Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis

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Urinary tract infections in young, healthy women frequently recur, despite their traditional classification as acute infections. Conventional wisdom dictates that uropathogens causing recurrent infections in such individuals come from the fecal or vaginal flora, in the same manner as the initial infection. However, recent studies of uropathogenic *Escherichia coli* have found that it can carry out a complex developmental program within the superficial epithelial cells of the mouse bladder, forming intracellular bacterial communities with many biofilm-like properties. These intracellular biofilms allow the bacteria to outlast a strong host immune response to establish a dormant reservoir of pathogens inside the bladder cells. Re-emergence of bacteria from this reservoir might be the source of recurrent infection.

Urinary tract infections (UTIs), considered among the most common of bacterial diseases, afflict a large proportion of the world population [1]. A majority of these infections occur in young, healthy women. In a large prospective study of young, sexually active women, the incidence of UTI was ~0.5 per person-year [2]. More recently, a population-based survey estimated that as many as 11 million women in the United States had at least one presumed UTI treated with an antibiotic in 1995, and that the cost for evaluation and management of UTIs was 1.6 billion dollars [1]. In this survey, the self-reported incidence of UTI in women 18 years and older was 10.8%, and the cumulative lifetime risk of UTI was 60%. Even episodes of acute, uncomplicated UTI are associated with considerable morbidity, including 6.1 days of symptoms, 2.4 days of restricted activity and 0.4 bed days [1]. Given that an estimated 130–175 million cases of UTI occur annually worldwide [3], the societal costs associated with UTI are huge. The most common isolates from patients are uropathogenic *Escherichia coli* (UPEC), accounting for ~80% of all acute, community-acquired UTIs [4]. Aside from UPEC, several other microorganisms can also cause UTI, most notably *Staphylococcus saprophyticus*, implicated in most of the remaining UTI cases [5]. Patients with complicating factors, such as diabetes, spinal cord injuries, urinary catheters, or hospitalization, can also present with UTI caused by *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, *Serratia marcescens* and group B streptococci [4]. Although traditionally thought of as acute and self-limiting, 27–44% of women with an initial UTI will experience at least one recurrence of symptoms within six months, despite antibiotic therapy [5,6]. Although these recurrent infections might occasionally be due to a persistent focus of infection, the majority have been thought to be reinfections caused by the initially infecting strain persisting in the fecal flora [7]. The bacteria associated with recurrent UTI often appear to be phenotypically or genetically identical to the bacterial strain that caused the initial infection, suggesting that selected *E. coli* strains might become uniquely adapted for colonizing and infecting their respective hosts [7]. The steady increase of antibiotic resistance [8] and occurrences of clonal outbreaks of UPEC-associated UTIs [9] highlight the need for greater understanding of the mechanisms of UPEC pathogenesis.

UPEC have proven to be quite stealthy in its pathogenesis of the urinary tract, using a complex pathway to mediate infection and persistence in the face of a strong innate host immune response. One of the most studied abilities of UPEC is binding host tissues and particular organ niches. UPEC accomplish this binding by assembling several different adhesive organelles on their surface, the most understood comprising P pili and type 1 pili [10]. These pili, assembled by the chaperone–usher pathway, display an adhesin molecule at their distal end [11]. P pili display the PapG adhesin, which has been shown to be required for pyelonephritic pathogenesis by binding to globoside present on human kidney cells [12]. By contrast, type 1 pili have been found to be vital for attachment to the bladder epithelium. The FimH adhesin on type 1 pili binds mannosse, and in the bladder FimH recognizes mannosylated residues present on uroplakin proteins that line the luminal surface of the bladder superficial epithelial cells [13]. FimH-mediated binding of the bladder epithelium is the initial step in an intricate cascade of events leading to
the myriad of symptoms associated with acute UTI and possible long-term residence of UPEC in the urinary tract.

**UPEC virulence determinants**

Other than type 1 and P pili, UPEC produce additional factors that influence disease progression (Table 1). Some of these virulence determinants are located on one of several UPEC specific pathogenicity-associated islands [14]. A recent genomic analysis of a UPEC strain revealed the presence of genes for ten putative chaperone–usher pilus systems, two putative type IV pili and at least seven putative autotransporter proteins [15]. The large repertoire of pilus systems might confer upon UPEC multiple binding specificities and the capacity to colonize various sites throughout the urinary tract and other environments. Autotransporter proteins can also have adhesive properties or they might fill other roles, such as toxins, proteases, invasins, serum resistance factors and motility mediators [16]. One UPEC specific autotransporter, Sat, appears to exert a toxic effect upon urinary tract cells in vitro [17]. Incubation of bladder and kidney cells with Sat-producing UPEC leads to extreme vacuolation in the cytoplasm of the host cells and possible loosening of cellular junctions. In a mouse model of UTI, Sat also induces cytoplasmatic vacuolation and severe histological damage in kidneys of infected laboratory animals [18]. Two other UPEC-expressed autotransporter proteins, Pic and Tsh, appear to have serine protease activity [19]. These two proteins are expressed during infection in laboratory mice and appear to be generally associated with pyelonephritis strains. UPEC also express an RTX (repeat in toxin) toxin called α-hemolysin (HlyA), often linked with the P pilus operon [20]. HlyA forms pores in a variety of host cell membranes and can induce calcium oscillations in proximal tubule cells of infected rat kidneys [21]. These fluctuations might serve as second messengers during immune activation of the host. Another toxin produced by UPEC, cytotoxic necrotizing factor 1 (CNF-1), influences the host cell cytoskeleton by targeting the Rho family of GTP-binding proteins [22]. CNF-1 has been shown to kill human bladder epithelial cells in vitro and inhibit phagocytosis of bacteria by human polymorphonuclear leukocytes (PMNs) [23]. In addition to proteases and toxins, UPEC produce several iron acquisition systems, including aerobactin [24] and the more recently described IroN system [25,26]. These iron siderophores scavenge available iron and enhance UPEC survival in the nutrient-limiting bladder environment. Finally, most UPEC strains produce an acidic polysaccharide capsule, which protects the bacteria from phagocytosis by human PMNs and inhibits activation of complement [24].

**Host response to UPEC infection**

The host counters the actions of UPEC upon the bladder by mounting a robust and dramatic response. Bladder superficial epithelial cells express Toll-like receptor 4 (TLR-4) on their membrane, which, along with CD14, recognizes lipopolysaccharide from the bacteria and activates an innate immune response [27,28]. A burst of inflammatory cytokines leads to a massive infiltration of neutrophils to fight the infection [29,30]. Further, increased transcription of inducible nitric oxide synthase by PMNs and the bladder epithelium results in high levels of nitric oxide and related breakdown products, which could have toxic effects on the bacteria [31,32]. Upon infection, the bladder epithelium is triggered to exfoliate superficial facet cells [33]. This aids clearance of any tissue-associated bacteria [34]. The underlying epithelium, normally extremely inert [35], rapidly undergoes a renewal process wherein underlying epithelial cells proliferate and differentiate to replace the superficial layer of cells [36]. Despite the defensive immune arsenal, rapidly undergoing renewal, UPEC are able to colonize the bladder and establish a persistent reservoir which can last at least several months after infection in laboratory mice [37]. Bacteria persist in the bladder notwithstanding antibiotic treatment [38], and, periodically, bacteria arising from this reservoir are shed in the urine of these mice. Human patients have also been shown to shed bacteria in the urine, even in the absence of active UTI symptoms [39,40]. Therefore, UPEC appear to be quite adept at establishing a protective niche for themselves in the bladder and persisting for extended periods of time.

**Intracellular bacterial communities**

Recently, it was discovered that UPEC activate a complex developmental cascade upon their entry into superficial bladder cells [41,42]. This cascade was elucidated using state of the art microscopy, including scanning and transmission electron microscopy, immunohistochemistry and time-lapse videomicroscopy. For videomicroscopy, mouse bladders infected with green fluorescence protein-expressing UPEC were stretched on an incubation chamber and placed on an epifluorescence microscope [42]. Images were captured every 30 seconds to two minutes, and multiple overlapping time frames were examined to piece together a timeline of UTI progression. Using these techniques, it was found that, in the early stages, the bacteria invade the superficial cells and rapidly divide. As the bacteria grow, dramatic phenotypic switches result in the establishment of intracellular bacterial communities (IBCs) (Figure 1). IBCs progress through several stages, culminating in the formation of biofilm-like communities inside the superficial cells. Eventually, bacteria detach from the biofilm and burst.

**Table 1. Virulence determinants of uropathogenic *Escherichia coli***

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<td>CNF-1</td>
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out into the bladder lumen. These escaped bacteria then rebind to the epithelium and initiate another round of IBC formation. The downstream effects of this cascade might permit UPEC to evade the host immune response and persist in the urinary tract.

**Binding and early IBC formation**

Type 1 pili, with the FimH adhesin at the distal tip, are crucial for UPEC attachment to the bladder epithelium [43]. Two lines of evidence confirm the vital role of FimH for bacterial attachment to the epithelium. First, isogenic fimH mutants are defective for binding and colonization of the bladder [33]. Second, polystyrene latex beads coated with FimH readily associate with, and are internalized by, human bladder epithelial cells in vitro [44]. FimH binds to mannosylated uroplakins on the bladder epithelium via a deep acidic pocket formed at the tip of the lectin-binding domain [13]. Four different uroplakin molecules associate into hexameric rings, which are arrayed in crystalline plaques on the luminal membrane of the bladder superficial epithelial cells. This uroplakin plaque provides a permeability barrier to prevent toxic molecules concentrated in the urine from diffusing into deeper tissues [35,45]. High resolution freeze-fracture electron microscopy has revealed that the tips of type 1 pili, which contain the adhesin, are buried in the central cavity of the uroplakin hexamers, tethering the bacteria to the epithelium [33]. It was recently shown that shear force enhances FimH-mediated binding [46], and such forces when encountered in the bladder might increase the ability of type 1 piliated organisms to attach and colonize the urinary tract.

After bacterial attachment, UPEC quickly invade the bound epithelial cell [44,47]. This key event occurs one to three hours after initial inoculation. It has been shown using tissue culture models that binding activates the Rho family of small GTP-binding proteins, specifically RhoA, Cdc42 and Rac1, which then activate focal adhesion kinase, phosphoinositide 3-kinase, α-actinin and vinculin. These processes result in localized actin rearrangements and membrane extensions around the bacteria [47]. The membrane engulfs the bacteria, leading to UPEC internalization via zipper-like phagocytosis.

Once intracellular, UPEC rapidly grow and divide, forming small clusters of bacteria, termed ‘early IBCs’ [42]. Early IBCs have previously been called ‘bacterial factories’ owing to the ability of UPEC to usurp the superficial bladder cells and convert them into factories for bacterial growth [37]. A major benefit that invasion and establishment of an intracellular community confers upon UPEC is protection from killing by antibiotics, which has been shown both in vitro and in vivo [37,38]. In this way, UPEC form a protective niche in the bladder where they can hide and survive. The bacteria within the early IBC divide rapidly during the first six to eight hours after inoculation, with a doubling time of ~30 to 35 minutes. The bacteria at this stage orient themselves randomly in the cytoplasm of the host cell, such that they form an amorphous, loosely associated bacterial clump [42]. During this period of quick expansion, UPEC maintain their typical rod morphology, with an average length of 3 μm. This fast growth leads to a surge of microorganisms within the bladder early in infection.

**IBC maturation and middle IBCs**

Between six to eight hours postinoculation, early IBCs experience a dramatic phenotypic switch [42]. A remarkable drop in bacterial growth rate, resulting in doubling times greater than 60 minutes, takes place concurrent with a significant shortening of bacterial morphology to an average 0.7 μm. At the same time, the amorphous clump appears to come together to form a tight, highly organized sphere inside the host cell. These events represent a maturation of the early IBC into a middle IBC stage with many biofilm-like properties [41].

Bacterial biofilms are often associated with long-term persistence of organisms in various environments. Biofilms are collections of bacteria that are attached to a surface, or to each other, and that display community behavior [48]. Initial events in biofilm formation involve binding of planktonic bacteria to a surface and formation of small bacterial clusters called microcolonies. As these microcolonies grow into mature biofilms, the bacteria differentiate, undergo dramatic phenotypic changes and encase themselves in an extracellular polysaccharide matrix [49,50]. These actions result in a large three-dimensional structure, with subpopulations of bacteria at different regions of this structure displaying altered gene transcriptional patterns [48]. It is thought that these subpopulations represent a division of labor, where each group performs certain specialized functions that benefit

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Figure 1. Middle intracellular bacterial communities (ICBs) are seen as ‘pods’ on the surface of infected mouse bladder epithelium. (a) Numerous pods cover the bladder surface in this scanning electron micrograph. Formation of biofilm-like pods protects the bacteria from immune clearance, thus allowing uropathogenic *Escherichia coli* (UPEC) to build up to extremely high levels in the bladder. Scale bar, 50 μm. (b) Magnification of a pod reveals a smooth shell which is the uroplakin-coated luminal membrane of the bladder superficial epithelial cell. The pod consists of one cell that is completely filled with intracellular bacteria. Scale bar, 5 μm. (c) An IBC is visible inside the bladder epithelium by fluorescent microscopy. Scale bar, 20 μm. The green fluorescence protein-expressing bacteria form a dense sphere within the bladder cell. IBC progression can be monitored by time-lapse videomicroscopy, in which time-lapse images are captured by epifluorescence microscopy [42].
the whole community [51]. As the biofilm ages, a subset of bacteria detach from the biofilm and revert to a planktonic state, which allows for spread of organisms to other niches [48]. A biofilm lifestyle benefits the bacteria by providing protection from environmental insults and changes [49]. Bacteria in biofilms display dramatically increased resistance to antibiotics because of the inability of antibiotics to penetrate the biofilm matrix, the slow growth-rate of the bacteria in the biofilm, physiological changes in the bacteria and biofilm-state expression of factors that directly inhibit antibiotic activity [48,52]. In addition, biofilms confer increased resistance to host immune effects upon their constituent bacteria by concealing the bacteria inside the biofilm matrix. For instance, bacteria in biofilms are protected from opsonizing antibodies [48]. Phenotypic variation and differentiation might also enhance bacterial resistance to PMNs and immune effector molecules [48]. Antibiotic and immune resistances facilitate bacterial biofilm survival in vivo during many disease conditions, including native valve endocarditis, otitis media, chronic bacterial prostatitis, periodontitis and cystic fibrosis. Bacterial biofilms have also been described on inert medical devices, such as prosthetic heart valves, central venous catheters, contact lenses, intruterine devices, urinary catheters and dental unit water lines [48]. The presence of biofilms in disease or on medical devices leads to chronic symptoms, owing to persistence of bacteria, despite antimicrobial therapy.

An important factor in *E. coli* biofilm formation is type 1 pili, which aids stable attachment of the bacteria to a surface [53]. Flagella also assist biofilm formation by mediating initial weak surface contact and influencing expansion of biofilms across a surface [53]. Antigen 43, an outer membrane autotransporter protein, promotes auto-aggregation of *E. coli* and can assist interbacterial interactions during microcolony formation [54]. This might also be the function of secreted amyloid fibers called curli [55,56]. The regulator of the curli operon, CsgD, might also regulate several metabolic and other factors that influence formation of mature biofilms [57]. A key process during biofilm maturation, following attachment and microcolony formation, is secretion of colanic acid, an exopolysaccharide that creates a polysaccharide matrix consisting of a complex three-dimensional architecture [58].

The incredible organization and structure produced by UPEC in the middle IBC are highly reminiscent of bacterial biofilms [49]. As middle IBCs grow, the bacterial mass pushes against the epithelial cell membrane, creating a podlike protrusion on the surface of infected bladder epithelium (Figure 1) [41]. Pods correspond to a middle IBC. The bacteria in the middle IBC, although free in the cytoplasm, stay together as a spherical community, filling up much of the intracellular volume of a superficial umbrella cell. The surface of the pod is the luminal membrane of the bladder cell, which is stretched tight, appearing smooth by scanning electron microscopy, and is coated with an impermeable shell of uroplakin. The bacteria inside the pod are highly organized and surrounded by numerous fibers emanating from the bacterial surface. These fibers make extensive contacts with the surrounding matrix, creating individualized compartments for each bacterium that establish a spatial orientation of microorganisms inside the IBC. UPEC express several factors in the middle IBC which are important for in vitro biofilm formation, including type 1 pili and antigen 43 [41]. Similar to biofilms, expression of these factors is heterogeneous, with the bacteria setting up subpopulations with differential gene expression. Polysaccharides are also present throughout the middle IBC.

By differentiating into an intracellular biofilm, UPEC construct a safe haven where they can hide and wait out the tide of the host immune attack. PMNs recruited to the bladder are targeted to the IBCs and attempt to clear the bacteria [29,42]. However, the IBC remains largely unaffected. The uroplakin shell covering the IBC appears to protect UPEC by denying the PMNs access to the bacteria. The structural organization of the pod might also protect the bacteria from antibiotic susceptibility. The matrix surrounding the bacteria inside the cell might also add an additional layer of protection from antibiotics and the immune system. It is interesting that in vitro biofilm-state UPEC have been found to be resistant, even when antibiotics reach the bacteria [59], suggesting that the physiological state of the microbes in biofilms enhances bacterial survival. In this manner, UPEC are able to replicate to large numbers, unperturbed by the host and other insults.

**Late IBCs and fluxing**

Eventually, the IBC progresses to a late stage [42]. Starting around 12 hours postinoculation, bacteria on the edge of the intracellular biofilm detach from the group and differentiate into a typical rod morphology, with an average length of 2 μm. At this stage of the maturation cycle, the bacteria become highly motile. These motile bacteria swim toward the edge of the cell, burst out into the bladder lumen in a process termed ‘fluxing’ and spread out over the epithelium. Fluxing bacteria have also been observed erupting en masse from the epithelial cell, the entire community simultaneously emerging from its intracellular hiding place [37]. It is possible that fluxing initiates with a few bacteria, and as the host cell membrane integrity decreases, some signal leads to the concomitant bursting of the entire IBC. From the volume of mature IBCs and the size of the bacteria, it is estimated that an average IBC contains in the order of 10⁶ organisms. Fluxing of multiple IBCs, therefore, might result in the release of hundreds of thousands or millions of UPEC back into the bladder lumen, leading to spread within the bladder as well as dissemination back into the environment [42]. Escaped bacteria often filament, reaching up to 70 μm or greater in length [37,42]. Although the mechanisms and purpose behind filamentation remain unclear, PMNs appear to have little effect upon the filaments [42]. This allows UPEC to survive long enough in the inflamed bladder to reattach to the epithelium.

**UPEC persistence in the bladder**

A second round of IBC formation occurs as escaped bacteria rebind and reinvade the superficial cells [42].
However, this second round takes much longer to progress, with doubling times in the early IBC of greater than 60 minutes. Multiple IBC cycles probably occur but after several days, only a quiescent reservoir of small intracellular bacterial clusters (doublets, quadruplets, small groups) remains in the bladder. The bladder epithelium is intact at this stage, with the bacteria residing inside superficial cells which appear smaller than normal. These smaller superficial cells might result from growth and differentiation of the underlying epithelial cells following exfoliation of the original superficial cells. As infection progresses, a greater number of fluxed UPEC might bind and invade the underlying epithelium, exposed during exfoliation. Physiological differences between the superficial and underlying cells influence whether IBCs form. Indeed, IBCs have never been seen within the underlying bladder cells. During this reservoir stage, no apparent actions are taken either by the host or the bacteria, and UPEC can persist in this state in the bladder for months after an initial infection [37,38]. Periodically during this persistent stretch, large numbers of organisms are seen in the urine [38]. It is thought that reactivation of UPEC from their dormant reservoir state, and subsequent multiplication inside the bladder epithelium to re-form IBCs, lead to this bacterial spike in the urine.

Clinical impact

The discovery of the IBC maturation cascade suggests that UPEC possess a program that enables the bacteria to evade early innate immune defenses. Considering that extracellular bacteria are readily cleared by PMNs [42], the formation of intracellular communities of bacteria inside the superficial cells of the bladder appears key to the survival of UPEC in the urinary tract during the acute phases of infection (Figure 2). IBCs appear to permit UPEC to survive the immune onslaught that occurs at these early stages. In this haven, the bacteria can safely multiply, creating an overpowering legion which then escapes into the bladder lumen. Presumably, the sheer numbers of bacteria that escape overwhelm the immune system, leading to several potential downstream effects: transmission back into the environment, subsequent IBC cycles and establishment of a chronic bacterial reservoir. Alternatively, formation of the reservoir might occur through an entirely separate route. Continuing studies will provide answers to this and other fundamental questions relating to UPEC pathogenesis and persistence (Box 1). The elucidation of the IBC program was carried out using a mouse model of infection. However, UPEC also bind, invade and replicate inside human epithelial cells in vitro [37,44,47], and it will be vital for future studies to establish whether IBCs and quiescent reservoirs of bacteria form in human patients. As more is understood about the factors that are expressed during UTI, and their regulation, it will be essential to determine their role in the IBC program. Such knowledge will provide a comprehensive understanding of UTI progression and lead to new clues about how to abrogate UPEC infection. Further, because many different microorganisms invade host tissues and multiply inside host cells, it is possible that an IBC-like developmental program is important during various stages of other chronic recurrent infections. A greater understanding of the nature of intracellular bacterial communities in chronic or recurrent infections will aid the development of new and more effective treatments for these problematic diseases.

Box 1. Intriguing questions for future study

- Do intracellular bacterial communities (IBCs) form in human patients with cystitis?
- What genetic mechanisms do uropathogenic Escherichia coli (UPEC) use to form IBCs?
  - What accounts for polymorphonuclear leukocyte resistance by the filaments?
- Why do IBCs form only in the superficial epithelial cells of the bladder?
- What induces UPEC to become quiescent?
- Can stimulation of the dormant reservoir lead to a urinary tract infection recurrence?
- What signals activate the dormant reservoir?
- How and when do known virulence determinants influence the IBC cascade?
- What bacterial and/or host signals regulate IBC processes?
- How do IBCs relate to the chronic phase of infection?
- Are IBCs formed in other chronic recurrent infections?
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