Molecular Microbiology (2009) 71(1), 79–91 ■
doi:10.1111/j.1365-2958.2008.06509.x
First published online 31 October 2008

Haem acquisition is facilitated by a novel receptor Hma and required by uropathogenic *Escherichia coli* for kidney infection

Erin C. Hagan and Harry L. T. Mobley*
Department of Microbiology and Immunology, University of Michigan Medical School, Ann Arbor, MI 48109, USA.

Summary

Iron acquisition, mediated by specific outer membrane receptors, is critical for colonization of the urinary tract by uropathogenic *Escherichia coli* (UPEC). The role of specific iron sources *in vivo*, however, remains largely unknown. In this study, we identified a 79 kDa haem receptor, haem acquisition protein Hma, and established that it functions independently of ChuA to mediate haemin uptake by UPEC strain CFT073. We demonstrated that expression of *hma* promotes TonB-dependent haemin utilization and the Hma protein binds haemin with high affinity ($K_d = 8$ μM). Hma, however, lacks conserved His residues shown to mediate haem uptake by other bacterial receptors. In contrast, we identified Tyr-126 as a residue necessary for Hma-mediated haemin utilization. In a murine co-infection model of UTI, an isogenic *hma* mutant was out-competed by wild-type CFT073 in the kidneys ($P < 0.001$) and spleens ($P < 0.0001$) of infected mice, indicating its expression provided a competitive advantage in these organs. Furthermore, a *hma chuA* double mutant, which is unable to utilize haemin, was unable to colonize the kidneys to wild-type levels during independent infection ($P = 0.02$). Thus, we demonstrate that UPEC requires haem for kidney colonization and that uptake of this iron source is mediated, in part, by the novel receptor, Hma.

Introduction

Bacteria have evolved highly specialized systems to acquire iron, an essential nutrient, from their environment. For example, iron-chelating siderophores, secreted by many bacterial species, function to scavenge iron from host proteins or the environment. The near absence of free iron within mammalian hosts makes these uptake systems essential for bacterial pathogens during infection.

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 (Ferguson *et al.*, 1998; Buchanan *et al.*, 1999). To function, these receptors require the energy-transducing activity of an inner membrane-periplasmic protein complex composed of ExbB, ExbD and TonB (Fischer *et al.*, 1989; Skare *et al.*, 1993).

In addition to siderophore-mediated iron acquisition, many bacterial species can scavenge haem-bound iron. Specific outer membrane receptors bind host haemoproteins and transfer the co-ordinated haem molecule into the periplasm where an ABC transport system delivers it to the cytoplasm. Alternatively, haemophores scavenge haem and subsequently transfer it to specific outer membrane receptors in a process analogous to siderophore-mediated iron uptake (Wandersman and Stojiljkovic, 2000).

The majority of high-affinity haem or haemoglobin receptors share four conserved histidine residues and two motifs, the FRAP and NPNL domains (Bracken *et al.*, 1999). Two of these conserved histidines are required for HemR-, HmuR- or ShuA-mediated haem utilization in *Yersinia enterocolitica*, *Porphyromonas gingivalis* or *Shigella dysenteriae* respectively (corresponding to His-128 and His-461 in HemR) (Bracken *et al.*, 1999; Liu *et al.*, 2006; Burkhard and Wilks, 2007). Structural modelling has predicted these residues to reside extracellularly and recent evidence indicates they function to ligate haem (Burkhard and Wilks, 2007).

In pathogenic *Escherichia coli*, haem uptake is facilitated by the ChuA receptor, which shares > 99% amino acid sequence identity with ShuA, the *S. dysenteriae* haem-haemoglobin receptor (Torres and Payne, 1997). A study examining the distribution of *shuA* homologues in pathogenic *E. coli* by Southern hybridization found that, indeed, most haem-utilizing *E. coli* contain the *shu* locus

Accepted 17 October, 2008. *For correspondence. E-mail hmobley@umich.edu; Tel. (+1) 734 764 1466; Fax (+1) 734 763 7163.

(Wyckoff *et al*., 1998). However, several haem-utilizing strains were *shuA*-negative, even under reduced stringency conditions. Thus, the authors predicted the presence of an additional haem uptake gene in these strains whose sequence differs significantly from that of *shuA* (Wyckoff *et al*., 1998).

Like other bacterial pathogens, uropathogenic *E. coli* (UPEC), the primary cause of uncomplicated urinary tract infections (UTIs), requires TonB-dependent outer membrane iron receptors for host colonization (Torres *et al*., 2001). 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 (Welch *et al*., 2002). While several of these have been shown to contribute to the fitness of UPEC *in vivo* (Russo *et al*., 2001; 2002; Torres *et al*., 2001; Johnson *et al*., 2005), the importance of specific sources of host iron remains unknown.

Putative iron receptor c2482 was identified by our laboratory as an antigenic outer membrane protein expressed under iron limitation and induced during growth in human urine (Alteri and Mobley, 2007; Hagan and Mobley, 2007). 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. 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 (Alteri and Mobley, 2007). 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.

The c2482 gene appears to be conserved among pathogenic strains of *E. coli.* DNA dot blot analysis of a panel of *E. coli* strains showed that c2482 or a close homologue was present in 69% and 50% of uropathogenic and intestinal pathogenic isolates tested respectively. This differed significantly from the 17% of faecal-commensal *E. coli* strains that possessed c2482 (Hagan and Mobley, 2007). In addition, c2482 was among 131 genes present in all of 11 UPEC strains, but none of the six faecal-commensal strains examined by a recent comparative genomic hybridization study (Lloyd *et al*., 2007). These findings indicate that c2482 is present more frequently among pathogenic *E. coli* and suggest that this gene may contribute to the virulence of these pathogens.

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

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 wild type, even in the presence of high concentrations of iron chelator [600 μM 2′2-dipyridyl (DIP)] (data not shown). 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 wild type for limited nutrients. Wild type and mutant were inoculated approximately 1:1 into the same medium and continually re-passaged into fresh medium for 72 h. In rich medium, the *hma* mutant reached densities similar to those of wild type throughout the duration of the experiment, despite an approximately half log lower inoculum (Fig. 1A), 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 out-competed by wild type by 72 h ($P = 0.03$). 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 wild type and mutant in an effort to detect subtle differences in fitness. Total inoculum equalled $\sim 1 \times 10^8$ colony-forming units (cfu) per mouse. At 72 h post inoculation, the *hma* mutant was significantly out-competed by wild type in the kidneys (8-fold reduction; $P < 0.001$) and spleens (80-fold reduction; $P < 0.0001$) of infected mice (Fig. 1B). Moreover, the *hma* mutant was undetectable in the kidneys and spleens of infected mice significantly more frequently than wild type ($P = 0.021$, $P < 0.0001$ 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 out-competed 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.

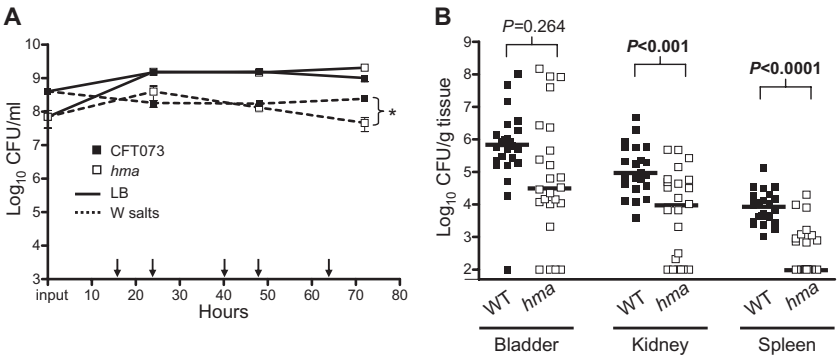

Fig. 1. Fitness of *hma* mutant *in vitro* and *in vivo*.
A. *In vitro* culture competition assay of wild-type CFT073 (filled symbols) and *hma* mutant (open symbols) cultured in Luria broth (solid line) or W salts minimal medium (dashed line). After inoculation (input cfu ml^{-1} plotted on *y*-axis), 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. *$P = 0.03$.
B. Seventy-two-hour CBA/J mouse co-infection with 10^8 cfu mixture of wild type (WT) and *hma* mutant. Data points represent cfu per gram of individual animals in the organs indicated; bars show median values ($n = 24$).

Expression of hma *promotes haemin 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 (Torres and Payne, 1997). 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*, haemin (10 μM) and haemoglobin (1 mg ml^{-1}) only promoted the growth of the strain expressing *hma* (Fig. 2A). 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. 2B). However, only growth of the strain expressing *hma* was enhanced by the addition of 10 μM haemin. Together, these data indicate that expression of *hma* promotes the utilization of haemin 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 haemin utilization by wild-type K12, not the *tonB* mutant (Fig. 2C). Thus, Hma was unable to function in the absence of TonB, indicating that it is indeed a TonB-dependent receptor.

Hma is a haemin-binding protein

To further test the hypothesis that Hma is a haem receptor, the ability of Hma to directly bind haemin was examined. *E. coli* K12 whole cells expressing *hma*, haem receptor *chuA*, or siderophore receptor *iutA*, or carrying empty vector were incubated with haemin and pelleted. Haemin bound by the pelleted cells was removed from solution, resulting in a measurable decrease in the haem concentration of the supernatant. Using the intrinsic peroxidase activity of haemin as an indirect measure of haem quantity, we found that cells expressing *hma* or *chuA* bound and removed significantly more haemin from the solution than did cells containing a vector control ($P = 0.001$, $P = 0.0004$ respectively) (Fig. 3A). While bacteria expressing *iutA* bound slightly more haemin 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 haemin per μg of protein, as compared with 171 ng haemin μg^{-1} bound by outer membranes from *E. coli* carrying empty vector ($P < 0.0001$) (Fig. 3B). These data indicate that haemin binds to cells containing Hma and that at least part of this haem binding activity is due to a component of the outer membrane.

To detect direct haem–Hma interaction, we incubated outer membrane proteins from *E. coli* K12 either expressing or not expressing *hma* with haemin and separated the haemin–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 colour in the presence of haem-associated peroxidase activity. This activity was localized to an ~80 kDa band, consistent with the size of Hma, which was

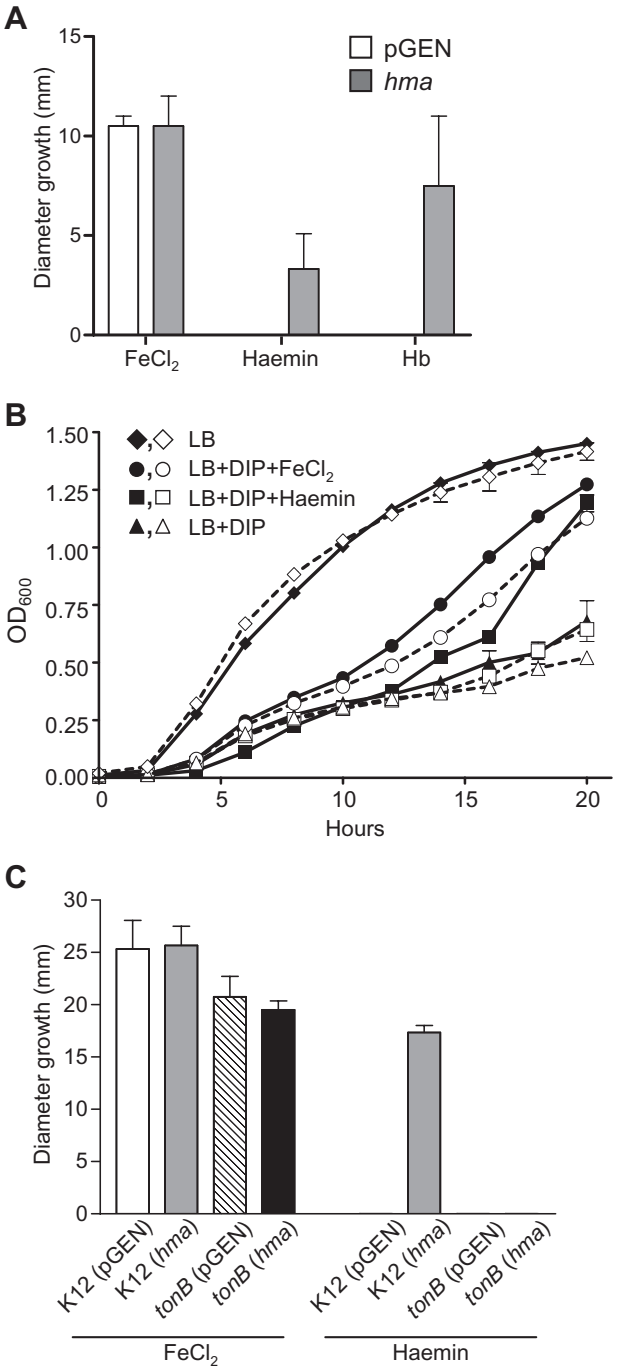

Fig. 2. Haemin utilization by *E. coli* strains expressing *hma*.
A. Growth of *E. coli* K12 carrying pGEN (open bars) or p_{native}*hma* (grey bars) on iron-depleted agar spotted with 1 mM $FeCl_2$, 10 μM haemin or 1 mg ml^{-1} haemoglobin (Hb). Bars represent mean diameter (mm) growth surrounding indicated 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 haemin (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 wild type and *tonB* mutant on iron-depleted agar spotted with 10 mM $FeCl_2$ or 10 mM haemin. Bars represent mean diameter (mm) growth of *E. coli* K12 pGEN (open bars), K12 p_{native}*hma* (grey bars), *tonB* pGEN (hatched bars) and *tonB* p_{native}*hma* (black bars) surrounding the indicated iron source ($n = 3$).

absent from the vector control lane (Fig. 3C). Together with our previous findings, these data demonstrate that Hma can function as a haem receptor.

Hma binds haemin with high affinity

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

Tyr-126 is required for Hma function

Previous studies have identified four histidine residues conserved among bacterial haem receptors, two of which are required for receptor function (Bracken *et al.*, 1999; Liu *et al.*, 2006; Burkhard and Wilks, 2007). However, while Hma contains a moderately conserved FRAP-NPNL domain characteristic of other haem receptors, it lacks these conserved His residues (Fig. 5A). 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 (Buchanan *et al.*, 1999), a prototypic *E. coli* outer membrane iron receptor, we predicted extracellular residues that may function in haem binding or transport. His-242, His-331 and His-337 are located in putative extracellular loops of Hma, while Tyr-126 is predicted to be on the extracellular face of the N-terminal plug domain of the molecule (Fig. 5B). Furthermore, amino acid alignment of Hma with other haem receptors indicates that Tyr-126 aligns with His-128 of *Y. enterocolitica* HemR (Fig. 5A), a residue necessary for receptor function (Bracken *et al.*, 1999).

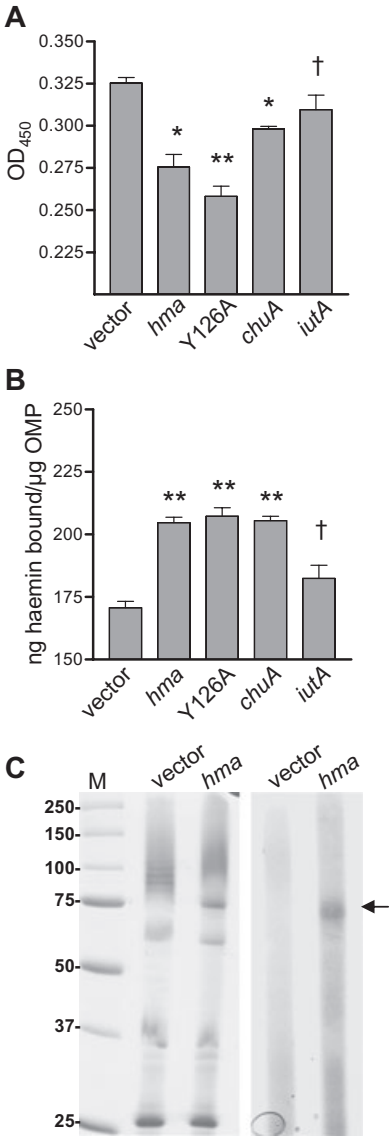

Fig. 3. Haemin binding activity of Hma.
A. Haemin binding to *E. coli* K12 carrying p*hma*, p*Y126A*, p*chuA*, p*iutA* or vector control. Induced cells were incubated with 50 μM haemin, pelleted, and haemin remaining in the supernatant detected with a peroxidase substrate. $^{**}P < 0.0001$, $^{*}P = 0.001$, †not significant (as compared with vector control).
B. Haemin binding to outer membrane proteins (OMPs) isolated from the strains in (A), as measured by microtitre plate assay. Wells were coated with 0.5 μg of protein and incubated with 50 μM haemin. Unbound haemin was removed by washing and, after the addition of a peroxidase substrate, haemin binding was calculated from a standard curve using the OD_{450}. Bars represent the mean ($n = 5$) and symbols are as in (A).
C. Haemin binding to Hma protein. OMPs isolated from the strains in (A) were incubated with 85 μM haemin and separated on a non-reducing SDS-PAGE gel. Left panel is Coomassie-stained gel and right panel is TMBZ stain of haem-associated peroxidase activity. Arrow indicates Hma band. M, molecular weight standards in kDa.

These four residues (His-242, His-331, His-337 and Tyr-126) were mutated to Ala in p_{native}*hma* and the resulting proteins were expressed in the *E. coli* K12 outer membrane at wild-type levels (Fig. 5C). 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 haem utilization was assessed by plating these strains on iron-depleted agar containing either $FeCl_2$ or haemin 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 haemin to the same extent as strains expressing wild-type Hma, indicating that these residues alone are not required for haem utilization (Table 1). 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 haem utilization to the same extent as wild type (Table 1). However, function of the Y126A protein was abolished, as the strain expressing this protein could not use even high concentrations of haemin (100 μM). The Y126A mutant Hma retained its haemin binding activity (Fig. 3A and B), though, suggesting the importance of this residue in the transport, rather than binding, of haem. Therefore, these data indicate that Tyr-126, but none of

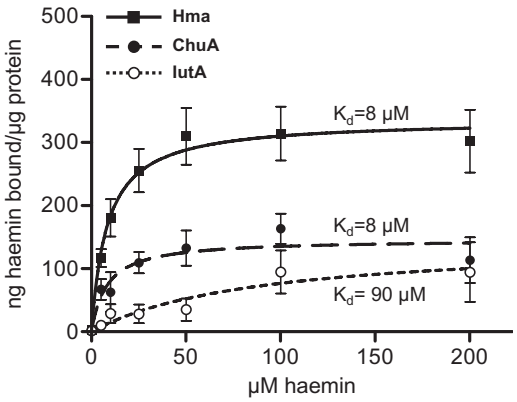

Fig. 4. Haem binding curve. Haemin bound by purified Hma-His_6 (solid squares), ChuA-His_6 (solid circles) or IutA-His_6 (open circles) as a function of substrate concentration. Protein (0.2 μg) was coated onto microtitre plate wells, incubated with haemin (0–200 μM), and bound haemin detected by addition of a peroxidase substrate. Haemin standards were used to calculate ng of haemin bound per μg of 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 non-linear regression analysis, are also plotted. Dissociation constant (K_d) values for each curve are indicated.

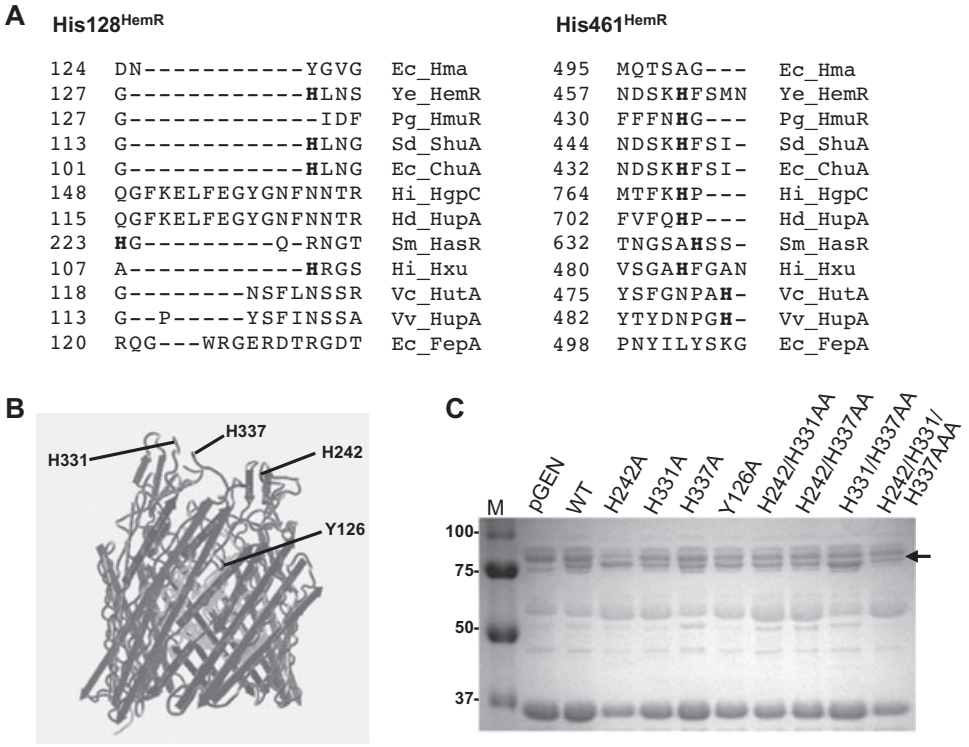

Fig. 5. Residues required for Hma-mediated haem utilization.
A. Partial amino acid alignment of Hma with bacterial haem receptors, indicating conserved His residues (bolded) critical for function of HemR (His-128^{HemR} and His-461^{HemR}). Ec, *E. coli* CFT073; Ye, *Y. enterocolitica*; Pg, *P. gingivalis*; Sd, *S. dysenteriae*; Hi, *Haemophilus influenzae*; Hd, *Haemophilus ducreyi*; Sm, *Serratia marcescens*; Vc, *Vibrio cholerae*; Vv, *Vibrio vulnificus*.
B. Structure alignment of Hma with FepA, showing the predicted locations of H242, H331, H337 and Y126 (black). Beta-barrel domain (dark grey) and N-terminal plug domain (light grey) 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 for ~7 h in LB with 200 μM DIP prior to outer membrane fractionation. Arrow indicates ~80 kDa Hma band.

the putative extracellular loop His residues, is required for the haem-uptake activity of Hma.

Both chuA *and* hma *contribute to CFT073 haem utilization*

In addition to Hma, *E. coli* CFT073 contains another haem or haemoglobin receptor, ChuA. To examine the contribution of each of these proteins to haem utilization by CFT073, a *hma chuA* isogenic mutant was constructed. Together with the single mutants, the ability of *hma chuA* to utilize haem as a sole iron source was assessed. Wild type, the single mutants and the *hma chuA* double mutant all required the same concentration of $FeCl_2$ for growth (Table 2). However, the *chuA* mutant required a higher concentration of haemin as compared with either wild type or the *hma* mutant (25 μM as compared with 1 μM) and the double mutant was unable to grow even with the highest concentration of haemin (100 μM). This defect could be complemented by expression of *hma* from

Table 1. Ability of Hma site-directed mutants to mediate haemin utilization.

Strain[a]	Minimum concentration (μM) required to support growth[b]	
	$FeCl_2$	Haemin
pGEN	10	> 100
Wild-type 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.

Table 2. Ability of CFT073 haem uptake mutants to utilize haemin as a sole iron source.

Strain	Minimum concentration (μM) required to support growth[a]	
	$FeCl_2$	Haemin
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.

p_{native}*hma*, but not with the pGEN empty vector. Therefore, although it appears that *chuA* contributes more to haem uptake, either *chuA* or *hma* is sufficient for haemin utilization by CFT073 *in vitro*.

Haem uptake is required for maximum kidney colonization

To identify the role of haem uptake for urinary tract colonization by CFT073, as well as define the relative contributions of *hma* and *chuA* to this process *in vivo*, the haem receptor mutants were tested in the mouse model of UTI. After a 72 h 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. 6A). This demonstrates that, in the kidney, the strain lacking *hma* was better able to compete for haem than was the *chuA* mutant, indicating that the ChuA receptor contributes more to haem 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 wild type (Fig. 6B). 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 haem uptake system for kidney colonization. While there was only an approximately one log difference between the median cfu per gram of kidney tissue of *hma chuA* and wild type, 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 with only 1/20 mice uninfected that were wild type-inoculated, $P = 0.044$). Together, these data demonstrate the requirement of a haem receptor (either *hma* or *chuA*) for efficient kidney colonization by CFT073, as well as provide evidence that haem is an essential source of iron for this pathogen during kidney infection.

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

As *chuA* and *hma* each encodes a haem receptor, we were surprised to note the phenotypic differences of these two mutants, both *in vitro* (Table 2) and *in vivo* (Fig. 6A). While *chuA* appears to contribute more to haem utilization, both receptors have similar affinities for haemin (Fig. 4). 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*. As compared with 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. 7A). In the presence of excess $FeCl_2$, transcripts for both genes were slightly decreased (–1.3-fold as compared with LB). Similarly, bacterial transcripts isolated from the urine of CFT073-infected mice showed that *chuA* was upregulated an average of 67-fold *in vivo*, while *hma* increased 24-fold as compared with LB-cultured bacteria (Fig. 7B). Urine samples from

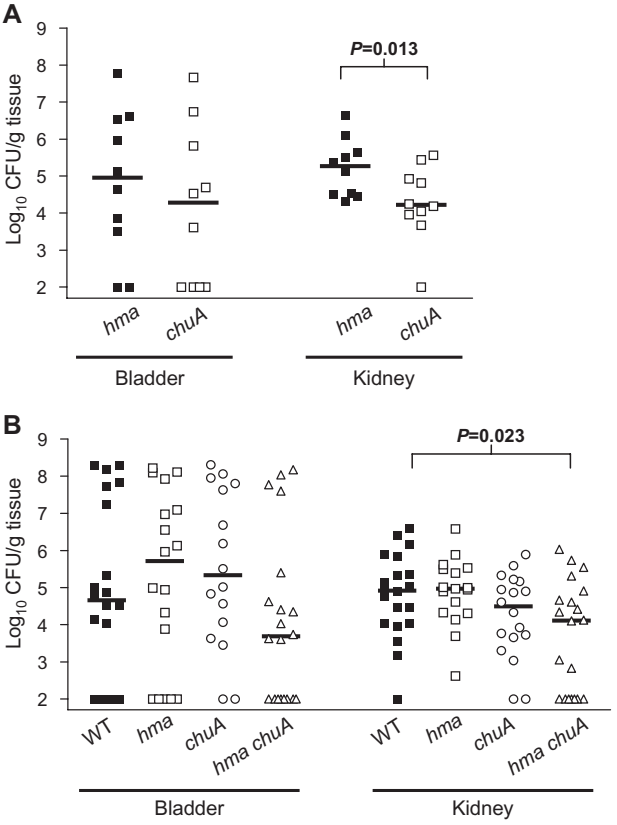

Fig. 6. Haem uptake mutants in a mouse model of UTI.
A. Seventy-two-hour CBA/J mouse co-infection with 10^8 cfu mixture of *hma* (solid symbols) and *chuA* (open symbols) mutants. Symbols represent cfu per gram of tissue in individual animals and bars indicate the median ($n = 10$).
B. Seventy-two-hour independent infections with 10^8 cfu of wild-type CFT073 (filled squares), *hma* (open squares), *chuA* (open circles) or *hma chuA* (open triangles) mutants ($n = 20$).

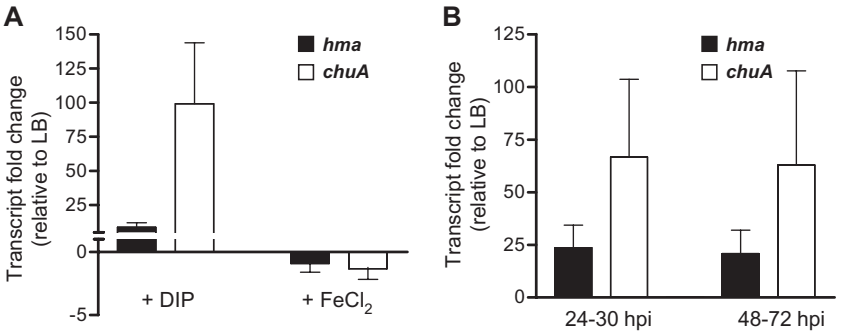

Fig. 7. *In vivo* expression of *chuA* and *hma* by real-time qPCR.
A. Fold change of *hma* (solid bars) and *chuA* (open bars) transcript levels in *E. coli* CFT073 cultured in LB supplemented with 200 μM DIP (left) or 10 μM $FeCl_2$ (right), relative to expression in LB alone. Bars represent the mean of four independent experiments.
B. Fold change 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, relative to expression in LB. Bars represent the mean of triplicate samples, each sample containing urine collected from five animals ($n = 15$) during the time points indicated [24–30 h post inoculation (hpi), right; 48–72 h post inoculation, left].

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 haem utilization.

Discussion

Hma functions as a haem receptor in UPEC and haem acquisition is necessary for upper urinary tract colonization by this pathogen. Expression of *hma* promotes TonB-dependent haemin utilization by a laboratory strain of *E. coli* and confers an ability to bind haemin. Furthermore, purified Hma binds haemin with high affinity ($K_d = 8$ μM). In UPEC, Hma functions independently of ChuA to mediate haem 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 haem receptors characterized to date, Tyr-126 is required for Hma-dependent haemin utilization. Therefore, we suggest that Hma represents a novel class of haem receptors that is distinct from the HemR family of bacterial haem receptors.

Hma has only limited homology to other characterized bacterial haem receptors. 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 sulphur-metabolizing bacteria. In addition to CFT073, copies of *hma* are present in all sequenced UPEC (F11, UTI89, 538) and enterohaemorrhagic *E. coli* strains (EDL933, Sakai, EC508, EC4042), and a close homologue (73% identical) is found in the infrequent uropathogen *Citrobacter koseri*. Furthermore, the G+C content of the *hma* ORF is considerably less than that of the CFT073 genome (45.3% as compared with 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*.

Previous structure–function studies identified two His residues conserved among haem receptors of Gram-negative bacteria that are required for haem uptake (Bracken *et al.*, 1999; Liu *et al.*, 2006; Burkhard and Wilks, 2007). Corresponding to His-128 and His-461 in *Y. enterocolitica* HemR, these residues are absent from Hma (Fig. 5A). As His-128 and His-461 are predicted to be located on the extracellular face of the N-terminal plug domain and on an extracellular loop, respectively (Burkhard and Wilks, 2007), we predicted the structure of Hma to identify putative haem-binding residues in these locations. Tyr-126 was predicted to reside extracellularly on the N-terminal plug domain (Fig. 5B) and in amino acid sequence alignments, Hma Tyr-126 aligned with HemR His-128 (Fig. 5A). Here we show that Tyr-126 is required for Hma-mediated haemin utilization (Table 1). While tyrosine is known to occasionally co-ordinate haem ligands (Arnoux *et al.*, 1999), the requirement of a Tyr residue at this location represents a significant difference between Hma and the previously studied haem receptors and provides further evidence that *hma* may have evolved independently of ChuA and HemR.

His-242, His-331 and His-337 are all located on putative extracellular loops of Hma (Fig. 5B); however, they are not required for receptor function, either alone or in combination (Table 1). As the Y126A mutant retained its haemin binding activity, it is likely that additional residue(s) function in binding/transport and compensated for the loss of Tyr-126 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 Tyr-126 in Hma-mediated haem uptake.

We estimated the affinity of Hma for haemin to be in the micromolar range ($K_d = 8$ μM). Observed haemin binding to IutA ($K_d = 90$ μM) likely represented binding to the His_6 tag present on the purified proteins or other non-specific interactions. While the affinities of most haem receptors, including HemR, are unknown, our result is similar to the $K_d = 5$ μM and $K_d = 24$ μM measured for *Serratia marcescens* HasR and *P. gingivalis* HmuR receptors respectively (Olczak *et al*., 2001; Izadi-Pruneyre *et al*., 2006).

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 (Torres *et al*., 2001). While no single uptake system has been found to be necessary for colonization, disruption of *chuA*-mediated haem uptake (Torres *et al*., 2001) or enterobactin (Johnson *et al*., 2005), salmochelin (Russo *et al*., 2002), or aerobactin (Torres *et al*., 2001) siderophore uptake resulted in out-competition by a wild-type strain *in vivo*. Thus, considerable functional redundancy exists among these systems.

Although siderophore and haem 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 haem utilization is unable to colonize the murine kidney to wild-type levels (Fig. 6B). This represents the first evidence that haem is a required source of iron for UPEC *in vivo*. The importance of haem uptake in the kidney is further supported by our and others' findings that both the *hma* and *chuA* mutants are out-competed by wild-type CFT073 in the kidneys of infected mice during co-infection experiments (Fig. 1B) (Torres *et al*., 2001). It is interesting to note that although ChuA appears to contribute more to haem uptake *in vivo* (Fig. 6A), Hma alone is sufficient for kidney colonization, as the *chuA* mutant independently colonized the kidneys at levels similar to wild type (Fig. 6B).

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 haem utilization. We propose that this difference is due, at least in part, to the relative expression levels of the two receptors. As compared with 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* (Fig. 7B). The qPCR results shown here are replicated at the protein level, as quantitative profiling of CFT073 cultured in urine measured considerably more ChuA than Hma in the outer membrane (Alteri and Mobley, 2007). Thus, a *chuA* mutant would likely contain significantly less haem receptor on its surface than would an *hma* mutant.

While haem uptake is critical for UPEC to colonize the murine kidney, it appears to play a lesser role in bladder colonization. In both co-infection (Fig. 1B) and independent infection (Fig. 6B) experiments, all haem uptake mutants infected the bladder to levels indistinguishable from wild type. Similarly, the *chuA* mutant, out-competed by the *hma* mutant in the kidneys of infected mice, colonized the bladders effectively in the presence of the competing strain (Fig. 6A). However, we show that both *hma* and *chuA* are highly upregulated in urine from infected mice (Fig. 7B), indicating that they are expressed in the iron-limited bladder. Instead, we hypothesize that non-haem sources of iron are more prevalent in the bladder and therefore more important during UPEC colonization of this site.

Free haem is not readily available in the host, as the majority is bound by haemoglobin and sequestered within erythrocytes or bound by other serum proteins (Wandersman and Stojiljkovic, 2000). Thus, it is unlikely that free haem is the substrate for Hma *in vivo*. Haemoglobin, which is utilized by both ChuA (Torres and Payne, 1997) and Hma, is a potential haem source *in vivo*, especially in the blood-rich kidneys. To facilitate use of this iron source, many UPEC strains secrete haemolysin, which lyses red blood cells (Gadeberg and Orskov, 1984) and releases haemoglobin. Additionally, in abscess-forming *E. coli*, a secreted haemoglobin protease (Hbp) degrades haemoglobin, binds the released haem and is hypothesized to transfer this haem to the bacteria for receptor-mediated import (Otto *et al*., 1998). A homologue of Hbp is found in UPEC strains, as well, although its protease activity remains unclear (Heimer *et al*., 2004). Further work is needed to elucidate the role these secreted proteins may play in ChuA- and Hma-mediated haem acquisition.

Experimental procedures

Bacterial strains and culture conditions

All strains used in this study are listed in Table 3. Bacteria were routinely cultured in Luria broth (LB) at 37°C with aeration and appropriate antibiotics. W salts minimal medium (Smith *et al*., 1971) consisted of 60 mM K_2HPO_4, 30 mM KH_2PO_4, 0.4 mM $MgSO_4$, 2% NaCl, 0.4% glucose, 0.005% thiamine and 10 mM NH_4Cl.

Mutant construction

Deletion of *hma* (in both wild-type CFT073 and *chuA*::*cat* backgrounds) and *tonB* (in MG1655) was achieved using the λ Red recombinase system (Datsenko and Wanner, 2000). 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 3). 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 EagI digestion.

Table 3. Bacterial strains and plasmids.

Strain/plasmid	Description	Reference or source
E. coli		
CFT073	Pyelonephritis isolate	Mobley *et al.* (1990)
K12	MG1655, laboratory strain	Blattner *et al.* 1997)
HB101 *ent*	HB101 *ent*::Tn*5* strain 1017*;* Kan^r	Torres and Payne (1997)
chuA	CFT073 *chuA*::*cat*; Cam^r	Torres *et al.* (2001)
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	Datsenko and Wanner (2000)
pKD46	Red recombinase helper plasmid, temperature-sensitive; Amp^r	Datsenko and Wanner (2000)
pGEN	pGEN-MCS, promoter-less expression vector, p15A ori (copy number ~15), *par hok sok mok parM parR*; Amp^r	Galen *et al.* (1999); Lane *et al.* (2007)
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-terminal His$_6$ tag	This study
p*chuA*-His	*chuA* in pBAD with C-terminal His$_6$ tag	This study
p*iutA*-His	*iutA* in pBAD with C-terminal His$_6$ tag	This study

Kan, kanamycin; Cam, cholamphenicol; Amp, ampicillin.

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 NdeI–EagI restriction sites of pGEN-MCS (p$_{native}$*hma*) (Table 3). The *hma* ORF (minus upstream region) was also PCR-amplified and cloned into the NcoI–BglII restriction sites of pBAD-*myc*-HisA (Table 3), both in and out of frame with the vector's C-terminal 6× His tag (p*hma*-His and p*hma* respectively). The *chuA* and *iutA* ORFs were PCR-amplified and similarly cloned into the NcoI–XhoI sites of pBAD, both in and out of frame with the C-terminal 6× 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), re-suspended 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 re-suspended in 2% sarcosine, incubated 30 min at room temperature and ultracentrifuged (30 min, 100 000 *g*, 4°C) to isolate the sarcosine-insoluble outer membranes. Outer membranes were re-suspended 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 (Lane *et al.*, 2005). Briefly, wild-type 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 h, cultures were passaged into fresh medium at 1:500 or 1:50 respectively. At 24, 48 and 72 h post inoculation, cultures were plated on LB agar and LB containing 25 μg ml^{-1} kanamycin to determine wild type and mutant cfu ml^{-1}. 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 (Torres and Payne, 1997), with modification. Prior to inoculation, bacteria were cultured in LB containing 200 μM DIP for at least 6 h 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 haemin, 1 mg ml^{-1} haemoglobin, 10 mg ml^{-1} lactoferrin, 10 mg ml^{-1} holo-transferrin and 10 mg ml^{-1} bovine serum albumin (Sigma)] and incubated for 48–72 h 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 haem titration experiments, 1–50 μM $FeCl_2$ and 10 nM–100 μM haemin were used. Prior to inoculation, bacteria were cultured in LB containing 200–400 μM DIP for at least 6 h and washed in PBS. The OD$_{600}$ was standardized to ~1.0 and approximately 200 cfu were spread per plate.

Table 4. 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′.

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 haemin, or no additional iron source. Growth curves were performed in a Bioscreen C Growth Curve Analyser (Growth Curves, USA) at 37°C with aeration.

Haemin-binding assays

Haemin binding to whole cells was determined as previously described (Olczak *et al*., 2001). MG1655 containing p*hma*, p*Y126A*, p*chuA*, p*iutA* or empty vector were induced for 3 h with 100 μM arabinose, washed and re-suspended in PBS. The OD_{600} was standardized to 1.0 and 800 μl of the cell suspension was mixed with 200 μl of 50 μM haemin. After 1 h incubation at 37°C, bacteria were pelleted at 16 000 *g* for 3 min and 20 μl of supernatant was incubated with 80 μl of 1-Step™ Turbo TMB-ELISA (Sigma) for 20 min at room temperature. Reactions were stopped by the addition of 100 μl of 1.0 N H_2SO_4 and the OD_{450} measured.

Haemin binding to outer membranes or purified protein was determined as previously described (Asuthkar *et al*., 2007), 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 microtitre 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 of haemin solution (for outer membranes 50 μM haemin, for purified protein 0–200 μM). Wells were washed 4× with PBS and 100 μl of 1-Step Turbo-TMB peroxidase substrate was added. After 20 min at room temperature, the OD_{450} was measured. Amount of haemin bound by each sample was calculated from a standard curve.

A modified in-gel TMBZ staining method was used to detect Hma-associated haem (Stugard *et al*., 1989). Outer membranes from MG1655 carrying p*hma* (10 μg) were incubated with 85 μM haemin for 1 h at 37°C. SDS-PAGE loading buffer lacking dithiothreitol was added to samples, which were electrophoresed in the presence of 0.1% SDS on a 10% acrylamide gel (3.75% stacking gel) at 200 V 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 haem-associated peroxidase activity, gels were stained as described in two parts freshly prepared 6.3 mM TMBZ (Sigma) in methanol, seven parts 0.25 M sodium acetate pH 5, and one part H_2O for 35 min (Thomas *et al*., 1976). Colour development was achieved by adding H_2O_2 to a final concentration of 0.1% and incubating for 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 and all reactions were carried out according to the manufacturer's instructions. All mutations were confirmed by sequencing (University of Michigan DNA Core Facility).

CBA mouse model of ascending UTI

Female 6- to 8-week-old CBA/J mice were transurethrally inoculated as previously described (Hagberg *et al*., 1983). A sterile 0.28-mm-diameter polyethylene catheter attached to an infusion pump (Harvard Apparatus) was used to deliver 50 μl of bacterial suspension containing 10^8 cfu per mouse. Cultures were grown overnight with aeration and re-suspended in PBS prior to inoculation. For co-infection experiments, re-suspended strains were mixed at a 1:1 ratio and inoculated into the same mouse. Seventy-two hours post inoculation, mice were euthanized and bladder, kidneys and spleens removed and homogenized in 3 ml of PBS using an Omni TH homogenizer (Omni International). Dilutions of this homogenate were plated on LB to determine cfu per gram of tissue. For co-infection experiments, homogenate was also plated on appropriate antibiotics to differentiate wild-type and mutant strains.

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 of LB or LB containing 200 μM DIP or 20 μM $FeCl_2$. Culture aliquots (200 μl) were mixed with 25 μl of cold 5% phenol-ethanol stop solution, pelleted (1 min, 10 000 *g*), and stored at –80°C for RNA isolation. Thawed pellets were re-suspended in 100 μl of RNase-free TE containing 1 mg ml^{-1} 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. Real-time qPCR was performed

using 30 ng of cDNA template and Brilliant SYBR® Green reagents (Stratagene). Data were normalized to *gapA* transcript and analysed 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 2 h intervals beginning at 24 h post inoculation, urine was collected and pooled from each cage of mice (five animals). Immediately after collection, cold 5% phenol-ethanol stop solution was added (0.125 µl of solution per µl of urine) and samples were pelleted (1 min, 10 000 *g*) and stored at –80°C. Pellets from five to seven time points were combined for RNA isolation, which was performed as described above.

Statistical analyses

Statistics were performed using GraphPad InStat® 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 test, and all others by the Student's *t*-test. GraphPad Prism® was used for non-linear regression analysis.

Acknowledgements

We would like to thank Alfredo Torres (University of Texas Medical Branch) for generously providing the CFT073 *chuA*::*cat* and HB101 *ent*::*kan* strains used in this study and Sara N. Smith and M. Chelsea Lane for assistance with our animal studies. Funding for this work was provided by Public Health Service Grant AI043360 from the National Institutes of Health.

References

Alteri, C.J., and Mobley, H.L. (2007) Quantitative profile of the uropathogenic *Escherichia coli* outer membrane proteome during growth in human urine. *Infect Immun* **75:** 2679–2688.

Arnoux, P., Haser, R., Izadi, N., Lecroisey, A., Delepierre, M., Wandersman, C., and Czjzek, M. (1999) The crystal structure of HasA, a hemophore secreted by *Serratia marcescens*. *Nat Struct Biol* **6:** 516–520.

Asuthkar, S., Velineni, S., Stadlmann, J., Altmann, F., and Sritharan, M. (2007) Expression and characterization of an iron-regulated hemin-binding protein, HbpA, from *Leptospira interrogans* serovar Lai. *Infect Immun* **75:** 4582–4591.

Blattner, F.R., Plunkett, G., 3rd, Bloch, C.A., Perna, N.T., Burland, V., *et al.* (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277:** 1453–1474.

Bracken, C.S., Baer, M.T., Abdur-Rashid, A., Helms, W., and Stojiljkovic, I. (1999) Use of heme–protein complexes by the *Yersinia enterocolitica* HemR receptor: histidine residues are essential for receptor function. *J Bacteriol* **181:** 6063–6072.

Buchanan, S.K., Smith, B.S., Venkatramani, L., Xia, D., Esser, L., Palnitkar, M., *et al.* (1999) Crystal structure of the outer membrane active transporter FepA from *Escherichia coli*. *Nat Struct Biol* **6:** 56–63.

Burkhard, K.A., and Wilks, A. (2007) Characterization of the outer membrane receptor ShuA from the heme uptake system of *Shigella dysenteriae*. Substrate specificity and identification of the heme protein ligands. *J Biol Chem* **282:** 15126–15136.

Datsenko, K.A., and Wanner, B.L. (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci USA* **97:** 6640–6645.

Ferguson, A.D., Hofmann, E., Coulton, J.W., Diederichs, K., and Welte, W. (1998) Siderophore-mediated iron transport: crystal structure of FhuA with bound lipopolysaccharide. *Science* **282:** 2215–2220.

Fischer, E., Gunter, K., and Braun, V. (1989) Involvement of ExbB and TonB in transport across the outer membrane of *Escherichia coli*: phenotypic complementation of *exb* mutants by overexpressed tonB and physical stabilization of TonB by ExbB. *J Bacteriol* **171:** 5127–5134.

Gadeberg, O.V., and Orskov, I. (1984) *In vitro* cytotoxic effect of alpha-hemolytic *Escherichia coli* on human blood granulocytes. *Infect Immun* **45:** 255–260.

Galen, J.E., Nair, J., Wang, J.Y., Wasserman, S.S., Tanner, M.K., Sztein, M.B., and Levine, M.M. (1999) Optimization of plasmid maintenance in the attenuated live vector vaccine strain *Salmonella typhi* CVD 908-htrA. *Infect Immun* **67:** 6424–6433.

Hagan, E.C., and Mobley, H.L. (2007) Uropathogenic *Escherichia coli* outer membrane antigens expressed during urinary tract infection. *Infect Immun* **75:** 3941–3949.

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

Heimer, S.R., Rasko, D.A., Lockatell, C.V., Johnson, D.E., and Mobley, H.L. (2004) Autotransporter genes *pic* and *tsh* are associated with *Escherichia coli* strains that cause acute pyelonephritis and are expressed during urinary tract infection. *Infect Immun* **72:** 593–597.

Izadi-Pruneyre, N., Huche, F., Lukat-Rodgers, G.S., Lecroisey, A., Gilli, R., Rodgers, K.R., *et al.* (2006) The heme transfer from the soluble HasA hemophore to its membrane-bound receptor HasR is driven by protein–protein interaction from a high to a lower affinity binding site. *J Biol Chem* **281:** 25541–25550.

Johnson, J.R., Jelacic, S., Schoening, L.M., Clabots, C., Shaikh, N., Mobley, H.L., and Tarr, P.I. (2005) The IrgA homologue adhesin Iha is an *Escherichia coli* virulence factor in murine urinary tract infection. *Infect Immun* **73:** 965–971.

Lane, M.C., Lockatell, V., Monterosso, G., Lamphier, D., Weinert, J., Hebel, J.R., *et al.* (2005) Role of motility in the colonization of uropathogenic *Escherichia coli* in the urinary tract. *Infect Immun* **73:** 7644–7656.

Lane, M.C., Alteri, C.J., Smith, S.N., and Mobley, H.L. (2007) Expression of flagella is coincident with uropathogenic *Escherichia coli* ascension to the upper urinary tract. *Proc Natl Acad Sci USA* **104:** 16669–16674.

Liu, X., Olczak, T., Guo, H.C., Dixon, D.W., and Genco, C.A. (2006) Identification of amino acid residues involved in heme binding and hemoprotein utilization in the *Porphyromonas gingivalis* heme receptor HmuR. *Infect Immun* **74:** 1222–1232.

Lloyd, A.L., Rasko, D.A., and Mobley, H.L. (2007) Defining genomic islands and uropathogen-specific genes in uropathogenic *Escherichia coli*. *J Bacteriol* **189:** 3532–3546.

Mobley, H.L., Green, D.M., Trifillis, A.L., Johnson, D.E., Chippendale, G.R., Lockatell, C.V., *et al.* (1990) Pyelonephritogenic *Escherichia coli* and killing of cultured human renal proximal tubular epithelial cells: role of hemolysin in some strains. *Infect Immun* **58:** 1281–1289.

Olczak, T., Dixon, D.W., and Genco, C.A. (2001) Binding specificity of the *Porphyromonas gingivalis* heme and hemoglobin receptor HmuR, gingipain K, and gingipain R1 for heme, porphyrins, and metalloporphyrins. *J Bacteriol* **183:** 5599–5608.

Otto, B.R., van Dooren, S.J., Nuijens, J.H., Luirink, J., and Oudega, B. (1998) Characterization of a hemoglobin protease secreted by the pathogenic *Escherichia coli* strain EB1. *J Exp Med* **188:** 1091–1103.

Russo, T.A., Carlino, U.B., and Johnson, J.R. (2001) Identification of a new iron-regulated virulence gene, *ireA*, in an extraintestinal pathogenic isolate of *Escherichia coli*. *Infect Immun* **69:** 6209–6216.

Russo, T.A., McFadden, C.D., Carlino-MacDonald, U.B., Beanan, J.M., Barnard, T.J., and Johnson, J.R. (2002) IroN functions as a siderophore receptor and is a urovirulence factor in an extraintestinal pathogenic isolate of *Escherichia coli*. *Infect Immun* **70:** 7156–7160.

Skare, J.T., Ahmer, B.M., Seachord, C.L., Darveau, R.P., and Postle, K. (1993) Energy transduction between membranes. TonB, a cytoplasmic membrane protein, can be chemically cross-linked *in vivo* to the outer membrane receptor FepA. *J Biol Chem* **268:** 16302–16308.

Smith, G.R., Halpern, Y.S., and Magasanik, B. (1971) Genetic and metabolic control of enzymes responsible for histidine degradation in *Salmonella typhimurium*. 4-Imidazolone-5-propionate amidohydrolase and N-formimino-L-glutamate formiminohydrolase. *J Biol Chem* **246:** 3320–3329.

Stugard, C.E., Daskaleros, P.A., and Payne, S.M. (1989) A 101-kilodalton heme-binding protein associated with Congo red binding and virulence of *Shigella flexneri* and enteroinvasive *Escherichia coli* strains. *Infect Immun* **57:** 3534–3539.

Thomas, P.E., Ryan, D., and Levin, W. (1976) An improved staining procedure for the detection of the peroxidase activity of cytochrome P-450 on sodium dodecyl sulfate polyacrylamide gels. *Anal Biochem* **75:** 168–176.

Torres, A.G., and Payne, S.M. (1997) Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. *Mol Microbiol* **23:** 825–833.

Torres, A.G., Redford, P., Welch, R.A., and Payne, S.M. (2001) TonB-dependent systems of uropathogenic *Escherichia coli*: aerobactin and heme transport and TonB are required for virulence in the mouse. *Infect Immun* **69:** 6179–6185.

Wandersman, C., and Stojiljkovic, I. (2000) Bacterial heme sources: the role of heme, hemoprotein receptors and hemophores. *Curr Opin Microbiol* **3:** 215–220.

Welch, R.A., Burland, V., Plunkett, G., 3rd, Redford, P., Roesch, P., *et al.* (2002) Extensive mosaic structure revealed by the complete genome sequence of uropathogenic *Escherichia coli*. *Proc Natl Acad Sci USA* **99:** 17020–17024.

Wyckoff, E.E., Duncan, D., Torres, A.G., Mills, M., Maase, K., and Payne, S.M. (1998) Structure of the *Shigella dysenteriae* haem transport locus and its phylogenetic distribution in enteric bacteria. *Mol Microbiol* **28:** 1139–1152.

