and IBC cycle kinetics of the

seven IBC-forming UPEC isolates were not characterized in-depth, it is apparent from the published images that the isolates differ in IBC morphology. These strain-specific phenotypes, combined with the relatively small number of strains assessed so far, Although a few general patterns have been identified loosely linking IBC phenotype to UPEC genotype or source (i.e. acute UTI, recurrent UTI, pyelonephritis, etc.), the small number of strains assessed means that these findings are not statistically significant and few conclusions can be drawn.

It is important to note that while Garofalo and Schreiber assessed a variety of strains, the majority of isolates studied were members of the B2 phylogroup[57,122]. The abundance of B2 strains used in these studies is reflective of the fact that in the United States, where the strains were isolated, the majority of UPEC are B2 strains. However, other phylogroups are still commonly isolated, and cause up to 50% of cystitis cases[222–226]. This abundance of B2 UPEC strains characterized by Garofolo and Schreiber in combination with the multiple strain-specific phenotypes observed, has meant that few conclusions could be made regarding a link between IBC phenotype and UPEC genotype. In this study, we sought to further characterize six additional UPEC isolates across multiple phylogroups for their ability to form IBCs to increase our understanding of the intracellular behaviors of diverse uropathogenic *E. coli*. Most of the clinical isolates could form IBCs. However, we found large differences between strains in IBC cycle kinetics and morphologies. These findings suggest the potential existence of multiple pathways or mechanisms, or of multiple regulatory mechanisms for one pathway, by which UPEC can complete the IBC cycle, and further highlight the importance of intracellular behavior to uropathogenesis as a whole. While individual isolates exhibited strain-specific idiosyncrasies in IBC phenotype, we did observe that the phylogroup A UPEC isolates performed the worst in IBC development.

3.3 Results

3.3.1 Selecting diverse UPEC strains.

We selected eight UPEC clinical isolates for study, based on the following criteria: **1)** Each strain has already been assessed for putative virulence factor (PUF) carriage, mannose-sensitive hemagglutination (MSHA) ability, and its ability to colonize a mouse bladder[10]; and **2)** Each phylogroup known to be associated with UPEC was represented; there were three phylogroup A strains, three phylogroup B2 strains, one phylogroup B1 strain, and one phylogroup D strain. Different types of infection were also well represented. Half of the strains came from acute UTIs, while the other half came from recurrent or chronic UTIs. Additionally, one strain came from a patient suffering from pyelonephritis and urosepsis. These characteristics are summarized in **Table 1,** below. As described by *Schreiber, 2016*, there were no clear patterns relating the measured characteristics with overall urovirulence in a mouse model[57].

Briefly, the nomenclature for each strain is as follows: [patient number].[UTI occurrence][UTI type where "a" is an acute, symptomatic UTI; "p" is a UTI where the patient provided a urine culture prior to the onset of symptoms; and "r" is a recurrent, symptomatic UTI][4]. For example, UPEC isolate 41.4p comes from patient #41's fourth UTI since the start of the study. The strain was isolated from a routine monthly urine culture provided by the patient prior to the onset of symptoms, rather than from a culture provided upon the onset of symptoms. Details for each strain are summarized in **Table 3.1**.

	2.1a	**11.2p**	**11.3r**	**41.4p**	**56.1a**	**9.1a**	**20.1a**	**41.1a**	**5.3r**	**UTI89**
Phylogroup	A	A	A	B1	B1	D	B2	B2	B2	B2
Patient number	2	11	11	41	56	9	20	41	5	N/A[x]
UTI occurrence	1st	2nd	3rd	4th	N/A[xx]	1st	1st	1st	3rd	N/A[x]
PUF score	2	4	4	7	2	13	16	20	18	17
MSHA titer (in PBS)	4.5	N/A[*]	N/A[*]	6	7	8	7	8	9[***]	7.5

MSHA titer (in mannose)	1	N/A**	N/A**	0	0	0	6	0	0***	2
Bladder Colonization Efficiency	deficient	N/A+	N/A+	variable	robust	robust	robust	robust	N/A++	robust

***Table 3.1. Previous Characterization of UPEC Strains by* Schreiber*, et al*, 2016**[57]

x Strain comes from a different study

xx Data indicates that although it is the patients' first UTI since enrolling in the study, it is a recurrent UTI and not the patients' first overall.

*Closest genetic neighbor, 11.1a, is 7

** Closest genetic neighbor, 11.1a, is 0

*** Data not in Schreiber, 2016; collected by Hazen in 2018

+ closest genetic neighbor, 11.1a, is 'deficient'

++ Closest genetic neighbor, UTI89, is 'robust'

3.3.2 Invasion capabilities of UPEC clinical isolates.

The strains were first assessed for the ability to invade mouse bladder epithelial cells using an antibiotic protection assay. Briefly, 10^8 CFU of each isolate was injected transurethrally into the bladders of young adult female C3H/HeN mice. One hour post infection the bladders were harvested, inverted, and washed in PBS. The processed bladders were then treated with the aminoglycoside gentamicin to kill any remaining extracellular adherent bacteria. As intracellular bacteria survive this treatment, the bladders were then homogenized and cultured. The clade A strain 2.1a was not tested in this assay, as it had been excluded based on the results of a concurrently-run experiment which demonstrated an extremely attenuated ability to form IBCs (see below, **altered IBC morphologies)**. Phylogroup B1 strains 41.1p; phylogroup D strain 9.1a; and phylogroup B2 strains 20.1a, 41.1a, and 5.3r all exhibited intracellular CFUs comparable to those of the prototypical laboratory isolates UTI89 and CFT073 and were classified as normal invaders **(Figure 3.1)**. The clade B1 strain 56.1a showed significantly higher intracellular CFUs at 1hpi than the laboratory strains. The isolate was thus classified as a robust invader.

The phylogroup A strains 11.2p and 11.3r had significantly lower invasion rates and were thus classified as poor invaders. Many bladders infected by these strains had no detectable

intracellular bacteria at 1hpi (LOD = 1000 CFU/bladder). These strains were also classified by Schreiber *et al* as 'deficient' bladder colonizers, further underlining the importance of the intracellular step of uropathogenesis. Data from Schreiber *et al 2016* suggests that 11.2p and 11.3r express normal levels of type 1 pili, and *in silico* analyses do not indicate any obvious genetic abnormalities in the two strains[10]. As these two strains could not invade the urothelium to form IBCs, they were excluded from further study.

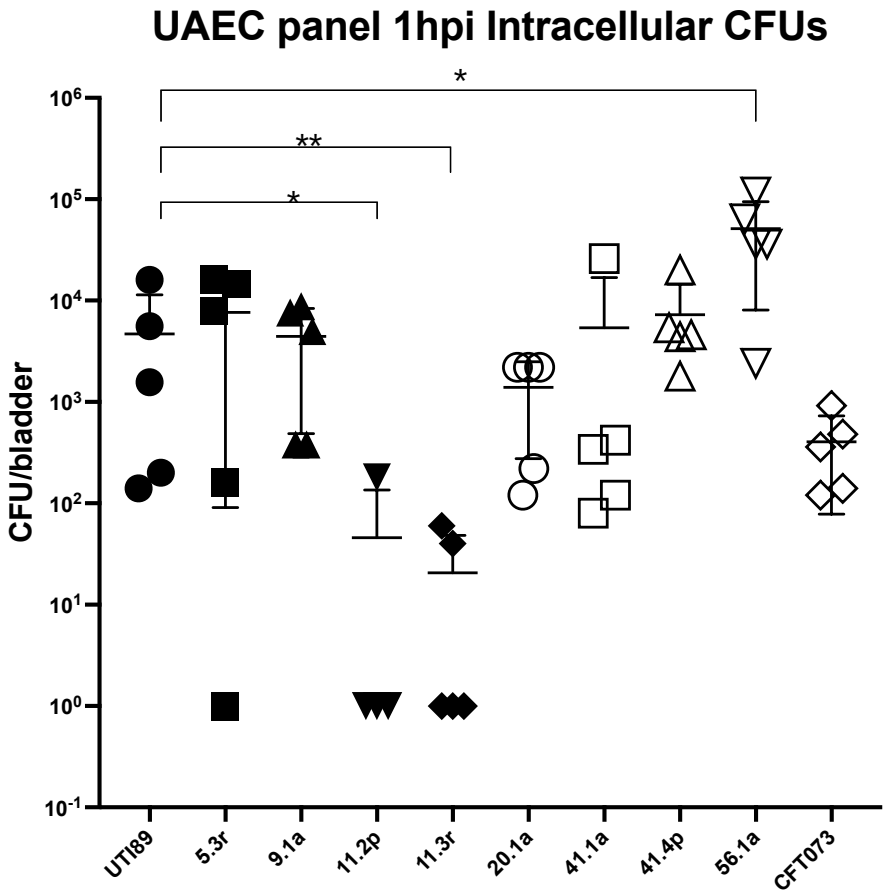

Figure 3.1. *In vivo* invasive capabilities of diverse UPEC strains. 6–8-week-old female C3H/HeN mice were transurethrally infected with 10^8 CFU of UPEC and 1hpi gentamycin protection assays were performed to quantify invasion. Strains tested include: 5.3r, 9.1a, 11.2p, 11.3r, 20.1a, 41.1a, 41.4p, and 56.1a. Prototypical lab isolates UTI89 and CFT073 were used as controls.

3.3.3 IBC cycle kinetics and IBC morphologies

The UPEC strains were next evaluated for their ability to form IBCs. Each isolate was first transformed with the plasmid pANT4, a high copy-number plasmid that constitutively expresses GFP under the tightly regulated control of the synthetic tac promoter (combination of tet and lacZ promoter)[22]. The clade D strain 9.3r could not be transformed and was thus assessed separately. The strains were inoculated transurethrally into the bladders of 6–8-week-old bl/6 female mice (5 mice/time point/isolate), and bladders were harvested at 3, 6, 12, 16, and 24hpi. The harvested bladders were subsequently bisected, splayed on silica dishes, stained with DAPI, and imaged under a confocal microscope. We found that each strain tested in our study was capable of invading bladder epithelial cells and forming IBCs, consistent with the results from Garofalo *et al*[122]. However, we found significant differences in IBC cycle kinetics and morphologies between strains. IBC cycle kinetics are summarized in **Table 3.2** and expanded on in further detail below.

	Clade	Invades by 1hpi	3hpi	6hpi	12hpi	16hpi	24hpi	Classification
UTI89	B2	√	Early-stage IBCs	Mid-stage IBCs	Late-stage IBCs	Late-stage IBCs, filaments begin to form	Second-gen IBCs	Comparator
2.1a	A	√	No IBC	No IBC	No IBC	1 IBC	No IBCs	Poor IBC former
11.2p	A	x	-	-	-	-	-	-
11.3r	A	x	-	-	-	-	-	-
41.4p	B1	√	Early-stage IBCs, have fewer bacteria overall	Mid-stage IBCs, resemble early-stage UTI89 IBCs	Late-stage IBCs	Late-stage IBCs, filaments begin to form	No IBCs	Altered IBC morphology, Deficient reinvasion
56.1a	B1	√√√	Early-stage IBCs	Large Mid-stage IBCs	Large Late-stage IBCs	Large, Late-stage IBCs, filaments begin to form	Second-gen IBCs	Robust IBC former
9.1a*	D	√	Early-stage IBCs	Mid-stage IBCs	Late-stage IBCs	Late-stage IBCs, filaments begin to form	Second-gen IBCs	Like comparator
20.1a	B2	√	Early-stage IBCs	Mid-stage IBCs	Late-stage IBCs	Late-stage IBCs, filaments begin to form	Second-gen IBCs	Like comparator
41.1a	B2	√	Small Early-stage IBCs	Small Mid-stage IBCs	Fewer, smaller Late-stage IBCs	Fewer Late-stage IBCs, filaments begin to form	No IBCs	Deficient Second-generation IBC former
5.3r	B2	√	Early-stage IBCs	Mid-stage IBCs, filaments begin to form	Fewer, smaller Late-stage IBCs, many filaments	Very few Late-stage IBCs, IBCs are smaller many filaments	No IBCs	Deficient IBC former

*Assessed separately, via X-gal staining

Table 3.2. IBC cycle kinetics of diverse UPEC strains. Individual timelines of IBC development for each strain. Strains are ordered based on their location on the phylogenetic tree, apart from the comparator strain UI89.

Deficient IBC formation

The two clade A strains 11.2p and 11.3r were classified as poor invaders and thus excluded from this analysis. Interestingly, the remaining clade A strain in the panel, 2.1a, only formed one single detectable IBC across all time-points, at 16hpi **(Figure 3.3)**. 2.1a is thus an extremely poor IBC former. Thus, all three phylogroup A strains tested in this paper were unable to robustly form IBCs in a naïve mouse model of acute UTI. These same strains were also previously characterized, or closely related to a strain that was previously characterized, as deficient bladder colonizer in mice[57]. Due to the low sample size represented (n=3 clade A strains) and to the similar PUF carriage patterns between these poor IBC formers and other non-IBC formers, further genetic analyses and *in vivo* testing is required to definitively identify any genetic factors underlying IBC formation. However, it is interesting to note that 2.1a's inability to form many IBCs does not reflect carriage of previously identified putative virulence factor (PUF) genes. According to Schreiber *et al,* 2.1a carries two of 31 identified PUFs. Strain 56.1a, a strain shown to be a robust IBC former, also carries the same two of 31 PUFs[57].

Altered IBC morphologies

The phylogroup D strain 9.1a could not be successfully transformed with the pANT4 plasmid and thus could not be assessed via confocal microscopy like its fluorescent-capable cohorts. IBCs formed by this strain were thus stained blue using X-gal and imaged under a dissection light microscope. The remaining clinical isolates displayed a breadth of strain-specific IBC phenotypes. Only one strain, the clade B2 strain 20.1a, formed IBCs that were indistinguishable from UTI89 at all timepoints **(Figure 3.2)**. The clade B2 5.3r isolate also formed filaments early in the course of infection at 6hpi, compared to UTI89's 16hpi. Conversely, the phylogroup B1 isolate 56.1a formed IBCs that trended larger than those of UTI89. This strain also had a unique

IBC morphology wherein a central, unevenly shaped, densely packed "nucleus" of UPEC was surrounded by a loosely-dispersed community of UPEC that took up the entirety of the bladder cell cytosol. The phylogroup B1 isolate 41.4p also displayed noncanonical IBC morphology, wherein the UPEC were still only loosely associated by 12hpi. Following the pattern established by Garofalo *et al* and Schreiber *et al,* this study also fails to identify a connection between PUF gene carriage and IBC morphology. Further investigation is required.

Deficient invasion of neighboring cells

The clade B1 strain 56.1a, the D strain 9.1a, and the clade B2 strain 20.1a underwent the IBC cycle at a similar timeline to UTI89 and formed second-generation IBCs. The strains 41.1a and 41.4p, which represent both acute and recurrent UTIs from the B2 and B1 phylogroups respectively and are further apart on the phylogenetic strains than other UPEC strains in the panel, also underwent the IBC cycle with a similar timeline to UTI89. However, they were unable to successfully invade neighboring bladder cells and form second generation IBCs by 24hpi. Furthermore, the B2 isolate 41.1a formed significantly fewer IBCs at 12hpi and 16hpi than the other strains in the panel. Again, these deficiencies in IBC cycle execution compared to UTI89 do not correlate with PUF genome carriage; 5.3r and 41.1a have an almost identical PUF carriage pattern to strain 20.1a, which carries two fewer PUFs than the two aforementioned strains and is able to successfully form second-generation IBCs. 41.4p carries fewer PUFs than the other two strains, but still carries the same PUFs as robust IBC-former 56[57]. Further testing is thus required to identify which pathways are being differentially utilized or expressed in both the bacteria and in the host cell that prevents re-invasion and formation of second-generation IBCs.

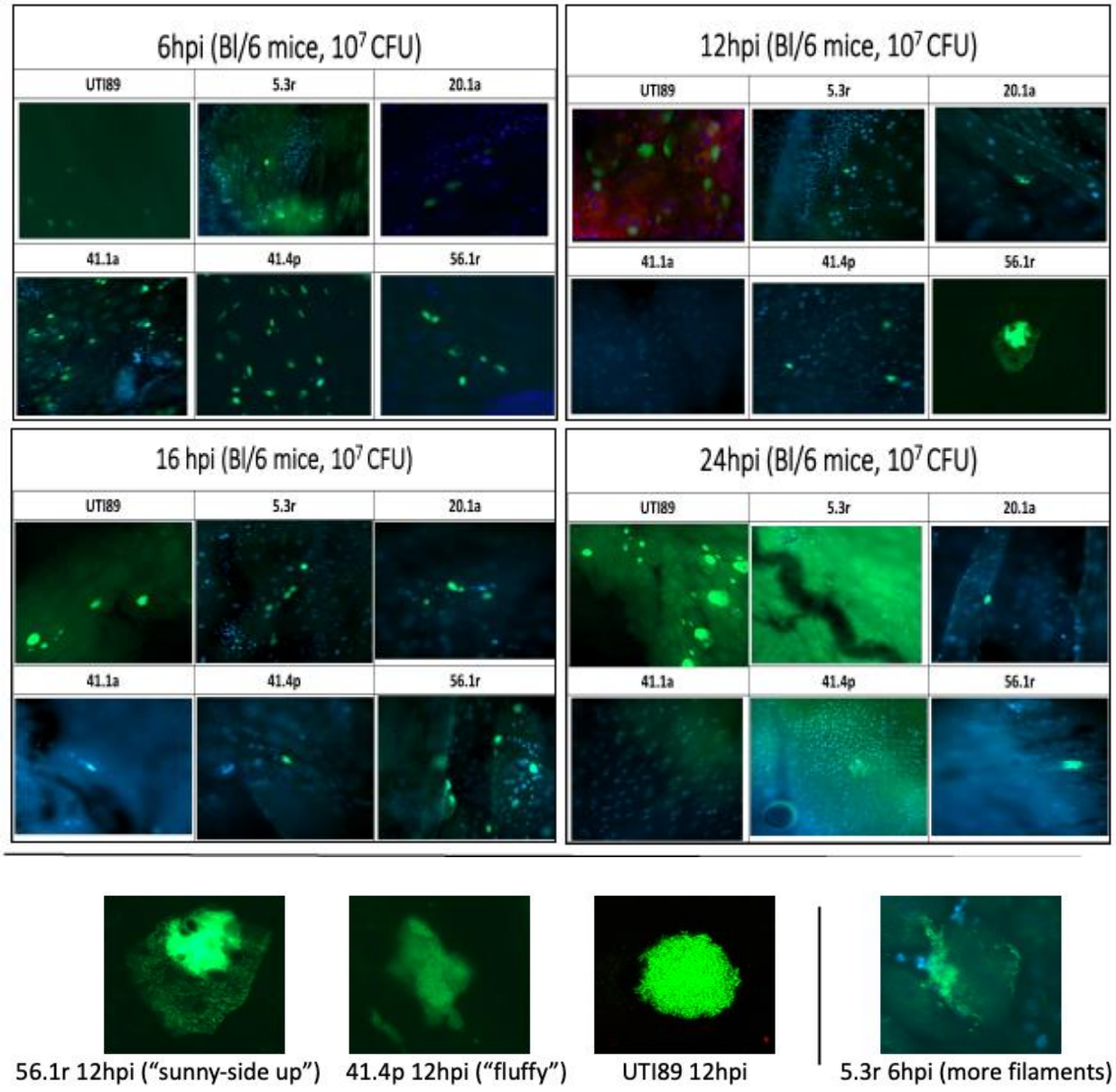

Figure 3.2 Representative images of IBCs formed by diverse UPEC clinical isolates at select time points. 6–8-week-old female C57bl/6 mice were transurethrally infected with 10^7 CFU of UPEC strain. **Top:** Bladders were excised, splayed, and imaged via confocal microscopy at 6hpi, 12hpi, 16hpi, and 24hpi. Green = E. coli, blue = DAPI. Images were taken at 40x. **Bottom:** Representative close-ups of IBCs with altered morphology compared to UTI89. Images were taken at 63x.

3.4 Discussion

Uropathogenic *E. coli* is a term that encompasses an extremely diverse group of pathogens. While UPEC strains share a pangenome of approximately 16000 genes, individual

isolates can vary in gene carriage by up to 40%[10]. Uropathogenic *E. coli* is also associated with a wide variety of UTI subtypes, such as acute cystitis, recurrent or chronic cystitis, pyelonephritis, urosepsis, catheter-associated UTIs, and more. Previous studies performed on phenotypically and genetically diverse UPEC isolates have revealed an important commonality in uropathogenesis, shared between members of this disparate group: the importance of intracellular steps to infection outcome, and the ability to successfully invade bladder epithelial cells and complete the loosely defined stages of the IBC cycle[10,12]. The IBC morphologies and IBC cycle kinetics differed between individual strains, suggesting the existence of multiple, strain-specific mechanisms by which UPEC can form and persist within IBCs[57,122]. However, so far there has been a relative lack of genetic diversity in the strains characterized for IBC behaviors. In this study, we further characterized the intracellular abilities and behaviors of eight diverse UPEC isolates, encompassing representatives from four distinct phylogroups. Following the established dogma, most strains assessed (6/8) were capable of invading bladder epithelial cells and forming IBCs, with strain-specific differences observed in IBC cycle kinetics and in IBC morphology. The strains that could not form IBCs at all both had defects in invasive abilities; any strain that was capable of invading bladder epithelial cells could form at least one IBC during uropathogenesis. When Garofalo *et al* characterized the intracellular behaviors of eleven UPEC isolates in 2007, they noted that all of the tested strains which were incapable of invading bladder cells or forming IBCs on their own were capable of doing so in a mixed inoculate with an IBC-competent uropathogen[122]. It is thus possible that the two non-IBC formers in our strain panel may be capable of forming IBCs in a mixed sample.

While our sample size was too small to support any broad conclusions, specific patterns were observed that invite follow-up. IBC morphology of the clade B1 strains, for example,

varied the most drastically from the morphology of canonical UTI89 IBCs, with 41.4p forming communities of loosely-associated bacteria during the mature stage of the IBC cycle, and with 56.1a forming IBCs consisting of loosely-associated rod-shaped bacteria surrounding a nucleus of tightly-associated coccoid bugs.

Surprisingly, the three clade A strains, were the least capable of executing the IBC cycle; only one 2.1a-strain-IBC was observed across 25 mice over 5 time points. 11.2p and 11.3r were unable to consistently invade the uroethelial cells in the first place. These strains, interestingly, did not appear to harbor a specific genetic signature that might confer this phenotype. The phylogroup A strain 2.1a, for example, has the same PUF carriage pattern as the robust IBC forming clade B1 isolate 56.1a[57]. The poor IBC phenotypes of these phylogroup A strains beg the question, how did these strains originally cause UTIs? While previous studies indicate that UPEC which cannot form IBCs tend to be less virulent, per the protocol by which these strains were isolated the patients experienced UTIs robust enough to notice and report their symptoms, and to submit a urine culture[4]. 2.1a was technically capable of forming at least one IBC in the mouse model, and thus had the potential to invade and colonize the lining of patient 2's bladder wall. However, the strains that caused patient 11's second and third UTIs, 11.2p and 11.3r, were incapable of invading urothelial cells on their own. It is possible that the recurrent nature of the UTIs themselves allowed these strains to cause a robust infection despite their inability to invade the uroethelium. Individuals who experience stronger inflammatory immune responses during an acute infection are, paradoxically, more susceptible to rUTIs. Mice that experience severe COX-2 – mediated inflammatory responses during the early hours of acute infection undergo significant bladder remodeling from neutrophil transmigration, which predisposes these mice towards chronic and recurrent UTIs[5–7]. Remodeled mouse bladders exhibit urothelial

hyperplasia, as indicated by an expansion of basal and intermediate cells within the pseudostratified urothelilum. Additionally, the superficial urothelial cells in these mice were significantly smaller and lacked many terminal differential markers compared with control bladders[5]. There is evidence that these translate to humans as well. Hannan *et al* demonstrated in 2014 that humans with elevated levels of inflammatory biomarkers in their serum were more prone to experiencing rUTI, and Ebrahimzadeh demonstrated in 2021 that women who suffer from rUTI have higher levels of prostaglandin E2 (a product of COX2) in their urine[7,8]. Additionally, patients with chronic and recurrent UTIs have demonstrated evidence of urothelial hyperplasia and incomplete terminal differentiation[9,10]. Mice who undergo this inflammation-induced bladder remodeling are classified as "sensitized" and are significantly more susceptible to chronic and recurrent UTI, even if the uropathogen cannot form IBCs[5]. It is possible that patient 11 is also sensitized, and thus more susceptible to infection via mechanisms where IBC formation is less required. Our findings thus invite further exploration into the pathogenic potential of diverse "poor colonizer" strains in the sensitized model of infection.

Our results also beg the question, why were none of the clade A strains tested competent IBC formers? As stated previously, preliminary genetic analyses do not reveal a specific pattern of putative urovirulence factor (PUF) gene carriage that separate competent from incompetent IBC formers. Further in depth analyses are therefore required to determine any genetic factors underlying IBC development. To this extent, we propose using the Mass Allelic Exchange (MAE) method developed by Khetrapal *et al* in 2022 to perform gain-of-function genetic screens[227]. Briefly, MAE facilitates direct sexual hybridization between two strains of *E. coli*, resulting in wild-type 'recipient strain' progeny with stable, scarless genetic insertions from a 'donor' strain. Khetrapal *et al* demonstrated that transfer of the chu operon into the IBC-

incompetent strain MG1655 conferred the ability to form intracellular bacterial aggregates, thus providing proof-of-concept for our proposal[227]. Specifically, generating hybrid libraries using UTI89 as a donor strain and the clade A isolates as recipient strains has the potential to yield insight into what genetic elements confer gain-of-function in IBC formation. It is important to note that while chu is sufficient for IBC formation, it is not necessary. Phylogroup B1 strains do not carry chuA by definition, but the B1 strains tested in this paper were robust IBC formers[59]. Thus, even if insertion of the chu operon does grant the clade A strains the ability to form IBCs, investigating other genetic determinants is still beneficial[59].

Regardless of the individual idiosyncrasies of each strain's intracellular behavior, the characterizations reported in this paper carry with them an important reminder: UPEC strains cannot all be treated the same. Virtually all prototypical UPEC isolates (UTI89, CFT073, NU14, 563) used in UTI studies are relatively similar, and come from the B2 phylogroup[11–14]. However, the continued trend of individual phenotypic variation in intracellular uropathogenesis in UPEC strains highlights the importance of studying multiple strains across multiple genetic backgrounds to gain a clear picture of the bacteria and the disease, especially because the genetic factors underlying IBC development have not yet been fully identified. Previous studies have identified a possible connection between gene expression, and uropathogenesis[15]. It is thus possible that genetic differences underlying IBC formation lie in patterns of gene expression, rather than of gene carriage. Further characterizations and studies are needed to better understand the intracellular step of uropathogenesis. Elucidating this understudied step has the potential to yield future knowledge and therapeutic targets to prevent and treat UTIs.

3.5 Materials and methods

3.5.1 Bacterial Strains and Growth Conditions

Urinary clinical isolates 2.1a, 5.3r, 9.1a, 11.2p, 11.3r, 20.1a, 41.1a, 41.4p, and 56.1a were collected in 2008 by Czaja *et al*[4]. The prototypical urinary isolate UTI89 was also utilized. All strains but 9.1a had been transformed with and carried the pANT4 plasmid. Cultures were started from freezer stocks and grown using a "2x24" system[16]. Bacteria was first grown statically in lysogeny broth (LB) at 37°C for 24 hours. The static cultures were then subcultured at 1:1000 into 20 mL of fresh LB and grown statically for another 24 hours at 37°C. The strains were grown in kan 50 or amp 20 to prevent plasmid loss.

3.5.2 Mannose-Sensitive Hemagglutination Assays

MSHA assays were performed as described in Hultgren *et al, 1986*[13]. Briefly, bacteria were grown statically using the "2x24" system. Bacteria was first grown statically in lysogeny broth (LB) at 37°C for 24 hours. The static cultures were then subcultured at 1:1000 into 20 mL of fresh LB and grown statically for another 24 hours at 37°C. Bacteria were then pelleted and re-suspended in phosphate-buffered saline (PBS), and subsequently re-concentrated in PBS to yield 100uL of OD_{600} = 1.0 bacteria (approximately 10^9 bacteria per 25uL). Two replicates of 25uL of suspension was serially diluted per strain in a v-bottom 96-well plates containing 25uL of PBS while the other two replicates of each strain was serially diluted in PBS + 4% mannose. 25uL of 3% guinea pig red blood cells, which had been washed in PBS and diluted to a concentration of OD_{640} ~ 1.9 – 2.0, were then added to the wells and the reaction was left overnight at 4°C. HA values were defined as the number of the first column in the dilution which a well did not have a clearly defined punctum of blood in the bottom of the plate.

3.5.3 Mouse Infections

Six-to-eight-week-old female mice were anesthetized via inhalation of 4% isoflurane and infected transurethrally, as previously described in Mulvey *et al,* 1998[17]. Bacteria cultures were grown in 2x24 conditions, described above, pelleted, resuspended in phosphate-buffered saline (PBS), and subsequently re-concentrated or diluted in PBS to yield 50 μl innocula of 1 x 10^7 CFU. All studies were approved of and performed in accordance with the guidelines set by the Committee for Animal Studies at Washington University School of Medicine, and the mice were housed in a facility with a 12-hour light/dark cycle where they had ad libitum access to standard food and water.

3.5.4 Gentamycin Protection Assays

Antibiotic protection assays were performed as described previously[18]. Infected mouse bladders were aseptically removed, hemisected to allow access to the bladder lumen, and washed 3x in PBS to collect adherent extracellular bacteria. The PBS washes were pooled and spun at 500 x g minutes to pellet any shed epithelial cells, after which bacterial loads of the wash were quantified. Simultaneously, the washed bladders were treated with 1 ug/ml of gentamycin in serum-free RPMI at 37°C for 75 minutes to kill any remaining adherent extracellular bacteria while leaving potential intracellular bacteria intact. Treated bladders were then homogenized in PBS, and the homogenate was serially diluted and plated on kan 50 plates to enumerate intracellular bacteria.

3.5.5 Histology and Immunohistochemistry

Mouse bladders were removed at the relevant time-points, hemisected, and splayed lumen-side up on silica plates containing PBS. The splayed bladders were fixed for one hour at RT in 4% paraformaldehyde, washed 3x5 min in PBS, and mounted on microscopy slides in Prolong Gold Antifade with DAPI. The bladders were then visualized under a confocal microscope (below).

3.5.6 X-gal Staining

Mouse bladders were aseptically removed, hemisected, splayed, and pinned lumen-side up on PBS-containing silica plates. The splayed bladders were fixed for 1 hour at RT in 3% paraformaldehyde, washed 3 x 5 min in PBS, and then washed 3 x 5 min in 2 mL/well lacZ staining buffer (2mM MgCl2, 0.01% NaDOC, and 0.02% Nonidet-P45 (Roche) in PBS, pH 7.4). The bladders were then incubated in 2 mL/well LacZ staining solution (9.5 mL lacZ wash buffer, 0.4mL of 25 mg/mL X-gal, 1mM K-ferrocyanide, and 1mM K-ferricyanide) for 6-8 hours at 30 degrees Celsius in a light-shielded environment. Bladders were then washed 3 x 5 min in PBS and post-fixed in 4% paraformaldehyde at 4 degrees Celsius overnight. The processed bladders were then imaged under a dissection microscope.

3.5.7 Bladder preparation for IBC analysis

Infected animals were euthanized 3, 6, 12, 16, and 24 hours post infection, after which their bladders were harvested, hemisected, splayed, and pinned flat in silica wells. The splayed bladders were then fixed overnight at 4°C, quenched in 2M glycine to remove any bulky adducts, washed in PBS, and mounted on microscope slides in Prolong Gold Antifade with DAPI. The bladders were visualized using a confocal microscope (see below). Total IBCs were counted, and snapshots were then taken of five random fields of view for each mouse bladder, totaling 25 snapshots per strain.

3.5.8 Confocal Microscopy

Samples were analyzed with a Zeiss LSM880 laser scanning confocal microscope (Carl Zeiss Inc. Thornwood, NY). The system is equipped with 405nm diode, 488nm Argon, 543nm HeNe, and 633nm HeNe lasers. Objectives used were: Plan-Apochromat 40X (NA 1.2) w korr objective, Plan-Apochromat 40X (NA 1.4) DIC objective, and Plan-Apochromat 63X (NA 1.4) DIC

objective. ZEN black 2.1 SP3 software was used for image acquisition. All microscopy was performed at the Washington University in St. Louis Molecular Microbiology Imaging Facility.

Chapter 4: Multiple redundancies in lactose metabolism in UPEC

By Jennie Hazen, Taylor Nye, Jesús Bazán Villicaña, Kent Kleinschmidt, and Scott Hultgren

4.1 Abstract

Urinary tract infections rank among the most common bacterial infections worldwide, and uropathogenic *E. coli* ranks as the most common bacterial species that causes UTIs. Previous studies have identified an important role for lactose metabolism in the intracellular steps of uropathogenesis: mutant UPEC strains lacking critical components of the lactose metabolism pathway were unable to properly develop intracellular bacterial communities and ultimately exhibited attenuated virulence compared to their wild-type counterparts. However, approximately 5% of UPEC isolates are atypical, lactose nonfermenting strains. The mechanisms by which these strains can mount robust infections, despite their lack of critical metabolic activities, are unknown. In the present chapter, I have identified and characterized a lactose nonfermenting UPEC isolate called 5.3r. The lactose-negative strain differs in its lac operon from that of prototypical lactose-positive strain UTI89 by six nucleotides in the lactose permease gene, lacY. UPEC isolate 5.3r resembles the UTI89ΔlacZ mutant in its intracellular phenotype. However, I have found that both lactose-negative strains can import the lactose-analog X-gal despite the lack of a functional import protein. Together, these data suggest the presence of a back-up lactose transport protein, and invite future research into what was previously thought to be a thoroughly studied field.

4.2 Introduction

Urinary tract infections (UTIs) are highly common diseases that are associated with high rates of recurrence; with several morbidities; and, in some cases, with relatively high rates of mortality[1–3]. Uropathogenic *E. coli* (UPEC) is the most common uropathogen, accounting for

over 85% of community-acquired UTIs and over 50% of nosocomial UTIs worldwide. During uropathogenesis, the majority successful UPEC strains follow a series of loosely defined intracellular steps during uropathogenesis known as the IBC cycle[122,163,228]. In murine models of acute cystitis, UPEC adhere to and invade their hosts' bladder epithelial cells and become intracellular pathogens. Once in the host cell cytosol, the bacteria form organized, biofilm-like pods called intracellular bacterial communities (IBCs). When an IBC forms, the bacteria within the IBC undergo multiple loosely defined stages of morphological change as they rapidly expand to overtake the volume of the cell. The IBC itself changes in its overall ultrastructure and composition during this time as well. Once the IBC is fully mature, some of the bacteria on the outer edge of the IBC adapt a filamentous morphology. Finally, bacteria in the IBC eventually flux out of the host cell into the bladder lumen, where they can attach to and invade neighboring epithelial cells[122,163] **(For more information, see chapter 1).**

Many animal studies suggest that the IBC cycle allows UPEC to gain a foothold in the bladder[167]. As part of an IBC, the uropathogens are protected by the host cell from the harsh environment of the bladder lumen. The IBCs are also inaccessible to many classes of antibiotics, to neutrophils, and to other host defense responses, which cannot penetrate the bladder cell[169,218,229]. The level of IBC formation in an individual mouse's bladder correlates with the severity and outcome of that mouse's infection[167]. IBCs have also been found to be shed in the urine of human patients experiencing UTIs [122,228]. The morphological and histological similarities observed between murine and human – derived IBCs, such as similarities in bacterial morphology and histological staining patterns, indicate the mouse model recapitulates human disease[122,228,230–232]. Collectively, these findings serve as evidence that the IBC cycle is a clinically relevant process to study.

One can make several well-informed hypotheses about certain genetic pathways involved in IBC formation based off the prevailing body of knowledge on the urothelial cell environment. For example, an intracellular bacterium would need to withstand host defenses like free radicals that cause oxidative stress. The intracellular bacterium would also have to utilize adhesion genes to adhere to the bladder cell in the first place, and it would need to express metabolic pathway genes and biofilm components to promote bacterial growth, aggregation and actual formation of the IBC. It is also logical to assume that the bacteria will express genes related to filamentation such as ftsZ. Through characterization of the mouse model, several microbial genes have been experimentally shown to play a role in the IBC cycle. As expected, genes involved in stress response, biofilm formation, and iron scavenging have been implicated[233–242] **(Table 4.1)**.

Gene	Function	IBC phenotype	Ref
IhfA	Transcription factor, regulates pap operon	Smaller IBCs	233
IhfB / HimD	Transcription factors regulating pap operon	Altered biofilm matrix; no rod-to-coccoid morphological change	233
FimA*	Major structural subunit of Type 1 pilus	Fails to transition from early-stage IBCs to mid-stage IBCs	234
SurA	Induces SOS response	Impaired filamentation**; no second-generation IBCs	165
DamX	Involved in cell division & cytokinesis	Impaired filamentation; no second-generation IBCs	235
Fur	Inhibits iron uptake pathways, iron dependent enzymes, in the presence of iron	Larger IBCs	236
OmpA	Outer membrane protein; contributes to structural integrity of the cell, phage & colicin receptor, conjugation	Impared ability to form mature IBCs; stunted filamentation	237
LacZ & GalK	Lactose metabolism	Smaller IBCs; fewer mature IBCs; stunted filamentation	238
Kps***	K1 capsule assembly region 1 (assembly & export)	Fail to transition from early-stage to mid-stage IBCs; neutrophils can infiltrate the IBCs	239

Neu	K1 capsule assembly region 2 (determine antigen)	Fail to transition from early-stage to mid-stage IBCs; neutrophils can infiltrate the IBCs	239
ChuA	Transmembrane heme transporter	Smaller IBCs	239
YeaR	Oxidative stress response, type 1 pilus regulator	Fewer IBCs form	239
cvpA-purF locus, or purF alone	Purine biosynthesis	Overall attenuated intracellular replication	240
cydAB	Cytochrome bd	Fewer IBCs; smaller IBCs; impaired ability to transition from early to mid-stage IBC	241
TolB, tolA, tolQ, tolR, pal	Components of the Tol-Pal system; colicin uptake, maintenance of the outer membrane	Failure to form a proper 'biofilm-like' community; failure to replicate within the bladder cell	242

* IBC phenotype occurs in a Tet-OFF induction model; full deletion of the gene results in failure to invade
** Wild-type filamentation patterns *in vitro*
*** When deleted in conjunction with NanR, IBC formation is restored

Table 4.1 Genes that play a role in the IBC cycle in UPEC. Genes whose mutant phenotype results in altered IBC morphology, IBC cycle kinetics, IBC numbers, etc. whose mutant phenotypes result in a complete lack of IBC formation are not included.

In this chapter, I specifically explore the subject of intracellular UPEC metabolism. The exact composition of a bladder cell is unknown, and thus there remains the question: from where do intracellular UPEC get their energy in the urothelium? Interestingly, as shown in **Table 4.1**, several of the genes utilized during IBC formation are involved in the breakdown and utilization of lactose. Microarray experiments performed on infected mouse bladder homogenates revealed an upregulation in several lactose-related genes such as the beta galactosidase lacZ and the downstream galactokinase galK[238]. These same genes, when deleted, also result in smaller intracellular bacterial communities, impeded ability to successfully complete the re-invasion steps of the IBC cycle, attenuated colonization of the urinary tract, and overall a milder infection outcome[238].

The lacZ gene is part of the tightly regulated lac operon, a group of genes which are under the control of a single promotor that encode the proteins responsible for the import and breakdown of lactose. The regulation of this operon has been extensively studied in various laboratory strains of *E. coli*[1]. Briefly, the operon encodes three main proteins necessary for the lactose catabolic pathway: **1.)** LacZ, the β -galactosidase that hydrolyzes the beta 1-4 glycosidic bond in the disaccharide lactose sugar to yield glucose and galactose; **2.)** LacY, the lactose permease that acts as a β -galactoside – proton symportor; and **3.)** LacA, the β-galactoside transacetylase that transfers an acetyl group from acetyl-CoA to thiogalactoside. The lac operon also contains a promoter, an operator, and a terminator. Upstream of the lac operon is the constitutively-expressed lacI, which encodes the lac operon repressor[243]. In the absence of lactose, the lacI-encoded repressor molecule binds to the operator of the lac operon and prevents transcription of lactose-catabolizing genes. In the presence of lactose, the lacI-encoded repressor preferentially binds the sugar; the lac operon is unbound, and lactose catabolism genes are expressed. It is important to note that the presence of lactose alone does not activate the lac operon. Regulation of the lac operon is also conferred by the cyclic-AMP (cAMP) binding protein, CAP[244]. When CAP is bound to cAMP, the CAP-cAMP complex binds to a sequence close to the promotor of the lac operon called the CAP binding sequence (CBS). When the CBS is bound, the RNA polymerase can bind to the promotor of the lac operon more easily and expression of the operon is increased. If glucose is available to the bacteria, it will reduce the expression of genes encoding the cAMP signaling molecule. This leads to a reduction of CAP-cAMP and thus to a reduction in lac operon expression. In this way, the lac operon is only expressed in the presence of lactose and in the absence of the preferred carbon source, glucose.

Even though lactose metabolism appears to play a role in intracellular uropathogenesis, many lactose-nonfermenting uropathogens are still capable of causing UTIs and indeed of invading bladder epithelial cells[152,245,245–250]. However, the intracellular phenotypes of these bacteria vary greatly from their lactose-fermenting counterparts. The gram-negative bacterium *A. baumannii,* for example, is a lactose nonfermenter that can cause a UTIs and can invade and persist within mouse bladder epithelial cells **(See chapter 2)**. However, the infection is attenuated compared to an infection mounted by UPEC, and in certain mouse strains *A. baumannii* cannot colonize the urinary tract at all[23]. Additionally, the number of intracellular bacteria observed in mouse bladders infected by *A. baumannii* is significantly lower than the number seen in *E. coli* or *K. pneumoniae* infected mice at similar timepoints (10^1 vs 10^{4-5}). *A. baumannii* invasive bacteria also do not form robust intracellular bacterial communities; rather, they remain quiescent within the cell.

However, even though lactose metabolism seems to be important to successful completion of the IBC cycle intracellular survival during uropathogenesis, instances of lactose nonfermenting uropathogenic *E. coli* strains have been isolated from the urine of patients suffering from UTIs[245,245–249]. Many of these lactose nonfermenting UPEC strains can cause robust UTIs, even though mutations in the lac operon of the prototypical UPEC strain UTI89 result in virulence defects. In fact, Versaolvic *et al* performed a comprehensive metaliterature analysis and estimated in the 2011 Manual of Clinical Microbiology that 5% of all *E. coli* clinical isolates, regardless of the source or sample site, should be lactose negative[4]. This estimation has been supported by multiple studies performed across the globe over the past several years. In India, multiple studies report a proportion of lactose-negative UPEC isolates ranging from 3.6% - 12.4%[5–7]. Studies in Angola, Hungary, and South Korea report lac- UPEC levels of 21%, 3.2%,

and 19.9%, respectively[8–10]. Alarmingly, these lactose negative UPEC isolates had significantly higher rates of multidrug-resistance (MDR) than their lactose-fermenting counterparts.[8–10] The bacteria were especially resistant to antibiotics more commonly prescribed for UTIs, such as norfloxacin, ciprofloxacin, Fosfomycin, and nitrofurantonin, thus making their resultant infections more difficult to treat[9,10].

The existence and global prevalence of lactose-negative UPEC strains is a cause for great alarm, as is their apparent proclivity for multidrug-resistance. Furthermore, the high isolation rates of these atypical *E. coli* strains challenge the prevailing understanding within the field that UPEC must metabolize lactose to successfully colonize the urinary tract and cause a robust infection. Further understanding of lactose-negative UPEC strains and their pathogenesis is thus necessary. In this work, we identified a lactose-negative UPEC clinical isolate whose intracellular behavior phenotypically resembles the previously-characterized UTI89ΔlacZ strain. We suspect that the strain was incapable of growth in lactose due to a two amino acid deletion in the active site of the lactose permease gene, lacY, in comparison to the lacY gene of UTI89. We also found that that while the strain could not grow *in vitro* using lactose as its sole carbon source, it could still import and break down the lactose analog, X-Gal. Further studies revealed that a clean UTI89ΔlacY deletion strain was also capable of importing x-gal. These results imply the presence of a secondary transport mechanism to import lactose-like sugars into the cell, which may be specific to uropathogenic strains of *E. coli.* While further investigation is needed to fully validate this hypothesis, this finding could potentially reveal novel therapeutic targets for urinary tract infections.

4.3 Results

4.3.1 The UPEC clinical isolate 5.3r is incapable of utilizing lactose as a sole carbon source

We first performed growth curve analysis of 23 clinical urinary *E. coli* isolates to identify any lactose-nonfermenting UPEC strains **(See chapter 3 for details about the strains)**[11]**.** These isolates were chosen because they have previously been extensively characterized *in vivo* and *in vitro*, and because their genome sequences are readily available[12]. These 23 UPEC strains were first grown overnight in M9 media containing 0.2% glucose. To mitigate future sugar contamination of the growth media and to 'prime' the bacteria for growth curve assays, the strains were subsequently subcultured and grown in sugar-starved conditions (M9 + 0.04% glucose) for approximately five hours or until they entered log phase (as determined by OD600 measurements). The bacteria were then separated from the media and any residual sugar by filtration and grown in M9 media containing 0.4% of either glucose, lactose, or galactose. Of the 23 clinical isolates one strain, named "5.3r" (patient number five's third recurrent UTI over the course of the study that isolated the strain)[251], was unable to grow utilizing lactose as its only source of carbon. The Lac- rate among our isolates is thus approximately 4%, and in line with Versaolvic's estimates[4].

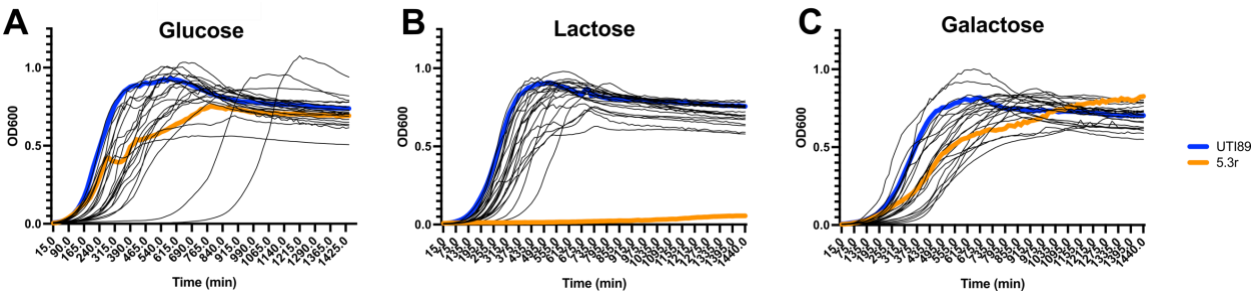

Figure 4.1. UPEC strain 5.3r is incapable of growing using lactose as its sole source of carbon. 23 clinical urinary isolates were screened alongside the prototypical urinary isolate UTI89. All strains were grown in shaking conditions at 37degC for 24 hours in minimal M9 media containing either glucose (**A)**, lactose (**B)**, or galactose (**C)** as the sole source of carbon. Strains were previously grown overnight in high

levels of glucoses before being subcultured in low glucose conditions and subsequently filtered away from any residual sugars. Lines representing lactose-fermenting laboratory clinical isolates are depicted in grey, UTI89 is depicted in blue and 5.3r in orange.

4.3.2 UPEC isolate 5.3r has similar *in vivo* intracellular phenotypes to that of UTI89ΔlacZ

Schreiber *et al* classified the UPEC strain 5.3r as a "robust" bladder and kidney colonizer[12]. However, the lactose-nonfermenting strain has not been evaluated for its ability to successfully invade bladder epithelial cells or execute the IBC cycle. We thus sought to characterize 5.3r's intracellular behaviors and compare it to those of lactose-negative mutants of the prototypical UPEC isolate, UTI89.

The prototypical urinary isolate UTI89 undergo a loosely defined, multistep-process of morphological change – both individually and as an overall intracellular bacterial community – during the IBC cycle. Briefly, this process includes: **i) Early-stage IBCs at 3hpi,** wherein the bacteria are rod-shaped, relatively slow to replicate, and loosely associated; **ii) Mid-stage IBCs at 6hpi**, wherein the bacteria undergo a coordinated "differentiation step" and adopt a small, coccoid morphology. The bacteria become densely-packed and replicate quickly. The community begins expressing markers indicative of a biofilm, such as extracellular bacteria and antigen 43[233];; **iii) Late-stage IBCs at 12hpi,** wherein the IBC has grown to overtake the volume of the bladder cell, after which the bacteria on the outer edge of the IBC begin to adopt a rod-shaped conformation; **iv) Filamentation and fluxing stages at 16-18hpi,** wherein the rod-shaped bacteria on the edges of the IBC quit dividing and become long and filamentous, and the IBC disperses with bacteria escaping from "fluxing" out of the infected bladder epithelial cell; and **v) re-invasion of neighboring umbrella cells and formation of new early-stage IBCs by 24hpi**[13].

Conover *et al* also demonstrated in their 2016 paper a significant decrease in IBC size when comparing UTI89ΔlacZ with its wild-type counterpart. They also report that UTI89ΔlacZ cannot progress through the IBC cycle properly, and do not reach the stage where they can form filaments at 16hpi[14]. Attenuated bladder titers during later stages of infection further suggest a failure to robustly form secondary IBCs. Similarly, the lactose-negative UPEC isolate 5.3r also exhibits an attenuated IBC phenotype compared to the wild type UTI89. While a subset of IBCs prematurely form filaments at 6hpi, the majority do not filament or flux from the cell nor do they form secondary IBCs **(See chapter 3) (Figure 4.2)**. Here, we report a similar result when quantifying the size of IBCs formed by 5.3r **(Figure 4.2)**.

Taken together, our results indicate that the lactose-nonfermenting UPEC strain 5.3r exhibits a similar intracellular phenotype to that of UTI89ΔlacZ.

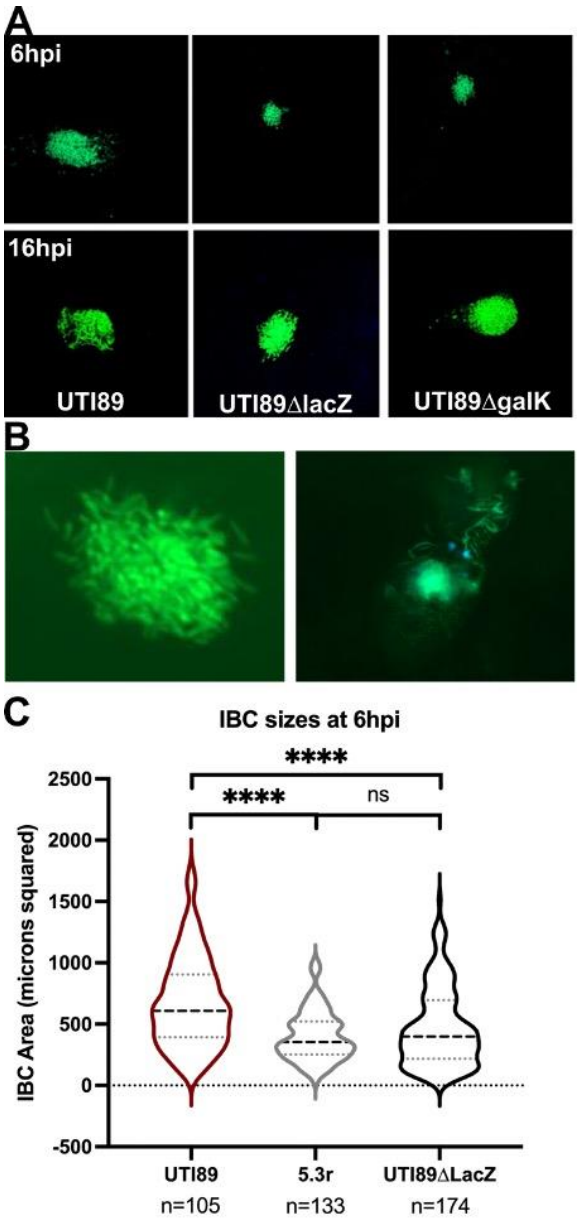

Figure 4. 2. Lactose-negative UPEC isolate 5.3r shares similar IBC characteristics to the UTI89ΔlacZ deletion mutant. 8-10 week old female c57bl/6 mice were transurethrally injected with 10^8 CFU of uropathogen. Each UPEC strain tested (UTI89 WT, UTI89ΔlacY, 5.3r) carried the plasmid pANT4, which encodes GFP under the control of the constitutive tac (tet-lac fusion) promoter. The mice were sacced and bladders removed at critical time-points related to the UTI89 IBC cycle. Bladders were hemisected, splayed on silica plates, and imaged under a confocal microscope to evaluate the IBC cycle kinetics, the morphology of the IBCs, and size of the IBCs at each time-point. n=5 mice per bacterial strain per time-point. **A)** Representative images of IBCs formed by UTI89, UTI89ΔlacZ, and UTI89ΔgalK at 6hpi and 16hpi, adapted from Connover *et al*, 2016. **B)** Representative images of IBCs formed by 5.3r WT at 6hpi. Deviations from the canonical UTI89 IBC phenotype are seen where (left) the IBC is immature and still contains rod-shaped bacteria and (right) the IBC is forming filaments prematurely. **C)** Quantification of IBC size for IBCs formed by UTI89 WT, UTI89ΔlacZ, and 5.3r WT at 6hpi.

4.3.3 Single two-codon deletion in the lacY gene of UPEC clinical isolate 5.3r is the only genetic difference in the lac operon when compared to protypical isolate UTI89.

We next investigated the genetic determinants underlying the *in vitro* and *in vivo* similarities in phenotype between 5.3r and UTI89ΔlacZ. Sanger sequencing and sequence alignment reveal that the lac operons of UTI89 and 5.3r share 99.9% nucleotide similarity; the only difference between the two strains is a six base pair deletion in the coding region of 5.3r's lacY gene, which encodes the lactose permease. Despite what its name suggests, the lactose permease's activity is two-fold: in addition to increasing the bacterial membrane's overall permeability for passive diffusion of lactose, the protein also facilitates active transport as a proton-beta-galactosidase symporter[252]. 3D modeling reveals that these six base pairs correspond with two amino acids that occupy the lactose permease's active site, suggesting that the observed lac phenotype can be attributed to a decrease in lacY function **(Figure 4.3)**.

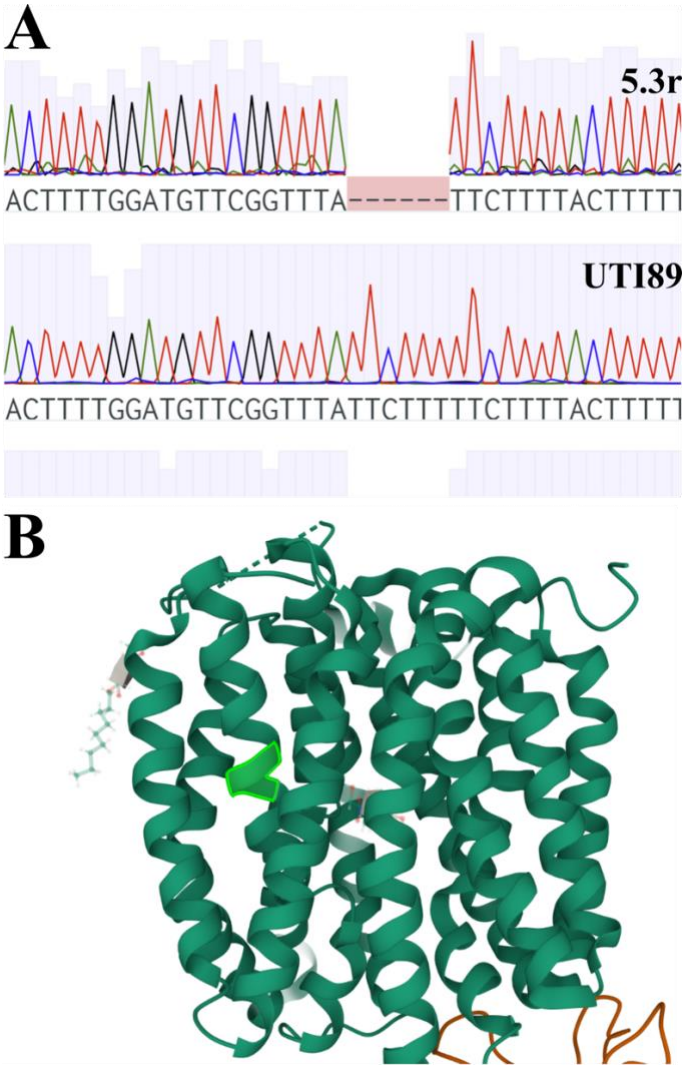

Figure 4.3. Lactose-nonfermenting UPEC strain 5.3r lacks two amino acids in the active site of its lactose permease compared to UTI89 A) Nucleotide sequence alignment of the lac operon between UTI89 (top) and 5.3r (bottom). **B)** 3D protein model of a ligand-bound UTI89 LacY protein. The two amino acids corresponding with 5.3r's six base pair deletion are highlighted in a brighter green.

4.3.4 Import of lactose analogues occurs independently of lacY permease in UPEC strains

After identifying the deletion in the lacY gene, we sought to assess 5.3r's ability to import lactose. We thus performed a beta galactosidase assay using the lactose analog X-Gal. Briefly, X-Gal (5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside) consists of a galactose molecule linked to a substituted indol via a beta 1-4 glycosidic bond. Beta galactosidase activity

is easily detected when the beta 1-4 linkage between the two molecules is broken, and the newly freed indole molecule is further processed to yield a chromogenic, blue molecule[253,254]. We wanted to specifically assay for extracellular, or secreted, beta galactosidase activity vs. intracellular, or cytoplasmic, beta galactosidase activity. We grew our *E. coli* strains in low-glucose conditions after which we filtered out any remaining sugars and pelleted the bacteria. We then separated the pellet (cell-associated) from the supernatant (secreted/extracellular) and added minimal M9 and X-gal media to both.

The positive control strain wild-type UTI89 and the negative control strain UTI89ΔlacZ behaved as expected, with the positive control cell pellet sample turning blue and the negative control cell pellet samples remaining clear. However, the cell pellet samples of the lactose-negative UPEC isolate 5.3r and the negative control UPEC strain UTI89ΔlacY both turned blue when exposed to X-gal **(Figure 4.4)**. The supernatants of these strains remained relatively clear compared to the pellet, indicating that even though these strains lack functional lactose permeases, they were still able to import the x-gal before cleaving the molecule's beta 1-4 glycosidic bond **(Figure 4.4)**. While some studies have demonstrated that *E. coli* can import low levels of X-gal via passive diffusion[252,255], this is to our knowledge the first experiment demonstrating the ability of an *E. coli* strain to import high levels of the lactose analog without the use of LacY.

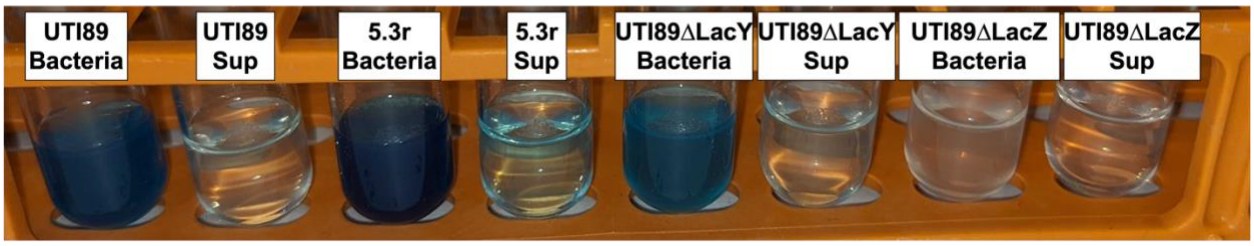

Figure 4. 4. UPEC strains 5.3r and UTI89ΔlacY can import X-gal via a lacY-independent mechanism. *E. coli* strains (left to right) UTI89, 5.3r, UTI89ΔlacY, and UTI89ΔlacZ were grown in

minimal media containing low levels of glucose, and were subsequently filtered from any residual sugars. The filtered bacterial cultures were spun down, and the pellets separated from the supernatant. X-gal was then added to the samples at a final concentration of 0.4% and the samples were incubated in low-light, shaking conditions at 37 degrees for 24 hours

4.4 Discussion

In this work, we identified a lactose fermentation-negative UPEC clinical isolate, 5.3r, whose intracellular behavior phenotypically resembles the previously characterized UTI89ΔlacZ strain. We determined that the strain was incapable of growth in lactose, and that the lac operon of 5.3r differs from the lac operon of UTI89 by only a two amino acid deletion in the active site of the lactose permease gene, lacY. We also found that that while the strain could not grow *in vitro* using lactose as its sole carbon source, it could still import and break down the lactose analog, X-Gal. Further studies revealed that a clean UTI89ΔlacY deletion strain was also capable of importing x-gal. Together, the data suggests the presence of a secondary transport mechanism to import lactose-like sugars into the cell, which may be specific to uropathogenic strains of *E. coli.*

These data raise an important question: why is lactose catabolism so important to bacteria within the bladder cell cytosol? Historically, in laboratory strains the lac operon is only expressed in the presence of lactose and the absence of glucose[15]; it thus stands to reason that intracellular UPEC only have access to lactose as a carbon source. While the bladder cell cytosol is not known to contain high amounts of lactose, further studies are required to determine the exact types and concentration and types of sugars in the cell. It is also possible that the lactose is not endogenous to the bladder cell cytosol, but rather is expressed on the surface of the cell. Complex sugar structures decorate the bladder cell surface as part of glycoproteins, specifically in uroplakin molecules[256–258]. Many uroplakins are decorated with different sugars with beta 1-4 glycosidic linkages either on their terminal ends, or as structural scaffolding[256,257,259–261]. When

the bladder contracts after voiding, the epithelial cells shrink and large portions of the surface of the cell are internalized via endocytosis[262–265]. Further studies are required to determine whether the internalized glycans can be broken down and utilized by the intracellular UPEC *in vivo*. Free-glycans containing 1-4 glycosidic linkages have been detected in human urine[266–268]. These molecules may also be available to UPEC.

Interestingly, there is precedent of of mucosa-resident bacteria catabolizing complex, lactose-like sugars. Gut-resident *Lactobacillus casei* utilize their lac operons to catabolize N-acetyllactosamine (Galβ1–4GlcNAcβ1–3 , or LacNac), a disaccharide found in human milk, in the gut mucosa, and in the bladder mucosa[269]. LacNac is comprised of a galactose molecule linked via a beta 1-4 glicosidic bond to a glucosamine molecule. There is also precedent of bacteria utilizing alternative, lac-independent pathways to import lactose and lactose-like sugars[270]. pBLAST of these molecules in *Aspergillus* results implicate the *E. coli* proton-galactose symporter GalP as a potential secondary import protein[270]. Interestingly, there is precedent for bacteria using the galactose-proton symporter to import lactose into the cell: the gut microbe *L. casei* lacks a canonical lac operon -- and thus a lacY gene -- but uses an analog of GalP to import lactose and other lactose-like sugars into the cell[269]. There is also evidence suggesting *Lactococcus lactis* utilizes a Galp-like protein to import lactose-like sugars[271]. To our knowledge no studies investigating the protein's ability to import lactose in *E. coli* have been performed. Thus, there is evidence supporting the hypothesis that UPEC carry multiple redundancies in lactose metabolism, specifically in lactose import, and that these redundancies might contribute to the urovirulence potential of UPEC. While further investigation is needed to fully validate this hypothesis, this finding has both the potential to reveal novel therapeutic

targets for UTIs and the potential to expand the current body of knowledge on lactose metabolism in *E. coli*.

4.5 Materials and Methods

4.5.1 Growth curves

UPEC were first grown in shaking conditions overnight at 37°C, in M9 media containing 0.2% glucose. To mitigate future sugar contamination of the growth media and to 'prime' the bacteria for growth curve assays, the strains were subsequently subcultured 1:1000 and grown shaking at 37°C in sugar-starved conditions (M9 + 0.04% glucose) for approximately five hours or until they entered log phase (as determined by OD600 measurements). The bacteria were then separated from the media and any residual sugar using 0.2 micron filters and concentrated in fresh M9 media to an OD of 1.0. The bacteria were then cultured 1:100 in a Corning 96-well flat bottom plate in wells containing 200uL M9 + 0.4% of either glucose, lactose, or galactose. The plate was read using a Tecan plate reader (settings: 37°C, shaking conditions, OD600 taken every 15 minutes), over a period of 24 hours.

4.5.2 Genetic analyses and visualization of the lac operon

Multiple overlapping fragments of the lac operon from ~20bp upstream of the inducer to ~20 downstream of lacA were cloned using PCR and sequenced via sanger sequencing. The FASTA files were aligned using nBLAST. The ligand-bound lacY structure used in Figure 4.3. was generated by the Mol* Viewer web application[272] and was obtained from the RCSB Protein Data Bank, structure 6C9W[273].

Primer #	F sequence	R sequence

01	GAATGTCGGCGAAATGTCCG	GGAAAACCCTGCAAACCGAC
02	GTATTCCGTGGCGATCTCGT	CGCAGCCATTGATAACCACG
03	AAATGCGATGCGGGCAATAC	GAGTTGCAACGCAAAGCTCA
04	CGTGGTTATCAATGGCTGCG	ATTACGATCTGGAGGCGCTG
05	TAAACGCCGGTTGCTTCTCT	ATTGAAGATTTACGCGCGGC
06	CATAAGCCGGGAACCACAGT	CTGGCAGCAAATATCACCGC
07	GAGGCGATCACATCGTCCAT	AGAGAAGCAACCGGCGTTTA
08	AGGTCACTTCATGCACCAGG	ACGAGATCGCCACGGAATAC
09	TTGTGATCCATGGTGGCGAA	TATTGCCCGCATCGCATTTG
10	GGGCAATACGCTCGATCTCA	AAATACAGCCTGACCGCCAA
11	GCCGCAAAATTCCACCACAT	GCACCCGATATCGCAGGTAA
12	TTGTCCAGACCTTCCGCTTC	GGTCGTATGACCCTGTGCAA

Table 4.2 Primers used to clone the lac operon

4.5.4 Mouse infections

Eight-week-old female mice were anesthetized via inhalation of 4% isoflurane and infected transurethrally, as previously described in Mulvey *et al,* 1998. Bacteria cultures were grown in 2x24 conditions, described above, pelleted, resuspended in PBS, and subsequently concentrated or diluted in PBS to yield 50 µl innocula of 1 x 10^7 CFU Bl/6 mice or 1 x 10^8 CFU for C3H/HeN mice. All studies were approved of and performed in accordance with the guidelines set by the Committee for Animal Studies at Washington Univeristy School of Medicine, and the mice were housed in a facility with a 12 hour light/dark cycle where they had ad libitum access to standard food and water.

4.5.5 Antibiotic protection assays

Infected mouse bladders were aseptically removed at 24hpi, hemisected to allow access to the bladder lumen, and washed 3x in PBS to collect adherent extracellular bacteria. The PBS washes were pooled and spun at 500xg for two minutes to pellet any shed epithelial cells, after which bacterial loads of the wash were quantified. Simultaneously, the washed bladders were treated with 1 ug/ml of gentamycin in serum-free RPMI at 37°C for one hour to kill any remaining adherent extracellular bacteria while leaving potential intracellular bacteria intact. Treated bladders were then homogenized in PBS, and the homogenate was serially diluted and plated on LB-kan50 plates to enumerate any intracellular bacteria.

4.5.6 Processing bladders for IBC imaging

Infected animals were euthanized 3, 6, 12, 16, and 24 hours post infection, after which their bladders were harvested, hemisected, splayed, and pinned flat in silica wells. The splayed bladders were then fixed overnight at 4°C, quenched in 2M glycine to remove any bulky adducts, washed in PBS, and mounted on microscope slides in Prolong Gold Antifade with DAPI. The bladders were visualized using a confocal microscope (see below). Snapshots were then taken of five random fields of view for each mouse bladder, totaling 25 snapshots per strain.

4.5.7 Confocal microscopy

Samples were analyzed with a Zeiss LSM880 laser scanning confocal microscope (Carl Zeiss Inc. Thornwood, NY). The system is equipped with 405nm diode, 488nm Argon, 543nm HeNe, and 633nm HeNe lasers. Objectives used were: Plan-Apochromat 40X (NA 1.2) w korr objective, Plan-Apochromat 40X (NA 1.4) DIC objective, and Plan-Apochromat 63X (NA 1.4) DIC objective. ZEN black 2.1 SP3 software was used for image acquisition. All microscopy was performed at the Washington University in St. Louis Molecular Microbiology Imaging Facility.

4.5.8 Image analysis

Z-projections were made based on maximum intensity using FIJI software[220]. 6hpi IBCs were traced using the "Freehand selections" option of FIJI, and measured using the "Measure" tool. Out of focus IBCs were excluded to optimize measurement accuracy. IBC sizes were only quantified in pictures taken with the 40x objective, whose metadata set a default scale of 3.0769 pixels per micron. Statistical analysis was performed in Graphpad Prism.

4.5.9 X-gal beta galactosidase assays

UPEC were first grown in shaking conditions overnight at 37°C, in M9 media containing 0.2% glucose. To mitigate future sugar contamination of the growth media and to 'prime' the bacteria for growth curve assays, the strains were subsequently subcultured 1:1000 and grown shaking at 37°C in sugar-starved conditions (M9 + 0.04% glucose) for approximately five hours or until they entered log phase (as determined by OD600 measurements). The bacteria were then separated from the media and any residual sugar using 0.2 micron filters and pelleted at 12000xg for 2 minutes. The supernatant was separated from the pellet and the pellet was resuspended in 1mL of fresh M9 media to an OD of 1.0. X-gal and IPTG were then added to both the pellets and the supernatants for a final concentration of 0.2% and 1uM, respectively. The samples were then shaken in the dark at 37 degrees C for 24 hours.

Chapter 5: Conclusions & Future Directions

By Jennie Hazen

While uropathogenic *E. coli* (UPEC) and *A. baumannii* differ greatly in terms of gene carriage, phenotypic characteristics, and disease manifestation, they share an unexpected commonality. Neither species is commonly associated with intracellular infections; however, these phylogenetically disparate species can both invade bladder epithelial cells *in vivo*. The ability to get inside bladder cells has important implications for acute and recurrent infections as well as efficacious treatment options. In this body of work, I have analyzed multiple aspects of intracellular uropathogenesis across these two diverse species of bacteria. My findings raise several outstanding questions in the field and highlight current gaps in the technology needed to answer said questions. This chapter will highlight some of these knowledge gaps and speculate on potential future directions to address these questions.

I have phenotypically characterized the intracellular behavior of multiple diverse UPEC strains, assessing IBC cycle kinetics, IBC morphology, and invasive abilities **(Chapter 3).** While I did see some general trends – for example, all three phylogroup A UPEC strains were the least capable of invading bladder epithelial cells and forming IBCs – more strains are needed before proper statistical analyses can be conducted. However, my work does corroborate hypotheses proposed by previously published papers, that IBC formation is strain-specific and is not strongly tied to any specific gene carriage pattern[57,122]. Together, these works underscore the uniqueness of individual UPEC strains. Each strain's idiosyncrasies have the potential to greatly affect its behavior in established UTI models. For example, if one had no prior knowledge of the UPEC strain 5.3r's inability to form second-generation IBCs and they looked for IBCs in a mouse bladder 24hpi, they may assume that the strain cannot form IBCs at all. Thorough, methodical characterization of novel UPEC isolates should be performed before using said isolates in *in vivo* experiments, and existing methods should be optimized for the specific strain in use.

I have also identified a potential secondary pathway that UPEC uses to import lactose and lactose-like sugars; my experiments indicate that it may be possible that UPEC strains can use the GalP galactose-proton symporter to transport these sugars into the cell. Multiple, rigorous analyses are needed before any conclusions can be made. For example, the identity of the potential redundant lactose import protein or proteins cannot be determined until experiments with double mutant ΔlacYΔgalP strains are performed. While mutagenesis efforts are ongoing, the difficulties involved in generating a ΔlacY double mutant are well documented[252]. However, regardless of its true identity, the single-deletion X-gal assays **(Chapter 4)** strongly imply the existence of at least one secondary mechanism of lactose import beyond diffusion in at least two UPEC strains. While some Lactic Acid Bacteria and *Aspergillus* species have been known to utilize non-lac dependent pathways to import lactose-like sugars[270,271], to our knowledge, this phenotype has not been observed in pathogenic *E. coli* strains. Improved understanding of the metabolic activities of UPEC during uropathogenesis will answer many outstanding questions in the field. This knowledge, in addition to yielding information about the composition of the bladder environment, has the potential to yield novel drug targets and therapeutics.

Finally, I have demonstrated that *Acinetobacter baumannii* is capable of invading bladder epithelial cells and forming ABIRs during acute UTI **(Chapter 2)** The ABIRs act as sleeper agents within the mouse bladder, persisting long after the initial colonization event and clearance of infection until they are "activated" by an external signal – in my experiments, catheterization – whereupon they escape into the bladder lumen and cause a resurgent infection in the form of CAUTI. While this phenotype has been observed in *E. coli*, the concept of same-strain, secondary or resurgent infections are under investigated in the *A. baumannii* field. *Acinetobacter*, like many other opportunistic pathogens, has to this point been primarily considered a "one and

done" bacterium; if the host clears their infection, the pathogen does not linger. In the majority of case studies, subsequent infections are thought to occur independently of the previous one through re-contamination with an external source[274]. Similarly, *A. baumannii* healthcare-associated infections are thought to be caused by contamination in the hospital setting[73], a notion that is reinforced by several case studies wherein sterilizing the medical ward stopped a hospital epidemic[274]. Novel strains of *Acinetobacter* are regularly detected in healthcare facilities, but their origins are not always known. The accepted dogma is that novel strains are brought in from poorly sterilized equipment, shipments, etc. brought in from other hospitals or facilities, and many papers assert that the community is not and cannot be a potential source to introduce new strains[73,74]. However, my findings challenge that dogma. More clinical studies are required to test the hypothesis arising from my mouse model work that following a community-acquired infection, individuals may carry bacteria into a hospital from reservoirs within their own bladder walls that can then cause a CAUTI in that individual and seed the hospital environment.

5.1 Outstanding questions and future directions

Question 1: (A) Does the inability of phylogroup A strains 2.1a, 11.2p, and 12.3r apply to other phylogroup A strains? (B) How do these strains cause robust UTI in their source patients if they cannot form IBCs? (C) Why can't these three strains invade and/or persist within the urothelium?

While data from three strains is not enough to make sweeping generalizations about an entire phylogroup, it is certainly suggestive of a broader pattern that warrants further study. Even if phylogroup A strains 2.1a, 11.2p, and 11.3r are outliers within their group, and other "A" strains are robust IBC formers, the three strains are still worth studying. Strain 2.1a is particularly

interesting, as unlike the other two strains it can invade bladder epithelial cells; its status as a poor IBC former is more likely a result of its failure to thrive in the bladder cell environment. Further study of these three strains specifically, and of additional phylogroup A strains, has the potential to provide great insights into how bacteria survive within a bladder cell.

Future directions to address question 1A: Further characterization of diverse UPEC isolates

At present, the majority of strains used to study intracellular uropathogenesis are from clade B2; very few are clade A[57,122,275–278]. To determine whether the inability to successfully complete the IBC cycle is a trait shared among members of phylogroup A, it is first necessary to test more members of phylogroup A following the protocols established by Justice, Garofalo, and myself[122,163] **(See chapter 3)**.

Future directions to address question 1B: In-vivo experiments in the bladder-sensitized model, and in vitro co-invasion assays

UPEC strains that cannot successfully complete the IBC cycle generally do not mount significant infections in their hosts. However, poor IBC formers 2.1a and 11.3r caused UTIs robust enough that the women from whom the strains were isolated felt significant distress[251].

One explanation for these strains' surprising uropathogenicity is that the patients were predisposed to UTI due to a mucosal imprint from a previous bladder infection. Individuals who experience extreme inflammatory immune responses to infection undergo significant bladder remodeling **(For more information, see chapter 3)**. UPEC are capable of robustly colonizing sensitized bladders and can do so even without forming IBCs[37]. While patient number two (from whom investigators isolated strain 2.1a) had never experienced a UTI before, patient number eleven (the source of strains 11.2p and 11.3r) suffered from recurrent UTIs[251]. It is possible that

her bladder was sensitized, and that bacteria did not need to invade the urothelium to gain a foothold in her urinary tract. For future studies, I propose infecting sensitized mice with non-invading 11.2p and 11.3r to determine whether the strains can cause robust infection without entering the IBC cycle.

Alternatively, it is possible that the poor IBC formers were able to cause significant infections in their hosts because they were part of a multi-species infection. Every single one of the strains Garofalo *et al* determined were incapable of forming IBCs in a standard murine model of infection, were able to enter the urothelium and complete the IBC cycle when co-inoculated along with another IBC former[122]. Perhaps patients two and eleven suffered from UTIs caused by multiple species of bacteria, but only the UPEC strains could be recovered. I thus propose co-inoculating mice with the phylogroup A strains and UTI89 to determine if the poor IBC formers gain the ability to execute the IBC cycle in a mixed-infection model.

Future directions to address question 1C: RNAseq and in-vitro gain-of-function screens

Preliminary genetic analysis of the strains characterized in this work focused primarily on the carriage of specific putative virulence factors (PUFs). However, no pattern was found between PUF carriage and virulence[57]. Similarly, my characterizations failed to yield a pattern between PUF carriage and IBC phenotype **(see chapter 3)**. For example phylogroup A strain 2.1a has the same PUF carriage pattern as the robust IBC forming clade B1 isolate 56.1a[57]. The tenuous link between PUF carriage and IBC phenotype is further underscored by recent publications. Khetrapal *et al* demonstrated in November 2022 that the gene encoding heme receptor molecule ChuA is sufficient to confer the ability to form IBCs[227]. Phylogroup A strains by definition do not carry that gene[59]. However, phylogroup B1 strains also do not carry chuA but the group includes many robust IBC formers such as 56.1a and 41.4p[57,59]. These strains thus must utilize

alternative genetic pathways. I thus propose utilizing mass allelic exchange, the same method used by Khetrapal *et al*, to generate a hybrid UPEC library using IBC competent clade B1 strain 56.1a as the donor strain, and IBC incompetent clade A strains as the recipient strain. These hybrid A-B1 strains can be tested in a high throughput *in vitro* gain-of-function screen to determine what genetic elements confer the ability to successfully invade the urothelium and form intracellular aggregates[170].

While it is possible to perform gain-of-function genetic screens *in vitro* to determine what genetic elements underlie the IBC cycle[227], there is a lack of *in vitro* models that allow for in depth study of IBC maturation. Future investigations into the pathways underlying the IBC cycle must focus on gene regulation in addition to gene carriage. Assessing gene regulation of the IBC cycle, while a logical proposal, is a very complex task. Successful studies must properly isolate IBCs, process the genetic material, and analyze the data. These methods and future directions are discussed in further detail in the response to **question two**.

Question 2: How can we improve isolation, processing, sequencing, and other analyses of bacteria in the bladder cells to overcome the "mixed sample problem" and the inherently low biomass of intracellular bacteria?

The relative lack of knowledge about genetic determinants of intracellular uropathogenesis is due to several complications that make the processes difficult to study. One of the main complications is that multiple stringent bottleneck events can randomly select founder bacteria between initial infection and the end of the IBC cycle – infecting a mouse with one hundred million individual bacteria tends to yield only a few hundred mature IBCs. In *Acinetobacter,* infection with 10^8 bacteria results in less than 20 ABIRs **(See chapter 2)**. These few bacteria in

each case that "found" the initial intracellular population have been shown in each case to be the ones responsible for ongoing acute (in the case of UPEC) and resurgent (in the case of *Acinetobacter*) infections. Thus, even if every bacterium in an initial inoculate culture is capable of binding to and invading bladder epithelial cells, only a fraction of these bacteria might happen to make physical contact with an epithelial cell. Those that do must then specifically bind and invade into the epithelial cell. The rest are lost during micturition or are killed by infiltrating immune cells[166]. This means performing traditional genetic screens to find genes involved in the important intracellular populations would require a prohibitive number of mice to overcome the founder effects caused by these genetic bottlenecks. These restrictions are problematic, because the field currently lacks a reliable *in vitro* method to study IBCs. Some *in vitro* models, such as Eto *et al*'s model of infecting saponin-treated 5637 cells, can be used to determine a bacterium's overall potential to form intracellular aggregates *in vivo*[170]. However, none of the available models recapitulate the IBC cycle. It is thus necessary to take an alternative approach.

One alternative approach to elucidating the pathways that IBC development has been via the direct observation and determination of the bacterial gene regulatory and transcription state, specifically via the use of microarray analyses, qRT-PCR, and RNA-seq[58,238,279]. However, these methods come with their own set of hurdles including isolating IBCs from mouse bladders, isolating bacterial nucleic acids from the mouse genetic material, and preserving the DNA and RNA during the isolation process. For UPEC, advances have been made in the isolation of individual IBCs[58,279]. However, directly studying an IBC, or even a group of IBCs, has its own set of complications. First, the bacterial biomass of isolated intracellular bacteria is extremely low. One UPEC-formed IBC might have 10^4 bacteria in it. Even pools of 20 IBCs give DNA and RNA yields in the order of picograms. Sequencing technologies have advanced and are now

capable of processing these low levels of material, but the low biomass means that it is easy for meaningful signals to be drowned out by noise. This is ~1000x worse for ABIRs, since one ABIR might have up to five bacteria. Unfortunately, the second complication in analyzing IBCs and ABIRs directly is that the bacterial DNA and RNA is overwhelmed by the eukaryotic genetic material from the surrounding bladder epithelial cell. Over 99% of genetic material from an individual UPEC IBC sample comes from the mouse bladder cell.

Future directions to address question 2: method development and optimization

Over the past several years, I have optimized a method to isolate IBCs. This method, which is a modified version of a protocol developed by Duraiswamy *et al*, involves harvesting and inverting infected mouse bladders, visualizing IBCs under a fluorescence-capable dissection microscope, and physically scraping off IBCs using a pair of fine forceps[279]. To preserve the gene expression and protein occupancy states of the bacteria within the IBC, which begin to change soon after the death of the mouse[279], all IBCs are harvested within the span of five minutes. My modified technique differs from the original in that I have made several attempts to enrich for bacterial materials. Previous attempts involved lysing the surrounding bladder cell with triton and then using anti *E. coli* antibodies to pull the bacteria down. While this increased the proportion of bacterial RNA to eukaryotic RNA, it was not enough. However, in the future it may be beneficial to try a multi-pronged approach. During IBC isolation, enriching for the bacteria may be improved by supplementing the antibody pulldown with an additional bacteria-specific pulldown. For example, it may be beneficial to take advantage of UPEC's affinity for mannose and run the sample across a mannose-decorated surface. It may also be beneficial to generate mutant UPEC that express an affinity hook on their surface to facilitate positive enrichment.

Bacterial recovery may also be improved via negative selection and depletion of the mouse material. For example, following IBC isolation, the sample may further be enriched by depleting eukaryotic RNA, for example by poly-A degradation to get rid of Eukaryotic RNA. The bacteria may also be better separated from the host bladder cell using improved lysis methods. While I have tested a combination of detergents, sonication, and micro-bead homogenization, I have not fully optimized any of these methods to lyse the bladder epithelial cell.

Future experiments may also benefit from the use of hybrid capture technology and targeted sequencing.

Successful development of IBC/ABIR isolation and analysis methods is critical to answering key knowledge gaps in the field including gene regulatory patterns governing intracellular behavior.

Question 3: What are the genetic determinants underlying ABIR formation, persistence, and resurgence?

One cannot assume that mechanisms utilized by UPEC will be utilized in *A. baumannii*, primarily because IBCs and ABIRs are distinctly unique intracellular structures. Furthermore, *A. baumannii* and UPEC appear to utilize unique mechanisms to successfully invade and persist within the bladder epithelial cell. For example, while the lactose metabolism pathway is important for intracellular UPEC to persist within bladder epithelial cells, *A. baumannii*, is not a lactose fermenter[72,238,280].

Future Directions to address question 3: broad in vitro mutant screen and narrow, targeted mutagenesis.

Due to the extreme bottleneck that occurs between infecting a mouse and ABIR formation, *in vivo* screens of mutant libraries are currently financially and physically impractical. However, unpublished data from the Feldman lab indicate that ABIRs can form and persist within cell lines *in vitro*, namely in 5637 bladder epithelial cancer cells. Thus, a transposon library or other mutant library of *A. baumannii* can be used to infect bladder epithelial cells in a 96-well plate, and mutants which exhibit deficient or altered ABIR phenotypes can be assessed.

Choi *et al* observed via SEM that *A. baumannii* utilize "fimbrial-like structures" to invade bronchial epithelial cells, and it is well documented that other uropathogens utilize fimbriae to invade bladder epithelial cells[161]. Thus, the first obvious potential virulence factors are adhesive pili. The Csu pilus, the most common and well-characterized pilus across *A. baumannii* strains; can be ruled out, as UPAB1, which I showed forms ABIRs **(Chapter 2)**, does not carry it[24]. However, it is possible that CUP1 and/or CUP2, which are utilized by *A. baumannii* during CAUTI[24], are important in invasion or persistence. Thus, a more targeted mutagenesis approach can also yield valuable information about *A. baumannii* uropathogenesis.

Question 4: Can ABIRs in the lung foster recrudescence upon mechanical manipulation to cause pneumonia?

It is well documented that *A. baumannii* can invade and persist within bronchial epithelial cells as ABIRs (though they are not referred to as such)[159,161]. It is also well known that *A. baumannii* can be a frequent cause of respirator-associated pneumonia[72,281,282]. Similarly to catheter-induced bladder injuries, which simulate cell proliferation and inflammatory responses in the bladder, ventilator-induced lung injuries stimulate increased inflammatory responses and cell turnover in the lung[283–285]. Future studies should explore whether device-associated

pneumonia can arise as a result of ABIR activation within the lung, like how urethral catheterization can activate ABIRs in the bladder.

Future directions to address question 4: Optimize murine models of community-acquired and respirator-associated pneumonia to assess ABIR resurgence in the lung.

The most logical approach to study the role of ABIRs in the lung is to give mice community-acquired pneumonia, allow their infections to resolve, simulate ventilator-associated lung damage, and then assess the disease state of the mice.

Many murine models of bacterial pneumonia exist, and optimizing them for a resurgence-specific model should be relatively straightforward . However, while murine models of ventilator-induced lung injury (VILI) exist, careful consideration is required to optimize them[285,288–292]. In fact, a 2021 literature review pointed out that in a collection of ninety-nine papers that utilized a murine model of VILI, there was no single standardized method used in the field[288].

VILI can take several forms including: **i.)** general mechanical damage caused during endotracheal intubation[292,293]; **ii.)** volutrauma, wherein excessive air volume pumped into the lung causes overdistention and stretch injuries in the alveoli[294–296]; **iii.)** biotrauma, wherein the immune system's severe inflammatory responses to a ventilator cause damage to the lung tissue[283,296]; and **iv.)** atelectotrauma, wherein the collapse and re-expansion of alveoli generates sheer force which in turn causes physical damage[295,297]. Some murine models specifically mimic damage caused by endotracheal intubation, while others focus more on the ventilation aspect of respirator-induced damage[285,288–292]. However, it is possible that these different models of VILI may act differently upon ABIRs in the lung. Generally, in optimizing a murine VILI model, specific criteria must be met. American Thoracic Society Committee concluded in 2010 that at

least three of the four following conditions must be met in a successful *in vivo* model for any of these traumas: **1.)** histological evidence of tissue injury, as evidenced by neutrophil accumulation, hyaline membrane formation, the presence of debris, alveolar wall thickening, and other gross macroscopic changes to the tissue; **2.)** alveolar capillary barrier alteration, as evidenced by an increase in extravascular lung water content, an increase in total bronchoalveolar proteins, and an increase in lung wet/dry ratio; **3.)** presence of an inflammatory response, as evidenced by increased neutrophil presence, lung myeloperoxidase activity, and increased cytokine activity; and **4.)** evidence of physiological disfunction, as evidenced by hypoxemia, or increased alveolar-arterial oxygen difference[298].

Successful optimization of these models, while complex, is crucial to investigate the relationship between ABIRs and pneumonia. If a prior history of symptomatic or asymptomatic *Acinetobacter* colonization in the lung does predispose individuals towards contracting respiratory pneumonia via activation and resurgence of ABIRs, then screening methods can be developed to identify and proactively treat at-risk patients.

5.2 Final Thoughts

My dissertation provides new data on the intracellular step of uropathogenesis, particularly in the context of intracellular UPEC and intracellular *A. baumannii*. I have identified a functional host reservoir of *A. baumannii*, which can seed resurgent infections. These findings greatly expand the scope of what the field believes *Acinetobacter* species are capable of, and open the door to entire new avenues of research in both the UTI and the pneumonia fields. With *E. coli*, I have characterized the intracellular behavior of several clinical urinary UPEC isolates. These characterizations enrich the current body of knowledge in the field of intracellular

uropathogenesis and provides important information on several interesting and often-used strains. The strain-specificity of each isolate reenforces the necessity of characterizing a strain before putting it in a mouse model that was optimized for a prototypical UPEC isolate such as UTI89. Additionally, my results invite future investigation into the intracellular abilities of phylogroup A strains. Finally, my observations of lactose import in UPEC have the potential to inform future studies about the metabolism of uropathogens, specifically by suggesting the existence of a secondary import protein.

These exciting results ultimately serve as a reminder that even the smallest and most overlooked aspects of pathogenesis, in this case invasion and persistence of epithelial cells in the first few hours of a days-long UTI, are as exciting, dynamic, and deserving of study as other, more well-established pathogenic steps. Paying attention to these intracellular bacteria in the research setting has the potential to yield novel insights into uropathogenesis and potential therapeutic targets. Paying attention to these intracellular bacteria in the clinical setting has the potential to improve the detection, treatment, and clearance of complex infectious diseases.

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