THE YERSINIABACTIN SYSTEM IS A UROPATHOGENIC *ESCHERICHIA COLI* VIRULENCE FACTOR AND NOVEL VACCINE TARGET TO PREVENT URINARY TRACT INFECTION

by

Ariel Ray Brumbaugh

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Doctoral Committee:

Professor Harry L.T. Mobley, Chair
Associate Professor Matthew R. Chapman
Professor Victor J. DiRita
Assistant Professor Irina L. Grigorova
Professor James B. Kaper, University of Maryland

To my families and pack.

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Ariel Ray Brumbaugh
University of Michigan
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ABSTRACT

Urinary tract infections (UTIs) are common, with nearly half of all women and 12% of men experiencing at least one UTI in their lifetime. While most UTIs seldom cause life-threatening or long-term health problems, the regularity with which they occur generates a substantial economic and public health burden. Over 80% of these infections are caused by a heterogeneous group of uropathogenic *Escherichia coli* (UPEC) strains, and patients are generally treated with a course of antibiotics to prevent more serious infection. However, the emergence and spread of antibiotic resistance mechanisms, such as extended-spectrum *beta*-lactamases and carbapenemases, complicate the successful long-term treatment of UTI and increase the potential for serious infection. As a result, novel therapeutics and preventive approaches to UTI, such as vaccination, are sought.

We hypothesized that targeting nutrient acquisition in pathogenic bacteria, specifically systems that acquire iron, could provide a novel vaccine target to prevent UTI. Using a murine model of infection and recombinant UPEC antigens, we identified the yersiniabactin receptor, FyuA, as a protective vaccine candidate. Intranasal immunization with FyuA induced a robust and long-lived humoral immune response that correlated with protection against UPEC colonization in the kidneys and development of acute pyelonephritis. In addition, using a UPEC mutant unable to acquire yersiniabactin during infection, we established the yersiniabactin receptor as a UPEC virulence factor during cystitis and pyelonephritis, a fitness factor during bacteremia, and the surface-accessible target of the experimental FyuA vaccine. We were also able to validate the

expression of iron acquisition systems, including the yersiniabactin system, during natural human infection by quantifying RNA transcripts, directly stabilized from *E. coli* in the urine of women with uncomplicated cystitis, by RNAseq analysis. These findings provide a foundation for the development of a vaccine to prevent UPEC UTI by targeting the yersiniabactin receptor, and reinforce support for the yersiniabactin system as a narrow-spectrum therapeutic target to combat multidrug-resistant pandemic clones of the family *Enterobacteriaceae*.

CHAPTER 1

INTRODUCTION

Author's Note: This chapter is a modified version of the previously published review article: Brumbaugh AR and Mobley HLT. Preventing Urinary Tract Infection: Progress toward an effective *Escherichia coli* vaccine. 2012. *Expert Rev Vaccines* 11**:**663-676

Urinary tract infection

A public health burden.

Despite the regular flow of urine, formidable physiological barriers, and a robust array of host defenses, the human urinary tract remains one of the most common sites for bacterial infection (1). Roughly half of all women and 12% of men will experience a urinary tract infection (UTI) in their lifetime (Figure 1-1) (2). Approximately a quarter of these women will have a recurrent infection within 6 to 12 months (3). The high incidence of infection results in 11 million physician office visits and 1.7 million emergency room visits annually in the United States alone (4). On a population scale, the consequence of frequent UTI amounts to a substantial fiscal public health burden. In 2000, Americans spent a staggering $3.5 billion treating UTIs (5).

Classifications.

The majority of UTIs begin as a bladder infection, clinically termed cystitis, which results from pathogenic bacteria, colonizing the perineum, traversing the urethra and successfully infecting the bladder. If cystitis is left untreated, colonizing bacteria can

ascend the ureters to cause a secondary infection in the kidneys, acute pyelonephritis, which can result in renal scarring and permanent kidney damage (6). In severe cases of pyelonephritis, invading bacteria can breach epithelial and endothelial barriers in the kidney to gain access to the bloodstream (bacteremia) leading to systemic infection and sepsis, a serious and sometimes fatal complication (7). UTIs occurring in individuals with no physical abnormalities of the urinary tract or medical devices that would circumvent natural host defenses, such as a catheter, are classified as “uncomplicated” and are the most common type of UTI (1). Uncomplicated UTIs are unique among bacterial infections in that they occur most frequently among otherwise healthy women between the ages of 18 and 29 years (1).

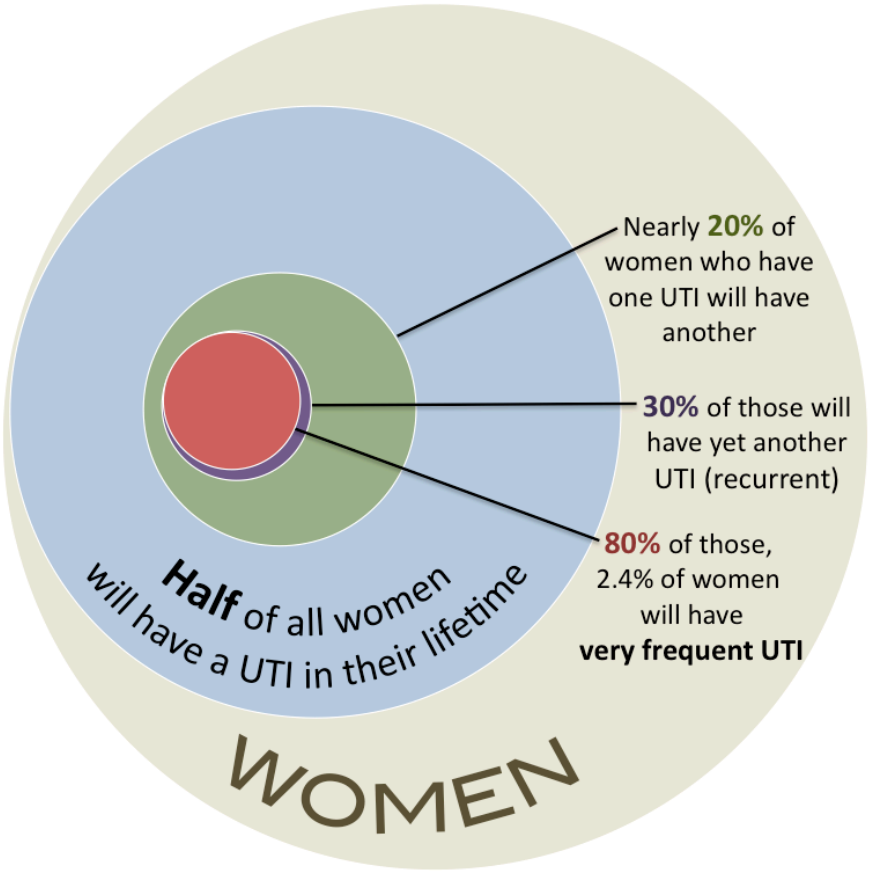


Figure 1-1. Urinary tract infection among women is extremely common. Roughly 13% of women between the ages of 18 and 90 years of age will have an annual incidence of urinary tract infection. Based on 2010 United States census data, an estimated 15 million women will have a urinary tract infection annually in the United States (8). Percentages are represented by circle area.

Uropathogenic *Escherichia coli*.

Although there are a number of bacterial genera capable of infecting the human urinary tract, including *Proteus*, *Enterococcus*, *Klebsiellae*, and *Staphylococcus spp.,* greater than 80% of uncomplicated UTIs are caused by a heterogeneous group of *Escherichia coli* strains, termed uropathogenic *Escherichia coli* (UPEC) (9). Genotypic and phylogenetic analyzes provide evidence that UPEC is actually part of a larger group of pathogenic *E. coli* strains collectively termed extraintestinal pathogenic *E. coli* (ExPEC) that include meningitis-associated *E. coli* and sepsis-associated UTI (10). UPEC are distinct from fecal commensal strains of *E. coli* found in the gastrointestinal tract, in that their genomes frequently encode a war chest of additional genes and virulence factors that facilitate infection of the host urinary tract (9, 11, 12). These additional genes may encode toxins, adhesins, iron acquisition systems, additional metabolic enzymes, and structural components that enable UPEC to take advantage of a unique and otherwise sterile host environmental niche (Figure 1-2)(13).

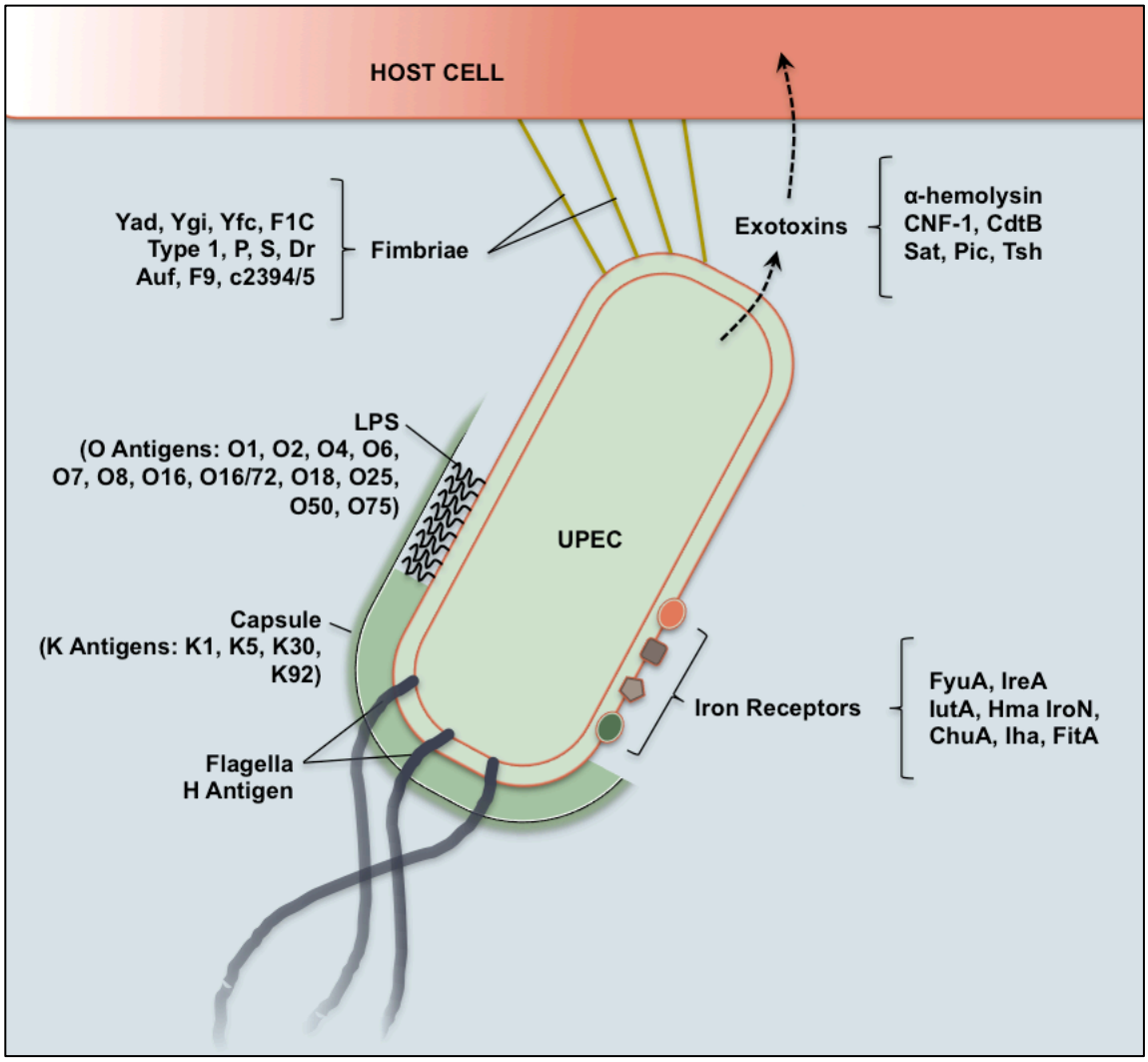


Figure 1-2. Classes of UPEC Vaccine Targets. Classes of UPEC vaccine targets include fimbrial adhesins, surface polysaccharides, outer membrane iron receptors, and toxins.

Current standard treatment for UTI: Antibiotics

The problem of decreasing efficacy.

Antibiotics currently represent the most commonly prescribed treatment for UTI and patients that suffer from recurrent infection, having three or more infections a year, may be prescribed antibiotics prophylactically (14). In individuals plagued with persistently recurring UTI, which includes roughly 3% of women, rising rates of antibiotic resistance to first- and second-line therapies can make UTI treatment especially challenging, forcing physicians to reach for more expensive and sometimes less effective drugs (15-17) (Figure 1-1). For example, in Turkey, the first-line antibiotic for UTI, trimethoprim-sulfamethoxazole (TMP-SMX), was used extensively beginning in the early 1980s. By the early 1990s uropathogen resistance rates to TMP-SMX had increased to above 50% leading to therapeutic failure and physicians to adopt quinolones as the preferred therapeutic for UTI (18). In the decade following the shift towards quinolones for UTI treatment, uropathogen resistance rates to quinolones in Turkey spiked almost 30% (18). In the United States and Canada, approximately 10-25% of uncomplicated UTI isolates are resistant to TMP-SMX, and in Spain and Portugal the resistance rate can be as high as 35% (16, 19, 20). Even more troubling is the rate of multidrug resistance among UPEC isolates, which has risen as physicians adapt prescription choices to address shifting microbial susceptibilities (21). A recent international Antimicrobial Resistance Epidemiological Study on Cystitis (ARESC) documented over 10% of cystitis *E. coli* isolates to be resistant to at least three different classes of antimicrobial agents (22).

Secondary infections and co-morbidities.

In addition to contributing to a rise in antibiotic resistance, repeat antibiotic treatment of UTI frequently results in added co-morbidities that drive-up medical costs and substantially diminish patient quality of life. Indeed, antibiotic therapy may deleteriously affect a patient's commensal microbiota and lead to secondary infections post-treatment such as vaginal yeast infection and gastrointestinal infection (23-25). Individuals that undergo repeat antibiotic treatment are also at increased risk for carrying additional pathogens resistant to multiple antibiotics, further complicating medical care (26). In an effort to provide a more effective and less costly alternative to antibiotic therapy for UTI management, progress has been made in the development of a preventive vaccine to target the most prominent uropathogen, UPEC.

UPEC Vaccine Development

Although it appears that a prior UTI fails to elicit a protective host immune response and uropathogen heterogeneity complicates vaccine design, data from animal model studies offer encouragement for successful UPEC vaccine development (27, 28). Immunization with UPEC antigens can stimulate a mucosal immune response that may be effective at preventing experimental UTI, and increases in urinary and serum antibody titers correlate with reductions in bladder bacterial load and infection duration (29-33). With continued effort, these data provide encouragement that an effective UPEC vaccine can be developed.

Design considerations.

A successful UPEC vaccine will likely require the following design

considerations. The target of a UPEC vaccine will need to be highly immunogenic, expressed by the bacterium *in vivo* (*i.e.*, during infection) and be surface-exposed on the bacterium in order to be accessible and recognized by the host immune system (34). In addition, ideal vaccine candidates should be pathogen-specific, as to avoid targeting host commensal *E. coli* in the gastrointestinal tract. The heterogeneous nature of the UPEC population will also need to be considered, as a required core set of virulence factors common to all UPEC isolates has yet to be identified. Designing a UTI vaccine that can be effective against such a diverse pathogen population as UPEC may prove to be a considerable challenge. Furthermore, an effective vaccine for UTI will need to generate a robust mucosal adaptive immune response in the urinary tract. To better enable a strong mucosal immune response, research into novel antigen delivery systems, routes of immunization, and adjuvants such as modified heat labile toxin, engineered outer membrane vesicles, and mast cell activators, among others, have been ongoing (35-39).

Modeling infection.

Much of our understanding of UPEC pathogenesis and our ability to evaluate UPEC vaccine designs has relied on the use of a mouse model of experimental UTI (for a recent review of mouse models of UTI see Hung *et al*. (40)). The use of mice to model UTI has proven to be an invaluable resource for advancing our knowledge of bacterial infection in the mammalian host. However, like all animal models of human disease, mouse models of UTI are not without limitations. Environmental, behavioral, and physiological differences between mice and humans, including differences in the urinary tract, such as urine concentration and contents (mouse urine contains more protein and is more concentrated compared to human urine), require that we avoid direct comparison

between human and mouse studies (41). Indeed, studies comparing UPEC gene expression between bacteria obtained from experimental UTI in the mouse and active human UTI have identified differences, indicating unsurprisingly that artificial infection of the mouse does not directly equate with natural infection in humans (42). Although mouse UTI models have been instrumental for UPEC vaccine design and we will include data from both animal models and human studies in this review, it is important to differentiate between data obtained through experimental infection in animals and those obtained through human studies. In the following sections, various UTI vaccination strategies as well as the future directions of the UPEC vaccine field will be discussed.

UPEC vaccine targets and formulations

Surface polysaccharides.

The carbohydrate-rich cell surface of UPEC, like all Gram-negative bacteria, contains an abundance of various polysaccharides. The external leaflet of the bacterium's outer membrane contains lipopolysaccharide (LPS; O antigen) and virtually all UPEC strains are covered in a protective polysaccharide coat, or capsule (K antigen). O-antigenic LPS, and to a greater extent, K-antigenic capsular polysaccharide are virulence factors that allow UPEC to evade host immune system assaults, such as opsonophagocytosis, complement-mediated killing, and damage by antimicrobial peptides (43, 44). Furthermore, recent work suggests that UPEC capsular polysaccharide and O antigen may cloak sub-capsular epitopes on the bacterial cell surface, obscuring them from recognition by host antibodies, demonstrating yet another mechanism by which surface polysaccharides may interfere with the host immune response (45).

Early UTI vaccine studies focused on surface polysaccharides as targets for immunization, and some O and K antigen-based UPEC vaccines did elicit a protective immune response in animal models of ascending UTI (Table 1-1)(46-50). However, substantial antigenic heterogeneity exists among *Escherichia coli* surface polysaccharides, including 167 different O serogroups and greater than 80 polysaccharide K antigens (51). Although certain K (K1, K5, K30, K92) and O (O1, O2, O4, O6, O7, O8, O16, O16/72, O18, O25, O50, and O75) antigenic groups are more prevalent among uropathogenic strains, designing a surface polysaccharide-based UPEC vaccine that can be effective against all UPEC serotypes is extremely challenging (52). In addition, many UPEC capsular polysaccharides are poorly immunogenic; camouflaged from the adaptive immune system by having a shared structural identity with the host. For example, the capsular antigen K1, which is present in an estimated 30% of pyelonephritis UPEC strains, is formed by repeating units of α-(2-8)-linked polysialic acid (52). An identical host structure is found in the carbohydrate portion of human neonatal neural cell adhesion molecule (n-CAM), which plays an important role in the organization of neural tissue (53). The adaptive immune system has protective mechanisms to prevent the generation of self-specific antibodies, and as a result, α-(2-8)-linked polysialic acid-specific antibodies are inefficiently produced, allowing K1-positive UPEC to avoid host adaptive immune detection (54). Other UPEC surface polysaccharide antigens, such as capsular antigen K5, display similar molecular mimicry, and their poor immunogenicity coupled with high heterogeneity pose a substantial barrier to the development of a polysaccharide-based UTI vaccine (55).

Table 1-1. Summary of surface polysaccharide vaccine studies targeting uropathogenic *Escherichia coli.*

Antigen	Species[a]	Route[b]	Adjuvant[c]	Method[d]/ Strain	Immunity[e]	Protection[f]	Study
O Antigen (O6)	R	SC, SP	None	IU/*E.coli* O6	IgG	B	(46)
K Antigen (K13)	M(BALB/c)	SC	None	IU/*E.coli* O6:K13:H1	IgG	NP	(48)
K Antigen (K13)	M(BALB/c)	SC	DT	IU/*E.coli* O6:K13:H1	IgG	K	(48)

[a]M, mouse; R, rat
[b]SC, subcutaneous; SP, surgical procedure
[c]DT, purified diphtheria toxoid
[d]IU, intraurethral
[e]IgG, significant increase of serum IgG
[f]K, significant reduction in kidney colonization; B, significant reduction in bladder colonization; NP, no protection

Whole cell and cell lysate formulations.

Vaccinating with whole or lysed fractions of inactivated pathogens can be an effective method to generate protective immunity and a number of successful vaccines against human pathogens including *Bordetella pertussis* (whooping cough), *Vibrio cholerae* (cholera), and *Salmonella typhi* (Typhus) contain killed whole bacteria (56). There are four standardized whole cell/cell lysate-based vaccines that have been tried for UTI with limited success (Table 1-2).

Urovac® (Solco Basel AG, Birsfelden, Switzerland & Protein Express, Cincinnati, Ohio, USA). The first such vaccine, Urovac®, was designed to provide broad protection by containing 10 heat-killed uropathogens: 6 UPEC strains, and one strain each of *Proteus mirabilis*, *Morganella morganii, Enterococcus faecalis,* and *Klebsiella pneumoniae.* The UPEC strains added to the Urovac® formulation possess several virulence factors, including hemolysin, type 1, P, and S fimbrial adhesins, cytotoxic necrotizing factor 1, several siderophores systems, the *E. coli* CFT073 pathogenicity island marker, and display the serotypes O: 1, 4, 6, 17, 75, 77; K: 1, 3, 5, 13, 95, and H: 1, 5, 7, 33 (57). Rodents immunized with Urovac® intramuscularly were protected for up to 20 weeks from experimental challenge by Urovac® homologous strains and some heterologous strains (strains that do not share any of the same O, K, or H antigens as the vaccine strains) (29, 30). Women volunteers with a history of recurrent UTI experienced a statistically significant reduction in recurrent infection (28 infections in 23 patients out of 202 immunized) in the 12 months following intramuscular injections of Urovac® (once weekly for 3 consecutive weeks), than women that did not receive the vaccine (84 infections in 47 patients out of 198) (58). Adverse side effects reported after Urovac®

injection were comparable to other bacterial vaccines, such as the DTaP vaccine for diphtheria, tetanus and pertussis, including redness at injection site (25% of patients), pressure (9%), pain (5%) and fever to 38ºC (3.5%) (58, 59). Subsequent trials delivered Urovac® vaginally, either with a vaginal suppository or an oil emulsion, to reduce the risk of endotoxin toxicity and adverse side effects, and to stimulate a more robust local mucosal immune response in the urinary tract (60). Phase II and extended Phase II clinical trials evaluating the efficacy of vaginally administered Urovac® found that women who received 6 total doses of vaccine, on weeks 0, 1, 2, 6, 10 and 14, gained short term protection from infection, having significant delays to reinfection during the first 8 weeks of the study in comparison to women who received placebos (61-64). However, over the full course of the six-month study, Urovac® immunization did not provide significant long-term protection from UTI or increase mean levels of UPEC-specific serum, urinary, or vaginal antibodies (61-64). Interestingly, although the average number of UTIs caused by any bacterial strain during the 6-month trial period was not significantly different between the vaccinated and placebo groups with 1.1 and 1.5 infections per patient respectively, the number of *E. coli*-caused UTIs was significantly decreased in the Urovac® vaccinated group (61-64). Of the women receiving Urovac®, 72% remained free from UTIs caused by *E. coli*, compared to women given placebos, of which only 30% remained free from *E. coli*-caused UTIs, suggesting that Urovac® may be successful in reducing the incidence of *E. coli*-caused UTIs in susceptible women. In addition, even with a vaginal route of delivery, some of the women reported adverse side effects, including single occurrences of low-grade fever 8% (4/50), a burning sensation

shortly after treatment 12% (6/50), nausea 8% (4/50), vaginal bleeding 8% (4/50), or vaginal rash 8% (4/50)(64).

OM-89/Uro-Vaxom® (OM Pharma, Myerlin, Switzerland). The second such standardized formula is Uro-Vaxom®, a lyophilized mix of membrane proteins from 18 UPEC strains that has been approved for use as a human therapeutic in Switzerland since 1988 and marketed and sold in almost 40 countries worldwide, excluding the United States and Canada. Uro-Vaxom® is prescribed as daily oral capsule and several double-blind, placebo-controlled clinical studies have evaluated the vaccine's safety and efficacy (65-69). A statistical meta-analysis of five clinical studies, involving 601 female participants given 90 days of treatment, showed Uro-Vaxom® to be significantly more effective than a placebo in preventing recurrent UTI (70). In a multicenter clinical trial funded by OM Pharma, women with a history of recurrent UTI were administered a daily oral capsule of Uro-Vaxom® for 90 days, given 3 months without treatment, and then boosted with a daily dose for 10 days in the beginning of months 7, 8, and 9 (68). After 12 months, women given Uro-Vaxom® experienced a statistically significant reduction in UTI recurrences (185 recurrences in 93 patients out of 220 total) in comparison to those given placebos (276 recurrences in 122 patients out of 215 total) (68). In addition, patients given Uro-Vaxom® experienced a reduction in the frequency in the signs and symptoms of UTI, including dysuria (painful urination), bacteriuria (presence of bacteria in the urine), and leukocyturia (presence of leukocytes in the urine) in comparison to women receiving placebos, although this reduction was only statistically significant during one of the 6 follow-up visits (68). Patient tolerance of Uro-Vaxom® is reported to be good with the most frequent adverse events being headache followed by

gastrointestinal events including pain and nausea and skin reactions (68, 69). Although Uro-Vaxom® does appear to reduce the incidence of recurrent UTI, with limited toxicity issues, the required daily administration may create problems with patient compliance.

Urvakol (Institute of Microbiology, Prague, Czech Republic) & Urostim (National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria). The third and fourth standardized whole cell vaccines, Urvakol and Urostim, are administered as daily oral tablets containing mixtures of inactivated uropathogens. Both formulations contain strains of *E. coli, P. mirabilis,* and *E. faecalis,* although Urvakol also includes a strain of *Pseudomonas aeruginosa*, whereas Urostim contains *K. pneumoniae.* Data from animal and patient studies demonstrate Urvakol and Urostim to have immunostimulating activity as measured by cytokine production and the presence of vaccine-specific antibodies in the serum, urine and saliva of patients after immunization (71-73). However, the ability of either vaccine to prevent recurrent UTI has not been established as well-structured clinical trials have yet to be completed.

Table 1-2. Summary of whole cell and cell lysate vaccine studies targeting uropathogenic *Escherichia coli*.

Antigen	Species[a]	Route[b]	Adjuvant[c]	Method[d]/ Strain	Immunity[e]	Protection[f]	Study
Urovac®	H	IM	None	---	---	Y	(58)
Urovac®	H	V	None	---	sIgA, IgG	Y	(61, 63, 64, 74)
Urovac®	P	V, IM	MO, None	VUR/JR1	IgG	NP	(75)
Urovac®	R	IM	AP	VUR/ATCC 23500, 23513, 23504, 9018	---	K	(30)
Urovac®	M(NMRI)	IP, IM	AP	IP/ATCC 23500, 23513, 23504, 9018	sIgA, IgG	LC	(29, 30)
Urovac®	M(BALB/c)(C57B1/6)	IP	None	VUR/1677	---	B, K	(31)
Urovac®	M(BALB/c)(C57B1/6)	V	MO	VUR/1677	ND	B	(31)
OM-89/ Urovaxom®	H	O	None	---	IgG	Y	(65, 67, 68)
Urostim	H	O	None	---	IgA, IgG	---	(73)

[a]M, mouse; R, rat; H, Human; P, non-human primates
[b]IM, intramuscular; V, vaginal; IP, intraperitoneal; O, oral
[c]MO, mineral oil; AP, Aluminum phosphate
[d]IP, intraperitoneal; VUR, intravesically per urethra reflux; ---, no challenge
[e]IgG, significant increase of serum; sIgA, significant increase in urinary IgA; IgA, significant increase in serum IgA; ND, not detected; ---, not determined
[f]K, significant reduction in kidney colonization; B, significant reduction in bladder colonization; NP, no protection; LC, significant protection from lethal challenge; Y, significant decrease in UTI incidence; ---, not determined

Genetically engineered strains.

In addition to the whole cell/cell lysate, inactivated UTI vaccines being designed and tested in Europe, vaccines based on genetically modified strains of UPEC are also in development (Table 1-3). To determine if UPEC's poorly-immunogenic surface polysaccharides impede the generation of an optimal host humoral response, Russo and colleagues immunized mice with a genetically modified isogenic strain of pathogenic *E. coli*, termed CP923, that is unable to produce capsule or the O antigen of LPS due to a transposon-insertion into a gene that encodes for an enzyme involved in rhamnose biosynthesis (76). Mice immunized intranasally with formalin-killed CP923 generated a significantly greater overall humoral immune response, as measured by serum antibody levels, than mice immunized with the wild-type parental strain CP9 (76). In addition, immunization with CP923 generated significantly greater levels of antibodies directed against non-capsular and non-O-antigenic epitopes, a desirable outcome for a vaccine directed against the highly heterologous UPEC population (76). However, when the formalin-killed CP923 vaccine was tested in an intravenous sepsis model of infection, mice intranasally immunized with CP923 were not significantly protected from experimental infection by CP9, in comparison to PBS-immunized controls (45). Data from *in vitro* binding and bactericidal assays suggest that the failure of the CP923 vaccine to protect against CP9 infection, despite generating high serum antibody titers, may be due to interference by CP9 surface polysaccharides (45). Since UPEC strains are generally positive for capsule and O antigen, the potential of surface polysaccharides to mask non-polysaccharide epitopes and reduce the efficiency of antibody binding, may

provide additional motivation to identify capsular antigens or vaccine targets that are external to the capsule.

Employing a vaccine strategy with a similar genetically modified *E. coli* strain with impaired surface polysaccharide expression, Billips and coworkers (77) assessed the use of a live-attenuated UPEC vaccine to prevent UTI. The authors produced an attenuated mutant UPEC strain unable to persist in the host urinary tract due to a targeted deletion of a gene that encodes an O antigen ligase (*waaL*), an enzyme required for joining the variable O antigen to the LPS lipid A-core during biosynthesis (77). Mice inoculated with the live-attenuated UPEC strain (NU14 Δ*waaL*), via a urinary catheter to the bladder, were protected from experimental UTI challenge with the wild type parental strain NU14, as well as a range of clinical UPEC isolates including CFT073 (77). Promisingly for UPEC vaccine development, although the live-attenuated vaccine strain, NU14 Δ*waaL*, failed to persist longer than two-weeks in the mouse bladder after inoculation, the protective immunity generated post-immunization lasted at least eight weeks after vaccination (77).

Table 1-3. Summary of genetically engineered vaccine studies targeting uropathogenic *Escherichia coli.*

Antigen	Species[a]	Route[b]	Adjuvant	Method[c]/ Strain	Immunity[d]	Protection[e]	Study
UPEC CP9	M(C57BL/6J)	IN	None	---	IgG	---	(76)
UPEC CP923	M(C57BL/6J)	IN	None	---	IgG	---	(76)
UPEC NU14	M(C57BL/6J)	TU	None	IU/NU14	---	B	(77)
UPEC NU14 Δ*waaL*	M(C57BL/6J)	TU	None	IU/NU14, CFT073	---	B	(77)

[a]M, mouse
[b]IN, intranasal; TU, transuretheral
[c]IU, intraurethral; ---, no challenge
[d]IgG, significant increase of serum IgG; ---, not determined
[e]B, significant reduction in bladder colonization; ---, not determined

Targeting UPEC adherence.

UPEC encode a wide variety of adhesins, a general term used to describe extracellular proteins that facilitate bacterial attachment to, or invasion of host cells, such as fimbriae or pili. Indeed, fimbrial adhesins were among the first recognized UPEC virulence factors and the genomes of UPEC isolates generally contain many more fimbrial operons (8.3 ± 1.3) than those of commensal *E. coli* strains (2.0 ± 2.3) (78). For example, the genome of the prototypical UPEC strain CFT073 encodes 12 putative fimbrial operons (12). Many of these fimbriae enable colonization of the host urinary tract and it was theorized that blocking bacterial adherence to host epithelial cells by the binding of antibodies would prevent colonization (79). Naturally, the critical role of adherence during infection has made fimbrial adhesins a prime target for UPEC vaccines. UPEC genomes generally encode multiple fimbriae. Thus far, however, only a small subset of UPEC fimbriae has been evaluated for use in a UTI vaccine, including P, Dr, and type 1 fimbriae (Table 1-4).

The UPEC adhesin, PapG, which is the tip adhesin on P fimbriae, binds to Gal(α1-4) Gal-specific glycosphingolipids on kidney epithelium (P blood group antigen) and likely plays an important role in UPEC human kidney colonization (80). Vaccination studies using P fimbrial subunits in murine and primate models of ascending UTI found it to be effective in preventing kidney infection (81-83). However, P fimbriae has a limited role during UPEC bladder colonization and as a result, vaccines targeting the P fimbrial adhesion, PapG, will need to be combined with additional components that can provide adequate protection from infection in the bladder.

Dr fimbriae bind to type IV collagen and decay-accelerating factor (DAF/CD55)

on the tubular basement membrane and Bowman's capsule of the human kidney and contribute to UPEC pathogenesis in animal models of UTI (84, 85). Mice experimentally infected with a strain of UPEC, positive for Dr fimbriae, maintained higher and more prolonged kidney bacterial loads and developed significantly more tubulointerstitial nephritis than mice infected with a Dr fimbriae knockout mutant (86). Vaccinating mice with purified Dr fimbriae produced high titers of serum anti-Dr antibodies and significantly reduced experimental UTI-associated mortality, but did not affect the rate of bladder or renal colonization (87). Pre-incubating Dr^+ UPEC with Dr-immunized mouse sera resulted in an observable reduction in bacterial adherence to mouse bladders and kidneys, although the same UPEC anti-binding activity was not observed when Dr^+ UPEC were pre-incubated with the urine from Dr-immunized mice (87). Further study is needed to fully test the potential of Dr fimbria as a UPEC vaccine target as it is possible that modifying the method or route of Dr antigen delivery may improve vaccine efficacy.

The type 1 fimbrial adhesion FimH mediates UPEC adherence to bladder epithelial cells by binding to mannose residues on uroplakin, a major structural component of the bladder epithelium (88). In the mouse, type 1 fimbria is a particularly important UPEC virulence factor, as its expression greatly enhances UPEC binding and colonization of the murine bladder during experimental UTI; a process that can be outcompeted by administering a competitive inhibitor of FimH, such as methyl α-D-mannopyranoside, with the challenging UPEC strain at the time of inoculation (89-91). A knockout mutant UPEC strain deficient in FimH production due to a targeted deletion of the *fimH* gene, failed to bind human and mouse bladder epithelial tissues, a phenotype that could be restored by complementing *fimH* expression off a plasmid (92). The

discovery that cystitis could potentially be prevented or treated through disrupting type 1 fimbria-mediated UPEC adherence to bladder epithelium, either pharmacologically or with host-generated antibodies, galvanized efforts to develop FimH-based vaccine designs. Indeed, mice immunized with FimHt, a mannose-binding truncated form of FimH, or a complex containing the periplasmic chaperone FimC bound to the full-length FimH protein, termed FimCH, were significantly protected from experimental UPEC infection in the bladder, exhibiting a 100- to 1000-fold reduction in the number of colonizing bacteria in comparison to adjuvant-only immunized controls (92). Immunization with the FimHt vaccine was able to generate a substantial and long-lasting humoral immune response as immunized mice had significantly higher levels of FimH-specific urinary IgG, an outcome that correlated with the levels of protection from infection in the bladder (92). Cynomolgus monkeys immunized with the FimCH vaccine elicited a strong systemic humoral response and three out of four were protected from experimental UTI 48 hours post inoculation, in comparison with zero out of four monkeys in the adjuvant-only control group (93, 94).

In 1999, the Maryland-based biotechnology company MedImmune, Inc. brought the UPEC type 1 fimbria subunit vaccine, FimCH, to phase II clinical trials with women volunteers. Unfortunately, MedImmune formally announced in early 2003 the discontinuation of further research and development of the FimCH vaccine, citing that the previous clinical trials failed to demonstrate a sufficient level of efficacy in prevention of UTIs to warrant additional larger Phase-III studies (95-97). Since the data from the FimCH Phase II clinical trials has not been published, the level of efficacy of the FimCH vaccine in humans remains unclear. In the decade since the start of FimCH

clinical trials, additional research on UPEC type 1 fimbriae has further complicated our understanding of its role during human infection. Microarray and transcriptional analysis data from women with active UTI suggest that genes encoding type 1 fimbrial subunits may not be highly expressed during UPEC infection of the human urinary tract (42, 98). In addition, the expression of type 1 fimbriae is phase variable, as its promoter region resides on an invertible element that switches *fim* expression between ON and OFF (99, 100). Variation in type 1 fimbriae expression may allow invading UPEC to avoid detection from an adaptive immune response targeting this organelle. Lastly, recent evidence suggests that antibodies generated against the adhesive tip of type 1 fimbriae, FimH, do not target the mannose binding pocket or block FimH adhesion, may actually enhance the binding affinity of FimH for mannose, and are shed as mannose-bound FimH undergoes a conformational shift during binding (101). While these results may be at odds with our previous understanding of FimH-mediated vaccine protection, they may suggest an alternative explanation for FimH-based vaccine efficacy in mice. The recent evidence suggesting UPEC gene expression may be different between mouse and human infection, and the experience with the FimCH vaccine, highlights our need to better understand UPEC antigen expression in the human host. Although the mouse model of ascending UTI is a valuable resource for testing vaccine efficacy and design, our ability to choose promising UPEC vaccine candidates would benefit enormously from having a more complete understanding of UPEC virulence gene expression during UTIs in humans.

Table 1-4. Summary of fimbrial vaccine studies targeting uropathogenic *Escherichia coli.*

Antigen	Species[a]	Route[b]	Adjuvant[c]	Method[d]/ Strain	Immunity[e]	Protection[f]	Study
P fimbria	M(BALB/c)	IM	CFA, IFA	IV/J96	IgG	K	(102)
PapDG	P	IP	AP	IU/DS17	IgG	NP	(83)
Dr fimbria	M(C3H/HeJ)	ND	CFA, IFA	IU/IH11128	IgG	NP	(87)
FimCH and FimHt	M(C3H/HeJ)	SC	CFA, IFA	IU/NU14	IgG	B, K	(92)
FimCH	P	IM	MF59	IU/NU14	IgG	U	(93)

[a]M, mouse; P, non-human primates
[b]IM, intramuscular; IP, intraperitoneal; SC, subcutaneous; ND, not disclosed
[c]CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant; AP, Aluminum phosphate
[d]IV, intravascular; IU, intraurethral
[e]IgG, significant increase of serum IgG
[f]K, significant reduction in kidney colonization; B, significant reduction in bladder colonization; NP, no protection; U, reduction of UTI as determined by analysis of urine

Toxins.

Vaccines containing inactivated bacterial toxins, termed toxoids, have been effective against a select number of bacterial pathogens and many of our current routine childhood vaccinations are toxoid-based, including the diphtheria, tetanus and acellular pertussis vaccine (DTaP) that contains both diphtheria and tetanus toxoids (56). UPEC genomes frequently contain genes that encode toxins including α-hemolysin (HlyA), cytotoxin necrotizing factor 1 (CNF1), cytolethal distending toxin (CdtB), and secreted autotransporter toxins Sat, Pic, and Tsh (103). Many of these toxins have been associated with symptom severity during urinary tract infection, such as increased inflammation, bladder epithelial cell shedding, and renal damage, but none have been shown to be required for infection and thus may represent less than ideal vaccine candidates (104, 105). When the pore forming cytolytic toxin, α-hemolysin, was administered intramuscularly to mice, which were then challenged with an intravesicular injection of UPEC, no significant protection was acquired (102). It was noted, however, that the HlyA-immunized mice did suffer significantly less renal damage than their PBS-immunized counterparts and although, toxoid-based vaccines may not be applicable for reducing the incidence of UTI, they may be effective at reducing infection severity in certain high-risk patient populations (Table 1-5).

Table 1-5. Summary of toxin vaccine studies targeting uropathogenic *Escherichia coli*.

Antigen	Species[a]	Route[b]	Adjuvant[c]	Method[d]/ Strain	Immunity[e]	Protection[f]	Study
Denatured α-hemolysin	M(BALB/c)	IM	CFA, IFA	IV/J96	IgG	NP*	(102)
Denatured α-hemolysin	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	ND	LC	(106)

[a]M, mouse
[b]IM, intramuscular; SC, subcutaneous
[c]CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant
[d]IV, intravascular; IP, intraperitoneal
[e]IgG, significant increase of serum IgG; ND, none detected
[f]NP, no protection; LC, significant protection from lethal challenge
*Did not prevent kidney colonization, but immunized mice had less renal damage.

Iron acquisition systems.

Nearly all forms of life require iron. Iron is an essential cofactor for enzymes involved in primary and secondary cellular metabolism, and a critical component of normal cell physiology. UPECs ability to colonize the host urinary tract is dependent on iron acquisition (107). Although the human body contains substantial iron, the majority is inaccessible to invading microorganisms as it is bound to the oxygen-carrying heme group within hemoglobin or sequestered by iron storage molecules such as ferritin and hemosiderin. The scarcity of free iron in the host is a major obstacle for microbial growth and the ability to circumvent this barrier is a hallmark of many successful bacterial pathogens (108).

UPEC survives in the iron-limited host urinary tract by upregulating the expression of molecular iron acquisition systems that synthesize and secrete small organic iron-chelating molecules called siderophores (42, 109). Siderophores have extremely high affinity for ferric iron and can successfully compete with host proteins for iron resources. Once iron is complexed, the ferri-siderophore is imported back into the bacterial cell through their respective outer membrane receptors, thereby allowing iron to be extracted from the host and used by the bacterium for its survival (110). In addition to expressing outer membrane iron receptors specific for its own siderophores, UPEC opportunistically expresses outer membrane receptors for iron-containing host molecules, such as heme, and the siderophores of other microorganisms, such as the fungal siderophore, ferrichrome (Figure 1-3).

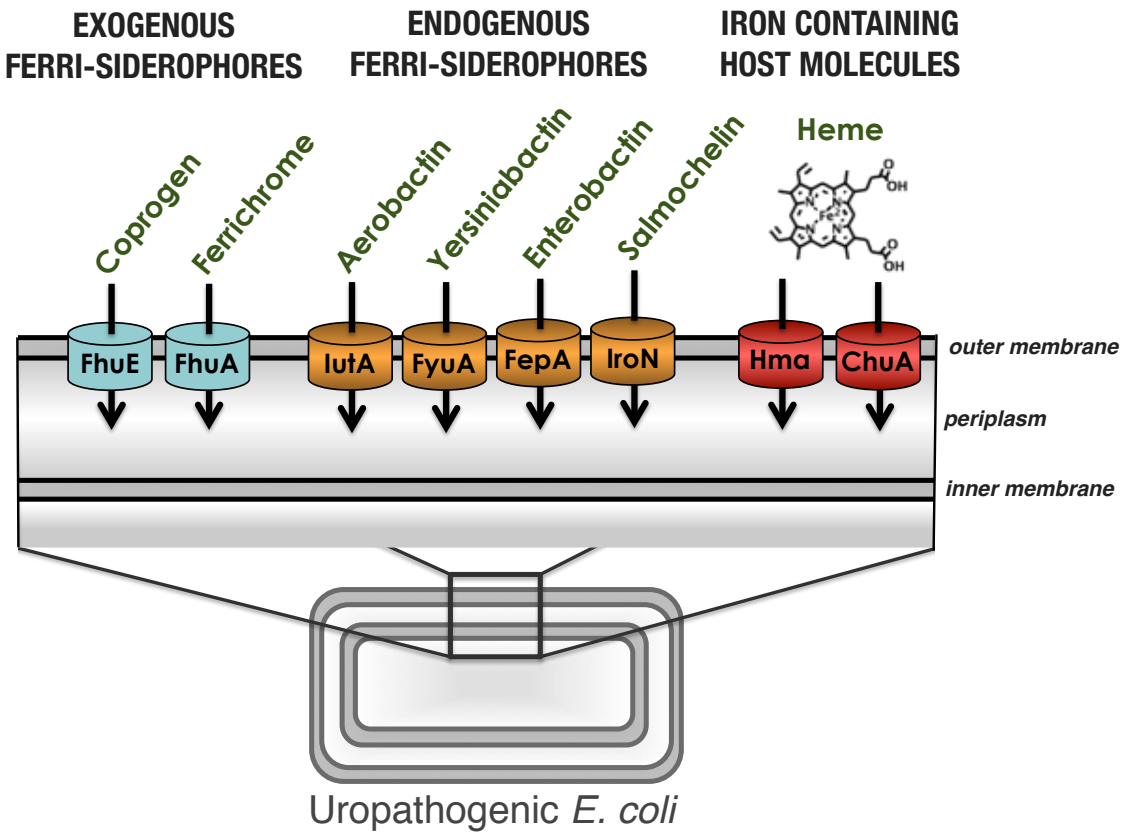


Figure 1-3. Classes of UPEC outer membrane iron receptors. A cartoon depicting the various classes of β-barrel outer membrane iron receptors used by UPEC strains. These include: exogenous siderophore receptors that import siderophores synthesized by microorganisims other than UPEC (blue), endogenous siderophore receptors that import UPEC-synthesized siderophores (orange), and heme receptors (red).

In contrast to most commensal *E. coli* strains that encode few iron-acquisition systems, UPEC expresses a large arsenal of iron-acquisition systems (111). For example, the genome of prototypical pyelonephritis UPEC strain CFT073 encodes 14 characterized outer membrane iron compound receptors, as well as the biosynthesis machinery for three different siderophores: catecholates enterobactin and salmochelin, and the hydroxamate aerobactin (12). Many outer membrane iron compound receptors share the characteristics of an ideal UPEC vaccine target: 1) they are surface-exposed on the bacterium, 2) expressed during infection, and 3) prevalent among pathogenic *E. coli* strains. Thus far, seven UPEC outer membrane iron compound receptors (IroN, IreA, IutA, FyuA, Iha, Hma, and ChuA) have been evaluated as vaccine candidates to prevent UTI, five of which (IroN, IreA, IutA, FyuA, and Hma) significantly protect in a mouse model of infection, demonstrating that outer membrane iron receptors are a promising new class of UPEC vaccine targets (Table 1-6) (32, 112, 113).

Of the four possible siderophores produced by *E. coli* clinical isolates, three are more often produced by pathogens: aerobactin, yersiniabactin, and salmochelin (111, 114). As a result, the outer membrane receptors for these siderophores have been investigated as prospective targets for vaccination. Subcutaneous immunization with a denatured form of the UPEC salmochelin outer membrane receptor, IroN, conferred significant protection from experimental UPEC infection in mouse kidneys and produced a significant IroN-specific serum IgG response (112). However, IroN immunization did not induce a significant systemic or mucosal IgA response or protect from experimental infection in the bladder, an outcome that could possibly be improved upon through the use of an adjuvant or alternative route of vaccine administration. Intranasal immunization

with the aerobactin receptor, IutA, conjugated to cholera toxin as adjuvant, significantly protected mice from experimental UPEC infection in both the bladder and kidneys, and induced a significant increase in IutA-specific urinary IgA (32). Although aerobactin production is pathogen-associated, gene expression data using mRNA isolated directly from bacteria in urine of women with active UTIs suggest that aerobactin production may not be widely abundant among UPEC strains, limiting the usefulness of an IutA-based UPEC vaccine (42). Subcutaneous immunization with the UPEC yersiniabactin receptor, FyuA, combined with Freund's adjuvant, significantly protected mice from death in a lethal sepsis model of pathogenic *E. coli* infection (113). Passive immunization with purified sera from FyuA-immunized rabbits demonstrated the protection to be immunoglobulin-mediated.

In addition to the outer membrane receptors that facilitate ferrisiderophore import, UPEC vaccines have also targeted bacterial outer membrane receptors for host heme, the iron containing prosthetic group of hemoglobin and other hemoproteins. Intranasal immunization with the heme receptor Hma conjugated to cholera toxin as adjuvant, significantly protected mice from experimental infection with UPEC in the kidney, but not the bladder (32). Surprisingly, immunization with an alternative UPEC heme receptor, ChuA, did not provide significant protection from experimental infection (32, 113).

Although iron acquisition is required for UPEC colonization of the host urinary tract, no single essential iron acquisition system has yet to be identified. Indeed, many UPEC iron acquisition systems may be functionally redundant and unequally expressed among UPEC strains, requiring a successful iron receptor-based UTI vaccine to target

more than one iron acquisition system to be broadly effective (42, 115, 116). Multiepitope vaccines that contain domains from many outer membrane iron receptors address this dilemma by generating protective immunity against several UPEC iron acquisition systems (Table 1-7). Recently, Wieser and colleagues (117) constructed two such multiepitope subunit vaccines (designated Vol1 and Vol2) containing domains from six *E. coli* outer membrane iron receptors (FyuA, IroN, ChuA, IreA, IutA and Iha) and the uropathogenic-specific protein (Usp) connected by spacer domains on single recombinant proteins. Intranasal immunization of mice with either Vol1 or Vol2, conjugated to cholera toxin as adjuvant, was able to significantly reduce UPEC bacterial loads in the liver 48 hours after experimental intraperitoneal UPEC challenge (117). However, only immunization with the Vol1 construct, containing domains from FyuA, IutA, Iha, and Usp, was able to significantly decrease experimental UPEC bacterial loads in the spleen (117). Similar results were obtained when the multiepitope vaccines were administered via a live bacterial antigen delivery system based on the *Salmonella* Type-III secretion system (118). In agreement with earlier results, immunization with the Vol1 (pST1) construct significantly reduced bacterial loads in the murine liver and spleen, in contrast to the Vol2 (pST2) that did not induce significant protection (118). These data from the multiepitope and iron receptor-based immunization studies are encouraging for UTI vaccine development and continued research into UPEC iron acquisition during infection may help inform future vaccine designs that target UPEC, as well as other pathogens that depend on iron acquisition to survive within the host.

Table 1-6. Summary of iron acquisition vaccine studies targeting uropathogenic *Escherichia coli*.

Antigen	Species[a]	Route[b]	Adjuvant[c]	Method[d]/ Strain	Immunity[e]	Protection[f]	Study
IroN denatured	M(BALB/c)	SC	None	IV/CP9	IgG	K	(112)
IroN denatured	M(BALB/c)	SC	CFA	IP/S26	IgG	LC	(113)
IroN renatured	M(CBA/J)	IN	CT	IU/CFT073	IgG	NP	(119)
IroN peptide	M(CBA/J)	IN	CT	IU/CFT073	IgG	NP	(32)
FyuA denatured	M(BALB/c)	SC	CFA	IP/S26	IgG	LC	(113)
FyuA renatured	M(CBA/J)	IN	CT	IU/536	sIgA, IgG	K	(119)
c0294 renatured	M(CBA/J)	IN	CT	IU/CFT073	IgG	NP	(119)
IutA renatured	M(CBA/J)	IN	CT	IU/CFT073	sIgA, IgG	B, K	(32)
IutA peptide	M(CBA/J)	IN	CT	IU/CFT073	IgG	NP	(32)
IreA renatured	M(CBA/J)	IN	CT	IU/CFT073	sIgA, IgG	B	(32)
Iha renatured	M(CBA/J)	IN	CT	IU/CFT073	sIgA, IgG	NP	(32)
Hma renatured	M(CBA/J)	IN	CT	IU/CFT073	sIgA, IgG	K	(32)
ChuA renatured	M(CBA/J)	IN	CT	IU/CFT073	sIgA, IgG	NP	(32)
ChuA truncated (C)	M(CBA/J)	IN	CT	IU/CFT073	IgG	NP	(119)

ChuA truncated (M)	M(CBA/J)	IN	CT	IU/CFT073	IgG	NP	(119)
ChuA denatured	M(BALB/c)	SC	CFA	IP/S26	ND	NP	(113)
FitA renatured	M(CBA/J)	IN	CT	IU/CFT073	IgG	NP	(119)
ECOK1_3457 (FitA)	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	ND	LC	(106)

[a]M, mouse
[b]SC, subcutaneous; IN, intranasal
[c]CT, cholera toxin; CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant
[d]IV, intravascular; IU, intraurethral; IP, intraperitoneal
[e]IgG, significant increase of serum IgG; sIgA, significant increase in urinary IgA; ND, none detected
[f]K, significant reduction in kidney colonization; B, significant reduction in bladder colonization; NP, no protection; LC, significant protection from lethal challenge

Table 1-7. Summary of multiepitope subunit vaccine studies targeting uropathogenic *Escherichia coli*.

Antigen	Species[a]	Route[b]	Adjuvant[c]	Method[d]/ Strain	Immunity[e]	Protection[f]	Study
Vol1 (FyuA, IutA, Iha, Usp)	M(BALB/c)	IN	CT	IP/CFT073	sIgA, IgG	S, L	(117)
Vol2 (IroN, ChuA, IreA)	M(BALB/c)	IN	CT	IP/CFT073	sIgA, IgG	L	(117)
pSTI (FyuA, IutA, Iha, Usp)	M(BALB/c)	O	T3SS	IP/CFT073	ND	S, L	(118)
pST2 (IroN, ChuA, IreA)	M(BALB/c)	O	T3SS	IP/CFT073	ND	NP	(118)

[a]M, mouse
[b]IN, intranasal; O, oral
[c]CT, cholera toxin; T3SS, *Salmonella* type 3 secretion system
[d]IP, intraperitoneal
[e]IgG, significant increase of serum IgG; sIgA, significant increase in urinary IgA; ND, none detected
[f]NP, no protection; S, significant reduction in spleen colonization; L, significant reduction in liver colonization

Challenges to UPEC vaccine design.

Strain diversity.

While select individual antigen-based UPEC vaccines may successfully prevent experimental infection in animal models, a broadly effective UTI vaccine may need to target more than one virulence factor to be clinically useful against the highly heterogeneous UPEC population. Substantial diversity exists within classes of UPEC virulence factors, such that, not every UPEC strain expresses the exact same set of virulence-associated genes during infection (42). Although the genomes of pathogenic *E. coli* frequently encode many more virulence factors than commensal *E. coli* strains, the absence of a required core set of virulence factors complicates UTI vaccine design (12, 78). Targeting a single virulence factor may only be effective against a select group of UPEC strains. Vaccine strategies that target multiple virulence factors, like the multiepitope vaccines constructed by Wieser and coworkers (117), provide a solution to the problem of UPEC strain diversity. Through targeting multiple members of a class of virulence factors, such as multiple fimbrial adhesins, or multiple iron receptors, rather than an individual, it may be possible to overcome UPEC diversity and design a clinically effective vaccine for UTI.

Renal immunology and UPEC pathogenesis is poorly understood.

In addition to tackling the ongoing challenge presented by UPEC strain diversity, UTI vaccine development could be better guided through further understanding UPEC pathogenesis and the host mucosal immune response to infection. It is still unclear why we are unable to generate an effective adaptive immune response after an initial UPEC infection, leaving us susceptible to repeat infection with the same UPEC strain. Future

UTI vaccine development strategies may need to include considerations for mechanisms by which UPEC may be impeding the host's generation or implementation of a protective adaptive immune response. Mechanisms by which UPEC may be subverting the host immune response include interference with Toll-like receptor signaling, the formation of intracellular bacterial communities, the suppression of cytokine secretion, impedance of antibody binding, and reduction of the secretion of secretory IgA (27, 45, 120-122). Understanding UPEC immune modulating mechanisms could help guide strategies to combat repeat infection. In addition, better understanding the mucosal immune response to infection could provide guidance to more effective vaccine delivery systems and adjuvants. Defining what specific factors influence the robustness and longevity of a mucosal immune response in the urinary tract would allow vaccine delivery systems and adjuvants to be tailored more effectively. The development of novel adjuvants to improve the mucosal immune response to vaccines, as well as increasing our knowledge of how UPEC interacts with the host immune system could help inform rational UTI vaccine design.

Shift towards a targeted approach to vaccine design.

As well as further exploring the potential of already identified promising vaccine candidates; novel vaccine candidate discovery screens are helping to identify previously unrecognized UTI vaccine targets. Vaxign, a web-based vaccine design program developed by He and colleagues (123), was used to predict new UPEC vaccine targets based on a reverse vaccinology strategy. In addition to Vaxign, other discovery screens have been employed to identify novel vaccine targets. In a "subtractive reverse

vaccinology screen" by Moriel and coworkers (106), vaccine antigens were predicted using a bioinformatic analysis of three ExPEC strains (CFT073, 536, IHE3034). Antigens from CFT073, 536 and IHE3034 predicted to be surface associated or secreted, with three or fewer transmembrane domains were selected and compared against non-pathogenic *E. coli* strains (MG1655, DH10B, W3110) for exclusion. By this approach, 230 potential antigens were identified and tested in a mouse model of sepsis, nine of which were newly identified and found to protect against experimental ExPEC infection (Table 1-8) (106). Despite ongoing challenges, the progress towards discovering and testing novel vaccine candidates, adjuvants and delivery methods is promising and we are hopeful for the future development of a vaccine to prevent UTI.

Table 1-8. Summary of hypothetical protein vaccine studies targeting uropathogenic *Escherichia coli*.

Antigen	Species[a]	Route[b]	Adjuvant[c]	Method[d]/ Strain	Immunity[e]	Protection[f]	Study
c1275	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	---	LC	(106)
c5321	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	---	LC	(106)
c0975	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	---	LC	(106)
ECOK1_3385	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	---	LC	(106)
ECOK1_3473	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	---	LC	(106)
ECOK1_0290	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	---	LC	(106)
ECOK1_3374	M(CD1)	SC	CFA, IFA	IP/IHE3034, CFT073; IV/536	---	LC	(106)

[a]M, mouse
[b]SC, subcutaneous
[c]CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant
[d]IP, intraperitoneal; IV, intravascular
[e]---, not determined
[f]LC, significant protection from lethal challenge

CHAPTER 2

IMMUNIZATION WITH THE YERSINIABACTIN RECEPTOR, FYUA, PROTECTS AGAINST PYELONEPHRITIS

Author's Note: This chapter is a modified version of the previously published research article: Brumbaugh AR, Smith SN and Mobley HLT. Immunization with the Yersiniabactin Receptor, FyuA, Protects Against Pyelonephritis in a Murine Model of Urinary Tract Infection. 2013. *Infect Immun* 81**:**3309-3316

Abstract

Urinary tract infections (UTI) are common and represent a substantial economic and public health burden. Roughly 80% of these infections are caused by a heterogeneous group of uropathogenic *Escherichia coli* (UPEC) strains. Antibiotics are standard therapy for UTI, but a rise in antibiotic resistance has complicated treatment, making the development of a UTI vaccine more urgent. Iron receptors are a promising new class of vaccine targets for UTI as UPEC require iron to colonize the iron-limited host urinary tract, and genes encoding iron acquisition systems are highly expressed during infection. Previously, three of six UPEC siderophore and heme receptors were identified as vaccine candidates by intranasal immunization in a murine model of ascending UTI. To complete the assessment of iron receptors as vaccine candidates, an additional six UPEC iron receptors were evaluated. Of the six vaccine candidates tested in this study (FyuA, FitA, IroN, the gene product of the CFT073 locus *c0294,* and two truncated derivatives of ChuA), only FyuA provided significant protection (p = 0.0018) against UPEC

colonization. Intranasal immunization induced a robust and long-lived humoral immune response. In addition levels of FyuA-specific serum IgG correlated with bacterial loads in the kidneys ($\rho(14) = -0.72$, $p = 0.0018$), providing a surrogate of protection. FyuA is the fourth UPEC iron receptor to be identified from our screens, in addition to IutA, Hma and IreA, which were previously demonstrated to elicit protection against UPEC challenge. Together, these iron receptor antigens will facilitate the development of a broadly protective, multivalent UTI vaccine to effectively target diverse strains of UPEC.

Introduction

The human urinary tract is one of the most common sites for bacterial infection, second only to the respiratory tract (124). Most urinary tract infections (UTI) are caused when pathogenic bacteria, commonly found in the gastrointestinal tract, colonize the perineum and traverse the urethra to cause an infection in the bladder, clinically termed cystitis. Left untreated, cystitis can progress to pyelonephritis as colonizing bacteria ascend the ureters to cause a secondary infection in the kidneys (6). In severe cases, invading bacteria can breach epithelial and endothelial barriers in the kidney to gain access to the bloodstream leading to systemic infection and sepsis, a serious and sometimes fatal complication (7).

While most UTIs seldom cause life-threatening or long-term health problems, the regularity at which they occur generates a substantial economic and public health burden (125). An estimated half of all women and 12% of men will experience a UTI in their lifetime and almost a quarter of women who have one UTI will experience a second within 6-12 months (2). Commonly these infections become recurrent, with an estimated three percent of women suffering from very frequent and often constant UTI (3). At the community level, frequent UTIs tax healthcare and financial resources, requiring over five million physician office visits, two million emergency room visits (126), and 500,000 hospitalizations annually in the United States (127), with associated annual costs estimated at $3.5 billion (5).

To prevent more serious infection and speed recovery, patients with UTI are generally treated with a course of antibiotics, and individuals with recurrent infection may be prescribed antibiotics prophylactically (14). However, uropathogen resistance

rates to first- and second-line antibiotic therapies are steadily climbing, which can complicate treatment and lead to therapeutic failure (15-17). For example, the resistance rate of community-acquired UTI isolates to the first-line antibiotic trimethoprim–sulfamethoxazole (TMP–SMX) currently exceeds 20% in most areas (15). Likewise, between 6-11% of community-acquired UTI isolates are resistant to the second-line fluoroquinolone agents, ciprofloxacin and levofloxacin (128-130), and alarmingly, roughly 25% of catheter-associated UTIs are fluoroquinolone-resistant (131). Multidrug resistance is also on the rise, so that now over 10% of cystitis isolates are resistant to at least three different classes of antimicrobial agents (22). Together, frequent and recurrent infection along with rising rates of antimicrobial resistance compromise effective long-term treatment for UTI, making the development of alternative management therapies for UTI essential.

Vaccine development represents a rational alternative approach to UTI prevention, where the most common cause of UTI, uropathogenic *Escherichia coli* (UPEC), can be specifically targeted (132-134). UPEC represent a heterogeneous group of extraintestinal pathogenic *E. coli* strains that are responsible for roughly 75-80% of all uncomplicated, or community-acquired UTIs (1) and an estimated 60% of complicated UTIs (135), or UTIs that occur in individuals where natural barriers to infection have been eroded by underlying conditions such as pregnancy or catheterization. Although UPEC strains can reside in the human gastrointestinal tract without causing disease, once in the urinary tract they use an arsenal of virulence factors to colonize and survive in this alternative ecological niche, inducing a robust inflammatory immune response (52). While certain virulence factors can be more prevalent among UPEC strains, as of yet, no

core set of virulence factors required for UPEC to cause UTI have been determined, making the identification of optimal UPEC vaccine targets a challenge (57).

Given that conventional vaccinology approaches targeting established UPEC virulence factors have yet to produce a commercially available vaccine for UTI, we undertook an alternative and unbiased, functional vaccinology approach to vaccine discovery that has been employed successfully against other bacterial pathogens such as *Streptococcus pneumoniae* (136), *Salmonella* (137) and *Neisseria meningitidis* (138). By analyzing data compiled from a series of genomic (109, 139-141), proteomic (142-145) and metabolic (111) screens, we were able to select UPEC antigens that fit criteria hypothesized to be desirable in a vaccine antigen, which we describe as *PASivE*: Pathogen-specific, Antigenic, Surface-exposed, and *in vivo* Expressed (34). Screening vaccine antigens for *PASivE* criteria ensured that the vaccine targets selected would not be expressed by commensal *E. coli*, would be accessible to and recognized by the host immune system, and be highly expressed and likely important for UPEC pathogenesis during UTI. Of the 5379 predicted proteins encoded by the prototype pyelonephritic UPEC strain CFT073, only six proteins, all involved in iron acquisition met the rigorous *PASivE* criteria (146).

Of the vaccine candidates identified using PASivE criteria, three (Hma, IutA, IreA) were found to protect against experimental UTI, establishing outer membrane iron receptors as a practical class of UPEC vaccine antigens (146). The purpose of this study was to complete the characterization of iron receptors as vaccine antigens by evaluating an additional six UPEC outer membrane iron receptors. Here, we describe the identification of the yersiniabactin receptor, FyuA, as a protective antigen that elicits a

sustained and robust serum IgG response following intranasal vaccination that correlates with the protection of mice transurethrally challenged with UPEC.

Materials and methods

Bacterial strains and culture conditions.

E. coli strains used in this study were cultured in Luria broth (10 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl) at 37°C with aeration. *E. coli* strain 536 was isolated from a patient suffering from urinary tract infection (147) and *E. coli* strain CFT073 was isolated from the blood and urine of a hospitalized patient with acute pyelonephritis (148).

Plasmid Construction.

Genes encoding candidate vaccine antigens were PCR-amplified from *E. coli* CFT073 genomic DNA (*c0294, fitA, iroN chuA.m, chuA.c*) or 536 genomic DNA (*fyuA*) using the primers listed in Table 2-1 and cloned into the XhoI and HindIII restriction sites of pBAD-*myc*-HisA (Invitrogen) to produce C-terminal tagged myc-His_{6X} fusions. The resulting constructs were verified by sequencing.

Table 2-1. Primer sequences for cloning vaccine candidate genes into pBAD-*myc*-HisA expression construct.

Gene	Forward[a]	Reverse
fyuA	AAAAAACTCGAGCAAAATGACACGGCTTTATCC	AAAAAAAAGCTTGAAGAAATCAATTCGCGTATTGATACC
c0294	AAAAAACTCGAGCAAAACTCAAATAACTTTCGCTG	AAAAAAAAGCTTGAAATCGAAGGTGACGGATG
sitA	AAAAAACTCGAGCCACTCGATAAAAAAAGTAACCA	AAAAAAAAGCTTGTAGTGTGCGCCGGTTTCAC
fitA	AAAAAACTCGAGCGCTATGTTCACACC	AAAAAAAAGCTTAAATTCCATTTTCACCGTGAATTGCAC
chuA(N)	AAAAAACTCGAGCGCTGTTTCTGCC	AAAAAAAAGCTTAACGGTCTGCGGATTTTTTGG
chuA(M)	AAAAAACTCGAGCGCTTCTGATAGCAGC	AAAAAAAAGCTTAAATTCCAGAGCATCATTGGACAAC
chuA(C)	AAAAAACTCGAGCAAAGCCAGCTACTTTGATAC	AAAAAAAAGCTTCCATTGATAACTCACGAAAATTTTTCCG
iroN	AAAAAACTCGAGCAGAATTAACAAAATCCTCTGGT	AAAAAAAAGCTTGAATGATGCGGTAACTCCG
iroN(trun)	AAAAAACTCGAGCGACGAGACTCTGG	AAAAAAAAGCTTGAATGATGCGGTAACTCCG

[a]All sequences are listed 5' → 3', with XhoI and HindIII restriction sites underlined.

Vaccine Antigen Preparation.

Recombinant protein expression was induced in *E. coli* TOP10 (FyuA-His_{6x}, c0294-His_{6x}, ChuA.c-His_{6x}), *E. coli* BL21 (ChuA.m-His_{6x}), or *E. coli* C41(DE3) (IroN-His_{6x}, FitA-His_{6x}), cultured in Terrific broth (12 g/l tryptone, 24 g/l yeast extract, 4 ml/l glycerol, 100 ml/l filter sterilized 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) at 37°C with aeration to an OD_{600} = 0.5-1.0 by the addition of 1 mM L-arabinose. Induced cultures were incubated at 37°C with aeration for 4 h before being harvested by centrifugation (8,000×*g*, 10 min, 4°C).

Bacterial pellets were resuspended in 10 mM HEPES, pH 7, and 100 U Benzonase nuclease (Sigma-Aldrich). Bacterial suspensions were lysed by two passages through a French pressure cell (20,000 psi) and the lysate was cleared by centrifugation (8,000×*g*, 10 min, 4°C). Bacterial membranes and insoluble aggregates were separated from the cleared lysate by ultracentrifugation (112,000×*g*, 30 min, 4°C) and solubilized in 5 ml 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, 1% ASB-14, pH 8.0. His-tagged proteins were purified by affinity chromatography, using nickel-nitriloacetic acid agarose (Qiagen) under denaturing conditions according to the manufacturer's instructions (The QIAexpressionist). Eluted protein was renatured by four successive dialysis steps at 4°C (dialysis buffer 1: 100 mM NaH_2PO_4, 5 mM Tris, 6 M Urea, 50 mM NaCl, pH 5.5; dialysis buffer 2: 100 mM NaH_2PO_4, 2.5 mM Tris, 4 M Urea, 100 mM NaCl, 0.005% Zwittergent, pH 6.5; dialysis buffer 3: 100 mM NaH_2PO_4, 2 M Urea, 150 mM NaCl, 0.01% Zwittergent, pH 7.4) into a final solution containing 0.05% Zwittergent in PBS, pH 7.4 and quantified using the BCA protein assay (Pierce).

Vaccination.

Purified antigens were chemically cross-linked to cholera toxin (CT) (Sigma-Aldrich) at a ratio of 10:1 using *N*-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) (Pierce) according to the manufacturer's recommendations. Cross-linked antigens were administered to six- to eight-week-old female CBA/J mice intranasally 20 μl/mouse (10 μl/nare). Animals received a primary dose on day 0 of 100 μg antigen cross-linked to 10 μg CT or 10 μg CT alone. Two booster immunizations of 25 μg antigen cross-linked to 2.5 μg CT or 2.5 μg CT alone were administered on days 7 and 14. When appropriate, mice were transurethrally challenged with a UPEC strain on day 21 as described below for the murine model of ascending UTI.

Murine model of ascending UTI.

Female CBA/J mice were inoculated transurethrally as previously described (149), with modification. Bacteria were cultured overnight in LB at 37°C with aeration. The inoculating strain was harvested by centrifugation (3500×*g*, 30 min, 4°C) and resuspended in PBS to $OD_{600} \approx 4.0$ (~1 x 10^9 CFU/ml) and 50 μl of this suspension (10^8 CFU) was delivered to each mouse via a sterile 0.28 mm polyethylene catheter attached to an infusion pump (Harvard Apparatus) with a flow rate of 100 μl/min. The inoculum was quantified by plating dilutions onto LB agar. Forty-eight hours post-inoculation (hpi), organs were removed from euthanized mice and homogenized in 3 ml PBS using a GLH homogenizer (Omni International). Using an Autoplate 4000 spiral plater (Spiral Biotech), tissue homogenates were plated onto LB agar. Colonies were enumerated with a QCount automated plate counter (Spiral Biotech) and CFU/g tissue determined

(output). Protocols involving CBA/J mice complied with all relevant federal guidelines and institutional policies.

ELISA.

For the indirect enzyme-linked immunosorbent (ELISA) assay, 50 µl of 10 µg/ml purified protein diluted in carbonate buffer (100 mM, pH 9.6) was coated onto 96-well EIA/RIA high-binding polystyrene plates (Corning, Costar #9018) and incubated at 4°C overnight. Plates were washed by flooding all wells three times with wash buffer (0.05% Tween 20 in PBS) using an ELx405 (Bio-Tek Instruments, Inc.) Microplate Washer. Non-specific binding sites were blocked with SuperBlock blocking buffer (Pierce) as recommended by the manufacturer and the plates were washed with wash buffer. Wells were coated with serum diluted 1:250 in blocking buffer or 50 µl undiluted urine and allowed to incubate for 1 h at 23°C. Plates were washed with wash buffer and coated with the secondary antibody, goat anti-mouse IgG (Abcam ab97265), or goat anti-mouse IgA (Abcam ab97235) diluted 1:10,000 in blocking buffer and allowed to incubate a 23°C for 1 h. Plates were washed and 50 µl of substrate, 1-Step Ultra TMB-ELISA (Thermo Scientific, 34018) was added and allowed to incubate at 23°C until color developed (≤20 min). Reactions were stopped by the addition of sulfuric acid and the absorbance of each well was read with a µQuant plate reader (Bio-Tek Instruments, Inc.) at a wavelength of 450 nm.

Statistical analyses.

Graphing and statistical analyses were performed using Prism® version 6 (GraphPad Software, Inc.) and *R* version 2.14.1 (*R* Development Core Team, 2011) (150). Significance was determined using a one- or two-tailed Mann-Whitney test where appropriate and correlates of protection were determined using a two-tailed Spearman's rank test with linear regression to generate a best-fit line. Outliers as determined to be three times the interquartile range by boxplot analysis were removed. All statistics were conducted using 95% confidence intervals where applicable.

Results

Candidate antigen expression and purification.

In preparation for immunization, the genes for six vaccine antigens, FyuA, IroN, c0294, FitA, ChuA (M) (middle fragment of ChuA; amino acid residues 260-493), and ChuA (C) (C terminal fragment of ChuA; amino acid residues 494-660) were cloned as His_{6X} translational fusions, expressed, and purified under denaturing conditions as His_{6X} affinity-tagged recombinant proteins. Bacterial cultures expressing recombinant vaccine antigens were lysed and bacterial membrane proteins were harvested by ultracentrifugation. The pelleted membrane proteins were solubilized in 8 M urea and passed over a nickel-affinity column to enrich for vaccine antigen. Fractions with concentrated vaccine antigen were pooled and visualized by SDS-PAGE (Figure 2-1). Urea was removed stepwise to allow outer membrane iron receptor vaccine antigens to refold by a series of dialysis steps to regain a *beta* barrel configuration as has been demonstrated by circular dichroism (146).

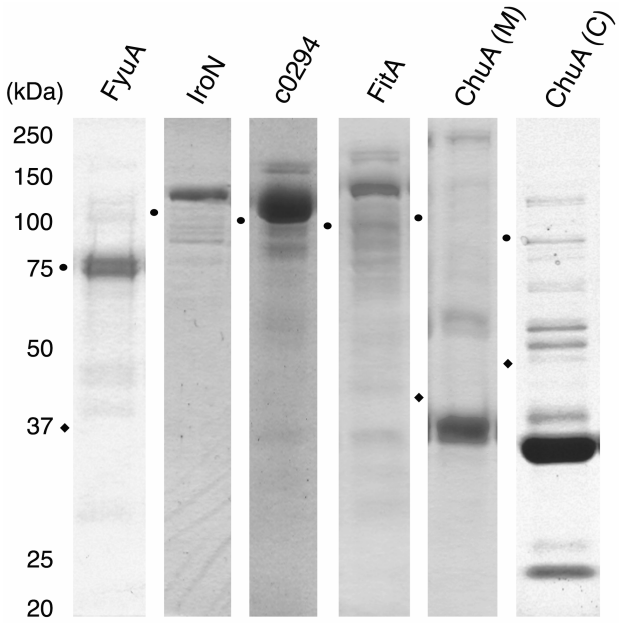


Figure 2-1. Expression and purification of UPEC outer membrane iron receptors. Genes encoding outer membrane iron receptors were cloned from UPEC strains 536 and CFT073. Iron receptors were recombinantly expressed with a His-tag and purified using immobilized nickel affinity chromatography. Purified protein fractions were separated by SDS-PAGE and stained with Invitrogen SimplyBlue™ SafeStain. Predicted size of tagged FyuA, 77 kDa; IroN, 83 kDa; c0294, 82 kDa; FitA, 82 kDa, (ChuA Middle fragment) ChuA (M), 30 kDa; (ChuA C Terminal fragment) ChuA (C), 22 kDa. Circle (•) and diamond (◆) symbols indicate the location of the 75 kDa and 37 kDa standard bands respectively for each individual gel.

Immunization with FyuA confers protection against pyelonephritis.

To induce a robust mucosal immune response, purified vaccine antigens were biochemically cross-linked to cholera toxin (CT) as adjuvant, at a ratio of (10:1) (antigen:CT). Mice were immunized intranasally with either adjuvant-conjugated vaccine antigen (antigen:CT) or adjuvant alone (CT). Following primary immunization with 100 μg antigen cross-linked to 10 μg CT or 10 μg CT alone (day 0) and two booster doses of 25 μg antigen cross-linked to 2.5 μg CT or 2.5 μg CT alone (days 7 and 14), mice were transurethrally inoculated on day 21 with 1 x 10^8 colony forming units (CFU) of UPEC. The prototypical pyelonephritis strain CFT073 was used as the challenge strain in all experiments, except when evaluating the vaccine antigen FyuA, where UPEC strain 536 was substituted as CFT073 does not express the siderophore receptor FyuA. The infection was allowed to progress for 48 hours before the mice were euthanized and their bladders and kidneys removed. Organs were homogenized and the UPEC bacterial load in the infected organs was quantified by determination of CFU (Figure 2-2). Of the six outer membrane iron receptor vaccine formulations tested, only the FyuA-based vaccine significantly protected (p = 0.0018) against experimental UTI, with vaccinated mice having a 29-fold decrease in median levels of UPEC kidney colonization in comparison to mice that only received adjuvant (Figures 2-2A). While data for FyuA are pooled in Figure 2-2A, vaccination with the antigen and control (CT alone) had been carried out in three independent trials. In each case, FyuA-vaccinated mice had at least a 12-fold reduction (12.92, 95.65, and 14.18) in the median level of UPEC kidney colonization in comparison to mice given only adjuvant with one of the three reductions being statistically significant (p = 0.2575, p = 0.0861, p = 0.0090). Although mice immunized

with the IroN- and FitA-based vaccines also had reduced median levels of UPEC kidney colonization, neither reduction was statistically significant (p = 0.270 and p = 0.188, respectively) (Figures 2-2B, D).

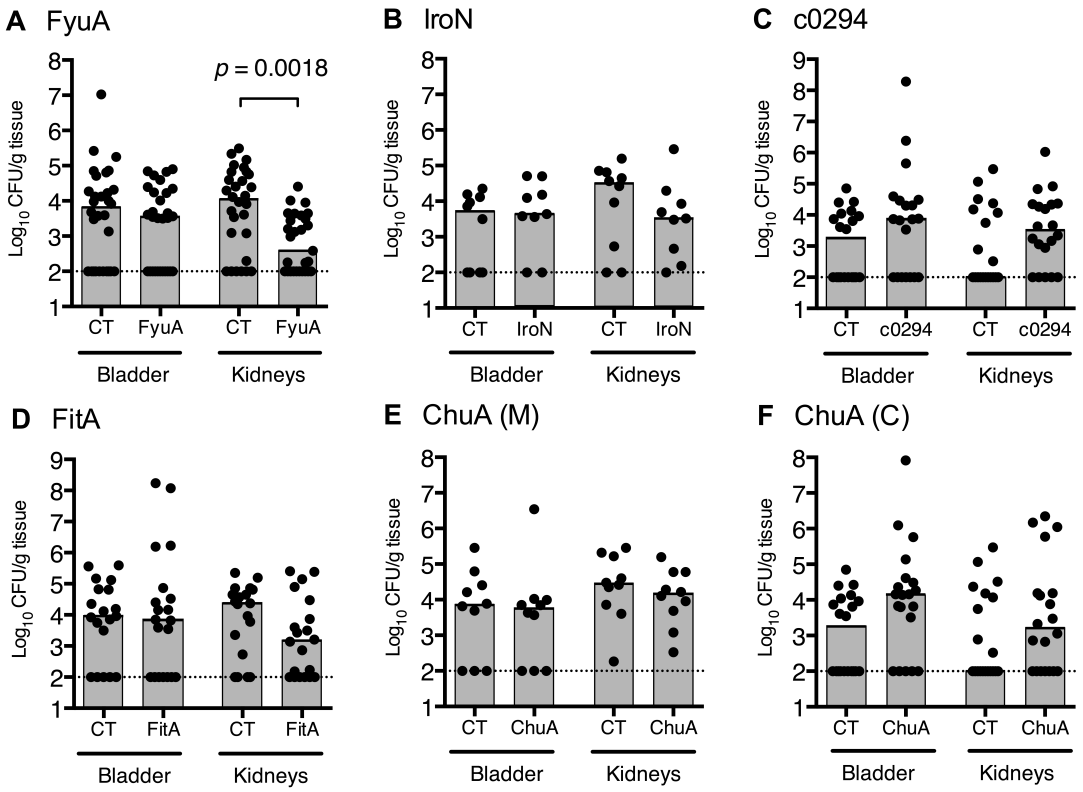


Figure 2-2. Immunization with the yersiniabactin receptor FyuA protects against experimental pyelonephritis. Female CBA/J mice were intranasally vaccinated as described with a primary dose of 100 μg purified protein crosslinked to 10 μg CT followed by two boosts of 25 μg antigen crosslinked to 2.5 μg CT. One week following the final boost, animals were transurethrally inoculated with 1×10^8 CFU of *E. coli* 536 **(A)** or CFT073 **(B-F)** and colonization was measured 48 hpi. Animals per group **(A)** CT: $n = 30$ FyuA: $n = 29$ in three independent immunization experiments (Kidneys P = 0.0430, 0.0045, 0.1287), **(B)** CT: $n = 10$ IroN: $n = 9$ in a single immunization experiment, **(C)** CT: $n = 20$ c0294: $n = 20$ in two immunization experiments, **(D)** CT: $n = 20$ FitA: $n = 20$ in two immunization experiments, **(E)** CT: $n = 10$ ChuA.m: $n = 10$ in a single immunization experiment, **(F)** CT: $n = 20$ ChuA.c: $n = 20$ in two immunization experiments. Symbols represent CFU/g tissue or /ml urine of individual mice and bars indicate median values. Dashed line shows the limit of detection for this assay, 100 CFU/g. Significance was determined using a two-tailed Mann-Whitney test. Only statistically significant differences are noted.

Immunized mice produce vaccine-specific serum IgG.

To verify that intranasal immunization with UPEC iron receptor-based vaccines induces a vaccine-specific humoral immune response, serum samples were taken from each mouse prior to the primary immunization (day 0) and again before UPEC challenge (day 21). The levels of vaccine antigen-specific serum IgG were quantified via indirect ELISA (Figure 2-3). All vaccine formulations induced a robust, antigen-specific serum IgG response following intranasal immunization, confirming that the failure of some vaccine antigens to significantly reduce median UPEC colonization levels was not due to the insufficient induction of a humoral immune response (Figures 2-3B-F).

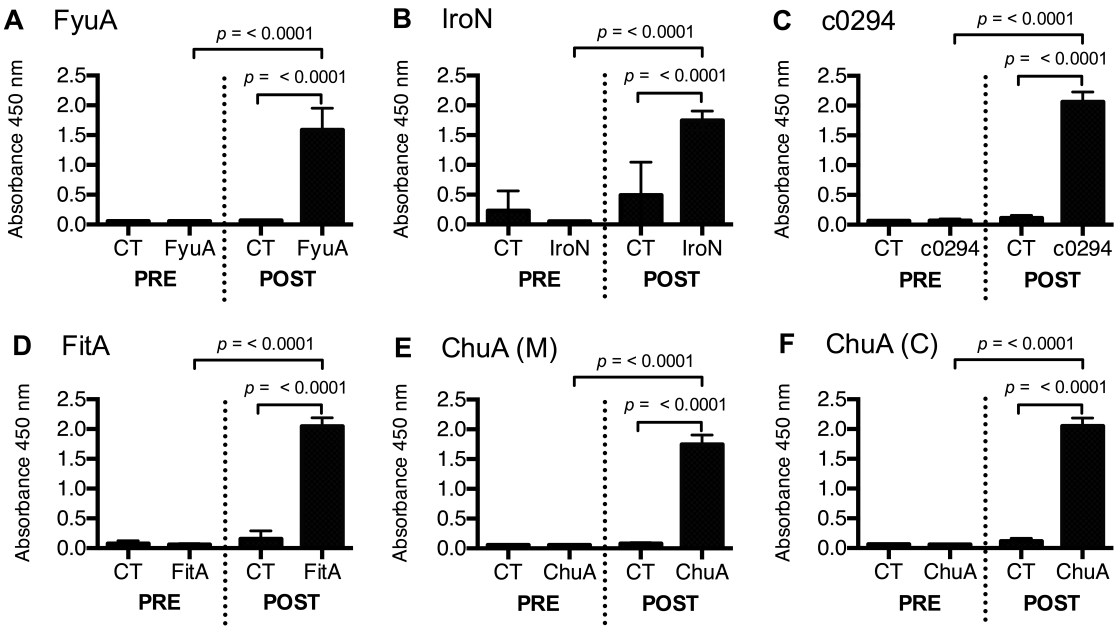


Figure 2-3. Intranasal immunization with the yersiniabactin receptor FyuA and all other antigens induces significant antigen-specific serum IgG expression in mice. Serum was collected from antigen-CT immunized or CT (CT) immunized mice prior to immunization (Pre-Immunization) and after immunization, but before UPEC challenge (Post-Immunization). Samples were plated in antigen-coated plates and probed for antigen-specific IgG via indirect ELISA. Absorbance reflects relative quantity of serum IgG. Each experimental group consists of 20 individual mice from two separate immunization experiments. Error bars indicate the mean +/- SD. Significance was determined using a one-tailed Mann-Whitney test.

Immunized mice secrete vaccine-specific IgA in urine.

To evaluate the humoral immune response at the site of UPEC colonization, urine samples were collected from individual mice following a course of immunization with either CT or FyuA-CT and the levels of FyuA-specific urinary IgA and IgG were quantified by indirect ELISA (Figures 2-4A, B). Intranasal immunization with the FyuA-based vaccine induced statistically significant levels of urinary IgA and IgG in comparison to CT-immunized mice (Figures 2-4A, B).

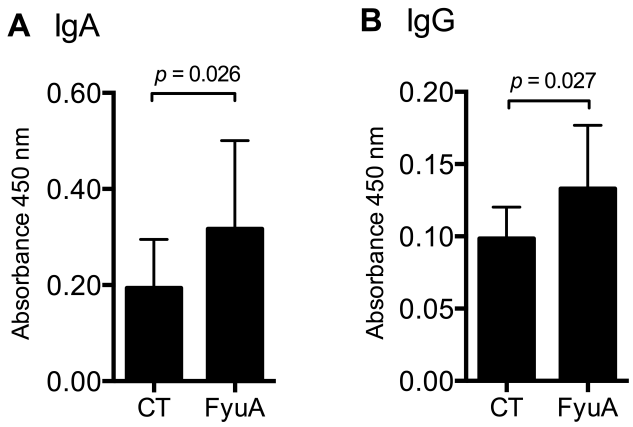


Figure 2-4. Mice immunized intranasally with the yersiniabactin receptor FyuA produce FyuA-specific urinary antibodies. Urine collected from FyuA-CT immunized (FyuA) or CT (CT) immunized mice was plated on FyuA-coated plates and probed for FyuA-specific IgA **(A)** or IgG **(B)** antibodies via indirect ELISA. Absorbance reflects relative quantity of immunoglobin. Each group (CT) or (FyuA) consists of 10 individual mice from a single immunization experiment. Error bars indicate the mean +/- SD. Significance was determined using a one-tailed Mann-Whitney test.

Vaccine-specific serum antibodies correlate with UPEC bacterial load.

Almost all current vaccines block infection by inducing pathogen-specific antibodies in the serum or mucosa and if a serological correlate of protection can be identified from a humoral immune response, it can provide a valuable tool to evaluate vaccine efficacy and design (151). Given our hypothesis that vaccine-mediated protection against UTI is dependent on a robust humoral immune response, it was important to determine whether antibody levels in vaccinated mice correlated with UPEC bacterial load in the urinary tract following transurethral challenge. To evaluate whether we could identify a serological correlate of protection for any of the antigens, we performed a Spearman's rank analysis comparing the levels of vaccine-specific serum IgG and the amount of UPEC bacterial load (CFU/g kidney) for each vaccine antigen using data from each vaccinated mouse (Figures 2-5A-F). Only in the group of mice immunized with the protective vaccine antigen, FyuA, did a reduction of UPEC bacterial colonization of the kidney strongly correlate with levels of vaccine-specific serum IgG ($\rho(14) = -0.72$, $p = 0.0018$) (Figure 2-5A). Levels of FyuA-specific urinary IgA or serum IgG did not significantly correlate with UPEC bacterial load in the bladder (data not shown).

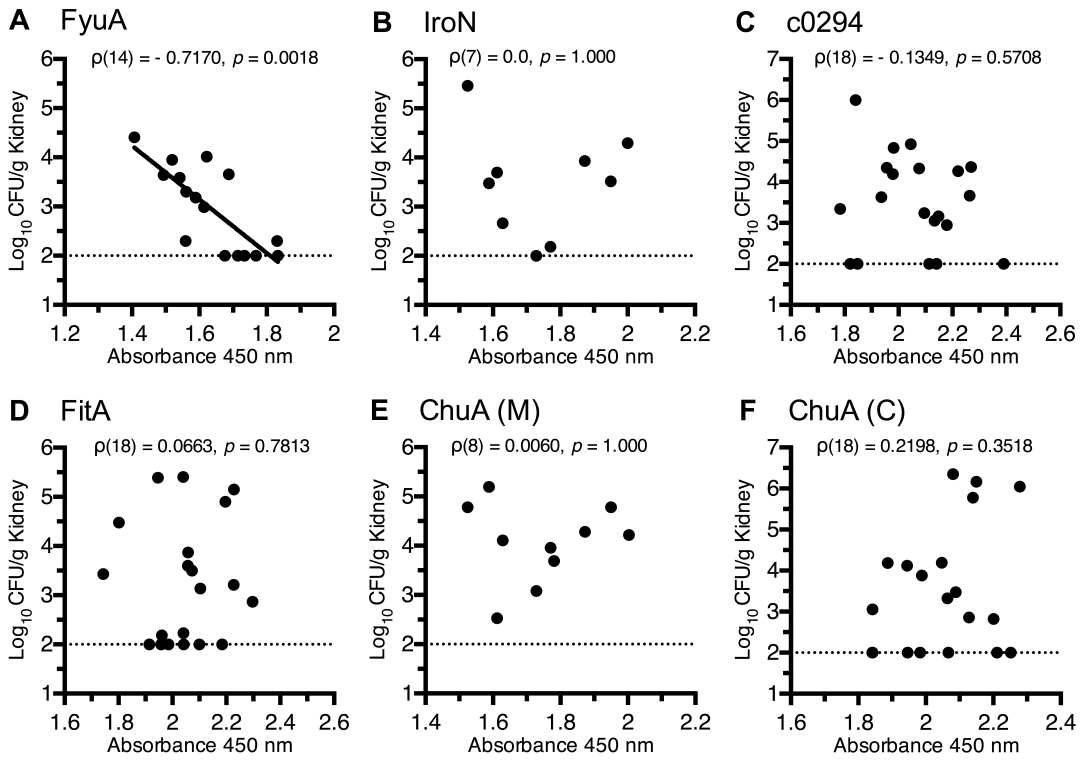


Figure 2-5. Correlation between vaccine-specific serum IgG titer and reduced bacterial counts is observed only in mice immunized with the protective vaccine. Normalized kidney CFU values from immunized and *E. coli* challenged mice are plotted against their respective vaccine-specific serum IgG levels as measured by indirect ELISA, where absorbance at 450 nm reflects the relative quantity of vaccine-specific serum IgG. Dashed line indicates the limit of detection for the immunization assay, 100 CFU/g kidney tissue. Correlative significance was determined using a two-tailed Spearman's rank correlation and the best-fit line was determined by linear regression; shown only when there is a statistically significant correlation ($p < 0.05$). Results of linear regression in **(A)** $R^2 = 0.60$, $F(1,14) = 20.6$, $p = 0.005$.

Immunization with FyuA generates long-lived plasma cells.

Upon initial antigen exposure, naïve B cells can progress through two different paths of cell development, differentiating into either short- or long-lived plasma cells. Short-lived plasma cells mediate the initial humoral response to antigen by secreting large amounts of antibodies, and typically appear 3-6 days after immunization and disappear after two weeks through programed cell death (152). Naïve B cells that differentiate into long-lived plasma cells, move to the bone marrow after maturation and maintain serological memory by continuing to secrete antibodies long after initial infection, thereby providing a critical first-line of defense against reinfection (152). To determine if vaccination with UPEC iron receptors has the potential to provide long-term immunity through the generation of long-lived plasma cells, we monitored vaccine-specific serum antibody levels for several weeks after immunization, to the point when serum antibody production could no longer be attributed to short-lived plasma cells. Per our defined dose and vaccination schedule we intranasally immunized five female CBA/J mice with CT-crosslinked FyuA and obtained weekly serum samples during the course of the immunization and for eight weeks following the last vaccine antigen booster dose. At the time when a transurethral challenge with UPEC would ordinarily be conducted, FyuA-specific serum IgG was at or near its highest level (Figure 2-6). Near peak levels of FyuA-specific serum antibodies were maintained for at least 70 days after initial vaccination and at least 56 days after the last booster dose (Figure 2-6). Given that the half-life for serum IgG subtypes averages 21 days or shorter, the sustained antibody response at 70 days in the absence of additional boosting suggests that immunization with FyuA generated FyuA-specific long-lived plasma cells.

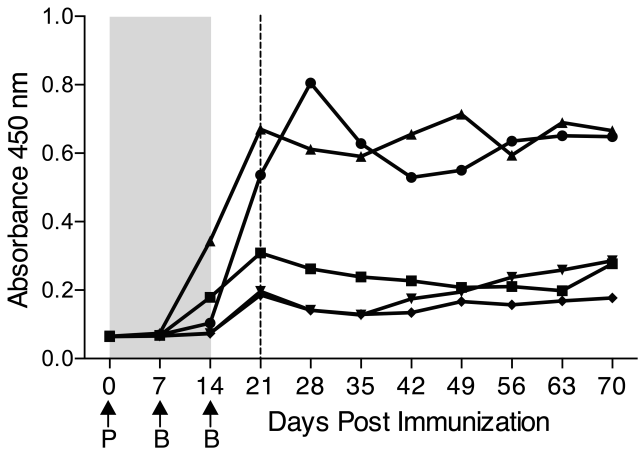


Figure 2-6. Immunization with the yersiniabactin receptor FyuA generates long-lived plasma cells. Five female CBA/J mice were intranasally vaccinated as described with a primary dose (P) of 100 μg purified FyuA crosslinked to 10 μg CT followed by two booster doses (B) of 25 μg FyuA crosslinked to 2.5 μg CT. Weekly serum samples were taken from mice (prior to same-day immunizations) and FyuA-specific serum IgG levels were quantified via indirect ELISA. The shaded area indicates the immunization period and arrows indicate when vaccine was administered. Dashed line indicates the time point when mice would ordinarily be challenged with UPEC to evaluate vaccine efficacy in a vaccine trial.

Discussion

UTIs are persistent in the general community and among hospitalized patients and, with rising rates of antibiotic resistance, are becoming increasingly more difficult to treat. The development of an effective UTI vaccine to lessen this substantial public health burden would be enormously beneficial to the population at large. Previously we identified iron acquisition proteins, including siderophore and heme receptors, as potent vaccine antigens that can protect the urinary tract from UPEC colonization. In a systematic screen, we tested six outer membrane components of distinct iron acquisition systems of *E. coli* CFT073 and identified three antigens that protect against infection (IreA, IutA, Hma) when used for intranasal vaccination (146). Having recognized iron acquisition proteins as a defined target for vaccination, here we described the evaluation of an additional six iron acquisition proteins for vaccination to prevent UTI.

Of the six candidate antigens described here, only the yersiniabactin receptor, FyuA, stimulated a protective response, whereas IroN, FitA, ChuA fragments, and the gene product of CFT073 locus *c0294* did not. FyuA-immunized mice elicited a vaccine-specific humoral immune response that strongly correlated with levels of UPEC kidney colonization after transurethral challenge with 10^8 CFU of *E. coli* 536. This humoral response was maintained for at least eight weeks following final antigen exposure, providing evidence for the generation of long-lived, vaccine-specific plasma cells, and demonstrates that an FyuA-based vaccine has the potential to provide robust and long-term protection against UTI. Not surprisingly, the other five antigens tested also elicited strong serum antibody responses following intranasal immunization; however, none

significantly protected from UPEC challenge or induced a correlative humoral immune response. Given the harsh denaturing conditions required for the purification of antigens, it is unlikely that the immune response we observe after immunization is the result of lipopolysaccharide (LPS) contamination in our vaccine preparations. When we tested our vaccine preparations previously we detected no contaminating LPS by limulus amebocyte lysis assay (146). In addition, given that five out of the six vaccine antigens failed to protect against infection, it is highly unlikely that the protection observed for one antigen could be the result of contaminating LPS.

Why five of these antigens failed to provide protection despite inducing high levels of vaccine-specific antibodies is unclear. It is unlikely that all the vaccine targets are equally accessible by the host's humoral immune system, and possible differences in the abundance or exposure of vaccine targets on the bacterial surface may offer an explanation for the observed differences in vaccine efficacy. In addition, although we hypothesize that bacterial clearance is mediated by pathogen opsonization or neutralization, another possible mechanism may be the generation of antibodies that bind to the receptors and inhibit their function. That is, a successful protective antigen might have to generate antibodies that both opsonize bacteria and fix complement as well as prevent the uptake of the cognate siderophore, in this case yersiniabactin. This hypothesis awaits testing for loss of function (iron uptake) studies in vaccinated mice.

Clearly, variations in mouse model, immunization route, adjuvant, antigen preparation and dose, challenge strain and inoculum dose, and challenge method all have an impact on the evaluation of vaccine efficacy. For example in previous studies, denatured IroN delivered subcutaneously with Complete Freund's adjuvant to BALB/c

mice protected against lethal challenge with the extraintestinal pathogenic *E. coli* (ExPEC) strain S26 delivered intraperitoneally (113), or if administered without adjuvant, protected BALB/c mice from kidney colonization by ExPEC strain CP9 delivered intravenously (112). The candidate antigen FitA, when administered intraperitoneally with Complete Freund's adjuvant or Incomplete Freund's adjuvant to CD1 mice, protected against lethal challenge from ExPEC strains CFT073 and IH3034 delivered intraperitoneally, and ExPEC strain 536 delivered intravascularly (106). Such differences in experimental design between our studies and those of others may account for the differences we observe in protection for IroN and FitA antigens.

During our previous UPEC vaccine antigen screen (146), we tested the potential of the vaccine antigen ChuA to protect against experimental UTI, but only 19 out of 30 ChuA-immunized mice survived immunization. To more clearly evaluate the potential of ChuA as a UPEC vaccine candidate, we designed and evaluated truncated derivatives of ChuA. Although none of the mice immunized with truncated derivatives of ChuA experienced toxic effects during the course of immunization as we had observed for intact ChuA, neither truncated ChuA derivative (M or C) significantly protected mice from transurethral challenge. Although it is possible that individual immunogenic epitopes of ChuA could have been disrupted by expressing only fragments of the protein, the results presented here are in accordance with our previous study (146) using whole renatured ChuA and work done by Durant and colleagues evaluating denatured ChuA as an ExPEC vaccine antigen (113).

The identification of FyuA as a protective vaccine antigen against UPEC adds a fourth antigen, in addition to Hma, IreA, and IutA identified previously (146), that can be

included in an intranasal vaccine to prevent UTI. Epidemiological studies and gene expression data indicate that *fyuA* is highly expressed during UTI in women (42), prevalent among UPEC strains, being carried on the High Pathogenicity Island (153, 154), and an important fitness factor during experimental UTI (155). In addition, protection from infection after FyuA immunization has been demonstrated in alternative models of ExPEC infection. Subcutaneous immunization with denatured FyuA significantly protected BALB/c mice against lethal challenge by the ExPEC strain S26 (113), and a multiepitope vaccine containing a fragment of FyuA protected mice from intraperitoneal challenge by CFT073 in the liver and spleen (117). Indeed, given that many bacterial pathogens require a source of iron to cause infection, FyuA is critical for the virulence of other enteric pathogens, such as *Yersinia* (156) and *Klebsiella* (157) species.

Interestingly, immunization with FyuA provided protection in a tissue-specific manner, significantly reducing UPEC infection in the kidneys, but not in the bladder. Tissue-specific protection has been observed before for antigens Hma, which also only protected in the kidney, and IreA, which only protected in the bladder and provokes questions of the role of these iron receptors during infection and whether differences in iron receptor expression or function between organs sites may account for the observed differences in tissue-specific protection. In addition, although we found levels of FyuA-specific serum IgG to be a surrogate of protection after immunization, it is still unclear whether the levels of FyuA-specific IgG mediates clearance of the urinary tract, or whether alternative mechanisms or components of the adaptive immune response are

providing UPEC clearance, such as urinary IgA or cellular immunity. These hypotheses await testing by passive protection and adoptive transfer assays in a mouse model of UTI. Clearly, targeting bacterial iron acquisition systems represents a rational approach to UPEC vaccine development. The four protective iron receptor vaccine antigens identified in our combined screens, Hma, IreA, IutA, (146) and FyuA described here, are highly expressed during infection and are more prevalent among uropathogenic strains, 69%, 20%, 65%, and 87%, than fecal commensal strains, 17%, 17%, 17%, and 59%, respectfully (142, 158). Because no single antigen is present in all UPEC strains, and absent from all commensal strains, targeting a single iron receptor is unlikely to yield the broad level of immunity required for an effective UTI vaccine. However, by combining the identified antigens we can develop an iron receptor-based multi-epitope vaccine with the potential to provide broad protection against the heterogeneous UPEC population.

CHAPTER 3

BLOCKING YERSINIABACTIN ATTENUATES PATHOGENIC *ESCHERICHIA COLI* IN CYSTITIS AND PYELONEPHRITIS AND IS A NOVEL THERAPEUTIC TARGET TO PREVENT URINARY TRACT INFECTION

Author's Note: This chapter is a modified version of the submitted research article: Brumbaugh AR, Smith SN, Subashchandrabose S, Himpsl SD, Hazen TH, Rasko DA and Mobley HLT. Blocking Yersiniabactin Attenuates Extraintestinal Pathogenic *Escherichia coli* in Cystitis and Pyelonephritis and is a Novel Therapeutic Target to Prevent Urinary Tract Infection.

Abstract

The emergence and spread of antibiotic resistance mechanisms such as extended-spectrum *beta*-lactamases and carbapenemases among common bacterial pathogens is threatening our ability to treat routine hospital- and community-acquired infections, such as urinary tract infection (UTI) and pneumonia. With the pipeline for new antibacterial drugs for Gram-negative enteric bacteria virtually empty, there is an urgent need to develop new therapeutics. Bacteria require iron to establish infection and specialized pathogen-associated iron acquisition systems like yersiniabactin, common among highly pathogenic species in the family *Enterobacteriaceae* including *Klebsiella pneumonia* and *Escherichia coli*, are potentially novel therapeutic targets. Although the yersiniabactin receptor, FyuA, was recently identified as a protective vaccine target for *E. coli* UTI, the contribution of yersiniabactin to uropathogenic *E. coli* (UPEC) pathogenesis is unknown.

Using an *E. coli* mutant unable to acquire yersiniabactin (536Δ*fyuA*) during infection, we established the yersiniabactin receptor as a UPEC virulence factor during cystitis and pyelonephritis, a fitness factor during bacteremia, and the surface-accessible target of the experimental FyuA vaccine. In addition, we determined through RNAseq analyses of RNA from *E. coli* causing cystitis in women that iron acquisition systems, including the yersiniabactin system, are highly expressed by bacteria during natural uncomplicated UTI. Yersiniabactin contributes to the virulence of several pathogenic species in the family *Enterobacteriaceae*, including UPEC, and its association with multidrug-resistance makes it a promising target to combat antibiotic resistant infections.

Introduction

Widespread and increasing antibiotic resistance among bacterial pathogens that cause some of our most common healthcare-associated and community-acquired infections is jeopardizing our ability to prevent and treat routine infectious diseases (159). Two pathogens of particular concern are uropathogenic *Escherichia coli* (UPEC), which causes the majority (80%) of uncomplicated urinary tract infections (UTI) (9) and *Klebsiella pneumoniae*, a frequent cause of hospital-acquired pneumonia and UTI (160). Without adequate treatment, both UPEC and *K. pneumoniae* can breach epithelial and endothelial barriers to gain access to the bloodstream, causing life-threatening bacteremia (161). *E. coli* resistance rates to fluoroquinolones and 3rd generation cephalosporins now exceed 50% in 5 of 6 global regions, and similar resistance rates were reported for *K. pneumoniae* worldwide (162). Unfortunately, this means that the treatment of severe infections caused by these species must rely on carbapenems, the last-resort to treat severe community- and hospital-acquired infections (129). Not only are these antibacterials more expensive and less available in resource-constrained settings, but their extended use has contributed to the emergence of carbapenem-resistant *Enterobacteriaceae* (CRE), a serious global public health concern (163).

Increasing rates of antimicrobial resistance and a lack of new therapeutics in the drug development pipeline has renewed interest in novel antibiotic discovery. We hypothesized that targeting nutrient acquisition in pathogenic bacteria, specifically systems that acquire iron, could provide a novel mechanism to prevent or treat infection. Iron is an essential cofactor for normal cell physiology and bacteria require a source of iron to establish infection (164). Most tissues in the body limit iron availability to

microorganisms, meaning that available ferrous iron is scarce, and most iron is sequestered in storage and carrier molecules such as transferrin, lactoferrin and ferritin, or bound to heme in hemoglobin and hemopexin (165). During infection, additional iron sequestration occurs as epithelial cells and neutrophils secrete lipocalin-2, a competitor for the bacterial iron-scavenging siderophore enterobactin, among others, and iron absorption and recycling pathways are repressed (166). Collectively these antimicrobial mechanisms are characterized as 'nutritional immunity' (167) and the ability to circumvent these barriers to iron acquisition is a hallmark of successful pathogens.

Many pathogenic species in the family *Enterobacteriaceae*, including UPEC and *K. pneumoniae*, have multiple and often redundant iron acquisition systems to facilitate infection (115). The genome of UPEC strain 536, for example, encodes two heme receptors (Hma, ChuA), three siderophore systems (enterobactin, salmochelin, and yersiniabactin), and receptors for two fungal siderophores (FhuA, FhuE), as presented in Table 3-1 (168). Of particular interest is the yersiniabactin system, which is often pathogen-associated (158) and encoded on the high-pathogenicity island (HPI), a horizontally acquired 30 kb chromosomal region common among highly pathogenic strains of *Yersinia pestis, Y. enterocolitica, Y. pseudotuberculosis, K. pneumoniae, K. oxytoca, Salmonella enterica* and *E. coli* (169). Animal studies confirm that yersiniabactin contributes to the virulence of *K. pneumoniae* during respiratory infection (157), and of *Y. pestis* during bubonic and pneumonic plague (170). Recently, we identified the receptor for yersiniabactin, FyuA, as a protective vaccine target against *E. coli*-mediated pyelonephritis in a murine model of UTI (171). Although the yersiniabactin system is pathogen-associated, a protective vaccine target in the urinary

tract, and more prevalent among UPEC isolates that cause pyelonephritis (94%) and cystitis (87%) as compared to commensal *E. coli* strains (59%), the contribution of the yersiniabactin system to *E. coli* pathogenesis during UTI is unknown (57).

The purpose of this study was to determine if the yersiniabactin system contributes to UPEC pathogenesis during UTI, and whether the yersiniabactin-mediated virulence is different between the kidney and bladder, which would clarify the kidney-specific protection of the experimental FyuA vaccine. Understanding yersiniabactin-mediated pathogenesis has the potential to provide a new therapeutic target for a number of highly pathogenic bacterial species that cause some of our most common community- and hospital-acquired infections. Here we describe the use of a yersiniabactin receptor mutant (Δ*fyuA*) to establish the yersiniabactin system as a UPEC virulence factor during cystitis and pyelonephritis, a fitness factor during bacteremia, and the surface-accessible target of the FyuA vaccine. In addition, we demonstrate through RNAseq analysis of RNA isolated directly from *E. coli* in urine from women with cystitis that iron acquisition systems, including the yersiniabactin system, are highly expressed by bacteria during natural uncomplicated UTI.

Table 3-1. Outer membrane iron receptor genes in prototypical UPEC strains

			Chromosomal Locus					
Gene	Receptor Substrate[a]	Substrate Type	CFT073	536	HM27	HM46	HM65	HM69
fepA	enterobactin	catecholate	c0669	ECP_0615	3441	3213	0994	0933
iha	enterobactin	catecholate	c3610	——	——	——	——	4356
ireA	unknown	catecholate	c5174	——	——	——	——	——
fiu	DBS, DHB	catecholate	c0890	ECP_0819	3833	3957	0791	0993
cirA	DBS, DHB	catecholate	c2690	ECP_2195	——	3284	1420	1803
iroN	salmochelin	catecholate	c1250	ECP_0302	2497, 3684	——	4837	——
fyuA	yersiniabactin	phenolate	c2436*	ECP_1947	1554	——	0518	3963
iutA	aerobactin	hydroxamate	c3623	——	——	——	——	4369
fhuA	ferrichrome	hydroxamate	c0185	ECP_0160	3849	0051	2318	2627
fhuE	coprogen, rhodotorulic acid	hydroxamate	c1374	ECP_1094	1904	1592	3888	4290
chuA	heme	NA	c4308	ECP_3597	1278	2713	4104	1172
hma	heme	NA	c2482	ECP_1994	3745	——	——	——
fitA	unknown	unknown	c3775	ECP_3121	2282	0938	1723	——
prrA	unknown	unknown	c1646	ECP_1239	2884	1723	2610	2869
yncD	unknown	unknown	——	ECP_1452	3313	1948	4376	1203
putative	unknown (may not be Fe^{3+})	unknown	c0294	——	2464	——	4860	——
putative	unknown (may not be Fe^{3+})	unknown	c2518	ECP_2036	——	——	——	——
putative	unknown (may not be Fe^{3+})	unknown	c1265	ECP_0314	——	——	——	5077

[a]DBS, dihydroxybenzolyserine; DHB, dihydroxybenzoate

*Nonsense mutations and a 711 bp insertion into genes encoding yersiniabactin biosynthesis machinery (*irp1 and irp2*) prevent yersiniabactin synthesis and as a result, *fyuA* is not expressed in UPEC strain CFT073.

Materials and methods

Ethical statement.

All protocols involving human subjects were approved by the Institutional Review Board of the University of Michigan Medical School (HUM00029910). Written consent was obtained from all subjects prior to enrollment and patient samples were de-identified. All mouse experimental procedures were conducted in accordance to protocols approved by the University Committee on Use and Care of Animals at the University of Michigan.

E. coli gene expression during human infection.

Urine samples collected from women with cystitis at the University Health Services Clinic were immediately stabilized with RNAprotect (Qiagen) to preserve bacterial RNA transcriptional profiles. Bacteria were pelleted by centrifugation, treated with Proteinase K (0.06 mAU/μl), and RNA was extracted with the RNeasy mini kit (Qiagen). DNA was removed by Turbo DNase (Ambion) treatment and RNA integrity assessed by Bioanalyzer (Agilent). Isolated *E. coli* strains were also cultured statically at 37°C in pooled, filter-sterilized human urine and LB to mid-log phase and processed following the same protocol. Cystitis RNA samples were depleted of human RNA using the MICROBEnrich kit (Ambion). Sequencing libraries were generated using the Ovation Prokaryotic RNAseq system (NuGen) and the Encore next generation sequencing library system (NuGen).

Libraries were sequenced using an Illumina HiSeq2000 at the Institute for Genome Sciences at the University of Maryland, Baltimore. Illumina reads were

analyzed using an automated pipeline (172) and Bowtie (173) was used to align the reads to the sequenced reference genome of the isolate (174). Gene expression was calculated as reads per kilobase of a gene per million mapped reads (RPKM) (175). Differences in gene expression exhibited by each *E. coli* strain in two samples (urine, UTI, or LB) were determined by calculating the $\log_2$ fold change (FC) of the RPKM values. A comprehensive report of this clinical study is presented by Subashchandrabose and colleagues (Subashchandrabose S. et al., submitted). A heat map of differential gene expression was produced in *R* version 2.15.1 (R Development Core Team) (176) with the package 'pheatmap' (177) and histograms of transcript abundance were generated with Artemis (Wellcome Trust Sanger Institute) (178).

Bacterial strains and culture conditions.

E. coli strains were cultured in lysogeny broth (LB; 10 g/liter tryptone, 5 g/liter yeast extract, 0.5 g/liter NaCl) at 37°C with aeration. *E. coli* strain 536 was isolated from the urine of a patient with UTI (168), and *E. coli* cystitis strains HM27, HM46, HM65, and HM69 were cultured from the urine of women presenting with cystitis at the University of Michigan University Health Services clinic, Ann Arbor, MI, USA.

Mutant construction and cloning.

An isogenic mutant unable to import yersiniabactin (536Δ*fyuA::kan*) was generated in *E. coli* 536 using the λ Red recombinase system (179) as described (115). Briefly, a kanamycin resistance cassette was PCR-amplified from the template plasmid

pKD4 using primers containing regions identical to the 5' and 3' ends of the gene *fyuA* from *E. coli* strain 536, shown underlined:

fwd: GGCTTTATCCTCTGGCCTTGGGGGGATTATTGCTCCCCGCCGTGTAGGCTGGAGCTGCTTC

rev: CGTATTGATACCGACGGTGCGACCCATATTGACCTGCGCGATGGGAATTAGCCATGGTCC.

The resulting PCR product was used to replace the *fyuA* gene by homologous recombination in bacteria expressing Red recombinase from pKD46. The mutant was confirmed by PCR.

Murine model of ascending UTI.

Six-to-eight week old, female CBA/J mice (Harlan Laboratories) were inoculated transurethrally as described (149) using a modification of the Hagberg protocol (180). Bacteria were cultured overnight in LB at 37°C with aeration, harvested by centrifugation (3,000 × *g* for 30 min), and resuspended in phosphate-buffered saline (PBS) (128 mM NaCl, 2.7 mM KCl, pH 7.4) to an OD_{600} of 4.0 (4×10^9 CFU/ml). 50 µl of this suspension (containing 1×10^8 CFU) was delivered to each mouse via a sterile 0.28 mm polyethylene catheter attached to an infusion pump (Harvard Apparatus) with a flow rate of 100 µl/min. In competition (cochallenge) experiments, mice were inoculated with 50 µl of a 1:1 mixture of the yersiniabactin receptor mutant (536Δ*fyuA::kan*) and wild type 536 *E. coli* for a total of 1×10^8 CFU of total bacteria/mouse. Total CFUs for each inoculum was quantified by plating dilutions onto LB agar with or without kanamycin (25 µg/ml) to differentiate inoculating strains when appropriate. After 48 h, mice were euthanized and bladders, kidneys, and spleens were removed and homogenized in 3 ml PBS using an Omni GLH homogenizer (Omni International). Homogenized organs were plated onto LB agar plates with or without kanamycin (25 µg/ml), using an Autoplate

4000 spiral plater (Spiral Biotech) and total CFU/g tissue was determined (output) for each inoculating strain. For cochallenge experiments with two inoculated strains, competitive indices (C.I.) were calculated by dividing the ratio of the mutant to wild type in the output by the ratio of the mutant to wild type in the inoculum [$(CFU_{\Delta fyuA}/ CFU_{536})_{output}/(CFU_{\Delta fyuA}/ CFU_{536})_{inoculum}$]. Coinoculated strains that colonize to a similar level would have a C.I.=1. Mutant strains with reduced colonization fitness in comparison to wild type would have a C.I.<1 and strains with enhanced colonization fitness would have a C.I.>1.

Murine model of bacteremia.

Six-to-eight week old, female CBA/J mice (Harlan Laboratories) were inoculated via tail vein injection with 100 µl of a 1:1 mixture of *E. coli* strains 536 and 536Δ*fyuA*::*kan* for a total of 1×10^7 CFU/mouse. After 21 h, mice were euthanized and kidneys, spleen, and liver were removed. Organs were homogenized in 3 ml PBS and plated onto LB agar plates with or without kanamycin (25 µg/ml), to quantify CFU/g tissue. Colonization fitness was calculated by a competitive index (C.I.) as described above.

Vaccine antigen preparation.

The gene encoding the yersiniabactin receptor, *fyuA,* was PCR-amplified from *E. coli* 536 genomic DNA and cloned into the expression vector pBAD-*myc*-HisA (Invitrogen). Recombinant FyuA expression was induced in *E. coli* TOP10 cells cultured in Terrific broth (12 g/liter tryptone, 24 g/liter yeast extract, 4 ml/liter glycerol, 100

ml/liter filter-sterilized 0.17 M KH_2PO_4 and 0.72 M K_2HPO_4) at 37°C with aeration to an OD_{600}=0.8 by the addition of 1 mM L-arabinose. After 4 h, the induced bacterial cultures were harvested by centrifugation (8,000 × *g*, 10 min, 4°C) and resuspended in 10 mM HEPES, pH 7, and 100 U benzonase nuclease (Sigma-Aldrich). Bacteria were lysed by two passages through a French pressure cell (20,000 psi) and the lysate cleared by centrifugation (8,000 × *g*, 10 min, 4°C). Bacterial membranes were pelleted from the cleared lysate by ultracentrifugation (112,000 × *g*, 30 min, 4°C) and solubilized in 5 ml of 100 mM NaH_2PO_4, 10 mM Tris-HCl, 8 M urea, pH 8.0. Recombinant His_6-tagged FyuA was purified on nickel-nitriloacetic acid-agarose columns (Qiagen) under denaturing conditions according to the manufacturer's instructions (The QIAexpressionist). Eluted FyuA-His_6 was renatured by four successive dialysis steps at 4°C (buffer 1: 100 mM NaH_2PO_4, 5 mM Tris-HCl, 6 M urea, 50 mM NaCl, pH 5.5; buffer 2: 100 mM NaH_2PO_4, 2.5 mM Tris-HCl, 4 M urea, 100 mM NaCl, 0.005% Zwittergent, pH 6.5; buffer 3: 100 mM NaH_2PO_4, 2 M urea, 150 mM NaCl, 0.01% Zwittergent, pH 7.4) into a final solution of 0.05% Zwittergent in PBS, pH 7.4, and quantified by the bicinchoninic acid (BCA) protein assay (Pierce).

Vaccination.

Purified, renatured, recombinant FyuA was chemically cross-linked to cholera toxin (CT) (Sigma-Aldrich) at a ratio if 10:1 using *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Pierce) according to the manufacturer's recommendations. Cross-linked FyuA-CT was administered to six-to-eight week old female CBA/J mice (Harlan Laboratories) intranasally at 20 µl/mouse (10 µl/nare). A

primary dose of 100 μg FyuA cross-linked to 10 μg CT was administered on day 0 and two booster doses of 25 μg FyuA cross-linked to 2.5 μg CT were administered on days 7 and 14. On day 21, mice were challenged transurethrally with *E. coli* as described in the murine model of ascending UTI.

Monoclonal antibody generation.

Three female BALB/c mice aged six-eight weeks were each immunized intraperitoneally with 10, 20, or 50 μg of purified, renatured, recombinant FyuA emulsified in complete Freund's adjuvant (Sigma-Aldrich) on day 0, followed by booster injections of 10, 20 and 50 μg (respectively) of FyuA with incomplete Freund's adjuvant (Sigma-Aldrich) on days 14, 42, and 56. The induction of FyuA-specific antibodies in serum was monitored by enzyme-linked immunosorbent assay (ELISA), and on day 60, the animal with the highest level of FyuA-specific antibodies was euthanized and the spleen removed. Harvested spleenocytes were fused to the murine cell line P3X63-Ag8.653 (181) using polyethylene glycol and conventional somatic cell hybridization techniques (182, 183) and the resulting hybridoma clones were screened for FyuA-specific antibody production by ELISA, Western blot, and flow cytometry. The monoclonal antibody (mAb) selected for the highest affinity for FyuA (5E7.22) was isotyped as IgG2bκ using a Pierce Rapid ELISA mouse mAb isotyping kit (ThermoFisher).

Flow cytometric analysis of yersiniabactin receptor surface accessibility.

Iron receptor expression was induced in wild type *E. coli* 536 and mutant

536Δ*fyuA*::*kan* by diluting overnight cultures 1:50 into fresh LB with 200 μM 2,2'-dipyridyl (DPD) (Sigma-Aldrich), and allowing cultures to incubate at 37°C with aeration for 6 h. Cultures were pelleted, washed with PBS, and incubated in undiluted supernatant from the FyuA-specific hybridoma clone 5E7.22 for 30 min at room temperature. FyuA-specific antibody binding was quantified by staining with the secondary antibody Fluorescein (FITC)-conjugated goat anti-mouse IgG (H+L) (Jackson ImmunoResearch) diluted 1:100 in PBS at room temperature for 30 min and analyzed by FACSCanto (Becton, Dickinson and Co.). *E. coli* cultured in iron-replete conditions, stably expressing GFP, or stained with only secondary antibody were used as controls for the assay. Histograms were produced with FlowJo software (Tree Star Inc.).

Statistical analysis.

Significance was assessed using a two-tailed Mann-Whitney test or a Wilcoxon signed rank test with a theoretical median of zero, when appropriate. All *P* values are two-tailed at a 95% confidence interval. Analyses were performed using GraphPad Prism, version 6.0d (GraphPad Software, San Diego, CA).

Results

The yersiniabactin system is highly expressed during uncomplicated UTI.

To determine if bacterial iron acquisition systems are expressed during natural, uncomplicated UTI, urine was collected and immediately stabilized with RNAprotect from women seeking treatment for cystitis at the University of Michigan University Health Services Clinic. Of the 86 urine samples collected, 42 were positive for bacteria by culture, and of those 38 (90%) contained *E. coli* at $\geq 10^5$ CFU/ml. An additional four species were isolated: *Citrobacter freundii, Citrobacter koseri, Enterobacter aerogenes,* and *Proteus mirabilis.* Bacterial RNA transcript levels from five *E. coli* samples were quantified by RNAseq analysis and four mapped sufficiently to their respective genomes for quantification (174). To provide comparison, RNAseq analyses were also performed on RNA isolated from the same four *E. coli* strains cultured in LB (an iron-replete medium) and filter-sterilized human urine, which is naturally iron-limited. All four UTI isolates during natural bladder infection had measurable RNA transcript levels for bacterial genes involved in iron acquisition, mirroring levels observed during culture in human urine, as compared to LB (Figure 3-1). Of the three *E. coli* siderophore systems that are pathogen-associated (salmochelin, aerobactin, and yersiniabactin), the yersiniabactin system was the most prevalent, found in three of the four isolates (Figure 3-1). Our observations support previous molecular epidemiological data on UPEC virulence factors, which found that 87% of cystitis *E. coli* isolate genomes encoded *fyuA*, compared to 74% and 35% for *iroN* (salmochelin) and *iutA* (aerobactin), respectively (184). Transcript levels for the gene *fyuA* were among the most abundant, as all three yersiniabactin-encoding isolates had *fyuA* in the top 15% of genes expressed during

cystitis, as compared to growth in LB (HM27:11.1%, HM69:10.4%, HM65:14.4%). Transcriptional start sites in the yersiniabactin operon could also be predicted by a histogram of transcript abundance, suggesting strong induction of the promoter upstream of *ybtA*, which encodes an AraC-like transcriptional activator of *fyuA* (185), and the promoter directly upstream of *fyuA* (Figure 3-2).

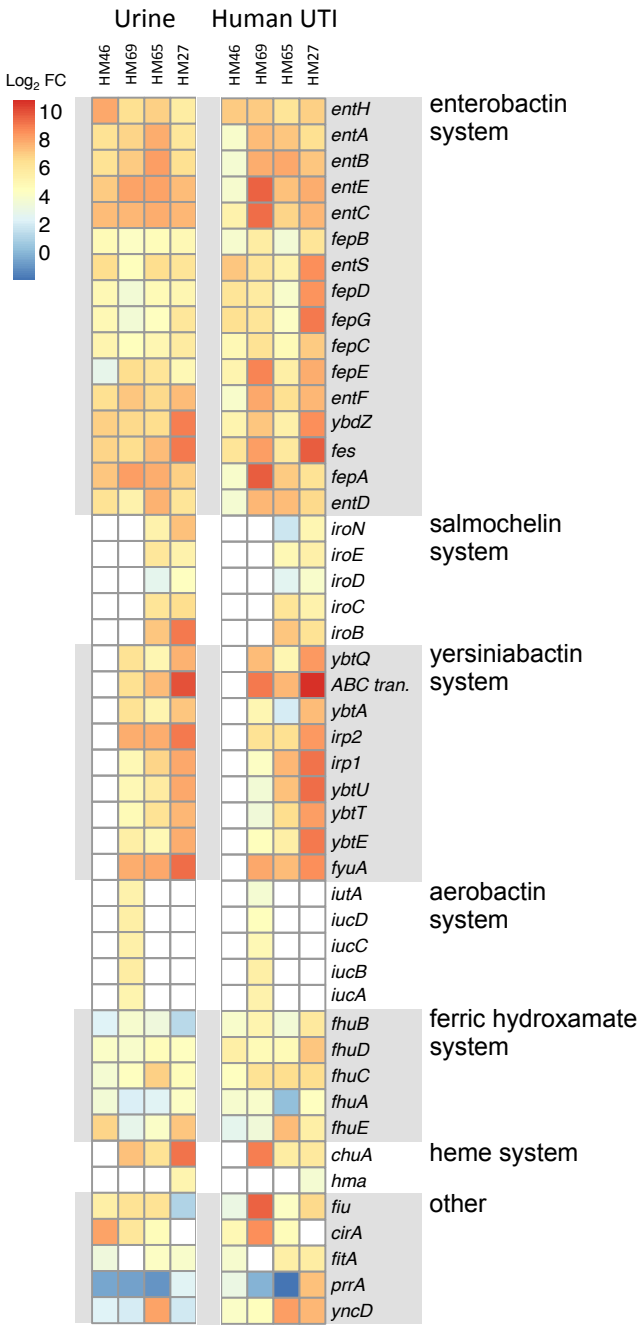


Figure 3-1. UPEC iron acquisition gene expression by *E. coli* strains during cystitis in women versus culture in urine. Fresh urine samples from four women with uncomplicated *E. coli* UTI were stabilized immediately and processed for bacterial RNA (HM46, HM69, HM65, HM27). RNA transcript levels were quantified by RNAseq and compared to transcript levels in the same strains during culture in human urine and LB. Differential transcript abundance between UTI or urine compared to LB for each gene is presented as the $\log_2$-fold change (FC) and visualized on a heat map. Genes with more abundant RNA transcripts during cystitis or cultured growth in urine in comparison to growth in LB are colored darker red. Genes not present in the genome of the isolate are left white.

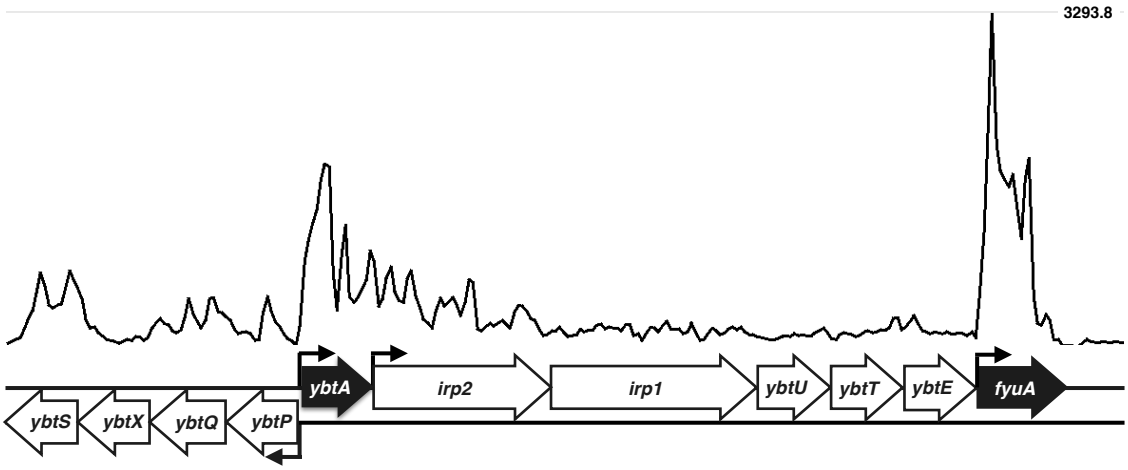


Figure 3-2. RNAseq read coverage of the yersiniabactin operon in *E. coli* cultured in human urine. Histogram displaying sequence coverage per nucleotide for reads mapped to the yersiniabactin operon of the *E. coli* HM27 genome from RNAseq data of *E. coli* HM27 cultured in urine. Open reading frames are illustrated below the histogram with known promoter regions identified. The genes *ybtA* and *fyuA*, which encode an AraC-like transcriptional activator of *fyuA* and the yersiniabactin receptor FyuA, respectively, are highlighted. Genes *irp2*, *irp1*, *ybtU*, *ybtT*, *ybtE* and *ybtS* encode proteins involved in yersiniabactin synthesis, *ybtP*, *ybtQ*, and *fyuA* encode proteins that facilitate yersiniabactin transport, and *ybtX* is of unknown function (186).

Blocking yersiniabactin attenuates UPEC during UTI.

The yersiniabactin receptor, FyuA, is an effective vaccine target to prevent pyelonephritis, but not cystitis (119). Given that gene expression studies from experimental infection in mice (109) and now natural uncomplicated infections in women indicate that iron acquisition systems are highly expressed by bacteria infecting the bladder, the lack of bladder protection by an FyuA-based vaccine is surprising. One possible mechanism for this organ-specific immunity is that the yersiniabactin system mediates infection of the kidneys, but not the bladder. To test whether the yersiniabactin system contributes to *E. coli* pathogenesis in the urinary tract, we evaluated the virulence of an *E. coli* mutant unable to acquire iron by yersiniabactin during infection. Mice inoculated transurethrally with wild type *E. coli* 536 had significantly higher bacterial colonization in the bladder and the kidneys compared to mice inoculated with an isogenic mutant deficient in the yersiniabactin receptor (Δ*fyuA*) (Figure 3-3). These data support yersiniabactin as a significant *E. coli* virulence factor in the bladder and kidneys and suggest that the failure of an FyuA-based vaccine to prevent cystitis may be due to immunological differences, such as antibody concentration, between the bladder and the kidneys, rather than differences in *E. coli* pathogenesis.

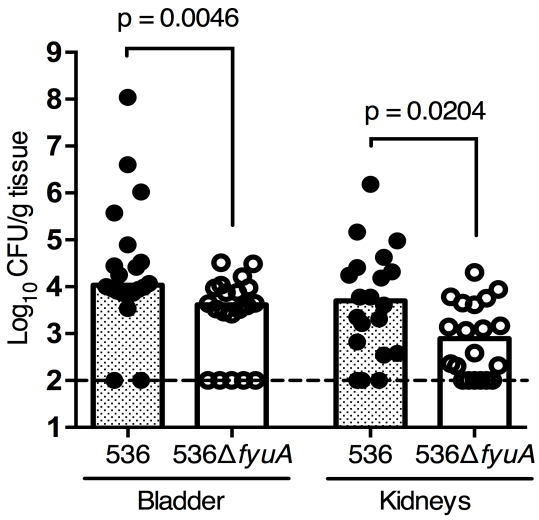


Figure 3-3. Blocking yersiniabactin import attenuates *E. coli* during UTI. Female CBA/J mice were transurethrally inoculated with 1×10^8 CFU of *E. coli* 536 or *E. coli* 536Δ*fyuA* in independent challenge and colonization was measured 48 hours post inoculation. Data from two independent experiments are presented with the total number of animals per group being *N*=20 (536) and *N*=20 (536Δ*fyuA*). Symbols represent CFU/g tissue from an individual mouse and bars indicate median values. Dashed line shows the limit of detection for the assay, 100 CFU/g. Significance was determined using a two-tailed Mann-Whitney test.

Recombinant FyuA vaccine is target-specific.

The yersiniabactin iron acquisition system facilitates *E. coli* infection of the kidneys and bladder, but immunization with a recombinant FyuA-based vaccine only protects against pyelonephritis, not cystitis (119). To confirm that the protective target of the FyuA vaccine is indeed the yersiniabactin receptor, mice were immunized and then cochallenged with a mixture of two isogenic *E. coli* strains: wild type 536 that produces FyuA, and 536Δ*fyuA*::*kan* that does not. *E. coli* expressing FyuA have a substantial fitness advantage in the urinary tract (Figure 3-3), but in the presence of an FyuA-specific immune response, we hypothesized that the advantage would be reduced or lost. Female CBA/J mice were intranasally immunized on day 0 with a primary dose of FyuA crosslinked to cholera toxin (CT) as adjuvant or CT alone, and then boosted with a quarter dose on days 7 and 14. On day 21, mice were transurethrally coinoculated with a 1:1 mixture of 536 and 536Δ*fyuA*::*kan*. After 48 hours post infection, mice were euthanized and organs were removed to quantify the level of bacterial infection from each strain. As expected, wild type *E. coli* outcompeted the Δ*fyuA* mutant when infecting the bladder and kidneys of control mice immunized with only adjuvant (Figure 3-4A) reinforcing the observation that FyuA acts as an UPEC virulence factor during UTI (Figure 3-3). Furthermore, in the five mice that had their infection reach the spleen, wild type *E. coli* was found exclusively, further supporting the role of the yersiniabactin system during systemic infection (Figure 3-4A). However, when mice were immunized with FyuA-CT prior to experimental infection, wild type *E. coli* lost its fitness advantage over the Δ*fyuA* mutant, exclusively in the kidneys (Figure 3-4B). The kidney-specific attenuation of wild type *E. coli* is consistent with the kidney-specific protection of the

FyuA vaccine previously observed (119) and confirms the yersiniabactin receptor, FyuA, as the protective target of the FyuA vaccine.

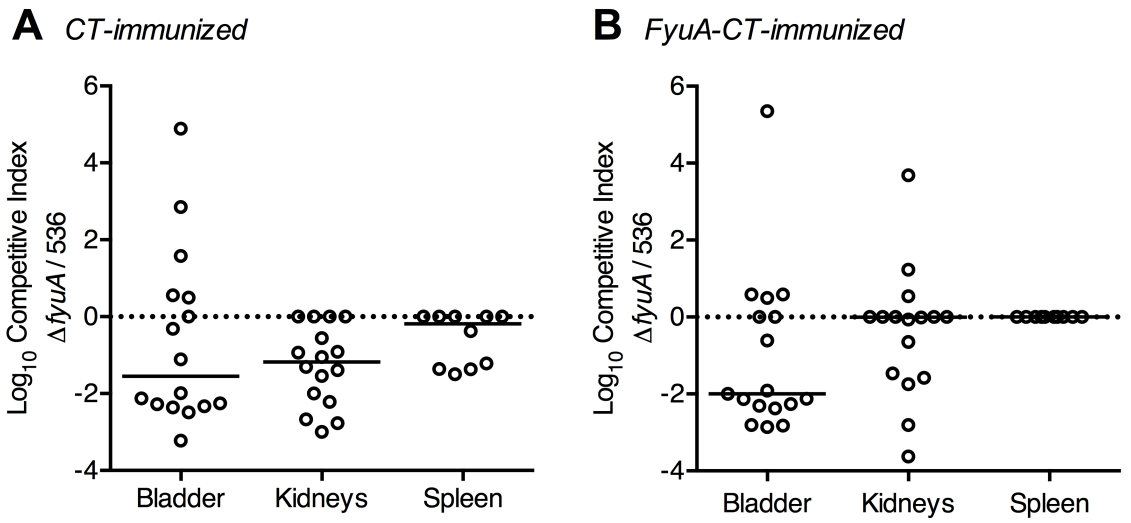


Figure 3-4. FyuA vaccine is specific for the yersiniabactin receptor. Female CBA/J mice immunized with CT **(A)**, or FyuA-CT **(B)** were transurethrally co-inoculated with a mixture of *E. coli* strains 536 and 536Δ*fyuA*::*kan*. Colonization was measured 48 hours post infection by plating for CFU/g tissue and competitive indices (C.I.) were calculated by dividing the fraction of the Δ*fyuA* mutant in the output (CFU/g tissue) by the fraction of the Δ*fyuA* mutant in the input (CFU/ml inoculum). Solid lines represent the median value for each group and the dashed line indicates a theoretical median of zero. Symbols represent the C.I. of an organ from a single mouse. Data were validated by two independent experiments for a total of 16 mice per group, with the exception of the spleen that only had 10 mice per group. Significance was determined by the Wilcoxon signed rank test with a theoretical median of zero. *P*-values for each group from left to right: **(A)** 0.2293, 0.0005, 0.0625, **(B)** 0.0245, 0.2402, NA (all spleens from the FyuA-CT-immunized mice were uninfected).

Yersiniabactin contributes to UPEC pathogenesis during systemic infection.

During severe infection, bacteria infecting the kidneys can breach epithelial and endothelial barriers to gain access to the bloodstream, causing systemic and life-threatening disease (161). Since blocking yersiniabactin attenuates *E. coli* in the urinary tract, and FyuA-immunized mice are protected from systemic infection (113), we hypothesized that the yersiniabactin system may contribute to *E. coli* pathogenesis during bacteremia. To test this, mice were challenged systemically by intravenous injection with a 1:1 mixture of wild type *E. coli* 536 and an isogenic mutant unable to import yersiniabactin. After 21 hours post infection, the *E. coli* mutant unable to acquire iron by the yersiniabactin system was significantly outcompeted by wild type *E. coli* in the spleen and kidneys (Figure 3-5), indicating that iron acquisition by the yersiniabactin system contributes significantly to UPEC pathogenesis during bacteremia.

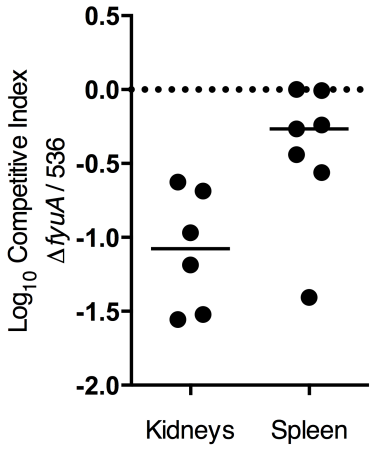


Figure 3-5. Blocking yersiniabactin import attenuates *E. coli* during systemic infection. Ten female CBA/J mice were intravenously co-inoculated with an equal mixture of wild type *E. coli* 536 and *E. coli* 536Δ*fyuA*::*kan* and organ colonization was measured 21 hours post inoculation. Competitive indices were calculated by dividing the fraction of the mutant in the output (CFU/g tissue) by the fraction of the mutant in the input (CFU/g inoculum). The median value for each group is represented as a solid line and the dashed line indicates a theoretical median of zero, which would occur if both wild type and mutant *E. coli* had equal representation in both the input and output. Three mice succumbed to the infection before being euthanized and are were excluded from the analysis. Significance was determined by the Wilcoxon signed rank test with a theoretical median of zero, and the *P*-value for each group was 0.0313 for the kidneys and 0.0313 for the spleen.

The yersiniabactin receptor is accessible on the bacterial cell surface.

Preventing iron acquisition by yersiniabactin attenuates UPEC in the urinary tract and bloodstream. Therapeutically blocking the yersiniabactin system, either with vaccine-induced antibodies or a pharmaceutical agent, would likely be simplest by targeting the siderophore receptor, FyuA, on the bacterial outer membrane. To confirm that FyuA is exposed and accessible on the cell surface, despite the presence of capsule and surface polysaccharides, we developed an FyuA-specific monoclonal antibody (mAb) and quantified cell surface binding by flow cytometry. *E. coli* 536 cultured under iron-limitation, mimicking conditions during infection, had a dramatic increase in median fluorescence intensity in comparison to an FyuA-deficient isogenic mutant (536Δ*fyuA*), indicating that a portion of FyuA is accessible to antibody binding on the bacterial cell surface (Figure 3-6).

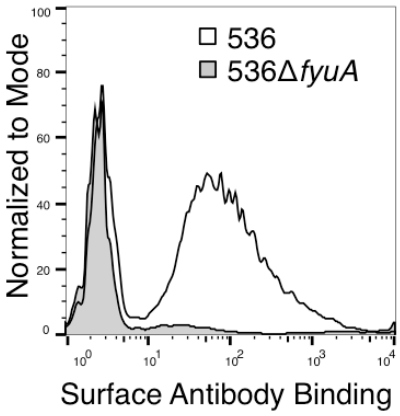
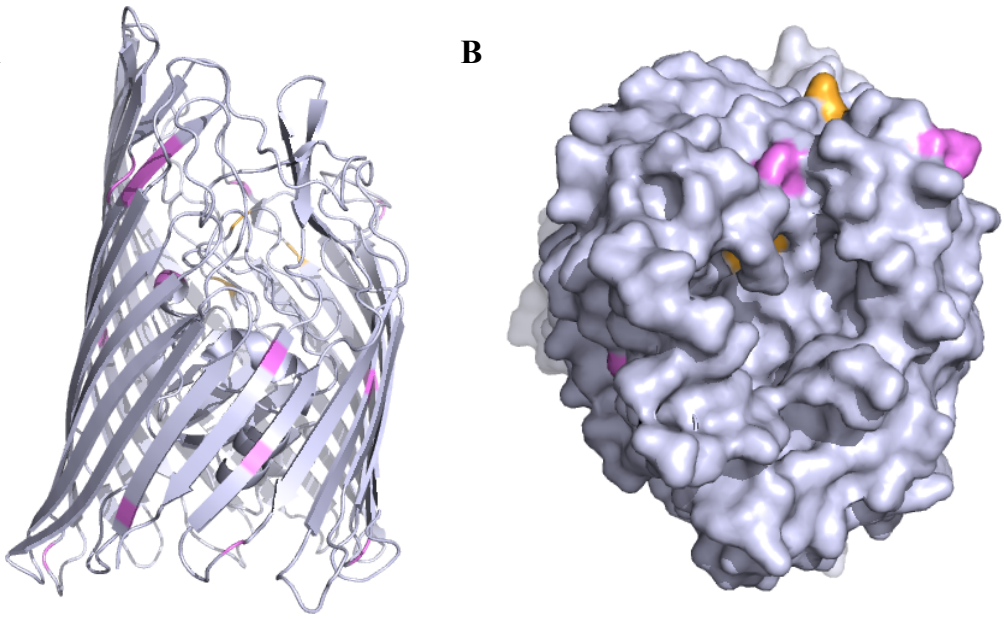


Figure 3-6. Yersiniabactin receptor is surface-exposed and accessible to antibodies. Histogram overlay of FyuA surface expression and antibody binding as assessed by flow cytometry. UPEC strains 536 and 536Δ*fyuA* were cultured under iron-limitation and incubated with an FyuA-specific mAb (5E7.22), followed by a secondary antibody conjugated to FITC. Shaded area represents a control *E. coli* strain unable to express FyuA (536Δ*fyuA*), and the unshaded area denotes antibody binding to wild type *E. coli*.

FyuA is highly conserved among pathogens in the family *Enterobacteriaceae*.

To determine the degree of conservation of the yersiniabactin receptor, FyuA, at the amino acid sequence level between *E. coli* and *K. pneumoniae* strains, sequences of FyuA from 20 *E. coli* strains and 17 *K. pneumoniae* strains chosen at random were aligned and compared using the Clustal Omega sequence alignment tool (187). A high level of conservation was observed, as pairwise alignments were greater than 99.3% identical for all 37 aligned sequences (Figure 3-7). In addition, a high level of sequence conservation between strains was observed on the surface-exposed face of FyuA, as predicted by the tool PRED-TMBB based on a Hidden Markov Model (188), and shown on the crystalized structure of FyuA (Figure 3-7A, B) (189).

To evaluate the level of FyuA amino acid sequence conservation between multiple pathogenic members of the family *Enterobacteriaceae*, consensus sequences were generated and compared between the species *E. coli*, *K. pneumoniae*, *K. oxytoca*, *Y. pestis*, *Y. enterocolitica*, *Y. pseudotuberculosis*, *S. enterica*, and *Citrobacter koseri* (Figure 3-8). Although increased variability exisits in the amino acid alignments for FyuA between the multiple species evaluated, a high degree of conservation can still be observed, as all pairwise alignments were greater than 92.0% identical (Figure 3-8).



C

1 MKMTRLYPLALGGLLLPAIANAQTSQQDESTLVVTASKQSSRSASANNVSSTVVSAPELSDA

63 GVTASDKLPRVLPGLNIENSGNMLFSTISLRGVSSAQDFYNPAVTLYVDGVPQLSTNTIQALTD

127 VQSVELLRGPQGTLYGKSAQGGIINIVTQQPDSTPRGYIEGGVSSRDSYRSKFNLSGPIQDGLL

191 YGSVTLLRQVDDGDMINPATGSDDLGGTRASIGNVKLRLAPDDQPWEMGFAASRECTRAT

251 QDAYVGWNDIKGRKLSISDGSPDPYMRRCTDSQTLSGKYTTDDWVFNLISAWQQQHYSR

310 TFPSGSLIVNMPQRWNQDVQELRAATLGDARTVDMVFGLYRQNTREKLNSAYDMPTMP

367 YLSSTGYTTAETLAAYSDLTWHLTDRFDIGGGVRFSHDKSSTQYHGSMLGNPFGDQGKSN

428 DDQVLGQLSAGYMLTDDWRVYTRVAQGYKPSGYNIVPTAGLDAKPFVAEKSINYELGTRY

489 ETADVTLQAATFYTHTKDMQLYSGPVGMQTLSNAGKADATGVELEAKWRFAPGWSWDI

547 NGNVIRSEFTNDSELYHGNRVPFVPRYGAGSSVNGVIDTRYGALMPRLAVNLVGPHYFDG

605 DNQLRQGTYATLDSSLGWQATERMNISVYVDNLFDRRYRTYGYMNGSSAVAQVNMGRTV

665 GINTRIDFF

Figure 3-7. FyuA amino acid sequence is highly conserved between *Escherichia coli* and *Klebsiella pneumoniae* strains. Cartoon **(A)** and surface view of the extracellular face **(B)** of the crystal structure of FyuA (PDB: 4EPA) (189) colored for conservation between a total of 20 *E. coli* and 17 *K. pneumoniae* strains sampled at random. Grey is 100%, violet >90%, and orange 85-90% conservation. **(C)** Sequence map of FyuA with extracellular exposed sequences as predicted by a Hidden Markov Model (PRED-TMBB) underlined in green (188). Individual amino acids are coded by the same conservation color scheme. Turquoise bar corresponds to FyuA plug domain, provided for reference.

Figure 3-8. FyuA amino acid sequence is highly conserved among pathogenic species in the family *Enterobacteriaceae*. FyuA consensus sequences generated with Clustal Omega from pathogenic species in the family *Enterobacteriaceae* are compared. Dark and light blue indicate complete or >80% identity respectively. A consensus sequence for all evaluated sequences is provided and the plug domain is highlighted in turquoise and extracellular exposed residues as predicted by PRED-TMBB are highlighted in green. Number of sequences used to generate consensus sequences: 20 *E. coli*, 17 *K. pneumoniae*, 13 *K. oxytoca*, 17 Y. pestis, 8 *Y. enterocolitica*, 3 *Y. pseudotuberculosis*, 2 *S. enterica*, 1 *Citrobacter koseri*.

Discussion

Here we demonstrate the yersiniabactin receptor to be a virulence factor for UPEC during cystitis and pyelonephritis, a fitness factor during bacteremia, and the specific, surface-accessible target of the experimental FyuA vaccine. Furthermore, we show that genes encoding bacterial systems for iron acquisition, including the yersiniabactin system, are highly expressed during natural, uncomplicated cystitis in women. Overall, our data support the yersiniabactin system as a therapeutic target to prevent or treat UPEC-mediated UTI, and reinforce the potential of the yersiniabactin system as a common target for several, increasingly multidrug-resistant, Gram-negative enteric pathogens (189, 190).

Given that UPEC strains frequently encode numerous, often-redundant iron acquisition systems, the ability to attenuate infection by targeting just a single system is surprising. While individual iron uptake mechanisms uniquely contribute to bacterial pathogenesis (115, 191), it seems reasonable to assume their similar function would allow for compensation. Of the four siderophores UPEC isolates produce, three are so-called 'stealth' siderophores (salmochelin, yersiniabactin, aerobactin) for their ability to avoid sequestration by lipocalin-2 (191, 192). Isolating UPEC strains with all three stealth siderophore systems is uncommon, possibly due to their outer membrane receptors being the frequent target of bacteriophages and bacteriocins (193). UPEC strain 536 synthesizes two of these stealth siderophores: salmochelin and yersiniabactin, but not aerobactin. It is possible that the less common aerobactin system may compensate for the loss of yersiniabactin during *E. coli* UTI, but this remains to be tested.

Although the protective mechanism of the kidney-specific FyuA vaccine is unknown, high *fyuA* expression and significant FyuA-mediated virulence during cystitis suggest that the absence of vaccine protection in the bladder (119) is not due to reduced yersiniabactin expression or pathogenesis in this organ. Thus immunological differences between the bladder and kidneys may explain the disparity in vaccine efficacy between tissues. Indeed, despite being prone to recurrent infection, the immunological networks of the bladder are distinct from the kidney and remain poorly defined (194). Therefore it may be possible to extend the protection of the FyuA vaccine to the bladder by modifying the immunization route, formula, adjuvant, or timeline to improve the adaptive immune response in the bladder.

Lastly, since yersiniabactin is predominantly associated with highly pathogenic strains and a major virulence factor for several pathogenic bacteria in the family *Enterobacteriaceae*, targeting FyuA may provide a mechanism to specifically eliminate disease-causing bacteria without harming the beneficial bacteria comprising the microbiome, which is a common side effect of conventional antibiotics (195). In addition, yersiniabactin is disproportionately associated with antibiotic-resistant strains, including >90% of the *E. coli* sequence type 131 (ST131) an alarming pandemic multidrug-resistant (MDR) clonal group (196-199), and >60% of respiratory carbapenemase-producing *K. pneumoniae* strains, as compared to 10% of susceptible *K. pneumoniae* strains (192). Novel antimicrobial agents or vaccines that target yersiniabactin iron transport, or other pathogen-associated nutrient acquisition systems may provide an effective strategy to combat the rising surge of antibiotic-resistant common infections.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Results

Here we presented studies that addressed the potential of select UPEC outer membrane iron receptors to be used as vaccine candidates to protect against UTI, and tested the contribution of the yersiniabactin receptor, FyuA, to UPEC pathogenesis during UTI and systemic infection. The major findings of this dissertation are briefly described:

- Intranasal immunization with the vaccine candidate FyuA protected CBA/J mice from experimental UTI, but IroN, FitA, ChuA fragments, and the gene product of CFT073 locus *c0294*, did not.
- Immunization with FyuA protected mice from experimental pyelonephritis, but not cystitis, and elicited a sustained and robust FyuA-specific serum IgG response that correlated with levels of UPEC colonization in the kidney, but not in the bladder.
- Bacterial RNA transcript levels for genes encoding proteins involved in iron acquisition were highly abundant in UPEC strains causing natural uncomplicated cystitis in women.

- The yersiniabactin receptor, FyuA, contributes to UPEC virulence during experimental cystitis and pyelonephritis, and to UPEC fitness during bacteremia.
- The experimental FyuA vaccine is specific for its target, FyuA, which is exposed and accessible to antibody binding on the *E. coli* outer membrane.
- FyuA is highly conserved at the amino acid level among *E. coli* and *K. pneumoniae* strains and provides a common narrow-spectrum therapeutic target for current outbreaks of carbapenem-resistant *Enterobacteriaceae*.

Conclusions and Perspectives

The data presented here strongly support a role for FyuA and yersiniabactin-mediated iron acquisition by UPEC during UTI. Of the six outer membrane iron receptor vaccine candidates tested in a mouse model of ascending UTI, the yersiniabactin receptor, FyuA, was the only protective candidate identified from the screen. In addition, a UPEC mutant unable to acquire yersiniabactin during infection was significantly outcompeted by wild type in the bladder, kidneys, and spleen. While we present strong evidence supporting the contribution of the yersiniabactin system to UPEC pathogenesis and its potential as a therapeutic target, a number of questions remain.

The specific mechanism(s) of protection for the experimental FyuA vaccine remain to be clearly defined. We hypothesize that vaccine protection is humorally-mediated, as there is ample evidence to suggest a role for antibodies during UPEC clearance. Naïve mice passively immunized with anti-ovalbumin (OVA) antibodies were protected from experimental UTI by a UPEC strain engineered to express the highly-antigenic OVA antigen (33), and naïve mice passively immunized with rabbit antisera

raised against UPEC iron receptors, including FyuA, were protected from lethal challenge by UPEC in a model of systemic infection (200). However, establishing that antibodies raised against natural UPEC antigens can passively protect against UPEC infection in the urinary tract has proven to be more difficult. An attempt to passively immunize naïve mice with mouse polyclonal FyuA antisera was unsuccessful (Figure A-1), as was an attempt to produce a therapeutic FyuA-specific monoclonal antibody (Appendix B).

We hypothesize that there are two reasons why passive protection in the urinary tract has been more difficult to achieve than in a model of systemic infection. First, the delivery of sufficient quantities of antibodies to the urinary tract may be a substantial hurdle. Antibodies delivered to mice by intraperitoneal injection quickly transferred from the peritoneal cavity to the bloodstream, within hours of injection (201), as validated by ELISA (Figure B-6). However, intraperitoneally-injected antibodies were not significantly detected in the urine of passively immunized mice 16 hours after injection (Figure B-6), suggesting that antibodies delivered to the peritoneal cavity are not efficiently transferred to the urinary tract. Poor or slow antibody delivery to the urinary tract may provide an explanation for why attempts to passively immunize mice against UTI were unsuccessful, including attempts with highly concentrated antisera raised against O-polysaccharide (O6), a UPEC antigen prevalent on the bacterial cell surface (data not shown).

Secondly, polyclonal FyuA antisera raised from intranasally-immunized mice may not provide sufficient antibody levels to passively protect naïve mice. Intranasal immunization with hydrophobic, insoluble iron receptors, such as FyuA, induced variable

levels of serum antibody (Figure 2-6), likely due to inconsistent dosing between animals. Pooled sera from multiple immunized mice may dilute the protective vaccine-specific antibodies generated, and low antibody levels coupled with poor or slow antibody delivery into the urinary tract from intraperitoneal injection may impede successful passive protection. Thus, the lack of UTI protection observed after passive immunization is likely the result of technical hurdles preventing the delivery of highly concentrated antisera into the urinary tract.

Understanding the specific role of vaccine-generated antibodies during infection can facilitate vaccine development by guiding the optimization of immunization route, dose, adjuvant, and timeline to better induce the specific protective component(s) of the vaccine-generated immune response. For example, although, we found FyuA-specific serum IgG levels to correlate with protection in the kidney, it is unclear if serum IgG plays a direct role in UPEC clearance from the urinary tract, or if it is only a marker for antibody class switching in B cells and the induction of an FyuA-specific adaptive immune response. If serum IgG plays a direct role in UPEC clearance, alternative routes of immunization such as intramuscular injection and adjuvants can be used to induce a more robust serum IgG response, and possibly improve vaccine protection. Identifying the specific immune system mechanisms that contribute to protection would be extremely beneficial, and immunization experiments using mouse knockout models deficient in specific components of the adaptive immune system may provide a more specific way to evaluate the role of the adaptive immune system in vaccine protection of the urinary tract.

Although we hypothesize that vaccine-induced bacterial clearance is mediated primarily by antibody opsonization, another possible mechanism may be the generation

of antibodies that bind to iron receptors and inhibit their function. Blocking yersiniabactin acquisition by FyuA attenuated UPEC during UTI, as did blocking heme uptake by the heme receptor Hma (202), and blocking aerobactin uptake by IutA (107). All three of these outer membrane receptors are protective vaccine targets for UPEC UTI (32, 119). The organ-specific protection induced by immunization closely correlates with the organ-specific attenuation observed by blocking receptor function. Immunizing mice with Hma protected against pyelonephritis, but not cystitis (32), and blocking Hma heme acquisition attenuated UPEC in the kidney, but not the bladder (202). Immunizing mice with IutA protected against both pyelonephritis and cystitis (32), and blocking aerobactin uptake by IutA attenuated UPEC in both the kidney and the bladder (107). Immunizing mice with FyuA protected against pyelonephritis and reduced cystitis, although not significantly (119), and blocking yersiniabactin acquisition attenuated UPEC in the kidney and bladder. Only the protective vaccine candidate, IreA, contravenes this trend. IreA is an iron regulated outer membrane receptor of unknown function (203), and mice immunized with IreA were protected against cystitis, but not pyelonephritis (32). However, IreA does not appear to contribute to the fitness of UPEC in the bladder or kidneys, as determined by coinfections with wild type UPEC and a Δ*ireA* mutant (Figure D-1). Although IreA-neutralization by vaccine-induced antibodies does not appear to be a possible protective mechanism against cystitis in IreA-immunized mice, it does not invalidate the possibility that blocking receptor function by antibody binding may contribute to vaccine-induced protection by the other vaccine candidates FyuA, Hma, and, IutA. Indeed, antisera raised against Hma was able to significantly reduce the level of heme binding to recombinant Hma *in vitro*, as compared to pre-immune sera (204).

The full evaluation of whether vaccine-induced antibodies contribute to UPEC UTI protection through blocking iron receptor function requires further testing by cross-feeding assays in the presence of vaccine-specific antibodies.

In addition to unanswered questions regarding the specific mechanism(s) of iron receptor vaccine protection, gene expression data from *E. coli* causing uncomplicated cystitis in women provoked new questions regarding UPEC pathogenesis during natural UTI. The quantification of RNA transcript levels with RNAseq from pathogenic *E. coli* directly stabilized from the urine of women with active infection supported a role for bacterial iron acquisition during human uncomplicated cystitis (Figure 3-1, 2), confirming studies in mice (205) and in patients with complicated UTI (206). In addition, the study revealed new UPEC UTI virulence determinants, such as a copper efflux system (Subashchandrabose et al., *submitted*), and confirmed previous UTI epidemiological studies that *E. coli* is the most prominent cystitis bacterial species (90%) and that antibiotic resistance to first-line therapeutics is prevalent (15.3% resistant to TMP-SMX, 7.7% resistant to Ciprofloxacin), though at lower levels than documented at the University of Michigan Hospital (27% resistant to TMP-SMX, 27% resistant to Ciprofloxacin) (Appendix E). However, RNAseq analysis of RNA transcripts allowed for very detailed and quantitative data on bacterial gene expression during infection, revealing substantial expression variability between isolates. RNA transcript levels for the gene *fimA*, which encodes the major structural subunit of type 1 fimbria, a virulence factor essential for UPEC colonization of the murine bladder (89), were among the top 10% most abundant in two of the isolates (HM27 and HM46), but among the bottom 50% in abundance in isolates HM69 and HM65. The variability of type 1 fimbria expression

was confirmed by evaluating the orientation of the *fim* promoter, located on an invertible element upstream of *fimA*, in 21 of the stabilized UPEC cystitis samples (Figure E-1). Similar expression variability between isolates was noted for multiple fimbrial systems (Luterbach et al., *unpublished*) and metabolic systems (Alteri et al., *unpublished*) leading to questions about host differences, such as patient hydration level or immunity, and strain differences, such as phylogenetic group. Alternatively, variations in UPEC gene expression may represent various stages of UTI progression, a possibility that needs to be explored through single infection time courses.

The conditions of the urinary tract upon initial bacterial inoculation are likely very different from conditions after logarithmic bacterial growth and colonization, and even more dissimilar after the initiation and recruitment of a damaging inflammatory immune response. Dissolved urinary oxygen levels drop significantly during UTI (207), likely due to exhaustive bacterial aerobic respiration and rapid growth. Environmental oxygen levels influence vast networks of *E. coli* gene expression, causing shifts in metabolism (208), fimbrial expression (209), and virulence (210). After the recruitment of innate immune cells and the initiation of an inflammatory immune response, host and bacterial cell damage and debris can change the metabolites available to invading bacteria. Oxidative bursts from host innate immune cells can cause oxidative stress, leading to substantial shifts in UPEC gene expression, such as the down-regulation of genes involved in aerobic respiration and an increase in the expression of mechanisms to protect against harmful oxidative radicals (211). Thus, variations in UPEC gene expression between cystitis isolates may reflect various stages of UTI progression. Temporal gene expression changes during the course of infection have been documented

for other bacterial human pathogens, including *Vibrio cholerae* (212) and *Mycobacterium tuberculosis* (213). Resolving the dynamics of UPEC gene expression through the course of a natural UTI could influence our choice of an ideal vaccine target (expressed upon initial infection) and an ideal therapeutic target (expressed during mature or late infection) and further our understanding of UPEC pathogenesis and the host immune response.

Finally, enrolling women in a clinical study with various histories of uncomplicated UTI presented an opportunity to evaluate the host adaptive immune response to UPEC at the time of infection. Serum collected from study participants was evaluated for UPEC-reactive antibodies by western blot and ELISA. Levels of serum reactivity to UPEC whole cell lysates by western blot did not correlate with patient UTI history (data not shown). Likewise, neither did serum reactivity to individual UPEC iron receptors (Figure E-2 and data not shown). These data support recent findings by Chan and colleagues identifying the bladder as an immune-privileged site (194), suggesting that poor adaptive immunity is generated after cystitis, leaving patients susceptible to recurrent infection. Interestingly, the serum from all patients assayed had detectable levels of FyuA-specific IgG antibodies, which raises the question of when the adaptive immune system has had contact with FyuA in patients with no history of UTI. The expression of iron receptors in other host sites, such as the gastrointestinal tract during periods of gut inflammation (214), may provide a possible explanation, but remains to be explored.

Future Directions

While the studies presented here provide a crucial first step in defining a role for yersiniabactin-mediated iron acquisition by UPEC during UTI and demonstrate FyuA as a promising vaccine target against UPEC, further work is needed to refine our understanding of the yersiniabactin system and iron acquisition as a therapeutic target for UTI, and a contributor to UPEC pathogenesis.

Screening for UPEC iron receptors that protect against infection in a mouse model of UTI is just the initial step towards vaccine development and additional progress needs to be made to optimize the route, dose, adjuvant, delivery system, antigen composition, and immunization timeline. In addition, since much of our understanding of UPEC pathogenesis and vaccine protection is dependent on mouse models of infection, it behooves us to fully investigate the similarities and differences between natural human infection and experimental infection in mice. For example, preliminary evidence suggests that UPEC fimbrial expression is more diverse during human infection, than during experimental mouse infection (206), information that could have informed early clinical trials with adhesin-based vaccines (132). Thus, acknowledging and understanding the strengths and weaknesses of our model, which guides therapeutic design, would be a prudent investment, potentially saving us substantial time and resources before advancing ultimately to clinical trials.

Finally, therapeutically targeting pathogen-associated bacterial iron receptors, such as yersiniabactin, provides a unique mechanism to eliminate predominantly disease-causing bacterial strains while sparing the beneficial commensal bacteria of the gastrointestinal tract. However, we have a limited understanding of the role of *E. coli* iron

acquisition outside of the context of infection. Given that the urinary tract is an evolutionary 'dead end' for pathogenic *E. coli*, frequent iron receptor redundancy and diversity among pathogenic *E. coli* strains suggests that *E. coli* must encounter selective pressure for iron receptor diversity in an alternative host niche.

The mammalian gastrointestinal tract, where *E. coli* resides as a member of the microbiota in healthy individuals, is a constantly changing environment with substantial microbial competition for resources, making it a logical candidate location for where the evolutionary expansion of resource acquisition systems could occur. Indeed, enteric bacteria that routinely compete for nutrients with a diverse and shifting array of microbial competitors commonly have substantial nutrient acquisition system redundancy. For example, the common gut bacterial species *Bacteroides thetaiotaomicron* has over 80 systems dedicated to acquiring and processing dietary starch (215). Although the gastrointestinal tract is commonly thought of as a strictly anaerobic environment that is rich in dietary iron, during conditions of inflammation, facultative anaerobic species like *E. coli* bloom and microbial competition for iron intensifies (216). Indeed, the 'probiotic' *E. coli* strain Nissle 1917 was able to reduce the intestinal colonization of the enteric pathogen *Salmonella enterica* serovar Typhimurium in a mouse model of acute colitis by competing for iron resources with similar iron acquisition systems (214). *E. coli* iron acquisition system diversity and redundancy may be favored during periods of gastrointestinal inflammation, which then may give rise to strains that are able to colonize extraintestinal host niches.

Although, large-scale gene expression studies in the gut are still difficult to accomplish due to the density of the microbiota, fluorescent markers driven by individual

gene promoters may provide a way to assess the expression of individual *E. coli* iron receptor genes in response to gut inflammation and provide a method to evaluate the requirement for iron acquisition in the gut. Clearly, effort needs to be made to understand the mechanisms that drive iron receptor acquisition and diversification among *E. coli* strains, which may inform why particular strains accumulate multiple iron acquisition systems, facilitating extraintestinal infection.

Overall substantial work remains to fully understand UPEC pathogenesis in the urinary tract and persistence in the gastrointestinal tract, but the progress in UPEC vaccine development appears promising. New emerging technologies such as next generation sequencing allow UPEC gene expression to be evaluated during active human infection, facilitating new insights into bacterial pathogenesis and the host immune response to infection. These tools will help guide the development of novel vaccine strategies and therapeutics to combat the increasing prevalence of antibiotic resistant strains and further our understanding of UPEC pathogenesis during UTI.

APPENDICES

Appendix A. Passive immunization with FyuA anti-sera

To determine if the protective mechanism of the experimental FyuA vaccine (119) was based on an FyuA-specific humoral immune response, naïve CBA/J mice were passively immunized with either pooled control sera, or FyuA-antisera before being challenged with a transurethral inoculation of UPEC. Mice given FyuA-antisera (FyuA) before UPEC challenge had no significant protection from UPEC infection in either the kidneys or the bladder, as compared to control mice (CT) (Figure A-1). We hypothesized that the lack of protection could be due to insufficient antibody transfer, given that passively immunized mice would have substantially less circulating anti-FyuA antibodies, as compared to mice actively immunized with the experimental FyuA vaccine.

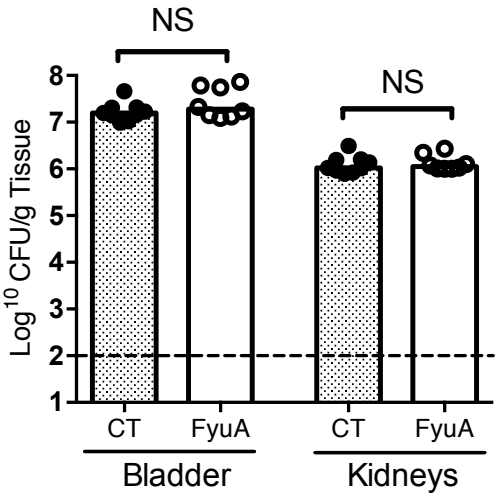


Figure A-1. Passive immunization of CBA/J mice using polyclonal FyuA-antisera. Six-to-eight week old female CBA/J mice were injected intraperitoneally with 200 µl of pooled serum from terminally bled female CBA/J mice, immunized with either purified recombinant FyuA-His_6 conjugated to cholera toxin (CT) or CT alone. After 16 h, passively immunized mice were inoculated intraurethrally with 1×10^8 CFU UPEC. Mice were euthanized 48 hpi and organs were homogenized and CFU/g tissue was enumerated by plating onto LB agar.

Appendix B. Developing a therapeutic FyuA-specific monoclonal antibody

Generating FyuA-specific spleenocytes

To determine if passively immunizing mice with sufficient FyuA-specific antibodies could protect against UPEC UTI, we attempted to develop a therapeutic FyuA-specific monoclonal antibody in conjunction with the University of Michigan Hybridoma Core facility. The FyuA-specific serum IgG response of FyuA-His_6-immunized mice was monitored by ELISA (Figure B-1). Mouse two was euthanized and its spleen removed to harvest spleenocytes.

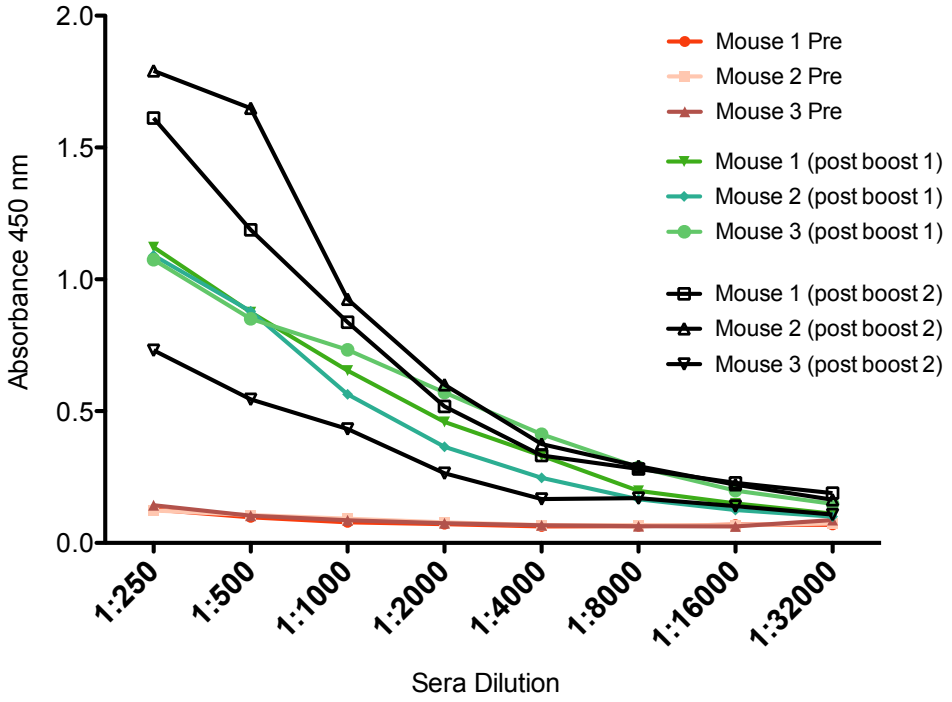


Figure B-1. Monitoring levels of FyuA-specific serum IgG of FyuA-immunized mice in preparation to generate an FyuA-specific monoclonal antibody. Three female BALB/c mice aged six-to-eight weeks were each immunized intraperitoneally with 10, 20, or 50 µg of purified, renatured, recombinant FyuA-His_6 emulsified in complete Freund's adjuvant (Sigma-Aldrich) on day 0, followed by booster injections of 10, 20 and 50 µg (respectively) of FyuA-His_6 with incomplete Freund's adjuvant (Sigma-Aldrich) on days 14, 42, and 56. The induction of FyuA-specific antibodies in serum was monitored by enzyme-linked immunosorbent assay (ELISA), and on day 60, the animal with the highest level of FyuA-specific antibodies (mouse 2) was euthanized and the spleen removed.

Screening hybridoma clones for FyuA-specificity by ELISA

Fused spleenocytes yielded 466 initial hybridoma clones that were screened by ELISA for FyuA-specific antibody production. Of these, 151 clones produced antibody that was specific to FyuA-His_6, and of these, 80 clones maintained growth after two weeks. Following this initial screen, the remaining 80 viable clones were re-screened a second time for FyuA-specificity by ELISA, and 62 remained positive for FyuA-specific antibody production (Figure B-2). Of these, 36 clones maintained growth four weeks following fusion.

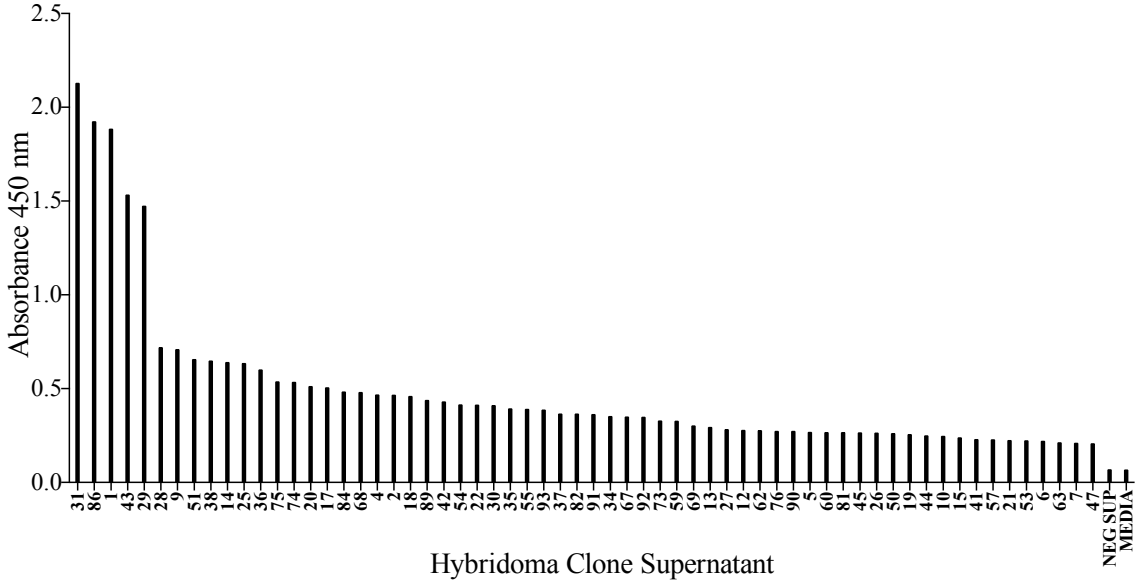


Figure B-2. Screening hybridoma clones for FyuA-specific IgG production by ELISA. Histogram of 62 clones positive for the production of FyuA-specific IgG as determined by ELISA. Wells incubated with unused cell culture media (Media) and spent media from the spleenocyte fusion partner, melanoma cell line P3X63-Ag8.653 (Neg Sup), were used as a negative controls for FyuA binding activity. Positive clones were defined as having an absorbance 450 nm signal greater or equal to three times the negative control (Neg Sup) signal.

Screening hybridoma clones for FyuA-specific binding by western blot

Purified recombinant FyuA-His_6 protein, used as vaccine and ELISA antigen, is enriched for FyuA-His_6 through nickle-affinity chromotography, and still contains a small amount of contaminating proteins. To be sure that the hybridoma clones selected by ELISA for FyuA-His_6-specific antibody production were specific for FyuA-His_6, and not a minority protein contaminating the antigen preparation, positive hybridoma clones were screened for FyuA-specific binding by western blot. Of the 21 clones screened, 13 clones produced antibody that bound to a 75 kDa band, the size of FyuA-His_6 (Figure B-3A), and 8 clones produced antibody that bound to a non-75 kDa band (Figure B-3B), or had no reactivity. Purified recombinant FyuA-His_6 was separated by SDS-PAGE and stained for total protein to demonstrate level of purity (Figure B-3C).

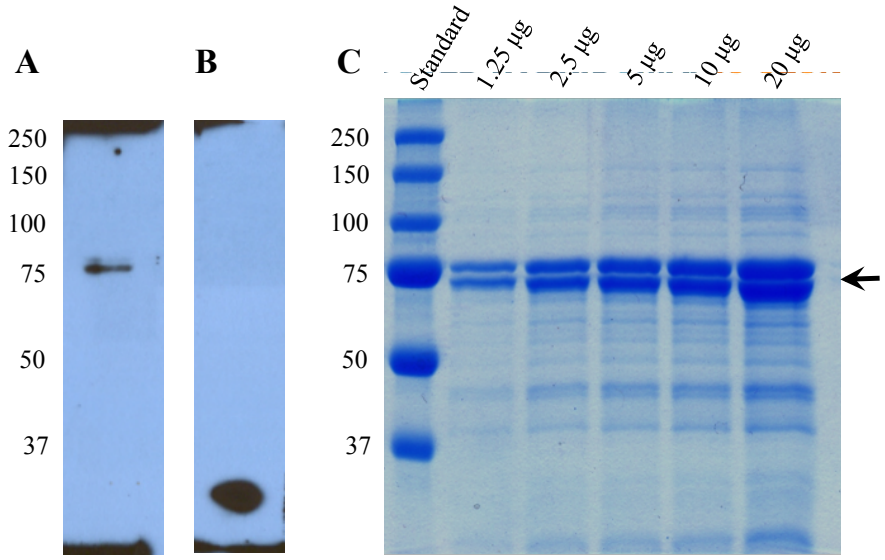


Figure B-3. Reactivity of hybridoma clone supernatant to FyuA-His_6 and purity of FyuA-His_6 protein preparation. Western blots demonstrating hybridoma supernantant reactivity to purified recombinant FyuA-His_6, using a goat-α-mouse IgG-HRP as the secondary antibody **(A, B)**. FyuA-specific clone 5E7 **(A)** and non-specific clone 8E10 **(B)** are used as representatives. A dilution curve of purified recombinant FyuA-His_6 was separated by 10% SDS-PAGE and stained with the coomassie-based SimplyBlue SafeStain (Life Technologies) **(C)**. Arrow indicates the location of FyuA-His_6 (doublet) and the molecular weight of the protein standard bands, in kDa, are shown on the left.

Screening hybridoma clones for cell surface binding by flow cytometry

We hypothesized that a therapeutic FyuA-specific monoclonal antibody would need to bind to the surface-exposed region of FyuA on the bacterial cell surface to be effective at preventing UPEC infection. To screen hybridoma clones for the production of antibodies with specificity for surface-exposed domains of FyuA, the supernatant from clones positive for FyuA-specific antibody production, as determined by ELISA and western blot, were incubated with live *E. coli* bacterial cells, which were then stained with a FITC-conjugated secondary antibody and screened by flow cytometry. Wild type *E. coli* 536 expressing FyuA had a substantial increase in median fluorescence intensity as compared to an FyuA-deficient isogenic mutant (536Δ*fyuA*), when incubated with the clone 5E7.22 supernatant, indicating that a portion of FyuA is accessible to 5E7.22 mAb binding on the bacterial cell surface (Figure B-4A). Of the 27 hybridoma subcolones screened, 5E7.22 produced antibodies with the most substantial surface-exposed FyuA binding activity. Unfortunately, the surface-specific binding activity of antibodies from subclone 5E7.22 diminished substantially, to the level of a small shoulder, while being cultured in the subcloning process (Figure B-4B). Despite the diminished levels of bacterial cell surface binding, subclone 5E7.22 was injected into the peritoneal cavity of a BALB/c mouse to generate ascites fluid, highly concentrated in 5E7.22 monoclonal antibody.

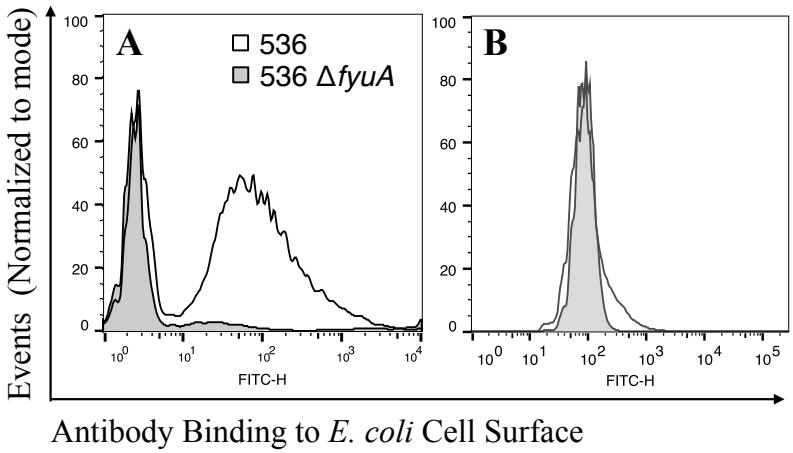


Figure B-4. Screening hybridoma supernatants for monoclonal antibodies specific for surface-exposed domains of FyuA. Histogram overlays of antibody binding as assessed by flow cytometry. UPEC strains 536 and 536Δ*fyuA* were cultured under iron-limitation by the addition of 200 μM 2'2-dipyridyl and incubated with the FyuA-specific mAb (5E7.22), followed by a secondary antibody conjugated to FITC. Shaded area represents a control *E. coli* strain unable to express FyuA (536Δ*fyuA*), and the unshaded area denotes antibody binding to wild type *E. coli*. Initial 5E7.22 mAb binding activity **(A)** is compared to its binding activity at the time of ascites preparation **(B)**.

Passive protection by FyuA-specific monoclonal antibodies (5E7.22)

Ascites fluid generated in BALB/c mice with hybridoma subclone 5E7.22 was harvested and used to passively immunize naïve six-to-eight week old female CBA/J mice. After 16 h, passively immunized mice were challenged with UPEC, either transurethrally, as an experimental model of UTI (Figure B-5A), or intravascularly, as an experimental model of system infection (Figure B-5A). Mice passively immunized with 5E7.22 were not significantly protected from experimental challenge with UPEC in either experimental infection model (Figure B-5).

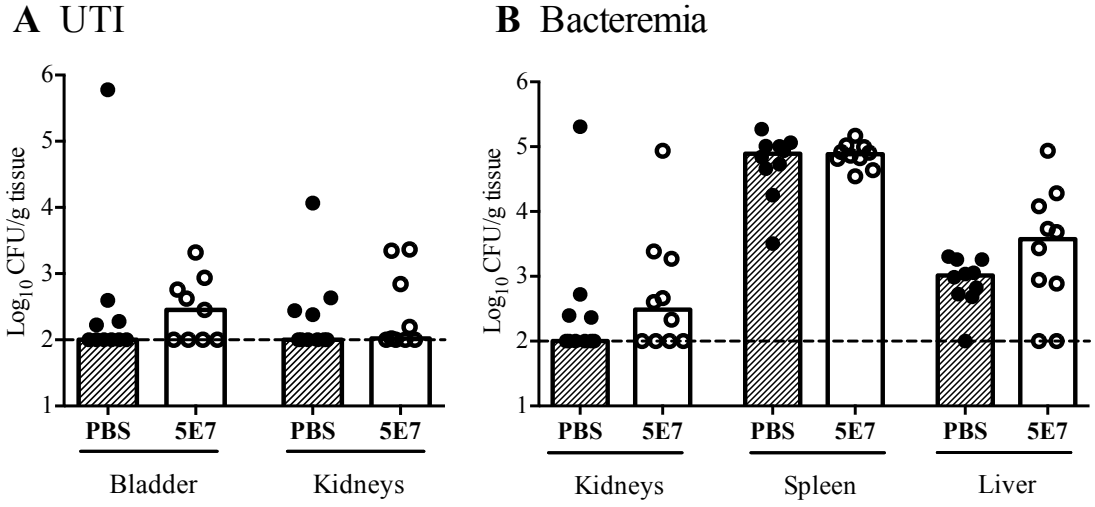


Figure B-5. Monoclonal antibody 5E7.22 fails to protect CBA/J mice from experimental UTI or bacteremia. Six-to-eight week old female CBA/J mice were injected intraperitoneally with 200 µl of 5E7.22 ascites fluid (5E7) or PBS (PBS). After 16 h, passively immunized mice were inoculated intraurethrally with 1×10^8 CFU UPEC 536 **(A)** or intravascularly with 1×10^7 CFU UPEC 536 **(B)**. Mice were euthanized 48 h **(A)** or 24 h **(B)** after UPEC inoculation and organs removed. Organs were homogenized in 3 ml PBS and plated onto LB agar to enumerate CFU/g tissue. Bars represent the median for each group and the dashed-line indicates the limit of detection for the assay (100 CFU/g tissue). Statistical significance was determined by a two-tailed Mann-Whitney test, and each experimental group contained ten mice.

Monoclonal antibody levels in the urine and serum of passively immunized mice

To determine if IgG antibodies injected into the peritoneal cavity of naïve mice could be detected in the urine and serum after 16 h, individual urine and serum samples were collected from 10 mice before intraperitoneal injection with 5E7.22 ascites fluid or PBS, and then 16 h following. FyuA-specific IgG in the urine and serum was quantified by ELISA. Significant levels of FyuA-specific IgG was detected in the serum of mice 16 h after intraperitoneal injection with 5E7.22 ascites fluid (5E7), as compared to PBS-injected control mice (PBS), however no significant difference was observed in the urine (Figure B-6).

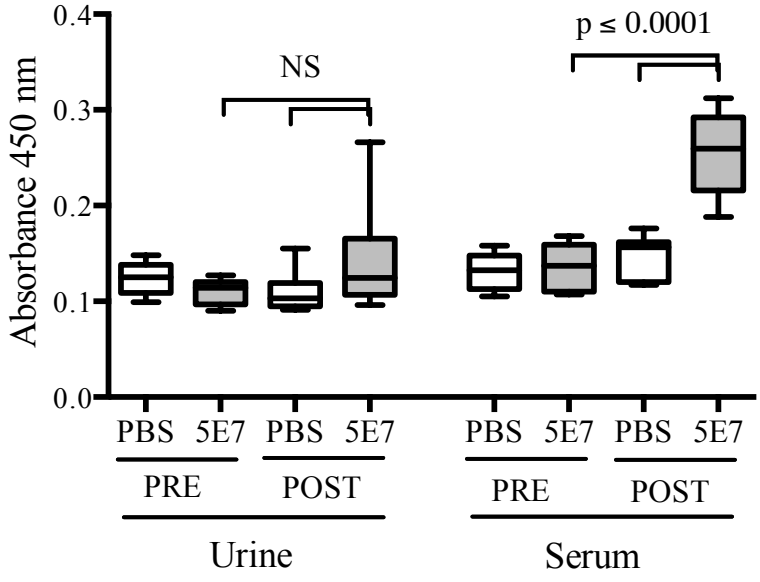


Figure B-6. Monoclonal antibody levels in the urine and serum of passively immunized mice. Six-to-eight week old female CBA/J mice were passively immunized with either 5E7.22 ascites fluid (5E7) or PBS (PBS) by intraperitoneal injection. Serum and urine samples were individually collected prior to passive immunization (PRE), and again 16 h following (POST). FyuA-specific IgG was quantified by ELISA and significance was determined by a two-tailed Mann-Whitney test. Each group contained ten mice.

Appendix C. UPEC mutant 536Δ*fyuA* cannot import yersiniabactin

To confirm that UPEC mutant 536Δ*fyuA* could not import yersiniabactin, we evaluated its growth on specific iron sources using an iron source utilization assay as described (217). PBS washed bacterial suspensions of 536Δ*fyuA*-pGEN$_{empty}$ (Figure C-1A) or 536Δ*fyuA*-pGEN$_{p\text{-}nativefyuA}$ (Figure C-1B) were added to molten sorbitol-MacConkey agar containing 350 μM 2'2-Dipyridyl to ~5 × 10^4 CFU/ml. Wells (~8-mm diameter) were punched into solidified plates using the ends of pipette tips, and 80 μl of an iron source was added to each well. Iron sources included 100 μM $FeCl_2$, the culture supernatant from a UPEC mutant that only produces yersiniabactin (536Δ*entF*), or the supernatant from a UPEC mutant that only produces enterobactin and salmochelin (536Δ*ybtS*). Siderophore donor strains were cultured overnight with 100 μM 2'2-Dipyridyl to induce siderophore synthesis and supernatants were filter-sterilized and concentrated 10-fold using a vacuum centrifuge. Plates were imaged and the diameter of growth surrounding each well was measured after 72 h at 37°C.

As expected, the UPEC 536Δ*fyuA* mutant could grow when $FeCl_2$ or the supernatant from 536Δ*ybtS* was provided as an iron source, but could not grow using the supernatant from a Δ$entF_{536}$ mutant (Figure C-1A). The defect in yersiniabactin use could be complemented by expression of *fyuA* off of its native promoter in *trans* (Figure C-1B).

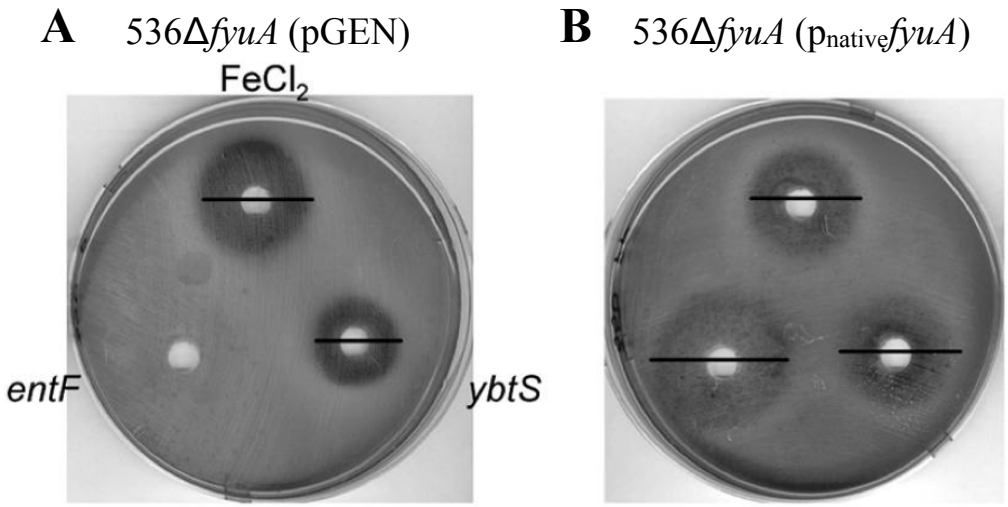


Figure C-1. UPEC mutant 536Δ*fyuA* cannot import yersiniabactin. Growth of the *E. coli* 536Δ*fyuA* mutant carrying empty pGEN vector **(A)** or pGEN-p_{native}*fyuA* **(B)** around wells containing 100 μM $FeCl_2$ (top), the concentrated supernatant from a UPEC mutant only able to siderophores enterobactin and salmochelin (536Δ*ybtS*) (*ybtS*-right), or the supernatant of a UPEC mutant only able to produce the siderophore yersiniabactin (536Δ*entF*) (*entF*-left). Bars indicate zones of growth.

Author's Note: Data in Appendix C is modified from the previously published research article: Garcia EC, Brumbaugh AR and Mobley HLT. Redundancy and Specificity of *Escherichia coli* Iron Acquisition Systems During Urinary Tract Infection. 2011. *Infect Immun* 79**:**1225-1235

Appendix D. Orphan receptor IreA does not contribute to UPEC fitness during UTI

To determine if the Fur-regulated orphan receptor and vaccine candidate IreA contributes to UPEC fitness during UTI, an isogenic mutant CFT073Δ*ireA* was tested in a murine coinfection model. At 48 hpi, the CFT073Δ*ireA* mutant was recovered at levels indistinguishable from wild type CFT073, from the bladder and kidneys (Figure D-1), indicating that *ireA* does not significantly contribute to UPEC fitness in the urinary tract.

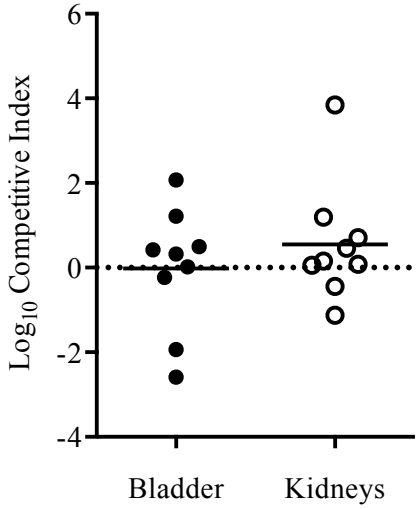


Figure D-1. IreA does not significantly contribute to UPEC fitness during UTI. Ten female CBA/J mice were transurethrally inoculated with a 1:1 mixture of *E. coli* CFT073 and *E. coli* CFT073Δ*ireA::kan* (1×10^8 total CFU/mouse) and collonization was measured 48 hpi. Competitive indices were calculated by dividing the fraction of the mutant in the output (CFU/g tissue) by the fraction of the mutant in the input (CFU/ml inoculum). Bars represent the median of value for each group and the dashed line indicates the theoretical median of zero. One mouse succumbed to the infection before being euthanized and is not included in the data. Significance was determined by Wilcoxon signed rank test with a theroretical median of zero.

Appendix E. Supplementary data from 2012 UHS *E. coli* UTI sample collection

Antimicrobial resistance profile of UHS *E. coli* cystitis isolates

The antibiotic resistance profile of 38 *E. coli* strains isolated from the urine of women with cystits at the University of Michigan University Health Services clinic in Ann Arbor, MI, during the spring of 2012 as determined by a Vitek2 Compact System (Table E-1). Notably, the antibiotic resistance rates of *E. coli* cystitis isolates from the UHS are lower than those documented at the UM Hospital during the same year (2012):

Ampicillin (*N* =1263)	**S**, 605 (48%)	**I**, 44 (3%)	**R**, 614 (49%)
Ciprofloxacin (*N* =1263)	**S**, 917 (73%)	**I**, 0 (0%)	**R**, 338 (27%)
TMP-SMX (*N* =1254)	**S**, 911 (73%)	**I**, 0 (0%)	**R**, 344 (27%)

Table E-1. Antimicrobial resistance profile of UHS *E. coli* cystitis isolates determined by a Vitek2 Compact System (38 isolates)

Antimicrobial Agent	Resistant *N* (%)	Intermediate *N* (%)
Amoxicillin/Clavulanic Acid	2 (5.1)	2 (5.1)
Ampicillin	12 (30.8)	0 (0)
Aztreonam	0 (0)	0 (0)
Cefalotin	4 (10.2)	6 (15.4)
Cefazolin	2 (5.1)	1 (2.6)
Cefepime	0 (0)	0 (0)
Cefoxitin	2 (5.1)	1 (2.6)
Cefpodoxime	0 (0)	0 (0)
Cefuroxime	1 (2.6)	0 (0)
Cefuroxime axetil	1 (2.6)	1 (2.6)
Ciprofloxacin	3 (7.7)	0 (0)
Gentamicin	2 (5.1)	0 (0)
Levofloxacin	3 (7.7)	0 (0)
Nitrofurantoin	1 (2.6)	2 (5.1)
Norfloxacin	3 (7.7)	0 (0)
Tetracycline	7 (17.9)	0 (0)
Tobramycin	3 (7.7)	0 (0)
TMP-SMX	6 (15.3)	0 (0)

Antibiotic resistance rates, iron systems, and patient history of sequenced UHS *E. coli* isolates

Table E-2. Antibiotic resistance rates, iron systems, and patient history of sequenced UHS *E. coli* isolates

Isolate	Group[a]	Antibiotic Resistance[b]	Patient History[c]	Siderophore Systems	Heme Receptors
HM27	B2	Cefalotin (16, I)	Frequent UTI	**Ent**$^{+}$ **Sal**$^{+}$ **Ybt**$^{+}$ Aer^{-}	*chuA, hma*
HM69	D	Ampicillin (≥32, R), Cefalotin (16, I), TMP-SMX (≥320, R)	Frequent UTI	**Ent**$^{+}$ Sal^{-} **Ybt**$^{+}$ **Aer**$^{+}$	*chuA*
HM46	D	None	First UTI	**Ent**$^{+}$ Sal^{-} Ybt^{-} Aer^{-}	*chuA*
HM65	B2	None	First UTI	**Ent**$^{+}$ **Sal**$^{+}$ **Ybt**$^{+}$ Aer^{-}	*chuA*

[a]Phylogenetic group
[b]TMP-SMX: trimethoprim/sulfamethoxazole
Cefalotin: first-generation cephalosporin, a class of β-lactam antibiotics
[c]Frequent UTI: Not recurrent UTI (three episodes in 12 months or two episodes in six months), but at least two episodes per year.

Orientation of the type 1 fimbrial promoter in UHS *E. coli* isolates during cystitis

Type 1 fimbria expression is regulated by a promoter on a 314 bp invertable element upstream of the gene *fimA,* a gene encoding the major structural subunit of type 1 fimbria. As an indicator of type 1 fimbriae expression during human cystitis, the orientation of the *fim* invertable element was determined from the genomic DNA of *E. coli* isolated and immediately stabilized from the urine of women with uncomplicated cystitis, using an Invertable Element (IE) PCR-based assay (Figure E-1) (218). Surprisingly, the orientation of the *fim* promoter varied between patient isolates, as the promoter was in the 'ON' orientation in 6 isolates, in the 'OFF' orientation in 13 isolates, and in a 'MIXED' orientation in 3 isolates (Figure E-1B).

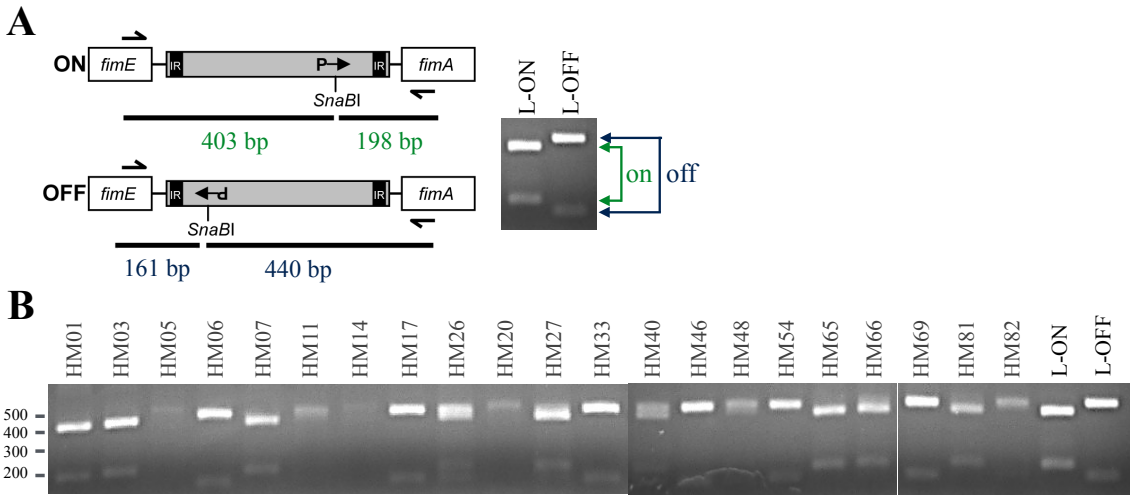


Figure E-1. Orientation of the *fim* promoter from *E. coli* directly stabilized from the urine of women with uncomplicated cystitis. A diagram of the invertible element (IE) PCR-based type 1 fimbriae assay, modified from Hagan and colleagues (42) **(A)**. The IE is in gray and an arrow indicated the orientation of the *fim* promoter (P). Black bars indicate the expected fragment sizes following PCR and asymmetrical digestion with *SnaB*I. CFT073 strains with mutations preventing IE switching are shown locked in the "phase-on" (L-ON) or "phase-off" (L-OFF) orientation. The orientation of the *fim* promoter in *E. coli* isolated from the urine of women with uncomplicated cystitis (HM01-HM82) **(B)**. Molecular mass standard base sizes are indicated.

FyuA-specific serum IgG from adults with or without a history of UTI

To determine if UPEC-reactive serum antibodies correlate with patient UTI history, we assessed the reactivity of patient serum to individual UPEC iron receptors by ELISA. Serum samples collected from women at the UHS were evaluated for serum IgG specific for iron receptors: Hma, IroN, IutA, IreA, FitA, and FyuA. Although all individuals had low levels of serum IgG specific to UPEC iron receptors, no correlation between antigen-reactive antibody levels and UTI history was found (data not shown). Serum from healthy adult males, aged 18-35, was used as a control for non-UTI induced UPEC-specific serum IgG, and FyuA-specific reactivity was evaluated. No significant difference was found between the FyuA-specific serum IgG levels between men or women with no history of UTI, and women with recurrent UTI, supporting data suggesting the bladder is an immune-privileged body site (194)(Figure E-2).

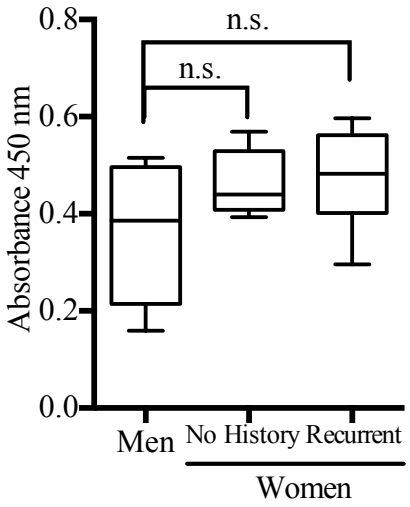


Figure E-2. FyuA-specific serum IgG reactivity of men and women. Serum samples from eight women with no history of UTI, eight women with recurrent UTI, and eight healthy men were probed for FyuA-specific IgG by indirect ELISA. Significance was determined by a two-tailed Mann-Whitney test.

REFERENCES

1. **Foxman B.** 2010. The epidemiology of urinary tract infection. Nat Rev Urol **7:**653-660.

2. **Foxman B, Brown P.** 2003. Epidemiology of urinary tract infections: transmission and risk factors, incidence, and costs. Infect Dis Clin North Am **17:**227-241.

3. **Foxman B, Gillespie B, Koopman J, Zhang L, Palin K, Tallman P, Marsh JV, Spear S, Sobel JD, Marty MJ, Marrs CF.** 2000. Risk factors for second urinary tract infection among college women. American journal of epidemiology **151:**1194-1205.

4. **DeFrances CJ, Lucas CA, Buie VC, Golosinskiy A.** 2008. 2006 National Hospital Discharge Survey. Natl Health Stat Report**:**1-20.

5. **Litwin MS, Saigal CS, Yano EM, Avila C, Geschwind SA, Hanley JM, Joyce GF, Madison R, Pace J, Polich SM, Wang M.** 2005. Urologic diseases in America Project: analytical methods and principal findings. The Journal of Urology **173:**933-937.

6. **Muhldorfer I.** 2001. Emerging bacterial pathogens. Preface. Contrib Microbiol **8:**XI-XIV.

7. **Mehnert-Kay SA.** 2005. Diagnosis and management of uncomplicated urinary tract infections. Am Fam Physician **72:**451-456.

8. **Foxman B.** 1990. Recurring urinary tract infection: incidence and risk factors. Am J Public Health **80:**331-333.

9. **Zhang L, Foxman B.** 2003. Molecular epidemiology of Escherichia coli mediated urinary tract infections. Front Biosci **8:**e235-244.

10. **Johnson JR, Russo TA.** 2002. Uropathogenic Escherichia coli as agents of diverse non-urinary tract extraintestinal infections. The Journal of infectious diseases **186:**859-864.

11. **Wiles TJ, Kulesus RR, Mulvey MA.** 2008. Origins and virulence mechanisms of uropathogenic Escherichia coli. Exp Mol Pathol **85:**11-19.

12. **Welch RA, Burland V, Plunkett G, 3rd, Redford P, Roesch P, Rasko D, Buckles EL, Liou SR, Boutin A, Hackett J, Stroud D, Mayhew GF, Rose DJ, Zhou S, Schwartz DC, Perna NT, Mobley HL, Donnenberg MS, Blattner FR.** 2002. Extensive mosaic structure revealed by the complete genome sequence of uropathogenic Escherichia coli. P Natl Acad Sci USA **99:**17020-17024.

13. **Wright KJ, Hultgren SJ.** 2006. Sticky fibers and uropathogenesis: bacterial adhesins in the urinary tract. Future Microbiol **1:**75-87.

14. **Kodner CM, Thomas Gupton EK.** 2010. Recurrent urinary tract infections in women: diagnosis and management. American family physician **82:**638-643.

15. **Gupta K, Hooton TM, Naber KG, Wullt B, Colgan R, Miller LG, Moran GJ, Nicolle LE, Raz R, Schaeffer AJ, Soper DE.** 2011. International clinical practice guidelines for the treatment of acute uncomplicated cystitis and pyelonephritis in women: a 2010 update by the infectious diseases society of america and the European society for microbiology and infectious diseases. Clin Infect Dis **52:**e103-120.

16. **Moura A, Nicolau A, Hooton T, Azeredo J.** 2009. Antibiotherapy and pathogenesis of uncomplicated UTI: difficult relationships. Journal of applied microbiology **106:**1779-1791.

17. **Foxman B, Ki M, Brown P.** 2007. Antibiotic resistance and pyelonephritis. Clin Infect Dis **45:**281-283.

18. **Karaca Y, Coplu N, Gozalan A, Oncul O, Citil BE, Esen B.** 2005. Co-trimoxazole and quinolone resistance in Escherichia coli isolated from urinary tract infections over the last 10 years. International journal of antimicrobial agents **26:**75-77.

19. **Gupta K, Hooton TM, Stamm WE.** 2001. Increasing antimicrobial resistance and the management of uncomplicated community-acquired urinary tract infections. Annals of internal medicine **135:**41-50.

20. **Zhanel GG, Hisanaga TL, Laing NM, DeCorby MR, Nichol KA, Palatnik LP, Johnson J, Noreddin A, Harding GK, Nicolle LE, Hoban DJ.** 2005. Antibiotic resistance in outpatient urinary isolates: final results from the North American Urinary Tract Infection Collaborative Alliance (NAUTICA). International journal of antimicrobial agents **26:**380-388.

21. **Karlowsky JA, Hoban DJ, Decorby MR, Laing NM, Zhanel GG.** 2006. Fluoroquinolone-resistant urinary isolates of Escherichia coli from outpatients are frequently multidrug resistant: results from the North American Urinary Tract Infection Collaborative Alliance-Quinolone Resistance study. Antimicrob Agents Chemother (Bethesda) **50:**2251-2254.

22. **Schito GC, Naber KG, Botto H, Palou J, Mazzei T, Gualco L, Marchese A.** 2009. The ARESC study: an international survey on the antimicrobial resistance of pathogens involved in uncomplicated urinary tract infections. International journal of antimicrobial agents **34:**407-413.

23. **MacDonald TM, Beardon PH, McGilchrist MM, Duncan ID, McKendrick AD, McDevitt DG.** 1993. The risks of symptomatic vaginal candidiasis after oral antibiotic therapy. Q J Med **86:**419-424.

24. **Bartlett JG.** 2006. Narrative review: the new epidemic of Clostridium difficile-associated enteric disease. Annals of internal medicine **145:**758-764.

25. **Dethlefsen L, Huse S, Sogin ML, Relman DA.** 2008. The pervasive effects of an antibiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. PLoS Biol **6:**e280.

26. **Van Eldere J, Mera RM, Miller LA, Poupard JA, Amrine-Madsen H.** 2007. Risk factors for development of multiple-class resistance to Streptococcus pneumoniae Strains in Belgium over a 10-year period: antimicrobial consumption, population density, and geographic location. Antimicrob Agents Chemother (Bethesda) **51:**3491-3497.

27. **Billips BK, Schaeffer AJ, Klumpp DJ.** 2008. Molecular basis of uropathogenic Escherichia coli evasion of the innate immune response in the bladder. Infection and Immunity **76:**3891-3900.

28. **Sivick KE, Schaller MA, Smith SN, Mobley HL.** 2010. The innate immune response to uropathogenic Escherichia coli involves IL-17A in a murine model of urinary tract infection. Journal of immunology **184:**2065-2075.

29. **Kruze D, Holzbecher K, Andrial M, Bossart W.** 1989. Urinary antibody response after immunisation with a vaccine against urinary tract infection. Urological research **17:**361-366.

30. **Kruze D, Biro K, Holzbecher K, Andrial M, Bossart W.** 1992. Protection by a polyvalent vaccine against challenge infection and pyelonephritis. Urological research **20:**177-181.

31. **Uehling DT, James LJ, Hopkins WJ, Balish E.** 1991. Immunization against urinary tract infection with a multi-valent vaginal vaccine. The Journal of Urology **146:**223-226.

32. **Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HL.** 2009. Mucosal immunization with iron receptor antigens protects against urinary tract infection. PLoS Pathog **5:**e1000586.

33. **Thumbikat P, Waltenbaugh C, Schaeffer AJ, Klumpp DJ.** 2006. Antigen-specific responses accelerate bacterial clearance in the bladder. Journal of immunology **176:**3080-3086.

34. **Sivick KE, Mobley HL.** 2009. An "omics" approach to uropathogenic Escherichia coli vaccinology. Trends Microbiol **17:**431-432.

35. **Lycke N.** 2005. Targeted vaccine adjuvants based on modified cholera toxin. Curr Mol Med **5:**591-597.

36. **Holmgren J, Czerkinsky C, Eriksson K, Mharandi A.** 2003. Mucosal immunisation and adjuvants: a brief overview of recent advances and challenges. Vaccine **21 Suppl 2:**S89-95.

37. **Scavone P, Rial A, Umpierrez A, Chabalgoity A, Zunino P.** 2009. Effects of the administration of cholera toxin as a mucosal adjuvant on the immune and protective response induced by Proteus mirabilis MrpA fimbrial protein in the urinary tract. Microbiol Immunol **53:**233-240.

38. **Chen DJ, Osterrieder N, Metzger SM, Buckles E, Doody AM, DeLisa MP, Putnam D.** 2010. Delivery of foreign antigens by engineered outer membrane vesicle vaccines. P Natl Acad Sci USA **107:**3099-3104.

39. **McLachlan JB, Shelburne CP, Hart JP, Pizzo SV, Goyal R, Brooking-Dixon R, Staats HF, Abraham SN.** 2008. Mast cell activators: a new class of highly effective vaccine adjuvants. Nat Med **14:**536-541.

40. **Hung CS, Dodson KW, Hultgren SJ.** 2009. A murine model of urinary tract infection. Nat Protoc **4:**1230-1243.

41. **Fox JG.** 2007. The mouse in biomedical research, 2nd ed. Elsevier, Amsterdam ; Boston.

42. **Hagan EC, Lloyd AL, Rasko DA, Faerber GJ, Mobley HL.** 2010. Escherichia coli global gene expression in urine from women with urinary tract infection. PLoS Pathogens **6:**e1001187.

43. **Whitfield C.** 2006. Biosynthesis and assembly of capsular polysaccharides in Escherichia coli. Annu Rev Biochem **75:**39-68.

44. **Buckles EL, Wang X, Lane MC, Lockatell CV, Johnson DE, Rasko DA, Mobley HL, Donnenberg MS.** 2009. Role of the K2 capsule in Escherichia coli urinary tract infection and serum resistance. The Journal of infectious diseases **199:**1689-1697.

45. **Russo TA, Beanan JM, Olson R, MacDonald U, Cope JJ.** 2009. Capsular polysaccharide and the O-specific antigen impede antibody binding: a potential obstacle for the successful development of an extraintestinal pathogenic Escherichia coli vaccine. Vaccine **27:**388-395.

46. **Uehling DT, Wolf L.** 1969. Enhancement of the bladder defense mechanism by immunization. Invest Urol **6:**520-526.

47. **Kaijser B, Ahlstedt S.** 1977. Protective capacity of antibodies against Escherichia coli and K antigens. Infection and Immunity **17:**286-289.

48. **Kumar V, Ganguly N, Joshi K, Mittal R, Harjai K, Chhibber S, Sharma S.** 2005. Protective efficacy and immunogenicity of Escherichia coli K13 diphtheria

toxoid conjugate against experimental ascending pyelonephritis. Medical microbiology and immunology **194:**211-217.

49. **Kaijser B, Larsson P, Olling S, Schneerson R.** 1983. Protection against acute, ascending pyelonephritis caused by Escherichia coli in rats, using isolated capsular antigen conjugated to bovine serum albumin. Infection and Immunity **39:**142-146.

50. **Kaijser B, Larsson P, Nimmich W, Soderstrom T.** 1983. Antibodies to Escherichia coli K and O antigens in protection against acute pyelonephritis. Prog Allergy **33:**275-288.

51. **Whitfield C, Roberts IS.** 1999. Structure, assembly and regulation of expression of capsules in Escherichia coli. Molecular microbiology **31:**1307-1319.

52. **Johnson JR.** 1991. Virulence factors in Escherichia coli urinary tract infection. Clin Microbiol Rev **4:**80-128.

53. **Hoffman S, Sorkin BC, White PC, Brackenbury R, Mailhammer R, Rutishauser U, Cunningham BA, Edelman GM.** 1982. Chemical characterization of a neural cell adhesion molecule purified from embryonic brain membranes. The Journal of biological chemistry **257:**7720-7729.

54. **Jann K, Jann B.** 1987. Polysaccharide antigens of Escherichia coli. Reviews of infectious diseases **9 Suppl 5:**S517-526.

55. **Vann WF, Schmidt MA, Jann B, Jann K.** 1981. The structure of the capsular polysaccharide (K5 antigen) of urinary-tract-infective Escherichia coli 010:K5:H4. A polymer similar to desulfo-heparin. Eur J Biochem **116:**359-364.

56. **Rappuoli R, Bagnoli F.** 2011. Vaccine design : innovative approaches and novel strategies. Caister Academic, Norfolk, UK.

57. **Johnson JR, Kuskowski MA, Gajewski A, Soto S, Horcajada JP, Jimenez de Anta MT, Vila J.** 2005. Extended virulence genotypes and phylogenetic background of Escherichia coli isolates from patients with cystitis, pyelonephritis, or prostatitis. The Journal of infectious diseases **191:**46-50.

58. **Grischke EM, Ruttgers H.** 1987. Treatment of bacterial infections of the female urinary tract by immunization of the patients. Urol Int **42:**338-341.

59. **Knuf M, Habermehl P, Faber J, Bock HL, Sanger R, Bogaerts H, Clemens R, Schuind A, du Prel JB, Schmitt HJ.** 2006. Assessment of nine candidate DTP-vaccines with reduced amount of antigen and/or without adjuvant as a fourth (booster-) dose in the second year of life. Vaccine **24:**5627-5636.

60. **Mestecky J.** 1987. The common mucosal immune system and current strategies for induction of immune responses in external secretions. J Clin Immunol **7:**265-276.

61. **Uehling DT, Hopkins WJ, Balish E, Xing Y, Heisey DM.** 1997. Vaginal mucosal immunization for recurrent urinary tract infection: phase II clinical trial. The Journal of Urology **157:**2049-2052.

62. **Uehling DT, Hopkins WJ, Beierle LM, Kryger JV, Heisey DM.** 2001. Vaginal mucosal immunization for recurrent urinary tract infection: extended phase II clinical trial. The Journal of infectious diseases **183 Suppl 1:**S81-83.

63. **Uehling DT, Hopkins WJ, Elkahwaji JE, Schmidt DM, Leverson GE.** 2003. Phase 2 clinical trial of a vaginal mucosal vaccine for urinary tract infections. The Journal of Urology **170:**867-869.

64. **Hopkins W, Elkahwaji J, Beierle L, Leverson G, Uehling D.** 2007. Vaginal Mucosal Vaccine for Recurrent Urinary Tract Infections in Women: Results of a Phase 2 Clinical Trial. The Journal of Urology **177:**1349-1353.

65. **Frey C, Obolensky W, Wyss H.** 1986. Treatment of recurrent urinary tract infections: efficacy of an orally administered biological response modifier. Urol Int **41:**444-446.

66. **Hachen HJ.** 1990. Oral immunotherapy in paraplegic patients with chronic urinary tract infections: a double-blind, placebo-controlled trial. The Journal of Urology **143:**759-762; discussion 762-753.

67. **Schulman CC, Corbusier A, Michiels H, Taenzer HJ.** 1993. Oral immunotherapy of recurrent urinary tract infections: a double-blind placebo-controlled multicenter study. The Journal of Urology **150:**917-921.

68. **Bauer HW, Alloussi S, Egger G, Blumlein HM, Cozma G, Schulman CC.** 2005. A long-term, multicenter, double-blind study of an Escherichia coli extract (OM-89) in female patients with recurrent urinary tract infections. Eur Urol **47:**542-548; discussion 548.

69. **Kim KS, Kim JY, Jeong IG, Paick JS, Son H, Lim DJ, Shim HB, Park WH, Jung HC, Choo MS.** 2010. A prospective multi-center trial of Escherichia coli extract for the prophylactic treatment of patients with chronically recurrent cystitis. J Korean Med Sci **25:**435-439.

70. **Bauer HW, Rahlfs VW, Lauener PA, Blessmann GS.** 2002. Prevention of recurrent urinary tract infections with immuno-active E. coli fractions: a meta-analysis of five placebo-controlled double-blind studies. International journal of antimicrobial agents **19:**451-456.

71. **Koukalova D, Krocova Z, Vitek P, Macela A, Hajek V.** 1999. [Immunostimulatory activity of the vaccine used in the treatment of recurrent urinary infections. II]. Bratisl Lek Listy **100:**215-217.

72. **Koukalova D, Reif R, Hajek V, Mucha Z, Vesely J, Viktorinova M, Lochman I.** 1999. [Immunomodulation of recurrent urinary tract infections with Urvakol vaccine]. Bratisl Lek Listy **100:**246-251.

73. **Marinova S, Nenkov P, Markova R, Nikolaeva S, Kostadinova R, Mitov I, Vretenarska M.** 2005. Cellular and humoral systemic and mucosal immune responses stimulated by an oral polybacterial immunomodulator in patients with chronic urinary tract infections. Int J Immunopathol Pharmacol **18:**457-473.

74. **Uehling DT, Hopkins WJ, Dahmer LA, Balish E.** 1994. Phase I clinical trial of vaginal mucosal immunization for recurrent urinary tract infection. The Journal of Urology **152:**2308-2311.

75. **Uehling DT, Hopkins WJ, James LJ, Balish E.** 1994. Vaginal immunization of monkeys against urinary tract infection with a multi-strain vaccine. The Journal of Urology **151:**214-216.

76. **Russo TA, Beanan JM, Olson R, Genagon SA, MacDonald U, Cope JJ, Davidson BA, Johnston B, Johnson JR.** 2007. A killed, genetically engineered derivative of a wild-type extraintestinal pathogenic E. coli strain is a vaccine candidate. Vaccine **25:**3859-3870.

77. **Billips BK, Yaggie RE, Cashy JP, Schaeffer AJ, Klumpp DJ.** 2009. A live-attenuated vaccine for the treatment of urinary tract infection by uropathogenic Escherichia coli. J Infect Dis **200:**263-272.

78. **Spurbeck RR, Stapleton AE, Johnson JR, Walk ST, Hooton TM, Mobley HL.** 2011. Fimbrial Profiles Predict Virulence of Uropathogenic E. coli Strains: Contribution of Ygi and Yad Fimbriae. Infection and Immunity.

79. **Thankavel K, Madison B, Ikeda T, Malaviya R, Shah AH, Arumugam PM, Abraham SN.** 1997. Localization of a domain in the FimH adhesin of Escherichia coli type 1 fimbriae capable of receptor recognition and use of a domain-specific antibody to confer protection against experimental urinary tract infection. J Clin Invest **100:**1123-1136.

80. **Lane MC, Mobley HL.** 2007. Role of P-fimbrial-mediated adherence in pyelonephritis and persistence of uropathogenic Escherichia coli (UPEC) in the mammalian kidney. Kidney Int **72:**19-25.

81. **O'Hanley P, Lark D, Falkow S, Schoolnik G.** 1985. Molecular basis of Escherichia coli colonization of the upper urinary tract in BALB/c mice. Gal-Gal pili immunization prevents Escherichia coli pyelonephritis in the BALB/c mouse model of human pyelonephritis. The Journal of clinical investigation **75:**347-360.

82. **Roberts JA, Hardaway K, Kaack B, Fussell EN, Baskin G.** 1984. Prevention of pyelonephritis by immunization with P-fimbriae. The Journal of Urology **131:**602-607.

83. **Roberts JA, Kaack MB, Baskin G, Chapman MR, Hunstad DA, Pinkner JS, Hultgren SJ.** 2004. Antibody responses and protection from pyelonephritis following vaccination with purified Escherichia coli PapDG protein. J Urol **171:**1682-1685.

84. **Carnoy C, Moseley SL.** 1997. Mutational analysis of receptor binding mediated by the Dr family of Escherichia coli adhesins. Molecular microbiology **23:**365-379.

85. **Westerlund B, Kuusela P, Risteli J, Risteli L, Vartio T, Rauvala H, Virkola R, Korhonen TK.** 1989. The O75X adhesin of uropathogenic Escherichia coli is a type IV collagen-binding protein. Molecular microbiology **3:**329-337.

86. **Goluszko P, Moseley SL, Truong LD, Kaul A, Williford JR, Selvarangan R, Nowicki S, Nowicki B.** 1997. Development of experimental model of chronic pyelonephritis with Escherichia coli O75:K5:H-bearing Dr fimbriae: mutation in the dra region prevented tubulointerstitial nephritis. The Journal of clinical investigation **99:**1662-1672.

87. **Goluszko P, Goluszko E, Nowicki B, Nowicki S, Popov V, Wang HQ.** 2005. Vaccination with purified Dr Fimbriae reduces mortality associated with chronic urinary tract infection due to Escherichia coli bearing Dr adhesin. Infect Immun **73:**627-631.

88. **Zhou G, Mo WJ, Sebbel P, Min G, Neubert TA, Glockshuber R, Wu XR, Sun TT, Kong XP.** 2001. Uroplakin Ia is the urothelial receptor for uropathogenic Escherichia coli: evidence from in vitro FimH binding. J Cell Sci **114:**4095-4103.

89. **Connell I, Agace W, Klemm P, Schembri M, Marild S, Svanborg C.** 1996. Type 1 fimbrial expression enhances Escherichia coli virulence for the urinary tract. P Natl Acad Sci USA **93:**9827-9832.

90. **Iwahi T, Abe Y, Nakao M, Imada A, Tsuchiya K.** 1983. Role of type 1 fimbriae in the pathogenesis of ascending urinary tract infection induced by escherichia coli in mice. Infection and Immunity **39:**1307-1315.

91. **Aronson M, Medalia O, Schori L, Mirelman D, Sharon N, Ofek I.** 1979. Prevention of colonization of the urinary tract of mice with Escherichia coli by blocking of bacterial adherence with methyl alpha-D-mannopyranoside. The Journal of infectious diseases **139:**329-332.

92. **Langermann S, Palaszynski S, Barnhart M, Auguste G, Pinkner JS, Burlein J, Barren P, Koenig S, Leath S, Jones CH, Hultgren SJ.** 1997. Prevention of

mucosal Escherichia coli infection by FimH-adhesin-based systemic vaccination. Science **276:**607-611.

93. **Langermann S, Mollby R, Burlein JE, Palaszynski SR, Auguste CG, DeFusco A, Strouse R, Schenerman MA, Hultgren SJ, Pinkner JS, Winberg J, Guldevall L, Soderhall M, Ishikawa K, Normark S, Koenig S.** 2000. Vaccination with FimH adhesin protects cynomolgus monkeys from colonization and infection by uropathogenic Escherichia coli. The Journal of infectious diseases **181:**774-778.

94. **Langermann S, Ballou WR, Jr.** 2001. Vaccination utilizing the FimCH complex as a strategy to prevent Escherichia coli urinary tract infections. J Infect Dis **183 Suppl 1:**S84-86.

95. **Meiland R, Geerlings SE, Langermann S, Brouwer EC, Coenjaerts FE, Hoepelman AI.** 2004. Fimch antiserum inhibits the adherence of Escherichia coli to cells collected by voided urine specimens of diabetic women. J Urol **171:**1589-1593.

96. 2002. Medimmune, Inc. Annual Report.

97. Personal correspondence with the MedImmune Medical Affairs Department, January 9th 2012.

98. **Lim JK, Gunther NWt, Zhao H, Johnson DE, Keay SK, Mobley HL.** 1998. In vivo phase variation of Escherichia coli type 1 fimbrial genes in women with urinary tract infection. Infection and Immunity **66:**3303-3310.

99. **Eisenstein BI.** 1981. Phase variation of type 1 fimbriae in Escherichia coli is under transcriptional control. Science **214:**337-339.

100. **Nowicki B, Rhen M, Vaisanen-Rhen V, Pere A, Korhonen TK.** 1984. Immunofluorescence study of fimbrial phase variation in Escherichia coli KS71. Journal of bacteriology **160:**691-695.

101. **Tchesnokova V, Aprikian P, Kisiela D, Gowey S, Korotkova N, Thomas W, Sokurenko E.** 2011. Type 1 fimbrial adhesin FimH elicits immune response which enhances cell adhesion of Escherichia coli. Infection and Immunity.

102. **O'Hanley P, Lalonde G, Ji G.** 1991. Alpha-hemolysin contributes to the pathogenicity of piliated digalactoside-binding Escherichia coli in the kidney: efficacy of an alpha-hemolysin vaccine in preventing renal injury in the BALB/c mouse model of pyelonephritis. Infect Immun **59:**1153-1161.

103. **Nielubowicz GR, Mobley HL.** 2010. Host-pathogen interactions in urinary tract infection. Nat Rev Urol **7:**430-441.

104. **Rippere-Lampe KE, Lang M, Ceri H, Olson M, Lockman HA, O'Brien AD.** 2001. Cytotoxic necrotizing factor type 1-positive Escherichia coli causes increased inflammation and tissue damage to the prostate in a rat prostatitis model. Infection and Immunity **69:**6515-6519.

105. **Smith YC, Rasmussen SB, Grande KK, Conran RM, O'Brien AD.** 2008. Hemolysin of uropathogenic Escherichia coli evokes extensive shedding of the uroepithelium and hemorrhage in bladder tissue within the first 24 hours after intraurethral inoculation of mice. Infection and Immunity **76:**2978-2990.

106. **Moriel DG, Bertoldi I, Spagnuolo A, Marchi S, Rosini R, Nesta B, Pastorello I, Corea VA, Torricelli G, Cartocci E, Savino S, Scarselli M, Dobrindt U, Hacker J, Tettelin H, Tallon LJ, Sullivan S, Wieler LH, Ewers C, Pickard D, Dougan G, Fontana MR, Rappuoli R, Pizza M, Serino L.** 2010. Identification of protective and broadly conserved vaccine antigens from the genome of extraintestinal pathogenic Escherichia coli. P Natl Acad Sci USA **107:**9072-9077.

107. **Torres AG, Redford P, Welch RA, Payne SM.** 2001. TonB-dependent systems of uropathogenic Escherichia coli: aerobactin and heme transport and TonB are required for virulence in the mouse. Infection and Immunity **69:**6179-6185.

108. **Chu BC, Garcia-Herrero A, Johanson TH, Krewulak KD, Lau CK, Peacock RS, Slavinskaya Z, Vogel HJ.** 2010. Siderophore uptake in bacteria and the battle for iron with the host; a bird's eye view. Biometals **23:**601-611.

109. **Snyder JA, Haugen BJ, Buckles EL, Lockatell CV, Johnson DE, Donnenberg MS, Welch RA, Mobley HL.** 2004. Transcriptome of uropathogenic Escherichia coli during urinary tract infection. Infection and Immunity **72:**6373-6381.

110. **Faraldo-Gomez JD, Sansom MS.** 2003. Acquisition of siderophores in gram-negative bacteria. Nature reviews. Molecular cell biology **4:**105-116.

111. **Henderson JP, Crowley JR, Pinkner JS, Walker JN, Tsukayama P, Stamm WE, Hooton TM, Hultgren SJ.** 2009. Quantitative metabolomics reveals an epigenetic blueprint for iron acquisition in uropathogenic Escherichia coli. PLoS pathogens **5:**e1000305.

112. **Russo TA, McFadden CD, Carlino-MacDonald UB, Beanan JM, Olson R, Wilding GE.** 2003. The Siderophore receptor IroN of extraintestinal pathogenic Escherichia coli is a potential vaccine candidate. Infection and Immunity **71:**7164-7169.

113. **Durant L, Metais A, Soulama-Mouze C, Genevard JM, Nassif X, Escaich S.** 2007. Identification of candidates for a subunit vaccine against extraintestinal pathogenic Escherichia coli. Infection and Immunity **75:**1916-1925.

114. **Carbonetti NH, Boonchai S, Parry SH, Vaisanen-Rhen V, Korhonen TK, Williams PH.** 1986. Aerobactin-mediated iron uptake by Escherichia coli isolates from human extraintestinal infections. Infection and Immunity **51:**966-968.

115. **Garcia EC, Brumbaugh AR, Mobley HL.** 2011. Redundancy and specificity of Escherichia coli iron acquisition systems during urinary tract infection. Infection and Immunity **79:**1225-1235.

116. **Watts RE, Totsika M, Challinor VL, Mabbett AN, Ulett GC, De Voss JJ, Schembri MA.** 2012. Contribution of Siderophore Systems to Growth and Urinary Tract Colonization of Asymptomatic Bacteriuria Escherichia coli. Infection and Immunity **80:**333-344.

117. **Wieser A, Romann E, Magistro G, Hoffmann C, Norenberg D, Weinert K, Schubert S.** 2010. A multiepitope subunit vaccine conveys protection against extraintestinal pathogenic Escherichia coli in mice. Infection and Immunity **78:**3432-3442.

118. **Wieser A, Magistro G, Norenberg D, Hoffmann C, Schubert S.** 2012. First multi-epitope subunit vaccine against extraintestinal pathogenic Escherichia coli delivered by a bacterial type-3 secretion system (T3SS). International journal of medical microbiology : IJMM **302:**10-18.

119. **Brumbaugh AR, Smith SN, Mobley HL.** 2013. Immunization with the yersiniabactin receptor, FyuA, protects against pyelonephritis in a murine model of urinary tract infection. Infect Immun **81:**3309-3316.

120. **Anderson GG, Palermo JJ, Schilling JD, Roth R, Heuser J, Hultgren SJ.** 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science **301:**105-107.

121. **Rice JC, Peng T, Spence JS, Wang HQ, Goldblum RM, Corthesy B, Nowicki BJ.** 2005. Pyelonephritic Escherichia coli expressing P fimbriae decrease immune response of the mouse kidney. Journal of the American Society of Nephrology : JASN **16:**3583-3591.

122. **Cirl C, Wieser A, Yadav M, Duerr S, Schubert S, Fischer H, Stappert D, Wantia N, Rodriguez N, Wagner H, Svanborg C, Miethke T.** 2008. Subversion of Toll-like receptor signaling by a unique family of bacterial Toll/interleukin-1 receptor domain-containing proteins. Nat Med **14:**399-406.

123. **He Y, Xiang Z, Mobley HL.** 2010. Vaxign: the first web-based vaccine design program for reverse vaccinology and applications for vaccine development. J Biomed Biotechnol **2010:**297505.

124. **Schappert SM, Rechtsteiner EA.** 2008. Ambulatory medical care utilization estimates for 2006. Natl Health Stat Report**:**1-29.

125. **Foxman B.** 2002. Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. Am J Med **113 Suppl 1A:**5S-13S.

126. **Schappert SM, Rechtsteiner EA.** 2011. Ambulatory medical care utilization estimates for 2007. Vital and health statistics. Series 13, Data from the National Health Survey**:**1-38.

127. **Hall MJ, DeFrances CJ, Williams SN, Golosinskiy A, Schwartzman A.** 2010. National Hospital Discharge Survey: 2007 summary. Natl Health Stat Report**:**1-20, 24.

128. **Gordon KA, Jones RN, Groups SP.** 2003. Susceptibility patterns of orally administered antimicrobials among urinary tract infection pathogens from hospitalized patients in North America: comparison report to Europe and Latin America. Results from the SENTRY Antimicrobial Surveillance Program (2000). Diagn Microbiol Infect Dis **45:**295-301.

129. **Schito GC, Naber KG, Botto H, Palou J, Mazzei T, Gualco L, Marchese A.** 2009. The ARESC study: an international survey on the antimicrobial resistance of pathogens involved in uncomplicated urinary tract infections. Int J Antimicrob Agents **34:**407-413.

130. **Zhanel GG, Hisanaga TL, Laing NM, DeCorby MR, Nichol KA, Weshnoweski B, Johnson J, Noreddin A, Low DE, Karlowsky JA, Group N, Hoban DJ.** 2006. Antibiotic resistance in Escherichia coli outpatient urinary isolates: final results from the North American Urinary Tract Infection Collaborative Alliance (NAUTICA). Int J Antimicrob Agents **27:**468-475.

131. **Hidron AI, Edwards JR, Patel J, Horan TC, Sievert DM, Pollock DA, Fridkin SK, National Healthcare Safety Network T, Participating National Healthcare Safety Network F.** 2008. NHSN annual update: antimicrobial-resistant pathogens associated with healthcare-associated infections: annual summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2006-2007. Infect Control Hosp Epidemiol **29:**996-1011.

132. **Brumbaugh AR, Mobley HL.** 2012. Preventing urinary tract infection: progress toward an effective Escherichia coli vaccine. Expert Rev Vaccines **11:**663-676.

133. **Russo TA, Johnson JR.** 2006. Extraintestinal isolates of Escherichia coli: identification and prospects for vaccine development. Expert Rev Vaccines **5:**45-54.

134. **Dobrindt U, Hacker J.** 2008. Targeting virulence traits: potential strategies to combat extraintestinal pathogenic E. coli infections. Curr Opin Microbiol **11:**409-413.

135. **Nicolle LE, Committee* ACG.** 2005. Complicated urinary tract infection in adults. The Canadian journal of infectious diseases & medical microbiology = Journal canadien des maladies infectieuses et de la microbiologie medicale / AMMI Canada **16:**349-360.

136. **Giefing C, Meinke AL, Hanner M, Henics T, Bui MD, Gelbmann D, Lundberg U, Senn BM, Schunn M, Habel A, Henriques-Normark B, Ortqvist A, Kalin M, von Gabain A, Nagy E.** 2008. Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. J Exp Med **205:**117-131.

137. **Lee SJ, Liang L, Juarez S, Nanton MR, Gondwe EN, Msefula CL, Kayala MA, Necchi F, Heath JN, Hart P, Tsolis RM, Heyderman RS, MacLennan CA, Felgner PL, Davies DH, McSorley SJ.** 2012. Identification of a common immune signature in murine and human systemic Salmonellosis. Proc Natl Acad Sci U S A **109:**4998-5003.

138. **Pizza M, Scarlato V, Masignani V, Giuliani MM, Arico B, Comanducci M, Jennings GT, Baldi L, Bartolini E, Capecchi B, Galeotti CL, Luzzi E, Manetti R, Marchetti E, Mora M, Nuti S, Ratti G, Santini L, Savino S, Scarselli M, Storni E, Zuo P, Broeker M, Hundt E, Knapp B, Blair E, Mason T, Tettelin H, Hood DW, Jeffries AC, Saunders NJ, Granoff DM, Venter JC, Moxon ER, Grandi G, Rappuoli R.** 2000. Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. Science **287:**1816-1820.

139. **Lloyd AL, Rasko DA, Mobley HL.** 2007. Defining genomic islands and uropathogen-specific genes in uropathogenic Escherichia coli. Journal of bacteriology **189:**3532-3546.

140. **Chen SL, Hung CS, Xu J, Reigstad CS, Magrini V, Sabo A, Blasiar D, Bieri T, Meyer RR, Ozersky P, Armstrong JR, Fulton RS, Latreille JP, Spieth J, Hooton TM, Mardis ER, Hultgren SJ, Gordon JI.** 2006. Identification of genes subject to positive selection in uropathogenic strains of Escherichia coli: a comparative genomics approach. P Natl Acad Sci USA **103:**5977-5982.

141. **Bahrani-Mougeot FK, Buckles EL, Lockatell CV, Hebel JR, Johnson DE, Tang CM, Donnenberg MS.** 2002. Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic Escherichia coli virulence determinants in the murine urinary tract. Mol Microbiol **45:**1079-1093.

142. **Hagan EC, Mobley HL.** 2007. Uropathogenic Escherichia coli outer membrane antigens expressed during urinary tract infection. Infect Immun **75:**3941-3949.

143. **Walters MS, Mobley HL.** 2009. Identification of uropathogenic Escherichia coli surface proteins by shotgun proteomics. J Microbiol Methods **78:**131-135.

144. **Alteri CJ, Mobley HL.** 2007. Quantitative profile of the uropathogenic Escherichia coli outer membrane proteome during growth in human urine. Infect Immun **75:**2679-2688.

145. **Vigil PD, Alteri CJ, Mobley HL.** 2011. Identification of In Vivo-Induced Antigens Including an RTX Family Exoprotein Required for Uropathogenic Escherichia coli Virulence. Infection and Immunity **79:**2335-2344.

146. **Alteri CJ, Hagan EC, Sivick KE, Smith SN, Mobley HL.** 2009. Mucosal immunization with iron receptor antigens protects against urinary tract infection. PLoS Pathogens **5:**e1000586.

147. **Hacker J, Knapp S, Goebel W.** 1983. Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of an Escherichia coli O6 strain. Journal of bacteriology **154:**1145-1152.

148. **Mobley HL, Green DM, Trifillis AL, Johnson DE, Chippendale GR, Lockatell CV, Jones BD, Warren JW.** 1990. Pyelonephritogenic Escherichia coli and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. Infection and Immunity **58:**1281-1289.

149. **Johnson DE, Lockatell CV, Hall-Craigs M, Mobley HL, Warren JW.** 1987. Uropathogenicity in rats and mice of Providencia stuartii from long-term catheterized patients. J Urol **138:**632-635.

150. **R Development Core Team.** 2011. R: A language and environment for statistical computing, 2.14.1 ed. R Foundation for Statistical Computing, Vienna, Austria.

151. **Plotkin SA.** 2010. Correlates of protection induced by vaccination. Clin Vaccine Immunol **17:**1055-1065.

152. **Elgueta R, de Vries VC, Noelle RJ.** 2010. The immortality of humoral immunity. Immunol Rev **236:**139-150.

153. **Johnson JR, Johnston B, Kuskowski MA, Nougayrede JP, Oswald E.** 2008. Molecular epidemiology and phylogenetic distribution of the Escherichia coli pks genomic island. J Clin Microbiol **46:**3906-3911.

154. **Schubert S, Picard B, Gouriou S, Heesemann J, Denamur E.** 2002. Yersinia high-pathogenicity island contributes to virulence in Escherichia coli causing extraintestinal infections. Infect Immun **70:**5335-5337.

155. **Garcia EC, Brumbaugh AR, Mobley HL.** 2011. Redundancy and Specificity of Escherichia coli Iron Acquisition Systems during Urinary Tract Infection. Infect Immun **79:**1225-1235.

156. **Fetherston JD, Kirillina O, Bobrov AG, Paulley JT, Perry RD.** 2010. The yersiniabactin transport system is critical for the pathogenesis of bubonic and pneumonic plague. Infect Immun **78:**2045-2052.

157. **Lawlor MS, O'Connor C, Miller VL.** 2007. Yersiniabactin is a virulence factor for Klebsiella pneumoniae during pulmonary infection. Infect Immun **75:**1463-1472.

158. **Spurbeck RR, Dinh PC, Jr., Walk ST, Stapleton AE, Hooton TM, Nolan LK, Kim KS, Johnson JR, Mobley HL.** 2012. Escherichia coli isolates that carry vat, fyuA, chuA, and yfcV efficiently colonize the urinary tract. Infect Immun **80:**4115-4122.

159. **Howard SJ, Hopwood S, Davies SC.** 2014. Antimicrobial resistance: a global challenge. Sci Transl Med **6:**236ed210.

160. **Jones RN.** 2010. Microbial etiologies of hospital-acquired bacterial pneumonia and ventilator-associated bacterial pneumonia. Clin Infect Dis **51 Suppl 1:**S81-87.

161. **Miajlovic H, Smith SG.** 2014. Bacterial self-defence: how Escherichia coli evades serum killing. FEMS Microbiol Lett **354:**1-9.

162. 2014. Antimicrobial resistance: global report on surveillance, p. 256. World Health Organization.

163. **Moayednia R, Shokri D, Mobasherizadeh S, Baradaran A, Fatemi SM, Merrikhi A.** 2014. Frequency assessment of beta-lactamase enzymes in Escherichia coli and Klebsiella isolates in patients with urinary tract infection. Journal of research in medical sciences : the official journal of Isfahan University of Medical Sciences **19:**S41-45.

164. **Miethke M, Marahiel MA.** 2007. Siderophore-based iron acquisition and pathogen control. Microbiol Mol Biol Rev **71:**413-451.

165. **Nairz M, Schroll A, Sonnweber T, Weiss G.** 2010. The struggle for iron - a metal at the host-pathogen interface. Cell Microbiol **12:**1691-1702.

166. **Drakesmith H, Prentice AM.** 2012. Hepcidin and the iron-infection axis. Science **338:**768-772.

167. **Skaar EP.** 2010. The battle for iron between bacterial pathogens and their vertebrate hosts. PLoS Pathog **6:**e1000949.

168. **Berger H, Hacker J, Juarez A, Hughes C, Goebel W.** 1982. Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in Escherichia coli. J Bacteriol **152:**1241-1247.

169. **Schubert S, Cuenca S, Fischer D, Heesemann J.** 2000. High-pathogenicity island of Yersinia pestis in enterobacteriaceae isolated from blood cultures and urine samples: prevalence and functional expression. J Infect Dis **182:**1268-1271.

170. **Perry RD, Fetherston JD.** 2011. Yersiniabactin iron uptake: mechanisms and role in Yersinia pestis pathogenesis. Microbes Infect **13:**808-817.

171. **Brumbaugh AR, Mobley HL.** 2012. Preventing urinary tract infection: progress toward an effective Escherichia coli vaccine. Expert Rev Vaccines **11:**663-676.

172. **Orvis J, Crabtree J, Galens K, Gussman A, Inman JM, Lee E, Nampally S, Riley D, Sundaram JP, Felix V, Whitty B, Mahurkar A, Wortman J, White O, Angiuoli SV.** 2010. Ergatis: a web interface and scalable software system for bioinformatics workflows. Bioinformatics **26:**1488-1492.

173. **Langmead B, Trapnell C, Pop M, Salzberg SL.** 2009. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome biology **10:**R25.

174. **Subashchandrabose S, Hazen TH, Rasko DA, Mobley HL.** 2013. Draft genome sequences of five recent human uropathogenic Escherichia coli isolates. Pathogens and disease.

175. **Mortazavi A, Williams BA, McCue K, Schaeffer L, Wold B.** 2008. Mapping and quantifying mammalian transcriptomes by RNA-Seq. Nature methods **5:**621-628.

176. **R Development Core Team.** 2012. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

177. **Kolde R.** 2013. Pretty Heatmaps. R package version 0.7.7.

178. **Rutherford K, Parkhill J, Crook J, Horsnell T, Rice P, Rajandream MA, Barrell B.** 2000. Artemis: sequence visualization and annotation. Bioinformatics **16:**944-945.

179. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in Escherichia coli K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640-6645.

180. **Hagberg L, Engberg I, Freter R, Lam J, Olling S, Svanborg Eden C.** 1983. Ascending, unobstructed urinary tract infection in mice caused by pyelonephritogenic *Escherichia coli* of human origin. **Infect Immun 40:**273-283.

181. **Kearney JF, Radbruch A, Liesegang B, Rajewsky K.** 1979. A new mouse myeloma cell line that has lost immunoglobulin expression but permits the construction of antibody-secreting hybrid cell lines. J Immunol **123:**1548-1550.

182. **Gefter ML, Margulies DH, Scharff MD.** 1977. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic cell genetics **3:**231-236.

183. **Claflin JL, Hudak S, Maddalena A.** 1981. Anti-phosphocholine hybridoma antibodies. I. Direct evidence for three distinct families of antibodies in the murine response. J Exp Med **153:**352-364.

184. **Johnson JR, Kuskowski MA, Gajewski A, Soto S, Horcajada JP, Jimenez de Anta MT, Vila J.** 2005. Extended virulence genotypes and phylogenetic background of Escherichia coli isolates from patients with cystitis, pyelonephritis, or prostatitis. J Infect Dis **191:**46-50.

185. **Fetherston JD, Bearden SW, Perry RD.** 1996. YbtA, an AraC-type regulator of the Yersinia pestis pesticin/yersiniabactin receptor. Mol Microbiol **22:**315-325.

186. **Rakin A, Schneider L, Podladchikova O.** 2012. Hunger for iron: the alternative siderophore iron scavenging systems in highly virulent Yersinia. Frontiers in cellular and infection microbiology **2:**151.

187. **Sievers F, Wilm A, Dineen D, Gibson TJ, Karplus K, Li W, Lopez R, McWilliam H, Remmert M, Soding J, Thompson JD, Higgins DG.** 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. Molecular systems biology **7:**539.

188. **Bagos PG, Liakopoulos TD, Spyropoulos IC, Hamodrakas SJ.** 2004. PRED-TMBB: a web server for predicting the topology of beta-barrel outer membrane proteins. Nucleic acids research **32:**W400-404.

189. **Lukacik P, Barnard TJ, Keller PW, Chaturvedi KS, Seddiki N, Fairman JW, Noinaj N, Kirby TL, Henderson JP, Steven AC, Hinnebusch BJ, Buchanan SK.** 2012. Structural engineering of a phage lysin that targets gram-negative pathogens. Proc Natl Acad Sci U S A **109:**9857-9862.

190. **Branger CG, Sun W, Torres-Escobar A, Perry R, Roland KL, Fetherston J, Curtiss R, 3rd.** 2010. Evaluation of Psn, HmuR and a modified LcrV protein delivered to mice by live attenuated Salmonella as a vaccine against bubonic and pneumonic Yersinia pestis challenge. Vaccine **29:**274-282.

191. **Bachman MA, Miller VL, Weiser JN.** 2009. Mucosal lipocalin 2 has pro-inflammatory and iron-sequestering effects in response to bacterial enterobactin. PLoS Pathog **5:**e1000622.

192. **Bachman MA, Oyler JE, Burns SH, Caza M, Lepine F, Dozois CM, Weiser JN.** 2011. Klebsiella pneumoniae yersiniabactin promotes respiratory tract infection through evasion of lipocalin 2. Infect Immun **79:**3309-3316.

193. **Neilands JB.** 1979. The ironic function of bacteriophage receptors. Trends in biochemical sciences (Amsterdam. Regular ed.) **4:**115-118.

194. **Chan CY, St John AL, Abraham SN.** 2013. Mast cell interleukin-10 drives localized tolerance in chronic bladder infection. Immunity **38:**349-359.

195. **Keeney KM, Yurist-Doutsch S, Arrieta MC, Finlay BB.** 2014. Effect of Antibiotics on Human Microbiota and Subsequent Disease. Annual review of microbiology.

196. **Olesen B, Hansen DS, Nilsson F, Frimodt-Moller J, Leihof RF, Struve C, Scheutz F, Johnston B, Krogfelt KA, Johnson JR.** 2013. Prevalence and characteristics of the epidemic multiresistant Escherichia coli ST131 clonal group among extended-spectrum beta-lactamase-producing E. coli isolates in Copenhagen, Denmark. J Clin Microbiol **51:**1779-1785.

197. **Oteo J, Navarro C, Cercenado E, Delgado-Iribarren A, Wilhelmi I, Orden B, Garcia C, Miguelanez S, Perez-Vazquez M, Garcia-Cobos S, Aracil B, Bautista V, Campos J.** 2006. Spread of Escherichia coli strains with high-level cefotaxime and ceftazidime resistance between the community, long-term care facilities, and hospital institutions. J Clin Microbiol **44:**2359-2366.

198. **Johnson JR, Johnston B, Clabots C, Kuskowski MA, Castanheira M.** 2010. Escherichia coli sequence type ST131 as the major cause of serious multidrug-resistant E. coli infections in the United States. Clin Infect Dis **51:**286-294.

199. **Petty NK, Ben Zakour NL, Stanton-Cook M, Skippington E, Totsika M, Forde BM, Phan MD, Gomes Moriel D, Peters KM, Davies M, Rogers BA, Dougan G, Rodriguez-Bano J, Pascual A, Pitout JD, Upton M, Paterson DL, Walsh TR, Schembri MA, Beatson SA.** 2014. Global dissemination of a multidrug resistant Escherichia coli clone. Proc Natl Acad Sci U S A **111:**5694-5699.

200. **Durant L, Metais A, Soulama-Mouze C, Genevard JM, Nassif X, Escaich S.** 2007. Identification of candidates for a subunit vaccine against extraintestinal pathogenic Escherichia coli. Infect Immun **75:**1916-1925.

201. **Mattes MJ.** 1987. Biodistribution of antibodies after intraperitoneal or intravenous injection and effect of carbohydrate modifications. Journal of the National Cancer Institute **79:**855-863.

202. **Hagan EC, Mobley HL.** 2009. Haem acquisition is facilitated by a novel receptor Hma and required by uropathogenic Escherichia coli for kidney infection. Mol Microbiol **71:**79-91.

203. **Russo TA, Carlino UB, Johnson JR.** 2001. Identification of a new iron-regulated virulence gene, ireA, in an extraintestinal pathogenic isolate of Escherichia coli. Infect Immun **69:**6209-6216.

204. **Hagan EC.** 2009. Iron Acquisition by Uropathogenic *Escherichia coli*: ChuA and Hma Heme Receptors as Virulence Determinants and Vaccine Targets.

205. **Snyder JA, Haugen BJ, Buckles EL, Lockatell CV, Johnson DE, Donnenberg MS, Welch RA, Mobley HL.** 2004. Transcriptome of uropathogenic Escherichia coli during urinary tract infection. Infect Immun **72:**6373-6381.

206. **Hagan EC, Lloyd AL, Rasko DA, Faerber GJ, Mobley HL.** 2010. Escherichia coli global gene expression in urine from women with urinary tract infection. PLoS Pathog **6:**e1001187.

207. **Giannakopoulos X, Evangelou A, Kalfakakou V, Grammeniatis E, Papandropoulos I, Charalambopoulos K.** 1997. Human bladder urine oxygen content: implications for urinary tract diseases. Int Urol Nephrol **29:**393-401.

208. **Shalel-Levanon S, San KY, Bennett GN.** 2005. Effect of oxygen, and ArcA and FNR regulators on the expression of genes related to the electron transfer chain and the TCA cycle in Escherichia coli. Metabolic engineering **7:**364-374.

209. **Lane MC, Li X, Pearson MM, Simms AN, Mobley HL.** 2009. Oxygen-limiting conditions enrich for fimbriate cells of uropathogenic Proteus mirabilis and Escherichia coli. J Bacteriol **191:**1382-1392.

210. **Cai W, Wannemuehler Y, Dell'anna G, Nicholson B, Barbieri NL, Kariyawasam S, Feng Y, Logue CM, Nolan LK, Li G.** 2013. A novel two-component signaling system facilitates uropathogenic Escherichia coli's ability to exploit abundant host metabolites. PLoS Pathog **9:**e1003428.

211. **Imlay JA.** 2013. The molecular mechanisms and physiological consequences of oxidative stress: lessons from a model bacterium. Nat Rev Microbiol **11:**443-454.

212. **Lee SH, Hava DL, Waldor MK, Camilli A.** 1999. Regulation and temporal expression patterns of Vibrio cholerae virulence genes during infection. Cell **99:**625-634.

213. **Talaat AM, Lyons R, Howard ST, Johnston SA.** 2004. The temporal expression profile of Mycobacterium tuberculosis infection in mice. Proc Natl Acad Sci U S A **101:**4602-4607.

214. **Deriu E, Liu JZ, Pezeshki M, Edwards RA, Ochoa RJ, Contreras H, Libby SJ, Fang FC, Raffatellu M.** 2013. Probiotic bacteria reduce salmonella typhimurium intestinal colonization by competing for iron. Cell host & microbe **14:**26-37.

215. **Koropatkin NM, Cameron EA, Martens EC.** 2012. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol **10:**323-335.

216. **Winter SE, Lopez CA, Baumler AJ.** 2013. The dynamics of gut-associated microbial communities during inflammation. EMBO reports **14:**319-327.

217. **Torres AG, Payne SM.** 1997. Haem iron-transport system in enterohaemorrhagic Escherichia coli O157:H7. Mol Microbiol **23:**825-833.

218. **Lim JK, Gunther NWt, Zhao H, Johnson DE, Keay SK, Mobley HL.** 1998. In vivo phase variation of Escherichia coli type 1 fimbrial genes in women with urinary tract infection. Infect Immun **66:**3303-3310.