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Drug and Vaccine Development for the Treatment and Prevention of Urinary Tract Infections

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Abstract

Urinary tract infections (UTI) are among the most common bacterial infections in humans, affecting millions of people every year. UTI cause significant morbidity in women throughout their lifespan, in infant boys, in older men, in individuals with underlying urinary tract abnormalities, and in those that require long-term urethral catheterization, such as patients with spinal cord injuries or incapacitated individuals living in nursing homes. Serious sequelae include frequent recurrences, pyelonephritis with sepsis, renal damage in young children, pre-term birth, and complications of frequent antimicrobial use including high-level antibiotic resistance and *Clostridium difficile* colitis. Uropathogenic *E. coli* (UPEC) cause the vast majority of UTI, but less common pathogens such as *Enterococcus faecalis* and other enterococci frequently take advantage of an abnormal or catheterized urinary tract to cause opportunistic infections. While antibiotic therapy has historically been very successful in controlling UTI, the high rate of recurrence remains a major problem, and many individuals suffer from chronically recurring UTI, requiring long-term prophylactic antibiotic regimens to prevent recurrent UTI. Furthermore, the global emergence of multi-drug resistant UPEC in the past ten years spotlights the need for alternative therapeutic and preventative strategies to combat UTI, including anti-infective drug therapies and

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vaccines. In this chapter, we review recent advances in the field of UTI pathogenesis, with an emphasis on the identification of promising drug and vaccine targets. We then discuss the development of new UTI drugs and vaccines, highlighting the challenges these approaches face and the need for a greater understanding of urinary tract mucosal immunity.

The Urgent Need for New Therapies and Vaccines

Urinary tract infections (UTI) are one of the most common bacterial infections, with roughly eleven million cases reported in the U.S. each year that cost an estimated $3.5 billion annually ([1]). Nearly one in every two women will experience at least one UTI in her lifetime, and nearly one in three women will have received antibiotic treatment for a UTI before age 24 ([2]). The clinical manifestations of symptomatic UTI include infection-induced inflammation of the urethra (urethritis), urinary bladder (cystitis), and kidneys (pyelonephritis) and are diagnosed by the presence of high levels of bacteria in the urine (bacteriuria) with concomitant symptoms. Symptoms of cystitis include frequent urination, burning sensation and pain during urination (dysuria), suprapubic pain and/or lower abdominal discomfort, and cloudy and/or bloody, foul-smelling urine. Symptoms of pyelonephritis include the presence of bacteriuria and pyuria (white blood cells in the urine) that is accompanied by flank pain and fever, but may or may not include other symptoms of cystitis. The vast majority of UTI manifest as cystitis and urethritis, affecting primarily the lower urinary tract, but this can potentially lead to bacterial ascension to the kidneys and pyelonephritis, particularly in pregnant women and diabetics ([3]). As a result, renal scarring and loss of function is a potentially serious complication of any UTI, particularly in infants, where diagnosis of UTI may be delayed.

UTI are not only common, but also highly recurrent. In particular, sexually active women, the elderly and pre-pubertal children are highly susceptible to chronically recurrent UTI, resulting in increased use of antibiotics and negatively affecting quality of life ([2]). Approximately 20–30% of adult women with an initial UTI will experience a recurrence within 3–4 months ([4]). In children, about one in three experiencing a UTI before the age of one will experience a recurrence within three years, and 18% will have a recurrence within a few months ([5]). Uncomplicated UTI, which are infections that are not associated with urethral instrumentation or abnormal anatomy or physiology of the urinary tract, predominantly affect women, young children, and the elderly. Risk factors for uncomplicated cystitis in adult women include environmental factors such as frequent sexual activity, exposure to spermicides, menopause, and a history of childhood UTI, as well as genetic factors such as being a non-secretor of ABO blood-group antigens and a maternal history of UTI ([6]). In contrast, patients at risk for what is termed complicated UTI include patients with spinal cord injuries, patients undergoing urethral catheterization, diabetics, and individuals with underlying urologic abnormalities such as vesicoureteral reflux (VUR) ([2]). Uropathogenic *Escherichia coli* (UPEC) cause 85% or more of uncomplicated UTI cases, while other Gram-negative rods and Gram-positive cocci, such as *Staphylococcus saprophyticus* and enterococci, are responsible for the remaining 5–15% of cases ([7]).

The epidemiology of UTI changes significantly in the health care environment. Urethral catheterization is strongly associated with UTI, and the risk of infection increases with the length of catheterization ([8]). Catheter-associated UTI (CAUTI) account for 30–40% of health care-associated infections in the United States, making them the most common nosocomial infection, with more than one million cases occurring yearly in hospitals and nursing homes ([9]). Although enterococci contribute only minimally to the burden of uncomplicated UTI, data from a national surveillance network of 463 hospitals in the United States revealed that 15% of CAUTI are caused by enterococci, second among bacterial isolates only to *E. coli* (21%) ([10]). Furthermore, CAUTI affect both sexes, as long-term urinary catheterization of both men and women almost invariably leads to detection of bacteria in the urine (bacteriuria) and carries a daily risk of 3–7% for the development of symptomatic CAUTI ([11]). While CAUTI is most often asymptomatic, the high incidence in catheterized patients greatly increases their risk for relatively rare but serious sequelae such as bacteremia, urosepsis, and death ([12]). Furthermore, CAUTI serve as reservoirs for the dissemination of antimicrobial-resistant nosocomial pathogens in the health care environment ([13]).

Although antibiotic therapy has historically been very successful in combating both uncomplicated and complicated UTI, many individuals suffer from chronically recurrent cystitis, requiring long-term antibiotic prophylaxis ([14]). Furthermore, the widespread use of antibiotics has led to accelerating antibiotic resistance and the emergence and spread of multidrug-resistant (MDR) uropathogens ([15]). As early as 1957, Weyrauch and colleagues foresaw this problem in their discussion of the results of a UTI vaccine trial ([16]). In their study, they found that intramuscular injection with heat-killed *E. coli* was protective or partially protective against pyelonephritis in 12 of 16 rabbits. However, unvaccinated rabbits treated with prophylactic tetracycline were completely resistant to pyelonephritis, leading the authors to predict that prophylactic antibiotic treatment would remain the best strategy for preventing UTI in humans. Despite the authors' admonition that "every effort must be made to avoid indiscriminate use" of antibiotic prophylaxis in order to prevent resistance, drug-resistant UTI has exploded into a major public health concern. For instance, a recent five-year nested case-control study of drug resistance in uncomplicated febrile UTI in adults found that 12% of patients with UPEC UTI had fluoroquinolone-resistant urine cultures; fluoroquinolone use in the previous six months was a significant independent risk factor for being afflicted with a fluoroquinolone-resistant UTI ([17]). In another study, more than 9,000 patient urine samples were analyzed for drug resistance; of the samples containing uropathogens, 22.1% were multi-drug resistant (resistant to third-generation cephalosporins, ciprofloxacin, and aminoglycosides) ([18]). In the past decade, the *E. coli* clone O25:H4-ST131 (*E. coli* ST131) emerged globally as an important MDR UPEC strain ([19]). Unlike other antibiotic-resistant UPEC strains, ST131 is highly virulent in the urinary tract and is not only found in health care settings, but is also isolated from the community ([15, 20]).

In summary, urinary tract infection is a significant cause of morbidity in women throughout their lifespan, in infant boys and in older men. Serious sequelae include frequent recurrences, pyelonephritis with sepsis, renal damage in young children, pre-term birth, and complications of frequent antimicrobial use including high-level antibiotic resistance and *Clostridium difficile* colitis ([21, 22]). High recurrence rates and increasing antimicrobial

resistance among uropathogens threaten to greatly increase the economic burden of this common infection. It has become increasingly evident that prophylactic use of antibiotics to prevent UTI is not a sustainable solution. The high incidence and recurrence rate of UTI, along with the rapid rise of MDR uropathogens and CAUTI, necessitate new drugs and vaccine therapies for the prevention of these infections. In this chapter, we will review UTI pathogenesis, focusing on uropathogenic *E. coli* (UPEC) as a model organism for uncomplicated UTI and *Enterococcus faecalis* as a model organism for complicated, catheter-associated UTI (CAUTI). We will then describe the development of anti-virulence therapies, including new classes of small molecule inhibitors that target uropathogenic virulence factors. Finally, we will discuss vaccines for the prevention of recurrent UTI, including both whole cell and specific-antigen vaccines. It is our hope that this chapter will draw attention to recent advances in the field of UTI therapeutics while highlighting specific topics that require further study.

Recent Discoveries in UTI Pathogenesis

Since 90% of symptomatic UTI present as simple cystitis/urethritis, an ideal drug or vaccine target would be one that is critical for both establishing and maintaining bladder colonization, thus preventing UTI altogether. Quantum-leap advances in molecular biological and imaging technologies in the past 15 years, along with the maturation of genomic science, have led to an unprecedented expansion of our understanding of UTI pathogenesis, and to the identification of previously unknown virulence mechanisms. For example, we now know that UPEC invade bladder epithelial cells and have the capacity to rapidly replicate within the cytoplasm of superficial facet cells of the bladder urothelium, producing between 10,000 and 100,000 daughter cells from a single invasive bacterium within 12–16 hours ([23–26]). The establishment of this protected intracellular niche, known as the intracellular bacterial community or IBC, helps UPEC gain a foothold in the lower urinary tract. Although discovered in mice, exfoliated bladder epithelial cells containing IBCs have been observed in urine sediments obtained from women with recurrent UTI, but not in healthy controls or in cases of UTI caused by Gram-positive pathogens ([27]), indicating that the murine model is a relevant and powerful tool for studying UTI pathogenesis. Indeed, mice are naturally susceptible to UPEC UTI and recapitulate many of the known characteristics of UTI in humans ([28]).

While the translation of findings from animal models to the clinic is always fraught with difficulties, a major challenge in developing new therapeutics is not just the species differences between mice and humans in our animal models, but also the fact that these experimental infections are typically performed in naive animals. Experimental models of UTI in naive mice and primates have revealed similar pathogenic mechanisms of both cystitis and pyelonephritis ([29–34]). In contrast, recently developed models of recurrent UTI and post-menopausal UTI in mice have found that the mucosal immune response of the urogenital tract is very different from that seen in naive mice ([35–37]). The epidemiology of UTI suggests that in a damaged or sensitized mucosal environment the requirements for bacterial virulence factors are diminished, potentially making any therapeutic intervention that targets the bacteria a tremendous challenge. In this section, we will briefly summarize our current knowledge of acute UTI pathogenesis in the latest animal models of

uncomplicated UTI and CAUTI, highlighting the bacterial factors and host-pathogen processes that are promising drug and vaccine targets (Figure 1 and Table 1).

Uncomplicated UTI: UPEC

UPEC adhesins—Whole genome sequencing of several "prototypical" UPEC strains in the past 10 years has revealed the presence in each strain of multiple known and putative adhesins, a number of which have been demonstrated to contribute to UPEC's ability to colonize the urinary tract. These include adhesive fibers called pili (fimbriae). A molecular machine known as the chaperone-usher pathway (CUP) mediates the assembly of pili on the bacterial outer membrane of diverse genera of Gram-negative bacteria ([38–43]) (detailed in Chapter 15 and summarized in Figure 2). Pili are long fibers that extend beyond the bacterial capsule. They contain adhesins at their tips that are thought to play an important role in host-pathogen interactions ([44]). Each sequenced UPEC strain encodes a multitude of CUP operons ([45–47]). For example, the cystitis strain UTI89 encodes 10 CUP operons, but of those that are broadly conserved among UPEC isolates, only two, type 1 and P pili, have so far been strongly implicated in UTI pathogenesis. CUP adhesins are known to recognize specific receptors with stereochemical specificity. For example, FimH, the tip adhesin of the type 1 pilus, has been shown to bind mannosylated glycoproteins ([48–50]), as well as N-linked oligosaccharides on α1 and β3 integrins ([51]), and the pattern recognition receptor Toll-like receptor 4 (TLR4) ([52]), all of which are expressed on the luminal surface of human and murine bladders. In contrast, the P pilus adhesin, PapG, is known to bind to Gal-α-1,4-Gal in globosides in the human kidney ([53]).

Type 1 pili—Several lines of evidence point to the type 1 pilus as a critical virulence factor in the establishment of UTI by UPEC in humans. Type 1 pili have been shown to be expressed during human UTI. A number of studies investigating whether type 1 pili are expressed by UPEC during UTI have found that the frequency of positive type 1 pilus immunostaining in urine sediments from women with acute UTI ranged from 40% to 76% ([54–56]), comparable to what was found in acutely infected mice ([57]). Type 1 pili have long been known to play a critical and essential role in establishing cystitis in a murine model of experimental UTI, and vaccination of mice and cynomolgus monkeys with the type 1 pilus tip adhesin, FimH, protects against experimental cystitis ([29, 31, 32, 57–62]). Type 1 pili have also been shown to be required for UPEC adherence to human urothelial tissue culture cells, and expression of FimH was required for bacterial adherence to human bladder tissue *in situ* ([32, 48, 63]). Lastly, there is strong evidence that FimH has undergone pathoadaptive mutation in UPEC clinical isolates, with several amino acid residues found to be under positive selection ([64–68]). Mutation of these residues resulted in reduced virulence in a murine model of cystitis, providing further support that FimH plays an important role *in vivo* during human UTI ([64]).

FimH mediates UPEC adherence to and invasion of urothelial cells—During experimental bladder infection, the type 1 pilus-associated tip adhesin FimH mediates adherence and invasion of the superficial umbrella cells of the urothelium ([23, 57]). The specific receptor for type 1 pili appears to vary with the differentiation state of the urothelial cells. In mature superficial umbrella cells, the FimH receptor is the mannosylated uroplakin

protein UPIa ([50]). However, the immature urothelial cells generally used for *in vitro* studies, such as 5637 bladder transitional carcinoma cells, do not typically express uroplakins on the cell surface, and FimH was found to bind to mannosylated α3β1 integrins *in vitro* ([51]). Klumpp and colleagues have demonstrated that binding of FimH to the uroplakin receptor complex via UPIa leads to the phosphorylation of UPIIIa, the only one of the four major uroplakins with a potential cytoplasmic signaling domain, resulting in an increase in intracellular calcium and enhanced invasion ([69], [70]). However, in immature urothelial cells, bacterial invasion subsequent to FimH binding has been reported to involve components of clathrin-coated pits such as clathrin and the cargo adaptor protein AP-2 ([71]), caveolae and lipid rafts ([72]), the action of microtubules ([73]), and actin rearrangement involving focal adhesion kinase, phosphotidylinositol-3-kinase, and the Rho GTPases Rac1 and Cdc42 ([63], [74], [75]). The toxin cytotoxic necrotizing factor 1 (CNF1) has been reported to enhance UPEC invasion of urothelial cells *in vitro* by constitutively activating Rho GTPases ([76]), but CNF1 has not been shown to play a clear role *in vivo* ([77], [78]).

P pili—The role of P pili in UTI is complex and not fully understood. While P pilus-expressing UPEC are strongly associated with first time pyelonephritis in children ([79], [80]), they are less well conserved in women with acute and recurrent UTI, being expressed in only 40–50% of isolates, regardless of upper urinary tract involvement ([81]). This is likely because the chronic inflammatory changes that occur in the urinary tract of patients with a history of severe or recurrent UTI lessen the requirement for P pili in colonizing the kidney. P pili mediate adhesion to Gal-α-1,4-Gal-containing globoseries glycosphingolipids elaborated on the surface of urinary tract epithelial cells ([82]–[85]). Patients with upper urinary tract symptoms during UTI mount humoral antibody responses to P pili, indicating that they are expressed during infection ([86], [87]). Moreover, in humans who are non-secretors of ABO antigens, sialyl galactosyl globosides, which are P pilus receptors, are found more abundantly on the surface of epithelial cells in the kidneys and lower urogenital tract compared to "secretors," perhaps explaining why some studies have identified the non-secretor status as a significant risk factor for recurrent UTI ([88]–[91]). Concordant with these findings, P pili-expressing UPEC have a greater capacity to bind to vaginal epithelial cells from non-secretors than from secretors ([88]).

There are at least three alleles of the P pilus tip adhesin PapG, each of which differs in its binding specificity to globosides. Of these, $PapG_{II}$, which binds the human kidney receptor GbO4, is required for the establishment of pyelonephritis in cynomolgus monkeys ([92], [93]). Replacement of $PapG_{II}$ with $PapG_{III}$, which binds to Forssman antigen (GbO5) and is the predominant PapG allele found in cystitis strains ([94]), shortened the course of bacteriuria after bladder inoculation of primates and diminished both renal damage and the development of a serum titers against P pili. In a primate model of uncomplicated cystitis, neither $PapG_{II}$ nor $PapG_{III}$ were required for robust bladder infection ([95]). However, $PapG_{II}$ conferred a competitive advantage in the primate bladder when co-inoculated with an isogenic strain lacking P pili ($PapG_{III}$ was not tested in competitive infection). Deletion of P pilus operons, including one expressing $PapG_{II}$, from a virulent UPEC strain did not affect pathogenesis in a CBA murine model of infection ([96]). This may be because the UPEC strain used in the study contains alternative adhesins capable of colonizing the kidney epithelium, or perhaps

the CBA mouse model does not reflect the importance of $PapG_{II}$ - GbO4 interactions, since the GbO4 receptor is likely not as highly expressed in the murine kidney due to the presence of a functional Forssman synthetase in non-primate mammals ([97]). Furthermore, the C3H/HeJ strain, which is closely related to the CBA strain, has been reported to be genetically susceptible to vesicoureteric reflux ([98]), so the requirement for kidney adhesins may be diminished in this model. In contrast, a mutant UPEC strain lacking $PapG_{II}$ and other putative kidney colonization factors was defective in colonizing the kidneys of Balb/c mice. Live multiphoton studies of pyelonephritis in rats suggest that P and type 1 pili may work in concert to colonize the renal tubules by facilitating bacterial attachment and biofilm production, respectively ([99]). Therefore, although P pili are only expressed in about half of all recurrent UTI isolates, drugs that target both type 1 and P pili would likely have broadly protective effects in both the bladder and kidneys.

Other pili and non-pilus adhesins—Compared to commensal strains, UPEC have been shown to contain numerous CUP-assembled pilus operons, in addition to those encoding type 1 and P pili. These pilus operons were identified by homology to the four minimum essential components of CUP-assembled fimbrial systems: the major pilin, the pilus adhesin, the outer membrane usher, and the periplasmic chaperone. However, the functions and receptors of these additional UPEC-associated pili are poorly understood. S and F1C pili and Dr adhesins are enriched among UPEC, but are less well conserved than type 1 and P pili. They have been demonstrated to impart the capacity to bind to human kidney epithelia in frozen tissue sections ([100], [101]) and may play distinct roles in various stages of UTI ([102]–[104]). Recently, the Yad pilus was shown to contribute to adherence of UPEC to bladder epithelial cells *in vitro*, but was not required for experimental urinary tract infection in mice, whereas the Ygi pilus conferred a modest competitive advantage in kidney colonization ([105]). While pili are likely involved in the initial attachment of UPEC to the urinary tract mucosa, the bacteria elaborate numerous other outer membrane protein adhesins that may play an important role in disease pathogenesis. Recently, a novel adhesin, TosA, which is secreted by a cognate Type 1 secretion system, was described ([106]). TosA is found in about 25% of urinary tract isolates and is expressed during UTI ([106], [107]). However, the role of TosA in UTI is unclear. Although an isogenic UPEC mutant lacking this RTX protein is dramatically impaired in its ability to colonize the urinary tract, the authors of the study were unable to complement the mutant, and vaccination of mice with this protein had no impact on the course of UTI by a TosA-containing UPEC strain ([108]). Another recently identified adhesin, FdeC, is highly conserved among all *E. coli* pathotypes and intestinal commensals and is reportedly expressed only upon contact with host cells ([109]). The presence of FdeC conferred a competitive advantage in colonization of the bladder and kidneys of the mouse model, but vaccination of mice with FdeC antigen only protected against kidney infection, with no effect on bladder infection. Finally, the iron-regulated adhesin Iha has been shown to mediate adherence to bladder epithelial cells and confer a slight, but significant, competitive advantage to UPEC in the mouse model of UTI ([110]). Thus, despite the considerable progress in our understanding of UPEC pathogenesis over the past fifteen years, type 1 and P pili remain the most promising candidate adhesins for drug and vaccine intervention.

Cyclic AMP and UPEC expulsion—After internalization, UPEC have been found to reside within Rab27b/CD63/Caveolin-1-positive fusiform vesicles, which resemble secretory lysosomes and are normally involved in regulating the surface area of the apical plasma membrane. However, UPEC can be expelled by a mechanism that requires Toll-like receptor 4 (TLR4), cyclic AMP, Rab27b, and caveolin-1 ([75], [111–113]). Treatment of mice prior to infection with the drug forskolin, which increases cytosolic cyclic AMP, reduces the intracellular bacterial burden in the bladder. Furthermore, TLR4 signaling-incompetent C3H/HeJ mice have higher intracellular bacterial burdens than TLR4 signaling-competent C3H/HeN mice. Thus, TLR4-dependent antagonism of invasion and active expulsion of internalized bacteria by urothelial cells is an important early innate defense against acute infection of the bladder, and these expulsion pathways are attractive targets for drug prophylaxis.

UPEC escape the endocytic vesicle—Although UPEC may be expelled from host cells after invasion, it is clear that a fraction of invasive bacteria survive within the superficial umbrella cells, eluding expulsion and phagolysosomal death, and escape into the cytoplasm, where several groups have demonstrated that they can replicate rapidly to form intracellular bacterial communities (IBCs) ([25], [26], [114–117]). Although the mechanism of escape into the cytoplasm is not understood, IBC formation does not typically occur in undifferentiated urothelial cells unless they are treated with either membrane- or actin-destabilizing agents ([118], [119]). This suggests that the actin network, which is denser in undifferentiated urothelial cells compared to superficial umbrella cells, may restrict bacterial escape from the vesicle and/or proliferation of UPEC within the cytoplasm. It may also be that the difference in FimH receptors in undifferentiated (α3β1 integrins) and differentiated (UPIa) urothelial cells results in distinct and divergent UPEC invasion pathways and intracellular trafficking. While pore-forming toxins have been shown to be required for bacterial escape into the cytoplasm in other infectious disease model systems, α-hemolysin, a UPEC pore-forming toxin which is expressed in IBCs ([120]), is not required for vesicular escape, as a mutant strain of UTI89 lacking the α-hemolysin gene forms IBCs equally as well as the wild type strain ([121]).

IBC formation—Upon escape into the urothelial cell cytoplasm, UPEC replicate quickly to form IBCs, with a doubling time of 30–35 minutes ([26]). A survey of published studies finds that the number of IBCs detected at 6 hours post-infection (hpi) in the bladders of individual mice ranges from 3 to 700 (median: ~40) after infection of 7–10 week old C3H/HeN mice with 10^7 colony-forming units of the UPEC strain UTI89 ([24], [62], [64], [122–124]). Microscopy studies of mouse bladders after infection with a mixed inoculum of green fluorescent protein-expressing (GFP+) and non-expressing (GFP-) UPEC have demonstrated that IBCs are clonal, originating from a single invasive bacterium ([24]). As a result, IBC formation appears to constitute a population bottleneck that initially limits bacterial diversity, followed by a rapid expansion of the clonal IBC population. Novel anti-infective drugs that prevent IBC formation may thus target a vital molecular bottleneck, which is thought to be the Achilles' heel of a pathogen during infection ([125], [126]). UPEC aggregation into IBCs resembles biofilm formation, as it requires continued type 1 pilus expression after invasion ([62]) and is accompanied by the production of structural components otherwise associated

with UPEC biofilm, such as antigen 43 and a polysaccharide-rich matrix ([25]). Capsular synthesis genes also play a role, as a K1 capsule-deficient mutant of the human cystitis isolate UTI89 is markedly deficient in its ability to aggregate and form IBCs ([127]), and a K2 capsule-deficient mutant of the human pyelonephritis isolate CFT073 is significantly outcompeted by wild type CFT073 in the bladder and kidneys ([128]). Secreted amyloid fibers (curli) and several other UPEC autotransporter proteins, including UpaC, UpaG and UpaH, have been implicated in biofilm growth; however, their role in IBC formation is unknown ([129_133]). The IBC pathway has been observed in all mouse strains tested, and 15 of 18 human clinical UPEC isolates formed IBCs in experimental infections of C3H/HeN mice, including some isolates without common putative UPEC virulence factors such as α-hemolysin ([134]). Those strains unable to form IBCs were also unable to invade the mouse urothelium. UPEC within IBCs are protected from both phagocytosis by PMNs ([26]) and many antibiotics, particularly the first-line drug trimethoprim-sulfamethoxazole, which has increased efficacy against UTI because it concentrates in the urine but is relatively cell-impermeant ([116, 135]). A recent study demonstrated that 16 antibiotics capable of killing the virulent cystitis isolate UTI89 *in vitro* are relatively ineffective in eliminating intracellular bacteria either from bladder epithelial cells *in vitro* or from bladder tissue during *in vivo* infection, even though they achieved urine levels far exceeding the minimum inhibitory concentrations for UTI89 ([116]). Thus, harboring bacteria that are protected from antibiotics within IBCs or a persistent intracellular niche ([116, 118, 136]) may provide a source of surviving pathogens within the bladder that can cause a relapse (treatment failure) or recurrent cystitis, respectively, once antibiotics are removed.

The IBC pathway occurs in humans and with other Gram-negative uropathogens that express type 1 pili—IBC development is not limited to experimental UPEC infection in mice. Translational studies found evidence of IBCs in 18% of urine sediments from women with recurrent cystitis with UPEC (a rate of detection similar to that seen in the urine of mice acutely infected with UPEC), but never in urine from healthy controls or when the causative agent of the UTI was a Gram-positive organism ([27]). Furthermore, other Gram-negative uropathogens that express type 1 pili, such as *Klebsiella pneumonia*, *Enterobacter spp.*, and *Citrobacter freundii*, also utilize the IBC pathway (Rosen and Hultgren, unpublished data) ([124, 137]). Together, these findings suggest that the IBC pathway is an important mechanism for the establishment of UTI in mammalian bladders by Gram-negative uropathogens that express type 1 pili and invade the urothelium. Therefore, the IBC pathway is an important and relevant target for therapeutic intervention.

UPEC dispersal and further IBC formation—Dispersal of UPEC from the IBC is also critical for bacterial persistence. IBC maturation involves a partially understood differentiation program during the first 12–16 hours of experimental infection of the mouse bladder. During this time, the rapidly replicating bacteria first take on a coccoid morphology, become more rod shaped again as the IBC matures, and then begin to flux away from the IBC. UPEC then emerge out of the dying urothelial cells, often in filamentous form, and colonize and invade neighboring cells, thus initiating a second round of IBC formation ([26]). Flagellar motility does not appear to be required for UPEC dispersal or initiation of the second round of IBC formation ([138]). However, deletion of the cell division inhibitor gene,

sulA, disrupts the ability of UTI89 to filament, a property that has been associated with resistance to neutrophil attack. The *sulA* mutant is also defective in bladder colonization and IBC formation at 24 hpi, but not at 6 hpi, suggesting that UPEC filamentation is necessary for virulence after the first round of IBC formation in the immunocompetent host ([139]). IBCs are transient, cycling through formation and dispersal primarily during the first 2–3 days of experimental UPEC infection in the immunocompetent mouse ([26]). However, the immunodeficient mouse strain C3H/HeJ, which lacks the ability to sense bacterial lipopolysaccharide (LPS), had microscopic evidence of bladder IBCs 4 weeks after experimental infection, indicating that host responses to LPS during infection alter the susceptibility of the bladder urothelium to IBC formation ([35]). Thus, the IBC pathway is important for the establishment of acute infection in the host, and resembles biofilm formation in the sense that both aggregation and dispersal of UPEC are critical for acute pathogenesis.

Central metabolism and two-component systems—Recently, central metabolism pathways, such as the tricarboxylic acid cycle, have been shown to be important for acute UPEC virulence and IBC formation in the urinary tract, but not for planktonic growth in urine ([140]–[142]). The QseBC two-component system, which is found in many Gram-negative pathogens including UPEC, plays a critical role in regulating virulence factor expression ([143]–[145]). Two-component systems typically consist of an inner membrane sensor kinase and a cytoplasmic response regulator. In response to a stimulus, the sensor kinase regulates by phosphorylation the activation state of the response regulator, thereby regulating gene expression programs. Disregulation of the QseBC system by deletion of the sensor kinase QseC causes pleiotropic effects in the bacterial cell, including reduced expression of virulence factors (such as type 1 pili) and reduced virulence and IBC formation *in vivo* ([141], [142]). A *qseC* deletion mutant forced to express type 1 pili also had an acute virulence defect when in competition with wild type UPEC, suggesting that the misregulation of additional factors beyond type 1 pili was responsible for the attenuation ([146]). Surprisingly, we found that the altered virulence factor regulation in the Δ*qseC* mutant was due to defects in central metabolism, as two different mutants unable to complete the TCA cycle phenocopied the Δ*qseC* mutant ([141]). Other two-component systems also contribute to UPEC virulence. Cpx is an envelope stress response system known to regulate the expression of P pili ([147], [148]). It was recently shown that deletion of the Cpx system resulted in impaired UPEC colonization of the murine bladder and impaired virulence in zebrafish embryos ([149]). Finally, the PhoP-PhoQ and BarA-UvrY two-component systems have been found to contribute to UPEC virulence as well ([150], [151]). Therefore, compounds that alter UPEC virulence gene expression or central metabolism pathways, either directly or by misregulating two-component systems, are potential novel therapeutics.

Metal ions—Iron acquisition is another critical requirement for bacterial virulence ([152]–[154]). Iron acquisition-associated genes common to all *E. coli* strains are under strong positive selection in UPEC clinical isolates ([46]). UPEC typically have multiple, seemingly redundant iron acquisition systems, and these have been shown to be highly upregulated in the IBC ([120]). As many as four siderophores (small-molecule iron chelators) are commonly

produced by UPEC strains, and scavenging ferric iron (Fe3+) is thought to be their main function. Among the siderophores, enterobactin is broadly conserved among *E. coli* strains, while yersiniabactin, salmochelin, and aerobactin synthesis genes are enriched in UPEC. To prevent microbial iron scavenging, urothelial cells in close proximity to the IBC upregulate genes for the transferrin receptor and for lipocalin 2, host factors that are involved in preventing bacterial acquisition of iron ([120]). However, metabolomic studies have found that UPEC clinical isolates preferentially synthesize yersiniabactin and salmochelin, each of which is associated with resistance to the antibacterial effects of lipocalin 2 ([155_157]). Furthermore, UPEC can scavenge iron from heme, and a deletion mutant lacking the heme transporter ChuA (which is highly expressed in the IBC) forms significantly smaller IBCs *in vivo* ([120]).

The salmochelin receptor IroN may have multiple functions, as it has been shown to enhance bacterial invasion of bladder epithelial cells *in vitro*, and a mutant UPEC strain lacking IroN was attenuated in a mouse model of cystitis ([158], [159]). Yersiniabactin also plays a role in sequestering the toxic effects of copper (II) ions, possibly enhancing resistance to phagocyte killing ([160]). Interestingly, the broadly conserved siderophore enterobactin actually contributes to copper sensitivity, suggesting that the apparent redundancy of siderophores may actually be a bacterial adaptation to inhabiting different host niches. In the pyelonephritis isolate CFT073, which does not synthesize yersiniabactin, aerobactin appears to play an important role in bladder fitness, suggesting that these two siderophores may have overlapping functions ([161], [162]). Thus, bacterial iron acquisition by multiple systems has been selected for in UPEC, possibly in part due to their role in IBC formation. Their redundancy points to their importance, which may make targeting them with vaccines or therapeutics problematic including multiple siderophore receptor antigens in a single vaccine might possibly overcome this challenge. However, all siderophore receptors require the TonB inner membrane protein to transduce the energy needed for import. Deletion of TonB from a UPEC strain greatly reduced virulence in the kidney and, to a lesser extent the bladder during experimental infection of the mouse ([162]). Therefore, targeting TonB with small molecule inhibitors may be an effective anti-infective strategy.

Modeling the outcomes of acute cystitis—Experimental mouse models of infection have revealed that UPEC are capable of chronic colonization of the urinary bladder in several different ways. In immunocompetent mice, the outcome of cystitis is typically either resolution of acute infection with elimination of bacteriuria, or persistent bacteriuria and chronic cystitis ([35]). However, even with resolution of active infection, UPEC are capable of persisting latently within $Lamp1^+$ vesicles inside urothelial cells ([118], [136]). These latent reservoirs have been termed the quiescent intracellular reservoir (QIR) and have the capacity to seed recurrent infections ([136]). Treatment of mice with protamine sulfate has shown some promise in eliminating this bacterial reservoir from the bladder.

In contrast to a latent reservoir, some mouse strains (including the C3H/HeN and CBA/J strains, which are commonly used for mouse models of UTI) are prone to developing high-titer persistent bacteriuria and chronic cystitis, which appears to last for the life of the animal, in response to UPEC infection in an infectious dose-dependent fashion ([35], [163]). Inflammation is most severe during early acute infection in this model and plays a non-

productive role, actually contributing to the development of chronic cystitis. This is potentially a very interesting model, as placebo-controlled studies have found that approximately 50% of women remain bacteriuric several weeks after a symptomatic UTI if not treated with antibiotics, despite overall improvement of symptoms ([164], [165]). Antibiotic therapy readily cures the infection in mice. However, if chronic cystitis is allowed to ensue for at least 7–14 days prior to antibiotic therapy, the mice become highly sensitized to severe, recurrent cystitis upon a second bacterial challenge administered 6 months or more after antibiotic therapy to clear the initial infection (Hannan & Hultgren, unpublished data) ([35]). In contrast, mice that are treated with antibiotics after only 1 day of infection, or that spontaneously resolve bacteriuria during the first two weeks of infection, are more resistant to challenge than naive, age-matched mice. Though the mechanism of sensitization in these mice is unclear, this model may be an invaluable tool not only for understanding host mechanisms of chronic and recurrent UTI, but also for developing therapies and vaccines that combat recurrent UTI.

Ascension to and colonization of the kidneys—The main complication of untreated cystitis in humans is ascension of bacteria in the ureters and colonization of the kidney parenchyma (medulla and cortex), which can lead to marked kidney inflammation with progressive loss of nephron function and even sepsis. Flagella are highly expressed by UPEC *in vivo* during ascension to the murine kidney ([166]). However, the contribution of flagella to kidney colonization is unclear, as different mutants impaired in flagellar motility or chemotaxis were not all defective in kidney colonization in competition infection experiments ([138], [167]). This may be because the mouse strains used in these studies, C3H/HeN and CBA/J, are genetically susceptible to vesicoureteric reflux ([98]), and therefore flagellar motility may not be important for ascension in these models. The UPEC pore-forming toxin α-hemolysin is associated with renal damage and scarring ([168]). However, whether and how α-hemolysin enhances colonization of the urinary tract is unclear. Although it has the capacity to lyse cells, at smaller, physiological doses it induces Ca^{2+} oscillations in renal tubular epithelial cells and thereby potentially enhances ascension and colonization of the ureters and kidney parenchyma by disrupting the normal flow of urine ([169]). Other UPEC toxins, such as Sat, PicU, and Tsh, are also not required for infection, but may contribute to renal pathology ([170], [171]). Ygi pili, type II and IV secretion systems, and multiple iron and heme acquisition components have all been shown to contribute to kidney colonization in animal models ([105], [162], [172], [173]). Recently, a Toll/interleukin-1 receptor (TIR) domain-containing protein that is secreted by an unknown mechanism, TcpC, was discovered in a subset of UPEC ([174]). Loss of function studies in mice indicate that TcpC enhances bacterial virulence by suppressing the early innate immune response to UPEC infection, resulting in higher bacterial burdens in the kidney and more severe kidney pathology over time ([175]).

Complicated UTI: *Enterococcus faecalis*

CAUTI—In contrast to the healthy bladder environment affected by UPEC in uncomplicated UTI, the placement of a urinary catheter effects pathologic changes in the bladder that may contribute to the greater variety of competent uropathogens that infect the catheterized bladder. In addition to causing mechanical damage, urinary catheterization interferes with

micturition (urination), a natural impediment to bacterial colonization of the bladder. Even in the absence of infection, bladder catheterization may lead to tissue edema and hyperplasia of the urothelium, in addition to hematuria and alteration of urine composition ([176], [177]). Probably the most significant factor contributing to infection development is the presence of an abiotic surface in the bladder, the catheter, which promotes bacterial biofilm formation, recognized as an important component of CAUTI. UPEC and enterococci are the two most common isolates from symptomatic CAUTI ([10]). Enterococci, commensal gut bacteria, have emerged as important human pathogens in the last 40 years, especially in the health care environment. Several aspects of modern medicine have contributed to this recent rise in infections caused by *E. faecium* and *E. faecalis*, the most commonly isolated species. Widespread use of antibiotics has likely selected for enterococci which display intrinsic or acquired resistance to many common classes of antimicrobials. Furthermore, medical devices (such as indwelling urinary catheters) and invasive surgical procedures compromise natural barriers to infection and are being used more frequently in the health care setting. Despite the increasing incidence of enterococcal infection, little is known about the molecular mechanisms these bacteria use to cause disease. However, it is clear that biofilm formation and the elaboration of secreted and surface proteins and organelles contribute significantly to enterococcal pathogenesis.

The role of secreted and surface structures in enterococcal biofilm formation—Biofilm formation is a critical aspect of device-related infections, including enterococcal CAUTI. As extracellular pathogens, enterococci may also rely on growth in the biofilm state to infect host tissues. Thus, putative molecular determinants of enterococcal virulence, identified using a variety of methodologies, are typically examined *in vitro* in assays of biofilm formation. These determinants must be secreted and, in many cases, covalently linked to the cell wall. This latter function is carried out by a group of membrane proteins known as sortases, which recognize conserved cell wall sorting sequences (CWSS) on membrane-linked proteins and catalyze the attachment of their extracellular domains to the cell wall. Bacterial proteins well-studied in biofilm assays include enterococcal surface protein (Esp), of unknown function, and gelatinase, a secreted zinc metalloprotease that hydrolyzes gelatin, collagen and casein ([178]). Extracellular DNA, autolysin, the housekeeping sortase SrtA, the endocarditis and biofilm-associated (Ebp) pilus, and the Ebp pilus-associated sortase SrtC have also been shown to play a role in biofilm formation ([179], [180]).

The role of adherence and biofilm formation in CAUTI—Many of the same virulence factors involved in biofilm formation, including Ace, Esp, SrtA, Ebp pili, and SrtC, have been shown to play a role in a ureteric reflux model of ascending pyelonephritis ([180_183]). However, the contribution of these factors was not large, and robust bladder infection could not be achieved in rodent models of uncomplicated UTI ([184]). More recently, a more relevant model of foreign body cystitis has been developed in mice for testing the role of *E. faecalis* virulence factors in CAUTI ([185]). In this model, the presence of urinary catheter material in the urinary bladder allows for biofilm formation on the implant and robust, high-titer bladder infection that persists as long as the catheter remains. Transient immunosuppression of mice concurrent with catheter implantation exacerbates *E.*

faecalis infection in C57BL/6J mice, suggesting that the presence of the catheter and not the inflammatory response is driving CAUTI ([186]). Ebp pili, and specifically the metal ion-dependent adhesion site (MIDAS) motif found within the predicted von Willebrand factor A domain of the putative tip adhesin protein EbpA, are essential for both bladder and implant colonization in this model ([187]). However, the *in vitro* biofilm determinants autolysin and gelatinase were not required ([185]). Thus, enterococci utilize Ebp pili to take advantage of the presence of foreign abiotic surfaces and damaged bladder mucosal barriers to cause CAUTI.

Sortase-mediated pilus assembly—Ebp pilus assembly is directed by the action of two different sortases (Figure 2). These membrane-linked transpeptidase enzymes catalyze the covalent assembly of pilus subunits into a functional pilus that is covalently attached to the peptidoglycan cell wall. Sortases are nearly ubiquitous among Gram-positive organisms and have duplicated and diversified among and within species to perform specific functions ([188]). Therefore, the development of small molecules to inhibit these enzymes has the potential to yield a wide array of therapeutics that range from broadly anti-Gram positive to specifically targeting virulence processes of single species. A sortase inhibitor targeting Ebp pilus assembly could potentially be beneficial for preventing health care-associated infections, including CAUTI. The Ebp pilins also make attractive vaccine candidates for several reasons, particularly for those individuals in a long-term health care setting (e.g. a nursing home), which strongly increases the risk of acquiring CAUTI. The *ebp* locus is present in ~95% of *E. faecalis* isolates regardless of source and is highly conserved ([189]). These proteins are expressed and function on the bacterial cell surface and are important virulence factors. Indeed, immunization with pilins from Group B streptococci has been shown to be protective in relevant infection models ([190], [191]). However, the potential protective effects of immunization with *E. faecalis* Ebp pilus components in experimental disease models have not been tested.

Translating Discoveries in Pathogenesis: the Development of Anti-Virulence Therapies

Biarylmannose-Derivative FimH Antagonists (Mannosides)

The mannose binding pocket of FimH is invariant in all strains of uropathogenic *E. coli* ([48]), and mutations in these residues disrupt mannose binding and attenuate virulence ([48], [64], [66]). With information gained from the crystal structures of FimH bound to α-D-mannose and mannose derivatives called mannosides ([48], [192–194]), we and others have rationally designed biarylmannose-derivative FimH binding inhibitors ([193], [195], [196]). Using a reiterative process of structure-based design, combinatorial chemistry, and *in vitro* cell-based screening, lead compounds with excellent cellular potency, low molecular weight, and optimized oral pharmacokinetics were identified. Experimental and pre-clinical translational studies have demonstrated that these optimized mannoside compounds can be given orally to mice either to prevent cystitis or to successfully treat an established bladder infection ([135], [195]).

Since the mannose binding pocket of FimH is invariant, mannosides have potent efficacy in preventing acute UTI caused by divergent strains, including the trimethoprim-sulfamethoxazole (TMP-SMZ)-resistant UPEC strain PBC-1 ([135]) and the multi-drug resistant UPEC strain ST131([197]). Mannoside treatment prior to infection of C3H/HeN mice prevents UPEC invasion of the urothelium and IBC formation, a process that protects UPEC from the effects of many antibiotics ([116]). Thus, in mannoside-treated mice, UPEC are confined to the bladder extracellular niche, where they are left exposed to high levels of antibiotics that are commonly used to treat UTI. As a result, although TMP-SMZ alone had no effect on bladder colonization by the resistant strain PBC-1, mannoside-potentiated killing by TMP-SMZ (which concentrates in the urine to levels well above the minimum inhibitory concentration of PBC-1) to successfully prevent the establishment of UTI by this strain. In a similar way, mannoside also potentiated killing by TMP-SMZ to prevent CAUTI in a foreign body model of experimental infection ([198]). Finally, a recent study found that mannoside is also efficacious against the multi drug-resistant UPEC clone ST131 in acute and chronic experimental infection in C3H/HeN mice. The clinical isolate used in this study was EC958, which is an extended spectrum β-lactamse strain that is resistant to eight classes of antibiotics, including fluoroquinolones. One prophylactic mannoside dose significantly decreased acute bacterial burdens in the bladder and treatment of chronically infected mice with a single dose of oral mannoside reduced bladder bacterial burdens greater than 1000-fold ([197]). If translated to clinical practice, mannosides have tremendous and exciting potential to be an efficacious, safe, and cost-effective new therapy either used in combination with commonly used first-line antibiotics to successfully treat existing uncomplicated cystitis and CAUTI, or used alone as a daily prophylaxis against chronically recurrent cystitis. By reducing the use of antibiotics, and particularly the use of fluoroquinolone antibiotics, in the treatment and prevention of UTI, mannosides could have an immediate and long-term impact on the development of antibiotic resistance in UPEC clinical isolates, which is currently as high as 30% in some studies ([17]). Furthermore, the unique mechanism of mannoside action, i.e. inhibiting the function of the extracellular FimH pilus tip adhesin by blocking the invariant lectin pocket, likely circumvents the development of resistance due to mutation of the binding pocket, porin mutations or efflux.

Galabiose PapG Antagonists

In 1982, the efficacy of glycolipids in preventing P pilus binding and *in vivo* infection was established ([199]). Determination of the crystal structure of $PapG_{II}$ bound to its receptor, GbO4, elucidated critical details of the adhesin-receptor interaction and allowed for further rational design of galabiose-derived receptor analogs ([82], [200], [201]). Further studies have identified high-affinity multivalent inhibitors that also inhibit galabiose binding by *Streptococcus suis* ([202], [203]). To the best of our knowledge, these inhibitors have not been tested *in vivo*, but a therapy that combines bioavailable PapG antagonists with mannosides has tremendous potential to treat and prevent UTI.

Inhibitors of the chaperone-usher pathway: pilicides and curlicides

Since Gram-negative pili are assembled by the chaperone-usher pathway (CUP), inhibitors of this pathway could be broadly effective against a number of pathogens that require pili for pathogenesis. In collaboration with Fredrik Almqvist, a medicinal chemist, we developed

ring-fused 2-pyridone small molecule inhibitors that target the CUP periplasmic chaperones ([204], [205]). We have called these compounds "pilicides." By screening for inhibitors that prevented type 1 pilus-mediated hemagglutination and *in vitro* biofilm formation, we identified pilicides with activity not only against type 1 pili, but also against P pili ([206]). NMR and crystallographic studies of the interaction of a pilicide with the P pilus cognate periplasmic chaperone, PapD, found that pilicide compounds interacted with a highly conserved region ([207]) that interacts with the N-terminal domain of the outer membrane usher ([206]) (Figure 2). Curli are amyloid fibers produced by many Enterobacteriaceae that are assembled at the outer membrane by a nucleation pathway of fibrillization ([208]). By screening for inhibitors of curli-mediated biofilm, we identified 2-pyridone derivatives capable of inhibiting both curli and pili formation. One such "curlicide" that inhibits both type 1 pilus production and curli biogenesis rendered UPEC relatively avirulent in a mouse model of experimental cystitis ([129]). Further optimization of these compounds has increased their potency dramatically ([209–211]), and these lead compounds are promising candidates for future drug development.

Intravesical Therapy with ASB Strain 83972

Another strategy that is currently under investigation is the use of an avirulent asymptomatic bacteriuria (ASB) strain, 83972, which has adapted for long-term colonization of the human urinary tract without causing significant symptoms or pathology, as a therapy for recurrent UTI ([212]). Although ASB strain 83972 is discussed in more detail in chapter 6, we will briefly discuss its therapeutic potential here. Strain 83972 has lost the capacity to express type 1, P, and F1c pili, and can outcompete UPEC strains in human urine whether growing planktonically or in a biofilm ([213], [214]). Therefore, it is hypothesized that colonization of the bladder by ASB strain 83972 prevents virulent UPEC strains from colonizing the urinary tract, thereby preventing recurrent symptomatic UTI. This therapy is currently undergoing clinical trials and has shown promise in "at risk" populations, such as those with incomplete bladder emptying or neurogenic bladder from a spinal cord injury ([215–217]). A variation of this approach was recently employed by Schembri and colleagues. The $PapG_{II}$ receptor, GbO4, is also the receptor for Shiga toxin. Adapting existing technology, Schembri and colleagues engineered a strain of 83972 to synthesize a galabiose analog that is linked to LPS on the surface of the cell ([218], [219]). This strain is able to inhibit binding of $PapG_{II}$-expressing UPEC to kidney epithelial cells, and when co-inoculated into the mouse urinary tract with virulent UPEC, the galabiose-expressing strain significantly reduces the UPEC bacterial load in the urine compared to the wild type 83972 strain. Conversely, others have transformed strain 83972 with a plasmid expressing type 1 pili and have demonstrated that this new strain forms better biofilm on urinary catheters and as a result is more efficacious in preventing their colonization by enterococci ([220]).

Nutraceuticals

So-called "nutraceuticals" are foods or food products that are thought to provide medical benefits and are often sold in a medicinal form. Since use of these products typically does not require regulatory authority approval (e.g. approval by the Food and Drug Administration in the United States), the efficacy of these compounds is often based merely on anecdotal reports. Two common nutraceuticals that have been investigated for preventing

recurrent UTI are probiotic lactobacillus preparations and cranberry products. These products may be advantageous because they are generally safe and readily available. However, there exists only limited evidence for their effectiveness.

Probiotics—Clinical evidence suggests that the vagina and periurethral area, which is normally colonized by *Lactobacillus spp.*, can act as a UPEC reservoir that could potentially seed recurrent infections. Women with recurrent UTI are more likely to have vaginal or periurethral UPEC colonization than women without recurrent UTI ([221]), and periurethral UPEC carriage dramatically increases in the days prior to a recurrent episode ([222]). Vaginal *Lactobacillus* suppositories might help clear this UPEC reservoir, preventing recurrences. However, probiotic therapy with *Lactobacillus spp.* has had mixed results. A randomized, controlled, double-blind clinical trial studied the effects of *L. casei rhamnosus* vaginal suppositories vs. placebo in women with recurrent UTI, and found no difference in infection rates between the groups ([223]). However, another randomized, controlled trial found that vaginal *Lactobacillus* suppositories given after antibiotic therapy for UTI reduced reoccurrence compared to sterilized skim milk suppositories ([224]). Clinical trials for *Lactobacillus* prophylaxis for infectious diseases of the urogenital tract, including UTI, are ongoing both in the United States and abroad. A Phase I trial to test the safety of an *L. crispatus* vaginal suppository (LACTIN-V, Osel, Mountain View, California) found that no severe adverse events occurred, although seven women (out of 15) developed asymptomatic pyuria ([225]). In the randomized, placebo-controlled Phase II trial, women with a history of recurrent UTI received antibiotic therapy for acute UTI, and then either LACTIN-V or placebo. The probiotic was protective, with recurrent UTI occurring in 15% of women receiving LACTIN-V and 27% of women receiving placebo ([226]). The mechanism of *Lactobacillus*-mediated protection from UTI is not clear. Some *Lactobacillus* strains produce surfactants and anti-adhesive molecules ([224]). *Lactobacillus acidophilus* surfactant was shown to inhibit initial deposition rates and adhesion numbers for several uropathogens, including *E. coli*, *E. faecalis*, and *Proteus mirabilis* ([227]). This raises the possibility that some *Lactobacillus* strains might be more effective than others at preventing UTI.

Cranberry products—Cranberry products are a common folk-remedy for preventing recurrent UTI, but the efficacy of cranberry products in UTI prophylaxis is largely unproven. Cranberries contain two compounds that have been shown to inhibit UPEC adherence to eukaryotic cells *in vitro*: fructose (found in all fruits), which weakly blocks type 1 pilus-mediated binding, and A-type proanthocyanidins, which have been shown to block P pilus-mediated binding ([228_231]). UPEC that was grown in human urine collected after consumption of cranberry juice had significantly reduced adherence to human red blood cells, resin beads coated with P-receptor oligosaccharides, and urothelial bladder cells compared to UPEC grown in normal urine ([232, 233]). Cranberry products are also less expensive and better tolerated than antibiotics, and thus are an intriguing candidate for UTI prophylaxis. However, the literature regarding the clinical efficacy of cranberry prophylactic therapy remains inconclusive. For example, a recent meta-analysis of 10 randomized clinical trials found some benefit for women with recurrent UTI, but studies of protection in elderly or catheterized patients are lacking ([234]). A 2009 randomized controlled trial found that cranberry extract had similar efficacy to low-dose trimethoprim for preventing recurrent UTI

in older women ([235]), while two randomized controlled clinical trials showed no significant effect of cranberry on UTI recurrence in adult pre-menopausal women ([236, 237]). Comparisons of these different studies may be confounded by differences in type of cranberry product consumed (e.g. juice vs. extract) and dosage regimens. Therefore, more studies are necessary to determine the effectiveness of cranberry products for preventing recurrent UTI.

Estrogen Therapy

Another therapeutic approach is the intravaginal application of estrogen. In a controlled trial of post-menopausal women with rUTI, intravaginal application of a topical estriol cream significantly reduced the incidence of recurrence ([238]). The efficacy was attributed to a restoration of low vaginal pH and vaginal *Lactobacillus* colonization. In contrast, several studies have indicated that systemic estrogen replacement therapy is not protective against rUTI ([239]). A recent study found that vaginal estradiol therapy in post-menopausal women altered the expression of antimicrobial peptides and cell junction proteins in epithelial cells isolated from voided urine, suggesting that estrogen therapy modulates the mucosal barrier of the lower urinary tract ([240]). In support of these translational findings, several studies of experimental UTI in ovariectomized mice have demonstrated that altering estrogen levels has profound effects on UTI pathogenesis ([240–242]). Thus, vaginal estrogen therapy remains a safe and viable therapeutic option for post-menopausal women suffering from rUTI.

Possible Applications to CAUTI

In contrast to uncomplicated UTI, CAUTI is dependent upon the presence of a foreign body, and removal of the catheter is often curative. Although it is not clear whether the biofilm forms first on the catheter, which in turn allows colonization of the damaged bladder mucosa, or vice versa, biofilm formation is clearly strongly associated with CAUTI, and the most common CAUTI pathogens, UPEC and *E. faecalis*, are good at making biofilm. The use of antibiotic- or silver-impregnated catheters has shown some efficacy in reducing the occurrence of bacteriuria in catheterized patients, but it is unclear whether they lower the rate of symptomatic CAUTI and associated complications ([13]). For those individuals who require long-term catheter placement, and particularly those with spinal cord injuries or in nursing homes, the additional use of anti-infective drugs such as mannosides and sortase inhibitors in combination may help to prevent a large percentage of CAUTI, as well as infections of other implants. Furthermore, vaccines that target UPEC, *E. faecalis* and *Proteus spp*. may also benefit these patients, reducing the incidence of CAUTI and potentially also the risk for bloodstream infections.

UTI Vaccines

Historical Perspective

Vaccines have been used against UTI for more than a century, though initially their intended purpose was therapeutic rather than prophylactic. In 1909, two case reports of pregnant women with pyelonephritis described significant clinical improvement after therapeutic systemic vaccination with *E. coli* (previously known as *Bacillus coli*) isolated from the

urinary tract of the same patient ([243], [244]). Despite these anecdotal reports, by the 1920s therapeutic vaccination against UTI was largely seen as ineffective. A survey of a thousand American physicians reported little to no use of vaccine therapy for cystitis and pyelonephritis ([245]), and it was said that "the day of extravagant expectations from vaccine therapy [for UTI] is for the moment past ([246])." As our understanding of vaccines advanced, UTI vaccines as a prophylactic therapy (i.e. "clinical" vaccines) reemerged as a topic of interest by the 1950s ([16], [247]), and have been the focus of much research, refinement, and testing in animals for the past 50 years. Efficacy in animals has been shown for UTI vaccines in every classical category: attenuated, inactivated, subunit, toxoid, and conjugate (Table 2). However, few modern vaccines have been tested in humans, and only one is currently commercially available.

Challenges in Developing UTI Vaccines

Clinical and technical challenges associated with developing a clinical UTI vaccine include our lack of understanding of the mechanisms that induce protective immunity in the urinary tract; the diverse patient subpopulations that would benefit from a vaccine; the heterogeneity of UPEC strains, which complicates the choice of the best target antigens; the route of administration (mucosal v. systemic); and the choice of adjuvant, if needed. Furthermore, experimental conditions tightly control for genetics (often using inbred animals) and environment, whereas the human population is outbred and regularly encounters a broad diversity of environmental variables that could affect the mucosal immune system. When testing UTI vaccines in humans, care must be taken to assess potential confounding variables. It is known that many common vaccines (e.g. influenza, pneumococcal, and zoster vaccines) do not induce optimal immune responses in a large portion of infants and the elderly ([248]). In addition, hysterectomy may decrease the effectiveness of vaginal mucosal vaccines, as the cervix has been shown to be "the major inductive and effector site for cell-mediated immunity in the lower female genital tract ([249])."

Recurrent UTI and protective immunity—The high frequency of recurrent UTI indicates that many patients are unable to mount an effective adaptive immune response that prevents re infection. The reasons for this are unknown. One possibility is that uropathogens may mask themselves from and/or directly suppress the immune system. For example, the attenuated UTI vaccine NU14 Δ*waaL* stimulates the immune system much more than wild type NU14, which requires waaL O antigen ligase for host immune suppression ([250]). Alternatively, the body may downregulate its own immune response to uropathogens in order to maintain the integrity of the mucosal barrier ([35], [251]). Animal models of recurrent UTI have begun to shed light on the mechanisms of adaptive immunity in response to urinary tract infection. C57BL6/J mice that are repeatedly infected with UPEC become more resistant to experimental UTI ([37]). However, in C3H/HeN mice, which are genetically susceptible to chronic cystitis in an infectious dose- dependent manner, the outcome of the first infection in naive mice determines susceptibility to subsequent UTI ([35]). Mice that spontaneously resolve the first episode of cystitis are resistant to bacterial challenge, but those that develop chronic infection upon the first infection are highly susceptible to severe, recurrent infection after antibiotic therapy, even when challenged by less virulent strains that do not cause severe infection in naive mice (O'Brien, Hannan & Hultgren, unpublished data)

([35]). Understanding this puzzle will be critical in order to rationally design UTI vaccines with maximal therapeutic efficacy.

Who should receive a UTI vaccine?—Cystitis accounts for 90% of all UTI and recurs at high frequency, with 20–30% of women experiencing a recurrence within 3–4 months ([4]). These women are excellent candidates to receive a cystitis vaccine to lower the rate and severity of subsequent recurrences. The target population for a pyelonephritis vaccine is more limited. Children with vesicoureteral reflux (VUR), diabetics, and newly pregnant women or women of child-bearing age (pre-natal) might benefit from a pyelonephritis vaccine. Both children with VUR and diabetics are at higher risk for developing pyelonephritis ([252], [253]) and therefore might benefit from vaccination. Importantly, the use of antibiotic prophylaxis to reduce the frequency and severity of VUR-associated UTI in children is controversial among clinicians, and a systematic review of twenty randomized, controlled trials found no clear benefit for antibiotic prophylaxis ([254], [255]). Thus, new treatment strategies, such as vaccination, are needed for this patient population. Bacteriuria that progresses to pyelonephritis during pregnancy is associated with poor outcomes, including perinatal death, low birth weight, prematurity, and preterm low birth weight ([256]). However, aggressive treatment of asymptomatic bacteriuria in pregnancy ensures that only a small percentage of pregnant women progress to pyelonephritis ([257]). Pregnant women with asymptomatic bacteriuria could potentially benefit from a pyelonephritis vaccine, if it were extremely effective and safe for both the mother and fetus. Systemic vaccination that induces IgG antibodies could also have the potential benefit of conferring passive immunity to the developing fetus, which could protect the newborn during their first year or so of life. Indeed, a systemic P pilus subunit vaccine administered to pregnant rhesus monkeys protected the newborns from pyelonephritis and induced a significant antigen-specific IgG response in the sera of both the mothers and newborns ([258]).

Choice of immunogen(s)—Effective UTI vaccines should target one or more surface-exposed bacterial structures that are either uniformly expressed by the uropathogen in the host or are expressed during critical stages of infection (Figure 1). UTI vaccines in development can generally be categorized into two broad categories: "whole agent" or "whole cell" vaccines, which include whole bacteria (either live attenuated or inactivated) and bacterial lysates, and "specific-antigen" vaccines, which include one or more antigens (subunit, toxoid, or conjugate vaccines). Although the majority of vaccines currently licensed in the United States are whole agent vaccines, most of these target viral pathogens. Among the eight bacterial pathogens for which there are licensed vaccines (compared to 16 vaccines targeting viruses), only three are targeted with whole cell vaccines: anthrax, tuberculosis, and typhoid fever (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm093830.htm). This is because specific-antigen vaccines are generally much safer than whole bacterial cell vaccines, particularly when vaccinating systemically with Gram-negative bacteria, which can lead to endotoxemia. However, in the absence of whole organisms, isolated antigens typically do not elicit robust or long-lasting immune responses, and thus must be administered with adjuvants to increase the inflammatory response to the antigen, thereby enhancing immunogenicity ([259]).

Specific-antigen vaccines can only be as good as the antigen(s) selected. The process of selecting the best antigen(s) represents a critical and formidable challenge early in vaccine development. An ideal vaccine target antigen would be highly and broadly expressed and would be required either for the initiation and/or maintenance of infection or for disease symptoms. For example, toxoid (inactivated toxin) vaccines are highly successful against diseases in which the toxin itself is the main cause of disease, such as tetanus and diphtheria. The development of subunit (protein antigen), conjugated (carbohydrate antigen conjugated to antigenic protein), and DNA (protein expressed from a DNA vector inside the host) vaccines has been the focus of much research, but has been met with limited success. The challenge is that bacterial infections are much more complex than viral infections, involving multiple host niches and antigenic variation. Additionally, bacteria have evolved to have redundant virulence mechanisms, a fact highlighted by the multiple adhesins and iron acquisition systems in many UPEC isolates ([45–47]). Furthermore, mechanistic studies of bacterial pathogenesis must be carried out in human cell lines or in animal models, and may not accurately reflect the requirement for vaccine targets to initiate infection and cause human disease. Finally, with the exception of the iron acquisition factors, the majority of putative UPEC virulence factors that have been described are found in about 50% or fewer of all isolates (Table 1), and thus would only be useful in a multi-subunit vaccine.

Once a candidate antigen is chosen, it must be tested in animal infection models, but the results of such efficacy studies may be difficult to parse. Several potential confounders that can vary among studies must be assessed. Among vaccines comprised of whole bacteria, outer membrane vesicle, and membrane protein extract preparations, the method of bacterial preparation can have profound effects on the efficacy of the vaccine, as the antigens present will vary with the culture conditions. For example, UPEC grown statically at 37°C in LB liquid media will predominantly express type 1 pili, whereas UPEC culture on tryptic soy agar plates at 37°C will express P pili. This bias can be overcome with live attenuated vaccines, which can replicate, mimic the natural route of infection and change their gene expression accordingly once introduced into the host. Other variables in testing UTI vaccine efficacy in animal models include the animal infection model and choice of uropathogen used for bacterial challenge, the culture conditions of the challenge bacterial inoculum, and, as we discuss below, the vaccine inoculation regimen, including the route and frequency of immunization and choice of adjuvant.

Route of administration—Both mucosal (vaginal and intranasal) and systemic UTI vaccines have been effective in animal models. In general, mucosal vaccines elicit both IgA and IgG responses and systemic vaccines elicit only IgG responses. Since IgA is thought to be protective against intimate and invasive infection of the gut, and is found in high concentrations at mucosal sites, it has been traditionally assumed that IgA is the most effective means of inducing mucosal immunity. However, one group compared systemic and mucosal routes of vaccination with the FimH adhesin and found that although only the mucosal route induced elevated levels of vaginal wash and urine antigen-specific IgA in mice, both vaccine delivery routes resulted in similar serum and urine antigen-specific IgG responses and protection against experimental UTI ([260]). It is possible that this finding may be explained by the experimental model, in which UPEC were instilled directly into the

bladder, thus avoiding the initial stages of periurethral colonization and urethral ascension, where IgA may be more important for protection. However, systemic vaccination and serum IgG responses have provided protection against other mucosal pathogens, such as rotavirus and human papillomavirus ([261], [262]). Therefore, antigen-specific IgG may be equally or more important than IgA for host defense at some mucosal surfaces. Also, data from experimental UPEC infection in mice suggest that the urogenital mucosa may become "sensitized" to uropathogens subsequent to an initial chronic bladder infection, such as commonly occurs in infants with UTI ([35]). In these individuals, a vaginal mucosal route of vaccine delivery may exacerbate this sensitization.

Vaccine adjuvants—The choice of adjuvant can be critical for adequate stimulation of the immune system, but relatively little is known about how adjuvants work ([263]), and only a few are approved for use in humans. In order to be approved for use in clinical vaccines, adjuvants must have low toxicity. Adjuvants currently approved for use include aluminum salts (e.g. alum), the squalene-based MF59, the LPS-derived monophosphoryl lipid A (MPL), and liposomes. The aluminum-based adjuvants aluminum phosphate and aluminum hydroxide are commonly used in systemic vaccines in humans. The specific functions of aluminum adjuvants continue to be debated, but in general, it is accepted that they form a depot at the injection site, allowing for efficient uptake of antigen by antigen-presenting cells (APCs). They also stimulate the immune system by inducing eosinophilia and activating complement and macrophages ([264]). MF59 is a squalene-based oil-in-water emulsion. After intramuscular injection, the squalene emulsion droplets are internalized by dendritic cells and enhance antigen presentation ([265]). Gene expression analysis of mouse muscle found that, compared to alum and the TLR9 agonist CpG, MF59 induces more changes in gene expression and is a stronger inducer of genes involved with cytokine responses, leukocyte migration, and antigen presentation ([266]). As a result, MF59 elicited a more rapid influx of myeloid cells to the site of injection. Monophosphoryl lipid A has been modified to reduce its toxicity, while retaining its ability to induce inflammation. It is an agonist for TLR4, although it is unclear whether this agonism is the main cause of its efficacy as an adjuvant. Due to its hydrophobicity, it has a strong propensity to aggregate into microparticulates that are potent activators of the NLRP3 inflammasome ([267]). Liposomes are thought to enhance immunogenicity, both by enhancing phagocytosis by APCs and by enabling direct cytoplasmic delivery of antigens by membrane fusion ([268]). Recently, combinations of the above adjuvants have been the subject of much research. In particular, MPL in liposomes has showed great promise, as liposomes have the dual benefit of masking the residual toxicity of MPL while enhancing its potency as an adjuvant ([268]).

Mast cell-derived adjuvants—Mast cells are important players in the bladder innate immune response. Not only have they been implicated in early defense against UTI in mice, but mast cell-derived factors play an important role in directing the adaptive immune response ([269_271]). Mast cell-derived adjuvants are an interesting recent development in vaccinology. Nasal instillation of vaccine antigens along with small-molecule mast cell activators resulted in antigen-specific serum IgG and mucosal (nasal, vaginal, fecal) IgA responses that correlated with increased dendritic cell and lymphocyte recruitment to the lymph nodes ([270]). Recently, Abraham and colleagues described the synthesis of

submicrometer particles that model mast cell granules and showed their successful use as an adjuvant in a mouse model of influenza ([272]). An advantage of these particles is that they can be engineered to contain particular cytokines in order to skew the adaptive immune response. To the best of our knowledge, mast cell-related adjuvants have not yet been tested in UTI vaccines. However, they are an intriguing candidate for further study.

Innate Immunity and the Rise of Systems Vaccinology

In the past, vaccine development was most often a hit-or-miss venture, with little understanding of why some vaccines are efficacious and others are not. To a certain extent this is still true today, but in recent years, vaccinologists and immunologists have begun to understand the role of the innate immune system in vaccine efficacy. The innate immune system relies on pattern recognition receptors (PRRs) expressed by innate immune cells in order to detect pathogen-associated molecular patterns (PAMPs). An important category of PRRs are the Toll- like receptors (TLRs), which can detect molecular patterns commonly found in bacteria, viruses, fungi, and parasites; C-type lectins and nucleotide oligomerization domain (NOD)-like receptors are also important innate sensing receptors. Signaling by PRRs on innate immune cells can trigger an adaptive immune response that differs based on the PRR and the dendritic cell subtype ([273]). Recently, systems biology approaches have been used to assess the effects of vaccination on the immune system, with a particular focus on the early innate response, which can predict vaccine immunogenicity ([248]). This "systems vaccinology" approach was used by Pulendran and colleagues to investigate changes in the human immune system after vaccination with a live attenuated Yellow Fever vaccine. By performing multiplex cytokine assays and microarrays with blood collected at baseline and at different time points post-vaccination, the authors were able to characterize a "molecular signature" involving the innate sensing of viruses that very accurately predicted the development of antiviral immunity ([274]). Systems vaccinology approaches may be useful for assessing the immunological profiles of UTI vaccines, predicting efficacy in vaccinated individuals, and determining the best adjuvant for a given vaccine. It is interesting to note that the mechanism of protection of the only commercially available UTI vaccine, StroVac, is currently unknown. Systems vaccinology may be the key to elucidating the efficacy of this and other UTI vaccines.

Whole Cell Vaccines in Development

Whole cell vaccines have been among the most successful vaccines developed to date. Indeed, the only UTI vaccine currently available for use in humans is the polyvalent inactivated whole cell vaccine StroVac. Vaccines comprised of whole uropathogens, whether attenuated or inactivated, expose the host to a variety of virulence factors. These preparations may or may not include, depending upon how the preparation is grown and processed, pili and other adhesins, outer membrane proteins, toxins such as hemolysin and CNF1, siderophore receptors, and LPS (Figure 1). Of all vaccine types, live attenuated vaccines have the potential to most closely mimic natural infections, and thus elicit strong immune responses. However, they cannot be given to immunocompromised patients, and there may be a risk of reversion to virulence in healthy individuals. Inactivated vaccines are generally safer than live ones, but this can be accompanied by the tradeoff of eliciting a weaker immune response than live attenuated vaccines.

Inactivated Vaccines—Inactivated *E. coli* vaccines have been investigated since at least the 1950s and have been found to protect animals from UTI. An early UTI vaccine consisted of intravenously-injected, heat-killed *E. coli*, and was protective against pyelonephritis in rabbits ([16]). In the 1970s, systemic vaccination with heat-killed or formalin-killed *E. coli* strains grown in trypticase soy broth (TSB, which induces P pili expression) protected rats from retrograde *E. coli* pyelonephritis ([275]) and ascending UTI ([276]), but did not protect rabbits against hematogenous pyelonephritis ([277]). Rats that were vaccinated by intravesical instillation of formalin-killed *E. coli* were protected against ascending UTI and resolved UTI faster than non-vaccinated controls ([276], [278]). To the best of our knowledge, vaginal mucosal immunization with an inactivated UTI vaccine was first published in 1982, when vaginal instillation with formalin-killed *E. coli* protected rats from cystitis ([279]). In a later study, vaginally instilled, but not systemically injected, formalin-killed *E. coli* inhibited bacterial adhesion to rat bladder mucosae ([280]). In 1987, vaginal immunization with formalin-killed *E. coli* protected cynomolgus monkeys from cystitis ([281]), and in 1995, intramuscular injection of formalin-killed *E. coli* protected monkeys from pyelonephritis-associated renal scarring ([282]).

SolcoUrovac® and StroVac®: With several decades of research showing the efficacy of inactivated UTI vaccines in animals, Solco Basel Co. (now Legacy Pharmaceuticals, Birsfelden, Switzerland) developed SolcoUrovac for use in humans. SolcoUrovac is a polyvalent whole cell vaccine consisting of 10 strains of heat-killed uropathogens: six from *E. coli* of different serotypes and one each from *Klebsiella pneumoniae*, *Proteus mirabilis*, *Morganella morganii*, and *Enterococcus faecalis*. It was initially administered by three intramuscular (intragluteal) injections at weekly intervals. The first results of clinical trials with SolcoUrovac, which were performed in Europe, showed that the vaccine was protective against recurrent UTI ([283]). Current information about SolcoUrovac is not available as Solco Basel appears to be defunct and SolcoUrovac is unavailable in Europe at this time. However, another intramuscular polyvalent inactivated UTI vaccine, called StroVac (Strathmann AG, Hamburg, Germany), is currently approved for use in Europe. StroVac contains the same 10 strains in a different formulation [http://www.strathmann.de/index.php/en/component/content/article/112-pflichtangaben/367-strovac-pflichttext-, reference in German]. While these vaccines have shown promise, they have never undergone large phase III studies to demonstrate efficacy. Therefore the European Association of Urology's "Guidelines on Urological Infections" make no recommendation on the use of SolcoUrovac or StroVac (http://www.uroweb.org/gls/pdf/17_Urological%20infections_LR%20II.pdf).

Vaginal mucosal delivery of SolcoUrovac: In the initial human studies with intramuscular injections of SolcoUrovac, some women experienced adverse effects such as pain (5.4%), fever (3.5%) and swelling at the injection site (1.5%) ([283]). Solco Basel thus collaborated with David Uehling, a pioneer in the field of UTI vaccines from the University of Wisconsin, to test the efficacy of vaginally administered SolcoUrovac, hypothesizing that mucosal administration in the vagina would reduce adverse effects. Vaginal instillation was effective in mice ([284]), cynomolgus monkeys ([285]), and women ([286]), paving the way for phase II clinical trials of vaginally instilled SolcoUrovac in the United States. SolcoUrovac's

phase II clinical trials with the vaginal suppository form of the vaccine, which were published between 1996 and 2007, were only partially successful ([287–290]). The most effective treatment course was determined to be six vaginal suppositories given at weeks 0, 1, 2, 6, 10 and 14. With this vaccination regimen, the percentage of women having a recurrence during the six-month trial declined from 83–89% in the placebo-treated groups to 45–54% in the vaccinated, boosted groups. However, these differences were not always significant. In one trial, the authors identified six patient sub-populations with significantly lower re-infection rates after vaccination: "women younger than 52 years, without a childhood history of recurrent UTIs, [having] 6 or more UTIs in the previous year, without a hysterectomy, using estrogen, [or] using birth control pills ([287])." Adverse events (e.g. low grade fever, nausea, vaginal irritation) did occur, but no patient was unable to complete the treatment.

The adaptive response to SolcoUrovac: After intramuscular vaccination, mice had 10-fold more total IgG and 2-fold more total IgA in the urine; IgM was not found in the urine ([291]).Vaginal immunization of monkeys was protective but did not increase anti-*E. coli* serum, vaginal wash, or urine antibody levels (serum IgG, IgM, and IgA; vaginal wash and urine IgA and IgG) ([285]). Interestingly, although some vaccinated women did have increased antibody titers over time, there were no significant differences in any of the tested antibody levels among treatment groups in any phase II trial ([287–290]). As expected by the formulation, SolcoUrovac was most effective against UPEC uropathogens. In one study, the percentage of women who experienced a UPEC UTI after vaccination decreased significantly from 54% to 27.5%. However, the percentage of infections caused by uropathogens other than UPEC increased dramatically in vaccinated women: non-UPEC strains accounted for 16% of UTI in women receiving placebo and 49.1% in women receiving six doses of vaccine ([287]). Perhaps because of this shift in uropathogens, to the best of our knowledge vaginal SolcoUrovac has not progressed to stage III trials or beyond.

CP923: In 2007, Johnson and colleagues described a candidate vaccine consisting of a formalin-killed derivative of the *E. coli* sepsis strain CP9 that is deficient in capsule and O-antigen, termed CP923 ([292]). Compared to formalin-killed CP9, intranasal vaccination with formalin-killed CP923 resulted in a significantly greater systemic antibody response that was able to bind to a subset of heterologous UPEC and bacteremia strains. The mucosal immune response and protection against urinary tract infection were not assessed, but this study shows the potential benefit of using genetically modified UPEC for inactivated vaccines.

Attenuated Vaccines—Attenuated vaccines have the potential benefit of progressing through early steps in disease pathogenesis in the relevant host niche. Recently, the Klumpp group identified as a vaccine candidate a mutant of the UPEC strain NU14 that lacks the O antigen ligase *waaL* ([250]). This gene was identified in a screen of transposon mutants that had lost the ability to suppress IL-8 production by bladder epithelial cells ([293]). In a murine UTI model, NU14 Δ*waaL* was significantly more inflammatory and less virulent than wild-type NU14. Vaccination with NU14 Δ*waaL* protected mice from challenge infection with NU14, CFT073, and four UPEC isolates from the *E. coli* Reference Collection ([250], [294]).

However, protection waned over time and was absent by eight weeks. Interestingly, vaccination also significantly reduced the level of persistent bladder colonization (indicative of a QIR population) by NU14 14 days after challenge, even though the NU14 ΔwaaL (vaccine) strain itself is unable to persist past acute infection. The authors hypothesized that the lack of O antigen on LPS in NU14 Δ*waaL* may allow for Toll-like receptor 4 recognition of LPS lipid A, or may increase the exposure of bacterial surface antigens to antigen-presenting cells, thereby stimulating protective immunity ([250]). However, the recent finding that O antigen modulates infection-induced bladder pain, and that serial infections with NU14 Δ*waaL* result in chronic bladder pain diminishes the promise of NU14 Δ*waaL* as a vaccine candidate ([295]).

Specific-Antigen Vaccines in Development

Specific-antigen vaccines (such as toxoid, conjugate, and subunit vaccines) have become more popular in recent years due to advances in protein purification and the development of recombinant DNA technology. Single-antigen vaccines typically have lower rates of adverse events than whole cell vaccines, and several antigens may be combined in a single multi-epitope vaccine to increase efficacy. Candidate antigens may be revealed by UTI virulence studies *in vitro* and in animal models; alternatively, reverse vaccinology allows researchers to predict effective antigens computationally.

Conjugate vaccines—Conjugate UTI vaccines against UPEC capsule and LPS components have shown protection in animal models after same-strain challenge, but have not been tested clinically in humans. In early studies, intraperitoneal injection of *E. coli* endotoxin protected rats from pyelonephritis ([247]), and the protection was later determined to be mediated by antibodies against O antigen ([296]). Subcutaneous and bladder injection of purified O antigen from *E. coli* O6 serotype protected rats from bladder infection with the same O6 strain ([297]). Decades later, O polysaccharide prepared from *E. coli* O8 LPS, conjugated to bovine serum albumin, reduced renal scarring and intratubular neutrophil infiltration in rhesus monkeys that were challenged with an O8 UPEC strain ([298]). Purified *E coli* K13 polysaccharide conjugated to bovine serum albumin protected rats from pyelonephritis ([299]). A different group conjugated *E. coli* K13 to diphtheria toxoid and found that the vaccine decreased renal bacteria load and disease severity scores in mice after challenge with a K13 UPEC strain ([300]). A considerable challenge in formulating a vaccine targeting capsule or O antigen, the most exposed component of LPS, is the fact that there is a great heterogeneity of serotypes among *E. coli* isolates. For example, 6 different O serotypes account for only 75% of UPEC isolates ([301]), making the formulation of a broadly protective conjugate vaccine impractical. Furthermore, some capsule serotypes, such as K1, are thought to evade the host immune response by molecular mimicry, potentially making them poor vaccine candidates ([302]).

Toxoid vaccines and outer membrane vesicles—UPEC toxins have not been demonstrated to play a required role in UTI pathogenesis, so they are not ideal vaccine candidates. A purified α-hemolysin toxoid vaccine prevented renal injury, but not colonization, in mice after challenge with a hemolytic UPEC strain. Rather than being secreted as "naked" proteins, UPEC toxins such as α-hemolysin and CNF1 are associated

with outer membrane vesicles (OMVs), which bleb from the surface of Gram-negative bacteria during all stages of growth ([303]). OMVs also contain adhesins, enzymes, and nonprotein antigens like lipopolysaccharide (LPS) ([303]). OMVs may be a mechanism for UPEC and other bacteria to protect their toxins while they are *en route* to host cells, and to deliver "concentrated bursts of effector molecules" to modulate host cell processes ([304]). OMVs are intriguing vaccine candidates, and because they contain LPS and other pro-inflammatory virulence factors, they should not require adjuvants to stimulate the immune system. Several successful meningococcal OMV vaccines have been developed ([305_307]). UPEC OMVs are thus candidate vaccine antigens, though to our knowledge they have not been tested.

Pili as vaccine candidates: Pili are adhesive surface organelles that mediate the colonization of mucosal surfaces. Adhesins make an attractive antigen candidate, because antibodies raised against an adhesin should be able to block adhesin-host cell receptor binding, thus disrupting bacterial colonization of the host ([308]). Several types of pili have been investigated as UTI vaccine candidates. Pilus vaccines have been tested since well before the effects of growth conditions on pilus production were fully understood, and as such, many early pilus vaccine papers do not describe the bacterial growth conditions for the challenge inocula. When possible, we will report the relevant information. The first pilus UTI vaccine was published in 1979. Rats that were vaccinated intradermally with pili purified from two clinical isolates of *E. coli* were protected from pyelonephritis; anti-pili antibodies raised in rabbits were also protective in rats ([309]).

FimH—Vaccines targeting the type 1 pilus adhesin FimH, which plays a critical role in UTI pathogenesis in the lower urinary tract in animal models, have been tremendously effective in animals that are challenged with bacteria grown in type 1-pilus inducing conditions. Since the tip adhesin is functionally critical, but not highly abundant, purified adhesin was found to be better than whole pili at eliciting antibodies that blocked receptor binding ([32]). FimH can be purified bound to its periplasmic chaperone FimC, or as a naturally occurring, mannose-binding FimH truncate (FimHt) ([310]). Both antigens protected subcutaneously-vaccinated mice from cystitis ([32]). The FimCH vaccine also protected intramuscularly-injected cynomolgus monkeys from bacteriuria and pyuria though the number of animals tested was by necessity small ([31]). Of note, only one out of four FimCH-vaccinated monkeys developed bacteriuria and pyuria upon challenge infection with type 1 pili-expressing UPEC (compared to four out of four control monkeys), and this was also the only FimCH-vaccinated monkey without increased anti-FimH IgG in vaginal secretions. Another group compared the efficacy of a recombinant FimHt vaccine administered either intranasally or intramuscularly to mice. Both routes were protective against cystitis, but the intranasal vaccine induced greater anti-rFimHt IgA in vaginal washes ([260]). Yet another group demonstrated that subcutaneous administration of recombinant *fimH* fused to the flagellin subunit *fliC*, a TLR5 agonist and candidate adjuvant, protected mice against cystitis upon challenge with a type 1 pilus-expressing clinical isolate; vaccination with admixed FimH, FliC, and Montanide ISA 206 adjuvant was also protective ([311]). This study's investigation of the cellular immune response to vaccination is unique among UTI vaccine studies, which generally test the humoral response only. Immunization resulted in T_H1 and

T_H2 responses as assessed by cytokine responses in splenocyte proliferation assays and ratio of IgG1 to IgG2a. Lastly, another group has recently developed a mammalian codon-optimized *fimH* plasmid construct that could be used in a DNA vaccine, but they have not published results of trials in animals ([312]).

While it has been reported that monoclonal antibodies raised against FimH do not block, but rather enhance, adherence to bladder epithelial cells *in vitro* ([313]), the above studies demonstrate that polyclonal IgG raised against FimH block bacterial binding to bladder epithelium and is clearly protective *in vivo*. Possible explanations for this discrepancy include steric hindrance preventing the antibody-adhesin complex from binding to the uroplakin receptor pocket and the effects of opsonization. The FimCH vaccine was originally licensed by Medimmune (Gaithersburg, Maryland, USA) and entered Phase I and II trials in the early 2000s. The vaccine was found to be safe in Phase I trials, but was dropped from development during Phase II trials. Sequoia Sciences (St. Louis, Missouri, USA) has since acquired the license and the vaccine is set to re-enter clinical trials in women with recurrent UTI, using a new adjuvant. In two pre-clinical rabbit studies conducted by Sequoia, serum IgG anti-FimH titers were greater than 1:1,000,000, with no apparent adverse effects from the vaccination (personal communication, Gary Eldridge, Sequoia Sciences).

P pili—P pilus subunit vaccines to protect against pyelonephritis became a hot topic in the 1980s and the initial studies showed promise. Vaccination with purified recombinant P pili blocked renal colonization in mice when the challenge bacteria were grown under P pilus-inducing conditions ([30], [314]). Vaccination with purified P pili protected monkeys ([315], [316]) and the unvaccinated infants of vaccinated monkeys ([258]) from pyelonephritis when the challenge bacteria were grown under P pilus-inducing conditions. Finally, synthetic P pilus peptides that were prepared by solid-phase Merrifield synthesis and conjugated to carrier proteins prevented urine and renal colonization in mice ([317]). However, studies utilizing whole purified P pilus UTI vaccines have not been published since the late 1980s. This is likely due to the high degree of antigenic variation among UPEC strains in the major P pilin subunit, PapA, which is the most abundant pilin protein in P pilus preparations. Indeed, natural P pilus-specific antibodies from patients with pyelonephritis do not seem to target the binding pocket of PapG as they are unable to prevent P pili-mediated hemagglutination ([86]). Consistent with this, an inactivated whole-cell vaccine consisting of formalin-killed P-fimbriated *E. coli* offered only limited protection against renal dysfunction and scarring in monkeys ([282]). Thus, whole cell vaccines may not be an effective way of inducing anti-pilin antibodies, even if they are being expressed on the bacterial surface.

PapDG vaccine—By 1988, the composition of P pili had been determined, and the tip adhesin protein PapG was identified as a vaccine candidate ([53], [318]). Lund and colleagues suggested that PapG could be purified in a complex with its periplasmic chaperone protein PapD, analogous to the FimCH vaccine. In 2004 it was shown that intraperitoneal administration of a purified PapDG vaccine protected cynomolgus monkeys from pyelonephritis ([33]). The efficacy was presumed to be the result of PapG-specific antibodies blocking the pilus adhesins and thereby preventing colonization, though the specific

mechanism of protection is unknown. To the best of our knowledge, no further studies have been conducted with the PapDG pyelonephritis vaccine.

Other pili—Among the adhesins expressed by some UPEC strains are S pili and Dr adhesins (and others), each of which has been used as a vaccine antigen. Rats vaccinated with purified recombinant S pili had reduced kidney colonization ([319]). In the same study, an avirulent strain of *Salmonella typhimurium* was genetically transformed to produce S pili, and live bacteria were orally administered to rats, which had reduced kidney colonization compared to mock-vaccinated and purified S pilus-vaccinated mice ([319]). In addition, mice vaccinated with purified recombinant Dr adhesins had reduced UTI-associated mortality ([320]). However, these adhesins are even less broadly conserved among UPEC than are P pili antigens, and thus, these targets would only be useful in a multi-epitope vaccine.

Subunit Vaccines and Reverse Vaccinology: Recently, investigators have been able to use information gathered through bioinformatic, genomic, and proteomic analyses to identify novel candidate antigens, in an approach termed "reverse vaccinology." The first web-based reverse vaccinology program, Vaxign, was used to predict 22 UPEC outer membrane proteins as potential vaccine targets ([321]). Some of these targets had been previously shown to be immunogenic and protective in animal models, while others remain to be tested. A large-scale reverse vaccinology screen was used to identify vaccine antigen candidates in *E. coli* CFT073, which is predicted to encode 5379 proteins. The criteria for candidate antigens were pathogen specificity, high *in vivo* expression, induction during growth in human urine, antigenicity, and surface exposure. Six candidates were identified, each an outer membrane receptor protein involved in bacterial iron or heme acquisition. When purified and administered intranasally, the candidate antigens Hma, IreA, and IutA protected mice from challenge infection ([322]). Vaccination with Hma, a heme receptor, protected the kidneys; IreA, a putative siderophore, protected the bladder; and IutA, a siderophore receptor for aerobactin, protected both the bladder and the kidneys. The three antigens also induced antigen-specific IgA in the urine and class-switching from IgM to IgG in the serum ([322]). A subsequent study investigated additional UPEC outer membrane iron receptors as vaccine candidates. The yersiniabactin receptor FyuA, purified and administered intranasally, protected mice from developing pyelonephritis upon challenge with 536, a UPEC strain that expresses FyuA. Vaccination elicited anti-FyuA IgA in urine and IgG in serum, and serum antibody levels were correlated with kidney bacterial burden ([323]). Another group had previously found that systemic vaccination with the siderophore receptor IroN protected against renal colonization in mice ([159]). This last study did not explicitly use a reverse vaccinology approach, but IroN was chosen because of its prevalence among clinical UPEC isolates, its role in urovirulence, and its expression in bodily fluids.

Another reverse vaccinology approach employed by a group at Novartis (Siena, Italy) involved comparing the genome of a neonatal meningitis-associated K1 strain of *E. coli* with known pathogenic and nonpathogenic *E. coli* strains. Potential antigens were chosen if they were predicted to be surface-associated or secreted, with no more than three transmembrane domains, and were absent from nonpathogenic strains. Two hundred and thirty candidates were identified in this manner and tested for protection in a murine sepsis model; nine were

protective ([324]). One protective antigen, named FdeC for factor adherence *E. coli*, was found to be expressed by most UPEC, but only upon host cell contact, helping to mediate *E. coli* adhesion to mammalian cells. Intranasal vaccination with recombinant FdeC significantly reduced kidney colonization in mice that were challenged with UPEC strain 536 or CFT073 ([109]).

Vaccines Against Non-UPEC UTI

Uropathogenic *E. coli* cause approximately 85% of uncomplicated UTI, and so it is not surprising that most tested UTI vaccines have used UPEC strains and antigens. However, vaccines targeting other uropathogens have been protective in animal models. The polyvalent inactivated vaccine SolcoUrovac/StroVac (described in detail above) contains one strain each of *Klebsiella pneumoniae*, *Proteus mirabilis*, *Morganella morganii*, and *Enterococcus faecalis*. Vaginally instilled, formalin-killed *K. pneumoniae* inhibited bacterial adhesion to rat bladder mucosae ([280]). In addition, a recent immunoproteome analysis identified candidate antigens for a *K. pneumoniae* vaccine ([325]), but to the best of our knowledge, these antigens have not been tested in a UTI model. Most of the other non-UPEC vaccines have targeted *P. mirabilis*, which causes about 3% of uncomplicated and 13% of complicated UTI ([326]).

***Proteus* vaccines—**Vaccines against *P. mirabilis* infection have been tested since at least the 1960s, when heat-killed *P. mirabilis* protected rats from pyelonephritis by promoting renal clearance of bacteria ([327]). A preparation of *P. mirabilis* outer membrane protein promoted renal clearance in mice and protected mice from renal infection and death ([326]). Purified inactivated proteus toxic agglutinin (Pta), a cytotoxic surface-associated alkaline protease, was conjugated with cholera toxin and protected mice from kidney colonization ([328]). Finally, several *P. mirabilis* adhesins have been tested as vaccine antigens. *P. mirabilis* expresses MR/P (mannose- resistant, *Proteus*-like) fimbriae on the cell surface, and most of the bacterial population synthesizes MR/P fimbriae during UTI ([329]). Vaccination with purified MR/P fimbriae was protective against ascending *P. mirabilis* UTI in a murine model ([330]). An attenuated mucosal vaccine consisting of *Lactococcus lactis* expressing the recombinant MrpA subunit of MR/P fimbriae significantly reduced renal colonization in mice after *P. mirabilis* challenge ([331]). Systemically-injected, purified recombinant MrpA also protected mice from ascending *P. mirabilis* UTI ([332]). Intranasal vaccination with recombinant MrpA protected mice from ascending *P. mirabilis* UTI, and transurethral vaccination protected the kidneys ([333]); the addition of a cholera toxin adjuvant did not enhance protection ([334]). MrpH is the MR/P fimbrial tip adhesin, similar to FimH and PapG (described above). Vaccination with recombinant MrpH was protective against ascending *P. mirabilis* UTI in a murine model ([330]). Finally, vaccination with the urothelial cell adhesin subunit UcaA protected mice from *P. mirabilis* infection in a hematogenous infection model ([332]).

Immunotherapeutic Compounds

OM-89/UroVaxom®—The immunotherapeutic formulation OM-89 (marketed in Europe by EurimPharm GmbH as UroVaxom) is a bacterial extract prepared from 18 strains of *E. coli.* For the purpose of preventing recurrent UTI, it is administered orally, typically as a

daily dose for three months, and is recommended by the European Association of Urology for women with recurrent uncomplicated UTI. Several meta-analyses have assessed the effectiveness of OM-89 in preventing recurrent UTI in humans. A meta-analysis of five placebo-controlled double-blind studies found that OM-89 was superior to placebo with regards to reducing UTI frequency and dysuria, bacteriuria and leukocyturia ([335]). Another meta-analysis of five studies found that the mean number of UTI episodes and the use of antibiotics were reduced in patients treated with OM-89 ([336]). OM-89 is generally safe and well-tolerated; the most frequent adverse events are headache and gastrointestinal events. While early studies looked at UTI recurrence over just six months from the start of treatment, OM-89 is effective for up to 12 months when booster doses are administered for the first 10 days in months 7, 8 and 9 ([337]). Several other immunostimulatory compounds exist for the prevention of recurrent UTI. Uromune, a preparation of *E. coli*, *K. pneumoniae*, *P. vulgaris*, and *E. faecalis,* was recently evaluated in a multicenter retrospective observational study. Women with a history of recurrent UTI who received daily Uromune prophylaxis for three months had significantly fewer recurrences over a 15-month period than women who received daily trimethoprim-sulfamethoxazole for six months ([338]). Other similar formulations, such as Urostim and Urvakol, have been developed, but few controlled studies are available and they will not be discussed further here.

The mechanism by which immunostimulatory compounds protect against recurrent UTI remains unclear. In mice, OM-89 activated macrophages ([339]) and induced a T_H1-type immune response as determined by increased IgG2a in the serum and IFN-γ in spleen cell supernatant ([340]). OM-89 also increased IL-6 and IFN-γ levels and decreased inflammation in the mouse bladder ([341]). The antibody response to OM-89 varies in different studies. In mice, OM-89 mainly induced IgG in the serum, and increased IgM only weakly ([342]). Also in mice, strain- specific IgG and IgA were increased in immune sera, and total and strain-specific IgG and IgA were increased in the urogenital tract. In addition, the antisera could recognize other human uropathogens such as *E. faecalis*, *K. pneumoniae* and *P. mirabilis*. However, cross-reactivity was stronger with intraperitoneal injection of OM-89, rather than oral administration ([340], [343]). Interestingly, in a meta-analysis of two studies, there was no significant difference in urine and vaginal fluid anti-*E. coli* IgG and IgA between OM-89-treated and placebo-treated patients ([336]), which would suggest that OM-89's efficacy may not be antibody-mediated. As OM-89 is a lysate of *E. coli* administered daily for several months, its efficacy could be the result of induced lipopolysaccharide (LPS) tolerance. Similarly, a TLR4 polymorphism that is associated with reduced inflammatory signaling in response to LPS is also associated with protection from recurrent UTI ([344]).

CONCLUSIONS

The Challenge is Great

The progress in our understanding of UTI pathogenesis over the past 15 years has been truly remarkable and has begun to change how UTI is viewed and treated in the clinic. Sophisticated animal models of infection and translational studies have revealed that, rather than being a simple extracellular infection of the urinary tract mucosa, infection with UPEC and a number of other Gram-negative uropathogens (which together cause more than 80% of

all UTI) proceeds through dynamic intracellular and extracellular host niches during the course of acute and chronic infection. Whole genome sequencing and gene expression analysis of uropathogens have allowed an unprecedented look into the lifestyle of these versatile pathogens, thus enabling genetic and computational approaches to identify novel virulence mechanisms and vaccine candidates. The determination of structural details of uropathogenic adhesins has led to the rational design of anti-infectives and preventative strategies. However, with increased understanding comes knowledge of the imposing challenges facing scientists in the effort to develop broadly protective therapies. These challenges stem from the fact that the development of symptomatic UTI is exceedingly complex, hinging upon two factors that are highly variable and yet define the host-pathogen interaction: the virulence of the infecting uropathogen and the character of the bladder mucosal immune response. On the one hand, uropathogens are a very heterogeneous collection of bacterial isolates that have the capacity to inhabit diverse host niches, including the gut, the urogenital tract, the bloodstream, and the meninges. Therefore, even among isolates from a single species (such as UPEC), uropathogens differ widely in their genetic and epigenetic makeup, and thus are represented by a large and varied number of serotypes and virulence factor profiles. On the other hand, the host genetic and environmental variables that determine the extent and character of the bladder mucosal immune response to infection are considerable and very poorly understood. As a result, two individuals may be infected with the same strain but have very different responses to infection, ranging from an asymptomatic carrier state, asymptomatic bacteriuria, to severe cystitis and pyelonephritis with renal scarring.

A Call to Arms: Investigators in Bladder Mucosal Immunity Needed!

Developing new and efficacious therapies should be the highest priority in UTI research, as such therapies have the real potential to positively affect the quality of life of millions of individuals and decrease the overall use of antibiotics. However, many challenges must be overcome. The significant contribution of the host to recurrent UTI makes it unlikely that any new vaccine or therapeutic alone will completely eliminate recurrent UTI in all patients, unless it is also able to alter the innate mucosal immune response to uropathogens. As the clinical trials of the vaginal vaccine Urovac have demonstrated, even a whole cell vaccine broadly targeting several UPEC serotypes as well as four other uropathogens was only able to significantly protect against recurrent UPEC UTI, and then only in a subset of women. Importantly, what the researchers saw in many of these women was a shift towards recurrent UTI caused by less common uropathogens, suggesting a defect or sensitization of the mucosal immune response. In the absence of vaccination, UPEC were able to predominate in the urogenital niche of these subjects, but with vaccination, other uropathogens replaced UPEC ([287]). Thus, as new and more efficacious strategies are being developed to combat recurrent UTI by UPEC, such as pilus adhesin antagonists (e.g. mannosides) and UPEC subunit vaccines, we must anticipate the likelihood that a subset of patients will continue to suffer from recurrent UTI with less common uropathogens. For these patients, therapeutic interventions targeting the bladder mucosal immune response may provide additional benefit and relief from symptoms. However, our understanding of bladder mucosal immunity is currently insufficient to allow informed predictions, and there is a paucity of investigators in this field. Despite the pioneering efforts of Dr. Svanborg and others, the field is relatively

small. For example, of the 10 investigator teams of the Mucosal Immunity Study Team NIH-NIAID U01 consortium (mucosal.org), only one group is focused on investigating the urogenital tract. Until our understanding of bladder mucosal immunity matures, which will require a critical mass of investigators in this field, novel approaches to the treatment and prevention of UTI may be slow in coming.

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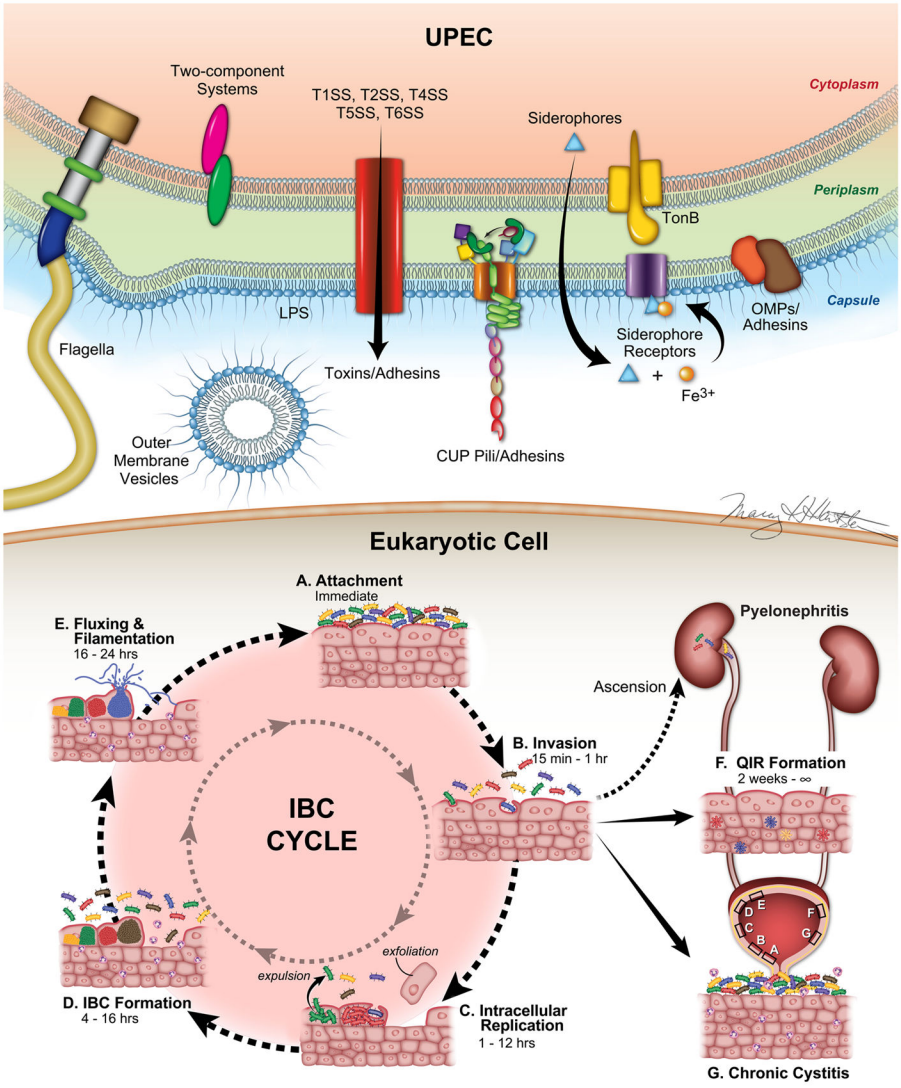

Figure 1. Targeting UPEC virulence factors that are critical for pathogenesis

Uropathogenic *E. coli* (UPEC) elaborate a variety of surface structures and two-component systems that play critical roles in UTI pathogenesis. The stages of pathogenesis, as determined from animal models and clinical data, include initial bladder colonization and the IBC cycle (*A*–*E*), the chronic bladder outcomes of quiescent intracellular reservoir (QIR) formation (*F*) and chronic cystitis (*G*), and ureteral ascension and pyelitis/pyelonephritis with increased risk for bacteremia/septicemia. UPEC surface structures that play a role in UTI pathogenesis include lipopolysaccharide (LPS), polysaccharide capsule, flagella, outer membrane vesicles, pili, non-pilus adhesins, outer membrane proteins (OMPs), toxins, secretion systems, and TonB-dependent iron uptake receptors, including siderophore receptors. These virulence components are attractive drug and vaccine candidates.

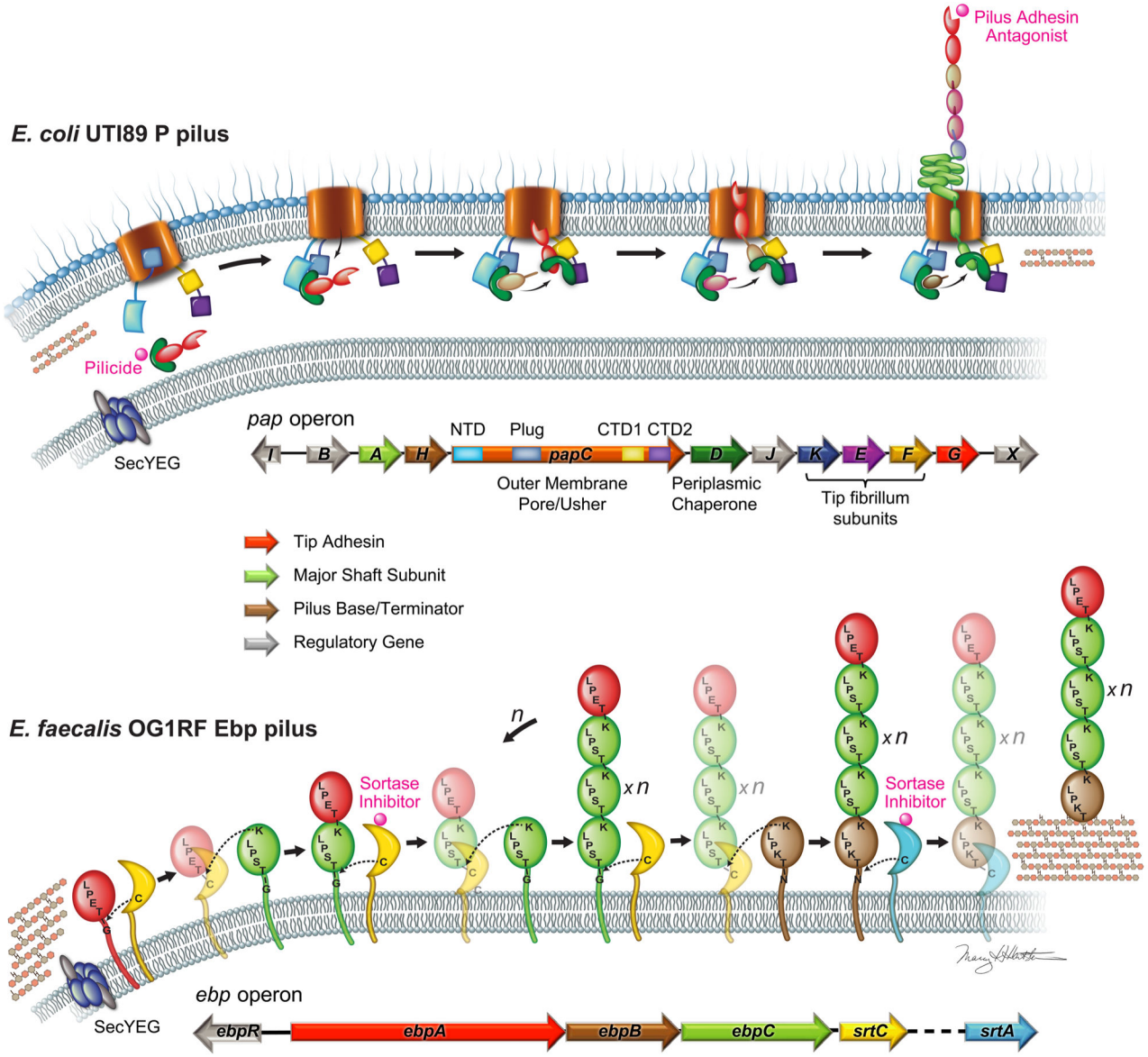

Figure 2. Models of pilus assembly in Gram-negative and Gram-positive pathogens

Top panel, Model of P pilus formation by the chaperone-usher pathway in uropathogenic *E. coli*. After secretion of pilus subunits into the periplasm via the general Sec machinery, periplasmic chaperones (*dark green*) serve as folding templates, providing a beta-sheet that enables proper folding of the pilin subunits into immunoglobulin-like domains, but in a non-conical orientation, in a mechanism called **donor strand complementation**. Assembly and anchoring of the pilus occurs at an outer membrane pore known as the usher (orange). The pilus tip adhesin (red) is the first subunit to interact with the usher, via a preferential interaction between the tip adhesin/periplasmic chaperone complex and the usher N-terminal periplasmic domain (NTD, *light blue*), and this interaction initiates assembly by causing a conformational change in the usher that "unplugs" (Plug, *dark blue*) the pore and displaces the tip adhesin subunit/chaperone complex to two C-terminal usher domains, CTD1 (*yellow*)

and CTD2 (*purple*) ([42], [367], [368]). The next pilin subunit/chaperone complex then binds to the NTD and if it has an N-terminal extension that is able to complete the immunoglobulin fold of the preceding subunit in a canonical fashion, this provides the free energy to displace the chaperone, in a process called **donor strand exchange**, and drive assembly ([39_41], [369]). In P pili, this occurs repeatedly, incorporating anywhere from hundreds to thousands of PapA major pilin subunits (green) in the pilus, until PapH (brown) is incorporated into pilus. PapH is a terminator because it is unable to undergo donor strand exchange ([370]). Small molecule inhibitors (*pink*) that disrupt pilus assembly ("pilicides") or adhesin binding to its receptor ("pilus adhesin antagonists") have been identified ([193], [206]). ***Bottom panel,*** Model of sortase-mediated assembly of the endocarditis- and biofilm-associated pilus (Ebp pilus) in *E. faecalis* ([371]). Unlike CUP pili in Gram-negative bacteria, sortase-assembled pilus subunits are covalently linked. Pilin subunits are first secreted to the outside of the cell via the general Sec machinery, and are retained in the membrane via a hydrophobic domain within their cell wall sorting sequence. Sortase C (SrtC, *yellow*) cleaves the EbpA (*red*) LPETG sequence, resulting in an EbpA-SrtC thioacyl intermediate that is resolved by the EbpC (*green*) Lys186 nucleophile. Pilus polymerization occurs when SrtC processes the EbpC LPSTG sequence at the base of a growing, membrane-associated pilus forming a pilus-SrtC intermediate that is resolved by the Lys186 of an incoming EbpC subunit. EbpB (*brown*) incorporates at the base of a pilus fiber when its Lys179 nucleophile resolves a pilus-SrtC intermediate. Sortase A (SrtA, *blue*) processing of the EbpB LPKTN sequence leads to eventual incorporation of the mature pilus into the cell wall. Sortase inhibitors (*pink*) may be useful for disrupting the virulence potential of Gram-positive uropathogens.

Table 1

Prevalence and sites of action of uropathogenic *E. coli* (UPEC) virulence factors and their use as candidate vaccine antigens ([34, 105, 107, 156, 322, 345–352]).

Category	Virulence Factor	Prevalence	Animal Model	Bladder	Kidneys	Refs
CUP Pili/Adhesins	Type 1	86–100	Mice	S,C,P	S,C	[29, 32, 57, 61, 260, 353]
			Monkeys	P	NR	[31]
	P	12–87	Rodents	N	S,P	[30, 34, 96, 309, 314, 317]
			Monkeys	C	S,P	[33, 92, 95, 258, 282, 315, 316]
	Ygi	57–66	Mice	N	C(R)	[105]
	Yad	36–53	Mice	N	N	[105]
	F1C	8–50				
	S	3–31	Rodents	NR	S(R),P	[104, 319]
	Dr	1–15	Mice	N	S, P	[320, 354]
Adhesins	FdeC	99	Mice	C	C, P	[109]
	TosA	25	Mice	S	S	[106, 108]
Toxins	α-Hemolysin	18–68	Rodents	L	S,P,L	[104, 168, 355]
	CNF1	13–54	Mice	S,C(R),L	C	[77, 78]
Endotoxin	WaaL	Conserved	Mice	S,P	P	[250, 295]
Autotransporter (T5SS) serine proteasess	Sat	26–52	Mice	N	L	[170]
	PicU	19–31	Mice	N	N	[171, 356]
	Vat/Tsh	54–68		NR	NR	[171]
Autotransporter (T5SS) adhesins	UpaG	21	Mice	N	N	[133]
	UpaC	47	Mice	N	N	[130]
	UpaB	58	Mice	S,C	N	[130]
	UpaH	76	Mice	C	N	[131]
Two-Component Systems	QseBC	Conserved	Mice	S,C	S,C	[142, 146]

Category	Virulence Factor	Prevalence	Animal Model	Bladder	Kidneys	Refs
	PhoPQ	Conserved	Mice	S(R)	N	(150)
	BarA-UvrY	Conserved	Mice	S	S	(151)
	Cpx	Conserved	Mice, zebrafish embryos	S,C		(149)
Iron Acquisition	FepA	Conserved		NR	NR	
	FyuA	71–96		NR	NR	
	IreA	20–26	Mice	N,P	NR	(322, 357)
	IroN	42–78	Mice	C	N,P	(159, 358)
	IutA	14–85	Mice	C,P	C,P	(162, 322)
	Iha	16–74	Mice	C	C	(110)
	Heme: ChuA	84–90	Mice	IBC,C	C	(120, 162)
	Heme: HmaA	61–81	Mice	N,P	S,P	(172, 322)
	TonB	Conserved	Mice	C(R)	S(R),C(R)	(162)
Capsule	K1	19–56	Mice	S(R)	NR	(127)
	K2	9–13	Mice	C(R)	C(R)	(128)
Motility	Flagellin	Conserved	Mice	S,C	S,C	(138, 167)
Secretion Systems	T2SS		Mice	N	N	(173)
	T4SS	NR	Mice	N	S(R)	(173)
	T6SS	NR	Mice	N	N	(359)
Immune Modulatory	TcpC	21–40	Mice	N	S(R),L	(174, 175, 360)
	SisA/B	A:67–86; B:22–28	Mice	S,C	S,D(R)	(361)
OMPs	OmpA	Conserved	Mice	S(R),C	S	(362)
	OmpT	70–94	Mice	S	NR	(363, 364)
Filamentation	SulA	Conserved	Mice	S	NR	(139)

S, mutant strain was attenuated in single infection studies

IBC, mutant strain formed smaller intracellular bacterial communities

C, mutant strain was attenuated in competition studies

(R), attenuation of mutant strain was rescued by complementation

P, vaccination with virulence factor was protective against challenge infection

L, mutant strain caused less organ damage in single infection studies

D, mutant strain caused more organ damage in single infection studies

N, no phenotype

NR, not reported.

Table 2

Candidate vaccines targeting uropathogens.

Multi-species Vaccines	Vaccine/Antigen	Animal Model	Human Trials	Site of Protection	Route	Adjuvant	Antibody Responses	Refs.
Whole Cell: Inactivated	**SolcoUrovac**®: 6 *E. coli* strains and 1 strain each of *P. mirabilis, M. morganii, E. faecalis* and *K. pneumoniae*	M[291, 365] R[365] NHP[285]	Trial in children[366] and women[283] Phase I & II clinical trials[286_290]	Rodent bladder and kidney[291, 365] Human bladder[287_290, 366]	IM[291, 366] V[285, 286] VS[287–290]	None	Increased total and antigen-specific urinary IgG & IgA in mice[291] Increased urinary sIgA[366] Increased total IgG and IgA in vaginal wash and urine[286]	[283, 285_291, 365, 366]
Whole Cell: Inactivated	**StroVac**®: *E. coli, P. mirabilis, M. morganii, K. pneumoniae* and *E. faecalis*	None reported in the literature	Distributed by Strathmann GmbH (Hamburg) in parts of Europe, Latin America and the Middle East	Bladder	IM	Aluminum phosphate	NR	Strovac Product Insert

UPEC-targeted Vaccines	Vaccine/Antigen	Animal Model	Human Trials	Site of Protection	Route of Delivery	Adjuvant	Antibody Responses	Refs.
Whole Cell: Inactivated	Heat- or formalin- killed *E. coli*	R[275, 276, 278, 279] Rb[16] NHP[281]	Led to the development of SolcoUrovac/Strovac	Bladder[278, 279, 281] kidney[16, 275, 276, 278, 279]	IV[16, 275] IP[276, 279, 281] B[276, 278] IM[279, 281] V[279, 281]	Freund's[279]	Anti-*E. coli* IgG and IgA in urine[281]	[16, 275, 276, 278_281]
Whole Cell: Attenuated	NU14 Δ*waaL*	M	NR	Bladder	B	None	NR	[250]
Whole Cell: Attenuated	*S. typhimurium* expressing S pili	R	NR	Kidney	Oral	None	Antigen-specific IgG and IgA in serum	[319]
Whole pili	P pili: purified[309, 315, 316] recombinant[30, 258, 314] synthetic peptides[317]	M[30, 314, 317] R[309] NHP[258, 282, 315, 316]	NR	Kidney[30, 258, 309, 314_317]	ID[309] IM[30, 317] IV[315] SQ[30, 314, 317] LN[258]	Freund's [30, 309, 314, 315, 317]	Anti-P IgG in serum[30, 258, 314, 316, 317] and urine[314] anti-P pilus IgM in serum[30]	[30, 258, 309, 314_317]
Specific Antigen: Subunit	FimH adhesin: FimCH[31, 32] FimH truncate[32, 260]	M[32, 260] NHP[31]	Phase I and II clinical trials performed by MedImmune License recently acquired by Sequoia Sciences	Bladder[31, 32, 260]	SQ[32] IM[31, 260] IN[260]	Systemic: Freund's[32, 260] MF59[31] Mucosal: CpG[260]	Anti-FimH IgG in urine[32] and serum[260] Anti-FimH IgA in vaginal washes[31, 260]	[31, 32, 260]
Specific Antigen: Subunit	PapDG adhesin	NHP	NR	Kidney	IP	Aluminum phosphate	Anti-PapDG IgG in serum	[33]
Specific Antigen: Subunit	IroN siderophore receptor	M	NR	Kidney	SQ	None	Anti-IroN IgG in serum	[159]
Specific Antigen: Subunit	FyuA siderophore receptor	M	NR	Kidney	IN	CT	Anti-FyuA IgG in serum (correlated with renal bacterial load) and IgA in urine	[323]

UPEC-targeted Vaccines	Vaccine/Antigen	Animal Model	Human Trials	Site of Protection	Route of Delivery	Adjuvant	Antibody Responses	Refs.
Specific Antigen: Subunit	FdeC adhesin	M	NR	Kidney	IN	CT	NR	(109)
Specific Antigen: Subunit	Dr adhesins	M	NR	Reduced mortality	NR	Freund's	Anti-Dr IgG in serum, but no significant effect on colonization	(320)
Specific Antigen: Subunit	S pili	R	NR	Kidney	SQ	None	Anti-S IgG in serum	(319)
Specific Antigen: Toxoid	α-hemolysin	M	NR	Kidney	IM	Freund's	Anti-α-hemolysin IgG in serum	(168)
Specific Antigen: Conjugated	O antigen	R(296, 297) NHP(298)	NR	Bladder(297) Kidney(296, 298)	SQ(297) SQ+IV(296) LN +SQ(298)	Freund's(296)	Anti-O8 IgG in serum(298)	(296_298)
Specific Antigen: Conjugated	K13 antigen	M(300) R(299)	NR	Kidney	SQ	DT(300) Conjugated to BSA (299)	Anti-K13 IgG and IgM in serum(299)	(299, 300)
Specific Antigen (Multi-Epitope) Subunit	IreA, Hma and IutA iron receptors	M	NR	Bladder and kidney	IN	CT	Antigen-specific IgG and IgM in serum and IgA in urine	(322)

Proteus-targeted Vaccines	Vaccine/Antigen	Animal Model	Human Testing	Site of Protection	Route of Delivery	Adjuvant	Antibody Responses	Refs.
Whole Cell: Inactivated	Formalin-killed *P. mirabilis*	M	NR	Bladder, Kidney	IN, SQ	SQ: Freund's IN: CT	Antigen-specific IgG and IgA in serum, urine, bladder, and vaginal washes	(330)
Whole Cell: Inactivated	Heat-killed *P. mirabilis*	R	NR	Kidney	SQ	None	NR	(327)
Whole Cell: Attenuated	*L. lactis* MrpA	M	NR	Kidney	IN	None	Anti-MrpA IgG and IgA in serum	(331)
Specific Antigen: subunit	MrpH adhesin	M	NR	Bladder, Kidney	IN, V	CT	High levels of IgG in serum, but no correlation with protection	(330)
Specific Antigen: subunit	MrpA pilus subunit	M	NR	Bladder(333) Kidney(332_334)	IN(333, 334), SQ(332), V(333)	Freund's(332) CT(334)	Anti-MrpA IgG in serum (332, 333) and urine(333) Anti-MrpA IgA in serum and urine(333), but no correlation with protection	(332_334)
Specific Antigen: subunit	UcaA adhesin	M	NR	Bladder, kidney	SQ	Freund's	Anti-UcaA IgG in serum, but no correlation with protection	(332)
Single Antigen: Toxoid	Proteus toxin agglutinin (Pta)	M	NR	Kidney, spleen	IN	CT	Anti-Pta IgG in the serum	(328)

NR, not reported. ***Animal Models:*** M, mice; NHP, non-human primates; R, rats; Rb, rabbits. ***Routes of delivery***: B, intravesical; ID, intradermal; IM, intramuscular; IN, intranasal; IP, intraperitoneal; IV, intravenous; LN, intra-lymph node; SQ, subcutaneous; V, vaginal instillation; VS, vaginal suppositories. ***Adjuvants***: CT, cholera toxin; DT, diphtheria toxoid.