Stony Brook University

Role of Type II Secretion and Type IV pilus Systems in the Virulence of Uropathogenic *Escherichia coli*

A Dissertation Presented

by

Ritwij Kulkarni

to

The Graduate School

in Partial Fulfillment of the

Requirements for

the Degree of

Doctor of Philosophy

in

Genetics

Stony Brook University

December 2007

Stony Brook University
The Graduate School

Ritwij Kulkarni

We, the dissertation committee for the above candidate for the
Doctor of Philosophy Degree, hereby recommend
acceptance of this dissertation.

Dr. David G. Thanassi- Dissertation Advisor
Associate Professor
Department of Molecular Genetics and Microbiology

Dr. James B. Bliska- Chairperson of Defense
Professor, Department of Molecular Genetics and Microbiology

Dr. Jorge L. Benach
Director, Center for Infectious Diseases
Professor and Chair, Department of Molecular Genetics and Microbiology

Dr. Matthew A. Mulvey
Associate Professor, Department of Pathology
University of Utah

This Dissertation is accepted by the Graduate School

Lawrence Martin
Dean of Graduate School

ABSTRACT OF DISSERTATION

Role of Type II Secretion and Type IV pilus Systems in the Virulence of Uropathogenic *Escherichia coli*.

by

Ritwij Kulkarni

Doctor of Philosophy

in

Genetics

Stony Brook University

2007

Type II secretion systems (T2SS) and the evolutionarily related type IV pili (T4P) are important virulence determinants in many Gram-negative bacterial pathogens. However, the roles of T2SS and T4P in the virulence of extraintestinal pathogenic *Escherichia coli* have not been determined. Extraintestinal pathogenic *E. coli* cause a variety of diseases, including urinary tract infections and neonatal meningitis. We investigated the function of T2SS and T4P in the model uropathogenic *E. coli* (UPEC) strains UTI89 and CFT073, which possess T2SS and T4P gene clusters homologous to clusters present in laboratory strains of *E. coli*. In addition, UTI89 contains a second T2SS gene cluster homologous to one present in enterotoxigenic *E. coli* (ETEC) strains that cause diarrhea. We constructed deletion mutations to disable

each T2S and T4P system in the UPEC strains, and compared these mutants with their wild type counterparts using tissue culture assays and the CBA/J mouse model of ascending urinary tract infection. No deficiencies were observed with any of the mutants in adherence, invasion or replication in human bladder or kidney cell lines, but UTI89 Δ*hofQ* (T4P) and UTI89 Δ*gspD* (ETEC-T2SS) exhibited approximately 2-fold defects in fluxing out of bladder epithelial cells. In the mouse infection model, each of the knockout mutants was able to establish successful infections in the bladder and kidneys by day one post-infection. However, UTI89 Δ*hofQ* and a CFT073 Δ*hofQ* Δ*yheF* (K12-T2SS) double mutant both exhibited defects in colonizing the kidneys by day seven post-infection. Furthermore, the plasmid-borne copies of *hofQ* and/or *yheF* were able to complement the kidney colonization defect of the deletion mutants. These results establish T4P and potentially also secreted T2SS effectors as virulence determinants of UPEC important for persistence in the urinary tract, particularly in renal tissues.

Dedication

To my grandparents,

Mrs. Tara and Prof. Vasant V. Upadhye.

"I owe so much of this to you..."

Table of Contents

List of Figures and Tables

Acknowledgements

In the past few years as a graduate student, I became friends with many people. Their whole hearted encouragements and timely rebukes have made this research endeavor and my survival in the US possible as well as fun.

I am deeply indebted to my thesis mentor Dr. David Thanassi. The reason I decided to join David's lab was his affable and optimistic nature. His positive attitude always meant a lot to me and in future that's something I am going to miss. Furthermore, David is a mentor in a true sense of the word. All Ph.D. advisors impart some scientific knowledge on their students. That's part of their job. But David took this idea of mentorship beyond the lab. Once he actually taught me how to change a flat tire on my car. In Utah, he taught me how to ski. In a restaurant, when I got grapefruit with my breakfast menu, he even taught me how to eat it. David has been my mentor both inside and outside the lab and I am certainly going to miss him.

I would also like to thank my committee members, Dr. James Bliska, Dr. Jorge Benach and Dr. Matthew Mulvey (Matt) for their insights, and all the simple and difficult questions that they asked me through the course of this project.

Matt is also our collaborator for this project. David and I went to Salt Lake City to his lab where Matt and his graduate student Bijaya K. Dhakal, taught me how to catheterize mice. They also carried out some of the initial mouse infection experiments. I thank them for being excellent troubleshooters of anything mice! I also thank Matt and his family for being the gracious hosts they were during our stay in Utah.

Catheterizing mice on my own in Stony Brook was a nerve wrecking experience. If Ms. Gloria Monsalve were not there to hold my hand and guide me patiently, I could not have done a single mouse experiment. I thank Gloria and Mr. Patricio Mena for their help in mouse work.

I became friends with Ms. Nadine Henderson, Ms. Dreania Levine and Ms. Kate Bell because of our shared interests in Harry Potter, movies, literature and/or politics. I thank them for their love and friendship.

Dr. Tony Ng, my best friend and former apartment mate, stood by me through ups and downs; always helping and advising in all matters scientific and non-scientific. Tony and other friends such as Ms. Zankhana Thakkar, Dr. Subhra Chakraborty and Mr. Zachary Kurtz made my graduate years and the Stony Brook experience memorable.

Ms. Jen Parla nee Choy and I are batchmates. We faced similar problems through the course of graduate school and hence were able to share our sorrows to reduce the pain. I will always cherish Jen's advice and how she would make me see the silver linings of the darkest of the clouds.

The support and friendship and all the gossip that we shared in our morning coffee group that included Mary, Betty and Kate (Kline), helped me survive the last two years of graduate school.

My parents, Ms. Mrinal and Mr. Purushottam Kulkarni have been a driving force in my life. Whatever I am today is because of them

And finally my wife, Ms. Priyadarshini Pathak (Priya): without her dedication, love and encouragement, I could not have faced the challenges both inside and outside the lab.

- RUDY (Ritwij Kulkarni)

Chapter One

Introduction

I. The Urinary Tract

The human urinary tract is a multi-organ complex consisting of the kidneys, ureters, urinary bladder and urethra; the organs involved in production, storage and excretion of urine. Blood is filtered in the glomerular filters of the kidneys at a rate of 125 ml/min (44). The glomerular filtrate consists of glucose, excess salts, urea and mainly water. Ninety-nine percent of water in the glomerular filtrate is reabsorbed into the blood to leave behind the urine. Urine is carried by the two ureters into the urinary bladder, where it is stored for excretion through the urethral opening (Fig. 1.1).

Since the urinary tract is open to the external environment, it is easy for pathogens to gain entry and establish infection. To prevent this, there are many innate defenses; for example, the gush of urine purges the urinary tract of any non-adherent particles as well as pathogens, the low pH and osmolarity of urine has a bacteriostatic effect and the sphincters at the bladder end of the ureters prevent back-flow of urine into the kidneys. These defense mechanisms can be breached by different pathogenic microorganisms to cause urinary tract infections (UTIs), including cystitis (infection of bladder) and pyelonephritis (infection of kidney). A descending UTI occurs as a consequence of bacteremia. Blood-borne bacteria enter the kidneys through the glomerular filters, an event more likely in neonates due to immaturity of the filters. An ascending UTI occurs due to the entry of the bacteria in the vicinity of the urethral opening into the urinary

tract, primarily due to fecal contamination. If left untreated, the pathogens may ascend the ureters into the kidneys establishing acute pyelonephritis. Presumably, because of their longer urethrae, males are less prone to ascending UTIs than females.

II. Uropathogenic *Escherichia coli*

In 1885, a German pediatrician and bacteriologist Dr. Theodore Escherich discovered the bacteria that were causative agents of infantile diarrhea and gastroenteritis (90). Later in 1919, these bacteria were grouped under the genus *Escherichia*. Most members of this genus colonize the lower intestines of mammals within a few hours after birth as commensals, with *E. coli* as the principal commensal species. These Gram-negative, facultative anaerobic, motile rod shaped bacteria belong to the γ suborder of the family *Proteobacteriaceae*. They cause disease rarely, except in immunocompromised individuals or where the normal gastrointestinal barriers are breached. There is a great diversity of *E. coli* strains in the environment, some of which are known to infect human beings while the others such as *E coli* K12 are commonly used research tools. Diseases caused by pathogenic *E. coli* in humans can be divided into three general clinical syndromes: enteric/diarrheal disease, UTIs, and sepsis/meningitis (52). The pathogenic *E. coli* causing UTI (uropathogenic *E. coli*; UPEC) and sepsis/meningitis (Neonatal meningitis associated *E. coli*; NMEC) in humans, and respiratory infections, pericarditis and peritonitis in birds (avian pathogenic *E. coli*; APEC), belong mainly to phylogenetic group B2 (intestinal pathogenic *E. coli* belong to groups A, B1 or D (49)) and exhibit a considerable overlap with respect to virulence factors and serotypes (21, 52). Owing to

these similarities, the UPEC, NMEC and APEC are grouped under an inclusive designation: extra-intestinal pathogenic *E. coli* (ExPEC) (83).

UTIs are of great interest because in North America alone, 40% of women suffer from a UTI at least once during their lifetime (25), leading to 7 million office visits every year with total monetary costs approaching $2 billion (27). The causative agents of UTIs come from different groups of microorganisms such as fungi (*Candida albicans*), Gram-negative (*E. coli*, *Proteus mirabilis, Pseudomonas aeruginosa*) and Gram-positive (*Enterococcus faecalis*, *Staphylococcus saprophyticus*) bacteria. Though these causative agents show a different distribution depending on whether the infection is hospital- or community-acquired, UPEC is the major culprit, causing 80% of the hospital- and 40% of the community-acquired infections (82).

The success of UPEC as pathogens can be attributed to the panoply of virulence factors that are produced by them. The virulence factors can be broadly categorized into the adhesive factors and toxins. They help UPEC to anchor in the urinary tract, invade the urothelium and establish infective foci for persistence and resurgence in the organs of the urinary tract.

III. Virulence Factors Produced by UPEC

Adhesins: UPEC produce a diverse range of adhesins such as P and type 1 pili along with other fimbriae such as F1C, S, M and Dr (48). Out of these, the roles of P and type 1 pili in the infection process have been studied in detail and hence are well understood. Both P and type 1 pili are composite, multiprotein, hair-like appendages

emanating from the surface of UPEC. With their adhesive tips (PapG and FimH are the adhesins for P and type 1 pili, respectively), they aid UPEC in colonizing the kidney and bladder epithelia (7, 67). The PapG adhesin binds to α-D-galactopyranosyl-(α1-4)-β-D-galactopyranoside moieties in the globoseries of kidney epithelium glycolipids while FimH binds to manosylatyed glycoproteins in the uroplakins of the bladder epithelium (69). Thus the bacteria get anchored in the urinary tract, an important advantage in face of the gush of urine that frequently washes the urinary tract of any non-adherent particles. Furthermore, FimH functions like a typical invasin by bringing about reorganization of the actin cytoskeleton, localized alterations in the host cell membrane and uptake of the bacteria (61). The different variants of PapG, namely PapG-I, -II and -III bind to different isoreceptors that vary in the number of N-acetylgalactosamine moieties added to the Gal-(α1-4)-Gal core or by the addition of sialic acid residues (57). These variants are proposed to aid in determining the host specificity of UPEC: PapGI and PapGII adhesins preferentially bind globotriaosylceramide (GbO_3) and globoside (GbO_4), both of which are common receptors on the human uroepithelium, respectively, whereas, PapGIII binds Forssman Antigen (GbO_5) receptor predominant on canine uroepithelium (57). P pili are strongly associated with pyelonephritis, although reports describing the importance and relevance of PapG adhesins and P pili in renal colonization and persistence are full of inconsistencies: an isogenic Pap mutant in the pyelonephritogenic UPEC strain CFT073 caused acute pyelonephritis just as well as its WT counterpart in a CBA/J mouse model of ascending UTI (66), whereas a PapG deletion mutant in another UPEC strain, DS-17, failed to cause acute pyelonephritis in a primate (cynomolgous monkeys) model of ascending UTI (80).

Toxins: Two important toxins that are coproduced and secreted by UPEC are hemolysin A (hlyA) and cytotoxic and necrotizing factor 1 (CNF1). CNF1 is a dermonecrotic, 155 kDa, single chain AB toxin that increases the invasiveness of UPEC by activating Rho GTPases (56). Active CNF1 is exported across the bacterial envelope in a complex with outer membrane vesicles (17). It impairs remodeling and clustering of CD11b receptors on the surface of polymorphonuclear leukocytes (PMNs), preventing phagocytosis of opsonized UPEC (18). HlyA is secreted by the type 1 secretion system (95). At high concentrations, HlyA targets erythrocytes and leukocytes to bring about cell lysis (52), while at sublytic concentrations, it induces intracellular Ca^{++} signaling in epithelial cells leading to pro-inflammatory responses such as production of IL-6 and IL-8 (99). Secreted autotransporter toxin (Sat) is a cytolytic vacuolating toxin that has been shown to damage the glomerular membrane and proximal tubule cells in the kidney, to create lesions in intestinal and renal epithelial cell tight junctions and contribute to the tissue damage associated with the UTIs (36, 37). In 2005, Aoki *et al* showed that UPEC strain EC93 inhibits the growth of *E. coli* K12 strain MG-1655 in a mixed culture and that the growth inhibition was dependent on the presence of surface protein contact dependent inhibitor A (CdiA) and a two-partner secretion family member CdiB (4).

IV. Secretion Systems in Gram-Negative Bacteria

The virulence factors described above are produced in the bacterial cytoplasm. For their assembly and/or secretion into the extracellular milieu, the virulence factors or their components must be transported across the bacterial envelope with the help of

different secretion systems. The secretion pathways that are employed by Gram-negative bacteria to transport cytoplasmic proteins across the bacterial envelope can be categorized into two types. The Sec-independent type I, III and IV secretion systems form a tunnel-like channel spanning the bacterial envelope such that the proteins are exported from the cytoplasm into the extracellular environment in a one-step process without the formation of periplasmic intermediates. On the other hand, Sec-dependent secretion systems such as type II, chaperone usher and autotransporter pathways require the Sec translocation machinery to transport cytoplasmic proteins across the IM into the periplasm to form a periplasmic intermediate. The periplasmic intermediate is then taken over by the secretion system for export across the OM (55). Figure 1.2 depicts different secretion pathways in Gram-negative bacteria. Many of these pathways are utilized by UPEC for the secretion of virulence factors. The chaperone usher pathway is utilized for the assembly and secretion of adhesive pili on the surface of UPEC (96), while Sat is secreted by means of the autotransporter pathway (37).

The type II secretion system (T2SS) is involved in the secretion of many important toxic effectors in a diverse variety of Gram-negative pathogenic bacteria. The protein components of the T2SS are similar to the structural and functional subunits of the evolutionarily related type 4 pilus (T4P) system, which assembles adhesive appendages on the bacterial surface that have been shown to aid bacterial colonization and virulence. The main purpose of this thesis dissertation is to analyze the importance of T2SS and T4P in the pathogenicity of the UPEC.

V. Type II Secretion System

In the 1980s, the T2SS was identified to be the mechanism involved in the trans-membrane export of pullulanase in *Klebsiella oxytoca* (85). Type II secretion systems (T2SS) are involved in the secretion of many important effectors in diverse Gram-negative pathogenic bacterial species (39, 47) that cause infections in humans, (cholera toxin secretion mediated by Eps T2SS in *Vibrio cholerae* (86)), animals (aerolysin secretion in the fish pathogen *Aeromonas hydrophila*) and plants (plant cell wall degrading enzyme secretion in *Erwinia caratovora*) (84). In the case of enterotoxigenic *E. coli* (ETEC), the T2SS secretes heat-labile enterotoxin (LT), which is structurally and functionally similar to cholera toxin (94). *E. coli* K12 strains possess a T2SS that is normally not expressed. However, in *E. coli* K12 mutants lacking the nucleoid structuring protein H-NS, the T2SS was shown to be involved in the secretion of chitinase (28).

The T2SS is regarded as the main terminal branch of the general secretory pathway (GSP). The T2S apparatus is a multiprotein complex that is coded for by a cluster of 12-15 different genes (Fig. 1.3). In most species, the letters A through O and S designate the T2SS genes and their protein products (see Fig. 3.1). The designations are based on the *K. oxytoca* Pul system and the general identifier for each protein is Gsp (i.e. GspA, GspB, etc.). The organization of the T2SS gene cluster is well conserved, with most of the genes arranged to form a single operon (85).

The entire T2S apparatus, called the secreton (78), spans the bacterial envelope (Fig. 1.3). It is interesting to note that even though the secreton is involved in the export of bacterial proteins across the OM, most of its component proteins are associated with the IM to form an IM platform of proteins (79). Structurally as well functionally the

subunits of a secreton are said to from three subassemblies: the OM pore formed by the secretin (GspD), an IM platform of proteins, and the pseudopilus. GspD is a member of the secretin family of gated pores that also includes proteins involved in T4P biogenesis, filamentous phage extrusion, and the type III secretion system (85). The C terminus of GspD is conserved across the secretin family of proteins and is embedded in the OM. On the other hand, the N terminus is variable, periplasmic and possibly interacts with other subunits of the secreton. An individual oligomer of GspD consists of 12-14 subunits. GspS is a small OM lipoprotein subunit that promotes stabilization and OM insertion of the secretin (GspD). GspE, a putative ATPase, is associated with the IM and provides energy for opening the gated secretin or for assembly of the secretion apparatus (84). GspG, H, I, J, and K exhibit homology with the structural subunit of type 4 pili, namely the N-terminal region encoding the prepilin peptidase cleavage site. This subset of T2SS proteins is called pseudopilins and they are N-terminally processed and methylated by the T4P prepilin peptidase GspO. The pseudopilins of *K. oxytoca* T2SS (PulG-H) were shown to assemble a pilus-like structure (pseudopilus) when the Pul secreton was over-expressed in *E. coli* K12 (89). Moreover, the high level expression of the endogenous Gsp secreton of *E. coli* K12 brings about the assembly of GspG (a PulG homolog) into bundled pili (100). Proteins GspF, L and M have been shown to interact with each other to form a multi-protein complex at the IM that possibly acts as an anchor for the pseudopilus (79).

The secreton is involved in the export of effector proteins across the OM only. The effectors are transported across the IM by the Sec translocon or the Tat pathway (101). In the periplasm, the effectors are completely folded. It is still unknown how a T2

secreton differentiates between its substrates and other periplasmic proteins. The secreted proteins are structurally as well as functionally diverse. For example, elastase secreted by *P. aeruginosa* is monomeric while cholera toxin is oligomeric; toxins like LT act inside the host cells while hydrolytic enzymes degrade molecules such as lipids, chitin, and pectin. The only defining characteristic of the T2SS effectors seems to be the relatively high β sheet content in their fully folded form prior to secretion across the OM (85). The failure to identify a linear signal sequence by comparing the primary amino acid sequences of the effector proteins has led researchers in the field to believe that the signal sequence is located at different parts of the linear protein and is brought together upon protein folding. The search for a T2 signal sequence is full of contradictory findings. In the case of PulA (pullulanase) secretion by *K. oxytoca*, the amino acid residues 1 to 78 (region A) and 734 to 815 (region B) were shown to retain ability to bring about the secretion of β-lactamase (54) while the simultaneous deletion of these two regions reduced the secretion of PulA to ≤ 25% (88). In a recent study, the PulA region between amino acid residues 234 and 324 was shown to be important for its secretion (29).

Even though the actual process of secretion through the T2 secreton involves participation by all three structural sub-assemblies; the exact role played by every single component is not yet deciphered. In the current model for T2SS functioning, the GspE ATPase is thought to provide the required energy for opening the OM gated pore GspD. Energy from GspE is transmitted to the members of the IM platform of proteins which span the periplasm contacting the GspD secretin and transporting the energy across the periplasm (6, 77). The extension and retraction of the pseudopilus is believed to bring

about opening and closing of the OM secretin pore while the protein cargo is carried on the tip of the pseudopilus (84).

VI. Type IV Pilus System

T4P are long, filamentous surface structures involved in myriad functions, including social gliding and twitching motility, adhesion, biofilm formation, virulence and horizontal genetic transfer by means of transformation, transduction and conjugation (8, 74). Their polar localization, sequence conservation among the different pilins and pilus assembly proteins, and role in bacterial motility sets them apart from other pili as a different group (103). The most distinguishing feature of T4P is their ability to retract through the bacterial envelope while their tips remain firmly adhered to a solid surface acting as firm anchors and translocating the bacterial cell forward in a flagella-independent manner (11, 64). T4P are known to play important roles in the virulence of a variety of Gram-negative pathogens such as *P. aeruginosa* (62), *Neisseria spp*. (97) and *Vibrio cholerae* (31). T4P of *P. aeruginosa* are a major virulence factor that aid bacterial adherence to the tracheal epithelium (108). In *N. gonorrhoeae* as well as *N. meningitidis*, association with the host epithelium followed by neisserial motility and dispersal over the epithelial surface as well as induction of the cytoskeletal rearrangements at the site of contact between *Neisseria* spp and host cells is mediated by T4P (63). Toxin-coregulated type IV pili (TCP) of *V. cholerae* help the bacteria form microcolonies on the human intestinal epithelium (42) and act as receptors for CTXΦ, the bacteriophage that carries genes for cholera toxin (102).

T4P are extremely long (1-4 μm) and thin (50-80 Å) (15), but very strong filaments that can withstand stress forces up to 100 pN (59). A type IV pilus fiber is a homopolymer of pilin. The type IV pilin family of proteins consists of 18-22 kDa polypeptides that are subdivided into two subgroups, A and B, based on whether the N-terminal amino acid in a mature protein is phenylalanine or methionine, respectively (92). A pilin is synthesized as a precursor protein with a short (6-7 amino acid long; for group A) or long (13-25 amino acid long; for group B) basic leader sequence that undergoes processing by the preprotein leader peptidase/transmethylase leading to the production of a mature pilin with an α-methylated phenylalanine or methionine residue at the N-terminus (72, 92, 93). Based on antibody mapping and X-ray crystallography studies, the T4P fiber from *P. aeruginosa* and *Neisseria spp*. (type A) is proposed to form a 3-layered structure: a central core of coiled conserved α-helices, surrounded by β-sheets, and the outermost, most exposed hypervarible region of β-hairpins and extended C-terminal tails (24).

The machinery involved in the biogenesis of T4P involves at least 16 protein components closely related to those of the T2SS (Fig. 1.3). The protein PilB is the major nucleotide binding ~62 KDa protein associated with the inner face of the cytoplasmic membrane. Inactivation of *pilB*, or site-directed mutagenesis in the conserved Walker box (consensus ATP-binding domain) domain ablates the assembly of T4P on the surface of *P. aeruginosa* cells (98). PilT (as well as PilU in *P. aeruginosa*), a protein associated with the cytoplasmic membrane is a PilB homolog and possesses the conserved Walker Box. PilT is conserved among different organisms such as *Myxocoxxus xanthus* and *N. gonorrhoeae*. Mutations in the ATP-binding domains of PilT (or PilU) result in

hyperfimbriated cells, which are unable to twitch (105, 106). This is reminiscent of flagellar motor protein mutants that can assemble flagella which fail to generate movement (103) and has given rise to a the idea of a bipartite motor in T4P with PilT transducing energy for pilus retraction while PilB provides the energy source for pilus extension by polymerization (103). PilQ belongs to the family of OM gated proteins similar to protein D of T2SS and is involved in the final passage of polymerized PilA pilins across the OM (60). Other protein components of the T4P biogenesis machinery such as PilC, Pil M-P, PilZ and PilV, are known to be involved in T4P assembly and secretion because mutants in any of these proteins are non-fimbriated, although the exact function of each of these is unknown (1).

E. coli K12 possesses genes coding for all proteins required for T4P biosynthesis, but under laboratory conditions the genes are transcribed at a very low level and pili are not assembled. The major pilin subunit from *E. coli* K12, PpdD (prepilin peptidase-dependent protein D) is assembled into pili if the Pul secreton is ectopically expressed (89), or if *ppdD* is ectopically expressed in *P. aeruginosa* (87). ETEC employ T4P (longus pili) as a colonization factor (34) while enteropathogenic *E. coli* (EPEC) use T4P (bundle-forming pili) for localized adherence in the intestinal tract and autoaggregation (33). Apart from the pseudopilins, the similarities between T2SS and T4P extend to include other structural components, such as the OM secretin GspD, the ATPase GspE, and GspF of the IM platform of proteins. In addition, a pilotin, GspS, is required for the assembly as well as the stability of the OM secretin in both the systems (76). All these observations indicate that T2SS and T4P are products of divergent evolution.

VII. Histology of the Urinary Tract

The major part of the urinary tract, from renal pelvis to the lumen of the bladder is lined by the urothelium, which normally consists of three different cell layers: basal, intermediate and superficial (81). The normal urothelium renews continuously but at a very slow, 40-week turnover rate. The luminal layer of the urinary bladder is made of highly differentiated, large and multinucleate facet or umbrella cells. Underlying this layer are intermediate epithelial cells, sitting atop a layer of small, relatively undifferentiated basal cells. A thin basement membrane and lamina propria separate the urothelium from the connective tissue and serous layers of the outer wall of the bladder (69). The apical face of facet cells is covered by a quasi-crystalline array of hexagonal complexes of integral membrane proteins called uroplakins (9) to create a rigid-looking asymmetric unit membrane (AUM) (81). So far five types of uroplakins (UPIa, UPIb, UPII and UPIIIa and UPIIIb) have been identified. Their expression is differentiation-related; i.e., the uroplakins are expressed in superficial and intermediate cells, but not in the undifferentiated basal cells. The facet cell permeability barrier is supplemented with a chemical barrier in form of the proteoglycans/ glycosaminoglycan that is known to interfere with bacterial adherence (75). Towards the bladder neck, there is a sharp transition from urothelium to truly stratified epithelium, which continues from this point into the distal urethra and the point of urethral exit out of the body.

The renal papilla (inner medulla) is the part of the kidney where most of the water from the glomerular filtrate is reabsorbed to form urine, which is delivered into the ureters. To facilitate the process of water resorption, the inner medulla is lined by a simple cuboidal epithelium that is one cell deep and devoid of uroplakins. The urothelium

extending into the other parts of the kidney such as outer medulla and cortex is 3-4 cells deep and shows the presence of uroplakins in the superficial cells. A similar, 3-4 cell deep, epithelial organization extends into the ureters with the umbrella cells and the uroplakins presented on their apical surfaces lining the ureteral lumen.

The urothelium lining of the urinary tract is adapted to withstand the frequent flushing by the urine. Apart from being a formidable permeability barrier that prevents unwanted/toxic constituents of urine from leaching into host tissue and circulation (9), the uroplakins also aid in adapting the bladder to the cycles of contractions and distensions (81). This important structural defense is subverted by UPEC to cause infections of the urinary tract.

VIII. UPEC Developmental Cascade and Urinary Tract Pathogenesis

The first important step in establishing a successful UTI is the attachment of UPEC to the urothelium. The adherent bacteria are able to escape the cleansing action of the gush of urine. Following adhesion, the UPEC can invade the superficial cells of urothelium and form intracellular biofilm-like structures called pods, intracellular bacterial communities (IBCs), or bacterial factories (68). The IBCs undergo a developmental cascade that involves bacteria going through different morphological changes (Fig. 1.5A) (50). Finally, the bacteria detach from the IBCs and flux out of the infected cells. The formation and development of IBCs has been studied in great detail in the bladder infection model and hence it is used as a paradigm for intracellular events leading to UTI.

FimH-mediated Adhesion and Invasion: Isogenic UPEC mutants lacking the FimH adhesin are defective in adhering to and invading the bladder in a mouse model of cystitis as well as in cell culture using the human bladder carcinoma cell line, 5637, in a gentamicin protection assay (61). 5637 cells also readily internalize FimH coated polystyrene beads (61). Based on these findings, FimH adhesin present at the distal tip of type 1 pili is considered to be important and sufficient for attachment of UPEC to the superficial umbrella cells of the bladder epithelium. Uroplakin, UPIa, binds to the FimH adhesin (69). Furthermore, FimH functions like a typical invasin by interacting with $\alpha_3\beta_1$ integrins on the surface of the urothelial cells (19), followed by FimH-mediated reorganization of actin cytoskeleton and localized alterations in the host cell membrane (61).

Cytochalasin D, an inhibitor of F-actin polymerization, as well as specific inhibitors of host protein tyrosine kinase, FAK (focal adhesion kinase) and phosphoinositide-3 kinase (PI-3K) reduce FimH-mediated invasion. This has led researchers to believe that FimH induces a pre-existing signal transduction cascade involving phosphorylation of FAK followed by formation of FAK and PI-3K complexes. This stimulates the generation of 3-phosphoinositide secondary messengers leading to actin polymerization and cytoskeletal rearrangement. This in turn leads to bacterial internalization (61).

In the superficial umbrella cells, UPEC spill out of the endosomes into the cytoplasm and start replicating to form small loosely packed clusters called “early IBCs” (50). The bacteria in early IBCs are rod-shaped (~3 μm in length), and divide rapidly (doubling time of 30-35 min) for the first 8 hours after inoculation (50). On the other

hand, UPEC invading the underlying, less differentiated intermediate cells are trafficked into vesicles that have properties of late endosomes or even lysosomes. An actin mesh surrounds these vesicles and may impede bacterial growth (20).

Middle IBCs: From 8-10 hours after inoculation, the UPEC undergo many changes; their growth rate drops, with doubling times of >60 min, the bacteria become coccoid, with their average cell length of ~0.7 μm, and the IBCs become more tightly packed (50). The growing IBCs push against the epithelial cell membranes giving it a pod-like appearance as shown in Fig. 1.5B (3). Immunofluorescent staining for type 1 pili and antigen 43, an autotransporter protein promoting agglutination, has shown a heterogeneous expression of these two factors in pods. This has given rise to the idea of IBCs being intracellular biofilms since the biofilms display community behavior with different tasks delegated to different subpopulations of cells. Pods also show a strong and uniform expression of polysaccharides. Presence of a polysaccharide matrix surrounding the bacterial community is reminiscent of biofilms (3).

Late IBCs: As early as 12 hours post infection the bacteria on the outer edge of IBCs differentiate into prototypical rod shape (average cell length 2 μm), become highly motile, and dissociate from their parent IBCs (50). The motile bacteria have been observed inside the umbrella cells as well as in the lumen of the bladder. Video-microscopic evidence suggests that the intracellular motility is flagella-based. To begin with, a few motile bacteria exit umbrella cells from a specific area on the cell surface. As IBCs grow in size, the infected cells become apoptotic (53). The reduction in membrane integrity of apoptotic cells might aid the motile bacteria to flux out. At some point the

entire IBCs burst out of the umbrella cells, releasing an estimated 10^5 bacteria into the lumen of the bladder (50).

Filamentation of Bacteria: At around 20 hours post inoculation (PI), a subpopulation of fluxed bacteria continues to grow without undergoing septation, giving rise to long (up to 70 μm) and filamentous bacteria (50). The exact signal for filamentation is unknown and some filamentous bacteria have been observed on the edge of late IBCs along with the motile, detaching cells (50).

Advantages of IBC Formation in Uropathogenesis: Formation of intracellular biofilm-like structures (IBCs) may provide the UPEC a safe haven away from the robust immune onslaught that ensues in the wake of bacterial infection. The IBC developmental cascade allows UPEC to establish replicating colonies in the infected bladders. Once the IBCs mature, bacteria flux out in such large numbers that PMNs fail to clear the entire bacterial population. Some of these bacteria are able to spread the infection. Filamentation also aids the survival of bacteria that are fluxing out of infected cells because PMNs do not respond to filamentous bacteria until the initiation of septation and PMNs are unable to engulf the filamentous bacteria (50). However, filamentation alone is not enough to subvert phagocytosis by PMNs, since bacterial filaments induced by overproduction of a cell division inhibitor, SulA, are efficiently engulfed by PMNs (51).

IX. Recurrent UTI (rUTI)

More than 25% of UTI recur within 6 months (26). Their recurrence is attributed to the ability of UPEC to inhabit underlying epithelial cells and to establish quiescent

reservoirs, from which bacteria can resurface at a later time point. In a murine model, upon interacting with an intact epithelial barrier, UPEC were shown to form rosette-like clusters inside early to late endosomes ($Lamp1^{+}/Rab5a^{-}$), called quiescent intracellular reservoirs (QIRs), located within the umbrella cells. The induction of facet cell exfoliation as part of host defense or as a result of the treatment with the cationic protein protamine sulphate (PS) led to complete removal of infected urothelial cells harboring the QIRs (70). Thus, at the end of 2 weeks post infection there was no evidence of bacteriuria (presence of bacteria in the urine) and pyuria, the two important features of an active infection. Concomitant with this, the superficial umbrella cell layer renewal process was also complete. On the other hand, if the bladder epithelial barrier was partially damaged (by administering PS) prior to the infection, it exposed the underlying transitional layers to bacterial onslaught and QIRs were established inside the subepithelial layers of cells (70). An actin mesh surrounds and impedes growth of UPEC within these vesicles (20). Upon differentiation of the underlying transitional epithelial cells, the composition of the actin mesh alters, leading to UPEC replication and eventually to the recurrence of UTI.

X. Host Response to Infection by UPEC

Introduction of UPEC into the bladder of a murine model of cystitis (C3H/HeN) induces a robust inflammatory response. The Toll-like receptor (TLR) family plays an important role in identifying LPS and other bacterial products. Different TLRs are expressed on cells of the immune system such as macrophages, as well as the epithelial cells. In mice lacking a functional *tlr4* gene (C3H/HeJ), the IBC developmental cascade

progresses normally but without any appearance of neutrophils at the site of infection (91). Also, UPEC emerging from infected umbrella cells of C3H/HeJ mice fail to form filaments 48 hours after infection (50). The inability of C3H/HeJ mice that lack a functional TLR-4 to mount an inflammatory response upon intravesicular challenge with *E. coli* suggests that TLR-4 is the primary TLR that generates an inflammatory response in the bladder (40). In 2004, TLR-11 was identified as a specific receptor for UPEC in mice (107). But a bacterial ligand for TLR-11 is yet to be identified and it is still unknown whether there is a human homologue of this receptor.

The cytoplasmic domains of TLRs (TIR) initiate a signaling cascade that ends in the activation of NF-κB. Activated NF-κB goes to the cell nucleus and induces transcription of anti-apoptotic and pro-inflammatory cytokines such as IL-6 and IL-8. The presence of IL-6 and IL-8 in the urine of patients suffering from cystitis and pyelonephritis indicates a possible role for these cytokines in UTI pathogenesis (69). The epithelial cells of the urinary tract are a major source of these cytokines found in the urine. Type 1 and P piliated UPEC have been shown to elicit stronger cytokine stimulation as compared to their isogenic mutants. IL-6 is a pro-inflammatory or an immuno-modulatory cytokine, which is known to facilitate transition from neutrophilic to a predominantly monocytic response in many infectious diseases, although its role in UTI is still unclear. IL-8 induction correlates with the appearance of PMNs in the urine (pyuria), which is a hallmark of UTI. In mice, it has been shown that induction of MIP-2 (a murine homologue of IL-8) correlates with pyuria, underlining a role of IL-8 as a neutrophil chemoattractant (41).

Many Gram-negative pathogens such as *Yersinia* and *Salmonella* spp. use the type III secretion system to deliver effector molecules to interrupt the NF-κB signal transduction cascade of the host (71, 73). It has been suggested that the ability of UPEC to suppress NF-κB signaling might be advantageous in establishing a pathogenic cascade in the urinary tract (especially in the bladder) despite a robust immune response. The mechanics of such suppression must be different from that of *Yersinia* and *Salmonella* spp. because UPEC lack the type III secretion system. The laboratory strain of *E coli* K12 (MG1655) activates IL-6 production in 5637 and T24 bladder epithelial cell lines in a dose dependent manner (45). In contrast, IL-6 production when these cell lines are treated with the UPEC clinical isolates UTI89 and NU14 is similar to unstimulated levels. These UPEC strains also abolish the induction of IL-6 even when the cell lines (5637 and T24) are treated with LPS or laboratory strains of *E. coli* (45).

XI. Model for UPEC Infections in the Bladder

A model depicting the various stages of UPEC infection in the bladder is shown in Fig. 1.4. The FimH tip adhesin of type 1 pili on the surface of UPEC mediates the attachment of bacteria to the uroplakins on the facet cells of bladder epithelium. The soluble and cell associated factors in the bladder such as Tamm-Horsfall proteins, secretory IgA, low molecular weight sugars and uromucoid act as anti-adhesive innate immunity mediators. The FimH adhesin together with LPS also activates the host signal transduction cascade involving protein tyrosine kinase, PI-3K and localized host actin cytoskeletal rearrangements. This leads to the AUM of facet cells zippering around the

bacteria to bring about their internalization. The inside of a facet cell provides a safe haven for the UPEC, away from the immune onslaught of the host. By an unknown mechanism, the internalized bacteria escape into the cytoplasm, replicate and establish the IBCs. In response to bacterial invasion, the facet cells, which otherwise have a very slow turnover rate, exfoliate and get washed off with the flow of the urine. To avoid being washed off with the exfoliating cells, the bacteria flux out of the infected cells and invade the underlying layers of the bladder epithelium. The ability to move in and out of the host urothelium can aid UPEC in disseminating to the other parts of the urinary tract. The bacteria emerging out of the facet cells are often filamentous in form. The exfoliation of the superficial facet cells is thought to expose lower levels of the urothelium to the bacterial infection. UPEC might persist in these cells for long periods, in a quiescent state, to reactivate at a late time point causing a recurrent UTI.

XII. The Model Organisms

This project uses two clinical isolates of UPEC, CFT073 and UTI89, for the diversity of clinical conditions they are known to cause. CFT073 was isolated from the blood of a woman suffering from acute pyelonephritis and this strain has very high cytotoxic and hemolytic activity (65). In contrast, UTI89 was isolated from a cystitis patient (68). The sequencing of both of these organisms has been completed (12, 104). Based on analysis of the genomic sequence, it is known that both CFT073 as well as UTI89 contain a T2SS (similar to the K12 T2SS), along with genes coding for components of T4P biosynthesis (12, 104). UTI89, but not CFT073, also possesses a

second T2SS gene cluster that is homologous to a T2SS present in enterotoxigenic *E. coli* (ETEC) strain H10407.

Stanley Falkow formulated molecular Koch's postulates in 1988 as guidelines to characterize whether a specific gene is an essential component of a microorganism's ability to infect and cause disease in a particular host (23). These postulates state that the specific inactivation of the gene(s) associated with the suspected virulence trait should lead to measurable loss in pathogenicity and that the re-introduction of WT genes should be accompanied by the restoration of virulence (22). Using the molecular Koch's postulates as guidelines, we generated deletion mutants in the essential T2SS and T4P genes, compared them with their parental WT strains using different *in vitro* and *in vivo* experiments, and complemented the defective phenotypes in mutants by introducing plasmid-borne copies of the deleted gene. In lower organisms such as bacteria, the C value (size of the haploid genome) (35) is proportional to the metabolic and morphological complexity of the organism (10). In other words, one can argue that a gene, let alone an operon, will not be present in the genome of a bacterium unless it has some functional importance. Thus, in case of CFT073 and UTI89, what remains to be found is the functions of the type II secretion and type IV pilus systems and their relevance to the disease process.

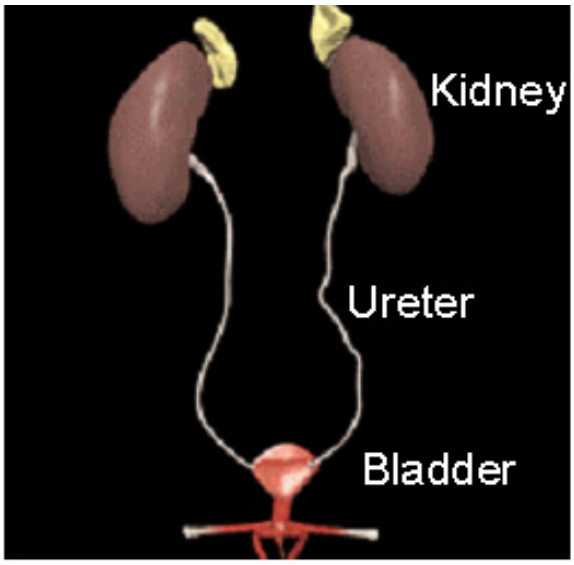

FIG 1.1. Human urinary tract. A 3-D space filled rendering of human urinary tract shows kidneys, which are the sites of blood filtration and urine formation; ureters, tubular structures that transport the urine to the urinary bladder, a muscular sac that stores the urine for excretion. (http://human-anatomy.net/urinary-tract-pictures.html)

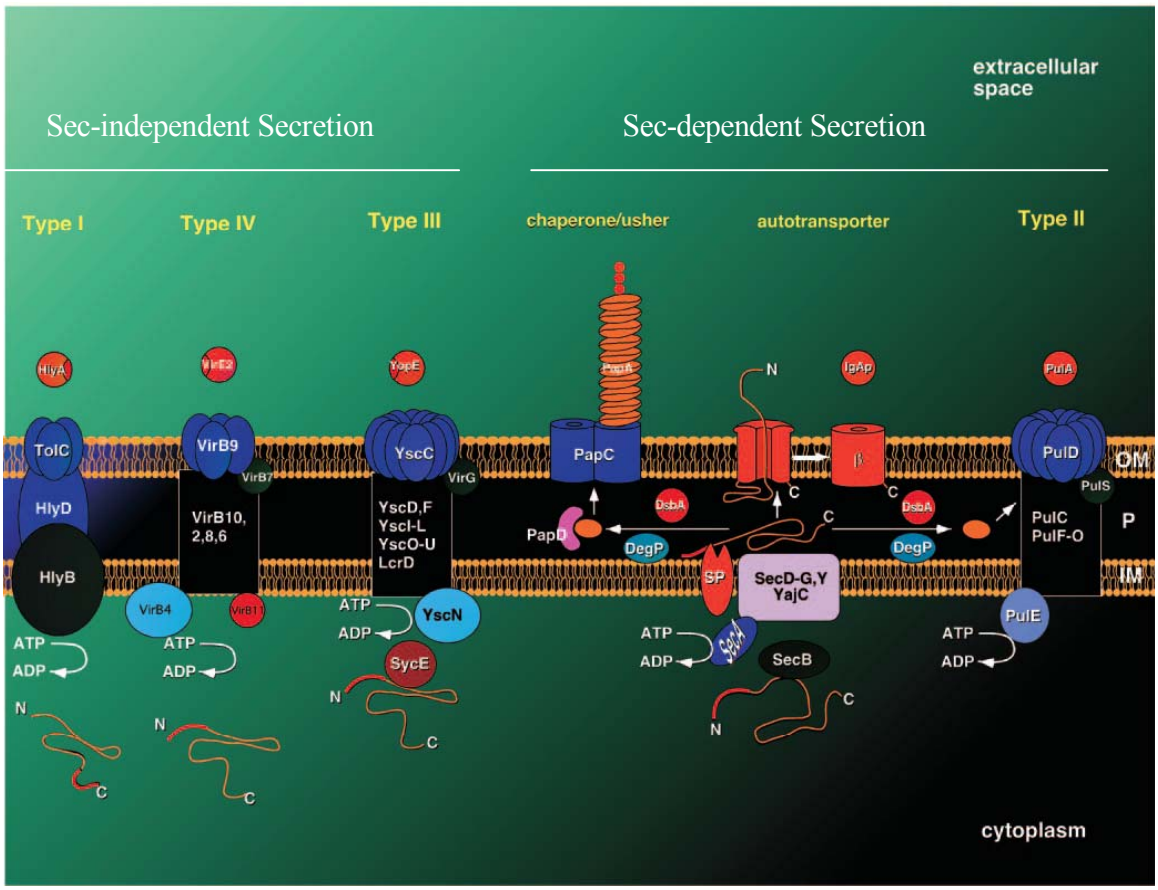

FIG. 1.2. Schematic representation of sec-independent and sec-dependent pathways. In Gram-negative bacteria, virulence effectors produced in the cytoplasm have to pass through the inner membrane (IM), periplasm and outer membrane to reach the extracellular space and interact with host cells. This is accomplished by a variety of secretion mechanisms. Well-known effector proteins secreted by these pathways are shown. Hemolysin (HlyB) is secreted by the type I pathway in pathogenic *E. coli*, YopE by type III in *Yersinia pestis*; VirE2 by type IV in *Agrobacterium tumefaciens*, P pili by chaperone-usher pathway in UPEC; Immunoglobin1A protease by autotransporter in *Neisseria gonorrohoeae*, and pullulanase (PulA) by type II in *Klebsiella oxytoca*. Figure adapted from reference (55).

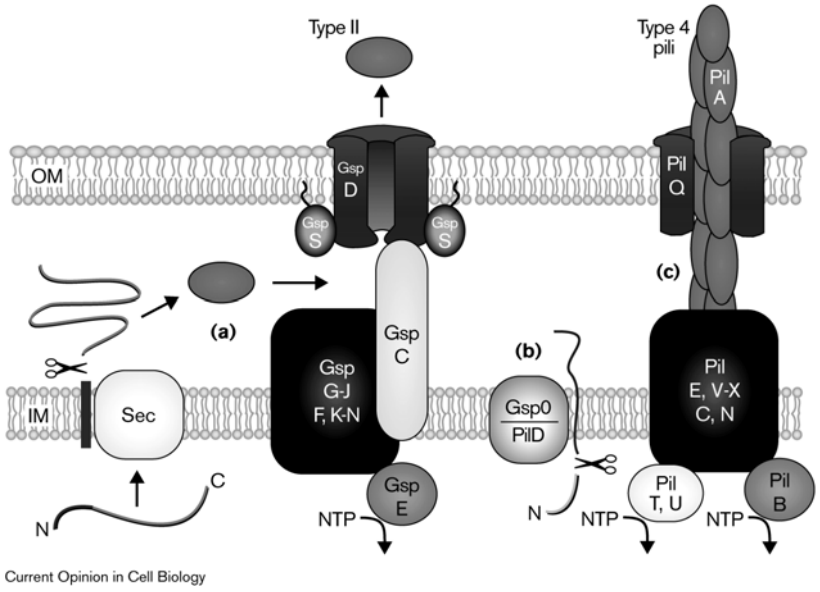

FIG. 1.3. Schematic representation of type II secretion and type 4 pilus biogenesis. The components of type II secreton and T4P proteins are indicated using Gsp and Pil nomenclatures, respectively. (a) T2SS effectors are transported across the IM in a Sec-dependent manner followed by the cleavage of the signal sequence, protein folding in the periplasm, and the transport of the folded periplasmic intermediate across the OM intermediate through the gated GspD secretin. GspC may transmit the energy generated by the nucleotide binding protein, GspE, across the periplasm. (b) GspO or PilD is the prepilin peptidase that is required for the processing of T2 pseudopilins (GspG-H) and T4P pilin (PilA) and pilin like components such as PilE, PilV-X. (c) T4P fibers are secreted across the OM through the PilQ secretin. The nucleotide binding proteins, PilT and PilU provide energy for twitching motility while PilB energizes the pilus assembly. Figure taken from reference (96).

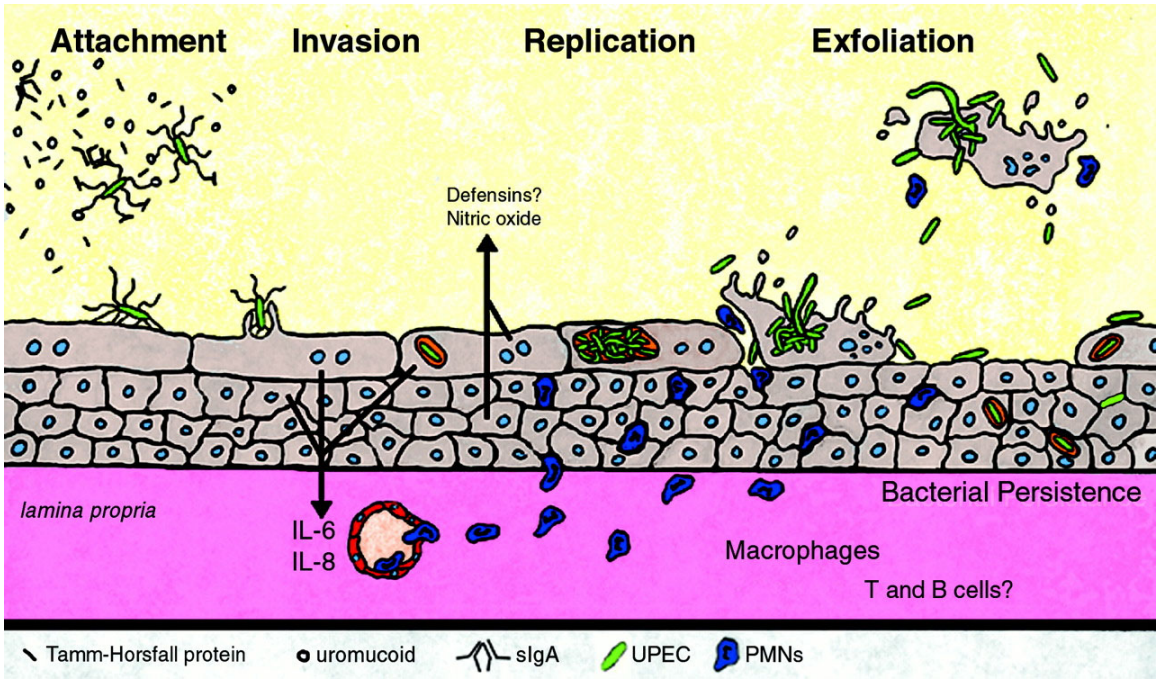

FIG. 1.4. Progression of bladder infection by UPEC. UPEC expressing surface adhesive pili adhere to uroplakins on the luminal surface of the superficial umbrella cells aiding the bacteria to avoid getting purged by the flow of urine. Various host factors such as Tamm-Horsfall protein competitively inhibit the interaction of the pilus adhesin with uroplakins. UPEC are internalized in a FimH dependent manner, and replicate to form IBCs. Apoptosis is induced and the infected umbrella cells are exfoliated. Meanwhile IBCs mature and filamentous, motile bacteria emerge and disseminate infection to other parts of the urinary tract. Figure taken from reference (69).

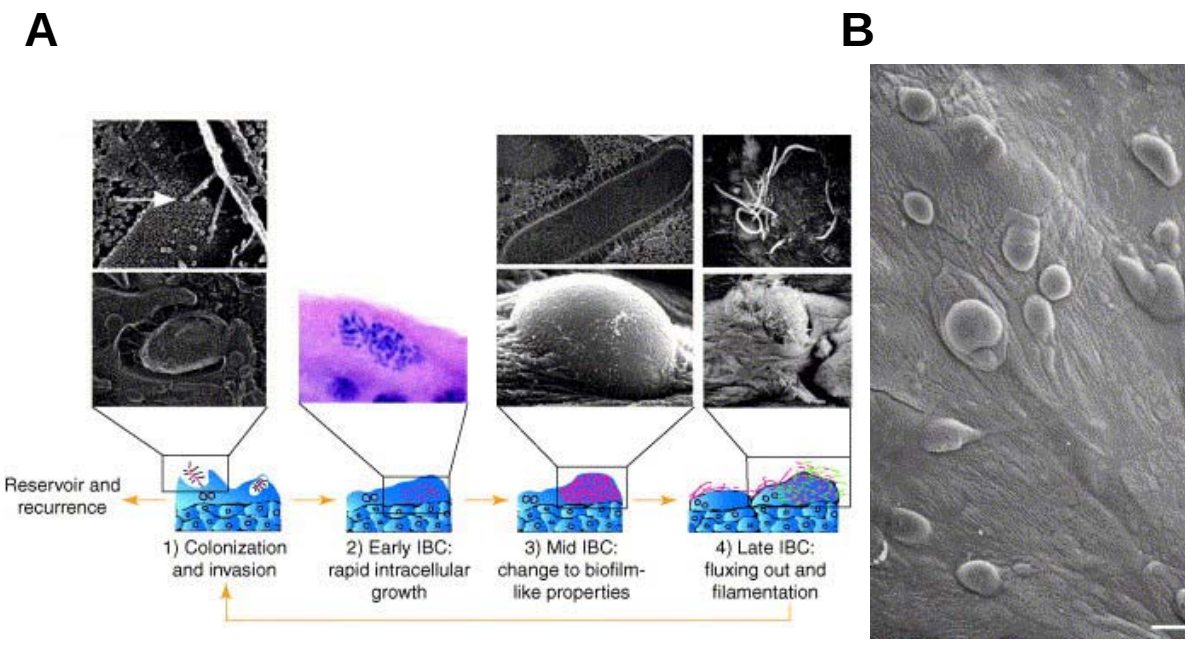

FIG. 1.5. UPEC developmental cascade in the host bladder. (A) Stages of IBC formation and maturation are shown in a figure taken from a review by Kau *et al* (53). 1) UPEC attachment to the uroplakins on the surface of facet cells (upper panel), and eventual zippering of epithelial cells around UPEC is followed by 2) the formation of IBCs as shown by hematoxylin and eosin (H&E) staining (upper panel). 3) During the mid-IBC stage, UPEC interacts intimately with its matrix (upper panel) and also forms luminal protrusions or pods (lower panel). 4) Late IBC stage is marked by the filamentation of UPEC (upper panel) which later burst out of IBCs (lower panel). (B) Scanning electron micrograph of pods formed on the surface of a mouse bladder. Scale bar is 50 μm. Figure taken from Anderson *et al* (2).

Chapter Two

Materials and Methods

Bacterial Strains and Cell Lines

UPEC strain CFT073 was isolated from the blood of a woman suffering from pyelonephritis (65), UTI89 from a cystitis patient (68), while MG1655 is a well-characterized laboratory strain of *E. coli* (5). The strains from the panel of pathogenic and non-pathogenic *E. coli* isolates are listed in Table 2.1. The bacteria were grown and maintained on LB agar plates or LB broth. When necessary, the medium was supplemented with ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (25 μg/ml) or arabinose (0.02%). Human bladder (5637 or ATCC#HTB-9) and human kidney (A498 or ATCC#HTB-14) cell lines were obtained from ATCC. 5637 cells were maintained in RPMI supplemented with 10% fetal bovine serum (FBS; Gemini) while A498 cells were maintained in modified Eagle's medium (MEM) supplemented with 10% FBS, 1X sodium pyruvate and 1X L-glutamine. For growth as well as during the steps of infection assays the cell lines were kept at 37^0C in presence of 5% CO_2.

Growth Curve Experiments

Bacterial cultures were grown overnight at 37^0C, 200 rpm in LB broth and diluted in LB broth or tissue culture medium (RPMI +10% FBS) to $OD_{600} < 0.1$. The cultures were maintained at 37^0C, 200 rpm for growth curve experiment in LB broth, or statically at 37^0C in presence of 5% CO_2 for tissue culture medium. The optical density of the

culture was measured at 600 nm using a spectrophotometer at indicated time points till reading reached a plateau near the value of 1.

Enumeration of Bacteria

Infected cell lines or organs were treated with 0.4 and 0.02% triton X-100 (TX100) detergent in PBS, respectively. The cell monolayers were then kept at 4^0C for 10 min to help lift the cells off plastic while the organs were ground in a tissue homogenizer. To obtain bacterial counts, the suspensions were vortexed, serially diluted in sterile LB broth (100 µl of bacterial suspension was added into 900 µl sterile LB) and 100 µl of appropriate dilutions were spread on LB agar plates. After incubating the plates at 37^0C overnight, single colonies were counted and CFU/ml estimated.

RNA Isolation and Semi-Quantitative Reverse Transcription (RT)-PCR.

Overnight cultures grown in LB were diluted 1:40 into LB or tissue culture medium (RPMI + 10% FBS) and grown to early-to-mid log phase at 37ºC. LB cultures were grown with aeration and bacteria in tissue culture medium were grown statically in 5% CO_2. Bacteria were harvested after mixing with twice the volume of RNAprotect reagent (Qiagen). RNA was isolated using RNeasy kit (Qiagen) as per manufacturer's instructions. Contaminating DNA was removed by on-column DNase treatment, performed twice, using RNase-free DNase (Qiagen). RNA was eluted in RNase-free water and the integrity of the RNA sample was checked by agarose gel electrophoresis and ethidium bromide staining.

14 µl of DNase treated RNA was then mixed with 1 µl random primers (Invitrogen), the mixture was kept at 95^0C for 2 min and then cooled to 42^0C over a period of 20 min. 15 µl of mastermix containing 5X AMV reverse transcriptase buffer (Roche), 0.3mM dNTPs and 50 U of AMV reverse transcriptase (Roche) was then added and the mixture kept at 42^0C for 150 min. The cDNA was PCR amplified using specific primers listed in Table 1. The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized using a Gel Doc 2000 gel documentation system (Bio-Rad).

Generation of KO mutants

The method described in (16) was used to generate genomic deletion mutants lacking the genes coding for OM secretins. This method harnesses the ability of the bacteriophage λ recombination system (*γ*, *β* and *exo*) to bring about homologous recombination as described in Fig. 2.2. Primers P0 and P1 were constructed such that they contained, at their 3' ends, 50 nucleotide long overhangs (H1 and H2) complimentary to regions at the two ends of the target gene and are able to amplify a region containing the antibiotic resistance cassette (ARC) carried on a template plasmid. The PCR products were gel purified and cleaned to electroporation grade (Step I). In parallel, a plasmid (pKD46, Red helper plasmid), carrying bacteriophage λ recombination genes under an arabinose promoter, was electroporated into the strain in which the KO was to be carried out. Replication of this plasmid is temperature-sensitive. In the presence of arabinose, enzymes necessary for recombination were ectopically produced inside the cell. These bacteria were then electroporated with the PCR products carrying the ARC.

Homologous recombination and concomitant replacement of the gene by the ARC was determined by the ability of bacteria to grow on medium containing antibiotic (Step II). The bacteria were then maintained in a medium lacking antibiotic at a temperature that does not allow pKD46 replication. Subsequently, drug resistant bacteria were made electro-competent and electroporated with a plasmid (pCP20) carrying the FLP gene under the control of a natural promoter (Step III). At the permissive temperature, the FLP recombinase was induced to eliminate the ARC. Cells were cured of pCP20 by growing them at a temperature non-permissive for its replication (Step IV).

Adhesion, Invasion and Efflux Assay

To cause a successful UTI, UPEC have to adhere to the host urothelium, invade it, form intracellular replication foci, escape the facet cells that are being shed as part of innate immune response and infect underlying layers of cells. These four cardinal steps in UPEC mediated UTI, namely adhesion, invasion, intracellular growth and efflux (extrusion) are recapitulated in a modification of the gentamicin protection assay (61) the procedure for which is described below. We used this assay protocol to compare different KO mutants with their WT counterparts in order to look for any differences in one or more of the four characteristic steps in UTI.

Human bladder or human kidney cell line monolayers in 24 well plates were infected with the appropriate bacteria grown in static cultures at 37^0C for 48 hours. Prior to infection, the number of bacteria was normalized to OD600~0.5 and enumerated further by plating the serial dilutions of bacteria on LB agar plates; **initial count (IC**). 10 µl/ml of the normalized bacteria were used for infection to achieve an MOI of 5-8. The

bacteria were spun down at 800 X g for 5 min to expedite their contact with host cell monolayers. The infected monolayers were kept at 37^0C in presence of 5% CO_2 for 2 h.

For the adhesion-invasion module of this assay, the monolayers from one set of wells were washed five times with sterile PBS containing $CaCO_3$ and $MgCO_3$ (Ca^{++}/Mg^{++}), lysed with 40 µl of 10% TX100. The intra-cellular as well as adherent bacteria in these lysates were enumerated; **adherent bacteria** (**AB).** The second set of wells that was left unwashed was lysed with TX100. The intracellular, adherent as well as non-adherent suspended bacteria in the lysates were enumerated; **total bacteria** (**TB**). Adherence frequencies were calculated as %AB ÷ TB.

The third set of wells was treated with 100 µg/ml gentamicin for 2 hours at 37^0C in the presence of CO_2. Further, this was washed five times with PBS (Ca^{++}/Mg^{++}), treated with TX100 and **intracellular bacteria** (**IB**) were enumerated. Invasion frequencies were calculated as %IB ÷ IC.

For the efflux assay module, the infection steps were carried out just as in the adhesion-invasion module except that after the 2-hour treatment with 100 µg/ml gentamicin, the infected monolayers were washed with PBS (Ca^{++}/Mg^{++}) once and were maintained in presence of 10 µg/ml gentamicin overnight at 37^0C in 5% CO_2. On day 2, the intracellular bacteria in one set of wells were enumerated after washing off gentamicin and lysing the cells with TX100; **overnight intracellular bacteria** (**OB**). The other two sets of wells were washed 5 times with PBS (Ca^{++}/Mg^{++}) and kept for 6 hours at 37^0C, 5% CO_2 either in the presence or absence of gentamicin. In the absence of gentamicin, the intracellular bacteria form the IBCs and start fluxing out of the infected cells. These cells were treated with TX100, and the suspensions plated to obtain the total

number of intracellular and **effluxed bacteria** (**EB**). Efflux index was calculated as %EB ÷ OB. The **intracellular bacterial counts** (**IC-6**) at the end of the 6 h incubation period were determined from the wells containing gentamicin. The intracellular growth index over a period of 6 h was determined as % IC-6 ÷ OB.

For an extended intracellular growth experiment, the infected steps were carried out as in the adhesion-invasion module, followed by washing with PBS (Ca^{++}/Mg^{++}) and maintaining the infected monolayers in presence of 10 μg/ml of gentamicin. The intracellular bacteria were enumerated after washing the monolayers and lysing the cells using TX100, at indicated time points.

Mouse Infections

Static cultures of bacterial strains were grown in 20 ml LB broth at 37^0C for 48 hours. On the day of animal infection, the bacteria were centrifuged and pellets resuspended in sterile PBS.

6-8 weeks old CBA/J female mice were infected with 50 μl of bacterial suspensions normalized to ~10^9 CFU/ml. The mice were anesthetized using isoflurane and the 50 μl bacterial suspension or sterile PBS (as negative control) was delivered to the bladder by transurethral catheterization using UV sterilized 0.28 mm inner diameter tubing (Becton Dickinson cat # 427401) mounted on 30 ½G needle tips. Dilutions of the inocula were plated on LB agar to determine input CFU/ml for all bacterial strains. 7 mice were infected with each bacterial strain. On day 7 PI, the animals were killed by CO_2 asphyxiation, dissected and organs (bladders and kidneys) harvested. Bladders and

right side kidneys from 5 infected mice were homogenized in sterile PBS containing 0.02% TX100, and serial dilutions were plated on LB agar plates.

Bladders and right side kidneys from the other two infected mice were stored in paraformaldehyde for tissue sectioning. Sections were stained by hematoxylin and eosin (H&E). The left side kidneys were homogenized, serial-diluted and CFUs enumerated. Each mouse experiment with the UTI89 and CFT073 knockout mutants was repeated at least once. Comparison of the UTI89 WT, Δ*hofQ*, and Δ*hofQ*/p*hofQ* strains was performed once. Comparison of the CFT073 WT, Δ*yheF* Δ*hofQ*, Δ*yheF* Δ*hofQ*/p*yheF* and Δ*yheF* Δ*hofQ*/p*hofQ* strains was performed twice.

All animal work was conducted according to the guidelines of the Stony Brook University institutional animal care and use committee (IACUC# 2005-1465).

Generation of Rescue Plasmids

The genes *hofQ* and *yheF* were PCR amplified using cloning primers listed in Table 2.2. The PCR products were gel purified, ligated into pGEM® T-easy vector (Promega) as per the manufacturer's instructions and the ligation products were transformed into DH5α. The transformants containing correct ligation products were identified by their ability to grow in the presence of 100 μg/ml ampicillin. The colonies containing pGEM-*hofQ* and pGEM-*yheF* were purified, the plasmids isolated and analyzed by DNA sequencing. EcoRI digested fragment from pGEM-*hofQ* and BamHI, HindIII digested fragment from pGEM-*yheF* were ligated into pMMB67HE pretreated with the same restriction endonucleases to obtain plasmids, p*hofQ* and p*yheF*. The correct orientation of *hofQ* gene in pMMB67 was confirmed by the DNA sequencing. These

plasmids were then transformed into appropriate deletion mutant strains for use in the rescue experiments.

IL-6 Suppression Assay

UPEC suppress the induction of IL-6, a pro-inflammatory or immunomodulatory cytokine, produced by the urothelium in response to Gram-negative bacterial LPS, laboratory strains of *E. coli* and IL-1 (45). We compared different KO mutants with their WT counterparts for ability to suppress IL-6 production by the human bladder cell line, 5637 using the method described by Hunstad *et al* (45).

Static cultures of bacteria were grown overnight at 37^0C in 20 ml LB broth. 48 hour prior to the experiment, 5637 bladder cells were treated with trypsin-EDTA, washed with tissue culture medium (RPMI+10%FBS) and seeded into 24-well plates to get confluent monolayers. On the day of experiment, bacteria were centrifuged, the pellet washed in pre-warmed sterile PBS, and resuspended in PBS containing such that there were 10^9cfu/ml (bacteria were enumerated by plating serial dilution on LB agar plates to determine the numbers of live bacteria added to each well). Bladder cell monolayers were washed with sterile PBS once and fresh tissue culture medium containing 5 µg/ml O157 LPS (Sigma) applied. 10µl normalized bacteria were added to each well. The bacterial contact with cells was expedited by centrifuging the plates at 400 X g for 3 min and the plates incubated at 37^0C in presence of 5% CO_2 atmosphere for 12 hours. At the end of incubation period, the supernates were extracted, spun at 13000 X g for 5 min to pellet bacteria as well as cell debris and stored at -80^0C until IL-6 estimation by ELISA (Antigenix America).

Cytotoxicity Assay

Cytotoxicity induced by different knockout mutants and their WT counterparts was compared under two different conditions; during the period of efflux after gentamicin removal and in the first three hours after infecting the 5637 human bladder cell line.

To measure cytotoxicity during bacterial efflux, 5637 human bladder cells were infected with WT or knockout UPEC strains for an efflux assay as described above. Upon removal of gentamicin on the second day of the infection, the supernatant fractions were collected at 2, 4 and 6 h time points. These fractions were tested for the presence of LDH using the Cytotox 96 non-radioactive cytotoxicity assay (Promega). In separate experiments, LDH release was determined after infecting 5637 cells for 3 h with different amounts (10^4 to 10^6 CFU/ml) of WT or knockout UPEC strains.

Biofilm Assay

Overnight grown (200 rpm, 37^0C.) bacterial strains were diluted 1:100 and 100 µl of diluted culture was added to a 96-well, U-bottom, polystyrene plate in quadruplicate. *P. aeruginosa* was used as a positive control, medium only wells as negative control, and 3 wells were left empty as blanks. The plates were then incubated under stationary growth conditions at 37^0C for 48 h. At the end of the incubation period, the wells were washed with PBS three times, vigorously, to remove all planktonic bacteria. 150 µl of 0.1% crystal violet was added to all the wells except the blanks and incubated for 10 min at room temperature to stain the biofilms. Later, the wells were washed three times with water, until no color was left in the negative control wells. Plates were then inverted on a

paper towel for 30 min to dry the wells completely. To solubilize the stained biofilms, 200 µl solvent solution (80% ethanol and 20% acetone) was added to all the wells including blanks, and the covered plates were incubated at RT, shaking for 15 min. 150 µl of the solubilized biofilms were transferred to fresh 96-well plates and the absorbance measured at 575 nm. $OD_{575} \geq 0.8$ is considered positive for biofilm formation.

Statistical Analysis.

The CFU/g of organ weight results from the mouse infection experiments were compared using the Mann-Whitney test for non-parametric data with one-tailed P value. The cell culture assay indices (efflux, adhesion, and invasion) were compared using Dunnett's multiple comparisons test. P values < 0.05 were considered significant.

Table 2.1 List of strains from the panel of pathogenic and non-pathogenic *E. coli*.

Clinical Isolate	Associated Pathological Condition	Source[a]
SNAC-396	First UTI	TMH
SNAC-403	First UTI	TMH
VVC-117	First UTI	TMH
C&C-1022	First UTI	TMH
C&C-1033	First UTI	TMH
Pylo-27	Pyelonephritis	TMH
Pylo-28	Pyelonephritis	TMH
Pylo-29	Pyelonephritis	TMH
Pylo-30	Pyelonephritis	TMH
Pylo-31	Pyelonephritis	TMH
Pylo-33	Pyelonephritis	TMH
Pylo-34	Pyelonephritis	TMH
Pylo-36	Pyelonephritis	TMH
Pylo-37	Pyelonephritis	TMH
Pylo-38	Pyelonephritis	TMH
Pylo-39	Pyelonephritis	TMH
Pylo-41	Pyelonephritis	TMH
Pylo-45	Pyelonephritis	TMH
Pylo-49	Pyelonephritis	TMH
Pylo-50	Pyelonephritis	TMH
TOP-9	Asymptomatic Bacteriuria	TMH
TOP-14	Asymptomatic Bacteriuria	TMH
TOP-31	Asymptomatic Bacteriuria	TMH
TOP-13	Asymptomatic Bacteriuria	TMH
TOP-21	Asymptomatic Bacteriuria	TMH
TOP-2	Recurrent UTI	TMH
TOP-11	Recurrent UTI	TMH
TOP-48	Recurrent UTI	TMH
TOP-49	Recurrent UTI	TMH
TOP-54	Recurrent UTI	TMH
TOP-80	Rectal Isolate	TMH
TOP-81	Rectal Isolate	TMH
TOP-82	Rectal Isolate	TMH
TOP-85	Rectal Isolate	TMH
TOP-86	Rectal Isolate	TMH
TOP-89	Rectal Isolate	TMH
U01-85	Cystitis	TMH
U01-87	Cystitis	TMH
U01-89	Cystitis	TMH
U01-90	Cystitis	TMH
U01-92	Cystitis	TMH
U01-93	Cystitis	TMH
U01-101	Cystitis	TMH

Clinical Isolate	Associated Pathological Condition	Source[a]
U01-105	Cystitis	TMH
U01-106	Cystitis	TMH
U01-107	Cystitis	TMH
U01-108	Cystitis	TMH
EC-42	Cystitis	MAM
EC-93	Cystitis	MAM
EC-45	Cystitis	MAM
EC-55	Cystitis	MAM
EC-56	Cystitis	MAM
B-217	Cystitis	MAM
B-212	Cystitis	MAM
B-220	Cystitis	MAM
B-223	Cystitis	MAM
M-1	Meningitis	Lab
M-2	Meningitis	Lab

[a] The clinical isolates from the panel were supplied by Dr. Thomas M. Hooton (TMH), University of Miami or by Dr. Matthew A. Mulvey (MAM), University of Utah.

Table 2.2 List of primers used in this study.

Primer	Sequence	Size of PCR Product (bp)	
		WT	Knockout
T4P Secretin (*hofQ*)			
Knockout forward (*P0*[a])	TTACTCACTGGAAACCAGTCGTGGCGTG ATAAACACCACTAACTCGCGTC*GTGTAG GCTGGAGCTGCTTC*		
Knockout reverse (*P3*[a])	ATGAAGCAATGGATAGCCGCACTACTGT TGATGCTGATACCCGGCGTACA*CATATGA ATATCCTCCTTAGT*		
Flank A Flank B	CATATTGAGTTGTTGAGCTAACTGG GACAATTTTACAGCTGACGCC	1724	726
RT-PCR forward	GATGGTGGATGACGTTCCG	650	
RT-PCR reverse	AACGTCCGTTGATGCGTCC		
Clone forward	CAAC<u>AAGCTT</u>GTGAGCCGTTTTTCGTTA AGCGTGG (HindIII)		
Clone reverse	GATA<u>AAGCTT</u>CATCATGGAACGCAGGCA GC (HindIII)		
ETEC-T2SS secretin (*gspD*)			
Knockout forward (*P0*[a])	TTAACGCGTTCTCCCGGCATTGAGGAAC GCGCGTACTTCCGGCGGTAAGGT*GTGTA GGCTGGAGCTGCTT*		
Knockout reverse (*P2*[a])	GTGTTTTGGCGTGATATGACGTTGTCTAT CTGGCGTAAGAAGACAACTGG*CATATGA ATATCCTCCTTA*		
Flank A Flank B	AGAGAGCGACAACGGATGAACATGG CGCCATTGCGCTAAATCAGC	2343	323
RT-PCR forward	CATACCGTCACGCAAAATGGTC	899	
RT-PCR reverse	CCAGCAAACACAGTAATGCCCTG		
K12-T2SS secretin (*yheF*)			
Knockout forward (*P0*[a])	GGACTCAATAAAATCACCTGCTGCTTGC TGGCAGCACTACTCATGCCTTGT*GTGTAG GCTGGAGCTGCTT*		
Knockout reverse (*P2*[a])	TCACCGTGACGATGGCGCAGGAGCGTG ACTGTTGAACGTGTTCTCATCCA*CATATG AATATCCTCCTTA*		
Flank A Flank B	GAACTTCAGGAATGAATGG TGAATTCTCATAAGAATGCC	2018	194
RT-PCR forward	TGCAGGACACGCTGAGAACG	350	
RT-PCR reverse	GAACGTTCTCAAGCGGTACG		

Primer	Sequence	Size of PCR Product (bp)	
		WT	Knockout
Clone forward	TGCTAAGCTTACAATTCGTCGCAACGGC (HindIII)		
Clone reverse	AGCGGGATCCTGGCGGGGTACGGTGAG TG (BamHI)		
Type 1 pilus usher (*FimD*)			
Knockout forward (*P0*[a])	ATGTCATATCTGAATTTAAGACTTTACC AGCGAAACACACAATGCTTGCA*GTGTAG GCTGGAGCTGCTTC*		
Knockout reverse (*P2*[a])	TTAACGACATTCAGGCTGATAGCTGGGT TAATAACTGCTGCTGACTCTCTG*CATATG AATATCCTCCTTAGT*		
Flank A	CGCACTTACCCCCAAAATGAC	2959	
Flank B	ATGGAACAACAGGAGTCGTCGC		
16S rRNA			
Forward	TGACGGGGGCCCGCACAAGC	222	
Reverse	CGGCCGGACCGCTGGCAACA		
E. coli Panel (ETEC-T2SS specific primers)			
J Forward	CAACCAGATGCGTTCAATTTCG	450	
J Reverse	TCTGACACAAATGATGCCGC		
D Forward	TTAAGCCTTGCTCATCGCGG	500	
D Reverse	CTTCTTCATGGTGGGCCAGG		

[a] The primers *P0*, *P1*, *P2*and *P3* are italicized.
[b] The restriction sites introduced in the primers are indicated in parentheses and their location within the primer sequence is underlined.

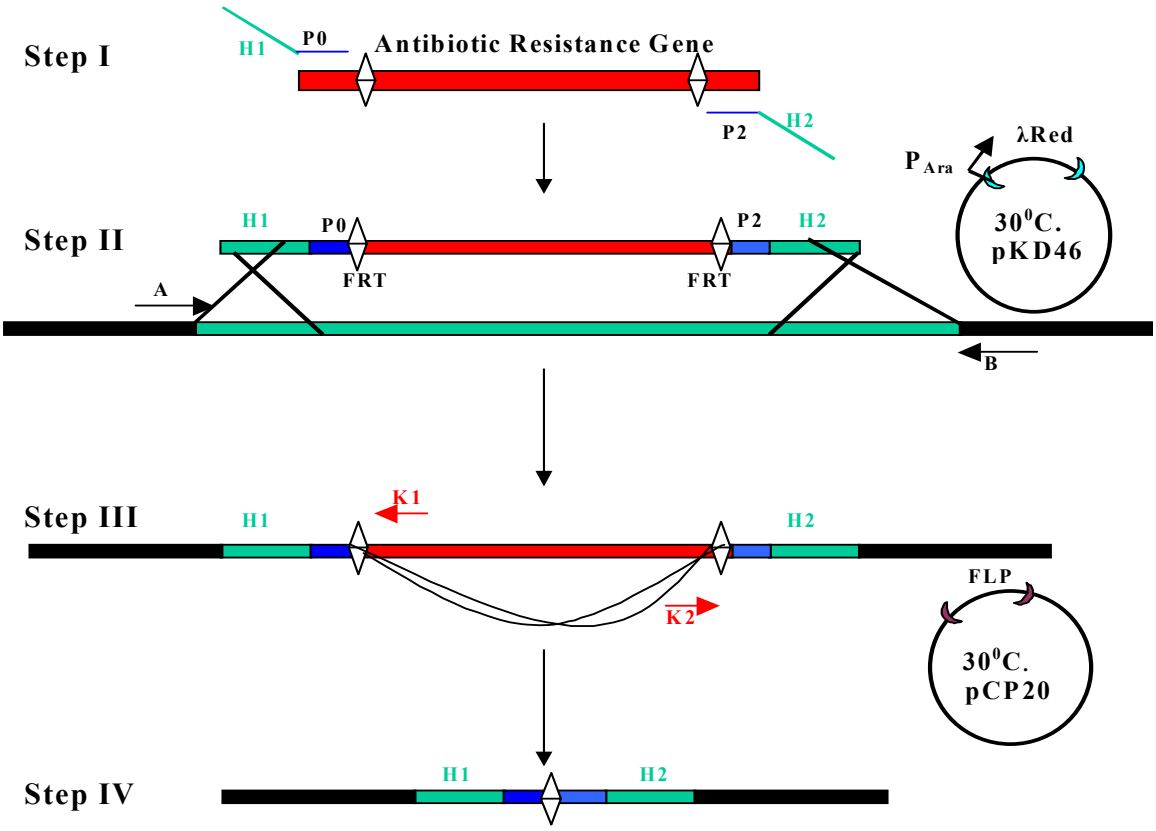

FIG. 2.1. Schematic representation of λ_{Red} recombinase-based gene deletion. P0 and P2 are priming sites in the antibiotic resistance cassette carried on a template plasmid. H1 and H2 are 50-60 nucleotide long regions homologous to regions of the target gene. A and B represent the flanking primer pair and K1 and K2 are ARC-specific primers. These were used for PCR verification of different variants and junction fragments formed during the procedure. Plasmid pKD46 carries bacteriophage λ recombination genes under the control of an arabinose promoter while pCP20 carries the FLP recombinase gene under the control of a natural promoter. Both of these plasmids replicate only at 30^0C (16).

Chapter Three

T2S and T4P Systems Contribute to the Virulence of UPEC

I. INTRODUCTION

E. coli are a diverse group of Gram-negative bacteria that can be subdivided into three major groups: commensals in the intestinal tract of warm-blooded mammals, agents of intestinal infections such as hemorrhagic colitis and hemolytic-uremic syndrome, and ExPEC (52). ExPEC are causative agents of a range of diseases, including UTI, nosocomial pneumonia, neonatal meningitis, and neonatal sepsis. UTIs comprise a group of disorders, including cystitis and pyelonephritis, which is defined by the presence of microorganisms in the otherwise sterile urinary tract and UPEC is the principal causative agent.

Many Gram-negative bacterial pathogens use type II secretion and the evolutionarily related T4P system to secrete and/or assemble virulence factors. Although both T2SS and T4P have well-studied roles in the virulence of intestinal pathogenic *E. coli* (33, 34, 94), their roles in the virulence of ExPEC had not been investigated. In this dissertation research, we investigated the roles of T2SS and T4P in the pathogenesis of the model UPEC strains CFT073 and UTI89, isolated from patients suffering from pyelonephritis and cystitis, respectively (65, 68). Both model strains, CFT073 and UTI89, possess a T2SS along with genes coding for components of T4P biosynthesis, which are similar to the K12 T2S and T4P systems (12, 104). Moreover, UTI89 possesses a second T2SS gene cluster that is homologous to a T2SS present in ETEC strain H10407. We

generated knockout mutations in OM secretin genes of the UPEC T2S and T4P systems and carried out direct comparison between wild type (WT) strains and knockout mutants *in vivo* using the CBA/J mouse model of ascending UTI. Our findings identify T4P as virulence factors of UTI89, and T4P and/or the T2SS as virulence determinants of CFT073. In particular, our results show that these systems are important for persistent infection of the upper urinary tract.

II. RESULTS

T2SS and T4P gene clusters in CFT073 and UTI89. UPEC strain CFT073 is a well characterized strain isolated from the blood of a woman suffering from pyelonephritis (65), whereas UTI89 is a UPEC strain isolated from a cystitis patient (68). Annotated complete genome sequences for both these model clinical isolates have been published (CFT073, accession NC_00431; UTI89, accession NC_007946) (12, 104). The T2SS and T4P genes present in the UTI98 and CFT073 genomes are shown schematically in Fig. 3.1. Both UPEC clinical isolates possess gene clusters homologous to a T2SS gene cluster present in *E. coli* K12 (K12-T2SS). Both UPEC strains also possess genes homologous to *E. coli* K12 T4P genes. The T2SS and T4P genes common to both UTI89 and CFT073 have very high amino acid homology to the corresponding *E. coli* K12 systems (91-99% identity, except for the *gspI* minor prepilin, which is 84% identical). In addition to the K12-homologous gene clusters, UTI89 possesses a second T2SS gene cluster with 84-97% amino acid homology to a cluster present in ETEC strain H10407 (ETEC-T2SS). This second UTI89 T2SS has relatively low homology to the K12-T2SS (16-60% amino acid identity). The ETEC-T2SS cluster is not present in strain CFT073.

RT-PCR was used to ascertain expression of the UPEC T2SS and T4P genes under various culture conditions, including growth to log phase in LB or tissue culture medium (RPMI + 10% FBS). Expression of T2SS and T4P genes in the *E. coli* K12 strain MG1655 was used for comparison. Fig. 3.2 shows the expression level of transcripts corresponding to the OM secretins. Both the *yheF* (K12-T2SS) and *hofQ* (T4P) secretin genes were expressed at low levels in UTI89 and CFT073, with expression

higher in LB than in tissue culture medium. Expression of these genes in the UPEC strains was comparable to expression detected for MG1655. Interestingly, the ETEC-T2SS secretin *gspD* was expressed at relatively high levels in both LB and RPMI, suggesting this system is activated under these culture conditions.

Construction of T2SS and T4P OM secretin deletion mutants. To analyze the importance of the T2SS and T4P genes in the pathogenesis of UPEC, we targeted the OM secretin of each system for deletion mutagenesis using the bacteriophage λ Red recombinase system (16). The secretin component forms a gated channel required for export of T2SS effectors or T4P across the OM. We made single knockout mutations of each of the three secretins present in UTI89, namely *yheF* for the K12-T2SS, *gspD* for the ETEC-T2SS, and *hofQ* for T4P (Fig. 3.1). For CFT073, we constructed single knockout mutations of the *yheF* (K12-T2SS) and *hofQ* (T4P) secretins, as well as a double deletion strain lacking both secretins (Fig. 3.1). Proper construction of the knockout mutations was verified by PCR (Fig. 3.3) using primers specific for the flanking regions of the targeted genes (Table 2.2). We also checked each of the knockout mutants for growth in LB, RPMI supplemented with 10% FBS (Fig. 3.4) and in M63 minimal medium (data not shown); growth of each mutant was similar to the parental WT strain.

Roles of the UPEC T2SS and T4P in pathogenesis. To determine the roles of the T2SS and T4P in the ability of UPEC to successfully infect the urinary tract, we tested the UPEC secretin knockout strains using the CBA/J mouse model of ascending UTI (38). For UTI89, we inoculated 10^7 CFU of the WT strain or one of the secretin mutants into the bladder by trans-urethral catheterization. At days 1 and 7 PI, animals

were sacrificed and CFU/g of bladder or kidney were determined. All knockout mutants colonized the bladder and kidneys to similar levels as WT UTI89 on day 1 PI (data not shown). On the other hand, while all knockout mutants were able to colonize the bladder similar to WT on day 7 PI (Fig. 3.5A), the Δ*hofQ* (T4P) mutant was significantly defective in colonizing the kidneys on day 7 PI (p=0.03, Fig. 3.5B). The Δ*yheF* (K12-T2SS) and Δ*gspD* (ETEC-T2SS) mutants colonized the kidneys similar to WT UTI89 (Fig. 3.5B). This defines a specific function for T4P in colonization of the kidney.

We next performed similar experiments with the CFT073 secretin mutants. 10^7 CFU of WT CFT073, the Δ*yheF* (K12-T2SS) or Δ*hofQ* (T4P) single secretin mutant, or the double secretin mutant (Δ*yheF* Δ*hofQ*) were inoculated into the bladder of CBA/J mice by trans-urethral catheterization. As found for UTI89, none of the CFT073 mutants showed defects in colonizing the bladder or kidneys compared to WT CFT073 on day 1 PI (data not shown). The single secretin knockout mutants of CFT073 also showed no defects in colonizing the urinary tract on day 7 PI (Fig. 3.5 C and D). However, CFU/g recovered from kidneys on day 7 PI of mice infected with the CFT073 Δ*yheF* Δ*hofQ* double mutant were significantly lower (p=0.015) compared to WT CFT073 (Fig. 3.7D). In addition, we noted consistently reduced, though not statistically significant, median CFU/g recovered from the bladders of mice infected with the double knockout strain (Fig. 3.5C). These results demonstrate a role for T4P and/or the T2SS in the ability of CFT073 to persist within the urinary tract.

Next, we checked whether the renal persistence of UTI89 Δ*hofQ* and CFT073 Δ*yheF* Δ*hofQ* mutant strains could be restored back to WT-like levels by introduction of the deleted genes in trans. The genes *yheF* and *hofQ* were cloned into the low-copy

number plasmid pMMB67HE as described in Materials and Methods. The resulting plasmids p*yheF* and p*hofQ* were used to complement the defective phenotype in UTI89 and CFT073. Complementation of UTI89 Δ*hofQ* with p*hofQ* restored the colonization of renal tissue in the CBA/J mouse infection model to WT-like levels (Fig 3.6A). To confirm the specificity of the kidney phenotype for the CFT073 Δ*yheF* Δ*hofQ* double mutant, we complemented this strain with either *yheF* or *hofQ* in trans (using plasmids p*yheF* or p*hofQ*, respectively). Complementation with p*hofQ* restored kidney colonization to WT-like levels (Fig. 3.6B). In contrast, although complementation with p*yheF* increased bacterial colonization in the kidney, this did not reach statistical significance (Fig. 3.6B). These results demonstrate a specific role for T4P in the ability of CFT073 to persist within the urinary tract, in particular the kidneys, and suggest the K12-T2SS may also contribute to kidney colonization.

Histological examination of the infected organs. To gain further insights into the defective nature of UTI89 Δ*hofQ* and CFT073 Δ*yheF* Δ*hofQ* in terms of the reduced levels of persistence in the kidneys, bladders and the right side kidneys from the animals inoculated with these mutant strains, their WT counterparts, or sterile PBS were fixed in formaldehyde, sectioned and stained with H&E. The CFUs from the corresponding left-side kidneys were enumerated to determine the extent of infection. As shown in Figs. 3.7 and 3.8, the kidney sections from mice infected with WT UPEC strains exhibited signs of severe inflammation, especially of the renal pelvis (the central part of the kidney where the urine accumulates prior to discharge), marked by the infiltration of papillary spaces and collecting ducts by inflammatory cells such as neutrophils. In contrast, kidneys obtained from mice inoculated with PBS exhibited normal tissue architecture as indicated

by clear papillary spaces, calyx, collecting ducts and the symmetrical nature of the glomerulus. The histological examination of renal sections obtained from mice infected with UTI89 Δ*hofQ* did not show any signs of inflammation (Fig. 3.7C). The kidney sections obtained from a mouse infected with CFT073 Δ*yheF* Δ*hofQ* looked free of inflammatory cells, similar to the PBS inoculated kidneys (Fig. 3.8C) and the left-side kidney from this animal did not yield CFUs. In contrast, the kidney section from the second CFT073 Δ*yheF* Δ*hofQ* infected mouse showed WT-like inflammation (Fig. 3.8D). The corresponding left-side kidney from this mouse yielded high CFUs (1.2×10^5 CFU/g of kidney). Thus, there was an obvious correlation between inflammation and the presence of detectable pathogens in the renal tissue. Consistent with the lack of detectable phenotype in the bladder, all bladder sections presented similar signs of inflammation, whether the mice were infected with WT or mutant strains (data not shown).

III. DISCUSSION

Many Gram-negative bacterial pathogens use T2S and T4P systems to secrete and/or assemble virulence factors. The two model clinical isolates CFT073 and UTI89 each possess gene clusters that have close homology to T2SS and T4P genes in *E. coli* K12 while UTI89 contains a second T2SS homologous to one found in ETEC strain H10407 (Fig. 3.1). To understand the importance of these systems in the virulence of UPEC, we constructed deletion mutations in T2SS and/or T4P OM secretin genes. We verified the specificity of the gene-deletion by carrying out DNA-PCR using different flanking and insertion-specific primers (Fig. 3.3). The deletion mutants did not exhibit any defects growing in LB, RPMI + 10% FBS or M63 minimal medium (Fig. 3.4). This is important because a general growth defect in a deletion mutant would have prevented its inclusion in the *in vivo* experiments.

We compared the secretin mutants to their WT counterparts using different animal model infection experiments. In the CBA/J mouse model of ascending UTI, we found that only the Δ*hofQ* (T4P) mutant of UTI89 was significantly defective in colonization of kidneys on day 7 PI as compared to WT UTI89 (Fig. 3.5B), although there was no apparent defect in the ability of this strain to colonize and persist within the bladder (Fig. 3.5A). Thus, T4P were required for UTI89 to colonize and persist in kidneys. The kidney colonization defect of the Δ*hofQ* mutant was rescued by reintroduction of WT *hofQ* gene (Fig. 3.6A), further establishing a specific role for T4P in the ability of UTI89 to persistently colonize the kidney. In the pyelonephritogenic isolate CFT073, mutants containing single deletions of Δ*hofQ* or Δ*yheF* were able to infect and colonize bladder and kidney tissues just as well as the WT strain. However,

similar to the UTI89 Δ*hofQ* strain, a CFT073 double knockout mutant lacking both *yheF* and *hofQ* showed significantly reduced colonization of the kidney on day 7 PI (Fig. 3.5D). Thus, the two OM secretins of CFT073, HofQ and YheF, may be able to replace one another functionally and hence the kidney defect was observed only when the mice were infected with a double deletion mutant. This possibility is further supported by the results of the complementation experiments, in which reintroduction of *hofQ* fully restored kidney colonization, but expression of *yheF* in trans only partly rescued the defect (Fig. 3.6B). Thus, T4P are likely the critical virulence factor for CFT073, as found for UTI89, but YheF is able to compensate at least partially for loss of the HofQ pilus secretin. Lack of full complementation by p*yheF* could be attributed to expression of *yheF* from an artificial promoter on the plasmid. Further investigation is required to determine the specific contributions of T4P and the T2SS to the pathogenesis of CFT073. The CFT073 double mutant also exhibited consistently reduced bacterial titers in the bladder on day 7 PI (Fig. 3.5C). Further investigation is required to determine the significance of this observation.

To analyze further the kidney colonization defects of the UTI89 Δ*hofQ* and CFT073 Δ*yheF* Δ*hofQ* deletion mutants in the CBA/J mouse model of ascending UTI, we sectioned bladders and right side kidneys from animals inoculated with mutants, WT or PBS alone for histological examination. The WT infected kidney sections showed various signs of inflammation, while the inflammation in mutant infected kidney sections correlated with the presence or absence of detectable CFUs in the corresponding left side kidneys (Figs. 3.7 and 3.8). Since colonization of kidneys occurred at similar levels with WT and mutant strains on day 1 PI, the mice with undetectable kidney CFUs on day 7 PI

were able to completely clear the mutant bacteria, with no signs of the prior infection. Unfortunately, the histological examination of the infected kidneys did not provide insights into the possible mechanism behind the defect of the mutants.

Overall, results from the animal infection experiments define a specific role for T4P and/or T2SS effectors in the virulence of UPEC model strains UTI89 and CFT073.

IV. FIGURES

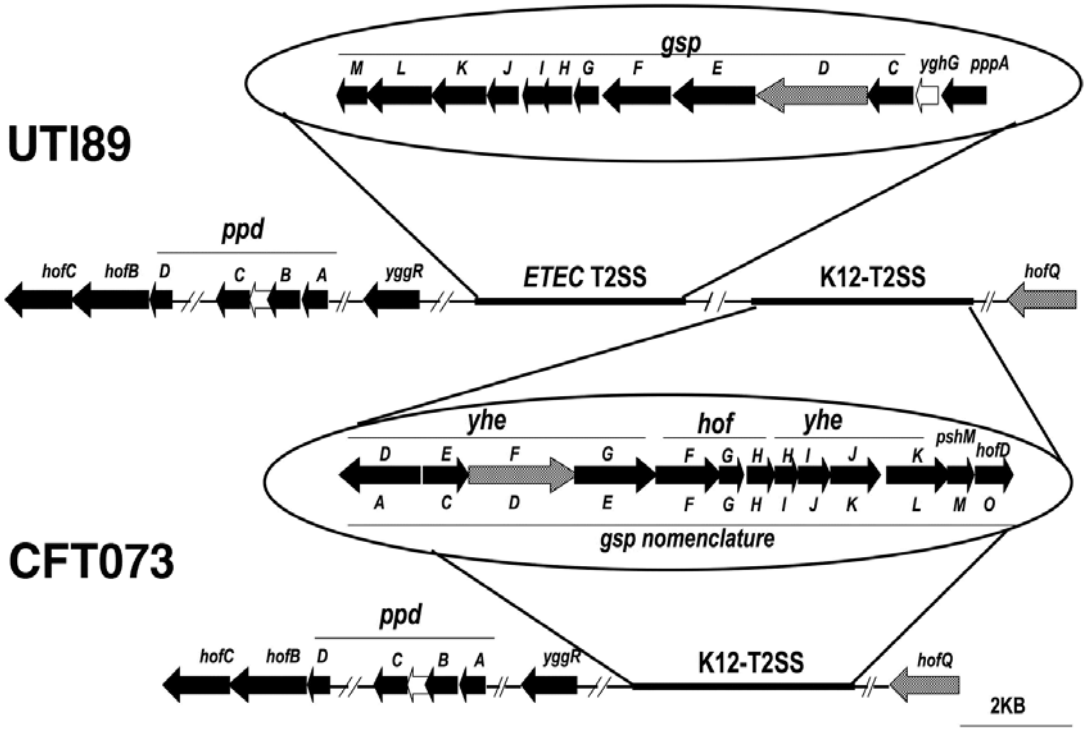

FIG. 3.1. Schematic representation of the T2SS and T4P gene clusters in UTI89 and CFT073. The different T2SS and T4P gene clusters in the genomes of UTI89 and CFT073 are shown. The detailed organization of the two T2SS gene clusters are shown in the ovals. For the K12-T2SS, the *E. coli* K12 gene names are listed above the arrows and the generic *gsp* nomenclature is given below. The OM secretin genes are indicated by the hatched arrows. The open arrows are genes that do not show homology to any of the known T2SS or T4P genes.

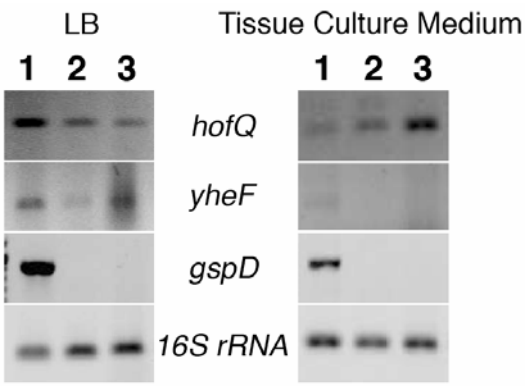

FIG. 3.2. Expression of the T2SS and T4P secretin genes. RNA was isolated from WT strains of UTI89 (1), CFT073 (2), or MG1655 (3) grown in LB or tissue culture medium, as indicated. RT-PCR was conducted using primer pairs (Table 1) specific for the OM secretin genes *hofQ*, *yheF* or *gspD*. RT-PCR of the *16S rRNA* gene was used as an internal control to compare expression levels among the three strains.

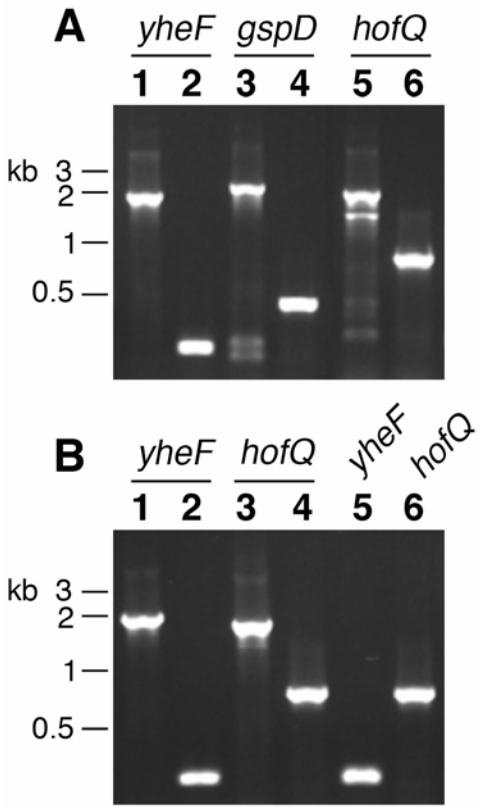

FIG. 3.3. PCR verification of the different secretin knockout mutants. PCR was performed on WT or knockout strains using primers flanking the indicated genes (Table 2.2). (A) UTI89 strains. Odd lanes, WT strain; even lanes, strains deleted for the indicated genes. (B) CFT073 strains. Lanes 1 and 3, WT strain; lane 2, Δ*yheF* strain; lane 4, Δ*hofQ* strain; lanes 5 and 6, Δ*yheF* Δ*hofQ* strain.

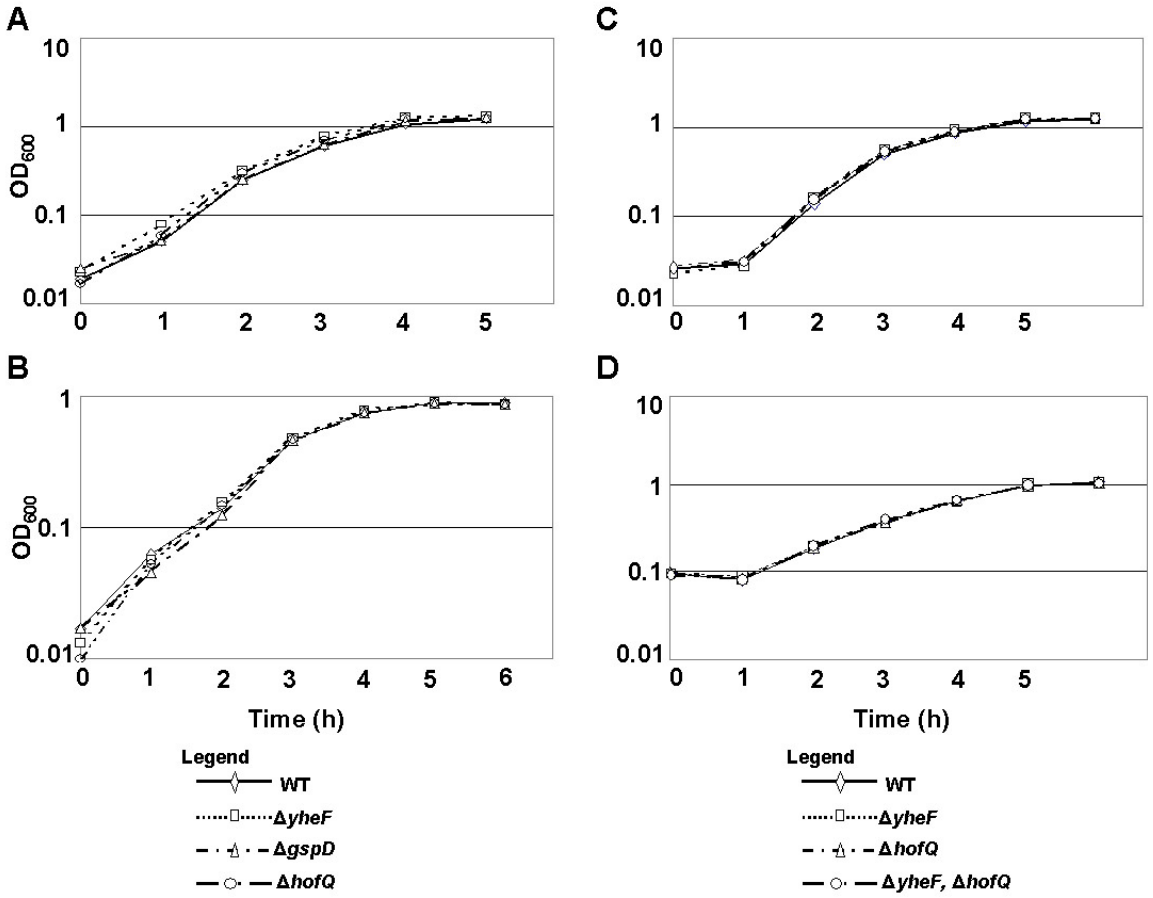

FIG. 3.4. Comparison of growth curves of the OM secretin deletion mutants and their WT counterparts. To confirm that the constructed deletion mutants did not exhibit any growth defects, the mutants and the parental WT strains of UTI89 (A and B) and CFT073 (C and D) were grown in LB (A and C) or RPMI + 10% FBS (B and D). Growth was monitored by measuring the absorbance spectrophotometrically at 600 nm. Growth of each mutant was similar to its WT counterpart.

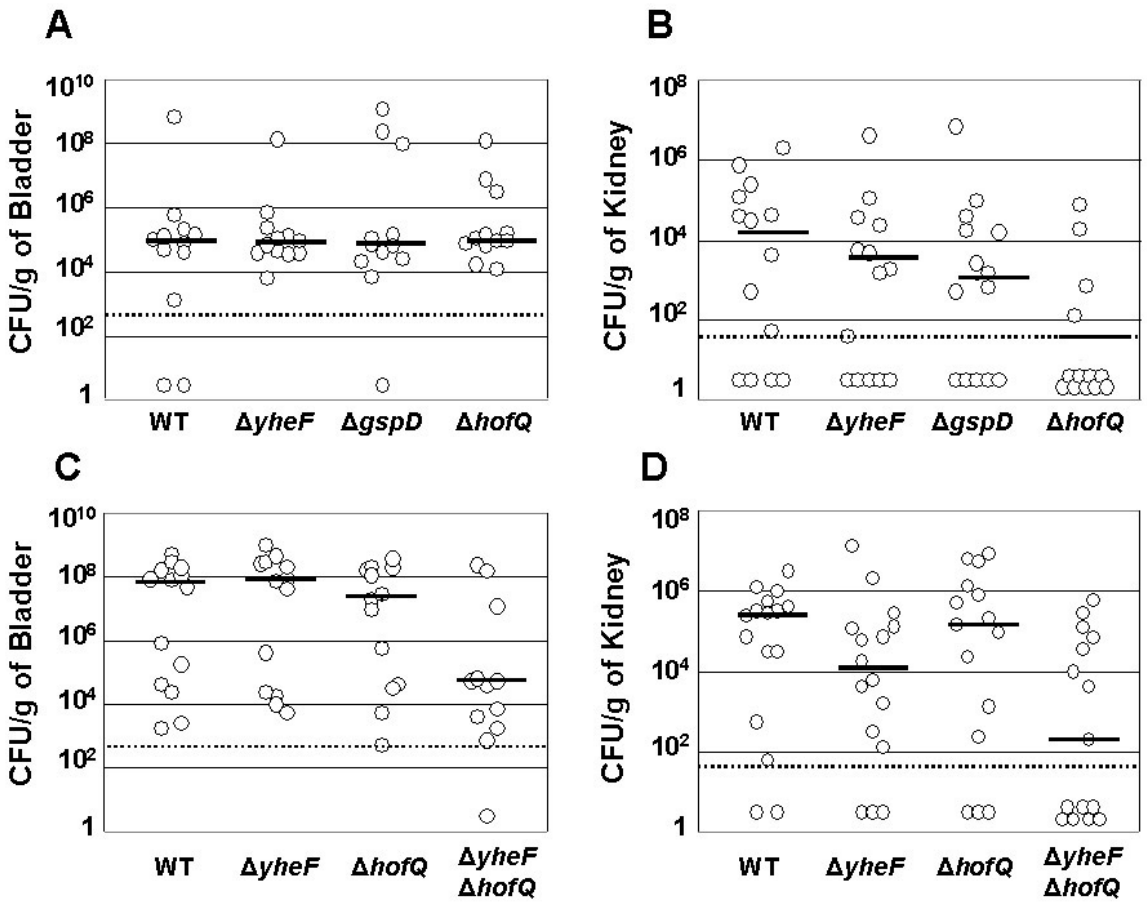

FIG. 3.5. Mouse infection experiments. Eight week-old, female CBA/J mice were infected by trans-urethral catheterization with WT or secretin knockout strains of UTI89 (A and B) or CFT073 (C and D). Bladders and kidneys were harvested on day 7 PI, weighed, homogenized and CFU/g of organ weight determined. The UTI89 Δ*hofQ* mutant was consistently defective in colonizing kidneys (p=0.03) (B), while no differences were observed in CFU recovered from the bladder (A). In CFT073, the Δ*yheF* Δ*hofQ* double mutant was defective in colonizing the kidneys (p=0.013) (C), while bladder CFU were slightly lowered as compared to WT (D). Median CFU values are indicated by the line. Dotted line indicates the limit of detection (50 CFU/ g of kidney and 500 CFU/g of bladder). The data were compared using Mann Whitney test for non-parametric data with one tailed P value. $P<0.05$ was considered significant.

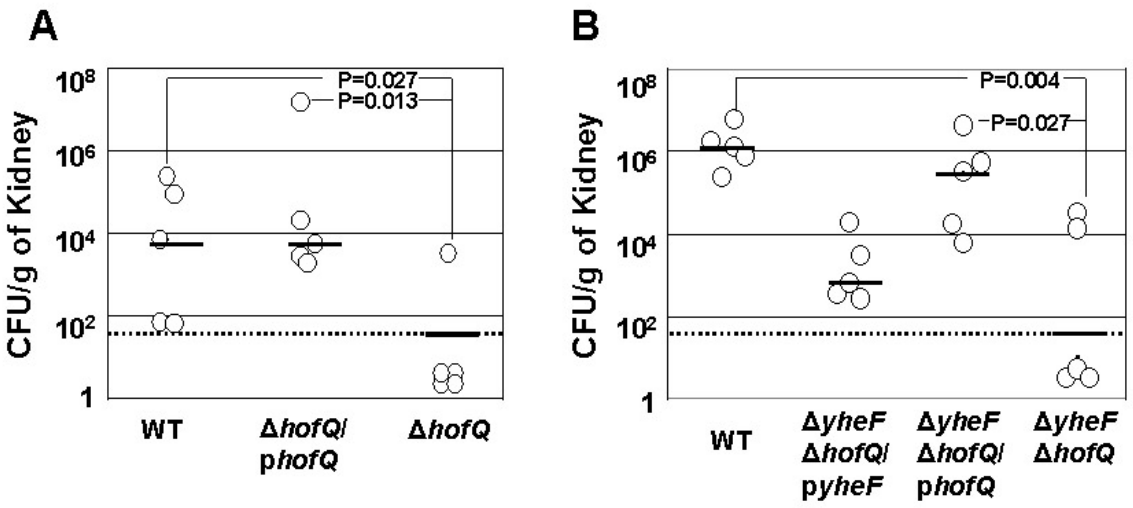

FIG. 3.6. Complementation of the UTI89 and CFT073 mutants. Plasmid-borne copies of *yheF* and *hofQ* were transformed into the mutants UTI89 Δ*hofQ* and CFT073 Δ*yheF* Δ*hofQ*. 8-week old female CBA/J mice were infected with the mutants, corresponding WT strains or the complemented strains by transurethral catheterization. On day 7 PI, the bladders and kidneys were harvested from the infected animals, homogenized, and CFUs enumerated. The complemented strains in UTI89 (A) as well as CFT073 (B) were able to colonize and persist in the renal tissues just as well as the WT strains. Median CFU values are indicated by the line. Dotted line indicates the limit of detection (50 CFU/ g of kidney). The data were compared using Mann Whitney test for non-parametric data with one tailed p value. $p<0.05$ was considered significant.

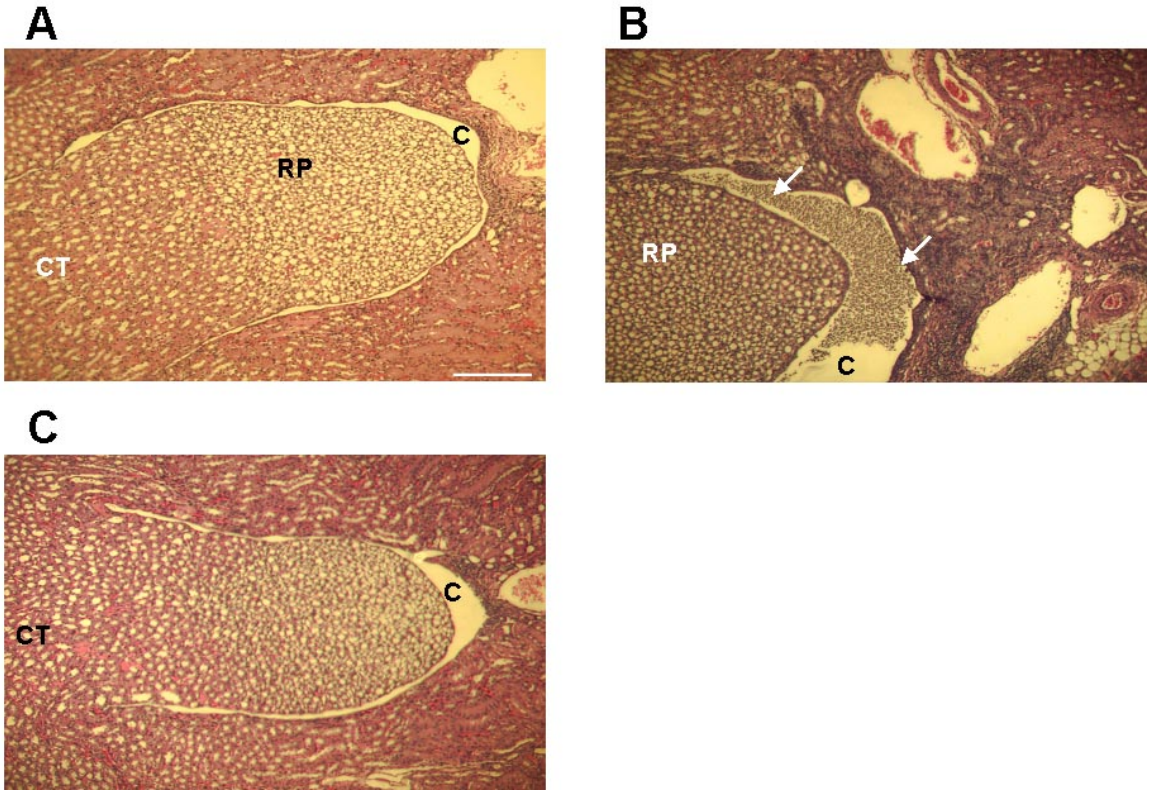

FIG. 3.7. Histological examination of the kidney sections from mice infected with mutant and WT strains of UTI89. Kidneys obtained from mice inoculated with PBS alone (A), UTI89 WT (B) or UTI89 *ΔhofQ* (C) were sectioned, H & E stained and examined microscopically. Different regions in the kidney are indicated as follows; C; calyx, CT; collecting tubule; RT; renal tubules, RP; renal papilla. The areas of leukocyte invasion are indicated by arrows. The images are presented at 100X magnification and the scale bar is 500 μm.

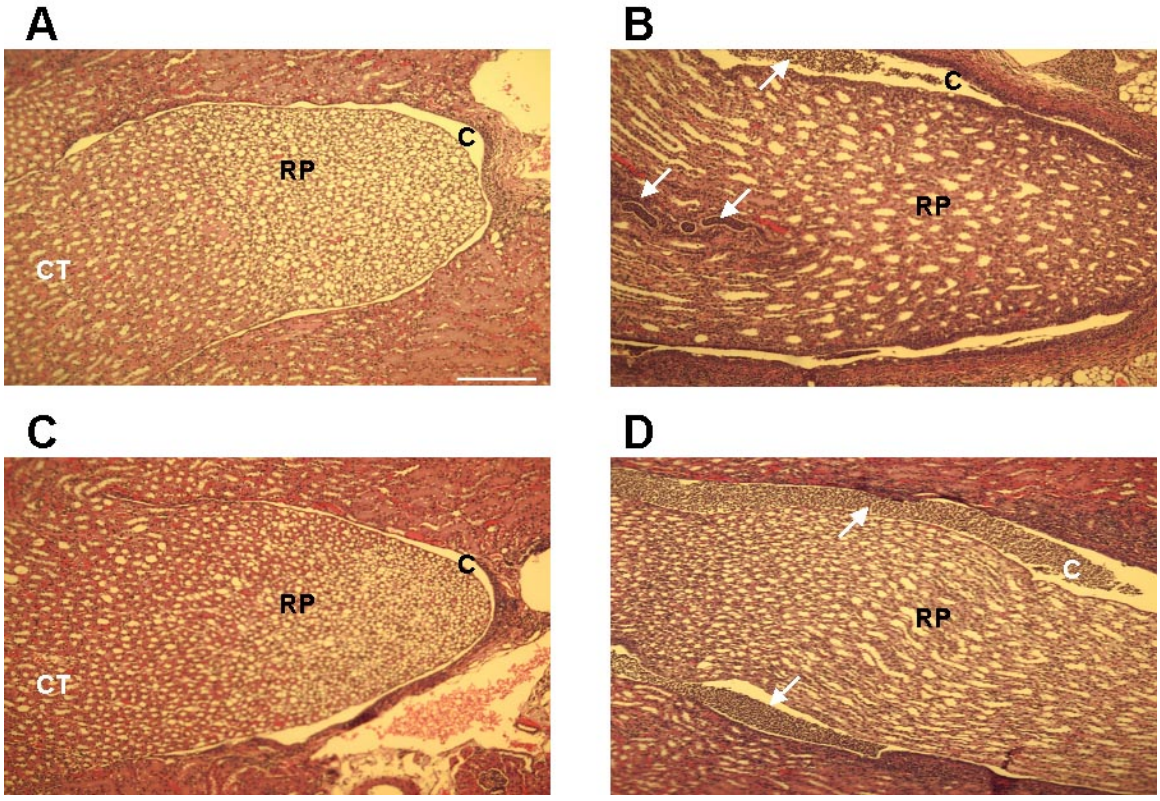

FIG. 3.8. Histological examination of the kidney sections from mice infected with mutant and WT strains of CFT073. Kidneys obtained from mice inoculated with PBS (A), CFT073 WT (B), or CFT073 Δ*yheF* Δ*hofQ* (C and D) were sectioned, H & E stained and examined microscopically. Different regions in the kidney are indicated as follows; C; calyx, CT; collecting tubule; RP; renal papilla. The areas of leukocyte invasion are indicated by arrows. The images are presented at 100X magnification and the scale bar is 500 μm.

Chapter Four

Investigation of the Molecular Basis for the Virulence Defects of UTI89 Δ*hofQ* and CFT073 Δ*yheF* Δ*hofQ*

I. INTRODUCTION

T2S and T4P systems are involved in the secretion and/or assembly of important virulence factors in a variety of Gram-negative pathogenic bacteria. Both of the model UPEC clinical strains- pyelonephritogenic isolate CFT073 and the cystitic isolate UTI89- possess T2SS and T4P gene clusters homologous to those found in *E. coli* K12 (12, 104). Moreover, UTI89 possesses a second T2SS gene cluster homologous to the ETEC-T2SS (12). To understand the importance of these systems in the pathogenesis of UPEC, we generated single and double deletion mutants in the OM secretin genes of CFT073 and UTI89 and compared them with their parental WT strains using animal infection experiments, as described in Chapter Three. These experiments showed that the kidney CFUs recovered from mice infected with UTI89 Δ*hofQ* or CFT073 Δ*yheF* Δ*hofQ* mutant strains were significantly lower than those of WT-infected mice (Fig. 3.7). This in turn indicates that T4P and probably T2SS effectors play some role in the persistence of UPEC in the upper urinary tract, especially the kidneys.

To gain further insight into the importance of T2SS and T4P in the virulence of UPEC, we studied interactions of different secretin mutants and their parental WT strains with human bladder and kidney epithelial cell lines, using a modified gentamicin

protection assay protocol (Materials and Methods) and examined the effects of the WT and mutant bacteria on the cell lines. A panel of UPEC clinical isolates was obtained and screened for the presence of the T2S and T4P systems to determine if the systems are correlated with specific UTI disease. We also assessed secretion and assembly of T4P indirectly by constructing UTI89 Δ*fimD* (type 1 pilus usher) deletion mutants in a Δ*hofQ* or WT background. These deletion mutants were compared with Δ*hofQ* and WT UTI89 strains for their ability to form biofilms and to adhere to the monolayers of bladder and kidney cell lines.

Overall, these experiments revealed a correlation between the UTI89 *hofQ* (T4P) and *gspD* (ETEC-T2SS) genes and ability to efflux out of bladder epithelial cells, but did not identify specific mechanisms to account for the virulence attenuation observed *in vivo*.

II. RESULTS

Interactions of the UPEC secretin mutants with bladder and kidney epithelial cells. The function of the T2SS and T4P in UTI89 and CFT073 was first assessed using a modified gentamicin protection assay (see Materials and Methods, (61)). This assay tests the ability of UPEC to adhere to and invade human urothelial cells, form intracellular replicating foci, and efflux out of the infected cells. The assay was carried out using the 5637 human bladder and A498 human kidney cell lines. None of the secretin knockout mutants in CFT073 or UTI89 exhibited defects in adhering to (Fig. 4.1) or invading (Fig. 4.2) the human bladder or kidney cells compared to the WT strains.

Bladder epithelial cells are exfoliated and washed off by urine as an innate immune response to infection by UPEC (69). To escape the exfoliating cells, UPEC efflux out of the infected cells and spread to other parts of the urinary tract. None of the CFT073 secretin mutants exhibited defects in effluxing out of the bladder or kidney cells (data not shown). In addition, the UTI89 secretin knockout mutants effluxed out of kidney cells similar to the WT strain. However, the UTI89 Δ*hofQ* (T4P) and Δ*gspD* (ETEC-T2SS) strains consistently exhibited approximately 2-fold reductions ($p<0.01$) in their ability to efflux out of human bladder cells as compared to the WT UTI89 strain (Fig. 4.3). Thus, both the ETEC-T2SS and T4P are required for efficient escape from bladder cells, at least for the UTI89 cystitis isolate.

An intracellular growth experiment for an extended period of 48 hours (as explained in Materials and Methods) was carried out. No significant differences were observed among the intracellular growth patterns of any of the OM secretin deletion mutants and the WT strains of UTI89 for the 5637-bladder cell line (Fig. 4.4). Moreover,

no difference was observed between the intracellular growth index (IGI) values for any of the deletion mutants and parental WT strain of UTI89 during the 6 h efflux period (Data not shown). These results established that the efflux defective nature of Δ*gspD* and Δ*hofQ* mutants in UTI89 is not a manifestation of defective growth inside the bladder epithelial cells.

Because the effluxed bacteria remain in the tissue culture medium for the 5637 bladder epithelial cell line for a period of 6 h during the efflux assay, we also compared the growth of the Δ*gspD* and Δ*hofQ* mutant strains with WT UTI89 in RPMI + 10% FBS (Fig. 3.4B); the mutants did not exhibit any growth defects, ruling out that the decreased efflux index values were due to lack of growth of the mutants in the tissue culture medium.

Effects of UPEC secretin mutants on IL-6 production and cytotoxicity. UPEC has been shown to suppress the induction of IL-6, a pro-inflammatory and immunomodulatory cytokine, produced by the urothelium in response to bacterial LPS and IL-1 (45). One hypothesis explaining the mechanism behind the cytokine-suppression is that UPEC secretes effectors that downregulate host cell NF-κB signaling to repress transcription of pro-inflammatory and apoptotic genes (45). To analyze whether T2SS and/or T4P are involved in the secretion of this unknown effector, we compared the different UTI89 and CFT073 knockout mutants with their parental WT strains for ability to suppress IL-6 production in response to LPS by the 5637 human bladder cell line. The knockout mutants were able to suppress IL-6 formation just as well as the WT strains (data not shown). We also tested whether the T2SS and/or T4P might be involved in the secretion of a toxic effector molecule. The UTI89 and CFT073

secretin knockout mutants were compared with their parental WT strains for differences in cytotoxicity toward the 5637 human bladder cell line. None of the mutants exhibited decreased cytotoxicity, as measured by LDH release during a 3-hour infection assay (Fig. 4.5). In addition, no differences were detected between WT UTI89 and the secretin knockout mutants in the amount of LDH released during the efflux assay carried out as described above (Fig. 4.6). This indicates that a defect in secretion of a cytotoxic effector molecule is not responsible for the decreased efflux of the UTI89 Δ*hofQ* and Δ*gspD* strains from bladder epithelial cells.

Prevalence of T2SS and T4P in a panel of pathogenic and non-pathogenic *E. coli*. The cystitic isolate, UTI89, possesses a second T2SS gene cluster homologous to one found in ETEC. In ETEC, the T2SS is known to be involved in the secretion of an important ETEC virulence factor, LT (94). Furthermore, the UTI89 Δ*gspD* deletion strain that was constructed in order to ablate the ETEC-T2SS was found to be defective in fluxing out of the 5637, human bladder carcinoma cell line (Fig. 4.3). Based on these findings we hypothesized that a correlation may exist between the presence of ETEC-T2SS in a UPEC isolate and the cystitic nature of that isolate.

We analyzed a panel of 58 different pathogenic and nonpathogenic *E. coli* strains (Table 2.1) by colony PCR using primers specific for ETEC-T2SS and T4P genes. The results from these experiments indicated that the ETEC-T2SS is widely distributed among pathogenic *E. coli* without any relation to the origin or tropism of a clinical isolate. Thus, the ETEC-T2SS does not appear to be specifically associated with cystitis. Furthermore, all the 58 isolates were observed to possess the three T4P genes, *hofQ*, *yggR* and *hofB*, that we tested for.

Next we checked whether an efflux-defect from bladder epithelial cells is directly related to the absence of the ETEC-T2SS. We analyzed strains from the panel that were negative by PCR for the ETEC-T2SS for their ability to flux out of monolayers of the 5637 human bladder epithelial cell line. From the results of this experiment, UPEC strains lacking the ETEC-T2SS do not appear to be generally efflux-defective with respect to the human bladder cell line (Fig. 4.7).

Analyzing the role of T4P in UTIs. As described earlier, T4P are long, surface-associated filaments that are involved in different virulence functions such as adhesion, twitching motility, biofilm formation, and horizontal genetic transfer. Since, the Δ*hofQ* mutant lacking the T4P OM secretin in UTI89, was defective in fluxing out of the human bladder epithelial cell line as well as in renal persistence in the CBA/J mouse model of ascending UTI, we decided to analyze this mutant further for any changes in the functions associated with T4P such as adhesion and biofilm formation.

The two well-characterized adhesins used by UPEC to bind to bladder and kidney epithelial cells are the FimH and PapG tips of type 1 and P pili, respectively. Moreover, the adhesion frequency of a *fimH* deletion mutant for 5637 bladder epithelial cells drops to negligible levels as compared to that of WT (61). Thus, FimH is the major adhesin that allows UPEC to adhere to and later invade the bladder epithelial cells. In many Gram-negative pathogens, T4P have been shown to play an important role as the major adhesive factors. However, Δ*hofQ* mutant showed no defect in adhesion to human bladder or kidney epithelial cells. To check whether the type 1 pili were masking the adhesive properties of the T4P, we generated type 1 pilus negative mutants in UTI89 by targeting the type 1 usher *fimD* for deletion in the WT as well as Δ*hofQ* backgrounds.

These two mutant strains, Δ*fimD* and Δ*fimD* Δ*hofQ*, were compared with the WT UTI89 for adherence to the monolayers of human bladder (5637) as well as kidney (A498) cell lines. As expected, the adhesion frequency of the Δ*fimD* mutant was lowered >20 times for both the cell lines. Quite interestingly, the double deletion mutant, Δ*fimD*/Δ*hofQ* was ~50% less adherent than Δ*fimD* with respect to both bladder and kidney epithelial cells (Fig 4.8 A and B). These results were consistent and statistically significant

Biofilms constitute a viscous phase composed of bacterial cells in consortium with other microorganisms and bacterial exopolysaccharide matrices, within which molecules and ions can occur at concentrations different from those found in the bulk phase (14). UPEC form replication foci with biofilm-like properties within the bladder epithelial cells (3), although nothing is known about their ability to form extracellular biofilms. To analyze biofilm formation by both UTI89 and CFT073 and to understand the role of T4P in this process, we compared different secretin deletion mutants with their WT counterparts for the ability to form biofilms. The mutant and WT bacteria were grown in LB, tissue culture medium (RPMI + 10% FBS), or M9 minimal medium in 96-well polystyrene plates at room temperature or at 37^0C in the presence of 5% CO_2. Formation of biofilms was determined by staining with crystal violet. Both UTI89 and CFT073 did not form biofilms in either tissue culture medium or LB. In contrast, strong biofilm formation was detected in M9 minimal medium, but no differences were detected between the biofilms formed by different deletion mutants and their parental WT strains (Fig. 4.9). Thus, T4P do not appear to contribute to biofilm formation by UPEC, at least under the *in vitro* conditions tested.

III. DISCUSSION

The cystitic strain UTI89 and the pyelonephritogenic strain CFT073, the two model UPEC isolates, possess chromosomal copies of T2SS and T4P gene clusters. To study the importance of these secretion pathways in the virulence of UPEC we generated OM secretin deletion mutants and as described in Chapter Three established a relationship between the presence of T4P and/or T2SS and the ability of UPEC to colonize and persist in the kidneys of CBA/J mouse model of ascending UTI. In this chapter, I have described experiments carried out to understand the mechanism governing this relationship.

Using a modified gentamicin protection assay, we compared different secretin deletion mutants with their WT counterparts for different aspects of uropathogenesis such as adhesion to and entry into cells of human bladder and kidney epithelia, intracellular replication and bacterial efflux out of the infected cells. Two deletion mutants in UTI89, Δ*hofQ* and Δ*gspD* were found to be defective in fluxing out of the 5637 human bladder epithelial cell line. Moreover, these deletion mutants were not defective in intracellular growth, ability to grow in the tissue culture medium, cytotoxicity or IL-6 suppression. This suggests that both T4P and the ETEC-T2SS contribute to the ability of UTI89 to efflux out of the bladder epithelial cells.

The results from the semi-quantitative RT-PCR indicating that the ETEC-T2SS was strongly expressed in vitro (Fig. 3.2), along with the results from the efflux assay and the fact that the ETEC-T2SS is absent from the genome of the CFT073 pyelonephritis isolate, raised the possibility that the ETEC-T2SS might allow secretion of a toxin or other virulence factor by UTI89. This also suggested that the ETEC-T2SS might be

specifically associated with the ability to cause cystitis. However, results from the analysis a panel of pathogenic and non-pathogenic *E. coli* isolates by DNA-PCR using ETEC-T2SS specific primer pairs indicated that the ETEC-T2SS gene cluster is widespread among *E. coli* clinical isolates, without any apparent correlation with the origin of the isolate. Thus, CFT073 appears to be an outlier among *E. coli* strains in lacking the ETEC-T2SS.

To examine the role of T4P in UTI89, we generated Δ*hofQ* deletion mutants in WT as well as Δ*fimD* (type 1 pilus usher) backgrounds. In the absence of type 1 pili (Δ*fimD*), the T4P seem to play a role in the adhesion of UTI89 to human bladder as well as kidney cell lines. The adhesion frequency values for the Δ*fimD* Δ*hofQ* double deletion mutants in 5637 bladder and A498 kidney epithelial cells, were consistently (~50%) lower than that for UTI89 Δ*fimD* strains (Fig. 4.8). Although this difference is statistically significant, the actual adhesion frequency values are so low (0.5-0.6%) that the role played by T4P in UPEC adherence to bladder and kidney epithelial cells can at best be described as minor. Ability to form biofilms is a property often associated with T4P. While CFT073 and UTI89 were able to form biofilms in M9 minimal medium, none of the deletion mutants exhibited reduced biofilm formation as compared to the parental WT strains (Fig. 4.9). This indicates that in UPEC the ability to form biofilms is not dependent on the presence of functional T4P. Note that these experiments also found no biofilm defects in strains containing deletions in T2SS or type 1 pili.

IV. FIGURES

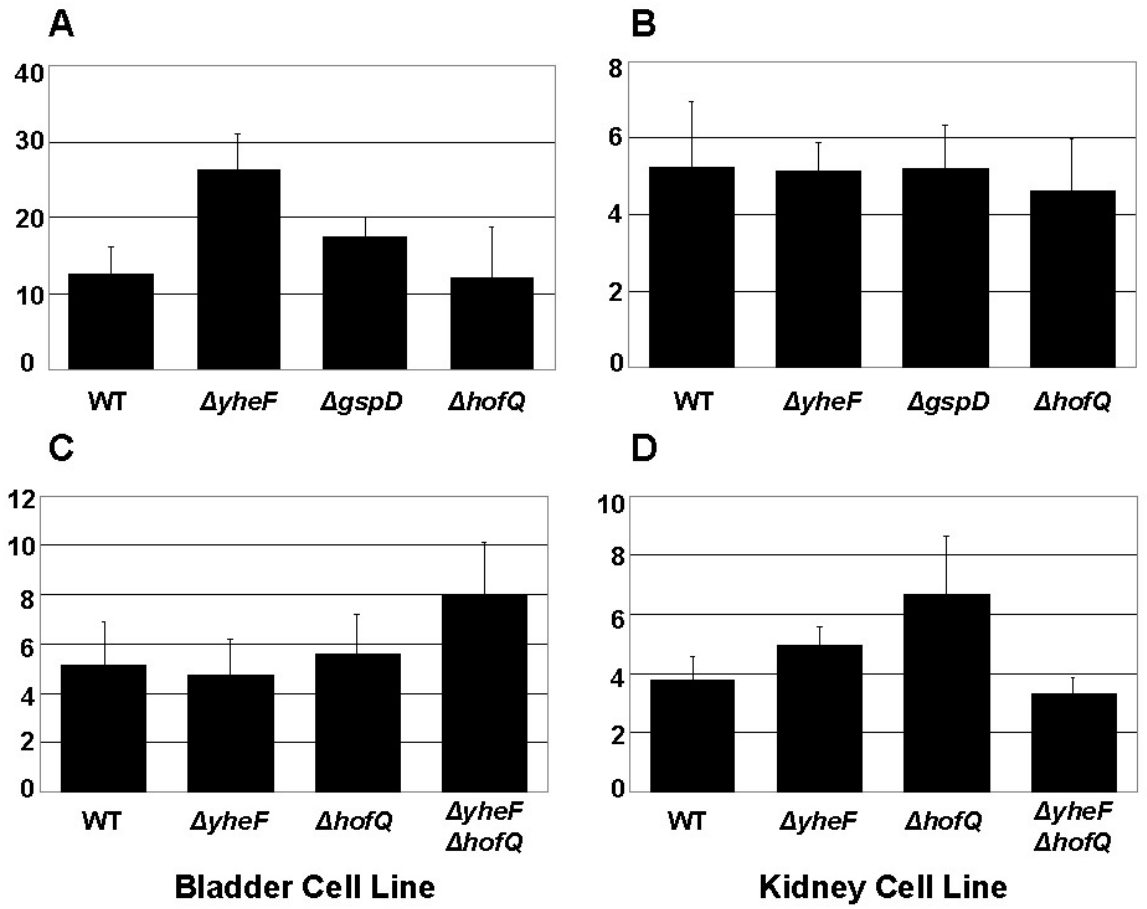

FIG. 4.1. Adhesion of secretin deletion mutants and WT strains to bladder and kidney epithelial cell lines. At the end of the two hour period of adhesion in the modified gentamicin protection assay, the infected cell lines were washed vigorously and the adherent bacterial counts enumerated. Different secretin deletion mutants in UTI89 (A and B) or CFT073 (C and D) were able to adhere to 5637 human bladder and A498 human kidney epithelial cell lines as well as their WT counterparts. Percent adhesion frequency values are indicated on the ordinate. All data are representative of three or more assays, each performed in triplicate.

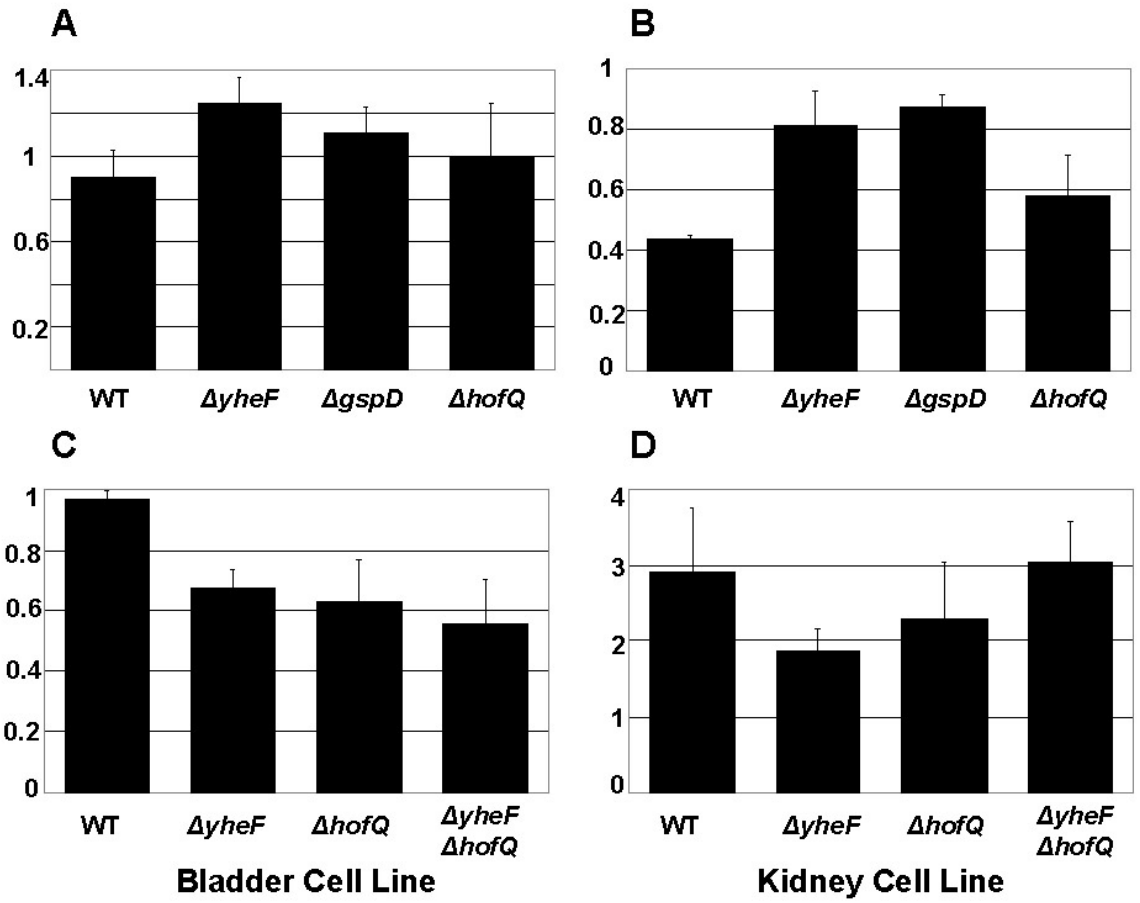

FIG. 4.2. Invasion of secretin deletion mutants and WT strains into bladder and kidney epithelial cell lines. In the modified gentamicin protection assay, following a period of adhesion, the infected cell lines were treated with 100 μg/ml gentamicin for two hours and the intracellular CFUs at the end of this period determined. Different secretin deletion mutants in UTI89 (A and B) or CFT073 (C and D) were able to invade the 5637 human bladder and A498 human kidney epithelial cell lines to WT-like levels. Percent invasion frequency values are indicated on the ordinate. All data are representative of three or more assays, each performed in triplicate.

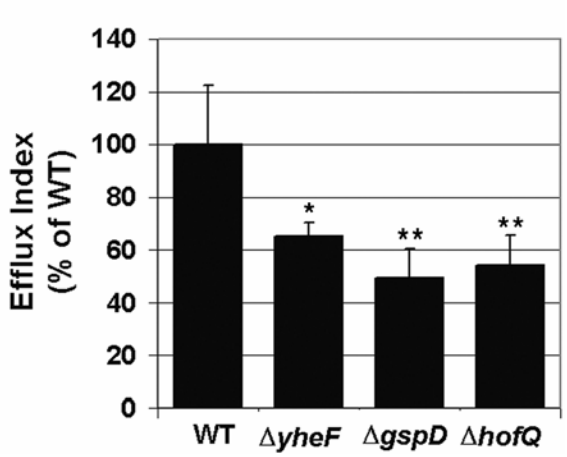

FIG. 4.3. Efflux of WT UTI89 and the indicated UTI89 secretin mutants from 5637 human bladder epithelial cells. The UTI89 Δ*gspD* and Δ*hofQ* mutants consistently exhibited significantly reduced efflux index values compared to bladder cells infected with WT UTI89. Efflux index values for the UTI89 Δ*yheF* mutant were not always reduced compared to WT. Each deletion mutant was compared with the WT strain at least 3 times, each in triplicate. Values are shown as a percentage of WT; error bars indicate SD. The efflux index values were compared by Dunnett's multiple comparison's test. *, $p<0.05$; **, $p<0.01$.

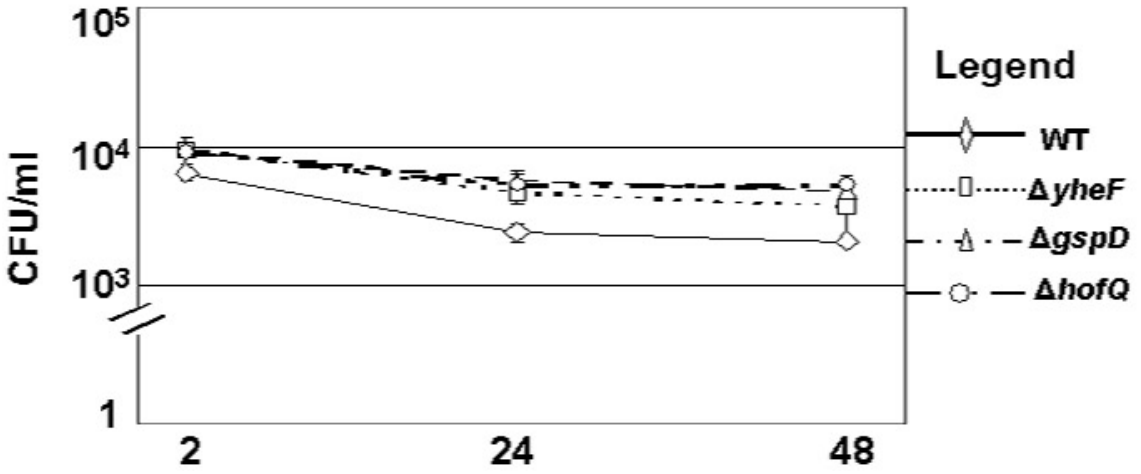

FIG. 4.4. Intracellular replication of secretin deletion mutants and parental WT strains of UTI89. The OM secretin deletion mutant and WT UTI89 strains were allowed to grow inside 5637 bladder cells over a period of 48 h. 10μg/ml gentamicin was maintained in the surrounding medium. At indicated times, the infected cells were washed, treated with TX100 and intracellular CFUs determined. The OM secretion deletion mutants in UTI89 were able to replicate inside 5637 human bladder cells in a WT-like manner.

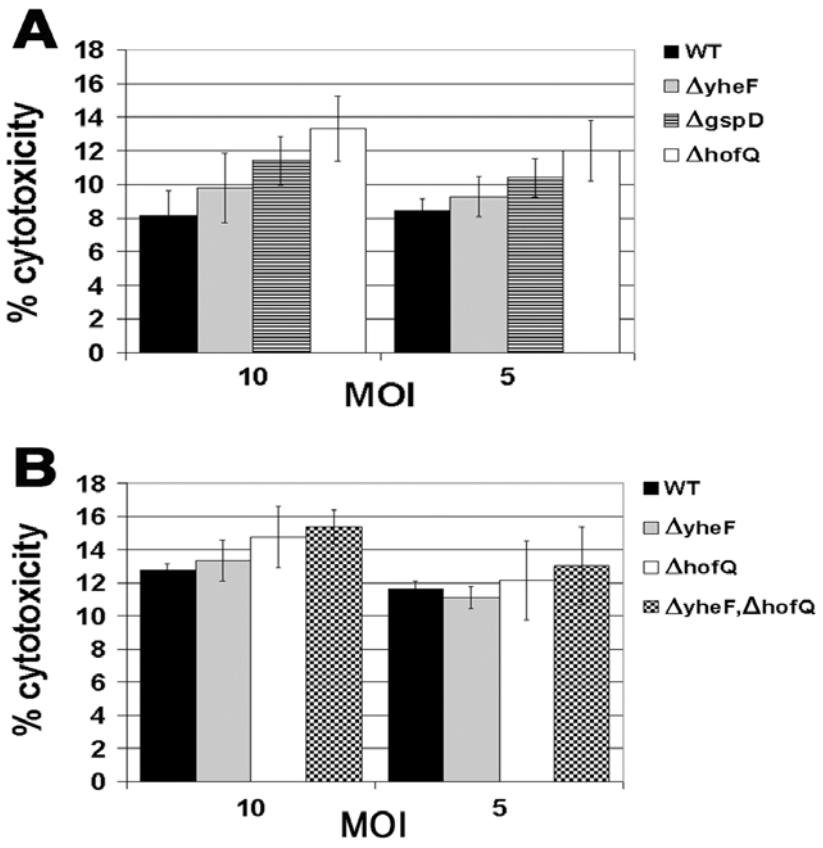

FIG. 4.5. Instant cytotoxicity of UTI89 (A) or CFT073 (B) for human bladder epithelial cells. WT strains or the different secretin knockout mutants were tested for their ability to cause cytotoxicity in 5637 human bladder cells over a period of 3 h, as measured by LDH release (% cytoxicity). Bacteria were added at an MOI of 5 or 10.

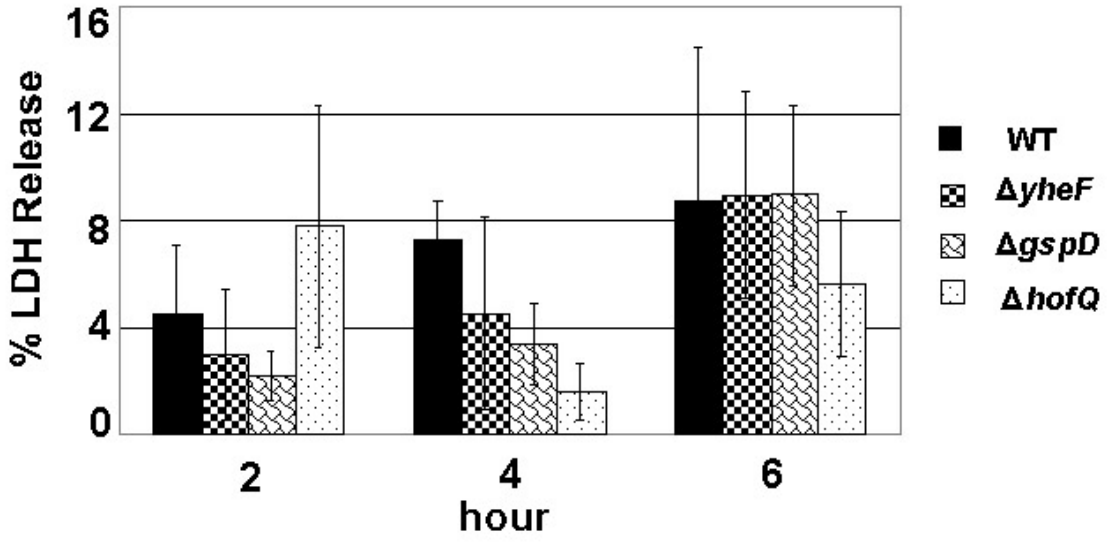

FIG. 4.6. Cytotoxicity caused by UTI89 during the 6 h efflux period. The supernatants were collected at indicated time points during the efflux assay carried out by infecting 5637 bladder epithelial cells with the T2S and T4P OM secretin deletion mutants and WT strains of UTI89. The cytotoxicity was assessed by estimating LDH released in the cell-free supernatants.

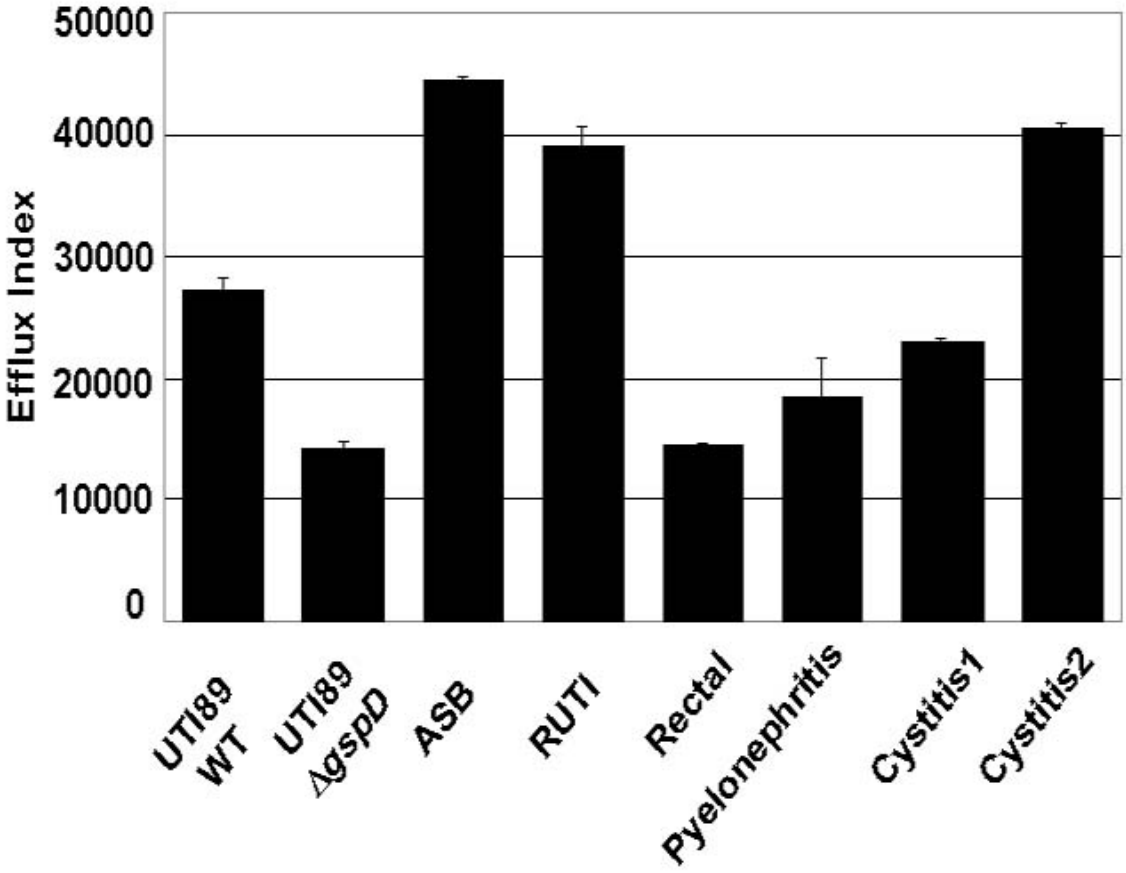

FIG. 4.7. Efflux index values for the Representative ETEC-T2SS negative strains. In the 6 h efflux assay module, representative strains from each clinical group of the pathogenic *E. coli* panel that were negative for ETEC-T2SS specific PCR did not exhibit defects in fluxing out of the monolayers of 5637 human bladder cell line. Error bars indicate SD.

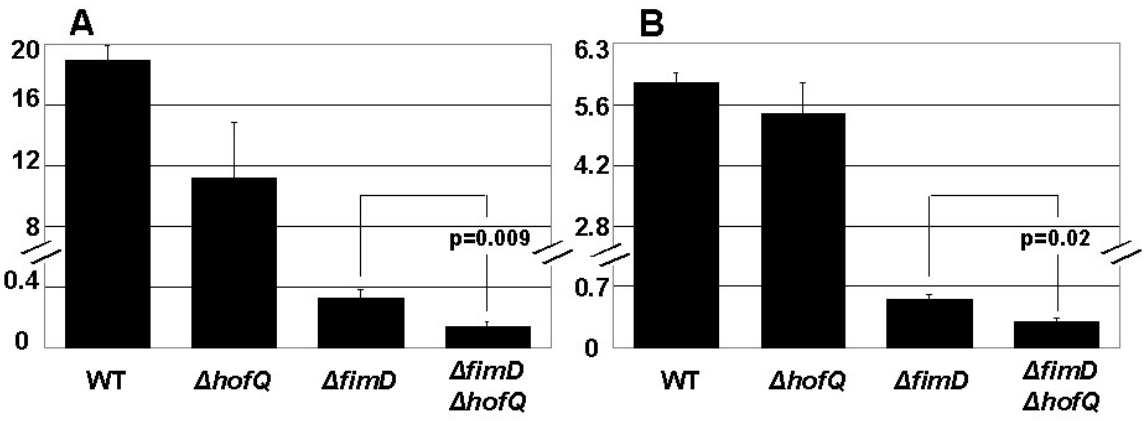

FIG. 4.8. Role of T4P in the adhesion to human bladder and kidney epithelial cells. Percent adhesion frequency for 5637 bladder (A) and A498 kidney (B) cells was determined for *hofQ* deletion mutants in WT as well as *fimD* deletion backgrounds in UTI89. All date are representative of two or more assays, each performed in triplicate. The results were compared by unpaired t test with one-tail P value. $p<0.05$ was considered significant.

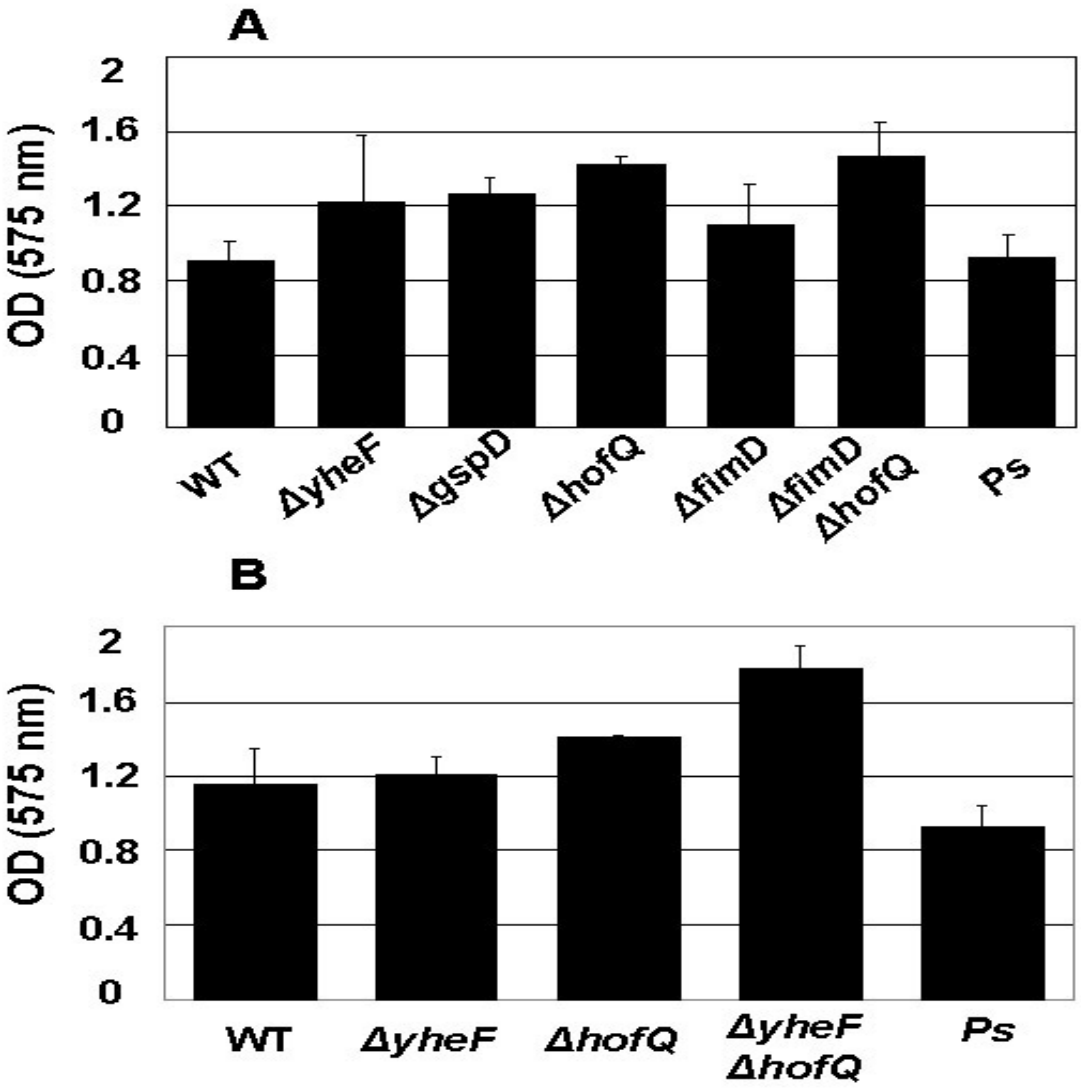

FIG. 4.9. Biofilm formation in M9 medium. Different OM secretin deletion mutants and parental WT strains of UTI89 (A) and CFT073 (B) were tested for their ability to form biofilms in M9 minimal medium in quadruplicate. *Ps* indicates the biofilm formed by *P. aeruginosa*. $OD_{575} \geq 0.8$ is considered positive for biofilm formation. Each histogram represents the average of four OD_{575} values +/- SD. Based on these results, the biofilm formation in UPEC strains UTI89 and CFT073 does not appear to be dependent on presence of T4P, T2SS or type 1 pili.

Chapter Five

Discussion and Future Directions

I. CONCLUSIONS

Many Gram-negative bacterial pathogens use type II secretion and the evolutionarily related T4P system to secrete and/or assemble virulence factors. Although functions for these secretion pathways are established for intestinal pathogenic *E. coli*, their roles in the virulence of ExPEC had not been investigated. In this report, we addressed this question by constructing deletion mutations to disable T2SS and T4P gene clusters present in the genomes of the model UPEC strains UTI89 and CFT073. We compared these mutants to their WT counterparts using different *in vitro* and *in vivo* assays. Our results demonstrate that T4P and potentially also the K12-T2SS are important to the pathogenesis of UPEC in the urinary tract, particularly in the ability to successfully colonize and persist within the kidneys.

Both the cystitis isolate UTI89 and the pyelonephritis isolate CFT073 possess T2SS and T4P gene clusters sharing very close homology (generally 91-99% amino acid identity) with genes present in *E. coli* K12. In K12 strains, the T2SS does not appear to be expressed under standard growth conditions, but this system was shown to be de-repressed in mutants lacking the nucleoid structuring protein H-NS and to function in the secretion of chitinase (28). Similarly, the *E. coli* K12 T4P genes are transcribed at low levels and T4P are not detected under standard laboratory conditions (87). However, the major pilin subunit PpdD (prepilin peptidase dependent protein D) was assembled into

T4P when ectopically expressed in *P. aeruginosa* (87). We found that the K12-homologous T2SS and T4P genes in UTI89 and CFT073 were expressed at low levels under in vitro culture conditions, similar to expression in a K12 strain. Since our infection studies indicate that these systems are functional in vivo, they are likely to be upregulated under yet-to-be-determined conditions. Notably, our results imply that the T4P and T2SS genes in *E. coli* K12 are also functional under the proper conditions.

Interestingly, in addition to the K12-homologous T2SS and T4P genes, the UTI89 cystitis isolate contains a second T2SS cluster homologous to genes present in ETEC. In ETEC this T2SS enables the secretion of LT, which is critical for pathogenesis in the intestinal tract (94). In contrast to the K12-homologous systems, we found that the ETEC-T2SS was strongly expressed *in vitro*. This raised the possibility that the ETEC-T2SS might allow secretion of a toxin or other virulence factor by UTI89. Furthermore, since the ETEC-T2SS is absent from the genome of the CFT073 pyelonephritis isolate, and because the UTI89 mutant lacking a functional ETEC-T2SS (Δ*gspD*) was specifically defective in fluxing out of the bladder epithelial cells, this suggested that the ETEC-T2SS might be specifically associated with the ability to cause cystitis. To check for a correlation between the cystitic nature of a UPEC strain and the presence of the ETEC-T2SS, we analyzed a panel of pathogenic and non-pathogenic *E. coli* isolates by PCR using ETEC-T2SS specific primer pairs. Results from this analysis indicated that the ETEC-T2SS gene cluster is widespread among *E. coli* clinical isolates, without any apparent correlation with the origin of the isolate. This observation was further corroborated by examination of the available genome sequences of *E. coli* strains by

BLAST analysis. Thus, CFT073 appears to be an outlier among *E. coli* strains in lacking the ETEC-T2SS.

In the CBA/J mouse model of ascending UTI, we found that the Δ*hofQ* (T4P) mutant of UTI89 was significantly defective in colonization of kidneys on day 7 PI as compared to WT UTI89, although there was no apparent defect in the ability of this strain to colonize and persist within the bladder. In addition, we did not detect any defects in the abilities of the UTI89 Δ*yheF* (K12-T2SS) or Δ*gspD* (ETEC-T2SS) mutants to colonize the bladder or kidneys at any time points. Thus, our experiments revealed a specific role for T4P in the ability of UTI89 to persistently colonize the kidney. In the pyelonephritogenic isolate CFT073, mutants containing single deletions of *hofQ* or *yheF* were able to infect and colonize bladder and kidney tissues just as well as the WT strain. However, similar to the UTI89 Δ*hofQ* strain, a CFT073 double knockout mutant lacking both *yheF* and *hofQ* showed significantly reduced colonization of the kidney on day 7 PI. The CFT073 double mutant also exhibited consistently reduced bacterial titers in the bladder on day 7 PI. These results demonstrate that T4P and/or the T2SS play important roles in the ability of CFT073 to persistently colonize the urinary tract. Further investigation is required to determine the specific contributions of T4P and the T2SS to the pathogenesis of CFT073. OM secretins are capable of functioning in more than one secretion pathway within a bacterium. For example, in *V. cholerae*, the EpsD secretin is able to secrete proteins as part of a T2SS while at the same time helping in the extrusion of filamentous CTX phage (84). In the case of CFT073, the two OM secretins, YheF and HofQ, might be able to replace one another functionally and hence the colonization

defect was observed only when the mice were infected with the double knockout mutant lacking both secretins.

In vitro experiments using model cell lines do not reflect the myriad aspects of host-pathogen interaction and our experiments to analyze interactions of the UTI89 and CFT037 mutants with human bladder and kidney epithelial cells did not point to a mechanism underlying the defects observed in the mouse infection experiments. UPEC adhere to cells of the host urothelium with the help of the type 1 and P pili, and in case of the bladder epithelium their entry into the superficial umbrella cells is mediated by the type 1 pilus tip adhesin, FimH (61). Hence it was expected that none of the knockout mutants, generated by specifically targeting the type II secretion and T4P pathways, would be defective in adhesion or invasion; although in the absence of type 1 pili (Δ*fimD*), T4P played a minor role in the adhesion of UTI89 to cells of the bladder and kidney epithelium. In addition, we did not detect defects in intracellular replication, cytotoxicity, or suppression of IL-6 expression by bladder epithelial cells. Using a modified gentamicin protection assay that mimics several aspects of UPEC pathogenesis, we did observe that knockout mutants in UTI89 lacking *hofQ* (T4P) and *gspD* (ETEC-T2SS) were defective in fluxing out of the bladder epithelial cells line. However, this defect in fluxing out of the epithelial cells did not correlate with the behavior of the mutants in the mouse UTI model, where only the Δ*hofQ* mutant exhibited decreased colonization. Observation of green fluorescent protein-expressing UTI89 in the cell culture efflux assay confirmed defects in the Δ*hofQ* and Δ*gspD* mutants, but indicated a delay in efflux rather than an absolute defect (data not shown). This could explain the lack of correlation to the in vivo results. Moreover, the CFT073 secretin mutants did not

exhibit any defects in fluxing out of bladder epithelial cells. Finally, the progression of UTI in the kidney is not documented in as many details as in the bladder. From our experiments, it is apparent that all knockout mutants in UTI89 as well as in CFT073 were able to form IBCs in the A498 human kidney cell line.

Based on my dissertation work, we propose that T4P aid in the colonization and persistence of UPEC in the urinary tract, particularly the upper urinary tract. The ascent of UPEC along the ureters is not well understood and different bacterial factors that might aid in this process are still undefined. In a number of Gram-negative pathogens, association with the host epithelium followed by motility and dispersal over the epithelial surface are mediated by T4P (63). The T4P in UPEC might play a similar role, although colonization of the urinary tract at early time points PI were similar in both the mutant and WT strains. Alternatively, T4P may specifically aid in the colonization of kidney tissues by UPEC, possibly by mediating bacterial-bacterial and bacterial-host interactions and/or the formation of biofilms. Another possibility is that in the absence of functional T4P and/or the T2SS, the mutant bacteria were defective due to the lack of an unknown secreted effector, leading to clearance of the bacteria by day 7 PI.

II. FUTURE DIRECTIONS

My dissertation research established a correlation between T4P and/or effectors of the T2SS with the ability of UPEC to colonize and persist in renal tissue. However, we do not currently understand the detailed mechanism behind this relationship. Future research should be directed toward elucidating the molecular basis of the virulence attenuation of the UPEC mutants. This will further our understanding of *E. coli* pathogenesis within the urinary tract. Potential future experiments are described below.

Study of the regulation of T2SS and T4P proteins in UPEC. As described in Chapter Three, the results from the semiquantitative RT-PCR experiments revealed that the K12-T2SS as well as T4P genes were transcribed at low levels, while the ETEC-T2SS was transcribed at relatively high levels under the *in vitro* conditions tested. To study the expression of the UPEC T2SS and T4P *in vivo*, and to understand the temporal aspects of their regulation, transcription fusion constructs to reporters such as β-galactosidase or luciferase could be generated. A new technique to generate specific reporter gene fusions in bacterial chromosomes is described by Gerlach, *et al* (32). This technique is very similar to the method used for generation of the secretin deletion mutants. It makes use of bacteriophage λ RED recombinase to insert a PCR-generated gene cassette containing a promoterless reporter gene together with a FRT-flanked antibiotic resistance cassette behind the ribosome binding site and the start codon of the target gene. These T2SS and T4P reporter fusions can be used in *in vitro* cell culture assays and *in vivo* animal model infection experiments to monitor gene expression. Once the correct condition(s) for the induction of T2S and T4P systems are identified, additional experiments such as analysis of secretion profiles of mutants and WT strains or

the visualization of T4P on the surface of UPEC by EM can be carried out. These experiments may also aid in understanding whether the K12 T2SS or T4P is more important in the renal colonization and persistence of CFT073.

***In vivo* imaging of host-UPEC interactions.** The ability to monitor the progress of infections in the mouse model will greatly aid understanding of the function of the T2S and T4P systems in UPEC pathogenesis. A first approach to comparing the WT and mutant strains during the course of an infection is to conduct detailed histological analysis during different time points post infection. A powerful new approach is the field of *in vivo* imaging. One method for this employs pathogens that are genetically engineered to express the *lux* operon from bacteria such as *Photorhabdus luminescence* (46). Although this technique has not been employed and standardized for research related to UPEC mediated UTI, there is ample precedent for the use of bioluminescent *in vivo* imaging in the study of infections caused by pathogenic bacteria such as *Salmonella typhimrium* (13), *Streptococcus pneumoniae* (30), viruses such as herpes simplex virus (HSV)-1 (58), and even protozoan parasites such as *Toxoplasma gondii* (43). The lux operon encodes genes to synthesize the enzyme luciferase and its substrate luciferin. Thus the pathogenic bacteria (UPEC) will constitutively emit light without need for an exogenous substrate. UPEC engineered to express lux (WT as well as OM secretin deletion mutants) can be used to infect the CBA/J mice by transurethral catheterization and the disease progression monitored by placing the infected mice under a charge-coupled device (CCD) camera that can detect the low levels of luciferase emitted by the UPEC *in vivo*. The *in vivo* imaging procedure using the bioluminescent UPEC will allow the progression of UTI in CBA/J mice to be monitored without killing the animals and to

gain spatial and temporal insights into the colonization of the upper urinary tract, especially the renal tissue, by the Δ*hofQ* deletion strain in UTI89, the Δ*yheF* Δ*hofQ* double deletion mutant in CFT073 and their WT counterparts.

Role of T2S and T4P systems in bladder infections caused by UPEC. As described in Chapter One, the UPEC developmental cascade in the bladder consists of bacterial adherence to the bladder epithelium followed by invasion, establishment and maturation of intracellular replicating foci and emergence of filamentous bacteria from the infected cells. The data from the *in vitro* bladder epithelial cell infection experiments showed that T4P and ETEC-T2SS are important for the efflux of UTI89 from bladder cells. However, no defects in bladder colonization and persistence were observed in animal infections with UTI89 mutants containing deletions in ETEC-T2SS or T4P. Further analysis of these mutants can be carried out by histological analysis of bladder samples obtained at different time points PI. The detailed study of the passage of UPEC through the maturation cascade in the bladder has been observed by the time lapse video-microscopic analysis of murine bladder mounted *ex vivo* on an anodized aluminum incubation chamber (50). This method can be used to analyze T2SS and T4P secretin deletion mutants for defects in steps of the developmental cascade.

Importance of T2S and T4P systems in intestinal colonization. UPEC (and other ExPEC) do not cause gastro-intestinal disease in humans, although they are able to establish prolonged intestinal colonization, at times even more effectively than the intestinal pathogenic *E. coli* (49). UPEC typically enter the urinary tract as a consequence of fecal contamination of the urethral opening and cause ascending UTI. Thus, UPEC must be able to successfully pass through the gastro-intestinal tract. As described earlier,

T2SS effectors such as LT in the case of ETEC or the bundle forming T4P of EPEC are known to play important roles in diseases caused by intestinal pathogenic *E. coli* (33, 34, 94). Moreover, UTI89 possesses a T2SS gene cluster that is homologous to one found in ETEC. Based on this information, it will be quite interesting to check whether the T2S and T4P systems in the model UPEC strains, UTI89 and CFT073, play a role in their ability to colonize and pass through the intestine. To address this, OM secretin mutants in which the antibiotic resistant cassette has not been removed can be used to infect mice orally along with their parental WT strains (marked by a different antibiotic resistance). The comparison of CFUs recovered from the feces of animals infected with different mutant and WT strains will indicate whether the lack of T2S and/or T4P systems has any effect on the ability of UPEC to colonize the intestine.

Concluding Remarks. ExPEC is an inclusive designation for a distinctive group of pathogenic *E. coli* based on their common virulence factors, serotypes and phylogenetic groups, and includes mainly the pathogenic strains causing UTI, sepsis/neonatal meningitis in humans and extra-intestinal (e.g.; respiratory) infection in poultry. ExPEC employ a broad array of virulence factors, including type 1 and P pili, and secreted toxins such as CNF1, hemolysin and Sat. Using the model UPEC isolates CFT073 and UTI89 we sought to understand the importance of the type II secretion and type IV pilus systems in the virulence of UPEC. The deletion mutants UTI89 Δ*hofQ* and CFT073 Δ*yheF* Δ*hofQ* showed reduced ability to colonize and persist in the upper urinary tract, especially kidneys. This defect was rescued by plasmid-borne copies of the specific

genes. These results fulfill the molecular Koch's postulates, in turn emphasizing that T4P are important virulence factors of UPEC and T4P and/or T2SS effectors aid in the colonization and persistence of UPEC in the upper urinary tract. Our results expand the impressive list of weaponry used by ExPEC to include T4P and possibly also the T2SS and open new avenues for understanding the molecular mechanisms of *E. coli* pathogenesis in the upper urinary tract.

Abbreviations

APEC	Avian Pathogenic *Escherichia coli*
AUM	Asymmetric unit membrane
Cdi	Contact-dependent inhibitor
CFU	Colony forming unit
CNF1	Cytotoxic necrotizing factor 1
ETEC	Enterotoxigenic *Escherichia coli*
ExPEC	Extra-intestinal Pathogenic *Escherichia coli*
FBS	Fetal bovine serum
GSP	General secretory pathway
GTP	Guanosine tri phosphate
H&E	Hematoxylin and eosin
HlyA	Hemolysin A
IBC	Intracellular bacterial community
IM	Inner membrane
KO	Knock out
LB	Luria Broth
LDH	Lactate dehydrogenase
LT	Heat-labile enterotoxin
MEM	Modified Eagle's medium
NMEC	Neonatal Meningitis-causing *Escherichia coli*
OD	Optical density
OM	Outer membrane
Pap	Pyelonephritis-associated pili
PI	Post-inoculation
PMN	Polumorphonuclear leukocyte
PS	Protamine sulphate
QIR	Quiescent intracellular reservoir
Sat	Secreted autotransporter protein
T2SS	Type II secretion system
T4P	Type IV pilus
TLR	Toll-like receptor
TX 100	Triton X 100
UP	uroplakin
UPEC	Uropathogenic *Escherichia coli*
UTI	Urinary Tract Infections
WT	Wild type

References

1. **Alm, R. A., and J. S. Mattick.** 1997. Genes involved in the biogenesis and function of type-4 fimbriae in *Pseudomonas aeruginosa*. Gene **192:**89-98.
2. **Anderson, G. G., K. W. Dodson, T. M. Hooton, and S. J. Hultgren.** 2004. Intracellular bacterial communities of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Trends Microbiol **12:**424-30.
3. **Anderson, G. G., J. J. Palermo, J. D. Schilling, R. Roth, J. Heuser, and S. J. Hultgren.** 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science **301:**105-7.
4. **Aoki, S. K., R. Pamma, A. D. Hernday, J. E. Bickham, B. A. Braaten, and D. A. Low.** 2005. Contact-dependent inhibition of growth in *Escherichia coli*. Science **309:**1245-8.
5. **Blattner, F. R., G. Plunkett, 3rd, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao.** 1997. The complete genome sequence of Escherichia coli K-12. Science **277:**1453-74.
6. **Bleves, S., M. Gerard-Vincent, A. Lazdunski, and A. Filloux.** 1999. Structure-function analysis of XcpP, a component involved in general secretory pathway-dependent protein secretion in *Pseudomonas aeruginosa*. J Bacteriol **181:**4012-9.
7. **Bock, K., M. E. Breimer, A. Brignole, G. C. Hansson, K. A. Karlsson, G. Larson, H. Leffler, B. E. Samuelsson, N. Stromberg, C. S. Eden, and et al.** 1985. Specificity of binding of a strain of uropathogenic *Escherichia coli* to Gal alpha 1-4Gal-containing glycosphingolipids. J Biol Chem **260:**8545-51.
8. **Bose, N., S. M. Payne, and R. K. Taylor.** 2002. Type 4 pilus biogenesis and type II-mediated protein secretion by Vibrio cholerae occur independently of the TonB-facilitated proton motive force. J Bacteriol **184:**2305-9.
9. **Bower, J. M., D. S. Eto, and M. A. Mulvey.** 2005. Covert operations of uropathogenic Escherichia coli within the urinary tract. Traffic **6:**18-31.
10. **Bruijn, F. J. d., Lupski, James R, Weinstock George M, .** 1998. Bacterial Genomes Physical Structure and Analysis, vol. Springer.
11. **Burrows, L. L.** 2005. Weapons of mass retraction. Mol Microbiol **57:**878-88.
12. **Chen, S. L., C. S. Hung, J. Xu, C. S. Reigstad, V. Magrini, A. Sabo, D. Blasiar, T. Bieri, R. R. Meyer, P. Ozersky, J. R. Armstrong, R. S. Fulton, J. P. Latreille, J. Spieth, T. M. Hooton, E. R. Mardis, S. J. Hultgren, and J. I. Gordon.** 2006. Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a comparative genomics approach. Proc Natl Acad Sci U S A **103:**5977-82.
13. **Contag, C. H., P. R. Contag, J. I. Mullins, S. D. Spilman, D. K. Stevenson, and D. A. Benaron.** 1995. Photonic detection of bacterial pathogens in living hosts. Mol Microbiol **18:**593-603.

14. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Annu Rev Microbiol **41:**435-64.
15. **Craig, L., R. K. Taylor, M. E. Pique, B. D. Adair, A. S. Arvai, M. Singh, S. J. Lloyd, D. S. Shin, E. D. Getzoff, M. Yeager, K. T. Forest, and J. A. Tainer.** 2003. Type IV pilin structure and assembly: X-ray and EM analyses of *Vibrio cholerae* toxin-coregulated pilus and *Pseudomonas aeruginosa* PAK pilin. Mol Cell **11:**1139-50.
16. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-5.
17. **Davis, J. M., H. M. Carvalho, S. B. Rasmussen, and A. D. O'Brien.** 2006. Cytotoxic necrotizing factor type 1 delivered by outer membrane vesicles of uropathogenic *Escherichia coli* attenuates polymorphonuclear leukocyte antimicrobial activity and chemotaxis. Infect Immun **74:**4401-8.
18. **Davis, J. M., S. B. Rasmussen, and A. D. O'Brien.** 2005. Cytotoxic necrotizing factor type 1 production by uropathogenic Escherichia coli modulates polymorphonuclear leukocyte function. Infect Immun **73:**5301-10.
19. **Eto, D. S., T. A. Jones, J. L. Sundsbak, and M. A. Mulvey.** 2007. Integrin-Mediated Host Cell Invasion by Type 1-Piliated Uropathogenic *Escherichia coli*. PLoS Pathog **3:**e100.
20. **Eto, D. S., J. L. Sundsbak, and M. A. Mulvey.** 2006. Actin-gated intracellular growth and resurgence of uropathogenic *Escherichia coli*. Cell Microbiol **8:**704-17.
21. **Ewers, C., G. Li, H. Wilking, S. Kiessling, K. Alt, E. M. Antao, C. Laturnus, I. Diehl, S. Glodde, T. Homeier, U. Bohnke, H. Steinruck, H. C. Philipp, and L. H. Wieler.** 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: how closely related are they? Int J Med Microbiol **297:**163-76.
22. **Falkow, S.** 2004. Molecular Koch's postulates applied to bacterial pathogenicity--a personal recollection 15 years later. Nat Rev Microbiol **2:**67-72.
23. **Falkow, S.** 1988. Molecular Koch's postulates applied to microbial pathogenicity. Rev Infect Dis **10 Suppl 2:**S274-6.
24. **Forest, K. T., and J. A. Tainer.** 1997. Type-4 pilus-structure: outside to inside and top to bottom--a minireview. Gene **192:**165-9.
25. **Foxman, B.** 2003. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Dis Mon **49:**53-70.
26. **Foxman, B.** 1990. Recurring urinary tract infection: incidence and risk factors. Am J Public Health **80:**331-3.
27. **Foxman, B., and P. Brown.** 2003. Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. Infect Dis Clin North Am **17:**227-41.
28. **Francetic, O., D. Belin, C. Badaut, and A. P. Pugsley.** 2000. Expression of the endogenous type II secretion pathway in Escherichia coli leads to chitinase secretion. Embo J **19:**6697-703.

29. **Francetic, O., and A. P. Pugsley.** 2005. Towards the identification of type II secretion signals in a nonacylated variant of pullulanase from *Klebsiella oxytoca*. J Bacteriol **187:**7045-55.
30. **Francis, K. P., J. Yu, C. Bellinger-Kawahara, D. Joh, M. J. Hawkinson, G. Xiao, T. F. Purchio, M. G. Caparon, M. Lipsitch, and P. R. Contag.** 2001. Visualizing pneumococcal infections in the lungs of live mice using bioluminescent *Streptococcus pneumoniae* transformed with a novel gram-positive lux transposon. Infect Immun **69:**3350-8.
31. **Fullner, K. J., and J. J. Mekalanos.** 1999. Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae*. Infect Immun **67:**1393-404.
32. **Gerlach, R. G., S. U. Holzer, D. Jackel, and M. Hensel.** 2007. Rapid engineering of bacterial reporter gene fusions by using red recombination. Appl Environ Microbiol **73:**4234-42.
33. **Giron, J. A., A. S. Ho, and G. K. Schoolnik.** 1991. An inducible bundle-forming pilus of enteropathogenic Escherichia coli. Science **254:**710-3.
34. **Giron, J. A., M. M. Levine, and J. B. Kaper.** 1994. Longus: a long pilus ultrastructure produced by human enterotoxigenic Escherichia coli. Mol Microbiol **12:**71-82.
35. **Gregory, T. R.** 2005. Synergy between sequence and size in large-scale genomics. Nat Rev Genet **6:**699-708.
36. **Guignot, J., C. Chaplais, M. H. Coconnier-Polter, and A. L. Servin.** 2007. The secreted autotransporter toxin, Sat, functions as a virulence factor in Afa/Dr diffusely adhering Escherichia coli by promoting lesions in tight junction of polarized epithelial cells. Cell Microbiol **9:**204-21.
37. **Guyer, D. M., S. Radulovic, F. E. Jones, and H. L. Mobley.** 2002. Sat, the secreted autotransporter toxin of uropathogenic *Escherichia coli*, is a vacuolating cytotoxin for bladder and kidney epithelial cells. Infect Immun **70:**4539-46.
38. **Hagberg, L., I. Engberg, R. Freter, J. Lam, S. Olling, and C. Svanborg Eden.** 1983. Ascending, unobstructed urinary tract infection in mice caused by pyelonephritogenic *Escherichia coli* of human origin. Infect Immun **40:**273-83.
39. **Hales, L. M., and H. A. Shuman.** 1999. *Legionella pneumophila* contains a type II general secretion pathway required for growth in amoebae as well as for secretion of the Msp protease. Infect Immun **67:**3662-6.
40. **Haraoka, M., L. Hang, B. Frendeus, G. Godaly, M. Burdick, R. Strieter, and C. Svanborg.** 1999. Neutrophil recruitment and resistance to urinary tract infection. J Infect Dis **180:**1220-9.
41. **Hedlund, M., M. Svensson, A. Nilsson, R. D. Duan, and C. Svanborg.** 1996. Role of the ceramide-signaling pathway in cytokine responses to P-fimbriated *Escherichia coli*. J Exp Med **183:**1037-44.
42. **Herrington, D. A., R. H. Hall, G. Losonsky, J. J. Mekalanos, R. K. Taylor, and M. M. Levine.** 1988. Toxin, toxin-coregulated pili, and the toxR regulon are essential for *Vibrio cholerae* pathogenesis in humans. J Exp Med **168:**1487-92.
43. **Hitziger, N., I. Dellacasa, B. Albiger, and A. Barragan.** 2005. Dissemination of *Toxoplasma gondii* to immunoprivileged organs and role of Toll/interleukin-1

receptor signalling for host resistance assessed by in vivo bioluminescence imaging. Cell Microbiol **7:**837-48.

44. **Holechek, M. J.** 2003. Glomerular filtration: an overview. Nephrol Nurs J **30:**285-90; quiz 291-2.
45. **Hunstad, D. A., S. S. Justice, C. S. Hung, S. R. Lauer, and S. J. Hultgren.** 2005. Suppression of bladder epithelial cytokine responses by uropathogenic *Escherichia coli*. Infect Immun **73:**3999-4006.
46. **Hutchens, M., and G. D. Luker.** 2007. Applications of bioluminescence imaging to the study of infectious diseases. Cell Microbiol.
47. **Jiang, B., and S. P. Howard.** 1992. The *Aeromonas hydrophila* exeE gene, required both for protein secretion and normal outer membrane biogenesis, is a member of a general secretion pathway. Mol Microbiol **6:**1351-61.
48. **Johnson, J. R.** 1991. Virulence factors in *Escherichia coli* urinary tract infection. Clin Microbiol Rev **4:**80-128.
49. **Johnson, J. R., and T. A. Russo.** 2002. Extraintestinal pathogenic *Escherichia coli*: "the other bad *E coli*". J Lab Clin Med **139:**155-62.
50. **Justice, S. S., C. Hung, J. A. Theriot, D. A. Fletcher, G. G. Anderson, M. J. Footer, and S. J. Hultgren.** 2004. Differentiation and developmental pathways of uropathogenic *Escherichia coli* in urinary tract pathogenesis. Proc Natl Acad Sci U S A **101:**1333-8.
51. **Justice, S. S., D. A. Hunstad, P. C. Seed, and S. J. Hultgren.** 2006. Filamentation by *Escherichia coli* subverts innate defenses during urinary tract infection. Proc Natl Acad Sci U S A **103:**19884-9.
52. **Kaper, J. B., J. P. Nataro, and H. L. Mobley.** 2004. Pathogenic *Escherichia coli*. Nat Rev Microbiol **2:**123-40.
53. **Kau, A. L., D. A. Hunstad, and S. J. Hultgren.** 2005. Interaction of uropathogenic *Escherichia coli* with host uroepithelium. Curr Opin Microbiol **8:**54-9.
54. **Kornacker, M. G., and A. P. Pugsley.** 1990. The normally periplasmic enzyme beta-lactamase is specifically and efficiently translocated through the *Escherichia coli* outer membrane when it is fused to the cell-surface enzyme pullulanase. Mol Microbiol **4:**1101-9.
55. **Kostakioti, M., C. L. Newman, D. G. Thanassi, and C. Stathopoulos.** 2005. Mechanisms of protein export across the bacterial outer membrane. J Bacteriol **187:**4306-14.
56. **Landraud, L., C. Pulcini, P. Gounon, G. Flatau, P. Boquet, and E. Lemichez.** 2004. *E. coli* CNF1 toxin: a two-in-one system for host-cell invasion. Int J Med Microbiol **293:**513-8.
57. **Lane, M. C., and H. L. Mobley.** 2007. Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic *Escherichia coli* (UPEC) in the mammalian kidney. Kidney Int **72:**19-25.
58. **Luker, G. D., J. P. Bardill, J. L. Prior, C. M. Pica, D. Piwnica-Worms, and D. A. Leib.** 2002. Noninvasive bioluminescence imaging of herpes simplex virus type 1 infection and therapy in living mice. J Virol **76:**12149-61.
59. **Maier, B., L. Potter, M. So, C. D. Long, H. S. Seifert, and M. P. Sheetz.** 2002. Single pilus motor forces exceed 100 pN. Proc Natl Acad Sci U S A **99:**16012-7.

60. **Martin, P. R., M. Hobbs, P. D. Free, Y. Jeske, and J. S. Mattick.** 1993. Characterization of pilQ, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol Microbiol **9:**857-68.
61. **Martinez, J. J., M. A. Mulvey, J. D. Schilling, J. S. Pinkner, and S. J. Hultgren.** 2000. Type 1 pilus-mediated bacterial invasion of bladder epithelial cells. Embo J **19:**2803-12.
62. **Mattick, J. S.** 2002. Type IV pili and twitching motility. Annu Rev Microbiol **56:**289-314.
63. **Merz, A. J., and M. So.** 2000. Interactions of pathogenic neisseriae with epithelial cell membranes. Annu Rev Cell Dev Biol **16:**423-57.
64. **Merz, A. J., M. So, and M. P. Sheetz.** 2000. Pilus retraction powers bacterial twitching motility. Nature **407:**98-102.
65. **Mobley, H. L., D. M. Green, A. L. Trifillis, D. E. Johnson, G. R. Chippendale, C. V. Lockatell, B. D. Jones, and J. W. Warren.** 1990. Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infect Immun **58:**1281-9.
66. **Mobley, H. L., K. G. Jarvis, J. P. Elwood, D. I. Whittle, C. V. Lockatell, R. G. Russell, D. E. Johnson, M. S. Donnenberg, and J. W. Warren.** 1993. Isogenic P-fimbrial deletion mutants of pyelonephritogenic *Escherichia coli*: the role of alpha Gal(1-4) beta Gal binding in virulence of a wild-type strain. Mol Microbiol **10:**143-55.
67. **Mulvey, M. A., Y. S. Lopez-Boado, C. L. Wilson, R. Roth, W. C. Parks, J. Heuser, and S. J. Hultgren.** 1998. Induction and evasion of host defenses by type 1-piliated uropathogenic *Escherichia coli*. Science **282:**1494-7.
68. **Mulvey, M. A., J. D. Schilling, and S. J. Hultgren.** 2001. Establishment of a persistent *Escherichia coli* reservoir during the acute phase of a bladder infection. Infect Immun **69:**4572-9.
69. **Mulvey, M. A., J. D. Schilling, J. J. Martinez, and S. J. Hultgren.** 2000. Bad bugs and beleaguered bladders: interplay between uropathogenic *Escherichia coli* and innate host defenses. Proc Natl Acad Sci U S A **97:**8829-35.
70. **Mysorekar, I. U., and S. J. Hultgren.** 2006. Mechanisms of uropathogenic *Escherichia coli* persistence and eradication from the urinary tract. Proc Natl Acad Sci U S A **103:**14170-5.
71. **Neish, A. S., A. T. Gewirtz, H. Zeng, A. N. Young, M. E. Hobert, V. Karmali, A. S. Rao, and J. L. Madara.** 2000. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science **289:**1560-3.
72. **Nunn, D. N., and S. Lory.** 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc Natl Acad Sci U S A **88:**3281-5.
73. **Palmer, L. E., A. R. Pancetti, S. Greenberg, and J. B. Bliska.** 1999. YopJ of *Yersinia* spp. is sufficient to cause downregulation of multiple mitogen-activated protein kinases in eukaryotic cells. Infect Immun **67:**708-16.
74. **Paranjpye, R. N., and M. S. Strom.** 2005. A *Vibrio vulnificus* type IV pilin contributes to biofilm formation, adherence to epithelial cells, and virulence. Infect Immun **73:**1411-22.

75. **Parsons, C. L., C. W. Stauffer, and J. D. Schmidt.** 1988. Reversible inactivation of bladder surface glycosaminoglycan antibacterial activity by protamine sulfate. Infect Immun **56:**1341-3.
76. **Peabody, C. R., Y. J. Chung, M. R. Yen, D. Vidal-Ingigliardi, A. P. Pugsley, and M. H. Saier, Jr.** 2003. Type II protein secretion and its relationship to bacterial type IV pili and archaeal flagella. Microbiology **149:**3051-72.
77. **Possot, O. M., M. Gerard-Vincent, and A. P. Pugsley.** 1999. Membrane association and multimerization of secreton component pulC. J Bacteriol **181:**4004-11.
78. **Pugsley, A. P., O. Francetic, O. M. Possot, N. Sauvonnet, and K. R. Hardie.** 1997. Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria--a review. Gene **192:**13-9.
79. **Py, B., L. Loiseau, and F. Barras.** 2001. An inner membrane platform in the type II secretion machinery of Gram-negative bacteria. EMBO Rep **2:**244-8.
80. **Roberts, J. A., B. I. Marklund, D. Ilver, D. Haslam, M. B. Kaack, G. Baskin, M. Louis, R. Mollby, J. Winberg, and S. Normark.** 1994. The Gal(alpha 1-4)Gal-specific tip adhesin of *Escherichia coli* P-fimbriae is needed for pyelonephritis to occur in the normal urinary tract. Proc Natl Acad Sci U S A **91:**11889-93.
81. **Romih, R., P. Korosec, W. de Mello, Jr., and K. Jezernik.** 2005. Differentiation of epithelial cells in the urinary tract. Cell Tissue Res **320:**259-68.
82. **Ronald, A.** 2002. The etiology of urinary tract infection: traditional and emerging pathogens. Am J Med **113 Suppl 1A:**14S-19S.
83. **Russo, T. A., and J. R. Johnson.** 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of Escherichia coli: ExPEC. J Infect Dis **181:**1753-4.
84. **Sandkvist, M.** 2001. Biology of type II secretion. Mol Microbiol **40:**271-83.
85. **Sandkvist, M.** 2001. Type II secretion and pathogenesis. Infect Immun **69:**3523-35.
86. **Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, V. J. DiRita, and M. Bagdasarian.** 1997. General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in Vibrio cholerae. J Bacteriol **179:**6994-7003.
87. **Sauvonnet, N., P. Gounon, and A. P. Pugsley.** 2000. PpdD type IV pilin of *Escherichia coli* K-12 can Be assembled into pili in Pseudomonas aeruginosa. J Bacteriol **182:**848-54.
88. **Sauvonnet, N., and A. P. Pugsley.** 1996. Identification of two regions of Klebsiella oxytoca pullulanase that together are capable of promoting beta-lactamase secretion by the general secretory pathway. Mol Microbiol **22:**1-7.
89. **Sauvonnet, N., G. Vignon, A. P. Pugsley, and P. Gounon.** 2000. Pilus formation and protein secretion by the same machinery in *Escherichia coli*. Embo J **19:**2221-8.
90. **Savage, D. C.** 2001. Microbial biota of the human intestine: a tribute to some pioneering scientists. Curr Issues Intest Microbiol **2:**1-15.

91. **Schilling, J. D., M. A. Mulvey, C. D. Vincent, R. G. Lorenz, and S. J. Hultgren.** 2001. Bacterial invasion augments epithelial cytokine responses to *Escherichia coli* through a lipopolysaccharide-dependent mechanism. J Immunol **166:**1148-55.
92. **Strom, M. S., and S. Lory.** 1993. Structure-function and biogenesis of the type IV pili. Annu Rev Microbiol **47:**565-96.
93. **Strom, M. S., D. N. Nunn, and S. Lory.** 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc Natl Acad Sci U S A **90:**2404-8.
94. **Tauschek, M., R. J. Gorrell, R. A. Strugnell, and R. M. Robins-Browne.** 2002. Identification of a protein secretory pathway for the secretion of heat-labile enterotoxin by an enterotoxigenic strain of *Escherichia coli*. Proc Natl Acad Sci U S A **99:**7066-71.
95. **Thanabalu, T., E. Koronakis, C. Hughes, and V. Koronakis.** 1998. Substrate-induced assembly of a contiguous channel for protein export from *E.coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. Embo J **17:**6487-96.
96. **Thanassi, D. G., and S. J. Hultgren.** 2000. Multiple pathways allow protein secretion across the bacterial outer membrane. Curr Opin Cell Biol **12:**420-30.
97. **Tonjum, T., and M. Koomey.** 1997. The pilus colonization factor of pathogenic neisserial species: organelle biogenesis and structure/function relationships--a review. Gene **192:**155-63.
98. **Turner, L. R., J. C. Lara, D. N. Nunn, and S. Lory.** 1993. Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J Bacteriol **175:**4962-9.
99. **Uhlen, P., A. Laestadius, T. Jahnukainen, T. Soderblom, F. Backhed, G. Celsi, H. Brismar, S. Normark, A. Aperia, and A. Richter-Dahlfors.** 2000. Alpha-haemolysin of uropathogenic *E. coli* induces Ca2+ oscillations in renal epithelial cells. Nature **405:**694-7.
100. **Vignon, G., R. Kohler, E. Larquet, S. Giroux, M. C. Prevost, P. Roux, and A. P. Pugsley.** 2003. Type IV-like pili formed by the type II secreton: specificity, composition, bundling, polar localization, and surface presentation of peptides. J Bacteriol **185:**3416-28.
101. **Voulhoux, R., G. Ball, B. Ize, M. L. Vasil, A. Lazdunski, L. F. Wu, and A. Filloux.** 2001. Involvement of the twin-arginine translocation system in protein secretion via the type II pathway. Embo J **20:**6735-41.
102. **Waldor, M. K., and J. J. Mekalanos.** 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. Science **272:**1910-4.
103. **Wall, D., and D. Kaiser.** 1999. Type IV pili and cell motility. Mol Microbiol **32:**1-10.
104. **Welch, R. A., V. Burland, G. Plunkett, 3rd, P. Redford, P. Roesch, D. Rasko, E. L. Buckles, S. R. Liou, A. Boutin, J. Hackett, D. Stroud, G. F. Mayhew, D. J. Rose, S. Zhou, D. C. Schwartz, N. T. Perna, H. L. Mobley, M. S. Donnenberg, and F. R. Blattner.** 2002. Extensive mosaic structure revealed by

the complete genome sequence of uropathogenic *Escherichia coli*. Proc Natl Acad Sci U S A **99:**17020-4.

105. **Whitchurch, C. B., M. Hobbs, S. P. Livingston, V. Krishnapillai, and J. S. Mattick.** 1991. Characterisation of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene **101:**33-44.
106. **Whitchurch, C. B., and J. S. Mattick.** 1994. Characterization of a gene, pilU, required for twitching motility but not phage sensitivity in *Pseudomonas aeruginosa*. Mol Microbiol **13:**1079-91.
107. **Zhang, D., G. Zhang, M. S. Hayden, M. B. Greenblatt, C. Bussey, R. A. Flavell, and S. Ghosh.** 2004. A toll-like receptor that prevents infection by uropathogenic bacteria. Science **303:**1522-6.
108. **Zoutman, D. E., W. C. Hulbert, B. L. Pasloske, A. M. Joffe, K. Volpel, M. K. Trebilcock, and W. Paranchych.** 1991. The role of polar pili in the adherence of *Pseudomonas aeruginosa* to injured canine tracheal cells: a semiquantitative morphologic study. Scanning Microsc **5:**109-24; discussion 124-6.