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Virulence and Fitness Determinants of Uropathogenic *Escherichia coli*

Sargurunathan Subashchandrabose¹ and Harry L T. Mobley¹

¹Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109

Abstract

Urinary tract infection (UTI) caused by uropathogenic *Escherichia coli* (UPEC) is a major global public health concern. Increasing antibiotic resistance found in clinical UPEC isolates underscores the immediate need for development of novel therapeutics against this pathogen. Better understanding of the fitness and virulence mechanisms that are integral to the pathogenesis of UTI will facilitate identification of novel strategies to prevent and treat infection with UPEC. Working towards that goal, the global UPEC research community has made great strides at unraveling various virulence and fitness genes. Here, we summarize major findings on virulence and fitness determinants that enable UPEC to successfully survive and colonize the urinary tract of mammalian hosts. Major sections of this chapter are devoted to the role of iron acquisition systems, metabolic pathways, fimbriae, flagella, toxins, biofilm formation, capsule, and strain-specific genes in the initiation and progression of UTIs. Transcriptomes of UPEC during experimental UTI in a murine model and naturally occurring UTI in women are compared to elucidate virulence mechanisms specifically involved in human UTI. Capitalizing on the advances in molecular pathogenesis research by translating these findings will help develop better clinical strategies for prevention and management of UTIs.

INTRODUCTION

Urinary tract infections (UTIs) are one of the most common bacterial infections affecting humans, and uropathogenic *Escherichia coli* (UPEC) is the etiological agent in 75% to 95% of UTIs in otherwise healthy individuals (1–4). Based on the presence or absence of anatomic abnormalities and recent history of instrumentation in the urinary tract, UTIs are divided into either complicated or uncomplicated cases, respectively. UPEC is the most common cause of uncomplicated UTIs in humans. In clinical settings, UTIs are described with specific reference to the site of inflammation; cystitis indicates inflammation of the urinary bladder and pyelonephritis indicates inflammation of the renal pelvis and the kidneys. Presence of bacteria (bacteriuria) and neutrophils in the urine are hallmarks of UTIs caused by UPEC. Some patients, however, are bacteriuric unaccompanied with symptoms of UTI for long periods. This condition is referred to as asymptomatic bacteriuria (ABU) and is the most benign form of *E. coli* colonization in the human urinary tract. In patients suffering

Correspondence: Harry L.T. Mobley, hmobley@umich.edu.

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from pyelonephritis, UPEC can gain access to renal capillaries, leading to bacteremia (presence of bacteria in blood) and sepsis with the latter being the most dangerous and potentially fatal complication of UTIs caused by UPEC. Numerous virulence and fitness factors confer advantages to UPEC within host urinary tract and are discussed in this chapter.

Incidence of UTIs is around four-times higher in women compared to men and this difference is mainly attributed to the shorter length of urethra and a shorter distance between anus and urethral opening in women (3). Sexual activity is the primary risk factor reported in patients with uncomplicated UTIs. Almost one in every two adult women (40%) will be affected by cystitis during their lifetime and there is a 25% risk for the development of recurrent UTI within the next year (4). In the United States alone, UTIs led to an estimated 11 million physician visits, 1.7 million emergency room visits and 470,000 hospitalizations in 2006, at an estimated cost of \$3.5 billion (5, 6). Better understanding of clinically relevant virulence factors is required for developing novel therapeutic and prophylactic strategies that will take us one step closer towards reducing the morbidity, loss of productivity, and healthcare costs associated with UPEC UTIs.

Most cases of uncomplicated UTIs are community-acquired and gut colonization precedes access to the urinary tract. UPEC colonizes the perineal region, followed by ascent along the urethra and subsequent colonization of the bladder resulting in cystitis (7). UPEC is adept at orchestrating two completely opposite phenomena, adhesion and motility, for successful colonization and ascending infection. Both extracellular and intracellular lifestyles are exhibited by UPEC within the urinary bladder (8–10) and intracellular growth phase is proposed to promote persistent infection of the urinary bladder. Within superficial facet cells of the urinary bladder, some strains of UPEC form intracellular bacterial communities (IBCs).

UPEC utilizes numerous virulence and fitness factors to gain entry, adhere, acquire essential nutrients, multiply in a hostile environment, cause tissue damage, and disseminate within the urinary tract. Fitness and virulence factors are related but distinct entities: factors that contribute to UPEC survival in a given niche are described as fitness factors and genes that satisfy molecular Koch's postulates are defined as virulence genes. Tenets of molecular Koch's postulates include the presence of a gene in the pathogenic members of a species, mutants lacking that gene should display a measurable loss of pathogenicity or virulence, and complementation with the wild-type copy of the gene should restore wild-type levels of pathogenicity or virulence (11). Here, we review the role of fitness and virulence factors in the pathobiology of urinary tract infections caused by UPEC. This book also contains chapters dedicated to specific virulence factors and readers will be directed to those chapters for additional information.

NUTRIENT REQUIREMENTS

Growth in Urine

Urine is a high osmolar, dilute mixture of amino acids and peptides. Continuous inflow of urine from the kidneys to the bladder via ureters results in relatively steady levels of

nutrients in urine. Several groups use urine as a culture medium for UPEC in an attempt to recapitulate conditions encountered within a human host, at least in part. Although urine is rich in amino acids and peptides, amino acid biosynthetic capacity appears to be important for growth in urine. Specifically, UPEC mutants auxotrophic for arginine, glutamine, or guanine exhibit a severe growth defect in human urine and leucine, methionine, serine, phenylalanine, or proline auxotrophs display a reduced growth rate (12). Auxotrophs are mutants that lack the biosynthetic capacity for a particular nutrient; in this study, chemical mutagenesis was used for generating the mutants defective in amino acid biosynthetic pathways. A recent study with defined mutants generated by allelic exchange revealed that mutants auxotrophic for arginine and serine did not exhibit growth defects in human urine *in vitro* or in a mouse model of UTI (13). However, whether this is true for mutants auxotrophic for other amino acids remains to be tested. Transcriptional profiling experiments revealed that genes involved in amino acid and carbohydrate metabolism are among the genes significantly up-regulated during growth in human urine (14). Strain-specific differences in generation time during growth in human urine have been observed, indicating that in addition to growth in urine, there are other factors that are collectively required for urofitness. ABU 83972, an asymptomatic bacteriuria strain, exhibits rapid growth in urine and outcompetes UPEC strains such as 536, CFT073, NU14, and UTI89 in human urine (15). Additionally, ABU 83972 outcompetes NU14 in mouse bladder (15). It is possible that rapid growth can compensate for the lack of specific adherence organelles and ensure persistence within host urinary tract for at least some strains.

Competition for Metals

Metals such as iron, zinc, and manganese are essential nutrients for most forms of life, including bacteria. Mammalian immune system exploits the essentiality of metal acquisition to retard the growth of bacteria during infection. This concept is known as nutritional immunity and is part of innate immune response activated during bacterial infection (16). Here we review the mechanisms used by UPEC to surmount sequestration of iron and zinc within host urinary tract.

Iron Acquisition

Although iron is one of the most abundant metals in the earth's crust, pathogens encounter iron limitation within hosts. Indeed, iron limitation is one of the innate defenses against survival of bacteria within hosts (17), where elemental iron is bound by host glycoproteins such as transferrin and lactoferrin or incorporated into the heme moieties of proteins such as hemoglobin and myoglobin. To ensure adequate levels of intracellular iron, UPEC up-regulates the expression of genes involved in iron acquisition in response to iron limitation encountered within the mammalian urinary tract (14, 18, 19).

UPEC can acquire ferric iron using siderophores, ferrous iron through iron transporters, and heme through outer-membrane heme receptors. A common player involved in iron uptake through all these mechanisms is the inner membrane-associated TonB-ExbB-ExbD complex. ExbB and ExbD proteins energize TonB with the proton-motive force generated at the inner membrane and TonB transduces this energy to the outer-membrane iron-receptor complexes leading to their translocation to the periplasm (Fig. 1). Iron-containing complexes

are bound to binding proteins within the periplasm and are transported across the inner membrane via ABC transporters using adenosine triphosphate (ATP) as an energy source (Fig. 1). A UPEC mutant lacking the *tonB* gene is attenuated in a mouse model of UTI, demonstrating the importance of TonB-mediated iron uptake within the urinary tract (20). A high-throughput screen devised to identify inhibitors of iron uptake systems in UPEC CFT073 has revealed novel small-molecule inhibitors of TonB (21).

Regulation of Iron Uptake

Excess levels of intracellular iron are detrimental to a bacterial cell and therefore iron homeostasis is subject to rigorous regulation. The ferric-uptake regulator (Fur) regulates the expression of genes involved in iron uptake and metabolism at transcriptional level (Fig. 1). Iron-containing Fur homodimer binds to 19-base pair inverted repeats, known as the “Fur boxes”, in the promoter region of Fur-regulated genes, such as siderophore biosynthesis and uptake systems and heme-uptake systems, and represses their transcription (22). When the intracellular levels of iron are low, Fur-mediated repression is relieved due to low affinity of free Fur for Fur boxes and derepression of iron-uptake systems facilitates iron acquisition (Fig. 1). Fur, however, is also known to positively regulate the expression of some genes including *acnA*, *ftnA*, *fumA*, and *sdhCDAB* (23). This paradoxical observation was reconciled after the identification of a Fur-regulated small RNA, RyhB; RyhB acts as a negative post-transcriptional regulator for some genes whose protein products either require iron as a cofactor or are involved in iron storage (23). The expression of *ryhB* is subject to negative regulation by Fur. Small-RNA-mediated regulation adds an additional layer of complexity to the regulatory scheme controlling iron homeostasis.

Siderophores

Siderophores are low-molecular-mass, high-affinity, iron-chelating molecules released into the environment by several bacteria, including UPEC. Siderophores bind ferric iron and iron-siderophore complexes are recognized by cognate outer-membrane receptors (Fig. 1). UPEC strains encode the proteins required for the biosynthesis and uptake of the following siderophores: aerobactin, enterobactin, salmochelin, and yersiniabactin. Some UPEC strains such as 536 and UTI89 produce and utilize yersiniabactin whereas another prototypical strain, CFT073, lacks the biosynthetic capacity to produce yersiniabactin. Enterobactin and salmochelin belong to the catecholate family whereas aerobactin and yersiniabactin are members of hydroxamate and five-member heterocyclic ring-containing siderophores, respectively. Independent of siderophore activity, yersiniabactin also binds copper and mitigates copper toxicity (24).

Intense competition between bacteria and host for iron is exemplified by the fact that mammalian hosts produce specific proteins that bind siderophores and prevent the reuptake of iron-siderophore complexes by bacteria. As part of innate immune response, neutrophils produce lipocalin-2 (25), a molecule that binds enterobactin and prevents enterobactin-mediated iron uptake by bacteria (26). Bacterial pathogens, including UPEC and *Salmonella enterica* subspecies Enterica, overcome this barrier by using salmochelin, a glycosylated derivative of enterobactin, which is not recognized by lipocalin-2 (27).

Production of siderophores and their correlation with the presence of genes encoding siderophore biosynthetic pathways in prototypical UPEC strains have been characterized using a quantitative metabolomics approach (28). UPEC isolates from urine and *E. coli* from corresponding rectal samples were tested for siderophore production. Enterobactin production was identical in strains isolated from both urinary tract and rectum indicating that enterobactin system does not respond to specific cues sensed by UPEC within the human urinary tract. However, salmochelin and yersiniabactin were both produced at significantly higher levels in UPEC isolated from urine compared to rectal isolates and co-expression of salmochelin and yersiniabactin siderophores were observed, indicating that these two siderophores might play a greater role compared to enterobactin during iron acquisition within the human urinary tract (28).

Receptor-Mediated Heme Uptake

Heme is an abundant source of iron within mammalian hosts. Receptor-mediated uptake of heme is another iron-uptake mechanism utilized by UPEC. Two outer-membrane heme receptors, ChuA and Hma, have been characterized (Fig. 1). Functional homologues of ChuA are found in enterohemorrhagic *E. coli* and *Shigella dysenteriae* whereas Hma appears to be a UPEC-specific heme receptor. Genes encoding both ChuA and Hma are expressed during growth under iron-limited conditions, including in human urine and during experimental mouse UTI (29, 30). Mutants lacking ChuA are out-competed by mutants lacking Hma, indicating that ChuA is the favored heme receptor *in vivo*. Nevertheless, both ChuA and Hma are required for optimal fitness in a mouse model of UTI (30). ChuA also appears to be important during IBC formation by UPEC; expression of *chuA* was upregulated during IBC formation within bladder epithelial cells and a *chuA* mutant forms significantly smaller IBCs compared to parent strain (31). In combination, these studies imply that the ability to acquire iron from heme is an important *in vivo* fitness factor.

Redundant Iron Acquisition Systems

Contribution of the apparently redundant iron-uptake systems to fitness was tested in a mouse model of ascending UTI (32). Mutants lacking *fepA*, *iha*, and *iroN* genes (encoding cognate receptors for catecholate siderophores) displayed similar fitness indices within the murine urinary tract indicating that they are a functionally redundant group. Aerobactin-receptor mutant and yersiniabactin-receptor mutant were both out-competed by mutants lacking either hydroxamate-siderophore receptors or catecholate-siderophore receptors, respectively. Aerobactin and yersiniabactin receptors contribute significantly to fitness *in vivo* indicating that these two systems are critical for uropathogenesis. Additionally, the *hma chuA* double mutant, defective in heme/hemin uptake, is out-competed by mutants lacking catecholate or hydroxamate receptors only within the kidneys (32). Taken together, these data demonstrate that despite being redundant, individual iron-uptake systems appear to play a key role within specific regions of the urinary tract.

Ferrous Iron Uptake

Manganese and ferrous iron are transported into the cytoplasm by the Sit system, comprised of SitA, SitB, SitC, and SitD proteins. In *Salmonella enterica* serovar Typhimurium, the *sit*

genes are regulated by intracellular levels of both iron and manganese via corresponding metalloregulators, Fur and MntR, respectively (33). The *sit* genes are up-regulated during murine UTI and are expressed during human UTIs (14, 19). Several UPEC strains, including CFT073, contain the Sit system. Cystitis isolate F11 utilizes FetMP, a bipartite system to acquire ferrous iron (34). The contribution of ferrous iron-uptake systems to urofitness remains to be evaluated.

Iron Receptors as Vaccine Candidates

Several independent lines of evidence indicate iron limitation within urinary tract: genes involved in iron uptake are highly expressed during murine and human UTIs; iron receptors are found at higher levels during growth in human urine; iron-uptake receptors can be detected with antisera from mice with chronic UTI; and a subset of iron receptors are more prevalent in UPEC strains compared to commensal *E. coli* strains (14, 18, 19, 29, 35). Since iron uptake is clearly required for successful infection, the potential of iron-uptake receptors as vaccine candidates was evaluated. Outer-membrane iron receptors FyuA, Hma, Iha, IreA, IroN, and IutA, were tested as vaccine candidates against UPEC infection in a murine model (35, 36). Purified proteins were cross-linked to cholera toxin, an adjuvant, and administered intranasally. Mice immunized with Hma, IreA, and IutA displayed significantly higher protection upon challenge infection in kidneys, bladder, and kidneys and bladder, respectively, compared to adjuvant-only controls. Vaccination with FyuA protected mice against UPEC only in the kidneys. Furthermore, vaccination with Hma and IreA protected mice during challenge with a heterologous strain of UPEC. Protective antigens Hma, IreA, and IutA demonstrated significantly higher serum immunoglobulin (Ig)G levels post challenge, compared to ChuA and Iha, antigens that did not confer protection. Antigen-specific IgA was found only in the urine of mice vaccinated with IreA and IutA, both protective antigens. Taken together, specific iron-uptake receptors appear to confer protective immunity in a mouse model of UTI and are certainly among the top candidates for developing a subunit vaccine against UTIs caused by UPEC.

Zinc Acquisition

Zinc is an essential nutrient involved in a gamut of cellular functions including iron homeostasis and resistance to oxidative stress. Neutrophil-derived protein calprotectin is involved in sequestration of zinc and manganese and stifles the growth of bacterial pathogens within mammalian hosts (16). UPEC strain CFT073 encodes at least two different zinc-transport systems encoded by *znuACB* and *zupT* (37). The ZnuACB system is an ABC transporter similar to the SitABCD system that is involved in the acquisition of ferrous iron and manganese. Zinc uptake through the ZupT system is conserved among bacteria and eukaryotes and, in addition to zinc, this system can also transport cobalt, iron, and manganese.

ZnuACB system is required for wild-type levels of growth in minimal medium, whereas ZupT system is dispensable during growth under zinc-limiting conditions; this observation suggests that there are redundant zinc-transport systems albeit, with varying levels of expression and/or affinity to zinc. Furthermore, mutants devoid of either ZnuACB or both ZnuACB and ZupT revealed fitness defect in a murine model of UTI indicating that the

ability to acquire zinc is critical for survival within urinary tract (37). Human urine does not appear to be a zinc-limited milieu because growth of mutants defective in zinc uptake is indistinguishable from wild-type strain during growth in urine. This observation, however, does not exclude the possibility that zinc levels in urine could be drastically altered during massive influx of neutrophils observed during UTIs. Zinc is required for defenses against oxidative stress both directly and indirectly. Indirectly, reduced levels of zinc results in the derepression of Fur-regulated genes resulting in increased intracellular levels of iron, which exacerbates the damage inflicted during oxidative stress. Not surprisingly, mutants lacking ZnuACB exhibit higher sensitivity to oxidative stress. It would be interesting to determine the protection, if any, offered by zinc-uptake receptors alone or in combination with iron-uptake receptors in a composite subunit vaccine against UPEC.

METABOLISM

Since many metabolic functions are not pathogen-specific, the role of metabolic potential of pathogens in fitness during infections remains underappreciated. Thorough understanding of the metabolic state of a pathogen is, however, of paramount importance to define the microenvironment at the site of infection and thereby facilitate the identification of novel antimicrobial agents that target specific metabolic pathways.

Proteins involved in the transport of short peptides, transport and catabolism of sialic acid, gluconate, xylose, and arabinose, and biosynthesis of arginine and serine are highly expressed in UPEC cultured in human urine (Fig. 2) (13). Despite the fact that arginine and serine biosynthetic proteins are selectively expressed during growth in urine, arginine and serine auxotrophs did not exhibit any fitness defect in a mouse model of UTI. However, mutants defective in the uptake of oligopeptides and dipeptides were outcompeted by the wild-type strain, indicating that peptide uptake is critical for fitness of UPEC during infection. Mutants lacking enzymes in the citric-acid cycle and gluconeogenesis exhibit fitness defect in both bladder and kidneys (13). Defects in Entner-Doudoroff pathway, glycolysis, or pentose-phosphate pathway, however, did not affect *in vivo* fitness. In combination, these findings assert the idea that amino acids and peptides are the primary carbon sources for UPEC within the urinary tract (Fig. 2).

D-serine is one of the most abundant amino acids found in human urine. A UPEC CFT073 mutant lacking the D-serine deaminase gene (*dsdA*) required for D-serine catabolism, revealed a long lag phase when cultured in human urine. Surprisingly, the mutant exhibits a hypercolonization phenotype, manifested by increased fitness during co-infection with parental strain, in a mouse model of UTI (38). Additionally, the *dsdA* mutant is hypermotile and hyperflagellated compared to parental strain (Fig. 3). A *dsdA fliC* double mutant lacking flagella loses the fitness advantage, indicating that hypervirulence phenotype of *dsdA* mutant is mediated by excessive production of flagella. In addition to flagella, hemolysin A, and both P fimbriae (CFT073 strain contains two P fimbrial-gene clusters) contribute to the hypervirulence phenotype of *dsdA* mutant, albeit in a non-redundant manner (39).

Serine is deaminated to produce ammonia and pyruvate by enantiomer-specific (D/L) serine deaminases. Catabolism of pyruvate leads to the production of acetyl coenzyme A (CoA);

acetyl CoA is converted to acetyl phosphate by phosphotransacetylase (Pta) and acetate kinase (AckA) catalyzes the conversion of acetyl phosphate to acetate (40). Due to its phosphate-donor role, acetyl phosphate acts as an intracellular signal that causes genome-wide changes in gene expression (41). Acetate is excreted into the medium and reuptake of acetate occurs only after depletion of easily assimilable carbon sources. The hypervirulent phenotype of D-serine deaminase (DsdA)-defective mutants was abrogated when L-serine deaminases (SdaA and SdaB) were also inactivated in CFT073 (40). Indeed, a mutant defective in catabolizing both D and L forms of serine displayed a fitness defect in the urinary tract. Genes up-regulated in UPEC upon exposure to D-serine during growth in urine includes *pta* and *ackA*. The mutant defective in reuptake of acetate, however, does not exhibit a fitness defect (40). Taken together, these data demonstrate that UPEC produces acetate during growth in urine and acetogenic potential contributes to *in vivo* fitness, but acetate reuptake does not affect *in vivo* fitness.

SURFACE STRUCTURES

Fimbriae

Fimbriae or pili are short filamentous organelles used by bacteria to adhere to various surfaces. In general, UPEC strains carry significantly higher number of fimbrial gene clusters compared to fecal/commensal strains (42). The genome of a prototypical pyelonephritis strain, CFT073, contains 12 fimbrial gene clusters: 10 belong to the chaperone-ushe family, including type 1, two complete P, F1C, F9, and Auf fimbriae and 2 putative-type IV pili (*c2394-c2395* and *ppdD-hofBC*) (43). The chaperone-ushe fimbrial-biogenesis pathway utilizes a periplasmic chaperone that binds and assists folding of the fimbrial subunits. Chaperone-fimbrial subunit complexes are targeted to the outer membrane, where the usher forms a pore through which pilus subunits are exported and assembled. Type IV pilus biogenesis requires a functional type II-secretion system and both type IV pilus gene clusters are involved in fitness during UTI (44, 45).

Type 1 and P fimbria, both assembled by the chaperone-ushe pathway, are among the best characterized bacterial fimbriae. While type 1 fimbrial genes are found in the fecal/commensal backbone of the *E. coli* genome, P fimbrial genes are exclusively found in pathogenicity islands distributed among UPEC strains. Additionally, there is evidence for regulatory cross-talk between these two fimbrial types; constitutive expression of type 1 fimbria leads to down-regulation of P fimbrial gene expression (46). A brief review of fimbriae found in UPEC is included here (reviewed in 124).

Type 1 Fimbria

Type 1 fimbria mediates mannose-sensitive agglutination of guinea pig erythrocytes. Uroplakin, a highly mannosylated protein found in the uroepithelial cells is the receptor for type 1 fimbria (47). Specifically, FimH (type 1 fimbrial-tip adhesin) mediates interaction with D-mannose on glycoproteins. Type 1 fimbria is required to cause cystitis and development of IBCs in a mouse model of UTI (48–51). Within renal tubules of live rats, type 1 fimbriae promote interbacterial binding resulting in biofilm-like communities within renal tubules that cause obstruction of tubular flow (52).

Expression of type 1 fimbria is regulated by an invertible element carrying the promoter and is flanked by inverted repeats. FimB and FimE are site-specific recombinases that change the orientation of the promoter from ON-to-OFF/OFF-to-ON and ON-to-OFF, respectively. Several global regulatory proteins, such as lipoprotein receptor-related proteins (Lrp), histone-like nucleoid-structuring (H-NS), integration host factor (IHF), and cyclic adenosine monophosphate (cAMP) receptor protein (CRP)/cAMP, modulate the expression of type 1 fimbrial genes by controlling the orientation of the promoter region (53). For instance, CRP acts as a repressor of type 1 fimbrial gene expression by negatively regulating *lrp* resulting in reduced FimB-mediated recombination that flips the promoter from OFF-to-ON orientation. Additionally, CRP enhances the activity of DNA gyrase leading to OFF orientation of the promoter. Thus, expression of type 1 fimbrial genes is coordinately regulated by multiple regulatory inputs converging on recombinases that control the orientation of the promoter.

P Fimbria

UPEC are endowed with a unique type of pili encoded by the *pap* genes that are known as pyelonephritis-associated pili or P fimbriae. P fimbriae are required for mannose-resistant agglutination of human erythrocytes and PapG is the fimbrial-tip adhesin that renders specificity to P fimbriae-mediated adherence. PapG binds to glycosphingolipids containing digalactoside moieties found in the renal epithelium, specifically the P blood-group antigen (54). Although P fimbriae are associated with adherence to renal cells, their contribution to the virulence of UPEC remains equivocal (55–57). P fimbriae are required for attachment of UPEC to Bowman's capsule in a live animal. P fimbriae-mediated adhesion minimizes the impact of shear stress induced by the flow of primary filtrate within Bowman's space (52). In addition to its role in adherence, P fimbriae are also involved in regulating motility. PapX, a nonstructural gene found in the P fimbrial gene cluster, is a critical player in the crosstalk between fimbria-mediated adherence and flagella-mediated motility in UPEC (58).

Expression of P fimbrial genes is regulated by a methylation-dependent epigenetic switch (59). Competition between Lrp and deoxyadenosine methylase (Dam) methylase for binding sites at the *papBA* promoter determines the status (ON/OFF) of the switch. Methylation of the guanine, adenine, thymine, cytosine (GATC) site closer to the *papB* gene leads to the expression of P fimbrial genes; in contrast, Lrp binding to this site precludes methylation resulting in transcriptional repression. Once the switch is turned ON, there is an innate mechanism that strives to sustain the ON phase. PapB activates the transcription of *papI* and PapI promotes Lrp binding to distal GATC site facilitating the expression of P fimbrial genes. In summary, expression of both type 1 fimbria and P fimbria are tightly regulated in UPEC.

Other fimbrial gene clusters found in UPEC strains include Auf, Dr, F1C, F9, Pix, S, Yad, Yeh, Yfc, and Ygi. Dr fimbria agglutinates human erythrocytes and promotes renal tropism by binding to the basement membrane of renal tubular epithelium and Bowman's capsule (60). F1C fimbria binds to glycosphingolipids found in kidney cells and induces the production of IL-8, a proinflammatory cytokine (61). IL-8 production is independent of LPS-mediated TLR4 signaling because that signaling cascade is absent in renal cells. F1C

fimbria was highly expressed in a strain lacking both type 1 and P fimbriae suggesting cross-talk between regulatory proteins that affect expression of various fimbriae (46). S fimbria mediates UPEC binding to primary human renal proximal-tubular cells by binding to α -sialyl-2-3- β -galactoside and S fimbrial gene clusters are more frequently found in *E. coli* isolated from patients with neonatal sepsis or meningitis (62, 63). Pix pilus is a filamentous adhesin capable of agglutinating human erythrocytes (64). Recently, Ygi fimbria was demonstrated to be involved in adherence to human kidney cells and a mutant lacking this fimbria exhibits a fitness defect in a mouse model of UTI (42). Yad fimbria mediates adherence to human bladder cells but does not appear to contribute to fitness, at least independent of other fimbriae. The ability to produce multiple fimbria appears to confer selective advantage within a particular niche and active cross-talk is apparent between regulators of various fimbrial types.

Pilicides

Agents that specifically target virulence factors without affecting the survival of bacterial pathogens have been proposed as a promising alternative for conventional antimicrobial compounds (65). Since both type 1 and P pili are implicated in the pathogenesis of UTIs, chemical inhibitors of pili biogenesis (pilicides) were developed. Pilicides in the 2-bicyclic-pyrimidone family inhibit the production of both type 1 and P pili in UPEC and are potential candidates for development as therapeutic agents (66).

Motility

The ascending nature of most cases of UTI led to a speculation that flagella might be a critical virulence factor for UPEC. Two groups independently determined that possession of flagella confers a significant fitness advantage to UPEC. Lane et al. (67) constructed aflagellate, flagellate but hypomotile, flagellate but nonmotile, and flagellate, motile but non-chemotactic, mutant strains of UPEC CFT073 and tested the contribution of motility and chemotaxis to virulence in a mouse model of ascending UTI. Flagellum-mediated motility was not required for successful colonization during individual infections indicating that this is not an urovirulence trait. Nevertheless, flagellum-mediated motility increased the fitness of mutant strains during mixed infections demonstrating that this is an important fitness factor. A UPEC mutant lacking flagella did not exhibit a defect in forming IBCs within mouse bladder-facet cells compared to the wild-type strain (68). Additionally, AL511 (pyelonephritis strain) invades mouse renal collecting-duct epithelial cells utilizing a flagella and motility-driven mechanism (69). This finding demonstrates that flagella-dependent invasion might be cell type-specific and might contribute to breaching the renal epithelial barrier to gain access to the bloodstream.

An *in vivo* imaging approach was utilized to determine temporo-spatial pattern of flagellar gene expression within the murine urinary tract (70). A luciferase reporter system was used, where expression of *fliC* gene was detected and measured by a corresponding increase in light production. Maximal expression of flagellar genes is coincident with UPEC ascension into ureters and kidneys, suggesting a role for flagella in the development of pyelonephritis. During the early stages of infection, the *fliC* mutant was defective in kidney colonization

despite successful colonization of the bladder, indicating an advantage conferred by flagella on the progression of ascending UTI.

Chemotaxis

The ability to sense and respond to chemical signals by altering motility is known as chemotaxis and bacteria use flagellum-mediated motility to respond to chemotactic cues. Since most UTIs arise from colonization of periurethral region with UPEC strains originating from the gastrointestinal tract, it is possible that UPEC strains could exhibit enhanced chemotactic behavior towards urine compared to fecal-commensal strains. *E. coli* strains express chemotactic genes *tap*, *tar*, *tsr*, and *trg* that respond to dipeptides, aspartate and maltose, serine, and ribose and galactose, respectively. The UPEC strains CFT073 and F11, however, lack *tap* and *trg* genes whereas the fecal/commensal strains harbor all 4 genes. Interestingly, both UPEC and fecal-commensal strains displayed a similar degree of chemotaxis towards urine, indicating that this is not an uropathogen-specific trait (71).

Motility at Population Level

Pyelonephritogenic strains ascend to the kidneys via ureters whereas cystitis strains remain within the bladder. Understanding the population dynamics involved in this phenomenon is important to understand how UPEC causes a spectrum of conditions, including cystitis, pyelonephritis, bacteremia, and sepsis. Walters et al. (72) used a collection of CFT073 strains that harbor unique traceable sequence tags to infect mice transurethrally and assessed the distribution of individual UPEC strain as a proportion of the population in bladders, kidneys, and spleen. Ascension to kidneys and entry into the bloodstream appear to result from multiple rounds of dissemination; progression from pyelonephritis to bacteremia represents a major bottleneck in the progression of pyelonephritis to sepsis.

Interplay Between Adherence and Motility

Motility, mediated primarily by flagella, and adherence, mediated primarily by fimbriae, represent two opposing phenomenon, yet both have been shown to contribute to uropathogenesis. Understanding how UPEC regulates these two phenomena is therefore crucial for better understanding of UTIs. Lane and coworkers demonstrated that constitutive expression of type 1 fimbria negatively affects motility and the levels of flagellin (Fig. 3), the major structural subunit of bacterial flagella (73). Conversely, overexpression of flagellar proteins or hypermotility phenotype did not alter the expression of type 1 fimbria. These results indicate that the expression of type 1 fimbria exerts a negative, unidirectional effect on flagellum-mediated motility.

Inhibitor of Motility, PapX

To understand the complex relationship between type 1 fimbrial gene expression and flagella, a transposon mutagenesis screen was conducted in a strain constitutively expressing type 1 fimbria to identify mutants that regained motility phenotype (74). This screen led to the identification of *papX* gene as a negative regulator of motility in UPEC (Fig. 3). Although the UPEC strain CFT073 has two gene clusters encoding the production of P fimbria, only the *pap1* gene cluster located on the PheV-associated pathogenicity island

contains *papX*. PapX was previously shown to inhibit bacterial motility and is a functional homolog of MrpJ, another bacterial motility inhibitor, found in *Proteus mirabilis* (75). PapX binds directly to the *flhDC* promoter, thereby inhibiting the transcription of master regulator of flagellar biosynthesis, motility and chemotaxis (58, 76). Overexpression of PapX led to reduced transcription of the genes involved in flagellar biosynthesis and chemotaxis; predictably, lack of PapX led to an increase in transcription of the same set of genes. Recently, the PapX binding site at the *flhD* promoter region was mapped to a 29-base-pair site upstream of the *flhDC* translational start site (76, 77).

TOXINS

UPEC strains are known to elaborate α -hemolysin, cytotoxic necrotizing factor-1 (CNF-1), secreted auto-transporter toxin (Sat), and protease involved in colonization (Pic) (78–87). Hemolysin A (HlyA) is one of the best characterized pore-forming toxins and is the founding member of the repeats in toxin (RTX) family of toxins. HlyA contains the characteristic glycine-rich nonapeptide repeats in the C-terminal domain. The *hlyCABD* operon encodes the proteins involved in the production, activation and export of HlyA. The toxin is encoded by *hlyA* gene and *hlyC* gene encodes an acyl transferase that is required for activation of the toxin. HlyA is secreted via type 1-secretion system. HlyB and HlyD act in concert with TolC, an outer membrane protein, for energy-dependent secretion of HlyA. HlyA is not only a hemolysin but also exhibits toxicity towards other cell types including leukocytes.

Functional genomic analysis of IBCs revealed that *hlyA* is among the genes highly expressed during UPEC growth within bladder cells (31). In addition to its cytolytic activity, HlyA is also involved in the communication between host and pathogen. HlyA degrades paxillin, a cytoskeletal scaffold protein, and other proteins involved in nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B)-signaling cascade (81). This study elucidated the role of HlyA in the exfoliation of bladder epithelial cells during UPEC UTIs. By interfering with NF- κ B-mediated proinflammatory-signaling pathway, HlyA dampens host immune response to infection. While most UPEC strains carry one copy of the hemolysin operon, pyelonephritogenic strains 536 and J96 harbor two copies of the *hly* operon and both loci are required for the full virulence (82). However, there is preferential expression of one locus over the other and selective expression could provide distinct advantages within different niches.

CNF-1 is elaborated by some UPEC isolates and it deamidates small Rho guanosine triphosphatases (GTPases) including RhoA and Rac1, resulting in constitutive activation of these proteins. Rho GTPases are critical for several cellular processes including phagocytosis and oxidative burst in neutrophils. A mutant lacking CNF-1 is outcompeted by the wild-type strain in a murine model of UTI, clearly indicating that CNF-1 enhances the fitness of UPEC during UTI (79). Individual infection with the CNF-1-deficient mutant induces less severe inflammation but the mutant colonizes at levels comparable to wild-type strain. CNF-1-mediated disruption of Rho GTPase signaling pathways leads to immune dysregulation and production of CNF-1 confers an advantage during survival in the presence of neutrophils (83).

Autotransporter Toxins

Pic, secreted autotransporter toxin (Sat), and temperature-sensitive hemagglutinin (Tsh) are members of the SPATE (serine-protease autotransporter toxins of *Enterobacteriaceae*) family of proteins elaborated by UPEC. Sat induces vacuolation in proximal renal-tubule cells and serine-protease activity is required for cytopathic effects of Sat (84, 85). However, Sat does not contribute to fitness of UPEC in a mouse model of ascending UTI. Pic is expressed during UPEC infection in a murine model and is a potential mucus secretagogue (86, 87). By targeting major human leukocyte-adhesion molecules CD43, CD44, CD45, and CD93, Pic deregulates leukocyte migration and inflammation (80). Tsh does not display serine-protease activity (86) and appears to be more closely related to Vat, a vacuolating autotransporter toxin found in avian pathogenic *E. coli*. In summary, toxins produced by UPEC not only inflict tissue damage but are also involved in the communication between pathogen and host.

BIOFILM

Biofilms are sessile, complex, differentiated, multicellular, bacterial communities that may be populated by a single or multiple species. A prominent feature that distinguishes biofilm from planktonic cells is the presence of extracellular matrix that could be composed of proteins, polysaccharides, and/or DNA. The composition of biofilm matrix differs between various species of bacteria; proteins (curli and fimbriae) and polysaccharides (cellulose, colonic acid, and poly-N-acetyl glucosamine) are found in the matrix of biofilms formed by *E. coli*. Cells in a biofilm exhibit higher degree of antibiotic resistance compared to planktonic cells, and biofilms formed on in-dwelling devices such as urinary catheters pose a huge problem in healthcare settings. Therefore, there is a considerable interest in understanding the biology of biofilm formation in UPEC. By screening a transposon-mutant library, multiple genes involved in biofilm formation were identified in the cystitis strain UTI89 (88). A subset of the genes involved in biofilm formation is also implicated in colonization of mouse urinary bladders.

Curli, amyloid-like bacterial proteins, are produced by several members of the family Enterobacteriaceae including UPEC. These complex insoluble proteins form the extracellular matrix that acts as a scaffold for cells during biofilm development. Curli are found at detectable levels in human UTI urine samples and promotes adherence of UPEC to epithelial cells derived from both human bladder and kidneys (89). Curliated strains induce production of proinflammatory cytokine IL-8 and exhibit higher levels of resistance to an antimicrobial peptide, cathelicidin (LL-37). LL-37 inhibits polymerization of curli-specific gene (Csg)A, structural subunit of curli fibers, and prevents curli-mediated adherence and biofilm formation *in vitro*. Chemical inhibitors of curli biogenesis, curlicides, were sought as a novel treatment option for UPEC (90). Molecules belonging to the ring-fused 2-pyridone class were detected to display bifunctional curlicide and pilicide activity. These findings shed light on the potential role of curli in the pathogenesis of UTIs.

UPEC CFT073 encodes a gene cluster (*c1931–c1936*) capable of producing detectable fimbria, known as F9 fimbria (91). *E. coli* K-12 strains overexpressing F9 fimbria produce denser biofilm on polystyrene surfaces than control strain. However, F9 fimbria does not

seem to be involved in hemagglutination or adherence to epithelial cells. It must be noted that several studies on biofilm formation are conducted under conditions vastly different from the microenvironment within host urinary tract and therefore results must be interpreted with due diligence.

Gene Expression Within UPEC Biofilms

Genes involved in the biogenesis of type 1, P, and F1C fimbriae are down-regulated in CFT073 biofilms grown in human urine, indicating that these fimbriae are not critical for biofilm formation, at least during growth in urine. In contrast, *Auf* fimbrial genes and *Yad* fimbrial genes are up-regulated, suggesting that these fimbriae could act as determinants for biofilm mode of growth in human urine (92). Transcriptional profiles of asymptomatic bacteriuria strain 83972 in biofilm, planktonic cells, and human volunteer colonization studies were compared to delineate biofilm-specific changes in gene expression (93). These studies elucidate the differences in expression of surface structures that might be involved in biofilm formation of various UPEC and ABU strains under specific conditions, such as growth in urine *in vitro* or during colonization of human bladder.

Regulation of Biofilm Formation

RfaH is an *E. coli* transcriptional antiterminator protein that affects the expression of genes involved in lipopolysaccharide biosynthesis, capsule production, hemolysin A, and CNF-I. Loss of *rfaH* leads to an increased biofilm phenotype in UPEC strain 536 and *E. coli* K-12. Upregulation of the *flu* gene that encodes antigen 43, a protein involved in autoaggregation and biofilm formation was observed in K-12 *rfaH* mutant. However, a precise mechanism for the regulation of biofilm formation by RfaH in UPEC 536 is yet to emerge (94). Nitric oxide-sensitive transcriptional repressor, NsrR, is yet another regulator of biofilm formation in UPEC. A mutant lacking NsrR displayed significantly reduced surface-attached growth and this effect might be mediated by derepression of NsrR-repressed genes involved in flagellar biogenesis and motility (95).

Inhibition Biofilm Formation

Prototypical UPEC strain CFT073 and all *E. coli* strains producing a group II type of capsule release a soluble high-molecular-weight polysaccharide into spent medium that reduces biofilm formation by a broad range of pathogens including *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *S. epidermidis*. Although it is not clear how capsular polysaccharides that are covalently attached to the membrane are released into the medium, this phenomenon could be harnessed to develop a coating material for catheters to minimize biofilm development (96).

OUTER MEMBRANE PROTEINS

Autotransporter Proteins

UPEC encodes several autotransporter (AT) proteins that are typically 70 to 100 amino acids in length, with a C-terminal membrane anchor region that forms the β -barrel through which the passenger domain is translocated to the cell surface; the latter phenomenon lends the name for this class of secretion system as AT proteins. AT proteins are also referred to as

type V-secretion system in Gram-negative bacteria and some AT proteins affect adherence and biofilm formation.

Urokinase-type plasminogen activator (Upa)B and UpaC are among several AT proteins found in UPEC (97). However, they are not uropathogen-specific, as their homologs are widely distributed among various strains of *E. coli*. UpaC does not affect the fitness of UPEC in a mouse model of UTI. However, UpaC promotes adherence to abiotic surfaces and biofilm formation. H-NS was identified as a transcriptional repressor of the *upaC* gene. UpaB promotes adherence to biotic surfaces such as components of extracellular matrix, including fibronectin, fibrinogen, and laminin. An isogenic mutant lacking UpaB displayed reduced fitness in murine bladders suggesting that UpaB-mediated adherence might be favorable for fitness *in vivo*. UpaG is a UPEC trimeric-autotransporter protein that promotes biofilm formation on abiotic surfaces and facilitates binding to extracellular matrix proteins, fibronectin and laminin (98).

Antigen 43 is an outer-membrane protein encoded by the *flu* gene that is widely distributed among *E. coli* strains, including UPEC and is subject to phase variation. Prototypical UPEC strain CFT073 contains two variant copies of this gene that were designated as *ag43a* and *ag43b*. Ag43a increased autoaggregation and biofilm formation on abiotic surfaces, when expressed in K-12 genetic background. A CFT073 strain overexpressing *ag43a* was more successful at persistent colonization for up to 5 days post inoculation, in a mouse model of UTI. Furthermore, it was demonstrated that in the presence of type 1 fimbriae, which prevents intimate bacterial cell-cell contact, the effect of Ag43 on autoaggregation and biofilm formation is diminished (99). Immunofluorescence analyses revealed that Ag43 is expressed within IBCs and is therefore proposed to play a role in the intracellular growth phase of UPEC (100). Taken together, these observations support a model in which Ag43-mediated autoaggregation and biofilm formation are important under conditions where close contact between bacterial cells can be achieved.

CAPSULE

The majority of the extraintestinal pathogenic *E. coli* strains, including UPEC, produce group II type of capsules (101). These capsular types are highly heterogeneous and resemble various glycoconjugates found within vertebrate hosts; this molecular mimicry is part of the bacterium's immune-evasion strategy. For instance, K1 and K4 capsular types mimic polysialic acid and substituted-chondroitin backbone, respectively. The assembly and biosynthesis of capsules in UPEC are closely related to the capsule-biogenesis systems found in the genera *Haemophilus* and *Neisseria*. Genes involved in the biosynthesis, serotype specificity, and export of UPEC capsules are located in 3 distinct gene clusters, designated as regions 1, 2, and 3, respectively.

Genes involved in the biosynthesis of capsule were up-regulated during murine infection (14). A signature-tagged mutagenesis screen of UPEC strain CFT073 revealed that a mutation in the *kpsC* gene that impairs capsular-polysaccharide transport was attenuated in a murine model of UTI (102). The role of K2 capsule in the pathogenesis of UTI was assessed using a mutant deficient in the biosynthesis of capsular polysaccharides in a murine model.

The mutant displayed a significant fitness defect in urine and kidneys that was reversed upon trans-complementation of the capsule-biosynthesis genes, clearly ascertaining the role of K2 capsule during UTI. Furthermore, this group also demonstrated that the K2 capsule was essential for protection from complement-mediated killing (103). The K5 capsule, also belonging to the group II family, was shown to prevent the association of neutrophils with an UPEC strain (104). In combination, these results establish that capsule is a bona-fide virulence factor in UPEC.

UTI89, another prototypical UPEC strain, produces a K1-type capsule and this capsule was shown to be required for successful development of IBCs within bladders from both wild-type and TLR4-deficient mice (105). Since defects in capsule synthesis and assembly increases the intracellular levels of sialic acid, the effect of N-acetyl-neuraminic acid regulator (NanR), a sialic acid-sensitive bacterial-transcriptional regulator, was tested in wild-type and capsule-mutant strains. Loss of NanR led to a partial increase in IBC formation in the capsule-mutant strain, indicating that perturbation in sialic acid levels has a profound effect on IBC formation.

GLOBAL REGULATORS OF GENE EXPRESSION

PhoP

Bacteria use two-component systems comprised of a sensor kinase and a cognate-response regulator to respond to external stimuli. PhoPQ is a two-component regulatory system that initiates a transcriptional program in response to environmental cues including low concentrations of calcium and magnesium. PhoQ is the inner membrane-associated sensor kinase that phosphorylates PhoP and phosphorylated PhoP acts as a transcriptional regulator of the PhoP regulon (genes regulated by PhoP). PhoP is a virulence factor in UPEC and controls the expression of around 11% of the genome (106). In CFT073, PhoP regulon includes the genes required for adaptation to acid stress and resistance to cationic antimicrobial peptides. UPEC is exposed to acidic pH in the stomach prior to gut colonization and in the urine. Cationic antimicrobial peptides are part of innate immune response to bacterial infections and play a critical role in controlling UTIs. Furthermore, PhoP represses the expression of flagellar genes and a mutant strain lacking PhoP is hypermotile (Fig. 3). In summary, PhoP affects acid resistance and virulence in UPEC by modulating polarization state of inner membrane.

Histone-Like Nucleoid Structuring Protein (H-NS)

H-NS, a DNA-binding protein, binds double-stranded DNA regions with intrinsic curvature. In UPEC, H-NS acts as a global transcriptional regulator; genes involved in motility (Fig. 3), hemolysin A production, type 1 (*fimB*, *fimE*) and S fimbrial expression, and iron uptake are repressed by H-NS. Not surprisingly, an *hns* mutant displayed increased virulence in mouse models of UTI and sepsis (107). StpA, another DNA-binding protein, however, partially compensates for the loss of H-NS function.

Sigma E

Sigma E facilitates transcription of genes required to combat envelope stress. Sigma E itself is regulated at several levels including sequestration by an inner membrane associated anti-sigma factor, RseA. During envelope stress, RseA is degraded by a protease (DegS) resulting in the expression of sigmaE-regulated genes. Mutants lacking DegS, therefore, constitutively express sigma E-regulated genes and are attenuated in a mouse model of UTI (108). Sigma E-regulated periplasmic chaperone (DegP) also contributes to urovirulence (109). These results demonstrate the critical need to maintain envelope homeostasis during colonization of urinary tract.

Host Factor Q-beta (Hfq)

RNA chaperone Hfq is a key player in riboregulation, small RNA-mediated regulation of gene expression, in bacteria. Hfq and small RNA-mediated circuits have been shown to affect global changes in transcript abundance and/or translation. Kulesus et al. (110) reported that an *hfq* mutant is highly attenuated in both bladder and kidneys concomitant with a reduced number of IBCs within the bladders of mice infected with the mutant strain. UPEC grows under acidic conditions in the presence of reactive oxygen and nitrogen species in the urinary tract. Hfq was required for wild-type levels of growth under acidic conditions as well as during oxidative or nitrosative stress. The *hfq* mutant exhibits impaired motility (Fig. 3) and resistance to cationic polypeptide, polymyxin B. These phenotypes and transcriptome analyses revealed that the *hfq* mutant is compromised in membrane-stress response, similar to an *rpoE* mutant. In summary, Hfq-mediated riboregulation is critical for orchestrating gene expression during infection.

Cell-Cell Communication

Population-level changes in gene expression that occur in a bacterial cell density-dependent manner are known as quorum sensing. Bacteria use small molecules or peptides, referred to as autoinducers, to determine cell density or quorum. UPEC strains, similar to several Gram-negative bacteria, are capable of sensing autoinducer 2. Recently, a novel quorum-sensing (Qse) system that uses catecholamines (epinephrine and norepinephrine) as signals has been discovered in enterohemorrhagic *E. coli* and has been proposed as an interkingdom-signaling system. A functional QseBC-signaling system is also found in UPEC. QseC is an inner membrane-associated sensor kinase that phosphorylates QseB, the cognate-response regulator. QseB is a transcriptional regulator that affects the expression of target genes including curli and flagella. In UPEC UTI89, a mutant strain lacking QseC is attenuated and is defective in differentiating into IBCs in a murine infection model (111). Interestingly, an isogenic mutant lacking *qseB* retained wild-type levels of virulence and the *qseBC* operon was constitutively expressed in the absence of QseC due to the loss of QseC-associated phosphatase activity. In summary, QseBC system is required for coordinated gene expression in UPEC during infection.

GENOME SEQUENCES

Complete, annotated genome sequences are available for the following strains: cystitis strains F11, IAI39, UMN026, and UTI89; pyelonephritis strain 536; pyelonephritis and

sepsis strain CFT073; and asymptomatic bacteriuria strains ABU 83972 and VR50. Sequences of UPEC strains not only provide genome-wide insights into the presence of fitness/virulence factors but have also ushered in a host of genome-enabled studies such as comparative genomics (comparing genome contents), functional genomics (transcriptional profiling, high-density mutational analysis) and proteomics (translational profiling). Due to rapid advances in sequencing technology, draft-genome sequences of several UPEC isolates are also available from GenBank (NCBI; National Center for Biotechnology Information) and the Broad Institute.

Comparison of genomes of pyelonephritis isolate CFT073, fecal/commensal strain K-12 MG1655, and enterohemorrhagic *E. coli* strain EDL933 revealed that genomes of pathogenic *E. coli* are mosaic in nature with large regions of horizontally transferred genes interspersed in between the core fecal/commensal *E. coli* genome (43). Only 39% of the protein-coding regions was conserved among these three strains of *E. coli*, demonstrating that plastic nature of the genomes could play a vital role in adaptation of these strains to the milieu encountered within their respective niches. Absence of a type III-secretion system and phage-encoded toxin genes in UPEC compared to enterohemorrhagic *E. coli* (EHEC) are among the remarkable differences between these two pathotypes. Recently, the presence of putative type III-secretion system genes in the genomes of UPEC isolated from patients with cystitis was reported (112). Its role in fitness during UTI, if any, remains to be determined.

Gene clusters encoding characterized or predicted fimbriae were overrepresented in the CFT073 genome (43). A total of 12 fimbrial gene clusters were identified; 10 belong to the chaperone-usher family of fimbriae that includes the type 1 fimbria and the P fimbria and two are reminiscent of the members of the type IV pilus family. A preponderance of FimBE-like recombinases (a total of five) was observed in the CFT073 genome and it was proposed that recombination-mediated regulation of gene expression is involved in orchestrating gene expression during various phases of UTI. In general, the genomes of pathogenic *E. coli* are larger than fecal/commensal strains. Specifically, the genomes of UPEC strains 536, CFT073, and UTI89 are 4.94 million base pairs (Mb), 5.23 Mb, and 5.07 Mb in length, respectively. The genome of the fecal/commensal strain K-12 MG1655 is 0.59 Mb shorter than the CFT073 genome (43) and these additional, UPEC-specific genes are thought to confer fitness advantage during survival within the urinary tract.

Pathogenicity Islands

Genomes of several pathogenic bacteria, including UPEC, harbor regions of DNA that are acquired by horizontal gene transfer (HGT). Such regions are referred to as genomic islands and are marked by signatures including the presence of direct repeats and evidence of mobile elements, such as transposons or phages. HGT is one of the fastest means of dissemination of fitness or virulence factors among bacteria. Sequenced genomes of UPEC strains reveal a mosaic structure with multiple horizontally transferred islands (43, 113). More often than not, such islands contain fitness or virulence genes and are known as pathogenicity islands (PAIs). First insights into the presence of PAIs in bacterial pathogens were garnered from studies using UPEC strain 536.

Genomes of 3 pyelonephritis strains, 4 cystitis strains, and 3 fecal/commensal strains were compared by comparative genomic hybridization and around 52% of genome was found to be conserved between these strains, indicating that there is high level of divergence among *E. coli* strains (114). Only 131 genes, however, were found in all the 11 UPEC strains, including 536, CFT073, F11, and UTI89, that were absent in fecal/commensal strains and were designated as uropathogen-specific genes. A subset of the uropathogen-specific genes (38 genes) is up-regulated during mouse UTI suggesting that they are involved in adaptation to growth within the host urinary tract (14). In agreement with the general trend of close proximity of PAIs to transfer RNA (tRNA) genes, 80% of the PAIs in CFT073 are associated with tRNA loci.

Pyelonephritis strain CFT073 harbors 13 genomic islands comprising 7 PAIs, 3 genomic islands (GI), and 3 phage-rich islands (PI) (115). Of these islands, PAI-*aspV*, PAI-*asnT*, and PAI-*metV* are required for *in vivo* fitness. In contrast, PAI-*serX*, GI-*asnW*, GI-*cobU*, GI-*selC*, PI-*b0847*, and PI-*potB* are dispensable without a measurable loss of fitness (115). This study led to the identification of a member of the RTX family of toxins, TosA. In an independent study, TosA was identified as an *in vivo*-expressed antigen that contributes to fitness both in the urinary tract and during bacteremia in a mouse model of UTI and bacteremia, respectively (116, 117). However, no toxic functions have been ascribed to TosA and TosA could very well belong to a growing subset of RTX family members that do not exhibit toxicity (118).

Studies on PAIs in UPEC CFT073 led to the identification of two proteins, homologs of *Shigella flexneri shiA*, that suppress host inflammatory response during UTI. The genes *sisA* and *sisB* are located in the PAI-*pheV* and PAI-*selC*, respectively (119). Strains lacking both genes incited a robust inflammatory response within mouse urinary tract compared to wild-type and this *in vivo* phenotype was reversed upon complementation with either of the two genes. Not surprisingly, the double mutant exhibited a colonization defect during the early stages of UTI that can be attributed to a severe inflammatory response. Interestingly, these anti-inflammatory proteins are localized within the cytoplasm and are predicted to be released only upon lysis of UPEC. Keeping in line with the genetic diversity observed among UPEC strains, *sisA* and *sisB* are not found in other model UPEC strains, including 536, F11, and UTI89.

The sequence of UPEC 536 is 292 Kb smaller than CFT073 genome and this difference is primarily due to PAIs that are unique to each strain. One unique feature of UPEC 536 genome is the presence of two hemolysin A operons, *hlyA1* and *hlyA2*, within PAI I and PAI II, respectively; both operons contribute to the virulence independently (113). A functional yersiniabactin biosynthesis and uptake system is found in a PAI in UPEC 536. In summary, availability of genome sequences has facilitated genome-wide comparisons that have delineated uropathogen-specific genes from the pangenome of *E. coli*.

UPEC GENE EXPRESSION *IN VIVO*

Dedicated research has led to the identification of numerous virulence factors in UPEC; however, we are just beginning to understand the response of UPEC to cues encountered

during infection. The availability of genome sequences for several UPEC strains and tools to determine genome-wide changes has shed new light on the molecular pathogenesis of UTIs. Genes that are either up or down-regulated under certain conditions are classified as differentially expressed genes. Approximately 9% of the UPEC CFT073 genes are differentially expressed during murine UTI (14). Genes involved in ribosome biosynthesis are among the most highly expressed genes, indicating that UPEC multiply rapidly, even during growth in the face of inflammation. Genes encoding the type 1 fimbria, siderophores, other factors involved in iron acquisition, and transporters of capsular polysaccharides are known virulence or fitness genes that are highly up-regulated within the murine urinary tract. Genes involved in glutamine biosynthesis and import, *glnA* and *glnPQ*, are up-regulated *in vivo* clearly indicating that UPEC experiences nitrogen limitation within host urinary tract. Although urine is replete with urea (~0.5 M), nitrogen derived from urea is unavailable for UPEC because of the absence of urease activity. Transporters of osmoprotectants, proline, and glycine betaine encoded by *proP* and *proVWX*, are up-regulated *in vivo*.

Conversely, many of the genes involved in flagellar biosynthesis and chemotaxis are among the most down-regulated genes within the murine urinary tract. Genes regulated by fumarate and nitrate reductase (Fnr), a global transcriptional regulator facilitating the adaptation to anaerobic conditions, such as *frdABCD*-encoding subunits of the fumarate reductase and *aspA* aspartase, are down-regulated, suggesting that UPEC is experiencing aerobic conditions *in vivo*. Taken together, the transcriptome paints the picture of the murine urinary tract as an iron and nitrogen-limited milieu, with high osmolarity and adequate oxygenation; UPEC utilizes amino acids and peptides along with carbohydrates such as altronate, fructose, glucitol, and mannionate, as sources of carbon and nitrogen to grow rapidly within the murine urinary tract (14).

While the *in vivo* transcriptome analysis elucidated the conditions encountered by UPEC during UTI, it also raised the question of parallels between a murine model and human infection. To precisely address that question, transcriptome analysis on UPEC samples directly derived from women with clinical UTIs was performed (19). General conditions appear to be conserved between murine and human UTI. Genes involved in iron uptake, peptide and amino acid transporters, ribosome biogenesis, transcription, and translation machinery were highly expressed in the human UTI-derived samples. This transcriptional profile reflects the rapid growth of UPEC under aerobic, nitrogen, and iron-limited environment. Despite the high levels of expression of genes involved in biogenesis of type 1 fimbria in the murine model, the *fim* genes were poorly expressed in human samples. The gene *fimH*, encoding the fimbrial-tip adhesin, was expressed only in 2 out of a total of 8 samples. These results suggest that type 1 fimbria might play significantly different roles in murine and human UTIs. Also, genes involved in the biogenesis of P, F1C, and Auf fimbriae were not expressed at detectable levels *in vivo*, at least in this group of patients. These findings emphasize the need to exercise caution when extrapolating information obtained from laboratory-animal models to human health.

OVERVIEW OF SELECT TECHNIQUES USED IN MOLECULAR PATHOGENESIS RESEARCH

Transcriptional profiling and transposon mutagenesis are widely used techniques that elucidate gene expression and genes required for growth and/or survival under specific conditions, respectively. Here we introduce these techniques and project how studies utilizing these techniques could impact research on the molecular pathogenesis of UPEC.

DNA microarrays, a well-established technique, and RNA-seq, an emerging technique, are utilized to determine transcriptional profile in bacteria. Hybridization based-detection of complementary DNA (cDNA) is the basis of DNA-microarray technology. Relative abundance of a particular transcript is used to determine the genes that are up or down-regulated under a specific condition. It is also possible to determine the absolute quantity of transcripts using specialized arrays such as gene chip (Affymetrix). DNA microarrays can also be used for comparative genomic-hybridization studies that analyze gene content. While DNA-microarray technology revolutionized the ability to gain a global perspective on UPEC gene expression during specific conditions (14, 19, 31, 93), it is slowly losing ground to RNA-seq, a sequencing-based technique.

Recent advances in high-throughput (HT) DNA-sequencing technology have been applied to transcriptome analyses. RNA-seq involves massively parallel sequencing of cDNA libraries utilizing HT-sequencing platforms such as Illumina (120). Both absolute and differential expression under various conditions can be evaluated using RNA-seq. Advantages conferred by RNA-seq over DNA microarrays include global analysis in an unbiased manner at a hitherto unprecedented resolution and dynamic range. Untranslated regions of the transcripts, promoter regions, novel transcripts, including small regulatory RNAs, and operon structure can be characterized using RNA-seq. Additional information offered by RNA-seq combined with decreasing cost associated with HT sequencing is poised to make RNA-seq the top choice for transcriptional analyses. Efforts are underway in the Mobley laboratory to define the transcriptome of UPEC during human infection using RNA-seq (unpublished results).

Transposon mutagenesis, specifically signature-tagged mutagenesis (STM), has been utilized to identify genes necessary for growth and survival under conditions of interest. An STM screen in a mouse model of UPEC UTI has unraveled several novel virulence factors along with validation of known virulence factors (102). To provide complete genome saturation, a major bottleneck in STM, several modifications that integrate state-of-the-art technologies in genome analysis with transposon mutagenesis such as transposon-site hybridization and transposon-insertion-site sequencing have been developed (121). Transposon-insertion libraries, comprised of genome-saturating numbers of transposon mutants, are subjected to selection under a condition of interest. The genomic DNA from the library prior to and post selection are sequenced to detect and determine the frequency of transposon insertion at a given locus. Since these approaches are similar to STM, a negative-selection strategy, the mutants that are less represented in the output pool, compared to the input pool, are delineated as putative virulence or fitness genes. Such genes warrant further characterization to determine their exact role in fitness or virulence. Genetic determinants of

fitness in UPEC during bacteremia in a murine model have been identified using transposon-directed insertion-site sequencing (TraDIS) (44). Recently, UPEC genes involved in serum resistance (122) and in colonization of a zebrafish model of infection were also identified using similar approaches (123). Conducting such forward genetic screens using UPEC in a mouse model of UTI has the potential to unravel hitherto unrecognized fitness and virulence mechanisms involved in the molecular pathogenesis of UPEC.

CONCLUDING REMARKS

The UPEC research community has made remarkable progress since the publication of the previous edition of this book. Access to advanced technology in the post-genome era has the potential to generate an enormous wealth of data that can be utilized to better understand the infection biology of UPEC and to identify pathways that can be targeted for developing novel therapeutics. Moving forward, a prime challenge is translating the knowledge gained in virulence and fitness mechanisms into developing therapeutics and vaccines that can alleviate the humongous burden imposed on human health by UPEC.

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FIGURE 1.

A simple model of iron uptake in UPEC. UPEC produces iron-scavenging molecules known as siderophores. Cognate outer-membrane receptors bind ferri-siderophore complexes or heme that are then imported into the periplasm. Within the periplasm, they associate with periplasmic-binding proteins. Translocation across the inner membrane involves ABC transporters. Iron is extracted from iron-siderophore complexes and heme via multiple reactions, denoted by broken arrows. In the presence of iron, Fur represses (X) transcription of the genes involved in iron uptake. Iron limitation within the host urinary tract results in the derepression of Fur-regulated genes, including siderophore-biosynthetic genes. UPEC, uropathogenic *E. coli*; OM, outer membrane; P, periplasm; IM, inner membrane; C, cytoplasm; Fur, ferric-uptake regulator; Fe, ferric iron; and TonB, ExbB, ExbD, energy-transduction complex for transport of iron-containing complexes to the periplasm. doi: [10.1128/microbiolspec.UTI-0015-2012.f1](https://doi.org/10.1128/microbiolspec.UTI-0015-2012.f1)

FIGURE 2.

Metabolism in UPEC during infection. Amino acids and di/oligo peptides are the primary sources of carbon and nitrogen for the UPEC within urinary tract. Mutants defective in peptide transport exhibit a fitness defect *in vivo*. Pyruvate is generated from amino acids and feeds into the citric-acid cycle and gluconeogenesis. Disruption of either citric-acid cycle or gluconeogenesis is detrimental for the survival of UPEC within the urinary tract. UPEC, uropathogenic *E. coli*; OM, outer membrane; P, periplasm; IM, inner membrane; and C, cytoplasm. [doi:10.1128/microbiolspec.UTI-0015-2012.f2](https://doi.org/10.1128/microbiolspec.UTI-0015-2012.f2)

FIGURE 3.

Regulation of flagellar motility in UPEC. *flhDC*, master regulator of the flagellar-biosynthesis cascade, is subject to both positive and negative regulation. Note that not all regulators affect the transcription of *flhDC* directly. Regulators such as PhoP and QseB are activated in response to specific cues encountered by UPEC. Various signals are integrated into the flagellar gene expression cascade via two-component regulatory systems and other signal-transduction cascades. UPEC, uropathogenic *E. coli*; OM, outer membrane; P, periplasm; IM, inner membrane; and C, cytoplasm. [doi:10.1128/microbiolspec.UTI-0015-2012.f3](https://doi.org/10.1128/microbiolspec.UTI-0015-2012.f3)