Role of Bacterial Biofilms in Urinary Tract Infections

J. K. Hatt and P. N. Rather(✉)

Abstract Bacterial urinary tract infections represent the most common type of nosocomial infection. In many cases, the ability of bacteria to both establish and maintain these infections is directly related to biofilm formation on indwelling devices or within the urinary tract itself. This chapter will focus on the role of biofilm formation in urinary tract infections with an emphasis on Gram-negative bacteria. The clinical implications of biofilm formation will be presented along with potential strategies for prevention. In addition, the role of specific pathogen-encoded functions in biofilm development will be discussed.

P. N. Rather
Department of Microbiology and Immunology, Emory University School of Medicine 3001 Rollins Research Center, Atlanta, GA, USA
prather@emory.edu

T. Romeo (ed.), *Bacterial Biofilms.*
Current Topics in Microbiology and Immunology 322.

1 Introduction

Urinary tract infections (UTIs) represent the most commonly acquired bacterial infection. These infections are prevalent in both outpatient and hospital populations and are responsible for an estimated seven million office visits, one million emergency room visits and 100,000 hospitalizations annually (Foxman 2003). Urinary tract infections account for an estimated 25%-40% of nosocomial infections and represent the most common type of these infections (Bagshaw and Laupland 2006; Foxman 2003; Kalsi et al. 2003; Maki and Tambyah 2001; Wagenlehner and Naber 2006). The annual healthcare costs of urinary tract infections are estimated at $1.6 billion per year (Foxman 2003).

The risk of developing a urinary tract infection increases significantly with the use of indwelling devices such as catheters and urethral stents/sphincters. Indwelling catheters are the primary contributing factor in the development of these infections. The use of catheters to manage urinary incontinence in nursing home and spinal cord injury patients makes these populations especially vulnerable to these infections. Remarkably, the risk of developing catheter-associated urinary tract infections increases 5% with each day of catheterization and virtually all patients are colonized by day 30 (Maki and Tambyah 2001). The ability of various bacteria to form biofilms on catheters is well documented and will be expanded on later in this chapter. For all these indwelling devices, a number of studies support the role of biofilms in the establishment of infection (Reid et al. 1992; Choog and Whitfield 2000; Silverstein and Donatucci 2003; Trautner et al. 2004; Tenke et al. 2006).

The predominant pathogens in urinary tract infections are *Escherichia coli* (25%), followed by *Enterococci* (16%), *Pseudomonas aeruginosa* (11%), *Klebsiella pneumoniae* (8%), *Candida albicans* (8%), *Enterobacter* (5%), *Proteus mirabilis* (5%), and coagulase-negative *Staphylococci* (4%) (Emori and Gaynes 1993) (Table 1). These pathogens are typically found in the lower intestinal tract and can be introduced into the urinary tract via contaminated indwelling devices.

This chapter will focus on the role of bacterial biofilms in the development of urinary tract infections. In the first section, we will discuss the role of biofilm

Table 1 Frequency of urinary tract infections by various pathogens

Organism	Percentage of infections
E. coli	25
Enterococcus spp.	16
P. aeruginosa	11
K. pneumoniae	8
C. albicans	8
Enterobacter spp.	5
P. mirabilis	5
Coagulase–negative *Staphylococci*	4

From Emori and Gaynes 2003

formation on indwelling devices with an emphasis on crystalline biofilms formed by urease-producing organisms. Next, the role of selected, uropathogen-specific factors that contribute to biofilm formation will be discussed. In the final section, the role of biofilms on, or in, host urinary tissues will be addressed.

2 General Aspects of Biofilm Formation on Indwelling Urinary Tract Devices

A common prerequisite to initial biofilm formation on indwelling urinary devices is the formation of a conditioning film from urinary components (polysaccharides and proteins) (Reid 1999; Trautner and Darouiche 2004; Tenke et al. 2006; Donlan 2001). This conditioning film facilitates the initial attachment of microorganisms, which normally adhere poorly to uncoated surfaces. The subsequent attachment of microorganisms and biofilm development is similar to that described elsewhere in this book and will not be described further. A variety of indwelling devices are commonly used in the urological setting, including open and closed urinary catheters, urethral stents and sphincters, and penile prostheses. For each type of device, biofilm formation has been documented from infection sites (Reid 1992; Nickel et al. 1993; Stickler et al. 1998; Morris et al. 1999; Choog and Whitfield 2000; Tenke et al. 2006). Of these devices, catheters are the primary culprit in the development of urinary tract infections (Morris et al. 1999; Stickler et al. 1998; Trautner and Darouiche 2004). Early studies documented that a catheter removed from a patient with recalcitrant urosepsis, who failed antibiotic therapy, contained a thick biofilm adherent to the catheter (Nickel et al. 1985). This work was followed up by additional studies that documented extensive biofilm formation on urinary catheters by scanning electron microscopy (Ohkawa et al. 1990), which primarily included studies of catheters from patients that failed antibiotic therapy (Nickel et al. 1989). A variety of Gram-negative bacteria were colonizing the catheters including *P. aeruginosa*, *Enterococcus faecalis*, *E. coli* and *P. mirabilis*, and these same organisms were isolated from infected urine (Nickel et al. 1989). Interestingly, this early study revealed the association of crystalline deposits with *P. mirabilis* biofilms and laid the groundwork for a large number of subsequent studies that revealed the relationship between urease production and crystalline biofilms on catheters.

3 Biofilm Formation by Urease-Producing Organisms

3.1 Crystalline Biofilms

Indwelling Foley catheters are used extensively to manage urinary incontinence in elderly patients or those with bladder dysfunction, such as spinal cord injury patients. However, these devices place a patient at high risk for the development of

urinary tract infections. A unique type of crystalline biofilm can form on catheters by urease producing organisms, such as members of the Proteeae (*P. mirabilis*, *Providencia stuartii*, *Morganella morganii*) and *K. pneumoniae* (Stickler et al. 1993, 1998). The production of urease by these organisms results in the cleavage of urea that occurs at concentrations of 0.4-0.5 M in urine (Li et al. 2002). The ammonia that is generated by urease activity raises the pH of the urine resulting in calcium and magnesium phosphate crystal formation within the biofilm matrix (Stickler et al. 1993, 1998). Studies by Nickel (1987) have demonstrated that biofilm development is a prerequisite for crystal formation as the matrix may act as a nucleation site for crystal development, and the higher concentration of cells in the biofilm allows for a greater localized concentration of urease. It has been proposed that crystalline biofilm formation is a multistep process as follows:

1. Introduction of a urease producing organism
2. Formation of a conditioning film on the surface of the catheter
3. Bacterial adherence to the catheter
4. Biofilm development and production of exopolysaccharides
5. Elevation of pH within the urine and biofilm by urease production
6. Crystallization of calcium and magnesium phosphate within the biofilm matrix (Morris et al. 1997)

Using a laboratory model for a catheterized bladder, Stickler and colleagues have demonstrated that with *P. mirabilis*, the development of biofilms on a catheter surface generally begins near the eye-hole with microcolonies forming at this site (Stickler et al. 2003b). Calcium and magnesium phosphate crystals begin to form and the biofilm then extends down the lumenal surface. Eventually the encrustations will block the catheter. This leads to bladder distension and urine leakage, or more serious complications such as pyelonephritis when urine from the distended bladder is refluxed into the kidney. In addition, crystalline biofilms that form on the outside of the catheter can cause irritation and trauma to the mucosa of the urethra. *P. mirabilis* appears to be the predominant organism in encrusted biofilms (Stickler et al. 1993) and appears to be the most effective organism at producing crystalline biofilms using in vitro models (Stickler et al. 1998). This is likely due to the fact that the *P. mirabilis* urease is six- to tenfold more active than other bacterial ureases (Tenke et al. 2006).

On solid surfaces, *P. mirabilis* undergoes a unique form of migration termed swarming. This process requires a complex cellular differentiation from a short vegetative rod to an elongated swarmer cell, reviewed in Rather 2005. The expression of urease in *P. mirabilis* is increased 30- to 50-fold in differentiated swarmer cells (Allison et al. 1992). However, although *P. mirabilis* is clearly capable of swarming on catheter surfaces (Sabbuba et al. 2002; Stickler et al. 1999), swarmer cell differentiation does not appear to be required for crystalline biofilm formation. Studies by Jones et al. (2005) examined a panel of *P. mirabilis* mutants and found that strains defective in swarming were actually more proficient at biofilm formation. The basis for this inverse relationship is unclear in *P. mirabilis*, but similar

findings have been reported in *Salmonella typhimurium* and involve decreased surfactin production (Mireles et al. 2001).

3.2 Control of Crystalline Biofilms

At the current time, all types of urinary catheters are subject to encrustation (Morris et al. 1997). A number of strategies have been used in an attempt to decrease crystalline biofilm formation on catheters, most of which have had limited success. In artificial bladder models, catheter material composed of silicone was blocked less effectively than silver gel-coated latex catheters (Morris et al. 1997). However, all materials tested were eventually encrusted. The use of chlorhexidine as an antiseptic was not effective at reducing Gram-negative infections and selected for resistant isolates that were also multidrug-resistant (Stickler 2002). In laboratory models, the use of triclosan to disinfect the retention balloon of catheters reduced crystalline biofilms significantly and may have utility in clinical settings (Stickler et al. 2003). An additional strategy, the use of silver impregnated catheters has also given mixed results and may have limited utility (Rosch et al. 1999) The direct role of urease and alkaline urine in crystalline biofilms has prompted studies to examine the effect of acidic drinks such as cranberry juice on crystalline biofilm formation; however, studies in catheterized patients revealed no difference in patients that had ingested this liquid (Morris and Stickler 2001). A separate strategy involves decreasing urine pH by the use of urease inhibitors. Using in vitro models, the addition of acetohydroxamic acid or flurofamide restricted the increase in urine pH in the presence of *P. mirabilis* and led to reduced deposits of calcium and magnesium salts on the catheters (Morris and Stickler 1998). At the present time, the utility of these treatments in a clinical setting has not been determined, but they offer promising options for the control of crystalline biofilms.

Recently, a more general strategy to control biofilm formation on urinary catheters has been described by Burton et al. and is based on the inhibition of GlmU, an *N*-acetyl-D-glucosamine-1-phosphate acetyltransferase (Burton et al. 2006). This enzyme synthesizes the activated nucleotide sugar UDP-GlcNAc, a precursor for synthesis of β-1,6-*N*-acetyl-D-glucosamine polysaccharide adhesin that is required for biofilm formation in *E. coli* and *Staphylococcus epidermidis*. Inhibitors of GlmU, such as iodoacetamide (IDA) and *N*-ethyl maleimide (NEM) inhibited *E. coli* biofilm formation. Moreover, derivatives of NEM such as *N*-phenyl malemide (NPM), *N.N*'-(1,2phenylene) dialeimide (oPDM), and *N*-(1-prenyl) malemide (PyrM) were effective at inhibiting biofilm formation by *P. aeruginosa*, *K. pneumoniae*, *S. epidermidis*, and *E. faecalis* (Burton et al. 2006). The addition of protamine sulfate to oPDM enhanced anti-biofilm activity and catheters coated with both compounds were virtually free of bacterial colonization (Burton et al. 2006). The promising combination of oPDM plus PS may have broad utility in the prevention of bacterial colonization of urinary catheters and this strategy may be

applicable to other indwelling medical devices colonized by organisms that require the β-1,6-*N*-acetyl-D-glucosamine adhesion for biofilm formation.

3.3 Infectious Urinary Stones

Approximately 15% of urinary stones are initiated by infections (Bichler et al. 2002). These stones can form in the bladder or kidney and are often associated with abnormalities of the urinary tract or obstructions (Bichler et al. 2002; Abrahams and Stoller 2003). These stones are composed of struvite (magnesium ammonium phosphate) or apatite (calcium phosphate). The formation of these stones is strongly correlated with urease-producing bacteria and for the purposes of this chapter, these stones will be considered a free-floating crystallized biofilm. In fact, bladder and kidney stones are remarkably similar to the crystals that form on catheters (Griffith et al. 1976). The first step in production of these stones was revealed from studies by Griffin et al. who established the key role of urease in the formation of these stones (Griffith et al. 1976). These in vitro studies demonstrated that the expression of bacterial urease increased the pH of urine, leading to the formation of calcium and magnesium crystals, a precursor to stone formation. Urine that was urea-free or contained urease inhibitors did not support crystal formation. McLean et al. proposed that the next step in stone formation is the interaction of crystals with bacterial exopolysaccharides (McLean et al. 1989). The O-antigen of LPS can be acidic due to uronic acid and this acidic nature can facilitate the binding of Ca^{2+} and Mg^{2+} (Knirel et al. 2003; Clapham et al. 1990; Dumanski et al. 1994). Studies by both Torzewski and co-workers and Dumanski et al. have extended these findings and found that the sugar composition of *P. mirabilis* lipopolysaccharide can influence the crystallization process (Torzewska et al. 2003; Dumanski et al. 1994). However, a paradox of these studies is that strains with the strongest cation binding supported the poorest crystal growth. It is proposed that the weaker binding is enough for concentration, but facilitates the release for crystal growth (Dumanski et al. 1994). Stone formation is facilitated by additional bacterial growth or interactions between stones, and the stone eventually comprises a matrix with imbedded bacteria (Nickel et al. 1987; McLean et al. 1989; Li et al. 2002; Takeuchi et al. 1984). Studies by Li et al. used *P. mirabilis* cells constitutively expressing green fluorescent protein (GFP) to visualize cells within urinary stones in a mouse model of urinary tract infection. Further examination by electron microscopy revealed that stones were compact in the core and had a loose structure in the outer layers (Li et al. 2002). Within the stone matrix, vegetative cells were the predominant type with some swarmer cells evident. The majority of bacteria resided in the outer layers of the stone. It was proposed that this location allows access to nutrients, yet may confer protection from immune responses.

A second possible mechanism of urinary stone formation involves nanobacteria (Kajander and Ciftcioglu, 1998). These organisms were capable of forming carbonite apatite at pH 7.4 in the absence of urease activity. In a survey of human kidney

stones, nanobacteria were found in all stones tested ($n = 30$). Therefore, stone formation can be initiated by urease-producing organisms and by urease-independent mechanisms at neutral pH involving nanobacteria.

4 *Escherichia coli* Factors Involved in UTI Biofilm Development

4.1 Type 1 pili in UPEC Pathogenesis and Intracellular Biofilm Formation

Uropathogenic *Escherichia coli* (UPEC) strains are the most common causes of urinary tract infections (UTIs) (Hooton and Stamm 1997). Moreover, UPEC biofilms are responsible for many catheter-associated and chronic UTIs (Nicolle 2005). UPEC strains can vary greatly in their ability to cause UTIs. This is most likely due to the different repertoire of virulence factors associated with each UPEC strain (Foxman et al. 1995; Johnson et al. 1998; Marrs et al. 2005). Virulence factors described for UPEC include α-hemolysin, cytotoxic necrotizing factor 1 (cnf1), lipopolysaccharide (LPS), capsule, the siderophores aerobactin and enterobactin, proteases, and a number of adhesive organelles (Johnson 1991; Oelschlaeger et al. 2002). However, no single virulence factor has been identified that is specific to or definitive of UPEC. Despite this fact and despite their presence in a majority of wild type *E. coli* strains (Hagberg et al. 1981; Langermann et al. 1997), perhaps the single most important virulence factor of UPEC is the expression of type 1 pili, which are key factors that are involved in the formation of UPEC biofilms in living tissue and on abiotic surfaces. This is due to the contribution of type 1 pili to adherence to, invasion of, and persistence within the bladder.

4.1.1 The *fim* Gene Cluster

Type 1 pili are peritrichously expressed proteinaceous cell surface structures found on many members of the *Enterobacteriaceae*, including the majority of both commensal and pathogenic strains of *E. coli* (Johnson 1991; Yamamoto et al. 1995). Type 1 pili of *E. coli* are thick rod-shaped composite structures approximately 7 nm wide and 1 μm in length (Brinton 1965). In *E. coli*, the nine genes of the *fim* gene cluster encode the structural and regulatory proteins that produce type 1 pili or fimbria. The *fimAFGH* genes are structural genes encoding the major and minor protein components of the pilus rod and tip fibrillum (Brinton 1965). The pilus is composed primarily of multiple copies of the FimA major subunit protein organized in a right-handed helical rod, the FimF adaptor protein, and the tip fibrillum composed of the minor protein component FimG and multiple copies of the mannose-specific adhesin FimH (Brinton 1965; Klemm and Christiansen 1987; Russell and Orndorff

1992; Jones et al. 1995). It has also been suggested that the FimH adhesin may be interspersed along the pilus rod (Krogfelt et al. 1990). The assembly of the pilus itself is dependent on the *fimC* and *fimD* genes that encode the periplasmic chaperone and the outer membrane usher proteins, respectively. These proteins mediate the translocation of the pilus structural components across the outer cell membrane during which time the pilus is assembled (Klemm and Christiansen 1990; Jones et al. 1993). The function of the *fimI* gene is as yet unknown; however, it has been implicated in pilus biogenesis (Valenski et al. 2003).

The final two genes of the *fim* gene cluster *fimB* and *fimE* encode regulatory proteins that control the phase variation of type 1 pili (Klemm 1986; Gally et al. 1996). Phase variation refers to the reversible on/off switch controlling the expression of type 1 pili in *E. coli*. Expression of type 1 pili varies as a result of the reversible inversion by site-specific recombination of a 314-bp regulatory element containing the promoter of the *fimA* gene (Abraham et al. 1985). The FimB and FimE proteins that function as site-specific recombinases mediate this DNA inversion (Klemm 1986; Gally et al. 1996). The FimB protein can turn the switch to both the on and off positions; however, FimE is specific for the switch from on to off (McClain et al. 1991; Gally et al. 1996). The importance of phase variation of type 1 pili in initiating infection of the bladder by UPEC has been well characterized using a murine model of ascending UTI (Hultgren et al. 1985; Schaeffer et al. 1987; Connell et al. 1996; Struve and Krogfelt 1999; Gunther et al. 2001; Bahrani-Mougeot et al. 2002; Gunther et al. 2002; Snyder et al. 2006). Type 1 expression was shown to be critical for infection of the lower urinary tract and strict control of phase variation was required for successful UPEC infection.

4.1.2 The FimH Adhesin

The FimH adhesin confers mannose-specific binding activity to type 1 pili (Abraham et al. 1987; Maurer and Orndorff 1987). FimH recognizes the terminal mannose moieties on many cell types and secreted glycoproteins, which include superficial bladder umbrella cells (Eden and Hansson 1978; Zhou et al. 2001; Duncan et al. 2004) and CD48 on mast cells and macrophages (Baorto et al. 1997; Malaviya et al. 1999; Shin et al. 2000). Colonization of the murine bladder requires the presence of FimH, and immunization with FimH was shown to protect against colonization and infection by UPEC in both murine and primate models of UTI (Langermann et al. 1997, 2000). Scanning electron microscopy (SEM) and high-resolution transmission electron microscopy (TEM) revealed that type 1 pili are in intimate contact with the uroplakin-coated superficial bladder epithelium via the tips of type 1 pili directly contacting the uroplakin-coated membrane, termed the asymmetric unit membrane (Mulvey et al. 1998). Uroplakins are proteins that cover the apical surface of superficial umbrella cells and are organized in hexagonal plaques that give strength to the bladder epithelium to help create a permeability barrier (Sun et al. 1996). Although earlier studies implicated the uroplakins UP1a and UP1b as targets for FimH binding

(Wu et al. 1996), recent in vitro studies using mouse uroepithelial plaques and purified recombinant FimH in a FimC-FimH complex showed unambiguously that uroplakin UP1a is the unique bacterial receptor for FimH adhesin binding to uroepithelial cells (Zhou et al. 2001).

Most commensal and pathogenic strains of *E. coli* with type 1 pili bind trimannose receptors via the FimH adhesin (Sokurenko et al. 1995). However, in about 70% of UPEC strains, higher tropism for the uroepithelium is conferred by the ability of FimH variants in these strains to bind monomannose as well as trimannose receptors (Sokurenko et al. 1995). Monomannose receptors are abundant on the uroplakin UP1a that coats the apical surface of superficial umbrella cells (Zhou et al. 2001). All UPEC strains with type 1 pili bind trimannose receptors with high affinity; however, affinities for monomannose binding by the FimH adhesin can vary greatly in these strains. FimH with low affinity for monomannose binding are found among fecal *E. coli* isolates, but naturally occurring variants with high affinity are most often found in UPEC strains (Sokurenko et al. 1995, 1997, 1998; Zhou et al. 2001). This ability to bind monomannose with high affinity is thought to provide a selective advantage during pathogenesis by increasing binding affinity specifically for the uroepithelium. Interestingly, an epidemiological study of a large panel of commensal and pathogenic *E. coli* strains indicated that UPEC isolates also have the highest frequency of mutator strains as compared to commensal strains and other pathogenic strain types (Denamur et al. 2002). No evidence was found to suggest that growth advantage in urine or increased antibiotic resistance provided a selective advantage that resulted in higher numbers of mutators among the UPEC strains. Although no difference was observed for the ability of a UPEC strain and its isogenic mutS$^-$ mutant to individually infect a mouse in an experimental murine model of UTI, in competition assays, the mutS$^-$ strain outcompeted the UPEC strain (Labat et al. 2005). Therefore, it was hypothesized that the increased mutation rate in mutator strains provides a mechanism by which mutations that alter binding specificity can accumulate and may confer a selective advantage to UPEC during pathogenesis.

4.1.3 UPEC Invasion of Host Cells

Not only are type 1 pili used by UPEC for adherence, they are also used for invasion of bladder epithelial cells. High-resolution TEM and SEM were used to show that bladder epithelial cells internalize UPEC in vivo via interactions between FimH and UP1a (Mulvey et al. 1998). In the same study, host bladder cells appeared to be zippering around and swallowing the attached bacteria, indicating that perhaps interaction between FimH and UP1a was the first step in internalization of UPEC strains. Gentamicin protection assays were subsequently used to show that UPEC and *E. coli* K12 strains expressing FimH but not isogenic FimH$^-$ mutant strains are internalized by a human bladder epithelial cell line (Martinez et al. 2000). Adherence alone was not sufficient to cause internalization, as binding of P-piliated strains was not able to mediate invasion. FimC-FimH complexes

conjugated to latex beads were then used to show that the presence of FimH was sufficient to cause uptake into a human bladder epithelial cell line. Beads coated with BSA or with FimC alone were not internalized. Therefore it was concluded that type 1-piliated strains mediate invasion of bladder epithelial cells via a mechanism dependent on the FimH adhesin. Moreover, internalization of type 1-piliated strains was dependent on localized host actin rearrangement as the actin polymerization inhibitor cytochalasin D could inhibit invasion of a bladder cell line in vitro (Martinez et al. 2000). Furthermore, FimC-FimH-coated latex bead uptake into bladder cells could also be blocked by the addition of cytochalasin D. In addition, inhibitors of tyrosine kinases and phosphoinositide 3-kinase (PI 3-kinase) blocked internalization of type 1-piliated bacteria or FimC-FimH-coated latex beads, indicating the involvement of signaling pathways in actin movement and bacterial engulfment. Inhibitors of bacterial invasion also inhibited the formation of complexes between focal adhesin kinase and PI 3-kinase, implicating this complex in the signaling cascade that results in actin cytoskeleton rearrangements during invasion.

More recent studies have indicated that type 1-piliated bacteria associate with plasma membrane microdomains known as lipid rafts (Duncan et al. 2004). Caveolae, a subtype of lipid raft with a cave-like appearance found in the plasma membrane was shown to be associated with intracellular bacteria during UPEC invasion and disruptors of the lipid rafts inhibited bacterial invasion. The involvement of the caveolin-1 protein indicative of caveolae was specifically determined via the use of RNA interference of caveolin-1 that led to reduced bacterial invasion of bladder epithelial cells in vitro. Finally, it was shown that UP1a was associated with lipid rafts in mouse bladder epithelial cells, thus demonstrating that lipid rafts mediate UPEC invasion of bladder cells. Ultimately, these data demonstrated that the FimH adhesin acts as an invasin to mediate bacterial invasion of host cells.

Type 1 piliated *E. coli* can also bind to and invade both macrophage (Baorto et al. 1997) and bone marrow-derived mast cells (Malaviya et al. 1999; Shin et al. 2000), which may be one source of bacteria causing chronic UTIs. FimH-mediated binding to macrophage cells via interaction with CD48 allows the bacteria to be internalized and survive within the macrophages (Baorto et al. 1997). Opsonized bacteria are generally phagocytosed and killed. The vesicles in which the UPEC strains are found after uptake differ between the opsonized cells and type 1 pili-expressing cells taken up via FimH-mediated invasion. Although the exact reason is unclear, this implies a fundamental difference between the two modes of entry, which allows for survival of the bacteria taken up during FimH-mediated entry into the macrophages. CD48 also mediates entry into mast cells (Malaviya et al. 1999; Shin et al. 2000). Binding of FimH to CD48 on mast cells triggers innate host immunity and the release of tumor necrosis factor alpha (TNFα), which can lead to clearance of the infection in a mouse model (Malaviya et al. 1999; Shin et al. 2000). However, in some cases the recruitment of caveolae has been documented for UPEC entry into both macrophage and mast cells, and caveolae disruptors inhibit FimH-mediated bacterial entry into both cell types (Baorto et al. 1997; Shin et al. 2000). Since intracellular compartments composed of caveolar material do not fuse

with endosomes, the bacteria contained in these caveolar vesicles can survive within both macrophage and mast cells and may function as a source of persistent bacteria.

4.1.4 Avoidance of Host Defenses by UPEC and Persistence

It was originally proposed that internalization of UPEC into bladder cells was an innate defense mechanism of the host cell. More recent studies, however, suggested that the process of internalization is used by UPEC to avoid host defenses (Mulvey et al. 1998, 2001). These defenses include urine flow, the secretion of adhesin-binding competitors such as the Tamm-Horsfall protein and secretory IgA, the secretion of chemokines resulting in recruitment of neutrophils to apical site of the bladder mucosa, and exfoliation of superficial bladder cells (McTaggart et al. 1990; Wold et al. 1990; Sobel 1997; Haraoka et al. 1999; Pak et al. 2001; Svanborg et al. 2001). Invasion of host cells provides a haven from most of these defenses. Moreover, UPEC sequestered in superficial bladder cells are protected from antibiotic treatments that sterilize urine (Mulvey et al. 1998; Hvidberg et al. 2000) and are provided a rich environment in which the bacteria can replicate (Mulvey et al. 2001). Therefore, all of the defenses except exfoliation of the superficial bladder cells can be avoided by UPEC by invasion of host cells.

Turnover of the cells lining the lumen of the bladder generally occurs very slowly (Jost 1989). However, the superficial bladder cells undergo a massive exfoliation in response to adherence of type 1-piliated strains of *E. coli* and large numbers of bacteria can be found on exfoliated bladder epithelium in the urine of humans and mice (Elliott et al. 1985; McTaggart et al. 1990; Mulvey et al. 1998). In a murine model of UTI, within 2 h after transurethral infection by UPEC, superficial bladder cells are infected by individual UPEC cells and begin to exfoliate (Mulvey et al. 1998, 2001). By 6 h after infection, large masses of intracellular bacteria are apparent within many of the superficial bladder cells and massive exfoliation was also apparent. Induction of exfoliation is dependent on FimH-mediated attachment because both UPEC and type 1-piliated *E. coli* K-12 strains caused exfoliation but P-piliated or Fim^- mutant strains did not (Mulvey et al. 1998). Bladder epithelial cell exfoliation observed in these studies occurred through an apoptotic mechanism involving both DNA fragmentation and the activation of proteolytic enzymes, termed caspases (cysteine-containing aspartate-specific proteases), and inhibitors of caspases prevented exfoliation, but also decreased the level of UPEC clearance from the bladder.

Although it initially appeared that exfoliation of infected bladder cells was an efficient method for removing UPEC from the bladder, this process is actually used by UPEC to form a persistent reservoir. By 24-48 h after invasion and subsequent exfoliation of superficial bladder cells, UPEC is found to resist gentamicin treatment ex vivo, indicating that it exists intracellularly within the immature basal cells of the bladder (Mulvey et al. 1998). Moreover, this ability to persist intracellularly was specific to UPEC isolates as UPEC titers remained constant throughout the

course of a 48 h infection in vitro despite gentamicin treatment, but titers of type 1-piliated *E. coli* K-12 strains decreased continuously (Mulvey et al. 2001). Furthermore, these studies showed that intracellular replication and the ability to escape from host cells was required for persistence of UPEC. Finally, TEM examination of a UPEC-infected bladder cell line maintained in gentamicin containing medium in vitro showed the presence of large intracellular inclusions in the tissue culture cells similar to foci of intracellular UPEC observed in mouse bladders in vivo (Mulvey et al. 2001). Type 1 piliated K-12 strains did not form these same inclusions, indicating that they do not multiply efficiently in bladder epithelial cells. These inclusions have been termed intracellular bacterial communities and form as a result of type 1 pili-mediated adherence and invasion of bladder epithelial cells. A detailed description of these intracellular communities can be seen below in Sect. 7.1.

4.2 Type 1 Pili Involvement in Abiotic Biofilm Formation

Many motile laboratory strains of *E. coli* are able to form biofilms on abiotic surfaces such as polyvinylchloride (PVC), polypropylene, polycarbonate, and borosilicate glass when grown statically in rich medium at room temperature (Pratt and Kolter 1998). Therefore, type 1-mediated biofilm formation may contribute to the ability of UPEC to withstand antibiotic treatments and host antimicrobial defenses in the urinary tract. To gain an understanding of the factors involved in formation of *E. coli* biofilms, Pratt and Kolter used transposon mutagenesis to generate mutants defective in biofilm formation on abiotic surfaces (Pratt and Kolter 1998). Mutants capable of motility but still severely defective in biofilm formation were isolated and determined to fall within the *fim* gene cluster. Independent insertions were found within *fimB*, *fimA*, *fimC*, *fimD*, and *fimH*. Microscopic analysis of PVC surfaces on which the *fim* mutants were grown statically in rich medium revealed that *fim* mutants were so severely defective in initial attachment that most of the surface contained no attached *E. coli*. This indicated that type 1 pili are essential in initial attachment to abiotic surfaces. Furthermore, FimH was shown to mediate this attachment as a nonmetabolizable mannose analog α-methyl-D-mannoside-inhibited biofilm formation by the type 1-piliated wild type *E. coli* strain 2K1056. FimH to surface interactions were concluded to be direct and involve nonspecific binding to abiotic surfaces. The role of FimH in mediating attachment to abiotic surfaces was supported by the isolation of both natural and engineered FimH variants that allow *E. coli* to form biofilms under hydrodynamic flow (HDF) conditions (Schembri and Klemm 2001a). *E. coli* with the wild type FimH adhesin and commensal *E. coli* strains could not form biofilms under the same HDF conditions. HDF shear force conditions are thought to better mimic the natural environment during UTI, and interestingly, a G73E variant of FimH that was identified as a functional alteration of FimH involved in biofilm formation was previously identified as a natural FimH variant pathoadaptive for UTI (Sokurenko et al. 1994).

The same FimH mutant library also yielded FimH variants that were capable of mediating autoaggregation of *E. coli* (Schembri et al. 2001). Phase contrast microscopy of cells expressing the FimH variants showed that they form large tight clusters of cells that might aid in forming microcolonies during biofilm formation. These results were again supported by the isolation of natural FimH variants from UPEC strains that exhibited the ability to autoaggregate, arguing that this phenotype may be relevant to UTI pathogenesis (Schembri et al. 2001). Furthermore, autoaggregation in general appears to be important for biofilm formation as other self-aggregating cell surface structures such as Antigen 43 (Ag43) and curli are also associated with biofilm formation in *E. coli* (Vidal et al. 1998; Danese et al. 2000; Kjaergaard et al. 2000; Prigent-Combaret et al. 2000). In support of this idea, DNA microarrays showed that both type 1 pili and Ag43 are more highly expressed in biofilm populations than in planktonic populations (Schembri et al. 2003b). Therefore, the presence of type 1 pili may facilitate the colonization of urinary tract catheters and other implants by mediating biofilm formation and autoaggregation. Finally, *E. coli* strains carrying transfer constitutive IncF plasmids were shown to form mature mushroom-shaped structures similar to those of *P. aeruginosa* biofilms in continuous-flow cell cultures in glucose minimal medium at 30°C (Reisner et al. 2003). The presence of type 1 pili was found to be dispensable for the biofilm maturation observed as neither fimbriated nor afimbriated variants had any effect on biofilm maturation by *E. coli* strains that were plasmid-free or carrying F plasmid.

The regulation of the FimB and FimE recombinases may also function as one regulatory checkpoint for biofilm formation in *E. coli*. Phase variation of type 1 pili is subject to multiple global regulators including LrpA (Blomfield et al. 1993; Gally et al. 1994), IHF (Blomfield et al. 1997), H-NS (Olsen and Klemm 1994; Donato et al. 1997; O'Gara and Dorman 2000) and LrhA (Olsen and Klemm 1994; Donato et al. 1997; O'Gara and Dorman 2000; Lehnen et al. 2002; Blumer et al. 2005). Both LrpA and IHF are required for the efficient switching by both the FimB and FimE recombinases, whereas H-NS affects only the FimB-mediated recombination event. LrhA, however, affects only the activation of the FimE recombinase that is required to turn off *fimA* production and subsequently the production of type 1 pili (Blumer et al. 2005). Mutation of LrhA therefore leads to decreased *fimE* expression and subsequent increased transcription of *fimA* that ultimately results in increased biofilm formation. Overexpression of LrhA abolishes biofilm formation.

4.3 Flagella and Motility

Many studies have implicated motility, flagella and/or chemotaxis in biofilm formation in bacteria (Deflaun et al. 1994; Korber et al. 1994; O'Toole and Kolter 1998a, 1998b; Watnick et al. 2001). To test their importance for biofilm formation by *E. coli*, Pratt and Kolter introduced defined mutations that affect flagellar function into the K-12 strain 2K1056 (Pratt and Kolter 1998). A microtiter dish

assay was used with flagellar mutations (*fliC*::*kan*, *flhD*::*kan*), motility mutations (Δ*motA*, Δ*motB* and Δ*motAB*) and chemotaxis mutations (Δ*cheA-Z*::*kan*) to assess their ability to form biofilms in LB medium grown at 30°C. Nonchemotactic strains were shown to be no different from wild type in formation of biofilms, indicating that chemotaxis was dispensable for biofilm formation by *E. coli*. In contrast, nonmotile strains containing motility and flagellar mutations were severely defective in initial steps of biofilm formation. Microscopic analysis indicated that this was due to the lack of adherence to PVC and that those cells that do attach form small clusters. Furthermore, biofilms did eventually form over time, indicating that motility appears to be most critical for initial attachment by *E. coli* on abiotic surfaces under static growth conditions in rich medium. Therefore, it was proposed that motility initially promotes cell-to-surface contact by overcoming repulsive forces and at a later stage is used to colonize a surface (Pratt and Kolter 1998). The role of motility during UTI pathogenesis, however, is still unclear. Flagella are not entirely required for colonization of the urinary tract during UTI by UPEC; however, motility does appear to contribute to UPEC fitness during pathogenesis in a mouse cystitis model (Lane et al. 2005; Wright et al. 2005).

4.4 Antigen 43

Many UPEC strains (~60%) express the cell surface adhesin Antigen 43 (Ag43) (Owen et al. 1996). Ag43 is an autotransporter protein encoded by the *flu* gene (also known as *agn43*), which is a self-recognizing adhesin (Diderichsen 1980; Hasman et al. 1999). Ag43 confers the ability to autoaggregate on *E. coli* in static liquid medium, resulting in settling of the cells (Diderichsen 1980). Because of this self-aggregative phenotype, Ag43 was tested for its ability to form biofilms (Danese et al. 2000). Ag43 presence was found to enhance biofilm formation of *E. coli* on PVC in glucose-minimal medium at 30°C by inducing microcolony formation while the *agn43* null allele was shown to have a limited ability to form biofilms compared to its wild type parental strain. Further studies indicated that Ag43 expression could mediate interactions between different bacterial species on glass under continuous flow growth conditions (Kjaergaard et al. 2000). These data therefore indicated that Ag43 is involved in providing both cell-to-surface as well as cell-to-cell contacts. Recent studies have shown that Ag43 is expressed specifically during the biofilm mode of growth (Schembri et al. 2003b). Interestingly, Ag43 was also shown to be expressed by UPEC within the intracellular pods that appear during IBC formation in a murine cystitis model (Anderson et al. 2003), suggesting that like type 1 pili, Ag43 may be involved in both abiotic biofilm development and biofilm formation in living tissue. Furthermore, Ag43-mediated aggregation was shown to be protective against oxidizing agents such as hydrogen peroxide in a manner characteristic of the protective nature of the biofilm mode of growth (Schembri et al. 2003a).

The expression of Ag43 is phase-variable and is controlled by the concerted action of OxyR (negative regulation) and Dam methylation (positive regulation) (Henderson and Owen 1999; Haagmans and van der Woude 2000). OxyR binds to a site found upstream of the *flu* gene to repress its transcription. Dam methylation of three GATC sequences overlapping the OxyR binding site blocks OxyR binding and thereby activates transcription of *flu* (Haagmans and van der Woude 2000; Waldron et al. 2002). OxyR is a sensor of cellular oxidative stress and responds to the redox status of the cell (Zheng et al. 1998). OxyR exists in one of two forms either oxidized or reduced, and only the reduced form can efficiently repress *flu* gene expression (Henderson and Owen 1999; Haagmans and van der Woude 2000). Moreover, fimbriation itself appears to influence expression of Ag43 as DNA microarray studies comparing *fim*$^+$ and *fim*$^-$ strains indicated that *flu* gene expression was increased by approximately 20-fold when type 1 pili expression was absent (Schembri et al. 2002). Indeed, it has been proposed that expression of thiol-disulfide containing fimbria such as type 1 pili results in a net cellular oxidation that is countered by the reduction of OxyR into the form that efficiently represses *flu* expression (Schembri and Klemm 2001b). Because the expression of Ag43 is mediated by the redox status of OxyR, mutant OxyR proteins locked in either a reduced or an oxidized state were used to study Ag43 expression on biofilm formation (Schembri et al. 2003a). The reduced OxyR protein repressed Ag43 expression and formed poor biofilms on polystyrene microtiter plates, whereas the oxidized form activated Ag43 expression and formed significantly better biofilms. A second gene *rfaH*, a transcriptional antiterminator, was also shown to regulate *flu* expression (Beloin et al. 2006). RfaH negatively regulates *flu* expression and therefore, inactivation of *rfaH* resulted in increased *flu* expression and better biofilm formation by both *E. coli* K-12 and UPEC strains in flow cultures in minimal medium. Although unclear exactly how RfaH influences *flu* transcription, it was shown by RT-PCR that a *rfaH* mutation has no effect on *oxyR* or *dam* transcript levels. Therefore, the mode of RfaH action on *flu* transcription does not occur via OxyR or Dam methylase action, and has yet to be elucidated. Additionally, both fimbriation and capsule formation by either *E. coli* K-12 and/or UPEC strains have been shown to sterically inhibit autoaggregation mediated by the shorter Ag43 adhesin (Hasman et al. 1999; Schembri et al. 2004). Capsule can protect bacteria from host defenses but then they cannot adhere or invade without adhesins, which suggests that regulation of both adhesin and capsule production may be required by UPEC during pathogenesis. Ag43 autoaggregation was also found to inhibit flagellar-based motility (Ulett et al. 2006) and this may also have implications for UPEC pathogenesis and biofilm formation, although the role of flagella in these processes remain somewhat unclear.

Ag43 is made up of two domains, an α-module that consists of a passenger domain and the β-module that is the autotransporter domain. Structure-function studies of Ag43 using domain swapping and linker scanning mutagenesis identified residues responsible for autoaggregation in the amino-terminal third of the passenger domain (α-module) (Klemm et al. 2004). Analysis of the primary sequence of the protein showed that it is likely that ionic interactions in interacting α-modules are responsible for the autoaggregation phenotype. Ag43 is found in many *E. coli*

strains and often in multiple copies in the genome (Roche et al. 2001). Although some natural variants do not mediate autoaggregation, all variants studied were found to promote biofilm formation with different efficiencies (Klemm et al. 2004). Interestingly, the two variants from the UPEC strain CFT073 did not mediate cell-cell aggregation; however, they formed more robust biofilms than those variants that did mediate autoaggregation. Therefore, it was proposed than cell aggregation and biofilm formation may be distinct features of the Ag43 protein but the primary function of Ag43 may be the formation of biofilms. In support of this idea, it was noted in this same study that in the CFT073 strain, both of the *flu* genes are associated with pathogenicity islands rather than the chromosomal locus of the *flu* gene found in the *E. coli* K-12 strain MG1655.

4.5 Curli

Curli are proteinaceous cell surface filaments composed of two proteins: a major subunit CsgA and a minor subunit CsgB (Olsen et al. 1989; Bian and Normark 1997). The insoluble curli filament consisting of curlin produced by the CsgA protein is formed at the bacterial cell surface by the CsgB protein that acts as a nucleator of filament formation (Bian and Normark 1997). The involvement of curli in biofilm formation was discovered as a result of the isolation of an *E. coli* K-12 strain carrying the mutant *ompR234* allele (Vidal et al. 1998). The presence of the mutant *ompR234* allele resulted in a biofilm-forming phenotype, and a knockout of either *csgA* or *ompR* caused the loss of adherence. The mutant OmpR protein activates expression of CsgD, a transcriptional activator of the *csgA* gene resulting in an increase in CsgA protein levels and promotion of biofilm formation. Furthermore, the overexpression of CsgA resulted in mature highly developed biofilm structures on polystyrene in glucose minimal medium at 30°C relative to biofilms formed by the isogenic wild type parent (Vidal et al. 1998). Similar results were observed when the same lesions were introduced into clinical isolates taken from patients suffering from catheter-associated bacteremia, indicating that curli involvement in biofilm formation may be relevant to UPEC pathogenesis and catheter-associated UTIs. In an attempt to understand what factors are involved in biofilm formation by curli, the role of flagellar motility and colanic acid production was examined (Prigent-Combaret et al. 2000). Biofilms formed by the *ompR234* strain with and without the flagellin gene *fliC* were examined using SEM. Both the $FliC^+$ and $FliC^-$ strains carrying the *ompR234* allele formed similar thick biofilms on plastic thermanox coverslips and polystyrene in glucose minimal medium. These data indicated that in curli-overproducing strains, flagella are dispensable for initial adhesion and biofilm formation. Examination of *ompR234* K-12 strains that either do or do not produce colanic acid (CA) indicated that while CA was necessary for a thick well-developed biofilm, strains that overexpress curli without CA production are able to support biofilm formation. Finally, SEM and TEM with negative staining indicated that the curli form a dense meshwork of intertwined fimbria

between cells and with the abiotic surface, suggesting that curli mediate cell-cell and cell-surface interactions. Examination of clinical isolates by SEM and TEM once again supported these results, indicating that curli involvement in biofilm formation may be relevant to UPEC pathogenesis. In addition, it was suggested that curli may be better adapted to form biofilms on abiotic surfaces such as catheters given that curli expression is favored by conditions that are found outside the host (i.e., poor nutrient availability and 30°C) (Prigent-Combaret et al. 2000). There is, however, some indication that although curli is not expressed on solid or liquid medium at 37°C, it is expressed in biofilms at 37°C and is able to support limited biofilm development at that temperature (Kikuchi et al. 2005). Furthermore, a comparison of the curli-expressing strain YMel and the curli-deficient strain YMel-1 showed that only the YMel strain was capable of forming mature mushroom-shaped biofilms, implying that curli are required for biofilm maturation (Kikuchi et al. 2005). The YMel curli-expressing strain was also shown to better adhere to human uroepithelial cells in vitro more than the curli-deficient strain YMel-1, once again implicating curli expression in UPEC pathogenesis.

5 *P. mirabilis*: Role of Mannose-Resistant Fimbriae

The MR/P fimbriae are surface appendages that confer hemagglutination in a mannose-resistant manner and are important in colonization and pathogenicity in mouse models of ascending urinary tract infections (Li et al. 2001). The Mrp locus is comprised of the *mrpA-J* genes (Bahrani and Mobley 1994). The *mrpA-H* genes encode Mrp structural components and other proteins necessary for assembly. The MrpJ product acts as a repressor of flagellin expression and acts to downregulate flagellin when Mrp fimbriae are being expressed (Li et al. 2001). The *mrpI* gene is divergently transcribed and encodes a recombinase that directs the inversion of a small promoter containing region upstream from *mrpA* (Li et al. 2002). In the ON position, the promoter drives expression of the *mrp* operon and in the OFF position, the promoter is in a divergent orientation (Li et al. 2002). In strains with MR/P locked in the ON orientation, *P. mirabilis* growing in sterile urine formed biofilms that were significantly more developed up to 48 h of incubation than either wild type or strains locked in the MR/P OFF state (Jansen et al. 2004). However, in 7-day-old biofilms, the wild type biofilm was significantly thicker than either the MR/P ON or OFF strains. Therefore, the expression of MR/P fimbriae promoted the early stages of biofilm development, possibly by facilitating the cell-cell interactions important for microcolony formation. In fact, MR/P ON strains are more autoaggregative in liquid than wild type (Jansen et al. 2004). However, expression of MR/P was detrimental to the later stages of biofilm development. This may be due to MR/P interactions between cells that serve to inhibit cell movement within the biofilm. Immunization of mice with the MR/P protein, protected animals against *P. mirabilis* in experimental urinary tract infections (Li et al. 2004). This emphasizes the importance of the MR/P fimbriae in the ability of *P. mirabilis* to cause urinary tract infections.

6 *K. pneumoniae*: Role of Type-3 Fimbriae

Approximately 8% of urinary tract infections are caused by *K. pneumoniae* (Tambyah et al. 2002). Two types of pili, type-1 and type-3, are produced in *K. pneumoniae* (Clegg and Gerlach 1987). The type-3 pilus is composed of a MrkA protein that comprises the fimbral shaft and an adhesion MrkD. In a search for mutations that affected biofilm formation, Langstraat et al. demonstrated that MrpA, but not MrpD was required for biofilm formation on abiotic surfaces (Langstraat et al. 2001). In a separate study, Di Martino et al. noted a strong correlation with expression of type-3 pili and the ability to form biofilms on abiotic surfaces (Di Martino et al. 2003). However, since indwelling devices become coated with host-derived substances (Donlan 2001), the above studies do not directly address the clinical relevance of biofilm formation. More recent studies examined the role of *Klebsiella*-specific factors on the ability to form biofilms on surfaces coated with extracellular matrix or collagen (Jagnow and Clegg 2003). The MrkD adhesin, but not the MrkA shaft protein was found to mediate biofilm formation on collagen and matrix-coated surfaces. Therefore, MrkA and MrkD may have distinct roles in biofilm formation on different surfaces. In a screen using signature-tagged mutagenesis to identify transposon insertions that caused a defect in biofilm formation on extracellular matrix material, insertions were identified in genes required for: (1) capsular biosynthesis, (2) transcriptional regulation (LuxR, LysR, and Crp-like genes) and (3) the sugar phosphotransferase (PTS) system. (Boddicker et al. 2006). The identification of a CRP-like protein is consistent with a previous study indicating that biofilm development in *Klebsiella* is under catabolite repression (Jackson et al. 2002).

7 Biofilm Formation on and in Urinary Tissues

7.1 Intracellular Bacterial Communities

7.1.1 Intracellular Pods

UPEC strains are not only capable of efficient adherence to and invasion of bladder epithelial cells, but they are also highly successful at forming persistent intracellular reservoirs that escape host defenses and antibiotic treatments (Mulvey et al. 2001). Recent work by Anderson et al. (2003) has indicated that UPEC strains accomplish these feats by the formation of biofilm-like pods or intracellular bacterial communities (IBCs) within the host bladder tissue (Fig. 1). Investigations of the replication of UPEC within the superficial bladder epithelial cells led to the discovery of these intracellular pods on the bladders of mice infected with UPEC. Bacterial replication within the superficial bladder epithelial cells resulted in tightly packed biofilm-like pods jutting into the

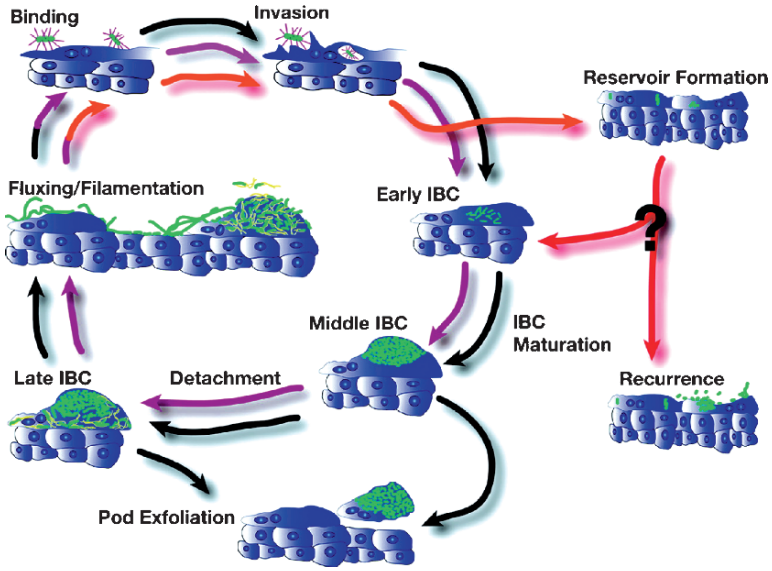

Fig. 1 Stages in intracellular pod formation in uropathogenic *E. coli.* (Reprinted from Justice et al. 2004, with permission form the National Academy of Sciences)

bladder lumen. Neither type 1-piliated *E. coli* K-12 strains nor *fimH* mutant strains produced pods, indicating that while type-1 pili are necessary for host-cell invasion, additional factors are required for UPEC pathogenesis (Anderson et al. 2003). Using time-lapse fluorescence videomicroscopy of mouse bladder explants infected with green fluorescent protein (GFP) producing UPEC, Justice et al. (2004) demonstrated that the bacteria within these intracellular pods undergo a continuous developmental program leading to maturation of the intracellular bacterial communities. This program can be divided into four distinct phases that result in dispersal to new sites of infection and establishment of a persistent reservoir, which closely parallels biofilm formation on abiotic surfaces (Fig. 1).

7.1.2 Early IBCs

The first phase of IBC formation begins at 1-3 h after infection with binding of type 1-piliated UPEC and invasion of superficial bladder epithelial cells (Fig. 1) (Justice et al. 2004). The bacteria in this phase are nonmotile rod-shaped cells that proliferate rapidly with a doubling time of approximately 30-35 min. Bacterial growth continues for up to 8 h postinfection to form loosely organized colonies free within the cytoplasm that resemble microcolonies of abiotic biofilms.

7.1.3 Middle IBCs

Middle IBCs form at 6-8 h postinfection producing characteristic pods on the mouse bladder lumenal surface (Fig. 1) (Justice et al. 2004). This stage is characterized by a reduction of cell growth and more strikingly, a reduction in cell size, resulting in a coccoid morphology for all of the UPEC within the pod. Each pod corresponds to a single superficial epithelial cell tightly packed with bacteria forming an intracellular biofilm. These pods share many of the definitive characteristics of abiotic biofilms. First, within the pods, a fibrous polysaccharide matrix reminiscent of a glycocalyx surrounds the bacteria (Anderson et al. 2003). Moreover, each bacterium is individually compartmentalized within this matrix and interacts with it via multiple fibers expressed on its surface. Next, immunofluorescent staining was used to show that both type 1 pili and Ag43 are expressed within the intracellular pods in a heterogeneous fashion reminiscent of gene expression within abiotic biofilms (Anderson et al. 2003). Finally, encasement of UPEC in these biofilm-like pods provides protection from antibiotic treatment and from host defenses similar to protection of bacteria found within abiotic biofilms. Polymorphonuclear leukocytes (PMNs) can discriminate with high accuracy cells infected by UPEC from cells uninfected by UPEC, but they cannot penetrate into the intracellular pod (Justice et al. 2004). Moreover, even when they gain access to the pod interior, the rapid proliferation during the early IBC formation provides sufficient numbers of bacteria to overwhelm the ability of the PMNs to engulf the entire bacterial population.

7.1.4 Late IBCs

During late IBC formation at approximately 12 h postinfection, the UPEC are found to flux out of the cells by regaining their rod-shaped morphology, becoming motile, and bursting out of the pods (Fig. 1) (Justice et al. 2004). The fluxing motility parallels the detachment of abiotic biofilms. The morphological change during this phase was not due to exposure to a rich medium environment because the same phenotype was also observed when the mouse bladder explants were exposed to saline buffer. Fluxing appeared to be necessary for UPEC to infect either adjacent superficial bladder cells or the underlying naïve bladder cells. Although the fluxing observed during this stage appeared characteristic of flagellar-based motility, subsequent studies showed that fluxing did not involve flagella because a UPEC mutant deficient in flagellin expression (Δ*fliC*) was able to form as many pods as a wild type UPEC strain in a murine cystitis model (Wright et al. 2005). The expression of flagella, however, did confer a subtle advantage in co-challenge infections with the wild type UPEC and Δ*fliC* mutant, although it was unclear exactly why. These results are supported by similar results in infection studies of flagellar and motility mutants of UPEC (*fliC*, *fliA*, and *motAB*) in a murine cystitis model (Lane et al. 2005).

7.1.5 UPEC Filament Formation and Reinfection

The final phase of IBC formation occurs between 24 and 48 h postinfection and results in the filamentation of UPEC (Fig. 1) (Justice et al. 2004). Although unclear if filamentation occurs within the IBC or on the bladder surface, filamentation was determined to be critical for escape from innate host defenses. Filamentous bacteria were observed to be resistant to engulfment by the PMNs recruited to infected bladder cells, while rod-shaped cells were easily engulfed and eliminated. Filamentous bacteria were also seen to septate and form rod-shaped daughter cells that were susceptible to elimination by the PMNs; however, those that escaped could also reinfect new cells. Previous studies have shown that the filamentous bacteria may interact with adjacent or underlying cells to cause new infections (Mulvey et al. 2001). The appearance of filaments also coincided with the appearance of small groups (usually pairs) of UPEC in newly infected healthy superficial bladder cells (Justice et al. 2004). These cells were indicative of a second round of infection that progressed through all of the stages, as indicated above, although with much longer kinetics of infection. Moreover, after exfoliation of the bladder cells at 36-48 h postinfection, most of the superficial bladder cells were observed to be smaller than normal and once again, many had small groups of bacteria intracellularly. These clusters appeared to be quiescent bacteria that continued to produce GFP for at least 12 days after infection. These quiescent bacteria are proposed to be the cells responsible for persistent and recurrent infections that characterize UTIs caused by UPEC.

Recently, it has been suggested that host actin cytoskeletal rearrangements during bladder epithelial cell maturation modulate growth and resurgence of quiescent UPEC found in naïve bladder epithelial cells (Eto et al. 2006). In terminally differentiated superficial bladder cells, actin is found mostly associated with the basolateral surface of the cell, whereas in immature bladder epithelial cells, the actin is found throughout the cell and along the cell periphery (Romih et al. 1999). In a murine cystitis model using immunofluorescent microscopy, IBCs in the cell cytosol were found to be only weakly associated with actin, whereas UPEC within naïve bladder cells were found within actin-lined vacuoles (Eto et al. 2006). For these reasons, it was thought that perhaps actin was involved in suppressing growth of UPEC in naïve bladder cells. Therefore, a bladder epithelial cell line 5637 resembling the immature basal or intermediate bladder epithelial cells with respect to their actin cytoskeleton was used to study the involvement of actin in UPEC growth and resurgence. This cell line rarely formed IBCs as large as those seen during infection of terminally differentiated bladder cells in a murine cystitis model (Mulvey et al. 2001; Anderson et al. 2003). Disruptors of actin polymerization and/or the actin cytoskeleton stimulated both intracellular growth and efflux of UPEC in the 5637 cell line in vitro as determined by gentamicin protection assays (Eto et al. 2006). Moreover, UPEC in the 5637 cell line was found sequestered within late endosome-like vacuoles. However, robust intracellular growth and IBC formation by UPEC usually occurs in the cytoplasm (Mulvey et al. 2001; Anderson et al. 2003; Justice et al. 2004). Disruption of host actin resulted in increased

clusters of bacteria and release from vacuoles into the host cytoplasm (Eto et al. 2006). However, these clusters of UPEC were smaller than the IBCs previously observed. To achieve more efficient release of UPEC from intracellular vacuoles, host membrane was treated with the membrane permeabilizing glycoside saponin. Saponin treatment stimulated IBC formation by UPEC but not by type 1-piliated *E. coli*, indicating that UPEC-specific factors are essential for pod formation. A model explaining how actin could affect the fate of UPEC during pathogenesis within the bladder suggested that infection of immature basal or intermediate epithelial cells results in UPEC trafficking into late endosome and lysosome-like acidic vacuoles enmeshed within actin. This process results in limited growth of UPEC until terminal differentiation of the bladder cells leads to host actin rearrangements that trigger the release of quiescent UPEC from the membrane-bound intracellular vacuoles. The alternate pathway in which UPEC infects superficial bladder cells directly results in the IBC formation described above (Mulvey et al. 2001; Anderson et al. 2003). This model suggests that both the interaction with actin and the membrane barrier of the intracellular vacuole results in quiescence of UPEC, and ultimately release into the host cytoplasm is required for growth and subsequent IBC formation (Eto et al. 2006).

7.2 Chronic Prostatitis

An additional infection of the urinary tract that is associated with biofilm formation is chronic bacterial prostatitis in men. The most commonly encountered bacteriological agent in prostatitis is *E. coli*, followed by other members of the *Enterobacteriaceae* (*Proteus* and *Klebsiella*) and coagulase-negative *Staphylococci* (Domingue and Hellstrom 1998). These infections are notoriously difficult to treat with antibiotic therapy. Studies by Nickel and Costerton demonstrated that prostate biopsy samples from chronically infected patients contained exopolysaccharide-encased microcolonies that were attached to the walls of the prostate ducts (Nickel and Costerton 1993). In chronic staphylococcal prostatitis, biofilm-like microcolonies were attached to the prostate in patients that were refractory to antibiotic therapy (Nickel and Costerton 1992). Finally, in a recent study, a total of 377 *E. coli* isolates obtained from a variety of urinary tract infections (cystitis, pyelonephritis, and prostatitis) were examined for biofilm-forming abilities by standard crystal violet staining of cells grown in microtiter wells. The isolates from prostatitis cases exhibited significantly greater biofilm formation than other isolates (Kanamaru et al. 2006).

8 Summary

Despite great strides in our understanding of the pathogenesis of UTI-causing organisms, urinary tract infections will continue to represent a major human health problem. Described in this chapter are some ways in which different UTI-causing

bacteria use the biofilm mode of growth to gain a foothold in and cause infection of the urinary tract. The classical antimicrobial therapies available that can effectively kill UTI-causing bacteria are designed for killing during the planktonic mode of growth and therefore are relatively ineffective against UTI biofilms. Awareness that biofilms are not only formed on abiotic surfaces, but can also be present in and on living tissues allows for a rational and concerted effort to devise strategies to counter the refractory nature of these infections. By understanding the molecular characteristics of device-related biofilms and chronic infections caused by biofilms associated with tissues, new preventative and therapeutic strategies can be targeted to specifically treat or promote the dissolution of biofilms. It seems clear that the biofilm phenotype must be considered in any future development of treatments to prevent the emergence and recurrence of UTIs.

Acknowledgements PNR is supported by grants MCB0406047 from the National Science Foundation, a Merit Review from the Department of Veterans Affairs, and a Research Career Scientist Award from the Department of Veterans Affairs

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