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Expression of the *Escherichia coli* IrgA homologue adhesin is regulated by the ferric uptake regulation protein

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Abstract

The IrgA homologue adhesin (Iha) is an adherence-conferring outer membrane protein of *Escherichia coli* associated with enterohemorrhagic and uropathogenic strains. Here, we used primer extension analysis to identify *iha* promoters in O157:H7 and uropathogenic *E. coli* strains. Transcriptional fusions demonstrated that *iha* transcription is repressed by iron. Gel shifts using purified ferric uptake regulator protein (Fur) demonstrated that repression involves a direct interaction between Fur and the *iha* promoter. We identified strain-dependent differences in *iha* expression and determined that single nucleotide polymorphisms upstream of the *iha* promoter, in particular position –85, contribute to differences in expression levels.

Keywords

Iha; enterohemorrhagic *E. coli*; uropathogenic *E. coli*; Fur

1. Introduction

Escherichia coli O157:H7 is a Shiga toxin-producing organism that causes diarrhea, bloody diarrhea, and the hemolytic uremic syndrome. A homologue of the locus of enterocyte effacement (LEE) pathogenicity island of enteropathogenic *E. coli* encodes proteins associated with the attaching and effacing lesions on intestinal epithelial cells colonized by enterohemorrhagic *E. coli* (EHEC) such as serotype O157:H7 [reviewed in 1]. However, the mechanism of initial adherence of these organisms is not well understood. Candidate EHEC adherence factors include long polar fimbriae (LPF) related to those of *Salmonella enterica* [2,3]; ToxB, encoded on plasmid pO157, which positively influences the adherence of O157:H7 [4]; EHEC factor for adherence (*efa1*) [5] in sorbitol-fermenting O157:H- (but which is not found in the closely related *E. coli* O157:H7); an *sfp* operon homologous to the P-fimbrial operon of uropathogenic *E. coli* also found in sorbitol-fermenting O157:H- [6]; and F9 fimbriae [7].

The IrgA homologue adhesin (Iha) encoded by *iha* is an adherence-conferring protein of *E. coli* O157:H7 [8] that is also a catecholate siderophore receptor [9]. *iha* is present in 25 of 25

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O157:H7 isolates of diverse origins but in only 3 of 20 commensal non-O157 fecal *E. coli* [8]. Additionally, *iha* is conserved among EHEC of a variety of serotypes [10]. Interestingly, an *iha* homologue is present on an island located at *selC* (where LEE is generally integrated into the chromosome) of LEE-negative STEC that produce activatable Stx2d [11].

iha is frequently found in uropathogenic *E. coli* (UPEC). Johnson *et al.* found that *iha* was present in 55% of urosepsis isolates and was more prevalent in isolates from immunocompetent hosts, suggesting that Iha confers some pathogenic advantage [12]. In a different study, Johnson *et al.* found that presence of *iha* predicted multiple same-strain recurrences of urinary tract infections (UTI) [13]. Also, *iha* was more frequent in UTI than fecal isolates [14]. Finally, *iha* is required for full virulence of the pyelonephritis isolate CFT073 in a mouse model of ascending UTI, defining it as a virulence factor, at least in this organism [15].

Some data have addressed expression of Iha. Tarr *et al.* found that a 78 kDa protein, which is the predicted size of Iha and which was reactive with an anti-Iha antibody, was expressed in outer membrane proteins (OMPs) from recombinant strain ORN172 transformed with a plasmid that expressed Iha (pIHA); however, they did not observe a 78 kDa protein in OMPs of *iha*-containing O157:H7 isolates which were grown in LB [8]. Schmidt *et al.* noted that Iha of non-O157:H7 STEC was more easily detected in OMPs of bacteria grown in glycerol-DMEM than LB [11]. Likewise, Iha is better expressed in CFT073 when grown in DMEM but not LB [15]. Also, *iha* was expressed *in vivo* in a mouse model of peritonitis during infection with CFT073 [16]. Following preparation of this manuscript, Leveille *et al.* reported that Iha of the uropathogen UCB34 is regulated by iron availability [9]. Finally, we have recently identified *iha* expression in *E. coli* O157:H7 in stools that have been freshly shed by infected children and experimentally inoculated cattle [17].

The purpose of this work was to characterize the mechanism of regulation of Iha expression in *E. coli* O157:H7 strain 86-24 [18] and UPEC O6:K2:H1 strain CFT073 [19], specifically, testing the hypothesis that *iha* is regulated at the transcriptional level by iron. This hypothesis is based on the presence of a ferric uptake regulator protein (Fur) binding site predicted upstream of the *iha* coding region [20].

2. Results

2.1. Identification of the *iha* promoter

To examine the regulation of expression of *iha*, we first identified its promoter. Primer extensions to determine the 5' end of *iha* mRNA in recombinant *E. coli* strain ORN172 (pIHA) [8] identified an extension product that is not present in the vector control. This product is also present in clinical isolates 86-24 and CFT073 when these organisms are grown in DMEM minimal medium (figure 1A). S1 nuclease protection of ORN172 (pIHA) confirmed that this product represents a true 5' end rather than a transcriptional pause site (data not shown). Though primer extension analysis identified two 5' ends, at G and T, only the T was identified using S1 nuclease analysis. Insertion of the omega fragment containing transcriptional terminators [21] in ORN172 (pIHA) upstream of the putative promoter region did not abolish the primer extension product, indicating this product is the result of transcription from the *iha*, and not a vector, promoter (data not shown). The omega fragment was inserted in the correct orientation to block transcription [22].

The -10 and -35 sequences upstream of the transcriptional start sites are identical in strains 86-24 and CFT073 (figure 1B). Homology between strains CFT073 and 86-24 upstream of *iha* begins 464 bp upstream of the transcriptional start site, and there are 5 single nucleotide polymorphisms in this interval, at positions -460, -414, -214, -85, and -51. The sequence

upstream of *iha* in the genomes of O157:H7 strains EDL933 and Sakai are identical to 86-24 at these five positions [23,24].

2.2. *iha* is repressed by iron

We hypothesized that Fur represses *iha* in the presence of iron, based on the prediction of Panina *et. al*, who identified a putative Fur binding site upstream of *iha* that contains 13 of the 19 bases of the consensus site [20] and spans the -35 locus. To test this hypothesis, we first examined *iha* transcriptional regulation by constructing single copy chromosomal transcriptional fusions of approximately 1.6 kb of *iha* with the firefly *luc* reporter gene. We chose to use chromosomal fusions so that any upstream regulatory elements would be included. A constitutively expressed sea pansy *Renilla reniformis luc* gene was utilized as a control, and luciferase activity was measured as the ratio of firefly:*Renilla* Relative Light Units (RLU) (figure 2). In both 86-24 and CFT073, the fusion was expressed in cells grown in DMEM minimal medium to a significantly greater extent than in DMEM medium supplemented with 10 μ M FeSO₄. Expression in CFT073 was approximately 25-fold higher in DMEM than iron-supplemented DMEM, and expression in 86-24 was approximately 2–5-fold higher in DMEM than iron-supplemented DMEM. *iha* expression in LB was approximately equal to *iha* expression in iron-supplemented DMEM for both strains. Addition of 200 μ M 2,2'-dipyridyl, an iron chelator, to LB increased expression.

To determine if *iha* gene expression correlates with levels of protein, Iha was detected by immunoblot analysis in outer membrane protein preparations of 86-24 and CFT073 grown in DMEM with or without added iron, and LB with or without added 2,2'-dipyridyl. As predicted, a 78 kDa band representing Iha was detected in cells grown under iron-limiting, but not iron-replete, conditions (figure 3).

2.3. Interaction of the *iha* promoter with Fur

The observed transcriptional repression of *iha* in the presence of iron is consistent with our hypothesized role for Fur in *iha* regulation. To further determine if Fur binds directly to the *iha* promoter region, Fur was isolated for use in gel-shift analysis.

A 150 bp PCR product surrounding the putative Fur-binding site was used as a probe in gel-shift analysis. The probe's migration through a polyacrylamide gel was retarded after adding increasing concentrations of Fur (figure 4A), indicating that Fur bound the *iha* promoter region. Multiple shift products were observed, as has been described for Fur [25,26]. Abundance of the higher molecular weight complex was decreased when the binding reaction was incubated in increasing (competitive) concentrations of unlabeled probe or of a short double-stranded oligonucleotide consisting of only the putative *iha* Fur-binding site flanked by 7 bp on either side (figure 4C). This suggests competition for Fur between the binding site on the labeled probe and on the competitor DNA. A second short oligonucleotide containing a 5 bp mutation previously demonstrated to reduce Fur binding to the FepA promoter [27] had diminished ability to inhibit formation of the higher molecular weight complex (figure 4C). Together, these data demonstrate that Fur binds to the *iha* promoter at the site predicted by Panina *et. al* [20].

2.4. Additional regulation of *iha*

During the course of this work, we observed that under iron-limiting conditions, CFT073 expresses *iha* to a greater extent than does 86-24, as demonstrated by primer extensions (figure 1A) and luciferase assays (figure 2); these observations correlate with expression of Iha antigen (figure 3). Northern analyses of 86-24 Δ *fur* and CFT073 Δ *fur* confirm that *iha* message is more abundant in CFT073 even in the absence of Fur (data not shown). As expected, *iha* expression in 86-24 Δ *fur* and CFT073 Δ *fur* is constitutive in iron-replete and iron-poor media; however,

we still observe higher *iha* expression in the CFT073 background, suggesting this difference is independent of Fur-mediated repression.

To test the hypothesis that this difference might be associated with the nucleotide polymorphisms closest to the promoter region (positions –51 and –85), we inserted the promoter regions of 86-24 and CFT073 into plasmid pRS415 to drive expression of the promoterless *lacZ* gene. β -galactosidase activities for these fusions were measured in strain CC1005, a host in which pRS415 copy number is reduced to approximately 10% [28]. By examining the fusions in identical backgrounds, we were able to determine the effects of these polymorphisms without the complications that might arise from differences in strain background. pCFT073*ihaP*, containing the promoter from CFT073, yielded approximately 1.3-fold higher activity than pO157*ihaP* (figure 5) ($P = 0.02$). This suggests that the nucleotide differences between the two strains at positions –51 and/or –85 contribute to differences in *iha* expression.

To elucidate which position is responsible for differences in *iha* expression, two additional *lacZ* promoter fusions were constructed: plasmid pihaP-G/T contains the –85 position G (identical to 86-24) and the –51 position T (identical to CFT073). Plasmid pihaP-A/C contains the –85 position A (identical to CFT073) and the –51 position C (identical to 86-24). CC1005 (pihaP-A/C) had expression similar to CC1005 (pCFT073*ihaP*), whereas CC1005 (pihaP-G/T) had expression similar to CC1005 (pO157*ihaP*) (figure 6). These data suggest that the –85 polymorphism most strongly influences *iha* expression.

3. Discussion

Iron is vital for bacterial growth; but it is essential that bacteria strictly regulate the amount of iron that enters, because excess iron catalyzes the production of the hydroxy radical (OH $\cdot$) [29]. One method to monitor and to respond to the iron in the bacterial cell is via the ferric uptake regulation (Fur) protein [30]. In the presence of iron, Fur dimerizes and can bind DNA to repress transcription. In the absence of iron, Fur does not bind DNA, and target genes are expressed. Fur regulates several genes involved in iron uptake, and it also provides the cell with a way to express other genes in an iron-limited environment. Because mucosal surfaces of the mammalian host are iron-poor environments due to the high affinity of lactoferrin for free iron [reviewed in 31], several bacterial virulence factors, including *stx1* of *E. coli* and *irgA* of *V. cholerae*, are transcribed under iron-poor conditions [32,33]. In this study, we demonstrate that *iha* is repressed by iron via a direct interaction between Fur and the *iha* promoter. This regulation might allow optimal expression of *iha* within the host, where its role as an adhesin would be useful to the bacterium. Consistent with this hypothesis, *iha* was upregulated in the mouse model of peritonitis [16] and is required for full virulence of UPEC [15]. Our results explain why Schmidt *et al.* [11] and Johnson *et al.* [15] found Iha to be more readily detected in minimal medium than in LB and why Tarr *et al.* [8] did not detect Iha in O157 isolates grown in LB.

Iron uptake systems are vital to bacterial survival within the host, and many bacteria secrete siderophores, which bind iron and are brought into the bacterial cell via a specific siderophore receptor. Iha has 33% identity to IrgA of *Vibrio cholerae* and 27% identity to FepA of *E. coli*. Both of these proteins are receptors for the siderophore enterobactin and, like Iha, are directly repressed by Fur [27,34–36]. In fact, Iha from the uropathogen, UCB34, was recently determined to be iron-repressed and to act as both a catecholate siderophore receptor and an adhesin [9].

Interestingly, our results demonstrate that *iha* is more highly expressed in CFT073 than in 86-24 under iron-limiting conditions. Although the –35 and –10 regions are identical in these

strains, it is possible that single nucleotide polymorphisms in other areas of the promoter region might affect *iha* transcription, in particular those closest to the promoter region, at positions –51 and –85. Our data demonstrate that these nucleotides do contribute to differential expression of *iha*, and that this is mainly the results of position –85, though it is unlikely to fully account for the observed differences in expression levels. It is likely that additional strain-specific factors play a role, for example a repressor in 86-24, an activator in CFT073, or additional 5' polymorphisms such as those at –460, –414, and –214.

In conclusion, regulation of *Iha* occurs at the transcriptional level via a direct interaction with *Fur*, which represses *iha* expression in the presence of iron. Though the mechanism of repression is the same, *iha* is expressed differently in 86-24 and CFT073 under iron-limiting conditions. Single nucleotide polymorphisms near the promoter, in particular that at position –85, contribute to this difference.

4. Materials and Methods

4.1. Media and growth conditions

Bacteria were grown at 37° in Luria-Bertani (LB) medium or in DMEM minimal medium prepared as described [15]. Ampicillin (100µg/ml), chloramphenicol (25 µg/ml), gentamicin (15 µg/ml), and nalidixic acid (15µg/ml) were added, if appropriate. To replete or restrict iron, FeSO₄·7H₂O was added to DMEM minimal medium to a final concentration of 10 µM, or 2,2' dipyridyl was added to a final concentration of 200 µM to LB, respectively.

4.2. Insertion of omega fragment

To insert the omega fragment upstream of *iha*, pUC19Ω [21] was digested with *Sma*I and the omega fragment moved into the *Sph*I site of pIHA that had been blunt-ended using T4 DNA polymerase (NEB). Correct orientation of the omega fragment was confirmed by sequencing with T7 Sequenase v2.0 (USB).

4.3. Primer extension analysis

RNA was extracted from bacteria [37] that had been grown to logarithmic phase in a shaking incubator at 37°C in LB containing ampicillin for recombinant strains, or in DMEM minimal medium for strains 86-24 and CFT073. Primer extensions were performed using primer *iha*.PE2 (5'CGAGACAGGGAATGACTACGGAAGC3') as described [38].

4.4. S1 nuclease analysis

End-labeling of probe, hybridization, and S1 nuclease digestion were performed as described [39] using primer *iha*.s1 (5' CGCATATTACTAATCTCCGATCATGTTAACCGGCAAGACAATACACAAATA CAAACAATAATGAGAATAATTTATATTTGCC3').

4.5. Construction of chromosomal *iha*-luciferase transcriptional fusions

pIHA was digested with *Eco*RI and *Nae*I, each of which cleaves within the *iha* open reading frame. A 567 bp fragment was gel-purified and ligated into vector pGPL01 [40], which had been digested with *Sma*I and *Eco*RI. pGPL01 is a suicide vector with a lambda/pir-dependent origin of replication that contains the promoterless *luc* gene encoding firefly luciferase. The resulting 6.5 kb construct, pGPL01R3, containing 567 bp of *iha* sequence in-frame with a TAA stop codon upstream of *luc*, was transformed into SM10λpir and used as the donor strain in matings where 86-24nal^R [41] or CFT073nal^R [15] was the recipient. Matings were performed as described previously [42] and nalidixic acid-resistant, ampicillin-resistant transconjugates, designated 86-24A3 and CFT073B5, respectively, were selected. Integration of the suicide

vector into the chromosome in the correct orientation was confirmed by PCR analysis using a forward primer in *iha* and a reverse primer in *luc*.

Vector pEM42 (gift of Dr. Samuel Miller) contains the tet promoter from pBR322 driving expression of the sea pansy *Renilla reniformis* luciferase in pRL-Pnull (Promega). pCRIICam (gift of Dr. Brad Cookson) contains *cat* in the *Age*I site of pCRII (Invitrogen). *cat* was excised from pCRIICam with *Age*I and blunt ends were created using T4 DNA polymerase as directed by the manufacturer (NEB). This product was inserted into the *Bam*HI site of pEM42, which had been blunt-ended with T4 DNA polymerase, to create pRL-PtetCm. This construct was electroporated into 86-24A3 and CFT073B5, where it served as a constitutively expressed control in the luciferase assays.

4.6. Luciferase assays

Overnight cultures of cells were diluted and grown to OD₆₀₀ = 0.6 in 3 ml of appropriate medium. 90 µl of culture was mixed with 10 µl 1 M K₂HP0₄ (pH 7.8)/20 mM EDTA (pH 8.0) and frozen at -80°C. Samples were thawed and analyzed with the Dual-Luciferase Reporter Assay System (Promega) per the manufacturer's recommendations. Data reported represent the ratio of firefly to *Renilla* luciferase activity in relative light units (RLU) for three experiments measured in duplicate.

4.7. Construction of *iha*-β-galactosidase transcriptional fusions

The *iha* promoter regions from 86-24 and CFT073, encompassing the -51 and -85 single nucleotide polymorphisms, were amplified using primers *iha*.BamF (5' GGGATCCAATTCTGGCATGCCGAGGCAGTCG3') [8] and RR10Bam-2 (5' GGGATCCTCAACATCCACACCCTCTAC3'). The resulting product was digested with *Bam*HI, ligated into the *Bam*HI site of vector pRS415 [43], and transformed into DH5α. pRS415 contains a promoterless *lacZ*; thus, colonies were selected that expressed β-galactosidase on iron-limiting plates containing X-gal and ampicillin. The correct sequence and orientation of the *iha* promoter was confirmed via sequence analysis using BigDye v3.1 (Applied Biosystems). Resulting vectors containing the 86-24 or CFT073 *iha* promoter driving β-galactosidase expression were named pO157ihaP and pCFT073ihaP, respectively. These plasmids were introduced into strain CC1005 (MC4100 *pcn*B^{Tn10} Δ *lac*U169 *ara*D139 *rps*L^{*thi*}) (gift of Dr. Colin Manoil). The number of ColE1-related plasmids is reduced in *E. coli* *pcn*B mutants to approximately 10% [28]; therefore, pRS415, which has an origin of replication from pBR322, is maintained at low copy number in CC1005.

To construct *piha*P-G/T and *piha*P-A/C, the *iha* promoter regions of 86-24 and CFT073 were amplified using two polymerase chain reactions. Primers RR15 (5' CGGGATCCCCATTCATGGTTGAC 3') and RR14 (5' CTTTACAAAAAATAAAGATTATTGAC 3') were used to amplify the region surrounding position -85. Primers RR13 (5' GTCAATAATCTTTATTTTGTAAAAG 3') and RR10-Bam.2 (5' GGGATCCTCAACATCCACACCCTCTAC 3') were used to amplify the region surrounding position -51. The resulting amplicons were column purified using the QIAquick PCR Purification Kit (Qiagen, Maryland), and 1 µL of the upstream and downstream amplicon were used as template to amplify the entire promoter region with primers RR15 and RR10-Bam.2. The program parameters were: (94°C 1 min, 55°C 1 min, 72°C 3 min) x 30 cycles. The resulting 468 bp product was gel-purified using the QIAquick Gel Extraction Kit (Qiagen, Maryland), digested with *Bam*HI, ligated into the *Bam*HI site of pRS415, and transformed into DH5α. Colonies were selected that expressed β-galactosidase on iron-limiting plates containing X-gal and ampicillin. Correct sequence and orientation of the *iha* promoter was confirmed via sequence analysis using BigDye v3.1 (Applied Biosystems). *piha*P-G/T contains the -85 region from 86-24 and the -51 region from CFT073. *piha*P-A/C contains the -85

region from CFT073 and the -51 region from 86-24. *pihA*P-G/T and *pihA*P-A/C were moved into the CC1005 background for use in β -galactosidase assays.

4.8. β -galactosidase assays

Overnight cultures of CC1005 (pO157ihaP) and CC1005 (pCFT073ihaP) were diluted in LB containing ampicillin and 2, 2' dipyridyl and grown to $OD_{600} = 0.5 - 0.6$. Cells were iced for 20 minutes, then pelleted before resuspension in 1x PBS. β -galactosidase activity was measured [42], and data reported represent the results of four experiments performed in triplicate for Figure 5. For Figure 6, data reported represent the results of one representative experiment performed in triplicate. Three additional experiments were performed with similar results.

4.9. OMP preparation and analysis

OMPs were prepared as previously described [44] from bacteria grown overnight at 37°C with shaking in 100 ml of medium, except that cells were lysed by French press. The protein concentration was determined using the Bradford Assay [45], and 2 μ g outer membrane proteins were separated on a 12% SDS-polyacrylamide gel. To observe total protein, gels were Coomassie stained and stored in acetate paper [46].

To observe Iha, proteins were transferred to a nitrocellulose membrane (PROTRAN; Schleicher & Schuell BioScience) for two hours at 7.5 volts/cm in Towbin transfer buffer (25 mM Tris, 192 mM glycine, 20% MeOH, 0.1% SDS). The membranes were blocked in PBS containing 0.05% Tween-20 and 5% (w/v) non-fat dried milk for two hours at room temperature, and then washed in PBS containing 0.05% Tween-20 (PBS-T) and incubated at 4°C overnight in rabbit polyclonal anti-Iha antibody [8] diluted 1:2000 in PBS-T. The membranes were then washed three times in PBS-T and incubated in goat anti-rabbit alkaline phosphatase (AP)-conjugated antibody (Pierce, Rockford, IL), diluted 1:2000 in PBS-T for one hour at room temperature. The membrane was washed three times and bound antibodies were detected in AP assay buffer (0.1 M Tris pH 9.5, 5 mM $MgCl_2$, 100 mM NaCl) by adding nitro blue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP).

4.10. Slot blot analysis

86-24 Δfur and CFT073 Δfur were constructed using the λ /Red system to create in-frame chromosomal deletions [47] using primers FurDelL2 (5' CAGGAGCCGACAACCATCACGTCAGTGCCTGTGTAGGCTGGAGCTGCTT C3') and FurDelR2 (5' GTGGTTAGTCAGGCGAATGCCATGTTTTGCCATATGAATATCCTCC3'). Analysis was performed on RNA immobilized by slot blotting [39]. Primers *iha*L2 (5' GAGGCTGGGGTGTATTACGA3') and *iha*R2 (5' CAGTGACAGCGTGACATCCT3') were used to amplify *iha* in 86-24, and the resulting amplicon was used to create a probe using the Random Primers DNA Labeling System (Invitrogen) following instructions of the manufacturer.

4.11. Fur Purification

pET21dFur (gift of Dr. Thomas O'Halloran) contains *fur* digested from pARC306FUR [48] using *Hind*III and *Nco*I and moved into the same sites of pET21d (Novagen). pET21dFur was transformed into BL21(DE3) (Novagen), and the purification strategy was based on previously published methods [48,25,26]. An overnight culture was diluted 1:100 and grown to $OD_{600} = 0.6$ while shaking at 37°C. 400 μ M isopropyl- β -D-thiogalactopyranoside was added to induce Fur expression, and the culture was grown for an additional 2.5 hours. Cells were harvested by centrifugation and washed in 20 mM Tris-HCL, pH 8.0, 50 mM EDTA. The cell pellet was frozen at -80°C overnight. On the next day, the pellet was suspended in an equal volume (to

the pellet) of ice-cold 20 mM Tris-HCL, pH 8.0 containing 1 mM of the protease inhibitor Pefabloc (Boehringer Mannheim), and sonicated. Cell debris was removed by centrifugation, and 20% w/v streptomycin sulfate was added to a final concentration of 1% to precipitate nucleic acids. Nucleic acids were removed by centrifugation at 20,000 x g for 15 minutes at 4°C, and ammonium sulfate was added to the supernatant to 80% saturation. The sample was incubated at 4°C overnight to precipitate protein. Protein was harvested by centrifugation at 20,000 x g for 30 minutes at 4°C. The protein pellet was dissolved in 6 ml 20 mM Tris-HCL, pH 8.0, 1 mM DTT and added to an iminodiacetate agarose (IDA) column that had a bed volume of 3 ml. The IDA resin was obtained from Novagen and equilibrated following the recommendation of the manufacturer using 5 bed volumes of 20 mM ZnSO₄. The column was then washed with 3 bed volumes of IDA Wash Buffer (50 mM Tris-Acetate, pH 7.9; 500 mM NaCl). The sample was run through the column at a flow rate of approximately 0.5 ml/min. The column was washed with 5 bed volumes of IDA Wash Buffer, and protein was eluted in wash buffer containing increasing amounts of L-histidine. Sample aliquots were mixed with Bradford Reagent, and samples containing protein were pooled and precipitated by adding ammonium sulfate to 80% saturation. Protein was centrifuged as before; the pellet was suspended in 1 ml HEPES buffer (20 mM HEPES, pH 7.0, 0.1 mM NaCl, 2 mM DTT, 5% glycerol) and added to a Sephadex G25 column to de-salt. Final protein concentration was determined using the Bradford Assay [45]. Recovered Fur shows a predominant protein band of 17 kDa on SDS-PAGE, which reacts with polyclonal anti-Fur antibody in immunoblot analysis (data not shown).

4.12. Gel-Shift Analysis

Primers *iha*.EcoR (5'CGGAATTCCGATCTCCGATCATGTTAACCG3') and *iha*.EcoL (5'CGGAATTCCGGCATGCCGAGGCAGTCGTTA3'), containing *Eco*RI sites at the 5' ends, were used to amplify an approximately 150 bp PCR product surrounding the putative Fur binding site of the *iha* promoter. PCR was performed using Amplitaq, buffer, and MgCl₂ from Applied Biosystems, and dNTPs from NEB. 4 pmol of this product was digested with *Eco*RI and end-labeled with ³²P using T4 Polynucleotide Kinase as directed by the manufacturer (NEB). Reaction products were cleaned using a Sephadex G50 column (Nick Column; Amersham) per the manufacturer's recommendations. Approximately 125 fmol/ml of probe was used in a binding reaction containing 40 mM KCl, 50 µg/ml polyIdC, 10 mM BisTris, pH 7.5, 100 µg/ml BSA, 100 µM MnCl₂·4H₂O, 1 mM MgCl₂·6H₂O, 5% glycerol, and increasing concentrations of Fur [49]. 20 µl of the binding reaction was electrophoresed on a 5% acrylamide gel containing 20 mM BisTris (pH 7.5), 100 µM MnCl₂·4H₂O. Running buffer was 20 mM BisTris (pH 7.5), 100 µM MnCl₂·4H₂O. The gel was run at 5.3 V/cm, and the buffer in the upper chamber was replaced with fresh buffer as it oxidized.

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Figure 1. Identification of the *iha* promoter region

(A): Identification of the 5' end of the *iha* transcript.

Primer extension products were analyzed on a 6% acrylamide sequencing gel. A product is observed in ORN172(pIHA) but not in the vector control ORN172(pSK+). A product of the same size is observed in clinical isolates CFT073 and 86-24 when grown in DMEM minimal medium.

(B): Promoter region identified by primer extension analysis.

Putative ribosome binding site, -10 and -35 sequences, and putative Fur binding site are noted. The transcriptional start site identified by primer extensions and nuclease protection assays is underlined.

Figure 2. *iha* transcription is repressed in the presence of iron

Cells containing single-copy chromosomal transcriptional fusions between *iha* and firefly *luc* were grown in DMEM minimal medium with or without addition of 10 μ M FeSO₄·7H₂O. Cells were grown in LB with or without the addition of 200 μ M of the iron chelator 2,2' dipyridyl. Results are shown as the ratio of firefly: *Renilla* RLU at OD₆₀₀ 0.6. (A) CFT073B5 grown in the presence or absence of iron. (B) 86-24A3 grown in the presence or absence of iron.

**p < 0.01

Figure 3. Iha is expressed under iron-limiting conditions

Iha expression was examined in outer membrane preparations of overnight cultures grown in iron-replete or iron-limiting conditions. 2 μ g total outer membranes were separated by SDS-PAGE and transferred to nitrocellulose for immunoblot analysis (left) or stained with Coomassie blue (right).

(A): Addition of 10 μ M $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ to DMEM abolishes Iha expression in the outer membrane.

(B): Addition of the iron chelator 2,2'dipyridyl to LB results in expression of Iha in the outer membrane.

Figure 4. Fur binds the *iha* promoter region

(A): A 150 bp DNA fragment encompassing the putative Fur-binding site was radiolabelled, incubated with increasing concentrations of Fur protein, and separated on a 5% acrylamide gel.

(B): Unlabeled competitor DNA sequences. Center 19 nucleotides represent the putative *iha* Fur binding site (underlined). Capital letters represent sequence that has been changed to decrease Fur binding.

(C): Fur binding in the presence of unlabeled competitor DNA. Lane 1, probe only; all other lanes, probe with 50 nM Fur. Lanes 3–5 contain 0.5 pMol, 2 pMol, and 5 pMol unlabeled probe, respectively. Lanes 7–10 contain 10 ng, 50 ng, 100 ng, and 150 ng, respectively, of unlabeled wt *iha* Fur binding site. Lanes 11–14 contain 10 ng, 50 ng, 100 ng, and 150 ng, respectively, of unlabeled mutated *iha* Fur binding site.

Figure 5. Role of single nucleotide polymorphisms in *iha* expression

Cells were grown to mid-log phase in LB containing 200 μ M 2,2'dipyridyl and assayed for β -galactosidase activity as expressed in Miller Units. CC1005(pO157ihaP) contains the promoter from 86-24 driving *lacZ* expression. CC1005(pCFT073ihaP) contains the promoter from CFT073 driving *lacZ* expression. CC1005(pRS415) is the vector-only control. **P = 0.02

Figure 6. Role of individual polymorphisms in *iha* expression

Cells were grown to mid-log phase in LB containing 200 μ M 2,2'dipyridyl and assayed for β -galactosidase activity as expressed in Miller Units. CC1005 (pCFT073ihaP) and CC1005 (pihaP-A/C) express significantly more β -galactosidase than CC1005 (pO157ihaP) and CC1005 (pihaP-G/T). $P < 0.05$

Table 1

Strains and Plasmids

<i>E. coli</i> strains	Description	Reference
86-24	EHEC O157:H7	[18]
CFT073	UPEC O6:K2:H1	[19]
SM10 λ pir	λ pir integrate with RP4 mob function	[50]
CC1005	MC4100 <i>pcnBTn10 DlacU169 araD139 rpsL thi</i>	gift of Dr. Colin Manoil
BL21(DE3)	F- <i>ompT hsdS gal dcm</i> (DE3)	Novagen
86-24nalR		[41]
CFT073nalR		[15]
ORN172		[51]
86-24A3	contains chromosomal <i>iha-luc</i> transcriptional fusion	this work
CFT073B5	contains chromosomal <i>iha-luc</i> transcriptional fusion	this work
plasmids	Description	Reference
pIHA	<i>iha</i> in pBluescript	[8]
pUFRO47	IncW replicon	[52]
pUC19 Ω	omega fragment in pUC19	[21]
pGPL01	λ pir-dependent origin of replication and promoterless <i>luc</i>	[40]
pRS415	contains promoterless β -galactosidase and origin of replication from pBR322	[43]
pEM42	<i>tet</i> promoter drives expression of <i>Renilla</i> luciferase in pRL-Pnull	gift of Dr. Samuel Miller
pCRIICam	<i>cat</i> in <i>AgeI</i> site of pCRII	gift of Dr. Brad Cookson
pET21dFur	<i>fur</i> in pET21d	gift of Dr. T.V. O'Halloran
pGPL01R3	<i>iha</i> sequence upstream of <i>luc</i> in pGPL01	this work
pRL-PtetCm	<i>cat</i> from pCRIICam in <i>Bam</i> HI site of pEM42	this work
pO157ihaP	86-24 <i>iha</i> promoter driving β -galactosidase expression in pRS415	this work
pCFT073ihaP	CFT073 <i>iha</i> promoter driving β -galactosidase expression in pRS415	this work
pihaP-G/T	same as pO157ihaP but with "T" at -51	this work
pihaP-A/C	same as pO157ihaP but with "A" at -85	this work