Chapter 3
Pathogenic Mechanisms of Uropathogens

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Abstract Urinary tract infections (UTIs) can affect both men and women at almost any stage in their lifetime. While the vast majority of these are not life-threatening, they cause significant morbidity to patients and place a heavy burden on healthcare systems worldwide. This is further complicated by the use of urinary drainage devices such as catheters and stents, which provide additional sites for bacterial/fungal attachment and biofilm development. Despite being exposed to a wide array of antagonistic environmental conditions and the host immune system, uropathogens are generally very successful at establishing infection. This is mainly due to the plethora of pathogenic mechanisms they utilize that provide an advantage over the host. In this brief review, we discuss a small subset of the mechanisms used by uropathogens including the appendages, proteins and sugars used to adhere to surfaces, the invasion into host tissues, immune evasion strategies and antibiotic resistance. This work illustrates the complexity of the interaction between the urinary tract and uropathogens, and supports the development and application of multi-faceted strategies for infection prevention and treatment.

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Introduction

Urinary tract infections (UTIs) are one of the most common diseases caused by bacteria, representing the most abundant cause of hospital acquired infections [1]. Roughly 50 % of all women will experience a UTI within their lifetime with 44 % of those experiencing recurrence [2]. A single mechanism to establish an active or recurrent UTI does not exist. Bacteria employ a plethora of mechanisms, often simultaneously, to assist in many key steps to survive in the host [3, 4]. In this chapter only a few select mechanisms can be discussed including adherence to the host epithelium, host immune modulation, nutrient acquisition, host-cell invasion, biofilm formation and antibiotic resistance. Although many different bacteria can cause UTI's, 80 % have been associated with *Escherichia coli* and as such, this species has been a major focus of study. Therefore, although other uropathogens will be discussed periodically, uropathogenic *E. coli* (UPEC) will be the primary focus.

Adherence to Epithelial Cells; Type I Fimbriae and P Fimbriae

An important step in infection establishment at almost any anatomical site is host cell adherence. For uropathogens, numerous mechanisms assist urothelial attachment, critical for avoiding clearance during micturition (urination). Some UPEC strains produce several long (~1 μm) extracellular fimbriae with adhesive tip proteins used to bind specific receptors on the mucosal surface [5]. In a model of cystitis, Type I fimbriae with the FimH adhesin bind to terminal mannose moieties on Uroplakin (UP) Ia found on umbrella cells of the urothelium ([6–13]). This binding induces numerous physiological changes in the host cell including an increase in intracellular calcium, the phosphorylation of a UP signalling complex and Rho-GTPase activation, leading to local rearrangement of the actin cytoskeleton and UPEC engulfment via a zippering mechanism [11, 14–18]. At the same time, bacterial attachment via FimH triggers host exfoliation of the terminally differentiated superficial umbrella cells in an effort to remove those infected [11, 19]; however, while this can help clear a large number of the invaders, it also exposes the underlying undifferentiated cells to attack. Further supporting the critical nature of Type I fimbriae in UTI development, FimH residues that enhance virulence have been shown to be selected for in UPEC strains [20], the fimbrial regulator *fimX* can be used as a molecular marker for identifying UPEC strains [21] and *Klebsiella pneumonia*, the second-most prevalent Gram-negative UTI pathogen, uses a virtually identical system during infection [22]. Once inside the host cell, the bacteria are sheltered from many extracellular host-defense mechanisms and can establish an active infection or a reservoir for recurrence.

Another fimbrial system associated with UTI is the P fimbrial system, strongly linked to ascending UTI and pyelonephritic infections [23–25]. While generally similar in structure to Type I fimbriae, their tip adhesin PapG binds specifically to

glycosphingolipids (Gal-(α1-4)-Gal) found on renal epithelial cells. PapG adhesion signals host release of ceramide, a Toll-like receptor 4 agonist which leads to local inflammation and pain. Although P fimbriae are not essential for lower UTI development, a synergy between Type I and P fimbriae has been shown to enhance the rate at which UPEC are able to invade renal epithelial cells and thus establish more serious infections [26]. This is a similar observation to the mannose-resistant *Proteus*-like (MR/P) fimbriae found on some uropathogenic strains of *Proteus mirabilis*; a cause of serious UTI's and acute pyelonephritis [27]. The MR/P fimbriae are not essential for infection but do contribute to virulence by eliciting a strong immune response *in vivo*. Akin to P fimbriae in UPEC, the role of MR/P fimbriae in urothelial adherence is not entirely determined. Strains lacking MR/P fimbriae are still able to adhere to urothelial cells after 1 h, albeit not as well as strains with MR/P fimbriae [27].

Although less studied, additional fimbriae have been implicated in UTIs. Both S and F1C fimbriae can bind epithelial and endothelial cells of the lower urinary tract and kidney [28–30]. S fimbriae have even been associated with *E. coli* strains that cause sepsis, meningitis and ascending UTIs [31]. Due to the implication of numerous fimbrial systems as strong virulence factors, a lot of research has gone into using inhibitors of fimbrial production and assembly as targets to increase sensitivity to many antibiotics that are no longer effective [32, 33].

Biofilm Formation on Implanted Devices

In addition to fimbriae, many uropathogens also produce adhesins expressed directly on the bacterial surface. These adhesins are not only used to attach to host cells but also to implanted devices such as urinary catheters and stents. When bacteria attach to devices they typically form biofilms, polymicrobial communities of bacteria enclosed within secreted extracellular polymeric substances (EPS) [34]. Due to biofilm structure and the reduced growth rates that typically occur within them, these communities are generally more resistant to harsh environmental conditions such as urine flow, pH changes, host immunity and antibiotic exposure. Furthermore, bacteria within biofilms become chronically exposed to sub-inhibitory concentrations of any clinically-administered antibiotic, inducing drug resistance, biofilm enhancement and the production of dormant persister cells; all key factors leading to chronic and recurrent infections [35], [36]). Several key adhesins linked to device adherence and biofilm formation are discussed herein.

Antigen 43 (Agn 43) is a self-binding, biofilm-promoting surface adhesin found on many UPEC strains [37]. This autoaggregating protein is so good at forming biofilms that its expression on the surface of non-biofilm-forming *Pseudomonas fluorescens* induced biofilm development similar to that of *E. coli* [38]. Glycosylated Agn 43 also binds to collagen and laminin, host proteins found within the urinary conditioning film that typically deposits on indwelling devices following placement [39].

As mentioned, host proteins and numerous other urinary constituents bind to the surface of any foreign device placed in the urinary tract within minutes. Many bacterial surface proteins (fimbrial and afimbrial) are subsequently able to utilize these factors for device attachment and biofilm formation. Examples include a number of fibrinogen-binding proteins such as Clumping factors A and B (ClfA and ClfB) and Iron-regulated surface determinant A (IsdA) on *Staphylococus aureus* [40, 41] and Fss1, Fss2, Fss3 and the Endocarditis and biofilm-associated pilus adhesion (EbpA) on *Enterococcus faecalis* [42]. Interestingly, EbpA has also been shown to directly promote biofilm formation on catheters and vaccination with the protein prevented biofilm development and catheter-associated UTI in a murine model [43]. Furthermore, Iron-regulated surface determinant A (IsdA) recognizes cytokeratins [40, 44] and heme [40] in addition to fibrinogen (all three compounds have been recovered from indwelling urinary devices), demonstrating the versatility of many of these bacterial proteins. Collectively, this data highlights how factors within the urinary conditioning film not only mask the exposed surface of any urinary device placed in a patient but also offer a plethora of microbial adherence options. These surface interactions represent a crucial step in the formation of indwelling device associated biofilms, and thus, is a significant area of study. Once formed, biofilms are extremely difficult to remove and typically require device removal alongside antimicrobial therapy to completely eradicate the infections.

Intracellular Biofilm Formation; Long Filament Formation; Quiescent Intracellular Reservoirs

As described previously, Type I fimbriae induce invasion into urothelial cells by inducing cytoskeletal rearrangement via binding to UPIa and UPIIIa. The natural response by the host is to shed the superficial umbrella layer; however, many UPEC strains are able to penetrate deeper into the underlying undifferentiated bladder cells to avoid clearance [45]. Upon engulfment into bladder epithelial cells, bacteria are generally enclosed within an acidic vacuole where they are unable to replicate efficiently [46]. Following this, the pathogen can then be expelled from the cell [47] or can escape from the vacuole into the cytosol. If escape occurs, the UPEC will begin reproducing quickly to form what is termed an intracellular biofilm community (IBC). Similar to extracellular biofilm formation, the cells are tightly packed, surrounded by EPS and contain subpopulations that undergo differential gene expression [48–50]. Numerous factors control this process including Type I fimbriae [51], capsular polysaccharide [52, 53] and five additional Type I-independent mediators whose functions are currently unknown [54]. IBC growth ultimately becomes limited based upon host cell size, triggering UPEC to alter its cellular morphology to a long filamentous cell type. These filaments are able to flux out of the cell to reach neighboring bladder epithelial cells and establish new IBCs. Naturally the host will try to exfoliate the superficial bladder epithelium to clear the infected cells. The invasion by UPEC of the underlying cells leads to the development of quiescent intracellular reservoirs (QIR).

Bacteria within the QIR are dormant, non-replicating cells that are highly resistant to antibiotics, and as such, are able to persist [55–57]. Occasionally a subset of these persister cells will become active and cause a new UTI [55].

Hemolysin and Cytotoxic Necrotizing Factor

In the bladder, exfoliation of the superficial umbrella cells can be beneficial or detrimental to the host. In one aspect, physical removal of infected cells is a quick and easy way to clear many of the bacteria in an infection. On the other side, it exposes undifferentiated cells below that, if infected, are more difficult to treat. α-Hemolysin (HlyA) is a pore-forming toxin produced by UPEC that is able to lyse many cell types including red blood cells, natural killer cells [58] and bladder epithelial cells (BECs). At subcytotoxic concentrations, HlyA activates serine proteases within BECs which lead to the degradation of a cytoskeletal scaffolding protein, paxillin [59]. This promotes the exfoliation of the bladder epithelium. HlyA also induces dephosphorylation of Akt which modulates signalling cascades, including a reduction in the NF-κB response [60]. This activation of proteases is not only limited to the bladder epithelium but has also been shown in macrophages, inhibiting proper function [59]. Cytotoxic necrotizing factor 1 (Cnf1) is another toxin secreted by UPEC via outer membrane vesicles [61, 62] that is able to induce bladder inflammation and submucosal edema in a murine UTI model [63, 64]. Cnf1 constitutively activates RhoA, Rac1 and Cdc42 which results in the formation of actin stress fibers, filopodia, lemellipodia and eventually apoptosis [65–67]. Overall, it is apparent UPEC isolates are able to use secreted toxins to promote exfoliation of host cells, establish an active infection and modulate the host immune response.

Siderophores

Iron is an essential cofactor required for many processes including electron transport and nucleotide biosynthesis. As the bioavailability of free iron in the host is limited, it is critical that bacteria develop methods for sequestering it to survive. The majority of iron in the host is bound to heme or heme-containing proteins such as hemoglobin and hemopexin. UPEC strains produce four different siderophores that sequester iron from these host proteins and transport it back into the bacterial cell [68]. Enterobactin is the strongest known siderophore produced by Gram-negative bacteria. Because of its strong binding, enterobactin practically "rips" the iron from heme and translocates it across the bacterial cell wall in a TonB-dependent manner [69]. Upon entry the iron is reduced, thereby decreasing its binding affinity for enterobactin and allowing its release and utilization. Lipocalin-2 is a secreted host-protein that binds bacterial enterobactin, essentially blocking its iron sequestration function and thus severely limiting bacterial growth and survival. While enterobactin is fairly widespread among

Gram-negative bacteria, other siderophores such as salmochelin, aerobactin and yersiniabactin are far more prevalent in pathogens. Salmochelins are a group of glucosylated forms of enterobactin that lipocalin-2 is unable to bind, thus allowing them to remain unaffected by host defenses [70]. Overall, the addition of glucosyl groups by the IroB glucosyltransferase and further modifications by other *iro* gene cluster members can result in the production of 9 structurally different salmochelins (3 cyclic and 6 linear), showing their diversity and importance in bacterial survival. Furthermore, the expression of the salmochelin receptor IroN has been shown to be upregulated in IBCs. Aerobactin is a siderophore strongly associated with UPEC that is generated through the oxidation of lysine. Unlike enterobactin, aerobactin delivers iron directly to iron-requiring sites within the bacterial cell and can be recycled without hydrolysis for immediate reuse. As its name implies, Yersiniabactin is a phenolate siderophore required for virulence in *Yersinia pestis* [71]. However, it is also strongly linked to UPEC isolates where it is associated with UTI establishment [72] as well as the development of more serious upper UTIs and pyelonephritis. Recent studies have shown that yersiniabactin can also bind copper [73] and vaccination with its receptor can prevent infection in animal models [74].

Urease

Urease production has been highly studied in the gastric ulcer-producing bacterium, *Helicobacter pylori*. Uropathogens such as *P. mirabilis, Klebsiella* species and some *E. coli* also produce urease [75], which has been linked to struvite stone formation and pyelonephritis. Urease hydrolyzes urea in the urine into ammonia and carbonate. This significantly increases the local pH resulting in the precipitation of ions that are normally soluble in urine. Magnesium ammonium phosphate (struvite) urinary stones can lead to calculus formation within the renal pelvis and result in the blockage of urine flow through catheters. Bacteria found within these stones are also more protected from the host immune response and antibiotic treatment leading to resistance and recurrence.

Antibiotic Resistance

There are many factors which contribute to antibiotic resistance or tolerance, a couple of which have already been mentioned such as metabolic dormancy and avoidance. Resistance is also acquired through additional strategies including antibiotic inactivation, efflux from the cell and target modification [76]. These strategies are typically acquired via random genetic mutation and/or novel gene acquisition. Examples of mutations include those that alter the active site of a critical bacterial enzyme where antibiotic binding typically occurs, such that it is no longer able to bind, as well as those that alter or upregulate bacterial efflux systems to promote or enhance antibiotic removal from the bacterial cell. Novel gene acquisition includes those encoding β-lactamase enzymes or antibiotic resistant transpeptidases. These novel genetic

elements can be picked up through natural transformation or transferred from other bacteria via mobile genetic elements (e.g., transposons and plasmids). For example, genes for β-lactamases like CTX-M-27 are commonly transferred between bacteria on plasmids. These enzymes degrade a large number of β-lactam antibiotics including cefotaxime, ceftazimide and aztreonam. CTX-M-27 originated in UPEC strains in Asia and can now be found spreading into the Czech Republic [77] showcasing the remarkable ability for these pathogens to transfer resistance.

Another example of resistance by UPEC involves the aminoglycosides. Many strains encode aminoglycoside-modifying enzymes that alter the active site such that it is unable to bind the bacterial ribosome and block protein synthesis. For example, aac(3)-IIa acetylates an amino group on the aminoglycoside while ant(2″)-la adenylates a hydroxyl group [78]. Both modify the antibiotic so it can no longer bind to the 30S ribosome, conferring resistance to multiple clinically-relevant aminoglycosides. These enzymes are of growing concern as Soleimani et al. [79] showed that nearly 40 % of UPEC strains isolated from urine samples in an Iranian hospital were resistant to gentamycin, kanamycin and tobramycin. To avoid or overcome these challenges, aminoglycosides and β-lactams are often prescribed together. These two classes have been shown to work well synergistically, attacking both bacterial peptidoglycan and protein synthesis simultaneously, in addition to decreasing resistance potential.

Trimethoprim and sulfamethoxazole are folic acid (folate) synthesis inhibitors that have been used in combination for decades in the treatment of UTI, as they effectively target different stages of the process. They are also both generally well tolerated and cost effective. However, resistance is increasing with one study demonstrating 86 % of UPEC isolates from patients resistant to the combination [80]. While random point mutations in the target genes have been shown to confer this resistance, it is most commonly acquired through plasmids encoding modified homologues of the genes that are functional but fail to bind the antimicrobials [81].

Briefly discussed above are the populations of bacteria within device biofilms and IBCs that develop into dormant persister cells. These subpopulations alter their metabolism such that many antibiotics that rely on active bacterial growth and replication for efficacy become ineffective. While these persister cells form naturally at low levels during general bacterial growth, under stressful growth/survival conditions their generation is greatly upregulated. It is important to note that these organisms are not antimicrobial resistant but instead are termed tolerant, waiting until the antibiotic treatment has subsided to resume metabolic activity and re-establish an active infection [82]. This phenomenon is a major driving force behind recurrence as there is currently no method to target and kill all persister cells.

Host Immune Modulation

In order to establish an infection, bacteria must be able to survive the host immune response. Many UPEC strains encode multiple mechanisms that modify the local environment to aid in this goal. A recent study demonstrated that several UPEC strains were able to significantly increase the secretion of anti-inflammatory

cytokines such as IL-5, IL-10 and IL-17 in a mouse model of infectious epididymitis [83]. In addition, UPEC strains have shown the ability to suppress the NF-kappaB pathway in urothelial cells, decreasing the secretion of several pro-inflammatory cytokines [84]. Both paradigms result in the suppression of a Th-1 mediated host immune response, resulting in a more suitable environment for UPEC to establish an infection. Finally, some UPEC strains have been shown to possess a variant of the periplasmic protein YbcL that when expressed inhibits the transepithelial migration of neutrophils to the site of infection [85].

It is important to note that only a small fraction of the pathogenic mechanisms of uropathogens have been discussed herein. Furthermore, it should be noted that many uropathogens will use multiple mechanisms simultaneously. Therefore, the success of a pathogen will rarely rely on a single trait but instead on a combination working synergistically. Generally speaking, pathogenic microorganisms are simply geared toward population survival and will exploit any weakness possible in the host's armour.

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