Iron Acquisition by Uropathogenic *Escherichia coli*: ChuA and Hma Heme Receptors as Virulence Determinants and Vaccine Targets

by

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To Mom, Dad, and Eric

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ABSTRACT

In Gram negative bacteria, specific outer membrane receptors facilitate the import of iron-chelating siderophores and iron from host organisms. Uropathogenic *Escherichia coli* (UPEC), the predominant cause of uncomplicated urinary tract infection (UTI), utilizes a wide array of these receptors to acquire iron, an essential nutrient from within the iron-limited urinary tract. However, the contributions of specific sources of host iron remain unknown. Furthermore, efforts to induce protective immunity against UPEC by targeting known virulence determinants have been largely unsuccessful, suggesting that novel targets are required. To address this, an immunoproteomics approach was used here to identify 23 outer membrane proteins that elicited an immune response during murine UTI. Among the most prevalent of these were TonB-dependent siderophore and iron compound receptors, including the putative receptor c2482 (heme acquisition protein, Hma). Intranasal immunization with Hma or the siderophore receptors IreA or IutA significantly protected mice from UPEC challenge, while vaccination against the heme receptor ChuA did not confer protection from UTI.

Additionally, genetic and biochemical approaches were used to demonstrate that the vaccine candidate Hma functions as a TonB-dependent heme receptor. Hma shares limited homology with known heme receptors and our structure-function studies identified a unique residue required for Hma-mediated heme utilization. We further establish that heme is an essential source of iron for UPEC in the kidney and present evidence for the importance of heme acquisition within the context of siderophore-

mediated iron uptake. The findings presented here provide a foundation for the development of an outer membrane protein-based vaccine against UPEC and suggest that iron receptor antigens may represent putative vaccine targets in other Gram negative bacteria. Furthermore, this work offers novel insight into the distinct contributions of specific UPEC receptors and highlights the necessity of iron acquisition for bacterial survival within the mammalian host.

CHAPTER 1

INTRODUCTION

Uropathogenic *Escherichia coli*

Urinary tract infection.

The urinary tract is among the most common sites of bacterial infection and *E. coli* is by far the most common species infecting the urinary (102). Infections in the urinary tract range in severity from asymptomatic bacteriuria to urosepsis, which can be fatal. Studies suggest that up to 95% of all urinary tract infections develop by an ascending route of infection (8), meaning that infection begins with colonization of the periurethral area, followed by an upward progression to infect the bladder, and, in some cases, continued progression of the bacteria through the ureters to infect the kidneys. After an initial episode of cystitis, there is a risk of about 25% that a woman will have a second symptomatic episode within six months (50). The bacterial source of these recurrent infections is unclear, with same-strain-episodes making up anywhere from 25-100% of cases, presumably due to reemergence of the initial infecting strain [reviewed in (91)]. *E. coli* is the predominant cause of the entire spectrum of UTI, accounting for more than 80% of community-acquired infections.

Virulence determinants of UPEC.

Uropathogenic *E. coli* strains are genetically distinct from commensal strains of *E. coli*. As compared to fecal isolates, cystitis and pyelonephritis isolates more frequently encode specific virulence-associated determinants (39), which are often encoded on pathogenicity-associated islands (PAIs) (19, 37, 66). To date, eight true UPEC virulence factors have been established that fulfill the major components of Molecular Koch's Postulates. These postulates require that an isogenic mutant lacking the virulence factor is attenuated in a model of infection and that virulence of the mutant can be restored upon complementation (41). For UPEC, these virulence factors are K2 capsule (21), type 1 fimbriae (33), Dr fimbriae (60), hemolysin (169, 198), CNF-1 (152), DegS (150), PhoU (22), and TonB (187). It should be noted that four of these factors (type 1 fimbriae, DegS, PhoU, and TonB) are present in both pathogenic and non-pathogenic *E. coli*, thus violating Molecular Koch's first postulate.

1. **Adherence and motility.**

Adherence to host tissues is a common mechanism of bacterial pathogenesis, but for UPEC, which must resist the strong shear forces of urine flow, the ability to adhere is particularly important. UPEC produces a number of adhesive organelles termed fimbriae, which are necessary for urinary tract colonization, including type 1 (33), P (202), Dr (60), and S/F1C fimbriae, as well as several afimbrial adhesins. Expression of both type 1 (1) and P fimbriae (16) is phase variable, providing versatility for the pathogen in terms of host tissue specificity and immune evasion. In contrast to adherence, flagella-mediated motility is also required for UPEC to establish an ascending (*i.e.* kidney) infection (104).

2. Toxin production.

UPEC secretes a complement of proteins which are toxic to eukaryotic cells, including alpha-hemolysin (127, 199) and cytotoxic necrotizing factor-1 (CNF-1) (152). Three secreted autotransporters, including homologues of Pic (protein involved in intestinal colonization) of *Shigella* and Tsh (temperature-sensitive hemagglutinin) of avian pathogenic *E. coli* (APEC) (77), as well as the UPEC-specific Sat (secreted autotransporter toxin) (65), have also been described in UPEC strains. Interestingly, Tsh shares 78% sequence identity with a secreted hemoglobin protease (Hbp) isolated from an abscess-forming *E. coli* strain (77). Hbp was shown to degrade hemoglobin and bind the released heme (143) and was required for intra-abdominal abscess formation in a murine mixed species infection model (142). While protease activity has not yet been demonstrated for Tsh, the gene is present in 63% of pyelonephritis strains and only 33% of fecal strains (77), suggesting its conservation confers a pathogenic advantage.

3. Biofilm formation.

Historically, most research has focused on *E. coli* that are either adherent to epithelial cells or free-floating planktonic bacteria in the lumen of the bladder. However, several laboratories have observed biofilm-like aggregates within superficial epithelial cells of both mice (4) and humans (157), termed intracellular bacterial communities. Bacteria may flux out of these pods as elongated cells (93, 131) and seed new invasive events. Thus, one hypothesis in the field is that recurrent UTI is due to reactivation of the sparsely remaining intracellular bacterial communities, presumably leading to a dramatic increase in bacterial load and a new episode of cystitis (132, 153, 163).

4. Nutrient acquisition.

Perhaps surprisingly, human urine provides a favorable growth medium for *E. coli* (2), despite the low pH and high osmolarity, which can be inhibiting factors for bacterial growth. Furthermore, in a screen of auxotrophic mutants, most were capable of *in vivo* colonization (26), suggesting that this environment, as well as the close association of the organisms with the urinary tract epithelium, provides sufficient nutrients.

Iron acquisition by Gram-negative bacteria

Acquisition of trace metals, especially iron, presents a unique challenge to many bacterial species. Iron is an essential nutrient, required as an electron donor and acceptor for many cellular proteins, including those involved in oxidative phosphorylation, metabolism, and oxidative stress. Although iron is the sixth most abundant element on Earth, ferric iron (Fe^{3+}), which is the dominant oxidation state under aerobic conditions, is poorly soluble at neutral pH. Bacterial pathogens encounter further difficulties, as iron is tightly sequestered within the mammalian host.

Bacterial iron uptake systems.

Bacteria have therefore evolved elegant systems to actively scavenge iron, either from the environment or host metalloproteins. Siderophores are small, iron-chelating molecules secreted by many bacterial species that bind ferric iron with high affinity (K_d=10^{-20}-10^{-50} M). Ferrisiderophores are then recognized by specific bacterial receptors and transported back into the cell, where the iron is dissociated and incorporated into the

necessary enzymes or stored for later use. Many distinct types of siderophores are produced by both fungi and bacteria, with a single species often synthesizing more than one. Siderophore synthesis is a multi-step process usually involving a number of enzymes and, in the case of *E. coli* enterobactin, the completed molecules are secreted directly from the cytoplasm to the extracellular space through the TolC complex (15).

Iron-loaded siderophores are transported across the outer membrane via specific receptors (Figure 1-1). Structurally similar, these 70-80 kDa proteins adopt a beta-barrel structure embedded in the outer membrane, with short periplasmic turns and longer extracellular loops, which are presumably involved in ligand-binding (20, 45, 120). These receptors require the activity of an inner membrane protein complex composed of TonB, ExbB, and ExbD to transduce energy from the proton motive force. The N-terminus of the receptor protein forms a "plug" domain that obstructs the pore of the beta-barrel during its ligand-unbound state. This region also interacts with TonB and, upon ligand binding, is thought to undergo a conformational change, allowing the ferrisiderophore to enter the periplasm (42, 147, 166). Structure-function studies have identified the consensus sequence D/EXXV near the N-termini of TonB-dependent outer membrane receptors that is required for TonB interaction (27, 139, 164). The precise mechanism of TonB action, however, is unknown. While some reports propose that it remains anchored in the inner membrane while spanning the periplasm to interact with the receptor (30), data from other studies suggest a "shuttling" mechanism, whereby it oscillates between the inner and outer membranes during receptor activity (110, 112).

Once in the periplasm, siderophores are bound by periplasmic substrate-binding proteins and actively transported to the cytoplasm via ABC transport systems. Unlike the

highly substrate-specific outer membrane receptors, these systems often transport a variety of structurally similar siderophores. For example, the FhuBCD system transports not only the FhuA-imported ferrichrome, but also other hydroxamates including aerobactin, coprogen, and rhodotorulic acid, which have their own outer membrane receptors (43).

In contrast, under anaerobic conditions, iron is in the ferrous (Fe^{2+}) state and is freely soluble. To transport this ferrous iron, *E. coli* uses an ATP-driven inner membrane permease system encoded by *feoAB* (96), a system that, unlike enterobactin, is required by *E. coli* K12 to colonize the murine intestine (175). Recently, an additional ferrous transport system, EfeUOB, was identified that functions in EHEC (and likely UPEC) at low pH (28, 63).

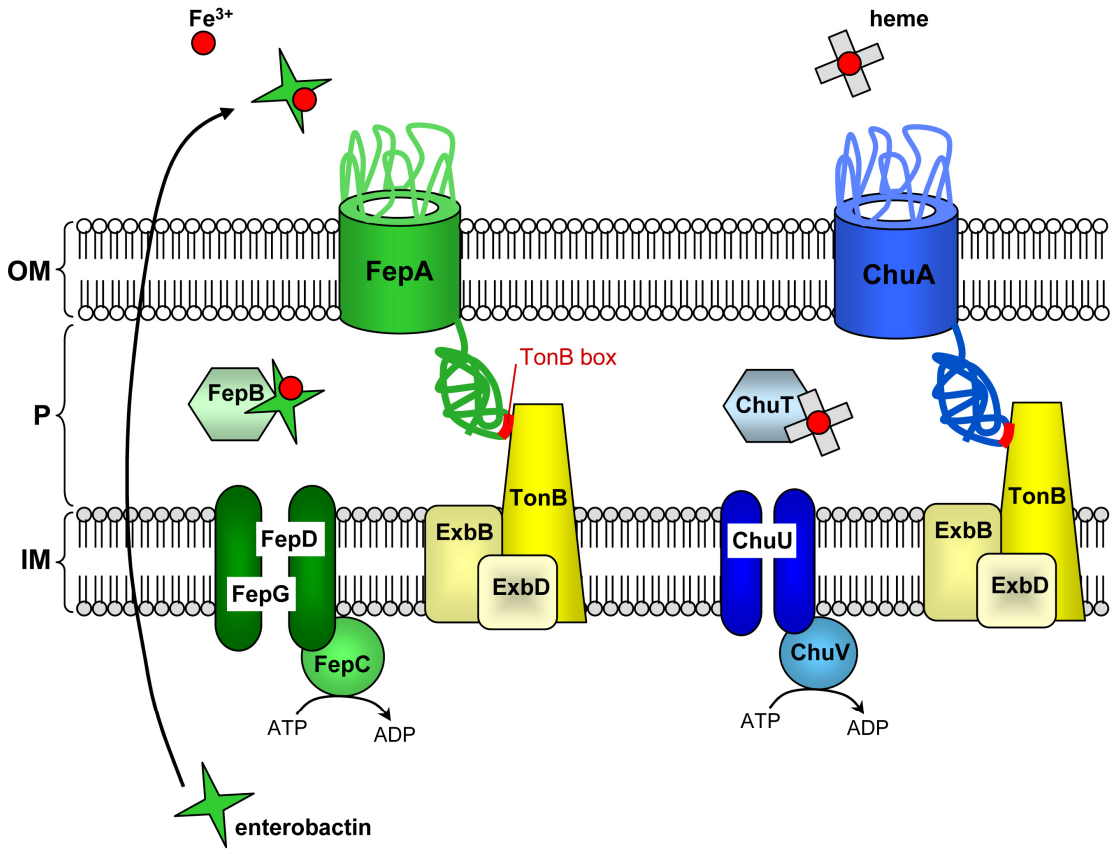

Figure 1-1. TonB-dependent iron transport in *E. coli*. Shown are the enterobactin (*fep*, green) and heme (*chu*, blue) uptake machinery. Iron-loaded enterobactin or heme is recognized by specific outer membrane receptors (FepA or ChuA, respectively) and transported into the periplasm with energy transduced from the TonB/ExbB/ExbD complex (yellow). Periplasmic binding proteins (FepB or ChuT) transfer the substrate to inner membrane permeases (FepDG or ChuU), where ATPases (FepC or ChuV) drive transport into the cytoplasm. Approximate locations of TonB boxes are shown on OMP receptors in red. OM, outer membrane; P, periplasm; IM, inner membrane.

Acquisition of host iron sources.

In addition to siderophore-mediated iron uptake, many bacterial pathogens have evolved specific mechanisms to directly acquire host iron sources. *Neisseria*, *Haemophilus*, *Pasteurella*, and *Moraxella* spp., for example, directly bind transferrin and remove its bound iron via a TonB-dependent outer membrane receptor and an outer membrane lipoprotein [reviewed in (34)]. Similar mechanisms exist for the removal of lactoferrin-bound iron. More widespread among bacterial pathogens is the acquisition of host iron in the form of heme. Present in all Proteobacteria classes, specific TonB-dependent outer membrane receptors bind heme, either directly or following its removal from hemoproteins, and transport it into the periplasm, where specific ABC transporters transfer it to the cytoplasm. These outer membrane receptors characterized to date share strong sequence identity and specific homologous residues have been identified in *Yersinia enterocolitica*, *Poryphyromonas gingivalis*, and *Shigella dysenteriae* receptors that are required for HemR-, HmuR-, and ShuA-mediated heme uptake, respectively (18, 24, 118). In certain species including *Serratia marcescens*, *Pseudomonas aeruginosa*, and *Y. pestis*, heme acquisition is further facilitated by the secretion of hemophores, small proteins that scavenge heme and present it to outer membrane receptors in a process analogous to siderophore uptake [reviewed in (196)].

In pathogenic *E. coli*, heme utilization is mediated by genes in the *chu* locus, which is homologous to the *Shigella* heme utilization locus, *shu*. This eight-gene cluster encodes proteins involved in both the transport of an intact heme moiety into the bacterial cell (125) and its subsequent processing (Fig. 1-1, 1-2). ChuA is a 69 kDa TonB-dependent outer membrane receptor that is more than 99% identical to *S. dysenteriae*

ShuA (186). *chuTUV* encode an ABC transporter shown to transport heme in an *in vitro* proteoliposome model of the cytoplasmic membrane (25). In this model, heme was delivered directly from the ShuUV membrane-spanning transporter to a cytoplasmic protein, ShuS. ChuS was initially described as a heme oxygenase, using cellular electron donors to catalyze the degradation of heme into biliverdin, carbon monoxide, and free iron (180). The crystal structure of ChuS indicates that it adopts a fold unique among bacterial heme oxygenases, a feature speculated to allow more efficient utilization of a wider range of electron donors (178, 180). In contrast, other studies report that ShuS and its homologs bind double-stranded DNA in addition to heme and speculate that this protein functions to traffic heme within the cytoplasm (109, 201). Regardless of the mechanism, ShuS is required by *S. dysenteriae* both for nutritional iron and to prevent heme toxicity (204). Function of the remaining *chu* genes is unknown, although *chuW* is predicted to encode a porphyrin oxidase and ChuX has been shown to bind heme (179).

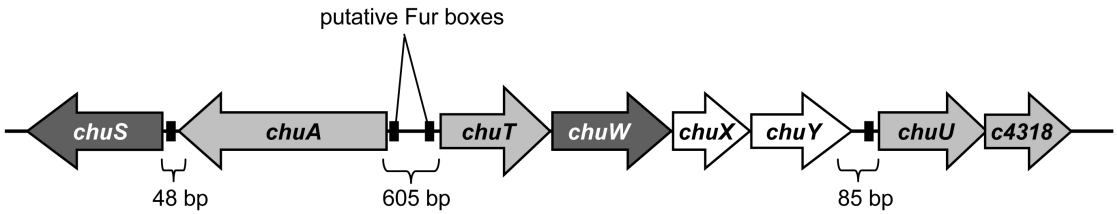

Figure 1-2. *E. coli chu* heme utilization locus. Arrows represent genes and are shaded according to function. Heme transport, light gray; heme processing, dark gray; unknown function, white. In strain CFT073, gene c4318 is not annotated as part of the *chu* locus, but is homologous to *S. dysenteriae shuV*. Four putative Fur binding sites, shown as black boxes, are located in intergenic regions (203). The Fur boxes upstream of *chuS* and *chuA* overlap the genes' -10 regions and the sites upstream of *chuT* and *chuU* overlap the -35 regions of the promoters. Distances between ORFs are indicated below map and unlisted intergenic regions ≤20 bp.

Regulation of iron acquisition gene expression.

While acquisition of scarce iron is indeed critical to bacterial survival, these systems must be tightly regulated, as free iron in the cell generates toxic oxygen radicals. Many species, including *E. coli*, achieve this homeostasis primarily through the action of Fur (ferric uptake regulator), an iron-binding protein that acts as a simple transcriptional repressor of genes involved in iron uptake. Under iron-replete conditions, equilibrium is shifted towards iron-bound Fur, which binds to specific target DNA sequences within the promoters of iron-responsive genes, physically blocking their transcription. When iron levels in the cell are low, however, Fur loses its bound iron and dissociates from the DNA, thus allowing gene transcription. Fur binds to a 19 bp inverted repeat sequence termed the Fur box (36) and recent analysis suggests that two Fur dimers may interact with these regions (10). Besides negatively regulating iron uptake genes, Fur indirectly positively regulates an array of genes involved in iron metabolism through a small RNA, RyhB (123). Thus under iron-limiting conditions, Fur simultaneously upregulates genes involved in iron acquisition and downregulates iron storage proteins.

In addition to Fur, other transcriptional regulators influence the expression of iron uptake genes. Expression of *chuA* is also regulated by RfaH (134), which enhances transcription of long operons including hemolysin by functioning as a transcriptional antiterminator (111). In other species, temperature (140) and oxygenation (17) have further been shown to affect iron uptake gene expression.

Iron uptake by uropathogenic *E. coli*.

UPEC possesses a broad repertoire of systems used to acquire iron within the iron-limited urinary tract and other environments. The genome of strain CFT073 encodes 14 characterized outer membrane iron compound receptors, four[1] siderophore biosynthesis systems, and a number of putative TonB-depenedent receptors that may also be involved in iron acquisition (Table 1-1) (197). Indeed, obtaining iron is a critical process for UPEC survival *in vivo*. The genes encoding these iron acquisition systems were among the most highly upregulated genes of CFT073 during infection of the murine urinary tract (171). Furthermore, a *tonB* mutant was severely attenuated in a mouse pyelonephritis model, indicating that TonB-dependent receptors are required for UTI (187). Interestingly, asymptomatic bacteriuria strains, while lacking classical UPEC virulence factors such as fimbriae, express the full complement of iron acquisition systems, providing further evidence for the necessity of iron uptake during successful urinary tract colonization (156).

Most pathogenic and nonpathogenic *E. coli* strains produce enterobactin, but aerobactin production has long been appreciated as an epidemiological marker of UPEC virulence (29, 89). While strains lacking one of these systems were able to colonize the murine urinary tract to wildtype levels, an *iucB entD* mutant defective for both enterobactin and aerobactin production was attenuated (187). More recently, UPEC genes located outside of the *ent*/*fep* enterobactin locus have been identified that transport this siderophore. Originally identified as an adhesive factor for enterohemorrhagic *E.*

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[1] The CFT073 genome encodes enzymes for the synthesis and export of four siderophores, but only produces three: enterobactin, aerobactin, and salmochelin. While CFT073 encodes yersiniabactin synthesis machinery and transporters (c2419-c2436), nonsense mutations in *irp1* and a 711 bp insertion element in *irp2* prevent yersiniabactin production by this strain.

coli (182), the IrgA homolog adhesin Iha was also shown to transport both enterobactin and its linear degradation product dihydroxybenzoylserine (DHBS) (113) and contribute to UPEC fitness in the urinary tract (87).

An enterobactin-like siderophore termed salmochelin was identified in *Salmonella* (13, 73) and later shown to be produced by a variety of pathogenic *E. coli* strains, presumably acquired by these strains via horizontal gene transfer (173). Salmochelin is a glucosylated form of enterobactin that represents a significant bacterial adaptation against innate immunity. The mammalian secreted protein lipocalin-2 binds and sequesters enterobactin, functioning to impair bacterial iron acquisition during infection (48). However, salmochelin's glucose moieties prevent lipocalin-2 binding, thus evading this particular host defense (46). In UPEC, the salmochelin receptor IroN contributes both to urinary tract colonization (160) and urothelial cell invasion (44).

Yersiniabactin synthesis and transport genes, encoded on the High Pathogenicity Island of *Yersinia* sp. are also present in many *Enterobacteriaceae* including ExPEC strains (32). Although its role in UTI is unknown, yersiniabactin synthesis was required for ExPEC virulence in a murine lethality model (165) and the yersiniabactin receptor *fyuA* was necessary for UPEC biofilm formation in human urine (69). Furthermore, both yersiniabactin and salmochelin production were correlated with urinary isolates, rather than fecal isolates, of patients with recurrent UTI (78).

In addition to these secreted siderophores, UPEC strains can utilize other iron sources, as well. Strain CFT073 encodes two receptors, FhuA and FhuE, shown to transport the fungal hydroxamate siderophores ferrichrome, coprogen, and rhodotorulic acid (43, 72). These receptors may be particularly important during intestinal

colonization or environmental survival, when diverse fungal species are present. Additionally, the previously-described heme receptor ChuA contributes to UPEC fitness *in vivo* (187) and intracellular bacterial community formation (151). Moreover, several iron-responsive putative TonB-dependent receptors have been described whose substrates have yet to be identified (144, 159).

Table 1-1. Ferric iron transport systems of uropathogenic *E. coli*.

Locus[a]	Iron substrate	% UPEC[b]
entD ***fepA*** *fes entF fepE fepDGC fepB entCEBE*[c]	enterobactin	100[d]
iucABCD ***iutA***[c]	aerobactin	66
chuA*S chuTWXYUV*	heme/hemoglobin	87
iroN *iroBCDE*[c]	salmochelin	74
fhuA*CDB*	ferrichrome	-[e]
fhuE	coprogen/rhodotorulic acid	-
fitA *fitBCDER*	unknown	-
ybtPQXS ybtA irp2 irp1 ybtUTE ***fyuA***[c]	yersiniabactin	91
cirA	catecholates	-
fiu	catecholates	-
iha	enterobactin	47
hma	heme/hemoglobin	69
ireA	unknown	34

[a] TonB-dependent outer membrane receptors are **bolded**.
[b] Percent of UPEC isolates carrying the gene encoding the indicated iron receptor. Shown are means derived from pooled data (88, 90).
[c] These loci encode siderophore synthesis, as well as transport, genes.
[d] Enterobactin prevalence determined by siderophore production as measured by mass spectrometry (78).
[e] -, not determined.

UPEC interactions with the host

Innate defenses of the urinary tract.

The urothelial layer of the bladder represents one of the first lines of defense against pathogen entry into the urinary tract. This transitional epithelium, which can readily expand and contract, is composed of basal cells, intermediate cells, and superficial umbrella cells [reviewed in (5)]. Coated apically by hexagonal arrays of semi-crystalline membrane proteins termed uroplakins (206, 207), terminally differentiated umbrella cells form a barrier between the bladder lumen and underlying epithelium. When damaged, these cells slough off and are eliminated with the voiding of urine, providing a means to eliminate adherent bacteria. Indeed, UPEC attachment to urothelial cells triggers apoptosis and exfoliation of the superficial layer in a type 1 fimbriae-dependent manner (130). This interaction also induces upregulation of urothelial proliferation, differentiation, and cell tight junction components (133) and observations indicate that the underlying epithelial cells differentiate rapidly to restore the superficial umbrella layer (52, 130).

In addition to these physical defense mechanisms, a number of secreted factors function to protect the urinary tract from infection. Tamm Horsfall protein (THP), the most abundant protein in mammalian urine, is a glycoprotein that prevents type 1-fimbriated *E. coli* from binding bladder uroplakins (145) and is required for UTI clearance in mice (11, 126). Expression of another secreted protein, lipocalin-2, is induced in *E. coli*-infected bladder cells (151). This protein has been shown to bind the siderophore enterobactin and inhibit enterobactin-dependent growth of *E. coli* (48, 57). In an intraperitoneal infection model, lipocalin-2-deficient mice sustained significantly

higher bacterial burdens, leading to a higher fatality rate and implicating the enterobactin-sequestering activity of lipocalin-2 as an important mechanism of bacterial control (48).

The classic innate immune response to UTI is initiated by toll-like receptors (TLRs), which recognize conserved pathogen-associated molecules. Mice lacking the LPS-responsive TLR4 (74) or the flagellin-responsive TLR5 (3) are each more susceptible to UTI than wildtype controls. Additionally, a novel receptor, TLR11, was identified in mice that mediates recognition of UPEC (208) and a parasite-derived profilin-like protein (205). However, as humans do not express this receptor, the role of TLR11 in innate resistance to UTI remains unclear (208). In addition to these classic TLR ligands, the type 1 fimbrial adhesin FimH was recently identified as a novel TLR4 ligand (129). Interestingly, ABU *E. coli* strains, which persist in the bladder without triggering an inflammatory response, do not encode functional type 1 or P fimbriae (84).

Signaling through TLRs initiates a local proinflammatory response in the urothelium. Cytokines are important mediators of this response and serve as markers for innate immune activation. Elevated levels of both interleukin-6 and IL-8 were detected in the urine of both patients with UTI (76, 98) and catheter-challenged human volunteers (75). Pyuria, or the presence of neutrophils in the urine, is a clinical hallmark of UTI and neutrophil infiltration has long been appreciated as a key innate response that is necessary for UTI resolution (56, 74).

Adaptive immunity.

While innate immune defenses are clearly necessary components of the host response to UTI, adaptive immunity is also induced upon UPEC infection. Humoral

responses appear to be particularly important. Indeed, secretory IgA that can block *E. coli* binding to urothelial cells was detected in the urine of patients with UTI, with higher levels observed in pyelonephritis patients than in those with lower UTIs (181, 188). Furthermore, B and T cell-deficient severe combined immunodeficient mice are significantly more susceptible to bladder and kidney infection. Nude mice, which have T cell defects, are equally resistant as wildtype animals, suggesting that humoral T cell-independent mechanisms are important for accelerated UTI clearance (80). Gamma delta T cells in these animals may contribute to clearance, though, as T cell receptor δ-chain knockout mice were found to be more susceptible to *E. coli* UTI (92). Furthermore, using an OVA-expressing UPEC strain as a model, Thumbikat and colleagues showed that adaptive responses contribute to UTI clearance, as they observed reduced bladder colonization after reinfection and the development of antigen-specific IgG and IgM (184). Further study of the adaptive immune response to UTI will be especially critical to refine our understanding and treatment of recurrent infections, as well as develop vaccines.

Vaccination against uropathogens

Capsule, O antigen and whole cell vaccines.

Due to the medical and economic cost of UTI, an estimated $2.4 billion annually (117), researchers have long sought to develop protective immunity against UPEC. Early studies demonstrated that active or passive immunization against capsule polysaccharide or LPS O antigen could provide protection from UTI (94, 95, 176). Similarly, when

chemically crosslinked to diphtheria toxoid, K13 capsule, one of the most common capsules among UTI-causing *E. coli*, provided renal protection and stimulated specific humoral and cellular responses (101). However, recent studies have suggested that the presence of capsule and O antigens may obstruct the development of protective immunity, as mucosal vaccination with a capsule- and O antigen-negative ExPEC strain generated a greater humoral response than immunization with the wildtype strain (158).

A mixture of 10 heat-killed uropathogens, including six *E. coli* strains, *Proteus mirabilis*, *Morganella morganii*, *Enterococcus faecalis*, and *Klebsiella pneumoniae* has been used in clinical trials as a vaginal suppository termed Urovac (79, 189-191). Early trials in mice demonstrated that this vaccine afforded protection against cystitis and pyelonephritis (99, 100, 193). In a phase II clinical trial, three doses of vaginal Urovac delayed the time to reinfection among women with recurrent UTIs, but did not significantly alter the number of patients remaining infection-free after six months (189). Greater and longer-lasting protection could be achieved by increasing Urovac doses to six (79, 190, 191), although no measurable change in *E. coli*-specific urinary antibodies was observed (79, 191).

Adhesin-based subunit vaccines.

Because of their abundance on the bacterial cell surface and role in UTI pathogenesis, fimbriae have been attractive targets for subunit vaccines against UPEC infection. For example, a FimH subunit vaccine composed of either the N-terminal portion of the protein or full-length FimH conjugated to its chaperone, FimC, was shown to induce a specific antibody response in immunized C3H/J mice and reduce bladder

colonization by two logs (108), as well as protect primates from symptoms of UTI (107). Though the precise mechanism of protection by FimH vaccination is unknown, it has been suggested that inhibition of bacterial binding to host epithelial cells plays a role in fimbrial vaccine function (59). Indeed, sera from FimCH-vaccinated mice blocked binding of *E. coli* to bladder epithelial cells (106).

Other fimbrial species have also been investigated as UTI vaccine candidates. Administration of a purified whole P fimbriae vaccine decreased renal colonization and invasion, protection which roughly correlated with specific anti-P fimbriae IgG titers (137). A subunit vaccine using PapG, the P fimbrial adhesin, complexed with its periplasmic chaperone, PapD, protected primates from histological indications of pyelonephritis (154). Mice immunized with purified whole Dr fimbriae displayed decreased mortality and antisera from vaccinated animals blocked UPEC binding to urothelial cells *in vitro* (59). Similarly, intranasal immunization against the uropathogen *P. mirabilis* with either the major fimbriae, MR/P, or the lectin-binding domain of the MR/P fimbrial adhesin each protected mice from urinary tract colonization (115).

Although a number of studies provide support for the use of UPEC adhesins as vaccine targets, other evidence suggests that these fimbriae-based vaccines may not be effective. Mutant strains lacking *fimH* or *papG* colonized the bladders of spinal cord injury patients, showing that these proteins are not required for colonization of the human neurogenic bladder and suggesting that vaccines targeting adhesion factors may not block bacterial adherence in all patients (83). Similarly, when no differences in bacteriuria or peripheral leukocyte levels were observed between PapDG-vaccinated primates and controls, the authors suggested that other adhesins may be compensating for the loss of P

fimbriae function, thus allowing bladder colonization (154). Indeed, when type 1 and P fimbrial genes are inactivated in strain CFT073, F1C fimbriae are upregulated to compensate (172). Antigenic diversity of adhesins also presents a major problem of vaccination with whole pili, potentially resulting in protection against only a small number of closely related strains (59).

Other vaccine targets.

While much effort has been given to developing a fimbriae-based vaccine for UTI, other targets in UPEC have also been investigated. For example, a combination vaccine including whole P fimbriae and purified alpha-hemolysin (HlyA) protected infected mice from renal colonization and injury (136). The *hlyA* gene is highly conserved among hemolytic *E. coli* strains and exhibits only local variation, suggesting that, as a vaccine, it would have broad cross-reactivity against many infecting strains. Indeed, an anti-HlyA monoclonal antibody was found that bound 94% of clinical isolates (138). Additionally, immunization with purified, denatured IroN, an outer membrane siderophore receptor prevented renal, but not bladder, colonization in infected animals, leading the authors to propose it as a complement to cystitis vaccine targets such as FimH (161).

Reverse vaccinology.

In contrast to these directed approaches to vaccine design, large-scale screening efforts have recently been used to identify and test novel vaccine targets against a number of bacterial pathogens. Pioneering these efforts is a technique termed reverse

vaccinology, which was used to screen the genome of serogroup B *Neisseria meningitidis* and identified a number of novel surface-exposed antigens that are conserved among *N. meningitidis* strains (148). The antigens that induced the strongest antibody response in immunized animals were then used successfully to develop a universal multivalent vaccine against this pathogen (55).

Statement of the Problem

As previous efforts to exploit known virulence factors for immunization against UPEC were of limited success, new antigen targets are needed. Furthermore, these novel vaccine targets may represent putative virulence determinants that contribute to the ability of UPEC to cause UTI.

Hypothesis 1: Outer membrane proteins, specifically heme and siderophore receptors, represent antigenic targets for immunization against UPEC.

Rationale: Because outer membrane proteins (OMPs) likely have surface-exposed domains that would be available to urinary or circulating antibodies, a proteomics screen was utilized to identify UPEC OMPs that elicited a humoral response during murine infection. We reasoned that these antigens were both expressed *in vivo* and capable of eliciting an immune response, important elements of vaccine candidates. Furthermore, previous work from our laboratory indicated that intranasal immunization generated a mucosal response in the urinary tract and protected from infection with the uropathogen *P. mirabilis* (115). Using this previously established model of immunization and challenge, we tested the hypothesis that vaccination with outer membrane iron receptor antigens identified in the proteomics screen, specifically the heme receptor c2482 (Hma), would protect from UPEC colonization.

Hypothesis 2: UPEC acquires heme via specific receptors, a process that contributes to its colonization of the urinary tract.

Rationale: One of the OMPs identified in the above screen and subsequently tested as a vaccine candidate was a putative iron receptor, c2482. These findings indicated that c2482 was expressed *in vivo* and, given the importance of iron uptake during UTI, we hypothesized that it may represent a functional iron receptor. Using genetics and biochemical techniques, we established that c2482 (Hma) serves as a heme receptor, distinct from the well-characterized ChuA. With this information, we were then prepared to investigate not only the roles of ChuA and c2482 (Hma) to UPEC colonization, but also the broader contribution that heme acquisition makes to UTI within the context of siderophore uptake.

CHAPTER 2

UROPATHOGENIC *E. COLI* OUTER MEMBRANE ANTIGENS EXPRESSED DURING URINARY TRACT INFECTION

Introduction

A number of virulence determinants facilitate the ability of UPEC to colonize the urinary tract and exert cytopathic effects, including adhesins (33, 60, 155), toxins (152, 198), extracellular polysaccharides (9), and TonB-dependent iron transport systems (187). Due to the medical and economic impact of UPEC and UTI, several of these virulence-associated factors have been tested as vaccine targets, with limited success (108, 154, 161). Therefore, there is a need to identify additional antigens that may be exploited for the development of a vaccine against UPEC.

While previous efforts to develop a UPEC vaccine were primarily based on specific virulence factors or whole cells, genomics and proteomics methods offer a broader approach to vaccine design. Immunoproteomics methods, for example, have been used to identify antigens in pathogens including *Campylobacter jejuni* (149), *Anaplasma marginale* (121), *Bartonella henselae* (31), and *Klebsiella pneumoniae* (103). An advantage of these genomics and proteomics techniques is the inclusion of novel proteins and non-virulence factors as candidates for immunization – proteins that are normally excluded from conventional vaccine design strategies.

Because an antibody response is likely a significant component of the adaptive immune response to UPEC (184, 194), ideal vaccine targets should be surface-exposed and accessible to circulating immunoglobulins. In Gram-negative bacteria such as *E. coli*, surface-exposed proteins are anchored in the outer membrane. Thus, the outer membrane proteins (OMPs) of UPEC represent a group of prospective vaccine candidates. Furthermore, ideal vaccine candidates should be specific to pathogenic *E. coli*, to avoid cross-reactivity with commensal strains. In this study, we utilized an immunoproteomics approach to identify potential vaccine targets in UPEC. By screening OMPs purified from *E. coli* CFT073 grown under conditions that mimic the urinary tract with antisera from chronically infected mice, we identified 23 antigenic outer membrane proteins that elicited an immune response during infection. Several UPEC-specific OMPs were identified, as well as a novel iron-induced protein. We suggest that these antigenic outer membrane proteins represent newly identified targets for the development of a multivalent vaccine against UPEC.

Materials and methods

Bacterial strains and culture conditions.

Uropathogenic *E. coli* strain CFT073 was isolated from the urine and blood of a patient with acute pyelonephritis (127). For hybridization studies, UPEC strains (n = 55) were randomly sampled from a collection of 67 isolates cultured from blood and urine samples of patients with acute pyelonephritis (127) and a collection of 38 isolates cultured from urine samples of women with cystitis (51, 174). Fecal-commensal *E. coli* strains (n = 30) included the well-characterized MG1655 and HS strains, as well as 28 isolates from fecal samples from healthy women (127). Ten diarrheagenic *E. coli* strains were randomly sampled from a collection provided by J. Nataro (University of Maryland, Baltimore, MD) (65).

All cultures were incubated at 37°C with aeration for ~16 hours unless otherwise noted. For iron limitation conditions, bacteria were cultured in Luria broth (LB) containing 10 mM deferoxamine mesylate (Sigma) or 400 μM 2'2'-dipyridyl (Sigma). Osmotic stress conditions were achieved by culturing bacteria in W salts medium (168) supplemented with 2% NaCl, 0.4% glucose, 0.005% thiamine, 10 mM NH_4Cl, and 15 μM $FeCl_2$. For nitrogen limiting conditions, W salts medium was supplemented with 0.4% glucose, 0.005% thiamine, and 1 mM NH_4Cl. Human urine was pooled from 10 male and female donors and sterilized by vacuum filtration through a 0.22 μm pore filter. Urine cultures were incubated at 37°C without aeration until the $OD_{600} \cong 0.4$. All LB or supplemented LB cultures were inoculated with a single colony, whereas minimal medium and urine cultures were inoculated 1:100 from an overnight LB culture.

Mice and sera.

The CBA/J mouse model of ascending UTI was used as previously described (68, 86). To achieve chronic infections, mice were transurethrally inoculated with 1×10^9 CFU of *E. coli* CFT073 three times over a 12-week period: on days 0, 6 and 65. After 12 weeks, the infection was assessed histologically, with all mice displaying signs of chronic pyelonephritis. Median bacterial loads at this time were 3.9×10^7 CFU/g bladder and 5.1×10^5 CFU/g kidney. Serum was collected at 12 weeks from each animal and equal volumes pooled from 20 individual mice. These anti-CFT073 sera were used as primary antibodies in subsequent experiments. Sera were also collected and pooled from three uninfected mice for use as non-immune controls.

Outer membrane isolation.

Outer membranes were isolated as described by Molloy *et al*. (128) with the following modifications. Briefly, bacterial cells were collected by centrifugation (8,000 × *g*, 10 min, 4°C) and the pellet was resuspended and washed in 10 mM HEPES, pH 7.0. After the addition of 100 U Benzonase Ultrapure™ nuclease (Sigma), bacterial cells were lysed by two passages through a French pressure cell at 20,000 psi. Unbroken cells and cell debris were removed by centrifugation of the lysate (8,000 × *g*, 10 min, 4°C). Supernatants were diluted in 0.1 M sodium carbonate, pH 11 to a final volume of 60 ml and stirred on ice for 1 hour. Carbonate-insoluble membranes were collected by ultracentrifugation (112,000 × *g*, 1 h, 4°C). Membrane pellets were washed with 10 mM HEPES, pH 7.0 and collected by ultracentrifugation (112,000 × *g*, 30 min, 4°C). To remove inner membrane contaminants, pellets were resuspended in 2% sodium lauryl

sarcosine in 10 mM HEPES, incubated 30 min at room temperature, and collected by ultracentrifugation (112,000 × *g*, 30 min, 4°C). The resulting outer membrane pellet was solubilized in 300-800 µl isoelectric focusing (IEF) solution (7 M urea, 2 M thiourea, 40 mM Tris-HCl pH 7.5, 1% ASB-14, 2 mM tributylphosphine, 0.5% BioLyte 3-10 [BioRad], 0.001% bromophenol blue). Soluble outer membrane proteins contained within the fraction were quantified using 2-D Quant (Amersham) according to the manufacturer's instructions.

Two-dimensional gel electrophoresis and western blot analysis.

Duplicate 17 cm, pH 4-7 immobilized pH gradient (IPG) strips (BioRad) were passively rehydrated overnight with 500 µg outer membrane sample in 325 µl IEF solution. Isoelectric focusing was performed at 250 V linear ramp for 20 min, 10,000 V linear ramp for 2.5 hr, and 10,000 V rapid ramp for 40,000 V·hr in a Protean IEF Cell (BioRad). Prior to second dimension separation, IPG strips were equilibrated 20 min with gentle shaking in ~5 ml buffer containing 6 M urea, 2% SDS, 20% glycerol, 5 mM tributylphosphine, and 2.5% acrylamide in 0.15 M bisTris/0.1 M HCl (128). Second dimension SDS-PAGE was completed on 10% polyacrylamide gels as previously described (128). One duplicate gel was stained overnight with colloidal Coomassie G-250, while the other was transferred to a PVDF membrane for western blotting. Membranes were probed with pooled antisera at a dilution of 1:5000. Horseradish peroxidase-conjugated goat anti-mouse secondary antibody was used at 1:100,000 and detected using the ECL Plus Western Blotting Detection System (Amersham).

Mass spectrometry.

Immunoreactive 2D gel spots were excised from a colloidal Coomassie-stained gel and submitted for peptide mass fingerprinting (PMF) or tandem mass spectrometry (MS/MS) at the University of Michigan Protein Structure Facility or Michigan Proteome Consortium (Ann Arbor, MI), respectively. Samples were subjected to in-gel trypsin digestion and mass spectra for PMF acquired on a Micromass TofSpec2E MALDI mass spectrometer. For MS/MS analysis, spectra were acquired on an Applied Biosystems 4700 Proteomics Analyzer (TOF/TOF) from 800-3500 Da and the eight most intense peaks in each spectrum selected for MS/MS. All peptide identifications were made using the MASCOT search engine and spectra were searched against the NCBInr database.

Enzymatic assays.

The activities of cytoplasmic, inner membrane, and outer membrane marker enzymes (glucose-6-phosphate dehydrogenase, NADH oxidase, and esterase, respectively) in the outer membrane preparations were determined. Glucose-6-phosphate dehydrogenase activity was assayed as described previously (200). Stock solutions of 45 mM NADP and 110 mM glucose-6-phosphate were diluted 1:100 into buffer containing 55 mM Tris-HCl, pH 7.5 and 11 mM $MgCl_2$. This substrate solution (950 µl) was added to 50 µl of sample (25-50 µg protein) and the increase in OD_{340} per min was measured at 25°C for 5 min. As described for NADH oxidase activity (185), 900 µl of substrate (50 mM Tris-HCl pH 7.5, 0.2 mM DTT, 0.12 mM NADH) was mixed with 100 µl of sample (50-100 µg) and the decrease in OD_{340} per min was measured at 25°C for 5 min. Esterase activity was determined as described previously (185). In a microtiter plate, 20 µl of

sample (25-50 μg) was mixed with 180 μl of substrate solution containing 10 mM $MgSO_4$, 1 mM *n*-hexanoic acid 4-nitrophenyl ether (*p*-nitrophenyl caproate) (Tokyo Kasei Kogyo Co., Tokyo), and 5% ethanol in 100 mM potassium phosphate buffer, pH 7. Reactions were incubated 10 min at 25°C and the OD_{410} measured. All enzymatic assays were standardized using commercially purified enzymes: glucose-6-phosphate dehydrogenase (Fluka), NADH oxidase (Calbiochem), esterase (Sigma).

Cell culture.

UM-UC-3 human urinary bladder epithelial cells (ATCC CRL-1749) were maintained at 37°C in a humidified 5% CO_2 environment. Cells were cultured in Dulbecco's modified Eagle medium (Gibco) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. For bladder cell infections, monolayers of ~80-90% confluency were washed in Dulbecco's phosphate buffered saline, pH 7.4 (PBS) and supplied with fresh medium lacking antibiotics. Cells were overlaid with *E. coli* CFT073 at an MOI of 100 for 2 h. To harvest cell-associated bacteria, monolayers were washed with PBS. To detach and lyse the uroepithelial cells, 20 ml of sterile water added to each 150 cm^2 flask and incubated at 37°C for 10-15 min. Eukaryotic cells were lysed by vortexing vigorously for 2 min and bacteria were collected by centrifugation (8000 × *g*, 10 min, 4°C). Outer membranes were isolated from the bacterial pellet as described above.

DNA dot blot hybridizations.

Dot blot hybridizations were performed as previously described (65). Overnight cultures of all UPEC, fecal-commensal, and diarrheagenic *E. coli* strains were standardized to an $OD_{600} \cong 4.0\text{-}5.0$. Equal volumes of lysis buffer (0.2 M NaOH, 0.6 M NaCl, 0.8% SDS) and culture were added to 96-well plates. After 10 min of lysis at room temperature, 90 µl of each sample was applied to ZetaProbe® (BioRad) nylon membranes using a BioDot vacuum apparatus (BioRad). Membranes were rinsed in 2X SSC and air dried. Probes were constructed by PCR amplifying 550-650 base pair fragments (near the 3' end of each gene) from CFT073 using the primers listed in Table 2-1. Probes do not share significant homology with any other genes in currently sequenced *E. coli* strains. Probe labeling, membrane hybridization and signal detection were performed using the ECL Direct Nucleic Acid Labelling and Detection System (Amersham) according to the manufacturer's instructions. All blots were performed in duplicate; ambiguities were validated by PCR. A two-tailed Fisher's exact test was used to compare the prevalence of each gene among groups.

Table 2-1. Primer sequences for dot blot probe construction.

Probe	Forward[a]	Reverse
chuA	GGAGCAAGGCTGGAGAAC	CGTCATCACATCCCAGCC
c2482	CAAGCAAATCTCTGACCG	AGTCCCCATGTTTTTGCC
iha	CATCTGGTTACGGTGGGG	CGCACCTTTGTTATCACC
ireA	GTGATGACTCAGCCACGG	CCCTTTCAGCGACTTGCC
iroN	CCTGACGGTGAATGACAG	GCTTCGATACCGTCCACC
iutA	CGGCTGGCAAATCACCTG	GTTGTCACCGGTAAAGCG

[a] All sequences are listed 5'→ 3'

Results

Specificity of antisera from chronically infected mice.

To identify *E. coli* antigens to which a humoral response was generated, antisera were pooled from 20 CBA/J mice chronically infected with the pyelonephritis strain *E. coli* CFT073. To determine the reactivity of these sera, whole cell CFT073 lysates separated on a 12% SDS polyacrylamide gel were probed with antisera from infected mice or non-immune sera from uninfected mice (Fig. 2-1). Antisera from infected mice reacted strongly with CFT073 lysate, while non-immune sera yielded minimal reaction. Thus, these antisera contain antibodies specific to protein and non-protein components of *E. coli* strain CFT073 that were elicited during chronic infection.

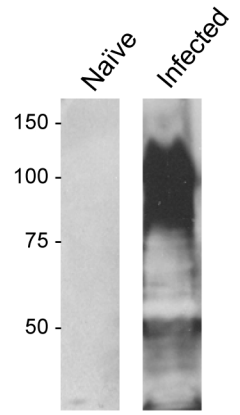

Figure 2-1. Specificity of antisera generated against *E. coli* CFT073. Western blots of CFT073 whole cell lysate electrophoresed on a 12% polyacrylamide gel and probed with non-immune sera from naïve CBA/J mice (left panel) or antisera from chronically infected mice (right panel). Molecular mass standards are shown in kDa.

Outer membrane proteins from UPEC are antigenic.

Outer membrane fractions were isolated from *E. coli* CFT073 cultured in Luria broth. To confirm successful fractionation of outer membranes from other cellular components, cell fractions were assayed for compartment-specific enzymes (Table 2-2). As expected, esterase, a marker enzyme for the outer membrane, showed significantly higher activity in the outer membrane fraction ($P<0.05$) than in other fractions. Esterase activity in the whole membrane fraction is due to the presence of outer membranes in this preparation, and indicates that esterase is enriched approximately 10-fold (2.0 U/mg compared to 17 U/mg) in these outer membrane preparations. NADH oxidase and glucose-6-phosphate dehydrogenase activities, markers for the inner membrane and cytoplasm, respectively, were significantly lower ($P<0.05$) in the outer membrane fraction, as compared to the native fractions of the enzymes. Together, these data indicate that outer membranes were successfully separated from cytoplasmic and inner membrane compartments and suggest that antigens subsequently identified from outer membrane fractions are indeed localized to the bacterial outer membrane.

Table 2-2. Activities of compartment-specific enzymes in *E. coli* CFT073 cell fractions.

	Specific Activity (U / mg) [a]		
Enzyme	Cytoplasm	Whole membrane [b]	Outer membrane
Esterase	0. 4 ± 0. 2	5.5 ± 0.1	16.5 ± 6.6*
NADH oxidase	0. 1 ± 0. 2	23.4 ± 3.9*	2.0 ± 3.5*
Glucose-6-phosphate dehydrogenase	1.7 ± 0. 8	ND [c]	ND [c]

[a] Values are given as mean ± standard deviation.
[b] Contains inner and outer membrane fractions.
[c] No detectable activity.
* $P<0.05$ by Student's t-test (as compared to activity in other fractions).

To identify antigens in the outer membrane of *E. coli* CFT073, proteins in outer membrane fractions were separated by 2D gel electrophoresis, transferred to PVDF membranes, and probed with pooled antisera from infected mice. The majority of outer membrane proteins detectable by colloidal Coomassie staining (Fig. 2-2A) reacted with the antisera by western blot (Fig. 2-2B). However, a minority of stained OMPs were not seroreactive, further demonstrating the specificity of this approach to identify antigens. Of the >60 ORFs predicted to encode outer membrane proteins in an *E. coli* genome (128), we identified 23 seroreactive proteins by mass spectrometry (Table 2-3). Seroreactivity did not always correlate with protein abundance (as determined by colloidal Coomassie stain intensity). For example, the intensely-stained OmpC spot (Fig. 2-2A) was weakly seroreactive (Fig. 2-2B), while OmpX displayed the opposite effect and was the most seroreactive antigen identified.

From CFT073 cultured in rich medium, porins were the most frequently recognized antigens, including OmpA, OmpC, OmpX, NmpC, and LamB. Outer membrane assembly factors, including YaeT and YeaF, as well as nucleoside and vitamin B12 receptors Tsx and BtuB were also identified as reactive antigens. Additionally, the secretion apparatus components TolC and a PulC homolog were also found to be antigenic. Therefore, the humoral response generated during UTI specifically targets these outer membrane proteins during chronic urinary tract infection in mice.

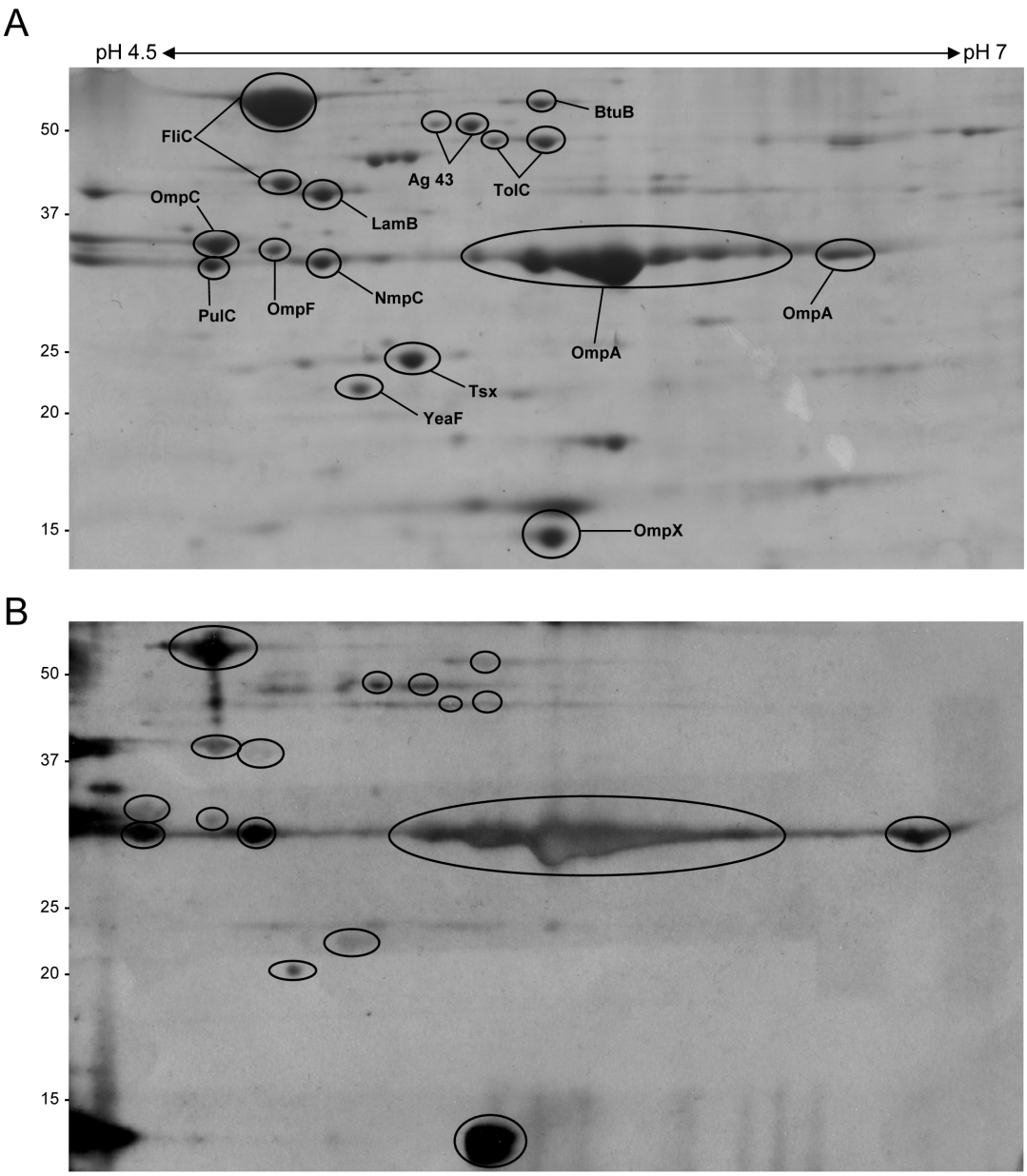

Figure 2-2. Identification of antigenic outer membrane proteins of *E. coli* CFT073. **(A)** Colloidal Coomassie-stained 2D-PAGE gel of outer membrane fractions isolated from CFT073 cultured in rich medium. **(B)** Western blot of 2D-PAGE gel probed with pooled antisera from chronically infected CBA/J mice. Proteins annotated in (A) are seroreactive and were identified by mass spectrometry. Molecular mass standards are shown in kDa.

Table 2-3. Antigenic outer membrane proteins identified by 2D-PAGE and mass spectrometry.

Locus tag	Protein	MW [a]	p*I* [a]	Function	# peptides matched	MASCOT score [b]	*in vivo* gene expression [c]	Culture conditions [f]					
								LB	OS	NL	IL	U	UEC
c0185	FhuA	82.2	5.3	ferrichrome siderophore receptor	8	34-100 [d]	3.60	+	+	+	+	+	+
c0214	YaeT	90.5	4.9	outer membrane assembly factor	8	20-125 [d]	3.46	+	+	+	+	+	+
c0652	OmpT	35.6	5.6	outer membrane protease	17	102	1.71	-	+	+	-	-	-
c0900	OmpX	18.8	6.6	putative adhesin	8	64	5.99	+	+	+	+	+	+
c1071	OmpF	39.3	4.8	porin	7	69	2.29	+	+	+	+	+	+
c1093	OmpA	41.2	6.2	porin	7	35-98 [d]	26.1	+	+	+	+	+	+
c1250	IroN	79.4	5.8	salmochelin siderophore receptor	24	102	2.11	-	-	-	+	+	+
c3655	Ag 43	107	6.0	nonprotease autotransporter adhesin	2	58, 113 [e]	2.16	+	+	+	+	+	+
c1560	NmpC	41.9	5.4	porin	4	68-164 [e]	0.27	+	+	+	+	+	+
c1722	OmpW	25.9	5.9	outer membrane biogenesis factor	4	63-92 [e]	4.78	+	+	+	+	+	+
c2187	YeaF	27.8	5.3	Mlt-interacting protein	26	134	0.53	+	+	+	+	+	+
c2338	FliC	60.8	4.7	flagellin	8	64-187 [d]	4.86	+	+	+	+	+	+
c2482		79.1	5.4	putative iron/colicin receptor	8	40-170 [e]	17.3	-	-	-	+	+	+
c2758	OmpC	41.2	4.6	porin	8	45-106 [d]	3.01	+	+	+	+	+	+
c3610	Iha	76.5	5.6	iron-regulated adhesin	7	41-131 [d]	7.84	-	-	-	+	+	+
c3623	IutA	84.1	5.2	aerobactin siderophore receptor	7	59-108 [e]	0.54	+	+	+	+	+	+
c3781	TolC	55	5.8	multidrug efflux channel	8	52-163 [d]	2.72	+	+	+	+	+	+
c4095	YheE	29.9	9.1	putative secretion component PulC	13	60	0.19	+	+	-	+	+	+
c4308	ChuA	71.1	5.0	heme/hemoglobin receptor	6	102-154 [e]	5.25	-	-	-	+	+	+

c4894	Tsx	33	6.0	nucleoside-binding OMP	2	92, 103 [e]	0.47	+	+	+	+	+	+
c4929	BtuB	70.3	5.5	vitamin B12 receptor	20	102	0.61	+	+	+	+	+	+
c5006	LamB	50	4.9	maltoporin	5	87-110 [e]	1.43	+	+	+	+	+	-
c5174	IreA	75.3	6.2	iron-regulated OMP	6	80-132 [d]	5.44	-	-	-	+	+	+

[a] Theoretical molecular mass (kDa) and isoelectric point were determined using the ExPASy Proteomics Server UniProt Knowledgebase (http://us.expasy.org/).
[b] Ranges represent MS/MS ion scores, single values indicate MS scores determined by peptide mass fingerprinting (where peptide mass fingerprinting scores greater than 60 are considered significant, $P<0.05$).
[c] Relative expression values represent average signal intensities from CFT073-specific DNA microarray used to quantify bacterial transcripts isolated during murine experimental UTI (170).
[d] Ion scores greater than 32 are considered significant ($P<0.05$).
[e] Ion scores greater than 51 are considered significant ($P<0.05$).
[f] (+) presence, (-) absence of the antigen following culture under the condition listed: LB = Luria broth, OS = osmotic stress, NL = nitrogen limitation, IL = iron limitation, U = urine, UEC = uroepithelial cell infection.

Antigens identified under conditions mimicking the urinary tract.

While 17 antigenic outer membrane proteins were identified from *E. coli* CFT073 cultured in LB medium, it is likely that every antigen expressed *in vivo* cannot be detected under these conditions. To identify additional antigens that may not be expressed in rich medium, CFT073 was cultured *in vitro* under conditions that more closely mimic the urinary tract. The recently described *in vivo* transcriptome of *E. coli* CFT073 demonstrated that genes involved in iron acquisition, osmoregulation, and nitrogen utilization are upregulated during infection of CBA/J mice, suggesting that the urinary tract is an iron- and nitrogen-limited environment for this pathogen, as well as hyperosmotic (170). Therefore, outer membrane antigens isolated from CFT073 cultured under iron-limiting, nitrogen-limiting, and high osmolarity conditions were identified (Table 2-3). An additional antigen, OmpT, was identified under nitrogen limitation and osmotic stress. Iron limiting conditions induced the expression of five additional antigens with known or putative roles in iron acquisition (Fig. 2-3). In addition to known UPEC iron compound receptors IroN, ChuA, Iha, and IreA, a novel iron-related OMP was also identified as an antigen. Hypothetical protein c2482, annotated as a putative receptor for iron or colicin, was identified at 75 kDa and an isoelectric point of 5.8. Therefore, the number of antigens identified using this immunoproteomics approach can be increased by culturing bacteria under conditions that more closely mimic the urinary tract environment.

To further simulate the urinary tract and induce the expression of antigens found *in vivo*, human urine was examined as a growth medium. Human urine represents an *ex vivo* system for culturing UPEC, as it contains the complex array of nutrients, salts,

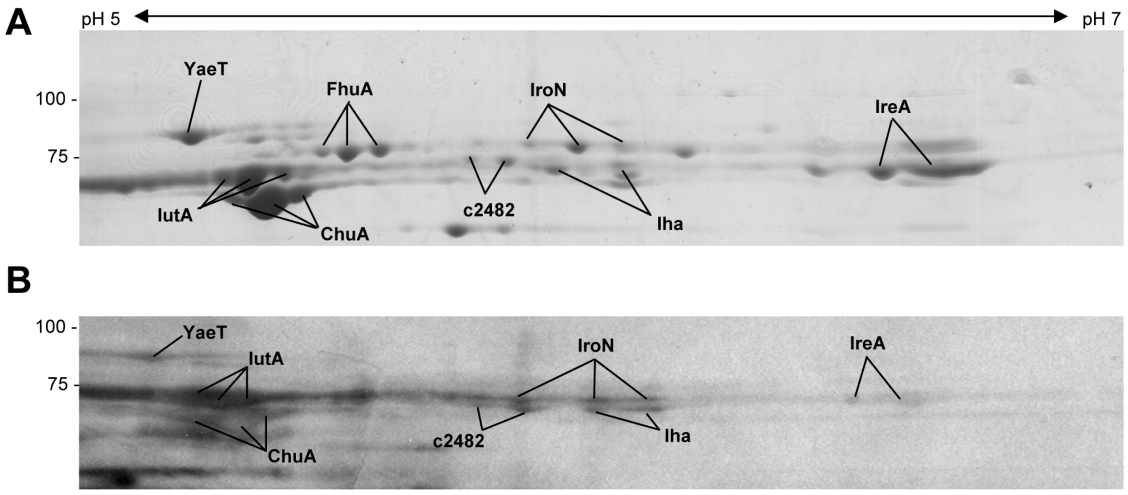

Figure 2-3. Antigenic outer membrane proteins identified from *E. coli* CFT073 cultured under iron limitation. (A) Colloidal Coomassie-stained 2D-PAGE gel of outer membrane fractions isolated from CFT073 cultured under iron-limiting conditions. **(B)** Western blot of 2D-PAGE gel probed with antisera from chronically infected CBA/J mice. Annotated proteins were identified by mass spectrometry. Note that FhuA, shown in (A), is visible on alternate exposures of the blot shown in (B). Molecular mass standards are shown in kDa.

minerals, and soluble cellular factors encountered by *E. coli* during infection. In view of this, CFT073 was cultured statically in pooled human urine and seroreactive outer membrane proteins produced during this growth were identified by mass spectrometry (Table 2-3). These antigens include the previously-identified porins, transporters, and receptors, as well as all of the iron compound receptors detected under iron limitation. The use of this *ex vivo* system is important not only to confirm the data acquired under iron limitation, but also because antigens produced during growth in urine are likely expressed by UPEC in the bladder.

While the studies described above attempt to reproduce the chemical nature of the urinary tract, the contribution of host cells may be equally important. Because adherence to uroepithelial cells is thought to be critical for the colonization of the urinary tract by *E. coli*, we next sought to identify outer membrane antigens that are expressed by CFT073 during interactions with host cells. A confluent monolayer of UM-UC-3 cells, a human bladder epithelial cell line, was inoculated with *E. coli* CFT073 at an MOI of 100. Two hours post-infection, cell-associated bacteria were harvested from the monolayer and outer membrane antigens from these bacteria were identified (Table 2-3). Interestingly, the antigen profile of CFT073 in contact with cultured uroepithelial cells is nearly identical to the antigenic OMP profiles during growth in either human urine or under iron limitation. Specifically, all of the iron-related antigens identified in our screen, FhuA, IutA, IroN, ChuA, Iha, IreA, and c2482, were detected during the exposure of CFT073 to host cells. These antigens were also detected during growth in spent tissue culture medium from bladder epithelial cells, but not fresh medium (data not shown), suggesting that uroepithelial cells sequester iron, making this environment iron-limited

for the bacteria. Although novel host cell association factors were not found in the outer membrane, the presence of numerous iron compound receptors provides further evidence that iron acquisition is critical for UPEC survival *in vivo* and confirms the relevance of these OMPs as antigens targeted by the immune system of the host.

Comparison of outer membrane antigen profiles under diverse culture conditions.

An additional application of this study was an analysis of outer membrane antigen profiles of *E. coli* CFT073 cultured under distinct conditions. Data in Table 2-3 demonstrate that the majority of antigens identified were detected in the CFT073 outer membrane in nearly all of the six culture conditions tested. Qualitative examination of Coomassie-stained 2D-PAGE gels indicated that while the level of each antigen varied tremendously with each environmental condition (data not shown), their presence remained constant among most conditions. Five outer membrane antigens with predicted iron-related functions are the exception to this observation. ChuA, IroN, Iha, IreA, and c2482 were only detected during growth in iron limitation, human urine, and during contact with bladder cells. Thus, with the exception of several iron-regulated OMPs, the range of urinary tract-mimicking conditions tested did not affect the identified antigens' presence or absence in the outer membrane. These data refine our previous findings, showing that using multiple culture conditions can only modestly increase the number of outer membrane antigens identified using this immunoproteomic approach.

Prevalence of genes encoding outer membrane antigens among *E. coli* strains.

We hypothesized that several of the outer membrane antigens identified in this study might be more prevalent among pathogenic strains of *E. coli* as compared to non-pathogenic commensal strains. Specifically, genes encoding the iron-related antigens ChuA, c2482, Iha, IreA, IroN, and IutA appeared to be absent from the prototypical commensal strain MG1655 *(E. coli* K12) by sequence analysis. To determine the prevalence of each of these genes among a larger collection of strains, dot blot hybridizations were performed. Genomic DNA in cell lysate from 95 *E. coli* strains, including uropathogenic (n=55), fecal-commensal (n=30), and diarrheagenic (n=10) isolates, was probed for each of the six genes encoding these antigens. *chuA*, c2482, *iroN*, and *iutA* were significantly more prevalent (P<0.01) among UPEC strains as compared to fecal-commensal isolates (Table 2-4). Furthermore, these four genes were detected in 65-87% of the UPEC isolates tested. Interestingly, *chuA* and *iutA* were also found significantly more frequently (P<0.01) in diarrheagenic isolates, suggesting that these genes might be more broadly conserved among pathogenic *E. coli*. These data clearly demonstrate the conservation of *chuA*, c2482, *iroN*, and *iutA* among UPEC strains, as well as demonstrate their absence from the majority of commensal *E. coli* strains, further supporting their potential for application as vaccine candidates.

Table 2-4. Prevalence of genes encoding iron-related outer membrane antigens among pathogenic and non-pathogenic *E. coli* isolates as determined by hybridization and PCR.

	Number (%) of isolates positive for gene		
Gene	Uropathogenic (*n* = 55)	Fecal-commensal (*n* = 30)	Diarrheagenic (*n* = 10)
chuA	48 (87) **	9 (30)	9 (90)*
c2482	38 (69)**	5 (17)	5 (50)
iha	25 (45)	11 (37)	4 (40)
ireA	11 (20)	5 (17)	2 (20)
iroN	39 (71)*	10 (33)	7 (70)
iutA	36 (65)**	5 (17)	8 (80)*

* $P<0.01$ by Fisher's exact test (as compared to fecal-commensal prevalence).
** $P<0.0001$ by Fisher's exact test (as compared to fecal-commensal prevalence).

Discussion

This study applies an immunoproteomics approach to identify outer membrane protein antigens produced during uropathogenic *E. coli* infection and represents the first broad screen for vaccine targets for this pathogen. Using immunoreactive antisera from chronically infected mice, we identified 23 outer membrane antigens from UPEC that are expressed *in vivo* and are capable of eliciting a humoral response. One protein identified in this screen, hypothetical protein c2482, is a novel antigen expressed under iron limitation. Furthermore, we demonstrated that the genes encoding at least four of these OMPs, *chuA*, c2482, *iroN*, and *iutA*, are significantly more prevalent among UPEC strains as compared to fecal-commensal *E. coli*. We suggest these conserved antigenic OMPs may be useful targets for a vaccine against UPEC.

The evaluation of UPEC virulence factors as protective immunogens for the prevention of UTI has been extensively investigated (58, 108, 136, 154, 161). However, the heterogeneous nature of UPEC isolates (19) suggests that additional vaccine targets will be required to ensure protection against a broad array of strains. Thus, we were interested to identify conserved outer membrane antigens of UPEC that may be exploited for use in a vaccine against UTI.

In addition to nutrient transporters, porins, and adhesins, our screen identified a novel UPEC outer membrane antigen. A putative iron receptor, c2482 was detected during iron limitation, growth in human urine, and contact with bladder epithelial cells. These findings confirm the predicted outer membrane localization of this protein, as well as provide indirect evidence of its involvement in iron acquisition. Recently, our laboratory also determined that expression of c2482 is upregulated during murine UTI

(170) and it is one of the most highly induced proteins during the growth of CFT073 in human urine (2), suggesting this antigen is a potential vaccine target. The discovery of novel antigens is a notable advantage of immunoproteomics approaches to vaccine design and the detection of this OMP is a major finding of this report.

This study also identifies proteins expressed by *E. coli* CFT073 during experimental UTI. While the recently determined *in vivo* transcriptome of murine UTI has described transcript-level gene expression during infection (170), the current findings support and extend this previous research by examining protein expression *in vivo*. Consequently, the 23 seroreactive OMPs identified are expressed in the UPEC outer membrane during infection. This agrees well with previous work, as 17 of these 23 seroreactive OMPs were among the top 30% of CFT073 transcripts detected *in vivo* (170). Indeed, 11 of these 17 OMPs were upregulated at least 2-fold during experimental UTI (170). In addition to potential applications for vaccine development, these findings also contribute toward understanding the pathogenesis of UPEC.

A notable advantage of the current study over other immunoproteomics analyses is the inclusion of multiple culture conditions designed to mimic the *in vivo* environment of the pathogen. While these various conditions only modestly increased the number of antigens identified, the approach nevertheless bolstered our confidence that few major outer membrane antigens were omitted from the screen. This approach also revealed several iron-related antigens, ChuA, Iha, IroN, c2482, and IreA, which were only detected during growth in three culture conditions: iron limitation, human urine, and exposure to bladder epithelial cells. As these environments likely induce iron deprivation, it is not surprising that they also induce the expression of additional proteins

involved in iron acquisition. A recent study from our laboratory confirmed these results, showing the induction of these five OMPs during culture in human urine, as well as their repression during growth in iron-replete medium (2). It is interesting to note that the genes encoding three of the iron-related antigens, *chuA*, *iroN*, and c2482 were also found to be both conserved and pathogen-specific by dot blot hybridization. Given the well-established role of iron acquisition in pathogenesis, we may speculate that UPEC, as compared to commensal *E. coli*, expresses a greater range of iron receptors in response to iron limiting environments.

Outer membrane-anchored surface structures, such as fimbriae, were largely absent from our western blots. We would predict, however, that the infected-mouse antisera used in the screen contain fimbriae-specific antibodies. Indeed, anti-P fimbriae IgG was detected in the serum of primates infected with uropathogenic *E. coli* (154). However, because fimbrial proteins often require additional steps to solubilize (70) and are easily sheared from the surface during preparation, they were likely absent from our 2D gels. Furthermore, a comprehensive 2D-PAGE analysis of the *E. coli* outer membrane proteome also lacked detection of fimbriae (128), indicating that this is an expected result.

While we are confident that we have identified the major outer membrane antigens expressed by *E. coli* CFT073 during UTI, there are several inherent limitations associated with our approach. Low abundance OMPs, which may nevertheless be seroreactive, could have been below the limits of detection of our 2D-PAGE colloidal coomassie staining and mass spectrometry analyses or lost during fractionation. Additionally, our screens were performed using anti-mouse IgG; thus we primarily

detected antigens that elicited an IgG response during infection. As UPEC is a mucosal pathogen, secretory IgA is thought to play a role in the clearance of UTI (81). Due to isotype switching, however, IgA-recognized antigens are likely also recognized by IgG. Therefore, while the immunoproteomics approach utilized in this study has limitations, we do not expect that these limits have adversely affected our findings.

Several outer membrane proteins identified as potential protective antigens have roles in the virulence of UPEC and in other pathogens. *E. coli* strains lacking the heme/hemoglobin receptor ChuA (187), aerobactin receptor IutA (187), salmochelin receptor IroN (160), iron-responsive element IreA (159), or iron-regulated adhesin Iha (87) were significantly outcompeted by the wildtype strain in a mouse model of UTI, demonstrating the importance of iron acquisition to the fitness of this pathogen *in vivo*. Additionally, isogenic mutants lacking the major flagella subunit FliC were similarly outcompeted by wildtype *E. coli* CFT073 (105). Therefore, our data provide evidence that a humoral response is generated against these virulence-associated factors during murine infection. Furthermore, while a role in pathogenesis is likely not a requirement for a vaccine target, it may be beneficial, as neutralizing antibodies may block critical functions of such targets upon infection.

Further validating our findings, several of the outer membrane antigens identified here have been previously implicated as immunogens. For example, OmpA, a strongly antigenic protein in our screen, was found to be one of the most antigenic outer membrane proteins of *Klebsiella pneumoniae* by a similar immunoproteomics approach (103). In addition, the most strongly seroreactive antigen identified, OmpX was previously demonstrated to have immunogenic properties, functioning as an

immunological carrier to elicit a humoral response against a co-administered hapten molecule (122). Moreover, Russo *et al*. demonstrated that immunization with purified IroN affords significant protection in the kidneys of mice infected with UPEC (161). Similarly, a recent study of vaccine candidates against extraintestinal *E. coli* showed that vaccination with IroN protected mice from lethal septicemic challenge with a neonatal meningitis strain (40). While immunization ChuA was not protective in this study, the authors note that ChuA does not contribute to the virulence of *E. coli* in their sepsis model, while it does contribute to the virulence of UPEC (40, 187). Thus, this evidence suggests that our approach has successfully identified antigens that may provide protective immunity against UPEC.

The outer membrane antigens identified in the present study have additional characteristics that support their putative roles as vaccine candidates. Many of these outer membrane antigens, including the iron compound receptors, are predicted to form β-barrel structures in the outer membrane. Even while the majority of each β-barrel protein will be embedded in the membrane, extracellular loops provide surface exposed regions (146). Because neutrophils are a significant component of the immune response to UTI (74), opsonizing antibodies against such surface exposed proteins may be important to increase phagocytosis at the site of infection. In addition, many of the OMPs identified in this screen are involved in cellular processes critical for bacterial growth *in vivo*, such as iron acquisition. Blocking the function of these proteins via neutralizing antibodies may also facilitate bacterial clearance from the urinary tract. Finally, at least four of the antigens, ChuA, c2482, IutA, and IroN, are conserved among UPEC isolates and absent from most fecal-commensal *E. coli* strains. Among sequenced

strains of pathogenic *E. coli*, little genetic diversity is observed for each of these antigens; at the amino acid level, each antigen is 90-100% identical between strains. This suggests that, if exploited as vaccine targets, these proteins may generate protection against a broad array of pathogenic strains with minimal cross-reactivity with the normal flora.

A vaccine that prevents uncomplicated UTI would have tremendous public health benefits. The data presented in this study represent a first step toward the development of such a broadly-protective vaccine against uropathogenic *E. coli*. Most outer membrane antigens identified here have not yet been examined as vaccine targets for UPEC. Additionally, a novel antigen identified in this screen, c2482, represents not only a unique vaccine target, but also a newly identified OMP that could function as an iron compound receptor. Clearly, much work is needed to extend these findings and investigate immunization with these antigens before an efficacious UTI vaccine can be developed.

CHAPTER 3

IMMUNIZATION AGAINST HMA AND OTHER OUTER MEMBRANE IRON RECEPTORS PROTECTS FROM URINARY TRACT INFECTION

Introduction

Over half (53%) of all women and 14% of men experience at least one urinary tract infection (UTI) in their lifetime (61, 62), leading to an average annual cost of over $2.4 billion in the United States alone (117). In addition to symptoms of acute cystitis and pyelonephritis caused by UTI, a number of more serious conditions are often associated with these infections. In young children, upper UTIs cause permanent kidney damage in an estimated 57% of cases (116). UTIs are classically treated with trimethoprim/sulfamethoxazole or ciprofloxacin to eradicate the infecting strain. However, there is documentation of increasing resistance to these antibiotics (64) and even following successful primary treatment, recurrent infections can occur (50). Consequently, these complications pose a significant challenge to UTI treatment and suggest that a vaccine to prevent UTI would alleviate this major source of morbidity and economic burden.

Indeed, a number of groups have sought to stimulate protective immunity against UPEC. Recently, whole cell or cell extract preparations have been shown to provide modest short-term protection in some individuals (12, 192). Surface-exposed molecules

such fimbriae (108, 154), alpha hemolysin (136), Dr fimbriae (59), the salmochelin receptor IroN (161), and capsule polysaccharide K13 (101) have also been demonstrated to induce at least some immune response in immunized animals. However, although much research has focused on the development of a vaccine against UPEC, none are available in the United States.

Due to the limited success of previous UTI vaccine design strategies, we hypothesized that a rational reverse vaccinology approach would, in an unbiased manner, identify vaccine targets of UPEC that could elicit protective immunity. In Chapter 2, an immunoproteomics approach was described that identified six outer membrane iron receptor proteins as putative vaccine candidates. One antigen, putative protein c2482, will be described in Chapter 4 as a heme receptor and will therefore be referred to as heme acquisition protein, Hma.

Each of these 71-84 kDa proteins is predicted to form a transmembrane beta-barrel in the outer membrane, with a series of loops extending extracellularly (20). Facilitating import of specific iron sources, these receptors mediate uptake of siderophores, secreted bacterial iron-chelating molecules, or host heme-derived iron. Because iron acquisition is necessary for bacterial pathogenesis and it is well known that the urinary tract is an iron-limited environment, iron acquisition via these receptors is crucial for UPEC infection (187). Consequently, deletion of any of these receptors decreases the fitness of UPEC in the murine urinary tract (87, 159, 160, 187).

In this study, we demonstrate that intranasal immunization with Hma and other UPEC outer membrane iron receptors provides protection from UTI. Although further attempts to enhance this protection were not successful, these studies provide key insights

into the use of these proteins as vaccine targets. Therefore, we propose that this class of molecules is promising as protective vaccine targets against UPEC.

Materials and methods

Antigen purification.

Genes encoding the selected antigens were PCR-amplified from CFT073 genomic DNA and cloned into either pBAD-*myc*-HisA (Invitrogen) or pET30b+ (Novagen). Recombinant protein expression from pBAD (Hma, IutA, ChuA) was induced in *E. coli* TOP10 (cultured to OD_{600} = 0.8 in LB) by addition of L-arabinose to 100 μM for 4 h. Protein expression from pET (IreA, Iha) was induced in *E. coli* BL21(DE3) pLysS cultured in Terrific broth (12 g/L tryptone, 24 g/L yeast extract, 2.3 g/L KH_2PO_4, 12.5 g/L K_2HPO_4, 4% glycerol) to OD_{600} = 1.0 at 37°C. Cultures were shifted to 25ºC to allow for maximum induction and induced overnight with 1 mM isopropyl β-D-1-thiogalactopyranoside.

Induced cultures were collected by centrifugation (8,000×*g*, 4°C, 10 min), resuspended in 10 mM HEPES, pH 7 containing 100 U Benzonase nuclease (Sigma). Bacteria were lysed by two passages though a French pressure cell (20,000 psi) and the lysate was cleared by centrifugation (8,000×*g*, 4ºC, 10 min). Bacterial membranes were pelleted from the cleared lysate by ultracentrifugation (112,000×*g*, 4ºC, 30 min) and the membrane pellet solubilized in 1 ml 10 mM HEPES, pH 7 containing 0.1 % Zwittergent (Calbiochem). A freeze-thaw cycle at -20ºC was used to increase solubility of resuspended membranes. To thawed membrane fractions, 9 ml Buffer B (100 mM NaH_2PO_4, 10 mM tris-HCl, 8 M urea, pH 8.0) was added and His_6-tagged proteins purified on nickel-nitriloacetic acid-agarose columns (Qiagen) under denaturing conditions according to the manufacturer's instructions (The QIAexpressionist) (Appendix A). Eluted purified proteins were renatured by dialysis at 4°C into a final

solution containing 0.05% Zwittergent in PBS, pH 7.5 and quantified using the BCA protein assay (Pierce). Native secondary structure (β-sheet) of purified renatured proteins was confirmed by circular dichroism (data not shown).

CtB-Hma chimera.

Hma tertiary structure was predicted using the PRED-TMBB program Viterbi method (http://bioinformatics.biol.uoa.gr/PRED-TMBB/) and modeling using the FepA crystal structure with Cn3D version 4.1 (NCBI). The region selected was predicted to reside extracellularly by both modeling analyses. Sequence corresponding to this putative extracellular loop (LSSKLTRGDGVSYTAGIISDTSLARESASDHE) was PCR-amplified from pBAD-Hma-His and cloned into an expression vector downstream of the cholera toxin B subunit (CtB). CtB-Hma fusions were affinity purified on galactose resin and dialyzed into PBS. Pentamer formation was confirmed by SDS-PAGE separation of denatured (boiled in the presence of β-mercaptoethanol) and nondenatured samples.

Vaccination.

Purified antigens were chemically cross-linked to cholera toxin (CT) (Sigma) at a ratio of 10:1 using *N*-succinimidyl 3-(2-pyridyldithio)-propionate (SPDP) (Pierce) according to the manufacturer's recommendations. CtB-Hma chimeric antigen was mixed 10:1 with CT holotoxin. All immunizations were administered intranasally in a total volume of 20 μl/animal (10 μl/nare). For single antigen and low dose divalent vaccinations, animals received a primary dose on day 0 of 100 μg crosslinked antigen (containing 10 μg CT) or 10 μg CT alone (CtB chimera controls received 100 μg CtB

plus 10 μg CT). Two boosts of 25 μg antigen (crosslinked to 2.5 μg CT) or 2.5 μg CT alone were given either on days 7 and 14 (Fig. 3-1A) or days 14 and 28 (Fig. 3-1B). For high dose divalent vaccinations (Fig. 3-1C), mice received 100 μg Hma primary dose on day 0, followed immediately by 100 μg IreA on day 1. Boosts of 25 μg Hma were administered on days 7 and 14 and 25 μg IreA on days 8 and 15. Control mice for high dose divalent vaccinations received the 10 μg or 2.5 μg CT on both days. One (Fig. 3-1A and C) or two weeks (Fig. 3-1B) following the final boost, mice were challenged as described below.

Murine model of ascending UTI.

Female CBA/J mice were transurethally inoculated as previously described (68). Prior to inoculation, overnight *E. coli* CFT073 cultures were harvested by centrifugation (3000×*g*, 30 min, 25°C) and resuspended in PBS to ~10^9 CFU/ml. Bacterial suspension (50 μl/mouse) was delivered transurethally using a sterile 0.28 mm inner diameter polyethylene catheter connected to an infusion pump (Harvard Apparatus), with total inoculum of 1×10^8 CFU/mouse. For determination of CFUs, organs were harvested from euthanized animals at 48 h post-inoculation and homogenized in 3 ml PBS with a GLH homogenizer (Omni International). Bacteria in tissue homogenates were quantified by plating on Luria-Bertani agar containing 0.5 g/L NaCl using an Autoplate 4000 spiral plater (Spiral Biotech). Colonies were enumerated using a QCount automated plate counter (Spiral Biotech). Significance for vaccination experiments was determined using the one-tailed Mann-Whitney test. Six- to eight-week old mice were used for these studies and animals were ≤15 weeks old at the conclusion of all experiments. All

procedures were conducted according to protocols approved by University Committee on the Care and Use of Animals at the University of Michigan.

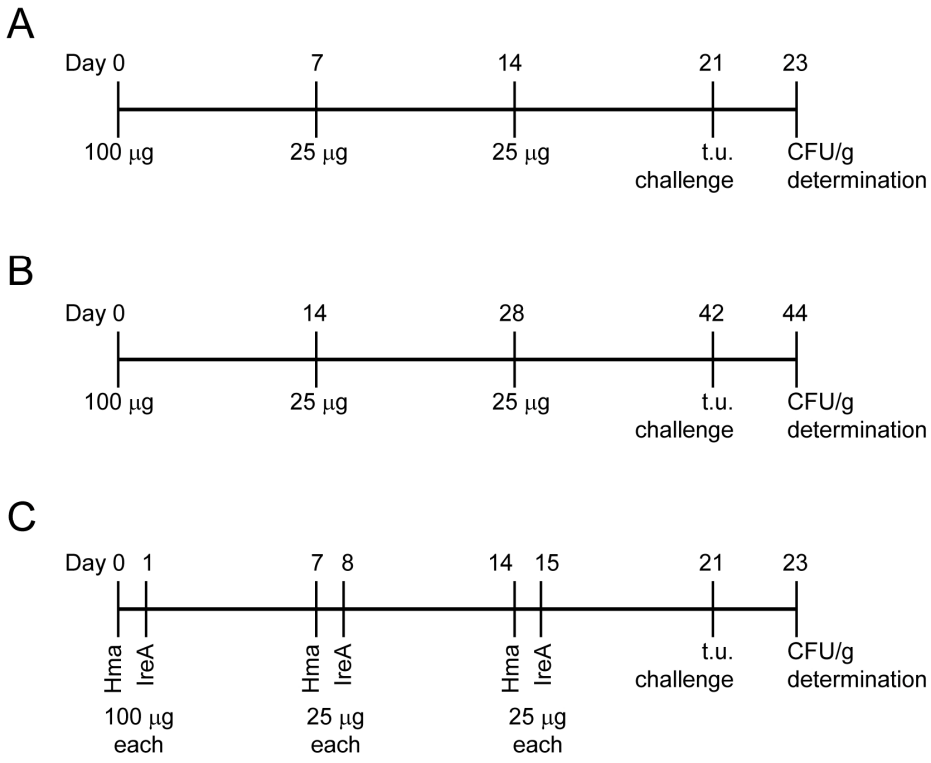

Figure 3-1. Intranasal immunization schedules used in vaccination studies. (A) One-week interval schedule. Mice received a primary dose intranasally of 100 μg, followed by two 25 μg doses at one-week intervals. One week after the final boost, animals were transurethrally (t.u.) challenged with 10^8 CFU and protection assessed 48 hpi. **(B)** Two-week extended schedule. Doses are as in (A), except all vaccinations were administered at two-week intervals and animals challenged two weeks following the final boost. **(C)** High dose combination schedule. Vaccination scheme is as in (A), except mice received back-to-back doses of Hma and IreA on successive days for both primary (100 μg) and boost (25 μg) administrations.

Results

Iron receptor antigens meet vaccine candidate criteria.

Six outer membrane iron receptor proteins were identified in Chapter 2 by their antigenicity during murine UTI (Table 3-1). Furthermore, the genes encoding several of these OMPs (*chuA*, *hma*, *iroN*, and *iutA*) were found significantly more frequently among pathogenic *E. coli* than non-pathogens, an important consideration as a vaccine must specifically target pathogenic strains.

Additional studies support the putative role for these proteins as vaccine candidates. A comparative genomics hybridization study from our laboratory identified 131 genes that were present in all UPEC strains analyzed ($n = 10$), but none of the fecal-commensal *E. coli* isolates tested ($n = 4$) (119). Among these highly UPEC-specific genes were two encoding the outer membrane heme receptors, *chuA* and *hma*, providing further evidence of their pathogen-specificity.

Furthermore, these iron receptor antigens appear to be highly upregulated *in vivo*, by a variety of measurements. An *in vivo* transcriptome study analyzing global gene expression of pyelonephritis strain CFT073 during murine UTI found that among the genes most highly upregulated *in vivo* were those encoding iron acquisition systems (170). When all genes were ranked in order of expression level *in vivo*, all six of these iron acquisition genes were among the top 18% most highly expressed in the murine urinary tract and the two heme receptors, *chuA* and *hma* were in the top 5.5% (Table 3-1). Similarly, when outer membrane proteins (OMPs), isolated from CFT073 cultured in human urine *ex vivo*, were compared with OMPs isolated from bacteria cultured in Luria broth using fluorescence difference in-gel electrophoresis, the iron receptors were the

most highly induced proteins (2). Indeed, the vaccine candidates listed in Table 3-1 comprised six of the top seven human urine-induced OMPs. These findings indicate that ChuA, Hma, IutA, IroN, Iha, and IreA are promising candidates for a vaccine against UPEC and strongly suggest that broadly targeting an entire class of molecules involved in iron acquisition could be an effective strategy to develop a protective UTI vaccine.

Table 3-1. Vaccine candidate profiles of iron receptor antigens.

Gene	Locus tag	Fold change *in vivo*[a]	*in vivo* percentile[b]	Fold change in human urine[c]	Antigenic[d]	% UPEC (*n*=55)[e]	% fecal (*n*=30)[e]
chuA	c4308	7.06	95.0	27.8	+	87[f]	30
hma	c2482	6.56	94.5	14.8	+	69[f]	17
iha	c3610	18.9	89.1	5.87	+	45	37
ireA	c5174	23.3	95.2	7.81	+	20	17
iroN	c1250	22.7	82.7	7.63	+	71	33
iutA	c3623	5.57	97.0	49.2	+	65	17

[a] Transcript fold change in the urine of experimentally infected mice as compared to growth in LB (170).
[b] Of 5379 genes ranked in order of *in vivo* transcript microarray signal intensity (170).
[c] Protein fold change following growth in pooled filter-sterilized human urine as compared to growth in LB (2).
[d] Reacts with sera from mice chonically infected with *E. coli* CFT073 (see Chapter 2).
[e] Results from Southern dot-blot hybridizations (see Chapter 2).
[f] Indicates genes present in 10/10 UPEC and 0/4 fecal strains analyzed by comparative genomic hybridization (119).

Vaccination confers protection against experimental UTI.

The five of the six iron receptor vaccine candidates (ChuA, Hma, IutA, IreA and Iha) were expressed and purified as affinity-tagged recombinant proteins (Appendix A). These purified protein antigens were biochemically cross-linked to cholera toxin (CT), a strong mucosal adjuvant, at a ratio of 10:1 (antigen:CT) and groups of mice (n=15-20) were intranasally inoculated with either antigen-CT complex or CT alone. Following primary immunization (day 0, 100 μg) and booster doses (days 7 and 14, 25 μg), the animals were transurethally challenged with UPEC strain CFT073 and protection assessed at 48 h post-inoculation (hpi).

Of the five tested candidates, three conferred protection against experimental challenge with UPEC. Hma-vaccinated mice had significantly less kidney colonization than CT-vaccinated controls (P=0.008), demonstrating nearly a three log reduction in median CFU/g in the kidney (Fig. 3-2B). Similarly, the putative siderophore receptor IreA showed significant protection and demonstrated a three log reduction in median CFU/g in the bladder (P=0.035) (Fig. 3-2C). For Hma and IreA, 13/20 and 10/15 vaccinated mice had undetectable levels (<100 CFU/g) of bacteria in the kidneys and bladder, respectively. While mice immunized with the aerobactin receptor IutA displayed a more modest one log CFU/g reduction, significant protection against UPEC challenge was generated in both the bladder (P=0.009) and kidneys (P=0.007) (Fig. 3-2D). Furthermore, antigen-specific serum IgG and urine IgA titers, as measured by reactivity with purified recombinant proteins and native proteins in membrane fractions, were significantly increased in vaccinated mice, as compared to CT controls

(K.E. Sivick and H.L.T. Mobley, unpublished data). Thus, mucosal immunization in the nares generates a protective effect against *E. coli* at distant sites, the bladder and kidneys.

When a lower primary dose of 30 μg was used, however, reduced protection was observed. While low-dose IreA immunization reduced bladder infection by approximately two logs, only 3/10 mice (30%) had undetectable bacterial levels (Fig. 3-2E), as compared to 10/15 mice (67%) vaccinated with a higher dose (Fig. 3-2D). Furthermore, kidney protection observed with high dose (100 μg, 25 μg boosts) Hma immunization (Fig. 3-2B) was completely lost when this lower dose was used. This indicates that antigen quantities greater than 30 μg in the primary dose and 10 μg in the boosts are needed for complete kidney or bladder protection by Hma or IreA vaccination.

Not all of the antigens selected as candidates provided protection against experimental UTI. Immunization with heme receptor ChuA or enterobactin receptor Iha recombinant proteins failed to elicit significant protection against challenge with UPEC strain CFT073 (Fig. 3-2A and data not shown). As compared to the protective antigens, vaccination with ChuA or Iha generally generated reduced serum IgG and urine IgA levels (K.E. Sivick and H.L.T. Mobley, unpublished data). All of the native protein vaccines were well-tolerated in immunized mice, with the exception of ChuA, which was lethal in 11/30 mice. Together, these findings show that targeting an entire functional class of molecules involved in iron acquisition may be an effective strategy to identify protective vaccine candidates that significantly reduce bacterial colonization during ascending UTI following experimental challenge.

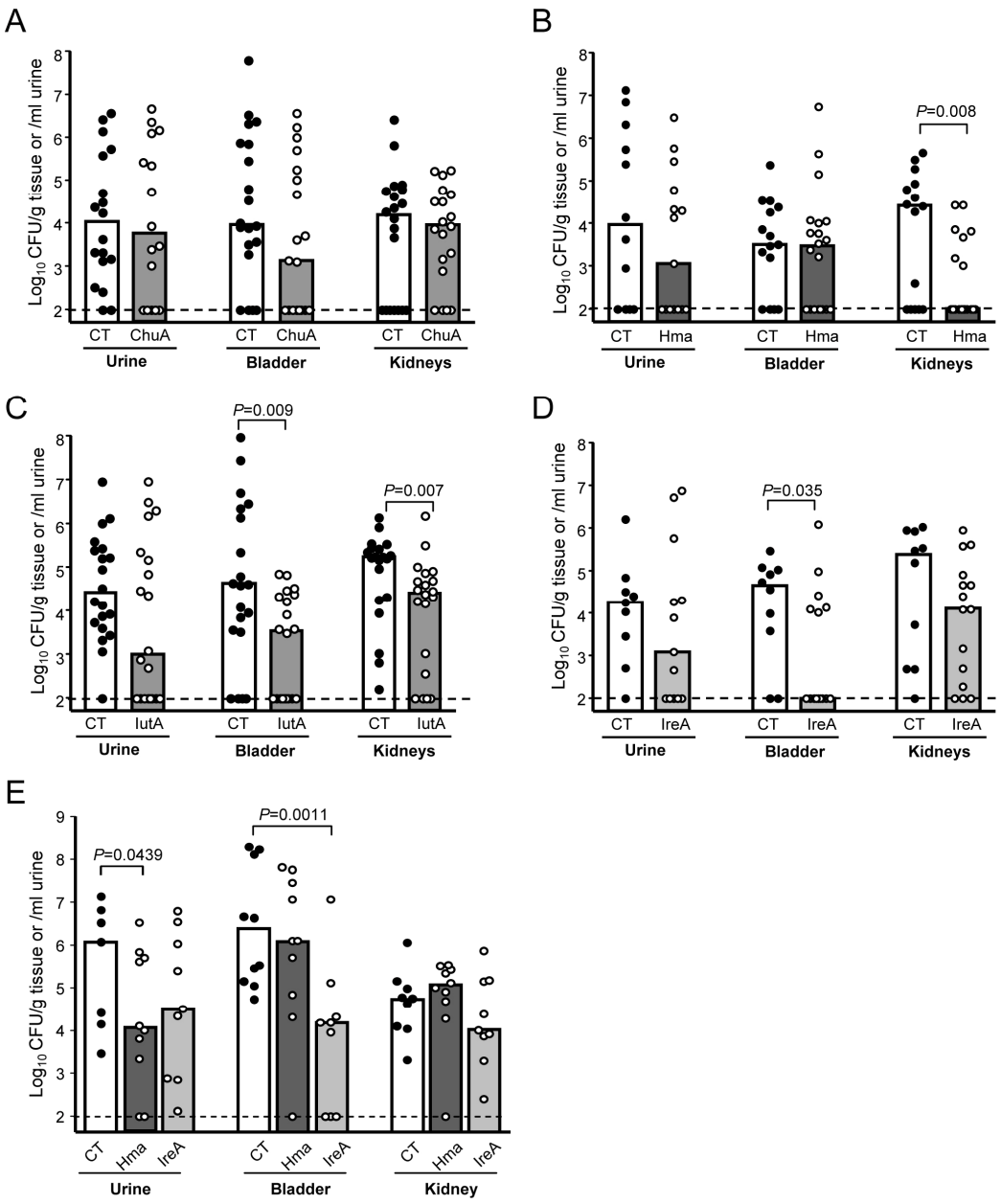

Figure 3-2. Urinary tract colonization following immunization with outer membrane iron receptor antigens and *E. coli* challenge. CBA/J mice (*n*=15-20) were intranasally vaccinated as described with a primary dose of 100 μg purifed protein crosslinked to 10 μg CT, followed by two boosts of 25 μg antigen crosslinked to 2.5 μg CT. One week following the final boost, animals were transurethally challenged with 1×10^8 CFU of *E. coli* CFT073 and colonization was measured 48 hpi. Symbols represent CFU/g tissue or /ml urine of individual mice and bars indicate median values. White bars, mock vaccinated with CT alone; gray bars, vaccinated with purified **(A)** ChuA, **(B)** Hma, **(C)** IutA, or **(D)** IreA. **(E)** Experiment as in (A-D) except mice (*n*=10) received a 30 μg primary dose, followed by two 10 μg boosts of either Hma or IreA. Dashed line shows the limit of detection for this assay, 100 CFU/g.

Increased time between Hma doses does not enhance protection.

It is generally accepted that the humoral response to an antigen takes approximately 14 days to peak. In light of this, we hypothesized that increasing the time between immunization doses may allow for greater development of immunity and increased protection from infection. To test this, mice (n=10) were vaccinated with Hma according to an extended schedule (Fig. 3-1B), with doses administered every two weeks and challenge occurring two weeks after the final boost. Unlike the previous one week-interval experiment, the urine of Hma-immunized mice contained significantly fewer CFU/ml (P=0.0406) (Fig. 3-3). However, the overall trends and median CFU/g levels remained the same. While not statistically significant, the median CFU/g kidney tissue was more than two logs lower in Hma-vaccinated animals as compared to CT-immunized controls. However, as previously, no protection was observed in the bladder. This suggests that, for Hma, increasing the time between vaccine doses does not lead to enhanced protection.

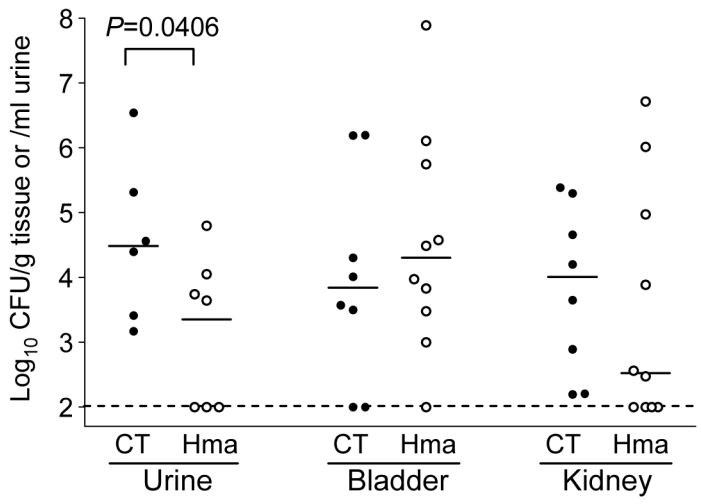

Figure 3-3. Urinary tract colonization following extended schedule immunization with Hma and *E. coli* challenge. Mice (*n*=10, two CT-immunized mice died) were immunized with 100 μg Hma and received two 25 μg boosts at two-week intervals. Two weeks following the final boost, animals were transurethrally inoculated with 10^8 CFU CFT073 and protection assessed 48 hpi by measuring CFU/g tissue. Bars represent the median and dashed line indicates limit of detection (100 CFU/g).

Immunization with a divalent vaccine does not protect from UTI.

When administered individually, Hma, IutA, and IreA provided partial protection from experimental UTI. However, no single antigen conferred complete protection in both the bladder and kidneys. We hypothesized that co-administration of Hma, a kidney-protective antigen, and the bladder-protective IreA or IutA may generate protection against UPEC in both urinary tract organs. To explore this, mice were vaccinated as described according to the one week interval schedule (Fig. 3-1A), receiving a primary dose of 50 μg each of Hma and IreA or Hma and IutA (100 μg total) and boosts of 12.5 μg each antigen (25 μg total). Not only were mice not protected in both the bladder and kidneys, but all protective effects of immunization with these antigens were lost by this vaccination scheme (Fig. 3-4A). While mice singly immunized with Hma displayed kidney protection (Fig. 3-2B), animals receiving Hma in combination with either IutA or IreA showed no significant difference in kidney colonization as compared to unimmunized controls.

Because the single-antigen vaccine preparations consisted of 100 μg of a particular antigen, while the combinations only contained 50 μg of that same antigen, we hypothesized that this apparent discrepancy may be explained by dosing. Consistent with this hypothesis are previous single-antigen experiments showing that an Hma primary dose of 30 μg is not protective and IreA is less protective (Fig. 3-4E). To test this, mice were immunized with 100 μg each of Hma and IreA on successive days followed by two successive boosts of 25 μg each antigen, according to the one week interval schedule described in Fig. 3-1C. Again, vaccinated animals were not significantly protected from CFT073 challenge in any organ, although there was a trend towards protection in the

urine and bladder (Fig. 3-4B). Because protection previously conferred by immunization with Hma or IreA alone was lost, this suggests that the co-administration scheme exerted negative effects by a yet-unknown mechanism on the generation of protective immunity in the urinary tract. These data demonstrate that murine co-vaccination with whole purified Hma and either IreA or IutA does not protect from UTI and suggest that, using these iron receptor antigens, a new approach will be needed to generate protective immunity in both the bladder and kidneys.

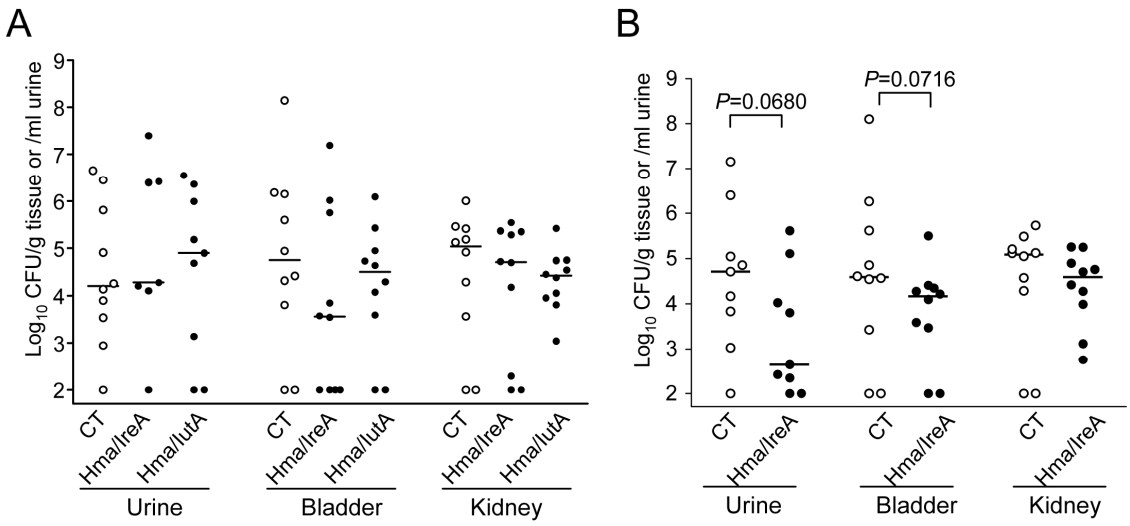

Figure 3-4. Urinary tract colonization following immunization with divalent Hma-IutA and Hma-IreA combinations. (A) Mice (*n*=10) were vaccinated with CT adjuvant alone or a mixture of Hma+IutA or Hma+IreA (50 μg each antigen, 100 μg total) and received two boost doses (12.5 μg each antigen, 25 μg total) at one-week intervals. All antigens were crosslinked to CT at 10:1 (antigen:CT). One week following the final boost, animals were challenged with 10^8 CFU CFT073. CFU/g tissue was measured at 48 hpi and is shown. **(B)** Experiment performed as in (A), except mice were vaccinated with 100 μg of Hma and 100 μg IreA the following day. Control mice received 10 μg CT on both days. Two boosts (25 μg each) on successive days were administered at one-week intervals.

Immunization with chimeric CtB-Hma does not protect from UTI.

An alternative to utilizing whole proteins as immunogens is the use of peptide antigens for vaccination. Hma is predicted to adopt a beta-barrel structure, with a number of extracellular loops extending above the outer membrane. Since these loops are highly surface-exposed portions of the molecule, we hypothesized that they may be protective epitopes. Furthermore, previous work from our laboratory has shown that a chimeric molecule consisting of a truncated fimbrial protein and cholera toxin A2 and B subunits is an effective vaccine target to prevent urinary tract infection by *Proteus mirabilis* (114). To test the ability of Hma peptide antigens to protect against UTI, we employed a similar approach. A 32-residue peptide corresponding to a putative extracellular loop of Hma was expressed as a fusion protein with cholera toxin B subunit (CtB) and the resulting chimera formed pentamers in solution (Fig. 3-5A). Mixed 10:1 with CT holotoxin, CtB-Hma was used to intranasally immunize mice according to the one week interval schedule (Fig. 3-1A). Control animals received an equivalent amount of CtB mixed 10:1 with CT. However, no significant difference existed between control and CtB-Hma-vaccinated mice (Fig. 3-5B). This indicates that this particular loop is not able to generate protective immunity against *E. coli* CFT073 and also suggests that this loop alone was not the protective epitope responsible for the protection observed after immunization with whole Hma.

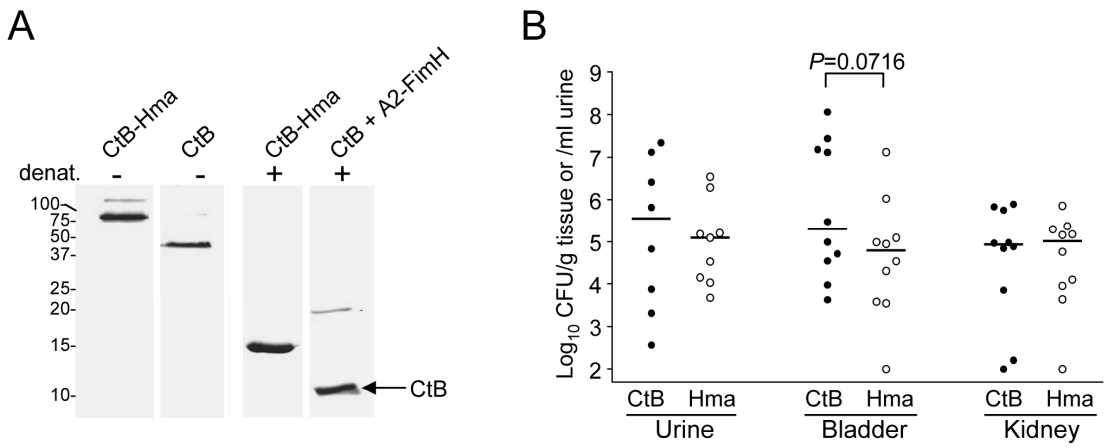

Figure 3-5. Vaccination with CtB-Hma chimera. (A) SDS-PAGE (15%) separation of CtB-Hma (lanes 1 and 3), CtB alone (lane 2), and CtB with A2-FimH fusion (lane 4). Samples were either heat-denatured in the presence of β-mercaptoethanol (+, lanes 3 and 4) or not (-, lanes 1 and 2) prior to gel loading. Molecular weight standards are shown in kDa and arrow indicates CtB monomer band. **(B)** Urinary tract colonization following immunization (100 μg) and two boosts (25 μg) with CtB or CtB-Hma and 48 h challenge with 10^8 CFU CFT073 (n=10). Bars represent the median and dashed line indicates limit of detection.

Discussion

The six candidate antigens identified in the previous chapter meet all of the following criteria for a vaccine against UPEC: antigenicity, predicted surface-exposure, conservation among UPEC strains (119), transcriptional upregulation *in vivo* (170) and induction during culture in human urine (2). Of these, we found that intranasal immunization with Hma, IreA, or IutA provides significant protection against experimental infection with UPEC. However, combinations of these antigens do not appear to elicit protective immunity.

Site-specific protection was observed for the antigens that provided the greatest reduction in bacterial counts, Hma and IreA. Mice immunized with the IreA vaccine were significantly protected from colonization only in the bladder, with 67% of mice having undetectable levels of bacteria within that organ. The high levels of kidney colonization in IreA-vaccinated mice may reflect early ascension of the inoculated bacteria, prior to immune targeting in the bladder. In contrast, Hma immunization significantly reduced the number of bacteria colonizing the kidneys and frequently (>50% of mice) prevented kidney colonization completely. Despite the significant protection in the kidney, immunization with the Hma vaccine did not reduce the level of bacteria able to colonize the bladder. The kidney-specific protection of the Hma vaccine may reflect the biological function of Hma, as UPEC that are unable to produce Hma have reduced fitness only within the kidney (see Chapter 4). The site-specificity for the Hma vaccine suggests that the immune response may perturb the normal function of this outer membrane heme receptor, perhaps by antibody interference with ligand-binding domains or selective immune targeting of bacteria that exhibit site-specific expression of

Hma. Thus, further work is needed to characterize the precise mechanism of protection via Hma or IreA immunization.

Mucosal immunization is considered the most effective means to develop a UTI vaccine and we have shown previously that intranasal immunization with MR/P fimbria is effective to provide mice protection against UTI caused by *Proteus mirabilis*, an agent of complicated UTI (115). Because local immunization of the urethra is not practical, the migration of immune cells between mucosal sites can be exploited during intranasal inoculation with antigen (97). We reasoned that intranasal immunization would generate a distant mucosal response in the genitourinary tract against UPEC in vaccinated animals. Consistent with this, we observed not only protection from infection in the urinary tract, but also generation of antigen-specific urinary IgA at the mucosa (data not shown, K.E. Sivick and H.L.T. Mobley). Interestingly, allowing additional time between Hma doses did not either enhance protection from kidney infection or generate protection in the bladder. This suggests that the maximum antibody response to this vaccine preparation is already occurring and that further optimization will require vaccine component, adjuvant, or route alterations.

Several of the vaccine candidates identified and tested here have been previously investigated as targets for immunization against pathogenic *E. coli*. Vaccination with denatured IroN, for example, was shown to provide modest protection from kidney infection in a murine UTI model, as well as stimulate IroN-specific IgG (161). Immunization with IroN was also shown to increase survival time using a murine intraperitoneal infection model of ExPEC infection (40). However, similar to the

findings presented here, immunization with ChuA failed to protect mice from ExPEC-induced death in this model.

While we hypothesized that vaccination with a combination of Hma and IreA might elicit protection in both the bladder and kidneys, this was not the case. In contrast, the combination immunizations appeared to protect even less effectively than single antigen vaccination with either Hma or IreA. For the lower dose combination (50 μg each component), it appears that this is simply not enough Hma antigen to stimulate the protective response observed with a higher dose. However, singly-administered IreA appears to provide a modest level of bladder protection with an even lower dose (30 μg). A 30 μg primary dose of IreA alone resulted in a significant two log reduction in CFU/g bladder tissue, while 50 μg of IreA in combination with Hma resulted in an only one log reduction. Thus it appears that the presence of Hma negatively affects the response to IreA antigen. One possibility for this effect is competition between IreA and Hma antigen for antigen-presenting cells available at the site of vaccination.

Further reduction in vaccine efficiency was observed when mice were co-immunized with high doses (100 μg primary dose) of Hma and IreA on successive days. While the biological explanations for these findings are unknown, it is possible that this unconventional vaccination strategy may have overwhelmed the recruited immune cells, resulting in a diminished response to the antigens. Furthermore, all vaccine preparations used in these studies contain unknown and likely variable amounts of LPS. As the role this molecule plays during these vaccinations is not known, another possible explanation is that the potentially large, successive LPS doses subverted the normal response to the antigens and/or induced LPS tolerance [reviewed in (38)].

While vaccination with whole Hma protein elicited kidney protection, immunization with a pentameric chimera protein consisting of a putative extracellular Hma loop fused to cholera toxin B subunit was not protective. As nine other putative extracellular loops exist, this result is not completely unexpected and demonstrates that antibodies directed against this loop (assuming they are generated) are not sufficient for protection. Because the Hma loop is fused to CtB, we were unable to measure loop-specific antibodies generated by vaccinated animals, but future studies should include the measurement of Hma (whole protein)-specific antibodies. Furthermore, while it is possible that antibodies directed against another loop may protect from infection, this result suggests that multiple epitopes may be required to generate protective immunity. Especially if the mechanism of protection involves inhibition of Hma function, inclusion of the yet-unknown ligand-binding region of the molecule in a vaccine preparation will be an important consideration for future work.

Consequently, these studies demonstrate that intranasal immunization with Hma, IreA, or IutA protects against experimental UTI. This protective response appears dependent upon antigen dose, generation of antigen-specific antibodies, and the presence of competing antigens in the vaccine preparation. To fully understand the capacity these proteins have as immunogens, further investigation of both their function as iron receptors, as well as their immunostimulatory properties will be necessary. However, these findings provide an important first step in the development of an iron receptor subunit vaccine designed to protect against UTI.

CHAPTER 4

HEME ACQUISITION IS FACILITATED BY A NOVEL RECEPTOR AND REQUIRED BY UROPATHOGENIC *E. COLI* FOR KIDNEY INFECTION

Introduction

In Gram negative bacteria, uptake of ferrisiderophores and other iron complexes is facilitated by specific outer membrane receptors. These 70-80 kDa proteins are structurally conserved, forming transmembrane beta-barrels with an N-terminal plug domain obstructing the pore of the protein (20, 45). To function, these receptors require the energy-transducing activity of an inner membrane-periplasmic protein complex composed of ExbB, ExbD, and TonB (47, 167).

In addition to siderophore-mediated iron acquisition, many bacterial species can scavenge heme-bound iron. Specific outer membrane receptors bind host hemoproteins and transfer the coordinated heme molecule into the periplasm where an ABC transport system delivers it to the cytoplasm. Alternatively, hemophores scavenge heme and subsequently transfer it to specific outer membrane receptors in a process analogous to siderophore-mediated iron uptake (196).

The majority of high affinity heme or hemoglobin receptors share four conserved histidine residues and two motifs, the FRAP and NPNL domains (18). Two of these conserved histidines are required for HemR-, HmuR-, or ShuA-mediated heme utilization

in *Yersinia enterocolitica*, *Porphyromonas gingivalis*, or *Shigella dysenteriae*, respectively (corresponding to His128 and His461 in HemR) (18, 24, 118). Structural modeling has predicted these residues to reside extracellularly and recent evidence indicates they function to ligate heme (24).

In pathogenic *E. coli*, heme uptake is facilitated by the ChuA receptor, which shares >99% amino acid sequence identity with ShuA, the *S. dysenteriae* heme-hemoglobin receptor (186). A study examining the distribution of *shuA* homologs in pathogenic *E. coli* by Southern hybridization found that, indeed, most heme-utilizing *E. coli* contain the *shu* locus (203). However, several heme-utilizing strains were *shuA*-negative, even under reduced stringency conditions. Thus, the authors predicted the presence of an additional heme uptake gene in these strains whose sequence differs significantly from that of *shuA* (203).

Like other bacterial pathogens, uropathogenic *E. coli* (UPEC), the primary cause of uncomplicated urinary tract infections, requires TonB-dependent outer membrane iron receptors for host colonization (187). Reflecting the importance of iron acquisition for UPEC pathogenesis, the genome of the representative pyelonephritis strain CFT073 encodes at least 14 different outer membrane iron receptors (197). While several of these have been shown to contribute to the fitness of UPEC *in vivo* (87, 159, 160, 187), the importance of specific sources of host iron remains unknown.

Putative iron receptor c2482 was identified in Chapter 2 as an antigenic outer membrane protein expressed under iron limitation. Like other outer membrane iron receptors, the 2148 bp c2482 gene encodes a 79,100 Da protein that is predicted to adopt a beta-barrel structure. No iron transport or processing genes are found in the sequences

flanking c2482 (Fig. 4-1). However, the promoter region of c2482 contains a putative Fur box and, indeed, work from our laboratory has shown that transcription of this gene is iron-responsive (2). Furthermore, the N-terminal region of c2482 contains a putative TonB box, suggesting that, like other iron receptors, the protein interacts with the inner membrane protein, TonB. Thus, initial evidence suggests that c2482 may function as a receptor for an iron compound. Moreover, the c2482 gene appears to be conserved among pathogenic strains of *E. coli*, suggesting that this gene may contribute to the virulence of these strains.

In Chapter 3, we established that vaccination with purified c2482 protein protected mice from kidney infection following experimental challenge with UPEC strain CFT073. Thus, further investigation of the biological function of c2482 will provide insight, both for understanding the pathogenesis of UPEC and also for the development of a vaccine to prevent UTI.

Here we show that c2482 functions as a high affinity receptor for heme and demonstrate that heme uptake is required by UPEC for kidney colonization. Thus, we will refer to c2482 as Hma, heme acquisition protein. Furthermore, we identify residues required for Hma-mediated hemin utilization and propose that this protein represents a novel class of heme receptors that are conserved among pathogenic *E. coli*.

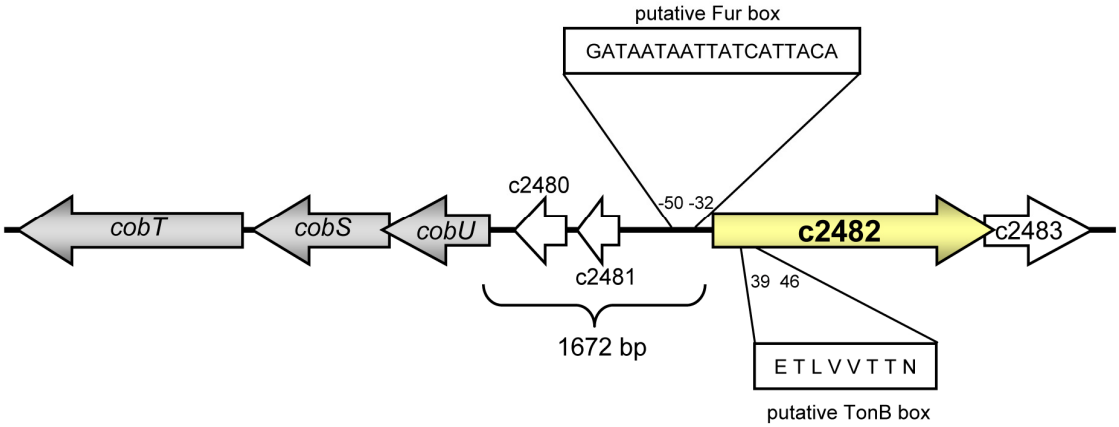

Figure 4-1. c2482 locus. Arrows represent genes located near c2482, which is highlighted yellow. White, hypothetical genes; gray, cobalamin synthesis genes. Approximately 1.5 kb separates c2482 from *cobU*. Insets indicate putative Fur and TonB boxes, located upstream (-50 to -32 bp) of c2482 and at the protein's N-terminus (residues 39-46), respectively.

Materials and methods

Bacterial strains and culture conditions.

All strains and plasmids used in this study are listed in Table 4-1.

Mutant construction.

Deletion of *hma* (in both wildtype CFT073 and *chuA*::*cat* backgrounds) and *tonB* (in MG1655) was achieved using the λ Red recombinase system (35). Using primers containing sequences in the 5' and 3' ends of *hma* or *tonB*, a kanamycin resistance gene was PCR amplified from the template plasmid pKD4 (Table 4-1). The resulting product was used to replace >80% of the *hma* or *tonB* gene by Red recombinase-mediated homologous recombination (recombinase expressed from pKD46). Mutants were verified by PCR and differential *Eag*I digestion.

Expression and purification of recombinant proteins.

An approximately 3 kb fragment containing the *hma* ORF plus 900 bp upstream was PCR amplified from CFT073 chromosomal DNA and cloned into the *Nde*I - *Eag*I restriction sites of pGEN-MCS (p_{native}*hma*) (Table 4-1). The *hma* ORF (minus upstream region) was also PCR amplified and cloned into the *Nco*I - *Bgl*II restriction sites of pBAD-*myc*-HisA (Table 4-1), both in- and out-of-frame with the vector's C-terminal 6x His tag (p*hma*-His and p*hma*, respectively). The *chuA* and *iutA* ORFs were PCR amplified and similarly cloned into the *Nco*I - *Xho*I sites of pBAD, both in- and out-of-

Table 4-1. Bacterial strains and plasmids.

E. coli strain	Description[a]	Reference or source
CFT073	Pyelonephritis isolate	(127)
K12	MG1655, laboratory strain	(14)
HB101 *ent*	HB101 *ent*::Tn*5* strain 1017*;* Kan^r	(186)
chuA	CFT073 *chuA*::*cat*; Cam^r	(187)
hma	CFT073 Δ*c2482*::*kan*; Kan^r	This study
hma chuA	CFT073 Δ*c2482*::*kan chuA*::*cat*; Kan^r, Cam^r	This study
tonB	MG1655 Δ*tonB*::*kan*, Kan^r	This study
Plamid		
pKD4	λ Red template vector; Kan^r Amp^r	(35)
pKD46	Red recombinase helper plasmid, temp-sensitive; Amp^r	(35)
pGEN	pGEN-MCS, promoter-less expression vector, p15A ori (copy number ~15), *par hok sok mok parM parR*; Amp^r	(54, 104)
p_{native}*hma*	*hma* with native promoter (900 bp upstream) in pGEN-MCS	This study
pBAD-*myc*-HisA	Expression vector, pBR322 ori (low copy), *araBAD* promoter (arabinose-inducible), *araC*; Amp^r	Commercial (Invitrogen)
p*hma*	*hma* in pBAD	This study
p*Y126A*	hma^{Y126A} in pBAD	This study
p*chuA*	*chuA* in pBAD	This study
p*iutA*	*iutA* in pBAD	This study
p*hma*-His	*hma* in pBAD with C-term. His_6 tag	This study
p*chuA*-His	*chuA* in pBAD with C-term. His_6 tag	This study
p*iutA*-His	*iutA* in pBAD with C-term. His_6 tag	This study

[a] Kan, kanamycin; Cam, chloramphenicol; Amp, ampicillin

frame with the C-terminal 6x His tag (p*chuA*-His, p*iutA*-His and p*chuA*, p*iutA*, respectively). Expression of *hma*, *chuA*, and *iutA* from P_{BAD} was induced by addition of L-arabinose to 100 μM. Using a nickel-nitriloacetic-agarose column (Qiagen), His_6 fusions were purified from *E. coli* TOP10® (Invitrogen) outer membrane fractions (see below) in the presence of 8 M urea. Buffer exchange at 4°C was used to solubilize the purified protein in PBS with 0.05% Zwittergent® (Calbiochem).

Outer membrane isolation.

Bacteria were harvested by centrifugation (10 min, 8000×*g*, 4°C), resuspended in 10 mM HEPES pH 7.0, and lysed by two passages through a French pressure cell (20,000 psi). After the lysate was cleared by centrifugation (10 min, 8000×*g*, 4°C), membranes were isolated from the cleared lysate by ultracentrifugation (30 min, 100,000×*g*, 4°C). The membrane pellet was resuspended in 2% sarcosine, incubated 30 min at room temp, and ultracentrifuged (30 min, 100,000×*g*, 4°C) to isolate the sarcosine-insoluble outer membranes. Outer membranes were resuspended in 10 mM HEPES pH 7.0 or solubilized in 0.2% Zwittergent® (Calbiochem).

***In vitro* competition assay.**

In vitro co-cultures were performed as previously described (105). Briefly, wildtype CFT073 and the *hma* mutant were grown to late exponential phase and the OD_{600} of each culture was standardized to 0.8. Standardized cultures were mixed 1:1, diluted 1:500 into LB or W salts minimal medium and incubated at 37°C with aeration. Every 8 or 16 hours, cultures were passaged into fresh medium at 1:500 or 1:50,

respectively. At 24, 48, and 72 hours post-inoculation, cultures were plated on LB agar and LB containing 25 μg/ml kanamycin to determine wildtype and mutant CFU/ml. All cultures were plated using an Autoplate 4000® (Spiral Biotech) spiral plater and enumerated with a Q-Count automatic colony counting system (Spiral Biotech).

Iron source growth assays.

Growth promotion assays were performed as described (186), with modification. Prior to inoculation, bacteria were cultured in LB containing 200 μM 2'2-dipyridyl (DIP) for at least 6 hours and washed in PBS. Approximately 10^5 CFU were plated onto LB supplemented with 375 μM DIP (Sigma). Iron sources (10 μl) were spotted directly onto the plate (1 mM $FeCl_2$, 10 μM hemin, 1 mg/ml hemoglobin, 10 mg/ml lactoferrin, 10 mg/ml holo-transferrin, and 10 mg/ml bovine serum albumin [Sigma]) and incubated 48-72 hours at 37°C.

All other plate assays utilized sorbitol-MacConkey agar (Difco) supplemented with 350 μM DIP and an iron source at the indicated concentration. For heme titration experiments, 1-50 μM $FeCl_2$ and 10 nM-100 μM hemin were used. Prior to inoculation, bacteria were cultured in LB containing 200-400 μM DIP for at least 6 hours and washed in PBS. The OD_{600} was standardized to ~1.0 and approximately 200 CFU were spread per plate.

For growth curves, strains were iron-limited overnight by culturing in LB containing 200 μM DIP. Prior to inoculation, strains were washed in PBS and ~10^5 CFU inoculated into LB containing 300 μM DIP and 20 μM $FeCl_2$, 10 μM hemin, or no

additional iron source. Growth curves were performed in a Bioscreen C Growth Curve Analyzer (Growth Curves, USA) at 37°C with aeration.

Hemin-binding assays.

Hemin binding to whole cells was determined as previously described (141). MG1655 containing p*hma*, p*Y126A*, p*chuA*, p*iutA* or empty vector were induced for 3 h with 100 μM arabinose, washed and resuspended in PBS. The OD_{600} was standardized to 1.0 and 800 μl samples of this cell suspension were mixed with 200 μl 50 μM hemin. After 1 h incubation at 37°C, bacteria were pelleted at 16,000×*g* for 3 min and 20 μl supernatant was incubated with 80 μl 1-Step™ Turbo TMB-ELISA (Sigma) for 20 min at room temp. Reactions were stopped by the addition of 100 μl 1.0 N H_2SO_4 and the OD_{450} measured.

Hemin binding to outer membranes or purified protein was determined as previously described (7), with modification. Purified proteins or outer membranes from MG1655 containing p*hma,* p*Y126A,* p*chuA, or* p*iutA* were prepared as described. Protein was diluted in coating buffer (50 mM Na_2CO_3, 50 mM $NaHCO_3$, pH 9.6) and coated onto a microtiter plate (0.1-0.5 μg per well) at 37°C overnight. Wells were blocked for 1 h with 2% BSA in PBS, washed with PBS and incubated at 37°C for 1 h with 100 μl hemin solution (for outer membranes 50 μM hemin, for purified protein 0-200 μM). Wells were washed 4x with PBS and 100 μl 1-Step Turbo-TMB peroxidase substrate was added. After 20 min at room temp, the OD_{450} was measured. Amount of hemin bound by each sample was calculated from a standard curve.

A modified in-gel TMBZ staining method was used to detect Hma-associated heme (177). Outer membranes from MG1655 carrying p*hma* (10 μg) were incubated with 85 μM hemin for 1 h at 37°C. SDS-PAGE loading buffer lacking dithiothreitol was added to samples, which were elecrophoresed in the presence of 0.1% SDS on a 10% acrylamide gel (3.75% stacking gel) at 200V for 1 h at 4°C in the dark. Gels were fixed for 1 h in a pre-chilled solution of 0.25 M sodium acetate pH 5-methanol-H_2O (6:3:1). To detect heme-associated peroxidase activity, gels were stained as described in 2 parts freshly-prepared 6.3 mM TMBZ (Sigma) in methanol, 7 parts 0.25 M sodium acetate pH 5, and 1 part H_2O for 35 min (183). Color development was achieved by adding H_2O_2 to a final concentration of 0.1% and incubating 30 min. Gels were washed in acetate-buffered 30% isopropanol and imaged immediately. All fixing/staining steps were performed at 4°C in the dark.

Site-directed mutagenesis.

The amino acid sequence of Hma was aligned with the structure of *E. coli* FepA using Cn3D version 4.1 (NCBI). Residue changes in Hma were made using the QuikChange® II Site-Directed Mutagenesis protocol (Stratagene), with p_{native}*hma* as the template. Mutagenic primers are listed in Table 4-2 and all reactions were carried out according to the manufacturer's instructions. All mutations were confirmed by sequencing (University of Michigan DNA Core Facility).

Table 4-2. Site-directed mutagenesis primer sequences[a].

Mutation	Forward	Reverse
H242A	GGTTATAACTCCGGAAACGCTCGTTTTGGCCTCTCGC	GCGAGAGGCCAAAACGAGCGTTTCCGGAGTTATAACC
H331A	CAGGCTCTGACCGTTGCTAACAAGACTGACACCCATG	CATGGGTGTCAGTCTTGTTAGCAACGGTCAGAGCCTG
H337A	CATAACAAGACTGACACCGCTGATAAGCAATACACTC	GAGTGTATTGCTTATCAGCGGTGTCAGTCTTGTTATG
Y126A	GCGCGCCGGAGATAATGCTGGTGTGGGACTGTTG	CAACAGTCCCACACCAGCATTATCTCCGGCGCGC

[a] all sequences listed 5'→ 3'

CBA mouse models of ascending UTI and bacteremia.

Mouse model of ascending UTI described in Chapter 3 was used with the following exceptions. For co-infection experiments, resuspended strains were mixed at a 1:1 ratio and inoculated into the same mouse. Seventy-two hpi, mice were euthanized and bladder, kidneys, and spleens were removed, homogenized and plated to determine CFU/g. For co-infection experiments, homogenate was also plated on appropriate antibiotics to differentiate wildtype and mutant strains.

For murine model of bacteremia, female six- to eight-week old CBA/J mice were intravenously inoculated with 10^6 CFU. Prior to inoculation, CFT073 and *hma chuA* mutant strains were cultured in overnight in LB or LB containing 200 μM DIP. Cultures were resuspended in PBS and either mixed 1:1 for co-infections or inoculated separately for independent infections. Bacterial suspensions were delivered via tail vein injection with a 27 ½ -gauge needle in a total volume of 100 μl. Spleens were harvested at 24 hpi and bacterial load quantified as described above.

RNA isolation and qPCR.

For *in vitro* RNA samples, CFT073 was cultured with aeration to late exponential phase (OD_{600}=0.5-0.6) in 100 ml LB or LB containing 200 μM DIP or 20 μM $FeCl_2$. Culture aliquots (200 μl) were mixed with 25 μl cold 5% phenol-ethanol stop solution, pelleted (1 min, 10,000×*g*), and stored at -80°C for RNA isolation. Thawed pellets were resuspended in 100 μl RNase-free TE containing 1 mg/ml lysozyme and RNA isolated using the RNeasy protocol (Qiagen). Samples were DNase-treated according to the Turbo™ DNA-Free procedure (Ambion) and cDNA synthesized using SuperScript™ II

First-Strand Synthesis reagents (Invitrogen) according to the manufacturers' instructions. PCR was performed on no-RT control reactions using *gapA* primers to ensure that cDNA samples were free from DNA contamination. Real-time qPCR was performed using 30 ng cDNA template and Brilliant SYBR® Green reagents (Stratagene). See Chapter 5 for qPCR primer sequences (Table 5-1). Data were normalized to *gapA* transcript and analyzed using MxPro 4.0 software (Stratagene).

For *in vivo* RNA samples, CBA/J mice were infected with CFT073 (or PBS control) as described above. At two-hour intervals beginning at 24 hpi, urine was collected and pooled from each cage of mice (5 animals). Immediately after collection, cold 5% phenol-ethanol stop solution was added (0.125 µl solution per µl urine) and samples were pelleted (1 min, 10,000×*g*) and stored at -80°C. Pellets from 5-7 timepoints were combined for RNA isolation, which was performed as described above.

Statistical analysis.

Statistics were performed using GraphPad InStat® version 3.05 statistical software. *P* values for co-infections and co-cultures were calculated by the Wilcoxon matched-pairs signed-ranks test, for independent infections by the Mann-Whitney and Fisher's exact tests, and all others by the Student's t-test. GraphPad Prism® version 3.00 was used for nonlinear regression analysis.

Results

hma contributes to the fitness of CFT073 *in vivo.*

To examine the role of *hma* in iron acquisition, we constructed a deletion mutant in UPEC strain CFT073. In independent culture in LB medium, the *hma* mutant had a growth rate similar to wildtype, even in the presence of high concentrations of iron chelator (600 μM 2'2-dipyridyl [DIP]) (Fig. 4-2A). Since subtle growth defects may not be detectable during independent culture, co-cultures were conducted to compare the ability of the *hma* mutant to directly compete with wildtype for limited nutrients. Wildtype and mutant were inoculated approximately 1:1 into the same medium and continually re-passaged into fresh medium for 72 hours. In rich medium, the *hma* mutant reached densities similar to those of wildtype throughout the duration of the experiment, despite an approximately half log lower inoculum (Fig. 4-2B), demonstrating that no growth defect exists in the mutant strain under these conditions. However in minimal medium (containing no supplemented iron), the *hma* mutant maintained similar cell densities initially, but was outcompeted by wildtype by 72 hours (P=0.030). Together, these data indicate that *hma* is not required for growth *in vitro* in rich medium, but may play a role during nutrient-depleted conditions.

Because iron acquisition is required for UPEC pathogenesis, we used a murine model of ascending UTI to investigate the contribution of *hma* to virulence during experimental infection. Given the redundancy of iron uptake systems in UPEC, we used a co-infection model, transurethrally inoculating mice with a 1:1 ratio of wildtype and mutant in an effort to detect subtle differences in fitness. Total inoculum equaled ~1×10^8 CFU per mouse. At 48 h post-inoculation, the *hma* mutant was slightly

outcompeted by wildtype in the kidneys (<2-fold reduction, P=0.0391) of infected mice (Fig. 4-2C). However, at 72 hpi, the *hma* mutant was more severely outcompeted in the kidneys (8-fold reduction; P<0.001) and spleens (80-fold reduction; P<0.0001) of infected mice (Fig. 4-2D). Moreover, the *hma* mutant was undetectable in the kidneys and spleens of infected mice significantly more frequently than wildtype (P=0.021, P<0.0001, respectively). Thus, *hma* contributes to the ability of CFT073 to colonize the kidneys and disseminate into the bloodstream. Interestingly, the *hma* mutant was not significantly outcompeted in the bladders of infected mice, suggesting either localized expression of this gene or localization of the receptor's iron substrate to the kidneys and bloodstream.

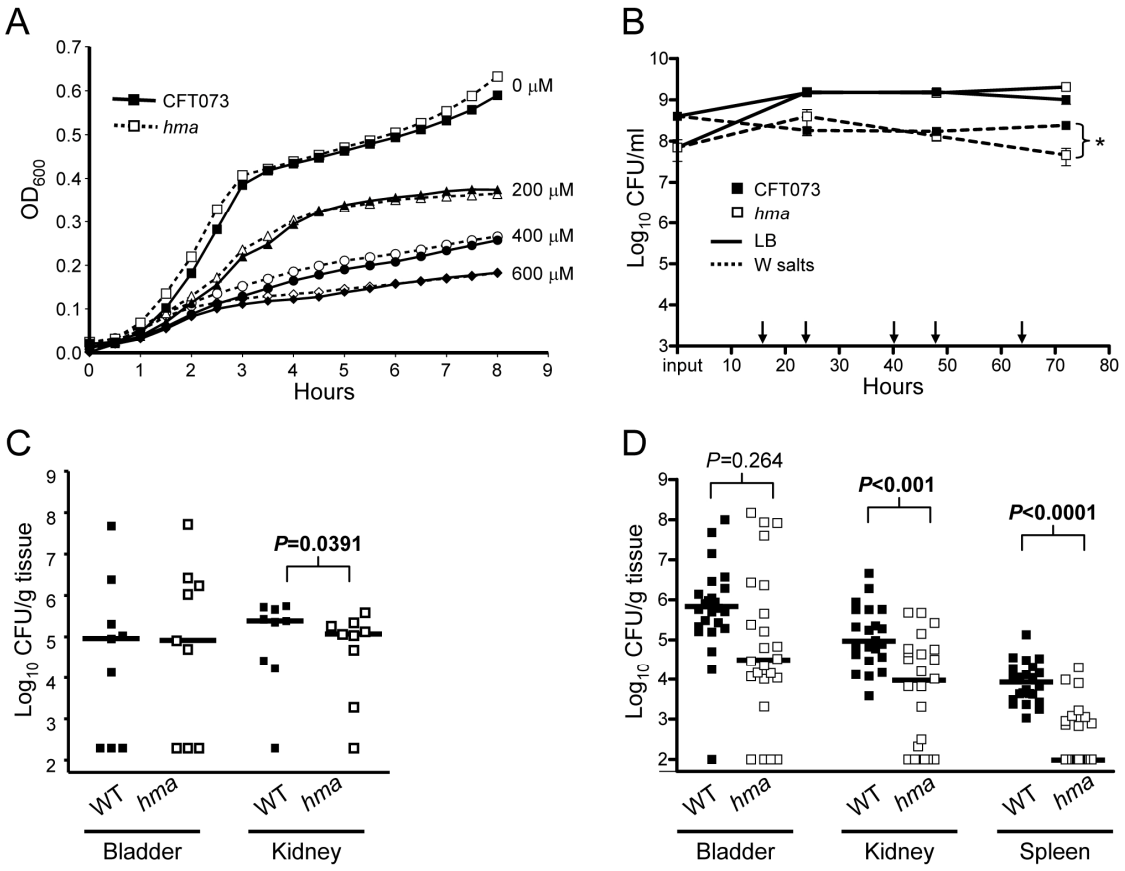

Figure 4-2. Fitness of *hma* mutant *in vitro* and *in vivo*. (A) Growth curves of wildtype CFT073 (filled symbols) and *hma* mutant (open symbols) in LB supplemented with indicated concentrations of DIP: 0 μM (squares), 200 μM (triangles), 400 μM (circles), or 600 μM (diamonds). **(B)** *in vitro* culture competition assay of wildtype CFT073 (filled symbols) and *hma* mutant (open symbols) cultured in Luria broth (solid line) or W salts minimal medium (dashed line). After inoculation (input CFU/ml plotted on y-axis), cultures were passaged into fresh medium every 8 (1:50 dilution) or 16 (1:500 dilution) hours. Arrows indicate culture passages. Means of triplicate cultures are plotted. **P*=0.030. **(C)** 48 hour (*n*=9) and **(D)** 72 hour (*n*=24) CBA/J mouse co-infection with 10^8 CFU mixture of wildtype (WT) and *hma* mutant. Data points represent CFU/g of individual animals in the organs indicated; bars show median values.

Expression of *hma* promotes hemin utilization.

To identify the iron substrate recognized by Hma, a gain-of-function approach was taken. *hma* was expressed from its native promoter (p_{native}*hma*) in *E. coli* HB101 *ent*, a laboratory strain deficient in the production of enterobactin, the major siderophore, making it highly susceptible to iron limitation (186). To screen iron compounds for putative substrates of Hma, iron sources (10 μl) were spotted onto iron-depleted agar overlaid with 10^5 CFU *E. coli* HB101 *ent*. While $FeCl_2$ (1 mM) supported the growth of strains carrying either empty vector or p_{native}*hma*, hemin (10 μM) and hemoglobin (1 mg/ml) only promoted the growth of the strain expressing *hma* (Fig 4-3A). Lactoferrin, transferrin, and albumin did not promote the growth of either strain (data not shown). A similar result was observed for growth of these strains in broth culture. HB101 *ent* containing vector control or p_{native}*hma* grew similarly in LB, chelated LB, and chelated LB supplemented with 20 μM $FeCl_2$ (Fig. 4-3B). However, only growth of the strain expressing *hma* was enhanced by the addition of 10 μM hemin. Together, these data indicate that expression of *hma* promotes the utilization of hemin and suggests that it likely functions as a receptor for this iron compound.

Hma function is TonB-dependent.

Other outer membrane iron transporters characterized to date are dependent on the energy-transducing function of the inner membrane protein TonB. Indeed, the N-terminal region of Hma contains a putative TonB interaction site (ETLVV, residues 39-43). To determine if Hma activity requires TonB, *hma* was expressed from p_{native}*hma* in an *E. coli* K12 *tonB* mutant. While $FeCl_2$ supported growth of both the parent and mutant

strains on iron-depleted medium, expression of *hma* only promoted hemin utilization by wildtype K12, not the *tonB* mutant (Fig. 4-3C). Thus, Hma was unable to function in the absence of TonB, indicating that it is indeed a TonB-dependent receptor.

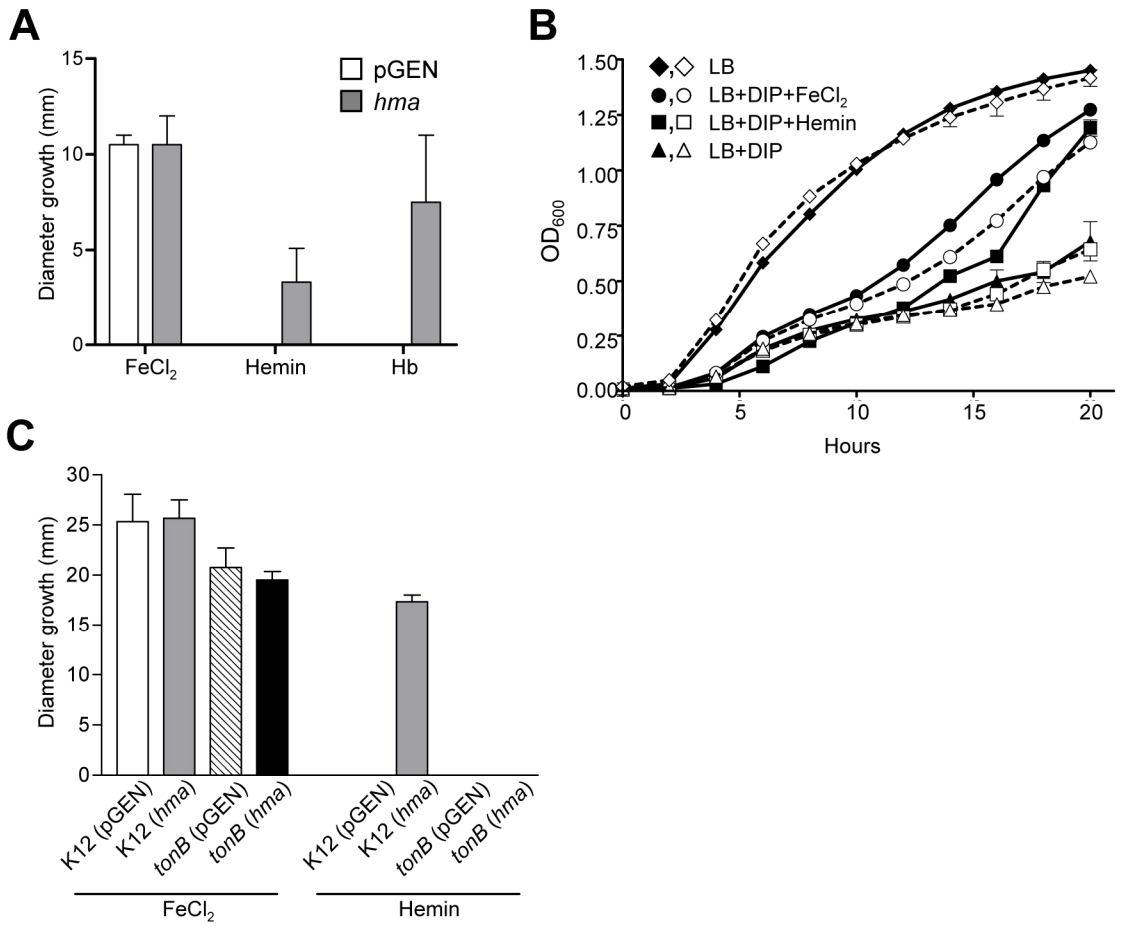

Figure 4-3. Hemin utilization by *E. coli* strains expressing *hma*. **(A)** Growth of *E. coli* K12 carrying pGEN (open bars) or p_{native}*hma* (gray bars) on iron-depleted agar spotted with 1 mM $FeCl_2$, 10 μM hemin, or 1 mg/ml hemoglobin (Hb). Bars represent mean diameter (mm) growth surrounding indicated iron source (n=3). **(B)** Growth of *E. coli* HB101 *ent* carrying pGEN vector control (open symbols, dashed lines) or p_{native}*hma* (filled symbols, solid lines) in LB (diamonds) or LB + 300 μM DIP supplemented with 20 μM $FeCl_2$ (circles), 10 μM hemin (squares), or no additional iron source (triangles). Cultures were iron-limited overnight prior to inoculation into the media indicated. The mean OD_{600} of triplicate cultures is plotted. **(C)** Growth of *E. coli* K12 wildtype and *tonB* mutant on iron-depleted agar spotted with 10 mM $FeCl_2$ or 10 mM hemin. Bars represent mean diameter (mm) growth of *E. coli* K12 pGEN (open bars), K12 p_{native}*hma* (gray bars), *tonB* pGEN (hatched bars), and *tonB* p_{native}*hma* (black bars) surrounding the indicated iron source ($n \geq 3$).

Hma is a hemin-binding protein.

To further test the hypothesis that Hma is a heme receptor, the ability of Hma to directly bind hemin was examined. *E. coli* K12 whole cells expressing *hma*, heme receptor *chuA*, or siderophore receptor *iutA*, or carrying empty vector were incubated with hemin and pelleted. Hemin bound by the pelleted cells was removed from solution, resulting in a measurable decrease in the heme concentration of the supernatant. Using the intrinsic peroxidase activity of hemin as an indirect measure of heme quantity, we found that cells expressing *hma* or *chuA* bound and removed significantly more hemin from the solution than did cells containing a vector control (P=0.001, P=0.0004, respectively) (Fig. 4-4A). While bacteria expressing *iutA* bound slightly more hemin than the vector control, this difference was not significant (P=0.145). Similarly, outer membranes isolated from *E. coli* expressing *hma* bound an average of 205 ng hemin/µg protein, as compared to 171 ng hemin/µg bound by outer membranes from *E. coli* carrying empty vector (P<0.0001) (Fig. 4-4B). These data indicate that hemin binds to cells containing Hma and that at least part of this heme-binding activity is due to a component of the outer membrane.

To detect direct heme-Hma interaction, we incubated outer membrane proteins from *E. coli* K12 either expressing or not expressing *hma* with hemin and separated the hemin-protein mixtures on a non-reducing SDS-PAGE gel. The gel was stained with 3,3',5,5'-tetramethylbenzidine (TMBZ), a chromogenic compound that changes color in the presence of heme-associated peroxidase activity. This activity was localized to an ~80 kDa band, consistent with the size of Hma, that was absent from the vector control

lane (Fig. 4-4C). Together with our previous findings, these data demonstrate that Hma can function as a heme receptor.

Hma binds hemin with high affinity.

To define the affinity for which Hma binds heme, we measured the amount of hemin bound by purified Hma-His_6 over a range of substrate concentrations. Hemin binding to Hma was saturable and each μg Hma protein bound a maximum of approximately 340 ng hemin (Fig. 4-5). Using nonlinear regression analysis ($R^2 \geq 0.820$) we estimated the dissociation constant (K_d) for Hma-hemin binding to be 8 μM. Although ChuA-His_6 maximally bound less hemin than Hma-His_6, it had an identical affinity constant in this assay. Because we were concerned about heme binding by the His_6 tag, we also tested purified IutA-His_6 and this protein bound hemin with approximately 10-fold lower affinity (K_d=90 μM) than Hma- or ChuA-His_6. Thus, heme binding to Hma is specific and occurs with high affinity.

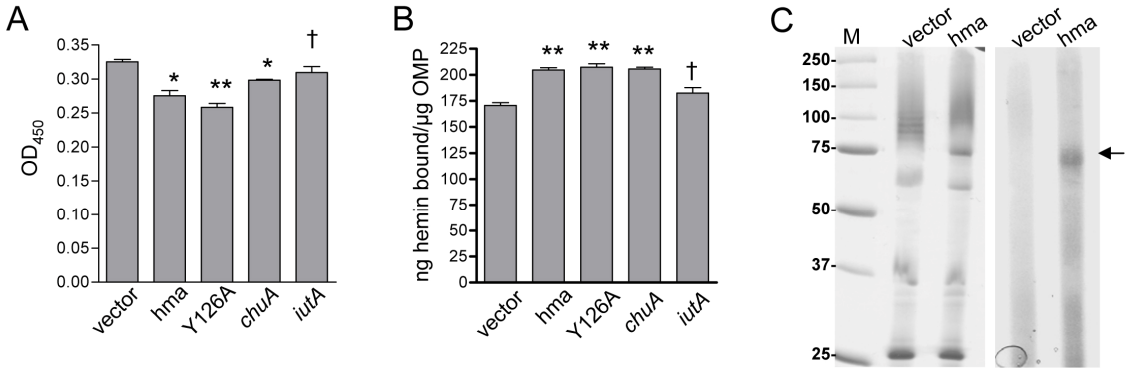

Figure 4-4. Hemin binding activity of Hma. (A) Hemin binding to *E. coli* K12 carrying p*hma*, p*Y126A*, p*chuA*, p*iutA*, or vector control. Induced cells were incubated with 50 μM hemin, pelleted, and hemin remaining in the supernatant detected with a peroxidase substrate. $^{**}P<0.0001$, $^{*}P\leq0.001$, † not significant (as compared to vector control). **(B)** Hemin binding to outer membrane proteins isolated from the strains in (A), as measured by microtiter plate assay. Wells were coated with 0.5 μg protein and incubated with 50 μM hemin. Unbound hemin was removed by washing and, after the addition of a peroxidase substrate, hemin binding was calculated from a standard curve using the OD_{450}. Bars represent the mean ($n\geq5$) and symbols are as in (A). **(C)** Hemin binding to Hma protein. OMPs isolated from the strains in (A) were incubated with 85 μM hemin and separated on a non-reducing SDS-PAGE gel. Left panel is Coomassie stained gel and right panel is TMBZ stain of heme-associated peroxidase activity. Arrow indicates Hma band. M, molecular weight standards in kDa.

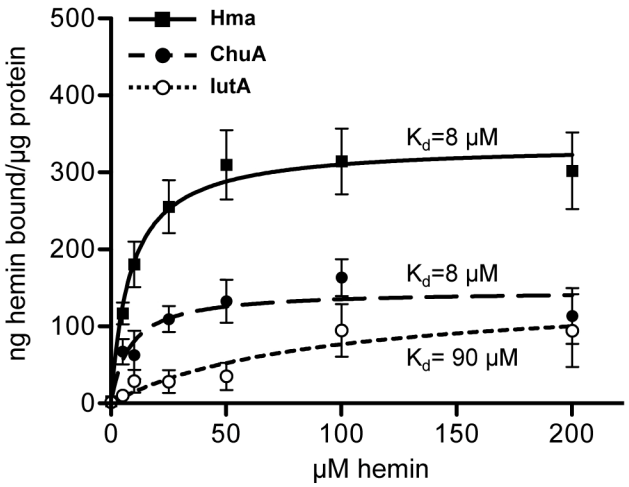

Figure 4-5. Heme binding curve. Hemin bound by purified Hma-His_6 (solid squares), ChuA-His_6 (solid circles), or IutA-His_6 (open circles) as a function of substrate concentration. Protein (0.2 µg) was coated onto microtiter plate wells, incubated with hemin (0-200 µM), and bound hemin detected by addition of a peroxidase substrate. Hemin standards were used to calculate ng hemin bound per µg purified protein. Mean values of triplicate samples are plotted. Saturation curves for Hma (solid line, R^2=0.820), ChuA (dashed line, R^2=0.594), and IutA (dotted line, R^2=0.455), determined by nonlinear regression analysis, are also plotted. Dissociation constant (K_d) values for each curve are indicated.

Hma is distinct from known heme receptors.

Hma has only limited homology to other characterized bacterial heme receptors (Fig. 4-6). While ChuA shares 70% amino acid sequence identity with HemR, Hma is only 18% identical. By BLAST analysis, Hma is most closely related to TonB-dependent receptors of *Dinoroseobacter* and *Desulfuromonas*, marine photosynthetic and sulfur-metabolizing bacteria. In addition to CFT073, copies of *hma* are present in all sequenced UPEC (F11, UTI89, 536) and enterohemorrhagic *E. coli* strains (EDL933, Sakai, EC508, EC4042), and a close homologs (73% and 51% identical, respectively) are found in the opportunistic pathogen *Citrobacter koseri* and plant pathogen *Pectobacterium* (*Erwinia*) *carotovora*. Furthermore, the G+C content of the *hma* ORF is considerably less than that of the CFT073 genome (45.3% as compared to 50.5%), implying that it may have been acquired horizontally. These findings suggest that *hma* likely evolved separately from *chuA* and *hemR* and may have been conserved among pathogens due to the selective advantage it conferred *in vivo*.

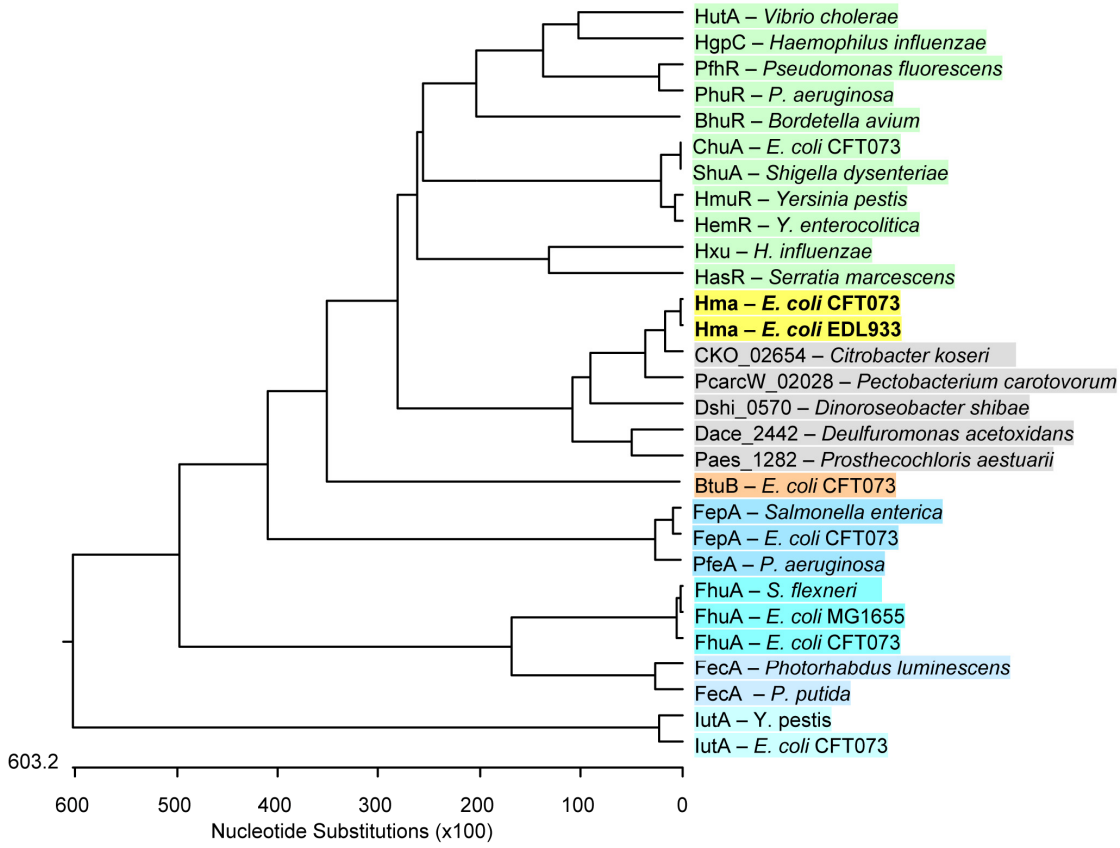

Figure 4-6. Phylogenetic tree showing relationship of Hma to heme and siderophore receptors. Amino acid sequences were aligned using the Clustal W algorithm in Lasergene Megalign. Protein (or locus tag) and species/strain names are indicated for each entry and shading indicates receptor function. Green, heme or hemoglobin receptor; orange, vitamin B12 receptor; blue/aqua, siderophore receptor; gray, unknown. Hma is highlighed in yellow.

Tyr126 is required for Hma function.

Previous studies have identified four histidine residues conserved among bacterial heme receptors, two of which are required for receptor function (18, 24, 118). However, while Hma contains a moderately-conserved FRAP-NPNL domain characteristic of other heme receptors, it lacks these conserved His residues (Fig. 4-7A). To identify other residues that may be important for Hma function, we employed site-directed mutagenesis. By aligning the Hma amino acid sequence with the crystal structure of FepA (20), a prototypic *E. coli* outer membrane iron receptor, we predicted extracellular residues that may function in heme binding or transport. His242, His331, and His337 are located in putative extracellular loops of Hma, while Tyr126 is predicted to be on the extracellular face of the N-terminal plug domain of the molecule (Fig. 4-7B). Furthermore, amino acid alignment of Hma with other heme receptors indicates that Tyr126 aligns with His128 of *Y. enterocolitica* HemR (Fig. 4-7A), a residue necessary for receptor function (18).

These four residues (His242, His331, His337, and Tyr126) were mutated to Ala in p_{native}*hma* and the resulting proteins were expressed in the *E. coli* K12 outer membrane at wildtype levels (Fig. 4-7C). We used this evidence of appropriate expression and membrane localization as an indirect indicator of correct protein folding, although it is possible that the mutation(s) disrupted Hma structure. The ability of the mutated Hma proteins to promote heme utilization was assessed by plating these strains on iron-depleted agar containing either $FeCl_2$ or hemin at various concentrations. The lowest concentration of iron compound capable of supporting growth was identified as the minimal supplementary concentration for each strain. *E. coli* K12 expressing the H242A,

H331A, or H337A mutants grew on hemin to the same extent as strains expressing wildtype Hma, indicating that these residues alone are not required for heme utilization (Table 4-3). To examine the possibility of functional redundancy with respect to the extracellular loop His residues (H242, H331, and H337), double and triple mutants of these residues were tested. Again, all of these mutant Hma proteins were able to facilitate heme utilization to the same extent as wildtype (Table 4-3). However, function of the Y126A protein was abolished, as the strain expressing this protein could not use even high concentrations of hemin (100 μM). The Y126A mutant Hma retained its hemin binding activity (Fig. 4-4A, 4-4B), though, suggesting the importance of this residue in the transport, rather than binding, of heme. Therefore, these data indicate that Tyr126, but none of the putative extracellular loop His residues, is required for the heme-uptake activity of Hma.

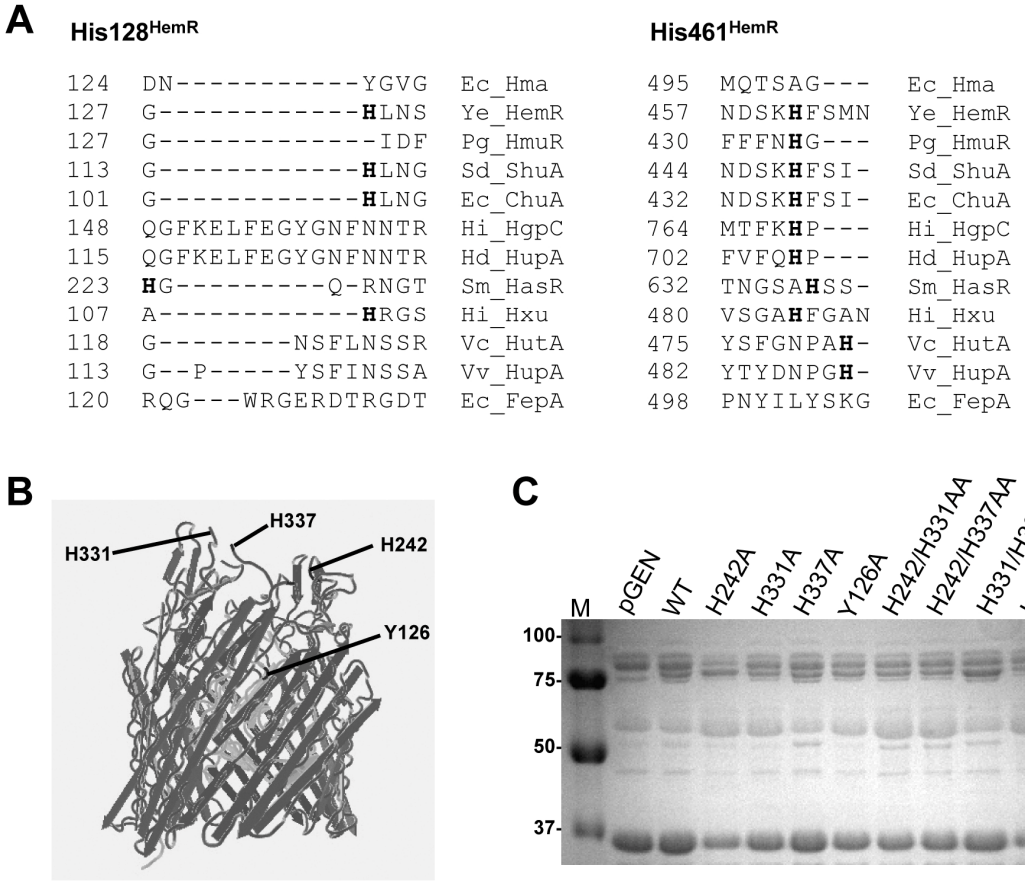

Figure 4-7. Residues required for Hma-mediated heme utilization. (A) Partial amino acid alignment of Hma with bacterial heme receptors, indicating conserved His residues (bolded) critical for function of HemR (His 128^{HemR} and His 461^{HemR}). Ec, *E. coli* CFT073; Ye, *Y. enterocolitica*; Pg, *P. gingivalis*; Sd, *S. dysenteriae*; Hi, *Haemophilus influenzae*; Hd, *H. ducreyi*; Sm, *Serratia marcescens*; Vc, *Vibrio cholerae*; Vv, *V. vulnificus*. **(B)** Structure alignment of Hma with FepA, showing the predicted locations of H242, H331, H337, and Y126 (black). Beta barrel domain (dark gray) and N-terminal plug domain (light gray) are also shown. **(C)** SDS-PAGE gel of outer membrane fractions (10 μg) isolated from *E. coli* K12 containing empty vector, p_{native}*hma*, or p_{native}*hma* with H242A, H331A, H337A, Y126A, H242A H331A, H242A H337A, H331 337A, or H242A H331A H337A mutations. Strains were iron-limited ~7 h in LB with 200 μM DIP prior to outer membrane fractionation. Arrow indicates ~80 kDa Hma band.

Table 4-3. Ability of Hma site-directed mutants to mediate hemin utilization.

Strain[a]	Minimum conc. (μM) required to support growth[b]	
	$FeCl_2$	Hemin
pGEN	10	>100
wildtype Hma	10	25
H242A	10	25
H331A	10	25
H337A	10	25
H242A H331A	10	25
H242A H337A	10	25
H331A H337A	10	25
H242A H331A H337A	10	50
Y126A	10	>100

[a] *E. coli* K12 containing pGEN vector alone or p_{native}*hma* with indicated mutation
[b] growth on sorbitol-MacConkey agar supplemented with 350 μM DIP

Both *chuA* and *hma* contribute to CFT073 heme utilization.

In addition to Hma, *E. coli* CFT073 contains another heme or hemoglobin receptor, ChuA. To examine the contribution of each of these proteins to heme utilization by CFT073, a *hma chuA* isogenic mutant was constructed. Together with the single mutants, the ability of *hma chuA* to utilize heme as a sole iron source was assessed. Wildtype, the single mutants, and the *hma chuA* double mutant all required the same concentration of $FeCl_2$ for growth (Table 4-4) and had similar growth rates under iron-limiting and iron-replete conditions (Appendix C, Fig. A-2A). However, the *chuA* mutant required a higher concentration of hemin as compared to either wildtype or the *hma* mutant (25 μM as compared to 1 μM) and the double mutant was unable to grow even with the highest concentration of hemin (100 μM). This defect could be complemented by expression of *hma* from p_{native}*hma*, but not with the pGEN empty vector. Therefore, although it appears that *chuA* contributes more to heme uptake, either *chuA* or *hma* is sufficient for hemin utilization by CFT073 *in vitro*.

Table 4-4. Ability of CFT073 heme uptake mutants to utilize hemin as a sole iron source.

Strain	Minimum conc. (μM) required to support growth[a]	
	$FeCl_2$	Hemin
CFT073	10	1
hma	10	1
chuA	10	25
hma chuA	10	>100
hma chuA (pGEN)	5	>100
hma chuA (p_{native}*hma*)	25	25

[a] growth on sorbitol-MacConkey agar supplemented with 350 μM DIP

Heme uptake is required for efficient kidney colonization.

To identify the role of heme uptake for urinary tract colonization by CFT073, as well as define the relative contributions of *hma* and *chuA* to this process *in vivo*, the heme receptor mutants were tested in the mouse model of UTI. After a 72 hour co-infection with a 1:1 mixture of 10^8 CFU of the *chuA* and *hma* mutants, the *chuA* mutant was found at significantly lower levels in the kidneys of infected animals ($P<0.05$) (Fig. 4-8A). This demonstrates that, in the kidney, the strain lacking *hma* was better able to compete for heme than was the *chuA* mutant, indicating that the ChuA receptor contributes more to heme uptake *in vivo* than does Hma. When the *chuA* and *hma* mutants were independently inoculated into separate mice, these strains colonized the bladder and kidneys to the same extent as wildtype (Fig. 4-8B). However, the *hma chuA* double mutant was found at significantly lower levels in the kidneys of infected mice during independent infection ($P=0.023$), suggesting the importance of an intact heme uptake system for kidney colonization. While there was only an approximately one log difference between the median CFU/g kidney tissue of *hma chuA* and wildtype, a significant number of mice inoculated with *hma chuA* failed to produce a kidney infection (7/20 *hma chuA*-inoculated mice were uninfected as compared to only 1/20 mice uninfected that were wildtype-inoculated, $P=0.044$). Together, these data demonstrate the requirement of a heme receptor (either *hma* or *chuA*) for efficient kidney colonization by CFT073, as well as provide evidence that heme is an essential source of iron for this pathogen during kidney infection.

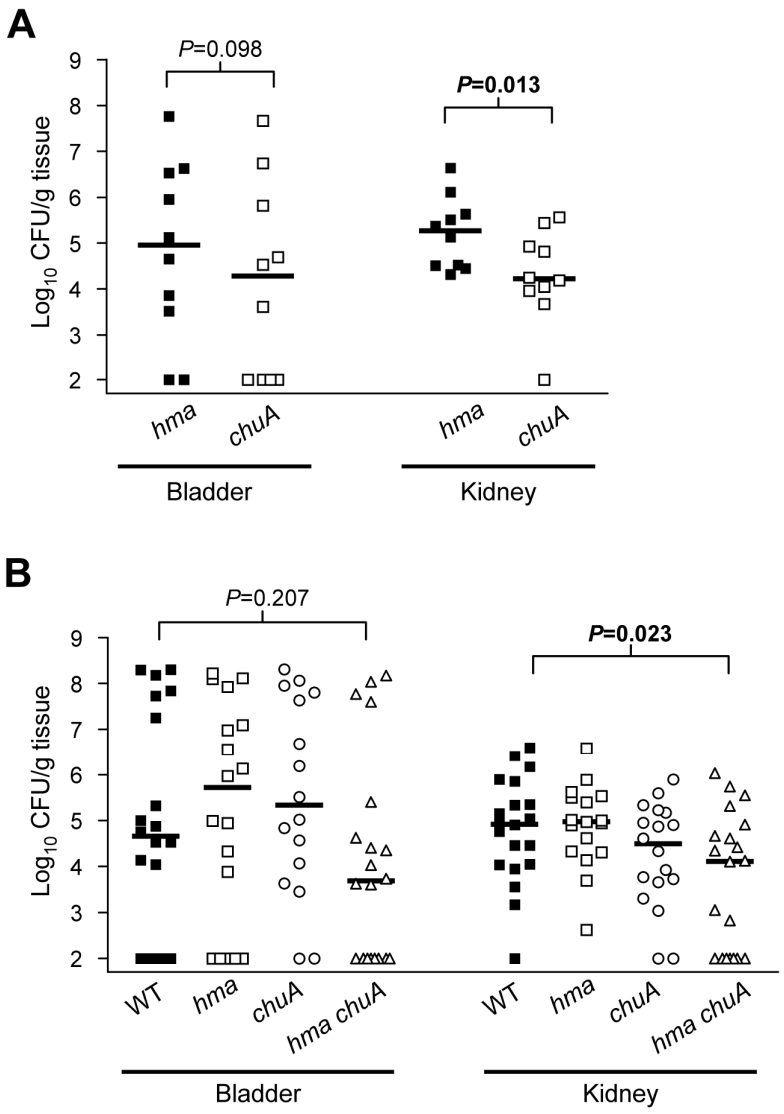

Figure 4-8. Heme uptake mutants in a mouse model of UTI. (A) 72 hour CBA/J mouse co-infection with 10^8 CFU mixture of *hma* (solid symbols) and *chuA* (open symbols) mutants. Symbols represent CFU/g tissue in individual animals and bars indicate the median (*n*=10). **(B)** 72 hour independent infections with 10^8 CFU of wildtype CFT073 (filled squares), *hma* (open squares), *chuA* (open circles), or *hma chuA* (open triangles) mutants (*n*=20).

chuA* and *hma* are differentially expressed *in vivo.

As *chuA* and *hma* each encodes a heme receptor, we were surprised to note the phenotypic differences of these two mutants, both *in vitro* (Table 4-4) and *in vivo* (Fig. 4-8A). While *chuA* appears to contribute more to heme utilization, both receptors have similar affinities for hemin under the conditions tested here (Fig 4-5). To examine potential differences in expression, we compared transcript levels using real-time qPCR of *chuA* and *hma* from bacteria cultured *in vitro* or isolated *in vivo*. In the presence of an iron chelator *in vitro*, relative transcript levels of *chuA* were ~10-fold higher than those of *hma* (Fig. 4-9A). As compared to LB-cultured CFT073, *chuA* transcript increased an average of 99-fold when bacteria were cultured under iron-limitation, while *hma* was just 8.8-fold upregulated (Fig. 4-9B). In the presence of excess $FeCl_2$, transcripts for both genes were slightly decreased (-1.3-fold as compared to LB). Similarly, bacterial transcripts isolated from the urine of CFT073-infected mice showed that *chuA* levels were nearly three-fold higher than *hma* transcript levels (Fig. 4-9C). Expressed relative to transcript levels from LB-cultured bacteria, *chuA* was upregulated an average of 67-fold *in vivo*, while *hma* increased 24-fold as compared to LB-cultured bacteria (Fig. 4-9D). Urine samples from PBS-infected control mice did not show significant amplification (data not shown). Together these data indicate that, under the iron-limiting conditions found *in vivo*, *chuA* is expressed more highly than *hma*. This difference in expression level is likely an important factor in the relative contributions of these two receptors to heme utilization.

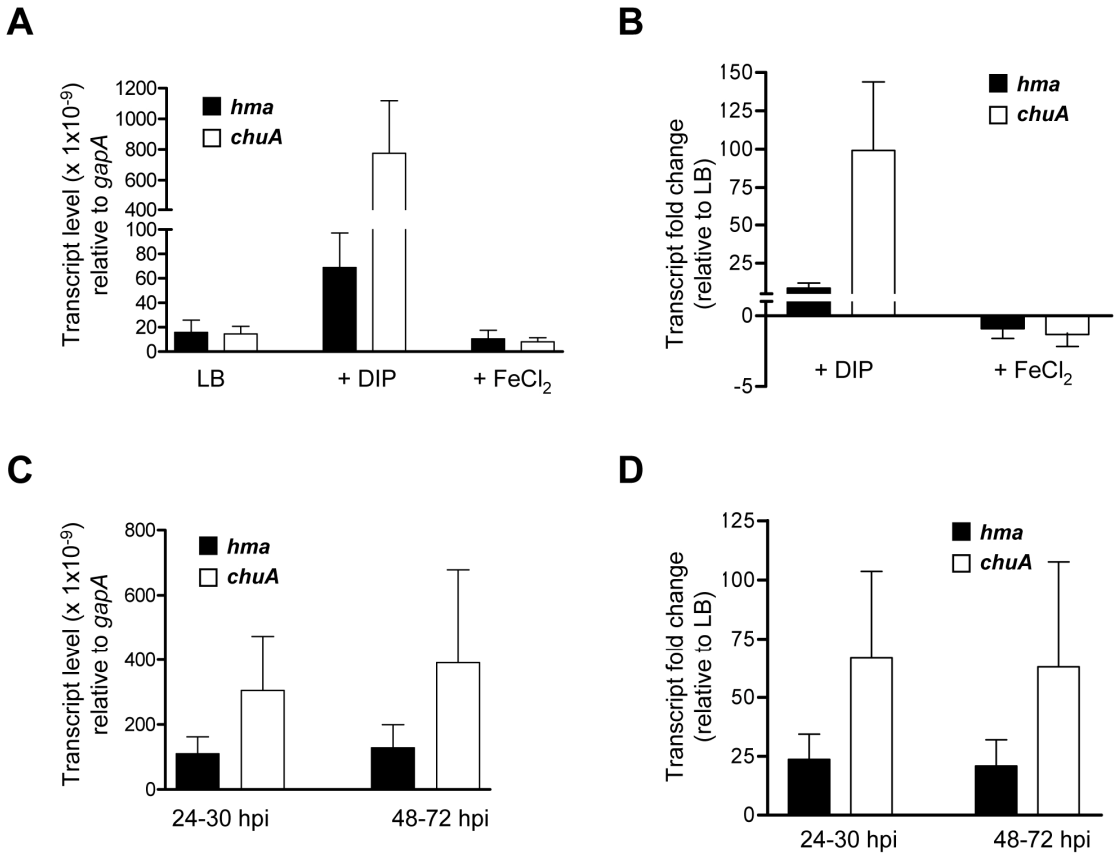

Figure 4-9. Expression of *chuA* and *hma* by real-time qPCR. (A) Relative transcript levels of *hma* (solid bars) and *chuA* (open bars) in *E. coli* CFT073 cultured in LB, or LB supplemented with 200 μM DIP or 10 μM $FeCl_2$, normalized to *gapA* transcript. Bars represent the mean of four independent experiments. **(B)** Data from (A) shown as fold change relative to transcript levels in LB. **(C)** Relative transcript levels of *hma* (solid bars) and *chuA* (open bars) transcript levels in the urine of CBA/J mice transurethrally inoculated with 10^8 CFU of *E. coli* CFT073, normalized to *gapA* transcript. Bars represent the mean of triplicate samples, each sample containing urine collected from five animals (*n*=15) during the timepoints indicated (24-30 hpi, right; 48-72 hpi, left). **(D)** Data from (C) shown as fold change relative to transcript levels in LB.

Heme uptake is required for bacteremia.

The *hma* single mutant was previously shown to be outcompeted by wildtype CFT073 in the spleens of infected mice (Fig. 4-2C). Because spleen infection requires prior kidney colonization, this result could reflect the reduced numbers of mutant bacteria in the kidneys or may indicate the requirement of *hma* for complete fitness during bacteremia. To investigate the requirement of heme uptake for UPEC survival in the bloodstream, a mouse model of bacteremia was utilized. Mice were intravenously injected with a mixture of *hma chuA* mutant and wildtype CFT073, with the total inoculum equaling 1×10^6 CFU. At 24 hpi, the heme uptake-deficient mutant was significantly outcompeted by wildtype (P=0.002), indicating that heme acquisition contributes to bacteremia (Fig. 4-10A). Groups of mice were also independently injected with either wildtype or mutant. Although the spleens of *hma chuA*-infected animals displayed only a half log lower median bacterial load than did those of wildtype-infected mice, this difference was nonetheless significant (P=0.0079) (Fig. 4-10C). While the biological significance of this modest difference is unclear, as the *hma chuA* strain was clearly able to survive relatively well in the absence of a competing strain, these data indicate that heme acquisition indeed plays a role during UPEC survival in the bloodstream.

During UTI, bacteremia occurs relatively late in the infection process and must be preceded by successful survival, if not colonization, in the bladder and kidneys. Thus, by the time UPEC reaches the bloodstream, is has likely been persisting *in vivo* for days. In light of this, we speculated that culturing these strains under iron limitation prior to inoculation would more closely mimic the *in vivo* environment encountered prior to their

entry into the bloodstream during a natural infection. When cultured under iron limitation, the *hma chuA* strain was more outcompeted by wildtype than when both strains were cultured in iron-replete medium (P=0.0288) (Fig. 4-10B). Furthermore, wildtype CFT073 reached significantly higher levels in the spleen when it was cultured under iron-limiting conditions prior to inoculation (P=0.0185) (Fig. 4-10A). These data suggest that the importance of heme uptake during the bacteremic phase of UTI may be even greater, given that the preceding environment of the urinary tract is iron-limiting.

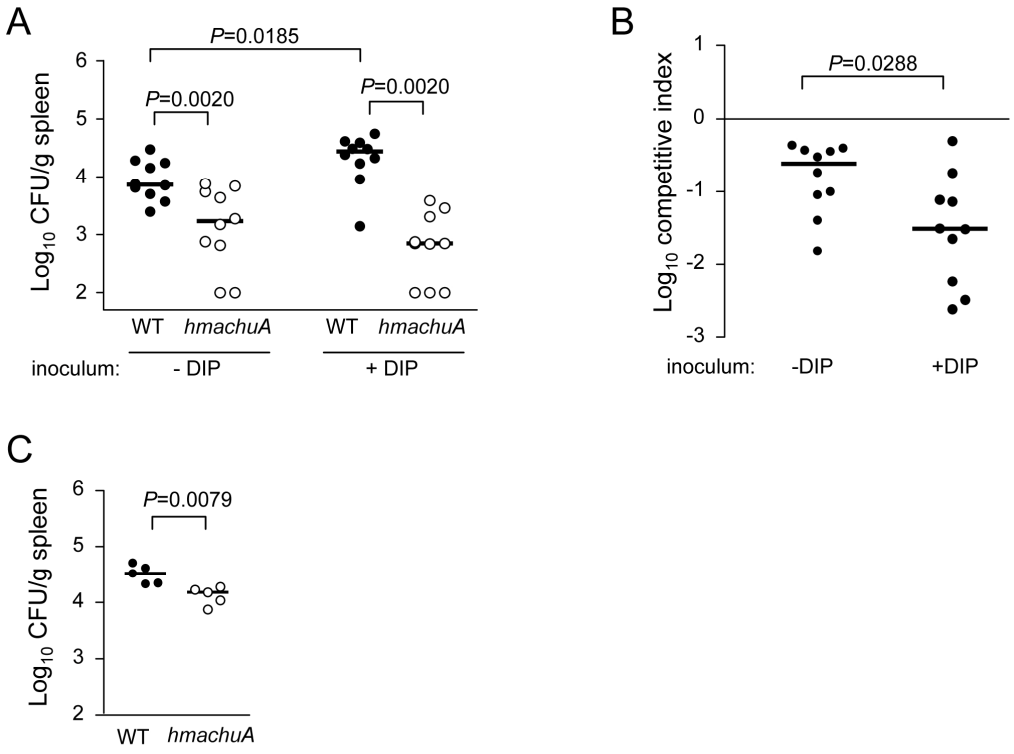

Figure 4-10. ***hma chuA*** **heme utilization-deficient mutant in a mouse model of bacteremia.** **(A)** Spleen colonization 24 hpi in mice i.v. co-inoculated with a mixture (10^6 CFU/mouse) of wildtype CFT073 (WT) and an *hma chuA* double mutant strain. Prior to infection, inocula were cultured in LB in either the absence (-DIP) or presence (+DIP) of 200 μM 2'2-dipyridyl. **(B)** Competitive indices (CFUmutant/CFU WT) of co-infection experiment shown in (A). **(C)** Spleen colonization 24 hpi in mice independently i.v. inoculated with either wildtype or *hma chuA* mutant bacteria (LB-cultured).

Discussion

In this study we establish that Hma functions as a heme receptor in uropathogenic *E. coli* and heme acquisition is necessary for upper urinary tract colonization by this pathogen. Expression of *hma* promotes TonB-dependent hemin utilization by a laboratory strain of *E. coli* and confers an ability to bind hemin. Furthermore, purified Hma binds hemin with high affinity (K_d=8 μM). In UPEC, Hma functions independently of ChuA to mediate heme uptake and a strain lacking both of these receptors is deficient for kidney colonization in a mouse model of UTI. Additionally, we demonstrate that, unlike the bacterial heme receptors characterized to date, Tyr126 is required for Hma-dependent hemin utilization. By protein sequence alignment, Hma clusters with TonB-dependent receptors from marine bacteria and appears more distantly related to HemR, ChuA, and HmuR. Therefore, we suggest that Hma represents a novel class of heme receptors that is distinct from the HemR family of bacterial heme receptors.

Previous structure-function studies identified two His residues conserved among heme receptors of Gram negative bacteria that are required for heme uptake (18, 24, 118). Corresponding to His128 and His461 in *Y. enterocolitica* HemR, these residues are absent from Hma. As His128 and His461 are predicted to be located on the extracellular face of the N-terminal plug domain and on an extracellular loop, respectively (24), we predicted the structure of Hma to identify putative heme-binding residues in these locations. Tyr126 was predicted to reside extracellularly on the N-terminal plug domain and in amino acid sequence alignments, Hma Tyr126 aligned with HemR His128. Here we show that Tyr126 is required for Hma-mediated hemin utilization. While tyrosine is known to occasionally coordinate heme ligands (6), the requirement of a Tyr residue at

this location represents a significant difference between Hma and the previously-studied heme receptors and provides further evidence that *hma* may have evolved independently of ChuA and HemR.

His242, His331, and His337 are all located on putative extracellular loops of Hma; however they are not required for receptor function, either alone or in combination. As the Y126A mutant retained its hemin-binding activity, it is likely that additional residue(s) function in binding/transport and compensated for the loss of Tyr126 in this mutant. A number of Tyr residues reside on the putative extracellular loops and additional work is needed to determine if they, or an alternative residue, participate with Tyr126 in Hma-mediated heme uptake.

We estimated the affinity of Hma for hemin to be in the micromolar range (K_d=8 μM). Observed hemin binding to IutA (K_d=90 μM) likely represented binding to the His_6 tag present on the purified proteins or other nonspecific interactions. While the affinities of most heme receptors, including HemR, are unknown, our result is similar to the K_d=5 μM and K_d=24 μM measured for *S. marsescens* HasR and *P. gingivalis* HmuR receptors, respectively (85, 141).

As for most bacterial pathogens, iron acquisition within the iron-limited host is crucial to the virulence of UPEC. A *tonB* mutant was severely attenuated *in vivo*, indicating that TonB-dependent systems are required for UPEC colonization (187). While no single uptake system has been found to be necessary for colonization, disruption of *chuA*-mediated heme uptake (187) or enterobactin (87), salmochelin (160), or aerobactin (187) siderophore uptake resulted in outcompetition by a wildtype strain *in vivo*. Thus, considerable functional redundancy exists among these systems.

Although siderophore and heme uptake systems contribute to the fitness of UPEC, the role of specific iron sources in the host remains largely unknown. Here we show that a CFT073 strain deficient for heme utilization is unable to colonize the murine kidney to wildtype levels. This represents the first evidence that heme is a required source of iron for UPEC *in vivo*. The importance of heme uptake in the kidney is further supported by our and others' findings that both the *hma* and *chuA* mutants are outcompeted by wildtype CFT073 in the kidneys of infected mice during co-infection experiments (187). Consistent with our data indicating that *hma* contributes more to virulence at later timepoints post-infection, Torres and colleagues found that a *chuA* mutant was only slightly outcompeted by wildtype in the kidneys at 48 hpi. This suggests that *hma* and *chuA* may be important for UPEC persistence in the urinary tract. Finally, it is interesting to note that although ChuA appears to contribute more to heme uptake *in vivo*, Hma alone is sufficient for kidney colonization, as the *chuA* mutant independently colonized the kidneys at levels similar to wildtype.

Both *in vitro* and *in vivo*, we observed a striking difference between the *hma* and *chuA* mutants, with *chuA* appearing to play a greater role in heme utilization. We propose that this difference is due, at least in part, to the relative expression levels of the two receptors. As compared to bacteria cultured in rich medium, bacteria cultured under iron limitation or isolated from the urine of infected mice upregulated *chuA* to a greater extent than *hma*. The qPCR results shown here are replicated at the protein level, as Fig. 2-3 shows significantly more ChuA than Hma in outer membrane preparations from bacteria cultured under iron limitation *in vitro*. Furthermore, quantitative proteomic profiling of CFT073 cultured in human urine measured an approximately two-fold higher

induction of ChuA than Hma (2). Thus, a *chuA* mutant would likely contain significantly less heme receptor on its surface than would an *hma* mutant.

Additionally, we cannot discount the possibility that other functional differences exist between Hma and ChuA. Although the two receptors have identical affinities for heme *in vitro*, they may differ in their abilities to extract heme from hemoproteins. The ShuA receptor of *S. dysenteriae*, for example, transfers heme from hemoglobin ~10^5 times more efficiently than it associates with free heme (24). This may be especially relavant *in vivo*, where free heme is likely unavailable and may not represent the physiological substrate of Hma and ChuA.

While heme uptake is critical for UPEC to colonize the murine kidney, it appears to play a lesser role in bladder colonization. In both co-infection and independent infection experiments, all heme uptake mutants infected the bladder to levels that did not significantly differ from wildtype. Similarly, the *chuA* mutant, outcompeted by the *hma* mutant in the kidneys of infected mice, colonized the bladders effectively in the presence of the competing strain. However, we show that both *hma* and *chuA* are highly upregulated in urine from infected mice, indicating that they are expressed in the iron-limited bladder. Instead, we hypothesize that non-heme sources of iron are more prevalent in the bladder and therefore more important during UPEC colonization of this site.

In addition to its role in kidney infection, we show that heme acquisition contributes to the fitness of UPEC during bacteremia. Our finding that the *hma chuA* mutant was only slightly attenuated in independent i.v. challenge is generally in agreement with results showing that *chuA* is not required by a neonatal meningitis strain

for bacteremia in a rat model (135). This suggests that, during ascending UTI, heme acquisition likely plays a greater role in kidney colonization by UPEC than for its survival in the bloodstream.

CHAPTER 5

RELATIVE CONTRIBUTIONS OF HEME AND SIDEROPHORE IRON ACQUISITION DURING MURINE UTI

Introduction

The entire length of the urinary tract is iron-limiting, but the dramatic anatomical differences from urethra to kidneys likely offer substantial differences in iron source availability. Protein-bound heme is a predominant form of intracellular iron, while lactoferrin and transferrin are abundant at mucosal surfaces. Transferrin and heme-containing carrier molecules like hemoglobin, hemopexin, and haptoglobin dominate the bloodstream and highly vascularized organs like the kidneys.

While both heme and siderophore iron acquisition contribute to the ability of UPEC to infect the urinary tract, evidence exists for distinct roles of specific systems. Heme uptake via ChuA was shown to play a role in bladder and kidney colonization (187), as well as in the formation of intracellular bacterial communities within superficial bladder epithelial cells (151). However, while mutants lacking the salmochelin receptor IroN were outcompeted by the wildtype strain in the bladders of infected mice, kidney colonization was unaltered (160). Conversely, a mutant deficient in both enterobactin and aerobactin synthesis was only modestly attenuated in the bladder, but showed a one-log reduction in kidney colonization (187). Not only do certain iron acquisition

systems appear more important than others for UPEC virulence, differences exist between mutant fitness in specific urinary tract organs.

In Chapter 4, we identified a novel heme receptor and demonstrated that heme utilization is a required mechanism for UPEC kidney colonization. However, the contribution of heme uptake *in vivo* within the context of siderophore iron acquisition is unclear. In this study, we assessed the relative contributions of heme and siderophore receptors to UTI and propose that aerobactin and heme uptake play critical roles at specific sites of the urinary tract.

Materials and methods

Mutant construction.

Mutants listed in Table 5-1 were constructed in *E. coli* CFT073 as described in Chapter 4 using the λ Red recombinase system (35). Linear PCR products containing kanamycin or chloramphenicol resistance cassettes were used to replace >80% of the *iutA*, or *iroN* genes. To remove the kanamycin resistance cassette from Δ*iutA*::*kan*, Flp recombinase was expressed from the temperature-sensitive pCP20. Δ*iutA*::*kan* cells transformed with pCP20 were recovered 1 h at 30°C and selected on ampicillin at 30°C overnight. Colonies were passaged three times on LB agar lacking antibiotic at 37°C to facilitate loss of pCP20. Removal of the kanamycin resistance cassette was confirmed by PCR and antibiotic sensitivity. Following cassette removal, an approximately 80 bp scar remained.

RNA isolation and qPCR.

RNA was isolated from wildtype and mutant strains cultured in LB containing 200 μM 2'2-dipyridyl in the presence or absence of 10 μM hemin to OD_{600}=0.5-0.6, cDNA synthesized, and qPCR performed as described in Chapter 4. Primer sequences are listed in Table 5-2.

CBA mouse model of ascending UTI.

CBA/J mice (*n*=10) were challenged as described for co-infection studies in Chapter 4, except that three or four mutant strains were mixed at equal ratios and co-inoculated. All CFU/g determinations were made at 72 hpi and mutants differentiated by

plating on LB containing appropriate antibiotics. For each mutant in each organ, percent total CFU/g tissue was calculated by dividing the CFU/g of the indicated mutant by the sum of the CFU/g levels of all mutants for the particular organ.

Statistical analysis.

Co-infection data were analyzed using the Wilcoxon matched-pairs signed-ranks test with GraphPad® InStat version 3.05.

Table 5-1. CFT073 siderophore receptor mutant strains.

E. coli strain	Description[a]	Reference or source
Δ*iutA*	CFT073 Δ*iutA*::FRT; $Kan^{Sensitive}$	This study
Δ*iroN*	CFT073 Δ*iroN*::*kan*; Kan^{r}	This study
Δ*fepA*	CFT073 Δ*fepA*::*cat*, Cam^{r}	This study

[a] Kan, kanamycin; Cam, chloramphenicol; FRT, Flp recombinase target site (scar)

Table 5-2. Real-time qPCR primer sequences[a].

Gene	Forward	Reverse
gapA	AAGTTGGTGTTGACGTTGTCGC	AGCGCCTTTAACGAACATCG
hma	ATCGTTCGGCAAGCAACCTTTG	ATGCGGATTTGTTTACGGCCTG
chuA	AGCGTGTTGAGATTGTTCGC	AAACCACTGCTTTGTCCTTCCTGC
iutA	AAAGAGCTGAAAGACGCACTGG	TGTCGGAACGTGAAGAGTTGAG
iroN	ATTACCAAACGTCCCACCAACG	AAACGCGTGGTAAGAGCATCAC
ireA	TCCTGTGGAAGCAATTGAACGC	AAGTACGCCATCCCATGTTTGG
fepA	ATTGATATTCGCGGCATGGGTC	ACGTTCAATCATTTCAGGCGGC
fhuA	ACGGCCAAAGCCAGAATAAC	TTACCGTAAAGCACGGAAACCG
btuB	TTGCGCCAACTACTGTTGTGAC	ACATGACTGGCATTTGTACCGC

[a] all sequences listed 5'→ 3'

Results

Iron receptor expression is unaltered in mutant strains.

To examine the contributions of heme and siderophore uptake to UTI, isogenic *iutA*, *fepA*, and *iroN* mutants were made. Previous work from our laboratory has shown that expression of these and other receptors is upregulated under iron-limiting conditions, both *in vitro* (2) and *in vivo* (171). To determine whether this expression was dysregulated in the receptor mutant strains, transcripts isolated from strains cultured under iron limitation were measured by real-time qPCR. None of the receptor genes examined differed by more than two-fold from wildtype expression levels (Fig. 5-1A). Expression of *ireA* and *iroN* appeared slightly elevated in the *iutA* mutant. However, as expression of *btuB*, the vitamin B12 receptor included as a control, was also slightly elevated, these differences are not significant. Receptor expression was also measured in *hma*, *chuA*, and *hma chuA* mutant strains (Fig. 5-1B). In either the presence or absence of excess hemin, receptor transcript levels were unaltered in the mutant strains. Thus, deletion of one or more outer membrane iron receptors does not affect transcription of the remaining receptors.

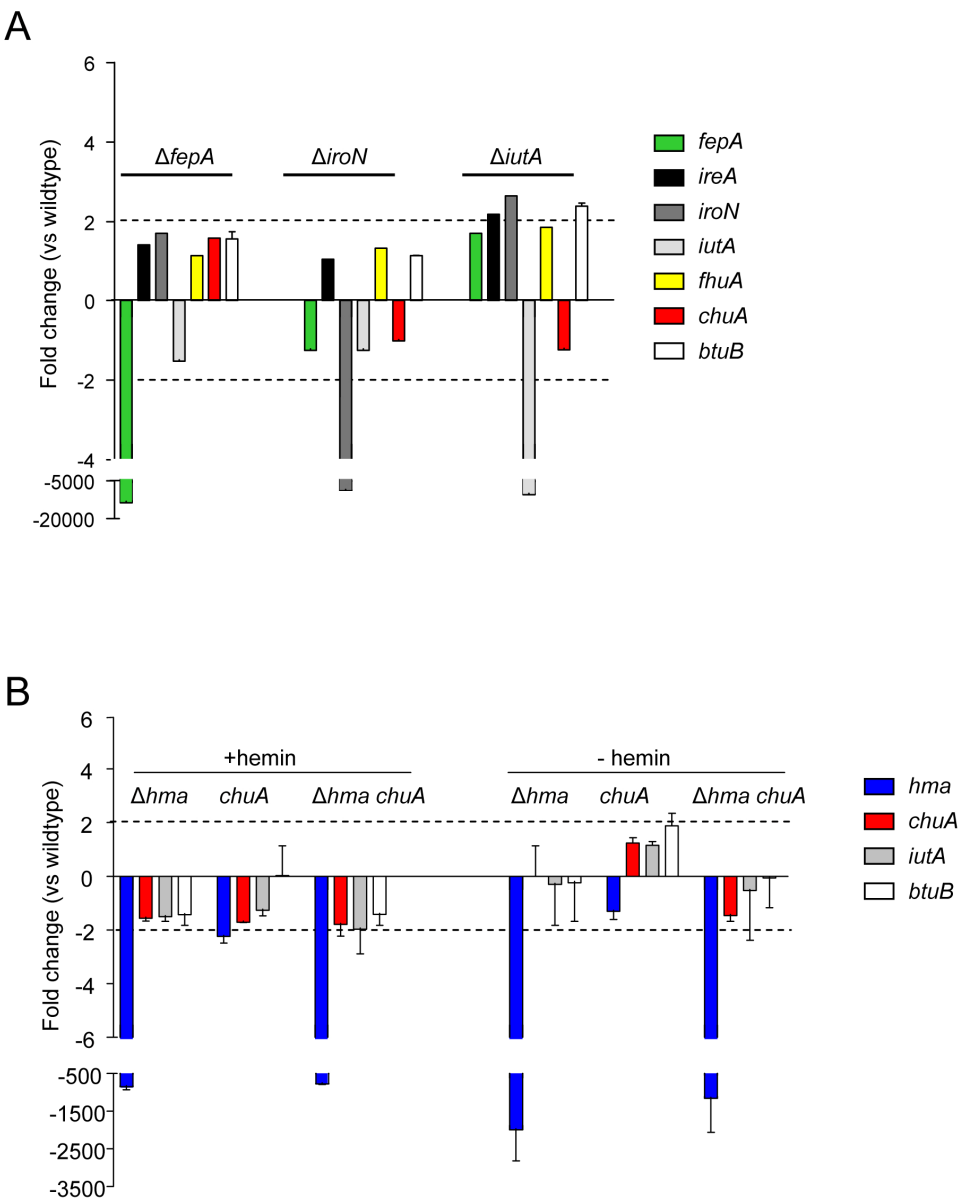

Figure 5-1. Heme and siderophore receptor expression levels by qPCR in receptor mutant strains. (A) RNA was isolated from wildtype and the indicated mutant strains cultured in LB containing 200 μM DIP. Transcript levels were measured in cDNA preparations from each strain, normalized to *gapA*, and shown as fold-change relative to wildtype levels. Mean and standard deviations of duplicate samples are shown. **(B)** Experiment as in (A), except strains were cultured in LB containing 200 μM DIP either in the presence (+hemin) or absence (-hemin) of 10 μM hemin.

***iutA* contributes more than *fepA* or *iroN* to bladder colonization.**

UPEC strain CFT073 secretes three distinct siderophores, aerobactin (*iuc*), enterobactin (*ent*/*fep*), and salmochelin (*iro*). To establish the relative contribution that uptake of each of these siderophores has to UTI, a three-way *in vivo* competition assay was used. Mutants defective in each siderophore receptor, Δ*iutA*, Δ*iroN*, and Δ*fepA*, were mixed at a 1:1:1 ratio and co-inoculated into CBA/J mice. At 72 hpi, Δ*iutA* was significantly outcompeted by Δ*iroN* in the bladders of infected animals (P=0.0371) (Fig. 5-2). Although Δ*fepA* comprised just 20% of the total CFU/g bladder, it was not significantly outcompeted by Δ*iroN*, which constituted almost 70% of the total CFU/g bladder. Similarly, while the median percent Δ*fepA* CFU/g kidney was approximately 60%, it did not significantly outcompete either Δ*iutA* or Δi*roN*. Examination of colonization levels in individual mice revealed that, while these median values suggested that differences existed, the seemingly defective mutants were not consistently outcompeted and occasionally even outcompeted the seemingly more fit strain. These data indicate although Δ*iutA*, Δi*roN*, and Δ*fepA* are equally fit in the kidney, aerobactin uptake via IutA contributes more strongly to bladder colonization by CFT073.

Heme uptake contributes most for kidney infection.

To determine the relative contribution that heme acquisition makes to the ability of CFT073 to colonize the urinary tract, a four-way *in vivo* competition assay was performed. A mixture of the previously examined Δ*iutA*, Δ*fepA*, and Δ*iroN* mutants, along with the heme utilization-defective *hma chuA* double mutant was co-inoculated into the bladders of mice. After a 72 h infection, the *iutA* mutant was outcompeted by all

strains in the bladder ($P<0.01$) and the *hma chuA* mutant was outcompeted in the same organ by both Δ*iroN* and Δ*fepA* ($P= 0.002$ and $P=0.0039$, respectively) (Fig. 5-3). In the kidneys, the *hma chuA* mutant was again outcompeted by both Δ*iroN* and Δ*fepA* ($P=0.0078$ and $P=0.0117$, respectively), while Δ*iutA* levels did not significantly differ from any strain. Together, these findings demonstrate again that aerobactin uptake via IutA contributes appreciably to UPEC colonization of the bladder. However, compared with IutA-, IroN-, and FepA-mediated siderophore uptake, heme acquisition via Hma and ChuA is the most critical mechanism of iron uptake during kidney colonization.

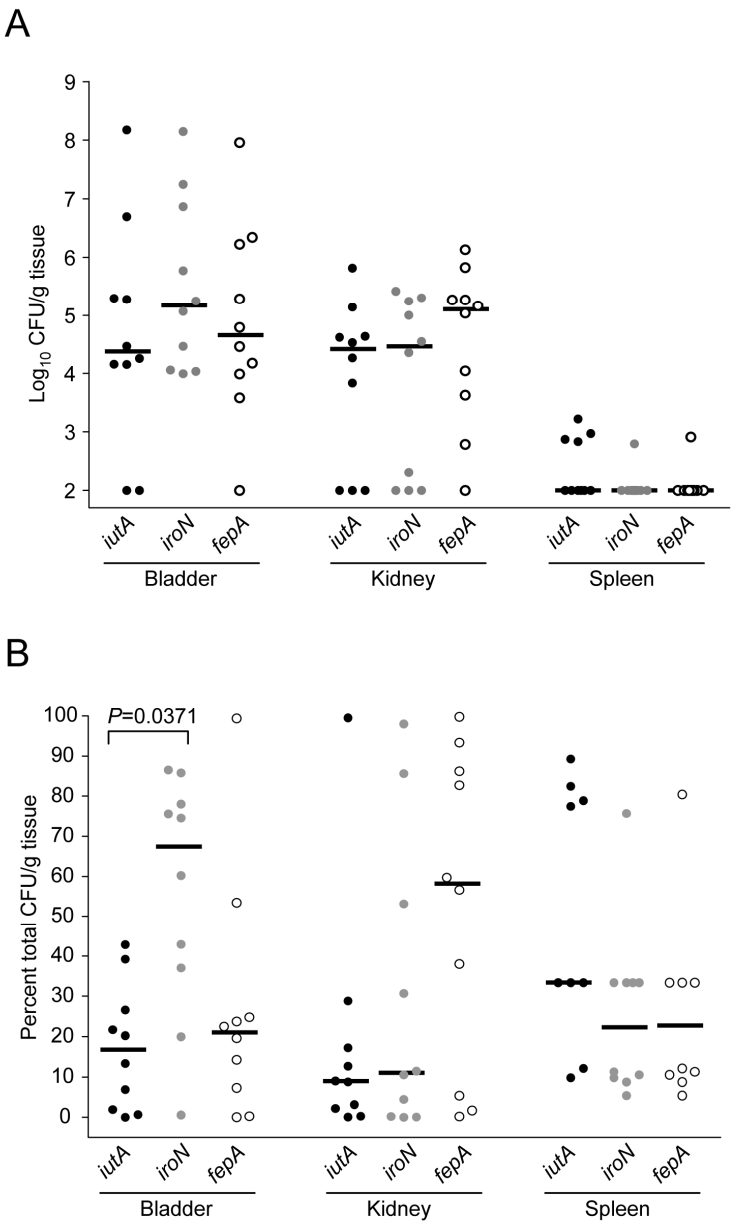

Figure 5-2. Siderophore receptor mutants in a murine co-infection model of UTI. Mice were transurethrally inoculated (10^8 CFU/mouse) with an equal mixture of Δ*iutA*, Δ*iroN*, and Δ*fepA* mutant strains. **(A)** Colonization levels at 72 hpi, shown as CFU/g tissue. **(B)** Relative colonization of each mutant, shown as percent total organ CFU/g. For (A) and (B), bars indicate the median.

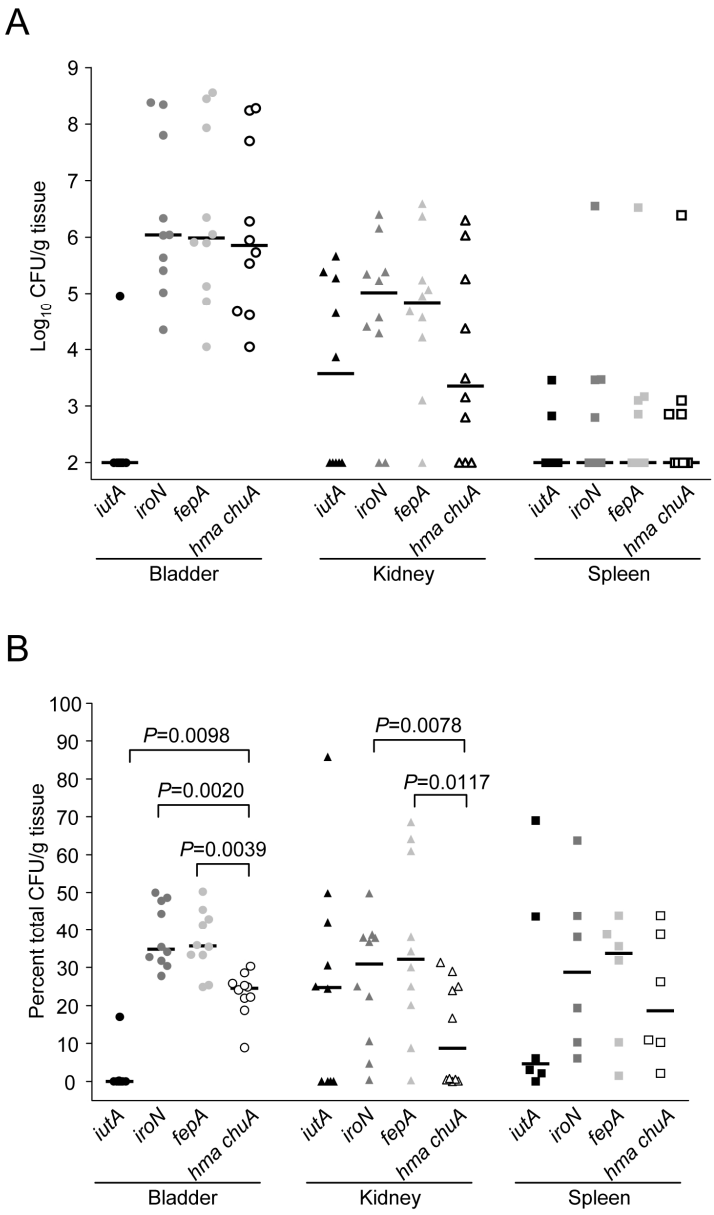

Figure 5-3. Heme uptake and siderophore receptor mutants in a murine co-infection model of UTI. Mice were transurethrally inoculated (10^8 CFU/mouse) with an equal mixture of Δ*iutA*, Δ*iroN*, Δ*fepA*, and Δ*hma chuA* mutant strains. **(A)** Colonization levels at 72 hpi, shown as CFU/g tissue. **(B)** Relative colonization of each mutant, shown as percent total organ CFU/g. For (A) and (B), bars indicate the median.

Discussion

Iron acquisition via heme and siderophore uptake is a critical process for UPEC colonization of the iron-limited urinary tract. However, previous examinations of specific iron uptake systems have generally assessed these systems' contribution to virulence independently of one another. Here we evaluate the relative contributions of FepA-, IroN-, and IutA-mediated siderophore uptake and ChuA- and Hma-mediated heme acquisition to UPEC virulence. We show that mutant strains lacking these receptors do not have altered expression of their remaining iron receptors and demonstrate that *iutA* and *hma chuA* mutants are outcompeted by strains lacking *fepA* or *iroN*. Furthermore, the data presented support prominent roles for aerobactin and heme uptake during UPEC colonization of the bladder and kidneys, respectively.

At least for the receptors tested here, there appears to be no transcriptional coordination between iron receptor expression. Disruption of one or more receptors does not result in measurable compensatory upregulation of other receptor genes. This is not unexpected, as these iron systems show characteristics of Fur regulation (2). However, it is unlike another class of surface molecules, the fimbriae, which are coordinately regulated in UPEC strain CFT073, marked by transcriptional upregulation of one fimbrial species upon genetic inactivation of another (172).

By considering the results of both co-infection experiments shown here, a model of iron receptor *in vivo* "importance" can be proposed (Fig. 5-4). In both co-challenges, Δ*iutA* was the least fit strain in the bladder, suggesting that the aerobactin receptor is the most critical at this site. The *hma chuA* mutant was also outcompeted in the bladder, but only by Δ*iroN* and Δ*fepA*, suggesting that it is of next importance. Finally, only the *iroN*

mutant outcompeted Δ*iutA* in the three-way co-challenge, indicating this strain was the most fit and, therefore, the salmochelin receptor of least importance in the bladder. Similar logic can be used to model kidney infection, with Hma- and ChuA-mediated heme uptake having the greatest impact, followed by IutA function. Because there were no differences between *iroN* and *fepA* mutants in the kidney, these two receptors cannot be distinguished at this site. Thus we propose that IutA and the heme receptors contribute most significantly to urinary tract colonization by UPEC strain CFT073, while salmochelin utilization by IroN plays a more subtle role.

This model is supported by previous studies examining UPEC siderophore and heme receptor function *in vivo*. In competition with a wildtype strain, an *iutA* mutant was outcompeted strongly in both the bladder and kidneys of infected mice (187). Unlike our findings, which suggest that IutA plays a greater role in bladder colonization, Torres and colleagues were unable to recover *iutA* mutant from infected kidneys. However, this difference is likely due to the relative enhanced fitness of Δ*iutA* in this study during competition against other mutants, as compared to a robust wildtype strain. Furthermore, ChuA has been shown to play roles both in bladder and kidney colonization (151, 187). A more subtle role for IroN has also been shown, as an *iroN* mutant was only slightly outcompeted by a wildtype UPEC strain in the bladders of infected mice and showed no defect in kidney colonization (160).

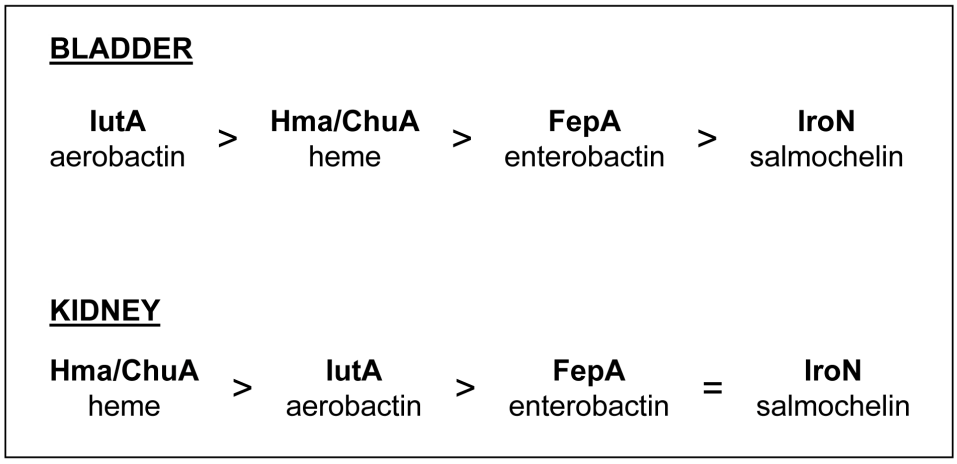

Figure 5-4. Model of heme and siderophore receptor contribution to urinary tract colonization by UPEC strain CFT073. Receptors are listed left to right, from greatest to least contribution to bladder (above) and kidney (below) colonization. >, receptor(s) to the left of symbol contribute more to colonization than receptor(s) to the right of symbol. =, contribution of receptors on either side of symbol cannot be distinguished.

A caveat to these studies is the presence of known and putative outer membrane receptors that may have overlapping specificities for the siderophores examined here. Catecholate siderophores especially are known to utilize multiple receptors with overlapping specificities. For example, in addition to the classical FepA receptor, Iha is also known to transport both enterobactin and its degradation product dihydroxybenzoylserine (DHBS) (113). Although DHBS is transported most efficiently by *E. coli* Cir and Fiu, it is also imported to a lesser extent by FepA (71). In *Salmonella*, while IroN is the major receptor for salmochelin, low amounts of this siderophore or its degradation products are also transported by Cir and FepA (73). Moreover, for other UPEC iron-responsive outer membrane receptors such as IreA (159) and FitA (144), a substrate has yet to be identified and could potentially overlap with the receptors tested. Therefore, the importance of enterobactin and salmochelin cannot be specifically addressed here, only the contributions of the IroN and FepA receptors.

A number of biological factors likely contribute to the differences observed *in vivo* between each iron receptor mutant. Host iron source availability presumably plays a role, especially with respect to the importance of ChuA and Hma. Heme is the largest source of iron *in vivo*, especially in the blood-rich kidneys; thus it is not surprising that uptake of this molecule is critical. Additionally, differences may exist in siderophore stability or synthesis at distinct urinary tract environments. Genes in the *iro* locus are maximally transcribed at high pH in *S. typhimurium* (49) and more secreted salmochelin is detected from *E. coli* cultured at high pH, while aerobactin production or stability peaks at slightly acidic pH (195). The pH of urine varies, but transcriptional profiling of UPEC suggests that it may be under acid stress *in vivo* (171). Receptor expression levels

may also contribute to the observed phenotype differences. By both transcript and protein measurements, the most important receptors to urinary tract colonization, *iutA* and *chuA* are also the most highly expressed outer membrane iron receptors (2, 171).

Approximately 91% of UPEC isolates synthesize and utilize a fourth siderophore, yersiniabactin (88, 90). Because strain CFT073 does not produce this chelator (although it expresses an intact yersiniabactin receptor) (19, 23), further work in a yersiniabactin-positive strain will be necessary to assess its relative contribution to virulence in the context of heme acquisition.

CHAPTER 6

CONCLUSIONS AND FUTURE DIRECTIONS

Summary of Results

The studies presented here address both the use of UPEC outer membrane iron receptors as targets for immunization, as well as the contribution of these proteins to UTI. Specifically, these studies focus on two heme receptors, Hma and ChuA. The major findings of this dissertation are briefly described:

- UPEC outer membrane iron receptors are antigenic, expressed *in vivo*, pathogen-specific, and represent putative vaccine targets.
- Immunization with purified Hma, IreA, or IutA, but not ChuA, protects CBA/J mice from experimental UTI in an organ-specific manner.
- Hma functions as a novel TonB-dependent heme receptor that contributes to UPEC fitness *in vivo*.
- ChuA contributes more to heme uptake than Hma, both *in vitro* and *in vivo*, at least partially due to its higher expression levels. However, Hma is sufficient for heme acquisition during murine UTI.
- Heme acquisition is required for kidney colonization and bacteremia by UPEC.

- ChuA- and Hma-mediated heme acquisition contributes more to kidney colonization than FepA-, IroN-, or IutA-mediated siderophore uptake. However, bladder colonization is more dependent on IutA-mediated aerobactin uptake.

Conclusions and Perspectives

The data presented here strongly support a role for Hma and heme acquisition by UPEC during pyelonephritis. The *hma* and *hma chuA* mutants were specifically outcompeted in the kidney, both by wildtype CFT073, as well as *fepA* and *iroN* mutants. Interestingly, immunization with Hma protected mice from kidney, but not bladder infection. As qPCR data indicate that *hma* is expressed in the bladder, this suggests that anti-Hma antibodies generated during vaccination may block its function. In the kidneys, where Hma is needed for heme uptake, this may reduce the fitness of UPEC, leading to poor bacterial growth or enhanced immune clearance. Indeed, preliminary data indicate sera from mice vaccinated with Hma block hemin binding by Hma (Fig. 6-1), suggesting that inhibition of receptor function at least partially contributes to the site-specific protection observed following Hma immunization.

There also appears to be a more subtle role for heme uptake during cystitis, as *hma chuA* was outcompeted by *fepA* and *iroN* mutants in the bladder. Consistent with this, other laboratories have found that mutants lacking *chuA* are outcompeted by wildtype in the bladders of infected mice at 48 hpi (187), as well as form smaller IBCs in the bladder (151). As an *hma* mutant was not impaired for bladder colonization at either 48 or 72 hpi, these findings suggest that *chuA* alone may play a minor role during bladder infection. Thus, we can speculate that, in the relatively low heme environment of the

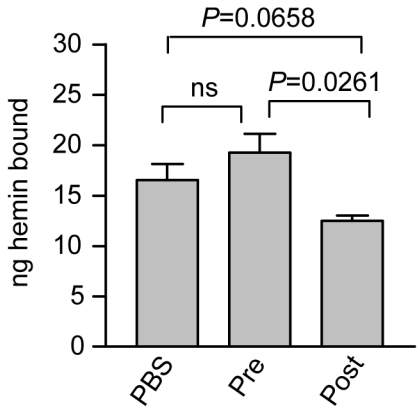

Figure 6-1. Hemin binding to Hma in the presence of sera from Hma-vaccinated mice. Microtiter plates, coated with 0.5 μg purified Hma-His_6, were blocked and incubated with either PBS, preimmune sera (Pre), or antisera from Hma-vaccinated mice (Post). Sera were added at a dilution of 1:2. Plates were washed, incubated with 50 μM hemin, and, following further washes, hemin bound to each well quantified by addition of a peroxidase substrate. Antisera were pooled from the ten mice with the strongest Hma-specific antibody response (as measured by difference in pre- to post-sera total Ig titer) and pre-immune sera were pooled from these selected mice. All sera were collected from two independent vaccination experiments, the results of which are shown in Fig. 3-2B. Mean and standard deviation of triplicate samples are shown.

bladder, the more highly expressed ChuA is able to transport a small amount of the scarce heme, which contributes to UPEC survival, whereas the heme taken up by the lesser-expressed Hma does not significantly affect bacterial growth (Fig. 6-3). However, in the heme-rich kidneys, both ChuA and Hma are able to transport their substrate in quantities sufficient to affect UPEC colonization. It is important to note that, although Hma and ChuA bind heme with similar affinities, substrate binding represents only the first step in heme uptake. Therefore, at this time we are unable to rule out heme transport efficiency as a contributing factor in the proposed model. Transport studies are necessary to assess the uptake efficiency for each receptor and show definitively that indeed the only functional differences between Hma and ChuA are expression levels.

Free heme is not readily available in the host, as the majority is bound by hemoglobin and sequestered within erythrocytes or bound by other hemoproteins (196). Thus, it is unlikely that free heme is the substrate for ChuA and Hma in the urinary tract. Hemoglobin, which can be utilized both by ChuA (186) and Hma, is a potential heme source *in vivo*, especially in the blood-rich kidneys. Hemolysin may facilitate use of this iron source, as it lyses red blood cells (53), among other cells types. Additionally, a secreted hemoglobin protease (Hbp) in abscess-forming *E. coli* degrades hemoglobin, binds the released heme, and is hypothesized to transfer this heme to the bacteria for receptor-mediated import (143). Many UPEC strains, including CFT073, encode a secreted autotransporter *tsh* (temperature sensitive hemagglutinin) that is 78% identical to Hbp (77), suggesting that these strains may also facilitate hemoglobin degradation. However, hemoglobin protease activity has not been detected for Tsh_{CFT073}.

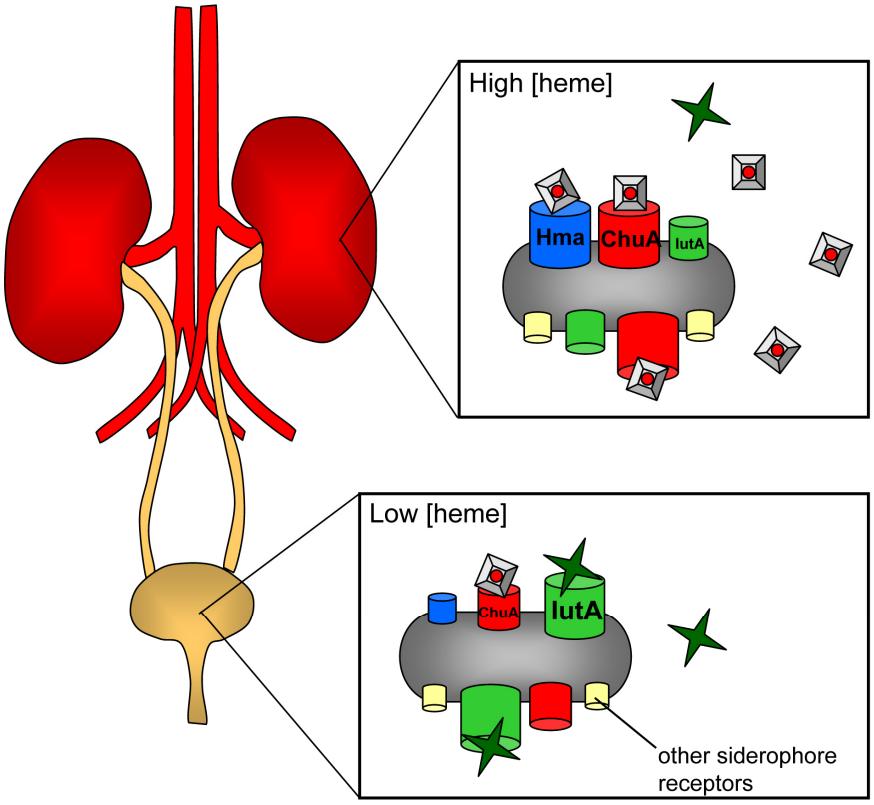

Figure 6-2. Model of heme acquisition by UPEC during UTI. Relative size of diagrammed iron receptors indicates their relative importance at a particular site in the urinary tract. Red, ChuA; blue, Hma; green, IutA; yellow, all other siderophore receptors. In the low heme environment of the bladder, IutA-mediated aerobactin transport is the predominant mechanism of iron acquisition. Because of its higher expression, ChuA is able to transport some of the scarce heme. However, in the heme-rich kidneys, both Hma and ChuA transport the molecule and are the dominant form of iron acquisition.

Furthermore, a *tsh* mutant was not outcompeted by wildtype CFT073 (Appendix D) as would be expected if Tsh were important for heme acquisition *in vivo*.

In the absence of a specific hemoglobin protease, we may speculate that hemolysin-, Sat-, and CNF-1-mediated tissue damage (67, 124) releases intracellular hemoproteins and hemoglobin that provide a substrate for ChuA and Hma. Indeed, hemolysin was shown to induce bladder hemorrhage (169). Close association of UPEC with the kidney epithelium may allow the bacteria to further exploit the heme content of these damaged host cells. Moreover, localized tissue damage in close proximity to UPEC likely provides microenvironments with elevated heme concentrations.

Despite the functional similarities between *chuA* and *hma*, we propose that Hma represents a novel class of heme receptors. While Hma is only 18% identical to ChuA, homologs are found in *Citrobacter koseri* (72% identical, 83% similar) and the plant pathogen *Pectobacterium* (*Erwinia*) *caratovorum* (51% identical, 70% similar). Similarly, ChuA and *Y. enterocolitica* HemR, which share heme-binding residues, are 70% identical. Indeed amino acid sequence alignment of Hma and its homologs indicates that Y126, which was required for Hma function, is conserved (Fig. 6-3). Furthermore, other conserved residues include four tyrosines (Y237, Y285, Y543, and Y569) that are both highly conserved among these receptors and are predicted to reside in extracellular loops of Hma. These amino acids are therefore candidates for the remaining unidentified heme coordinating residues in Hma.

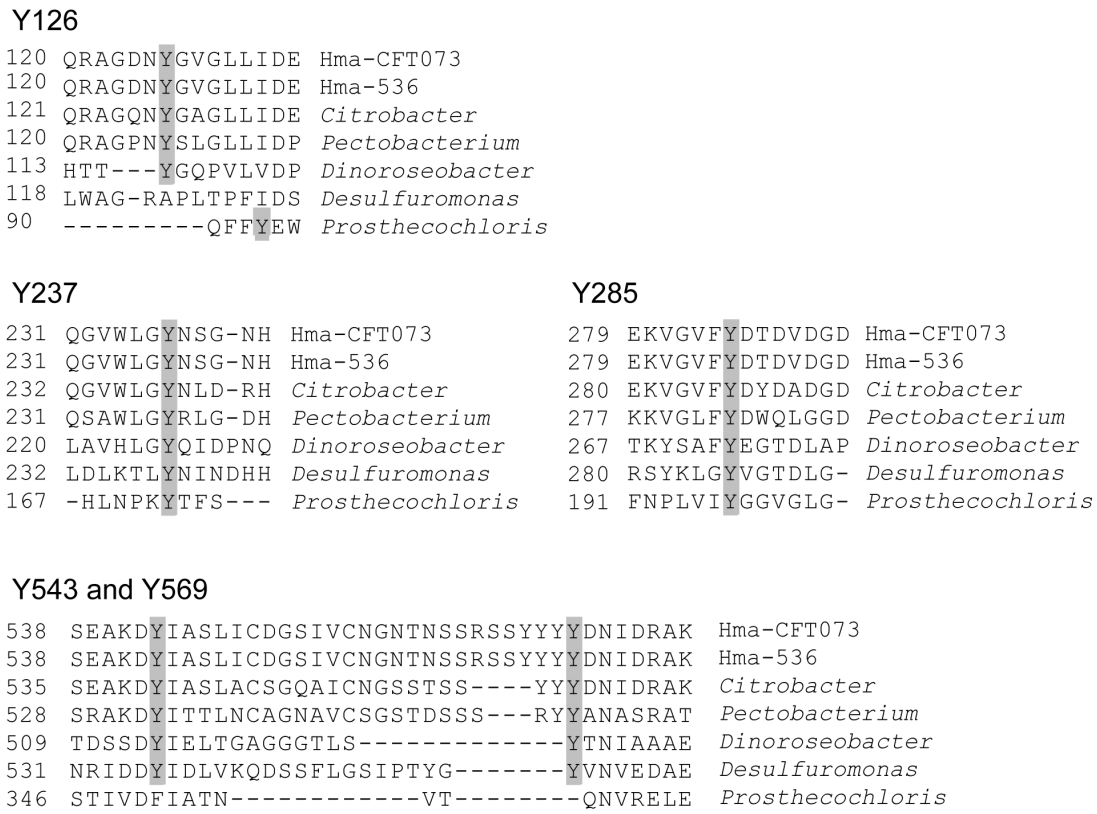

Figure 6-3. Residues conserved among Hma and its close homologs. Amino acid sequence alignments showing conservation of Tyr 126, 237, 285, 543, and 569 (highlighted gray) among the Hma-like receptors. The top two lines show of each alignment show Hma_{CFT073} and Hma_{536}, and the bottom rows indicate the related putative TonB-dependent receptors of the species listed (only genus name shown). Y237 and Y285 are absent in ChuA.

Furthermore, Hma appears to have evolved differently from ChuA and HemR, as it is more closely related to TonB-dependent receptors of environmental bacterial species. Similar receptors are found in *Desulfuromonas acetoxidans*, a marine sulfur-reducing species, *Dinoroseobacter shibae*, a marine phototroph isolated from dinoflagellates, and *Prosthecochloris aesturarii*, a green sulfur bacterium associated with coral disease. As both sulfur metabolism and photosynthesis require complex mechanisms of electron flow, an increased need for electron carriers such as heme would be expected. While heme acquisition is primarily associated with mammalian pathogenesis, there are examples of heme utilization among marine particle-associated bacteria (82). Thus, we speculate that these marine bacterial species encode Hma-like heme receptors and may share a common evolutionary ancestor receptor with Hma.

Indeed, *hma* has characteristics of a horizontally-acquired gene. Its G+C content differs significantly from that of the CFT073 genome (45.3% as compared to 50.5%). Furthermore, in strain CFT073 it is located on a 44 kb genomic island, GI-CFT073-*cobU* (119). Interestingly, commensal *E. coli* K12 encodes a truncated, nonfunctional Hma (1-167 of 715 residues). This implies that *hma* was acquired by this strain, but because it did not confer a fitness advantage, was lost due to a lack of selective pressure. In contrast, the presence of *hma* in the majority (69%) of UPEC isolates suggests that there is strong selective pressure to maintain the gene in these strains.

When considering UPEC genome evolution, it is important to note that the urinary tract does not represent an obligatory step in this pathogen's lifecycle. More likely, it is a way for strains possessing certain genetic traits to exploit a relatively competition-free niche, away from the tremendous bacterial burden of the intestinal tract.

Thus, UPEC-specific genes may also have been conserved for advantages they confer during UPEC intestinal colonization, allowing these strains to outcompete members of the microbiota. However, this is likely not the case for *hma*, as ferric iron receptors appear to be downregulated during UPEC growth in the anaerobic murine cecum (151).

Given the highly similar structure and function of these outer membrane iron receptors, it was somewhat surprising to observe striking differences in the protection they conferred upon immunization. Especially notable were the differences between ChuA and Hma. While ChuA is more important for heme acquisition and has higher *in vivo* expression, intranasal vaccination with this protein offered no protection against UTI. In contrast, immunization with the "secondary" heme receptor Hma significantly protected mice from kidney infection. Similarly, vaccination with Hma elicited a stronger antigen-specific IgG response. This suggests that differences between the ChuA and Hma antigens themselves, not solely the proteins' functions, are responsible for these results. The same may be true for IutA, the most highly expressed iron receptor which only modestly protected mice from UTI. In fact, we may speculate that the relatively low immunogenicity of ChuA and IutA has selected for the high expression of these receptors. Careful sequence and structure comparison of protective and non-protective antigens may provide insight into protective epitopes. Moreover, these non-protective, yet highly similar antigens will also provide useful controls when further examining immunological correlates of protection.

Future Directions

While the studies presented here provide a crucial first step in defining a protective vaccine against UPEC, further work is needed to refine Hma immunization. Rather than rationally selecting putative extracellular loops to include in vaccine preparations, epitope mapping of the Hma protein may be a useful tool to define protective regions of the molecule. Furthermore, identification of the ligand-binding domain(s) will provide key insight into regions that should be targeted by neutralizing antibodies. In addition, long-term studies are needed to determine whether persistent immunological memory is generated by Hma vaccination. Finally, despite our initial success with intranasal vaccination, alternative immunization routes should be explored using these antigens. For example, a systemic (intraperitoneal) primary dose followed by mucosal (intranasal) boosts may increase total antibody titers while still maintaining the mucosal response necessary for UTI clearance.

Although the only strain used for experimental challenge here was CFT073, it will be critical to know if Hma or IreA vaccination affords cross-strain protection. Among sequenced UPEC strains, these proteins appear to be well-conserved, with >99% sequence identity. Thus, we anticipate that vaccination will protect against any strain expressing these proteins. Close homologs of IreA are also encoded in the opportunistic uropathogens *P. mirabilis* (PMI1945; 56% identical, 74% similar) and *Providencia stuartii* (PROSTU_04194; 61% identical, 78% similar). It may be interesting to determine if immunization with *E. coli* IreA can protect against UTI by these species, as well.

Ultimately, we speculate that the greatest protection from UTI will be achieved when multiple iron receptors are targeted by a vaccine. First, by increasing the number of antigens included in an immunization, the array of UPEC strains targeted by the vaccine should be broadened. Thus, although IutA immunization was not strikingly protective, further work should be done to identify potentially immunogenic regions of this antigen that may be utilized, as it represents one of the most conserved pathogen-specific iron receptors (65% UPEC, 17% fecal-commensal). For the same reason, the yersiniabactin receptor, FyuA, should also be examined using a yersiniabactin-positive strain. Second, the inclusion of bladder- and kidney-specific antigens should provide protection against both cystitis and pyelonephritis. Finally, targeting multiple proteins involved in the same biological function will likely impair UPEC iron acquisition considerably, as well as decrease the likelihood of resistant strains emerging. The use of peptide or truncated antigens may be a useful approach to the development of a multivalent vaccine, as our initial attempts using full-length antigens were unsuccessful.

Both in the context of vaccination and biological function, it will also be important to appreciate how heme acquisition relates to siderophore-mediated iron uptake during UTI, at various timepoints post-infection. The co-challenge studies described in Chapter 5 begin to address these questions, but further work will likely be complicated by the fact that putative and uncharacterized iron receptors exist. Thus, putative iron receptors must be examined and substrates should be defined for existing receptors before a true hierarchy of UPEC iron acquisition can be modeled. Fortunately, the conserved structure of TonB-dependent receptors yields relatively straightforward predictions of putative receptors. Although, as examples of non-iron substrates of TonB-dependent

receptors are increasing (162), it is likely that not all predicted receptors will transport iron compounds. Once these putative receptors have been defined, the contributions that each gene makes to UTI can be determined, either by sequential complementation of a "super-deletion" strain lacking all receptors, or using a signature-tagged approach to conduct a multi-strain *in vivo* competition. Indeed, the presence of numerous iron systems makes UPEC a difficult model in which to examine iron acquisition. However, this very redundancy points to the significance of iron uptake for UPEC survival, indicating that understanding this process will be crucial to a more complete understanding of the pathogen.

APPENDICES

Appendix A. Hma-His_6 purification

Hma-His_6 was expressed and purified from outer membrane fractions of *E. coli* TOP10. Figure A-1 shows Ni-NTA purification of the protein from solubilized membrane fractions. The ~80 kDa band was confirmed by in-gel trypsin digestion and tandem mass spectrometry to be c2482 (Hma) (Michigan Proteome Consortium, data not shown). Elution fractions containing this band, but no contaminating bands, were pooled and dialyzed for use.

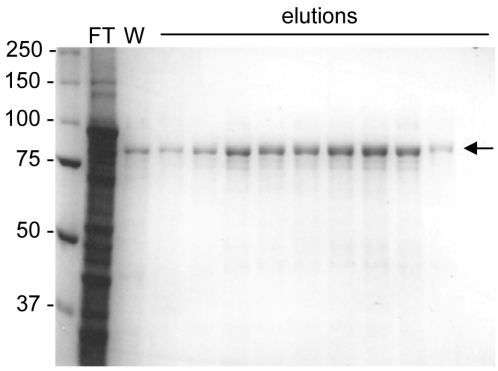

Figure A-1. Purification of Hma-His_6. SDS-PAGE (10%) separation of Ni-NTA column fractions: flow through (FT), final wash (W), and elutions. Molecular weight standards (kDa) are indicated on the left and arrow shows ~80 kDa Hma band.

Appendix B. Growth of siderophore and heme receptor mutants *in vitro*

To measure the growth of the heme and siderophore receptor mutants used in these studies, growth curves were performed (Fig. B-1). All strains grew similarly to wildtype CFT073, both in rich medium and under iron-limiting conditions.

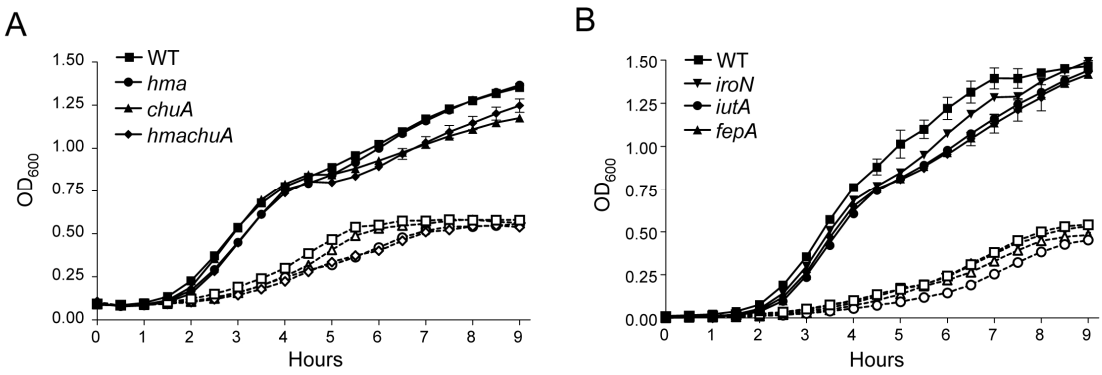

Figure B-1. Growth of iron receptor mutants under iron-limiting and iron-replete conditions. Standardized cultures were inoculated 1:100 into LB (solid symbols, solid lines) or LB containing 400 μM 2'2-dipyridyl (open symbols, dashed lines) and cultured at 37°C. **(A)** Heme receptor mutants: wildtype (squares), *hma* (circles), *chuA* (triangles), *hma chuA* (diamonds). **(B)** Siderophore receptor mutants: wildtype (squares), *iroN* (downward triangles), *iutA* (circles), and *fepA* (upward triangles).

Appendix C. Fitness of Δ*tsh in vivo*

To determine the role of *tsh* during UTI, an isogenic Δ*tsh* mutant was tested in a murine co-infection model. At both 48 and 72 hpi, Δ*tsh* was recovered at levels indistinguishable from wildtype CFT073 (Fig. D-1),indicating that this gene does not significantly contribute to UTI under these conditions.

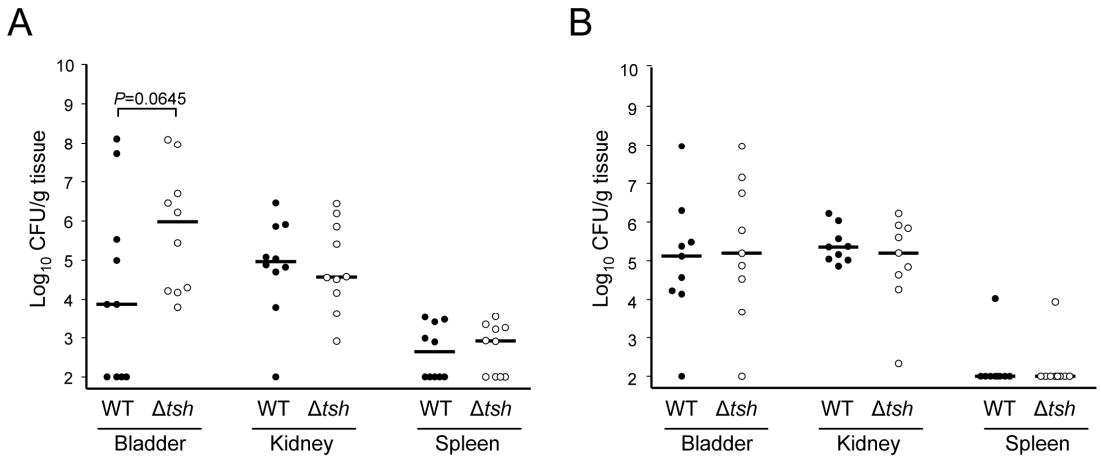

Figure C-1. *tsh* mutant in a co-infection model of UTI. CBA/J mice were transurethrally challenged with a 1:1 mixture of Δ*tsh* (open circles) and wildtype CFT073 (filled circles) (total inoculum equaled 1×10^8 CFU/mouse). Shown is CFU/g tissue at **(A)** 48 and **(B)** 72 hpi. Bars indicate the median.

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