The PAS domain-containing protein HeuR regulates heme uptake in

*Campylobacter jejuni*

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Short title: Regulation of heme uptake in *Campylobacter jejuni*
Abstract

Campylobacter jejuni is a leading cause of bacterial-derived gastroenteritis. A previous mutant screen demonstrated that the heme uptake system (Chu) is required for full colonization of the chicken gastrointestinal tract. Subsequent work found identified a PAS domain-containing regulator, termed HeuR, as required for chicken colonization. Here we confirm that both the heme uptake system and HeuR are required for full chicken gastrointestinal tract colonization, with the heuR mutant being particularly affected during competition with wild-type C. jejuni. Transcriptomic analysis identified the chu genes – and those encoding other iron uptake systems – as likely regulatory targets of HeuR. Purified HeuR specifically bound the chuZA promoter region in electrophoretic mobility shift assays. Consistent with a role for HeuR in chu expression, heuR mutants were unable to efficiently use heme as a source of iron in iron-limiting conditions and, mutants exhibited decreased levels of cell-associated iron by mass spectrometry. Finally, we demonstrate that a heuR mutant of C. jejuni is resistant to hydrogen peroxide, and that this resistance correlates to elevated levels of catalase activity.

Author Summary

Campylobacter jejuni causes millions of gastrointestinal infection every year. This is primarily due to its ability to reside in the gastrointestinal tract of
chickens. *C. jejuni* contaminates chicken meat during harvesting and processing. Following consumption of undercooked chicken or uncooked food that was contaminated with raw chicken juice, humans develop a debilitating illness that is characterized by diarrhea and abdominal cramps. As chickens are the source of most human infections, there is a need to understand how *C. jejuni* colonizes chickens so we can develop ways to reduce its presence in chickens and thereby improve food safety. Most organisms require iron to thrive and that some bacteria steal iron from host molecules, including hemoglobin. Here we demonstrate that *C. jejuni* may need to get iron from hemoglobin in order to colonize the chicken and that a regulatory protein, HeuR, controls the ability of the bacteria to do this. If we can understand how this protein works, we may be able to develop ways to inhibit its function and reduce the ability of *C. jejuni* to get iron during chicken colonization. This would limit the amount of *C. jejuni* in the chicken and make food safer.

**Introduction**

*Campylobacter jejuni* is a leading cause of gastrointestinal infection worldwide, with a projected 1.3 million annual cases in the United States (1). The prevalence of *C. jejuni* infection is due to its ability to reside asymptptomatically within the gastrointestinal tract of avians, especially chickens. During processing of poultry, *C. jejuni* is released from the gastrointestinal tract, contaminating meat products as a result. Patients with *C. jejuni* infection typically
develop mild to severe, bloody diarrhea that may be accompanied by fever. Additionally, several post-infectious disorders have been associated with C. jejuni infection, including Guillain-Barre Syndrome, post-infectious irritable bowel syndrome, and reactive arthritis (2, 3). Further highlighting these concerns is increasing resistance of C. jejuni to the clinically-relevant antibiotics, azithromycin and ciprofloxacin, prompting the Center for Disease Control and Prevention to list antibiotic-resistant C. jejuni as a “Serious Threat” to public health (1).

Due to the importance of chickens in human C. jejuni infection, much emphasis has been placed on identifying and characterizing factors that are required for colonization of the chicken gastrointestinal tract. Previously, our group has used both signature-tagged mutagenesis (STM) and insertion sequencing (TnSeq) approaches to identify colonization determinants of C. jejuni (4, 5). The earlier STM approach identified genes involved in chemotaxis and flagellar biogenesis, but several other colonization factors were identified using the TnSeq approach. One determinant identified to be reduced by 250-fold in cecal outputs by TnSeq was a component of the heme utilization gene cluster, chuB (Cjj81176_1602), indicating that acquiring iron from heme may be important during colonization of the chicken gastrointestinal tract (4). Due to the prominent redox potential of Fe^{2+}/Fe^{3+}, most organisms require iron as a redox cofactor for enzyme complexes. Since the host often restricts iron availability to limit microbial growth – a process termed nutritional immunity - many bacterial species have developed methods for obtaining iron from host molecules, including heme (6).
consists of genes encoding a predicted TonB-dependent heme receptor (chuA),
a hemin ABC transporter permease (chuB), a hemin ABC transporter ATP-binding protein (chuC), and a periplasmic hemin-binding protein (chuD) (7).

Divergently transcribed from the transport genes is a previously characterized heme oxygenase, chuZ, which directly binds heme and degrades it in the presence of an electron donor (7, 8). All components of the heme utilization gene cluster are required for efficient use of heme as a sole iron source and the ferric uptake repressor (Fur) protein directly binds to the promoter region of the gene cluster, implicating Fur as a chu locus regulator (7).

These data are consistent with observations that, in most organisms, intracellular iron levels are tightly regulated to avoid forming toxic hydroxyl radicals and superoxide anions via Haber-Weiss-Fenton catalysis (9). C. jejuni is exceptional in that it is also exquisitely sensitive to oxidative stress and, as a result, adequate intracellular iron levels must be maintained as a redox cofactor. While the above studies indicated that increased iron levels repress chu gene expression, likely via Fur binding, no positive regulators of heme and/or iron uptake have been identified. Due to the importance of iron in combating oxidative stress in C. jejuni, it would be surprising if Campylobacter did not have some mechanism for increasing iron uptake under unfavorable redox states. As such, we hypothesized that C. jejuni likely maintains intracellular iron levels using both negative regulation, as seen in previous work with Fur, and positive regulation.
In subsequent TnSeq experiments that aimed at finding additional chicken colonization determinants, a candidate regulator was identified, Cjj81176_1389 (unpublished data). This regulator is predicted to contain an N-terminal Per-Arnt-Sim (PAS) domain and a C-terminal helix-turn-helix DNA binding domain. The PAS domain is a ligand-binding domain that interacts with a chemically diverse array of small molecules (10). These domains, while highly divergent in primary sequence identity, maintain a conserved three-dimensional architecture (10).

When ligand binds to a PAS domain, the protein either directly initiates a signaling response or the protein-ligand complex becomes capable of responding to a secondary cue, including gas molecules, photons, or redox potential (10).

We reasoned that since the heme utilization gene cluster and Cjj81176_1389 appear to be required for full colonization of the chicken gastrointestinal, and that Cjj81176_1389 may be sensing intracellular redox potential, Cjj81176_1389 could be directly regulating chu gene expression.

Here we demonstrate that Cjj81176_1389 mutants are significantly reduced for chicken gastrointestinal tract colonization, particularly during competition with wild-type C. jejuni. Subsequent transcriptomic analysis found that this regulator, hereafter referred to as HeuR (for heme uptake regulator), positively influences chu gene expression, as well as other iron uptake systems.

Purified HeuR specifically binds the chuZA promoter region, indicating it may regulate chu gene expression directly. Further, heuR mutants are unable to efficiently use heme as a sole iron source and exhibit decreased levels of cell-
associated iron. Finally, we show that loss of heuR leads to hydrogen peroxide resistance, in turn due to elevated levels of catalase activity.

Results

*C. jejuni* lacking heuR are unable to efficiently colonize the chicken gastrointestinal tract. Colonization studies using defined insertion-deletion mutants were performed using day-of-hatch chicks. Animals were inoculated with: 2.5x10^8 cfu wild-type *C. jejuni*, 1.7x10^8 cfu heuR mutant, or 1.9x10^8 chuA mutant. Following 7 days of colonization, viable bacteria present within cecal samples were enumerated and the medians found to be: 5.8x10^9 cfu/g for wild-type *C. jejuni*, 6.6x10^8 cfu/g for heuR, and 5.6x10^7 cfu/g for the chuA mutant. The chicks that were mock infected with PBS did not yield detectable *C. jejuni*. Statistical analysis of the medians revealed that both heuR mutants were significantly decreased for colonization, but the chuA mutant was not (p = 0.1).

Competition analysis using the heuR mutant indicated a severe competitive defect of the mutant versus wild-type *C. jejuni*. The heuR mutant yielded a mean competitive index value of 0.004 ± 0.007, representing a 250-fold decrease in colonization potential compared to wild-type *C. jejuni* (Figure 1B). Statistical analysis of this competitive disadvantage was found to be significant.

Figure 1. Colonization of day-of-hatch chicks with heuR mutants. (A) Mono-colonization of day-of-hatch white leghorn chicks with either wild-type *C.
jejuni, the heuR mutant, or the chuA mutant. Cecal loads were determined using selective media following a 7-day colonization. The median values are noted and results compared using the Mann-Whitney U-test. (B) Competition analysis of wild-type C. jejuni versus the heuR mutant. Following correction for inocula, the ratio of heuR/wild-type was determined and presented as a competitive index. Statistical analysis was performed using a one-sample t-test against a hypothetical value of 1.

**Genes involved in inorganic ion transport are underexpressed in the heuR mutant.** To determine the extent of the HeuR regulon, we employed transcriptomic analysis of wild-type C. jejuni and heuR mutant cultures. RNAseq analysis of the heuR mutant identified 43 genes whose transcripts were decreased in abundance by at least two-fold compared to wild-type C. jejuni (Table 1). These values represented statistically significant decreases for all genes identified. The Microbial Genomic context Viewer (MGcV) identified the NCBI GI numbers for 31/43 loci and, using a cluster of orthologous group (COG) analysis, assigned a functional class to 19/31 loci. Of the 19 loci for which a function could be assigned, eight (42%) were involved in inorganic ion transport and metabolism, including all five chu genes (Figure 2A). Also, amino acid transport and metabolism were well represented with four loci (21%). The MGcV analysis did not identify NCBI GI numbers for the enterochelin system, which was also identified - iron acquisition by this system has been described in *Campylobacter* and multiple organisms (11, 12).
In addition to those genes that were found to exhibit reduced transcription in the heuR mutant, transcriptomic analysis identified 16 genes with elevated transcript abundance in the mutant (Table 2). The majority of this set included genes belonging to two gene clusters: Cjj81176_1390-1394 and Cjj81176_1214-1217. MGcV identified NCBI GI numbers for 11/16 genes and assigned functions to 10/11 loci. This group is fairly diverse with the largest representation belonging to the amino acid transport and metabolism COG (30%) (Figure 2B).

**Table 1. Genes with reduced transcript abundance in the heuR mutant.**

| Locus                 | Predicted Function                                | Fold-change (log2) | P-value' |
|-----------------------|---------------------------------------------------|--------------------|----------|
| CJJ81176_1389         | conserved hypothetical protein                    | -5.62              | 4.5E-108 |
| CJJ81176_1601         | TonB-dependent heme receptor                      | -3.34              | 1.2E-25  |
| CJJ81176_1603         | hemin ABC transporter, ATP-binding                | -3.32              | 1.0E-36  |
| CJJ81176_1604         | hemin ABC transporter, periplasmic binding        | -3.22              | 9.3E-33  |
| CJJ81176_1602         | hemin ABC transporter, permease protein           | -3.12              | 2.2E-38  |
| CJJ81176_1704         | 16S ribosomal RNA                                 | -2.48              | 5.1E-03  |
| CJJ81176_1727         | 23S ribosomal RNA                                 | -2.47              | 1.8E-04  |
| CJJ81176_1707         | 23S ribosomal RNA                                 | -2.46              | 2.1E-04  |
| CJJ81176_1714         | 23S ribosomal RNA                                 | -2.45              | 1.9E-04  |
| CJJ81176_1711         | 16S ribosomal RNA                                 | -2.45              | 6.0E-03  |
| CJJ81176_1724         | 16S ribosomal RNA                                 | -2.44              | 5.8E-03  |
| EBG00001201819        | Unknown                                           | -2.42              | 2.3E-04  |
| EBG00001201824        | Unknown                                           | -2.42              | 2.7E-04  |
| EBG00001201849        | Unknown                                           | -2.41              | 6.4E-03  |
| EBG00001201811        | Unknown                                           | -2.41              | 2.6E-04  |
| EBG00001201820        | Unknown                                           | -2.40              | 6.3E-03  |
| EBG00001201839        | Unknown                                           | -2.39              | 8.0E-03  |
| CJJ81176_1386         | conserved hypothetical protein                    | -2.39              | 3.6E-13  |
| CJJ81176_1385         | hypothetical protein                              | -2.33              | 6.4E-18  |
| CJJ81176_1600         | conserved hypothetical protein                    | -1.81              | 8.5E-12  |
| CJJ81176_1623         | putative periplasmic protein                      | -1.42              | 4.6E-08  |
| CJJ81176_0471         | TonB-dependent receptor, putative                | -1.28              | 2.4E-06  |
| CJJ81176_1333         | flagellin modification protein, PseA             | -1.28              | 2.0E-10  |
| CJJ81176_0761         | conserved hypothetical protein                    | -1.24              | 1.8E-07  |
| CJJ81176_0075         | cytochrome c family protein                       | -1.22              | 5.0E-05  |
Table 2. Genes with elevated transcript abundance in the *heuR* mutant.

| Locus            | Predicted Function                                      | Fold-change (log2) | P-value$^\dagger$ |
|------------------|--------------------------------------------------------|--------------------|-------------------|
| CJJ81176_1390    | endoribonuclease L-PSP, putative                        | 3.49               | 9.5E-51           |
| CJJ81176_1391    | cryptic C4-dicarboxylate transporter DcuD               | 3.32               | 3.1E-43           |
| CJJ81176_1394    | MmgE/PrpD family protein                               | 3.19               | 4.3E-42           |
| CJJ81176_1393    | adenylosuccinate lyase                                  | 3.17               | 5.8E-41           |
| CJJ81176_1392    | cystathionine beta-lyase                                | 3.16               | 5.0E-39           |
| CJJ81176_pVir0034| hypothetical protein                                    | 1.79               | 2.0E-07           |
| CJJ81176_1217    | 5,10-methylene tetrahydrofolate reductase              | 1.71               | 3.0E-28           |
| CJJ81176_1216    | methionine synthase MetE                               | 1.71               | 1.5E-14           |
| CJJ81176_1215    | lipoprotein, NLPA family                                | 1.71               | 1.4E-12           |
| CJJ81176_1214    | oxidoreductase, 2OG-Fe(II) oxygenase                    | 1.52               | 7.5E-13           |
| CJJ81176_0271    | MCP-domain signal transduction protein                 | 1.40               | 5.8E-03           |
| EBG000001201809   | Unknown                                                | 1.08               | 1.6E-03           |
| EBG000001201810   | Unknown                                                | 1.05               | 1.7E-03           |
| EBG000001201838   | Unknown                                                | 1.05               | 3.2E-05           |
| EBG000001201844   | Unknown                                                | 1.03               | 1.4E-02           |
| CJJ81176_0257    | conserved hypothetical protein                          | 1.01               | 7.5E-07           |

$^\dagger$False Discovery Rate corrected using Benjamini-Hochberg method
Figure 2. COG analysis of genes differentially expressed in the *heuR* mutant. (A) Identified COGs for genes in Table 1 that were found to be underexpressed in the *heuR* mutant. (B) COGs identified for genes in Table 2 that were overexpressed in the *heuR* mutant.

Purified HeuR binds specifically to the *chuZA* promoter. The putative structure of HeuR includes a C-terminal helix-turn-helix DNA binding domain. Given the transcriptome data demonstrating that *chu* RNA abundance is dependent on HeuR, we hypothesized that HeuR directly regulates *chu* gene expression. Purification of 6xHis-tagged HeuR was successful as soluble protein was obtained at concentrations of 4.3 mg/ml. Incubation of purified 6xHis-HeuR with radiolabeled *chuZA* promoter and subsequent resolution by native polyacrylamide gel electrophoresis indicated binding of the protein to the *chuZA* probe using 30 µg of 6xHis-HeuR (Figure 3). The shifted species increased in intensity until 125 µg of HeuR was used and the fragment representing unbound *chuZA* promoter was not detected. These results were in contrast to those obtained for a radiolabeled fragment that represented the intergenic region between *C. jejuni* 81-176 mapA and ctsW, which are not predicted to be regulated by HeuR. Mobility of this probe was relatively unaffected until 500 µg of HeuR was used, when unbound probe intensity was modestly decreased.
Figure 3. Binding of purified 6xHis-HeuR to the chuZA promoter.

Increasing amounts of purified 6xHis-HeuR were added to binding reactions containing approximately 0.25 nM of the chuZA promoter fragment and the mapA-ctsW intergenic region as a non-specific control.

Mutants of heuR are unable to use heme as a source of iron. To determine whether decreased expression of the Chu system affected the ability of the heuR mutant to use heme as an iron source, cultures of each strain were grown in iron-chelated conditions and supplemented with heme, hemoglobin, or ferric chloride. Both the heuR and chuA mutants were hypersensitive to iron-limiting conditions when compared to wild-type C. jejuni (Supplemental Figure 1). At 160 µM desferrioxamine (DFOM), the heuR mutant with empty vector and the chuA mutant with empty vector exhibited greatly reduced growth compared to their behavior in iron-replete medium (Figure 4A). Heme added to these cultures was unable to rescue growth of the heuR and chuA mutants. Complementation of the heuR mutant with cloned heuR restored the ability to use heme as an iron source. In contrast, cloned heuR introduced into the chuA mutant did not restore heme dependent growth. Similar results were also seen using whole hemoglobin (Figure 4B). That iron restriction is the explanation for these phenotypes was supported by the restoration of growth in all strains through the addition of ferric chloride (Figure 4C).
Figure 4. The heuR mutant is deficient in using heme as an iron source.

A) Growth of strains in media containing 160 µM desferrioxamine and 0.1 µg/ml purified heme. Represented as percent growth of same strains in media without desferrioxamine. (B) Growth of strains in media containing 160 µM desferrioxamine, but with 0.1 µg/ml purified hemoglobin. Represented as percent growth of strains in media without desferrioxamine. (C) Control growth of strains in media containing 160 µM desferrioxamine, but with 100 µM ferric chloride. Also represented as percent growth of strains in media without desferrioxamine. Statistical analysis performed using Student’s t-test; *** : p < 0.0001.

Mutation of heuR results in decreased cell-associated iron. The heuR mutant expressed lower abundance of mRNA from several iron uptake systems (Table 1), suggesting it may play a general role in regulating iron acquisition. To test this, we examined cell-associated iron levels using inductively coupled plasma mass spectrometry (ICP-MS) of wild-type C. jejuni, heuR mutant, and chuA mutant lysates. This analysis demonstrated that iron levels of the heuR and chuA mutants significantly decreased compared to those of wild-type C. jejuni (Figure 5A).

Iron levels in samples of the heuR mutant complemented with cloned heuR were similar to those of wild-type C. jejuni (Figure 5B) (p = 0.28). Similar to the results of the mutation alone, iron levels in the chuA mutant with pECO102 were significantly decreased compared to wild-type C. jejuni. Consistent with our
hypothesis that HeuR regulates iron acquisition through chu expression, when 
heuR was expressed in the chuA mutant, cell-associated iron remained 
significantly decreased compared to wild-type C. jejuni (Figure 5B).

Figure 5. ICP-MS analysis of C. jejuni cell-associated iron. (A) Levels of 
cell-associated iron in wild-type C. jejuni, the heuR mutant, and the chuA mutant. 
Parts-per-billion iron levels for each strain were expressed as a percentage of the 
average iron levels observed in wild-type C. jejuni. (B) Complementation 
analysis of iron levels in strains carrying the empty vector control (pECO102) or 
the complementation construct (pECOheuR). Parts-per-billion iron levels for 
each strain were expressed as a percentage of the average iron levels observed 
in wild-type C. jejuni with empty vector. Statistical analyses performed using 
Student’s t-test.

The heuR mutant exhibited increased resistance to hydrogen peroxide. We 
observed a lack of alpha-hemolysis on blood plates on which the heuR mutant 
was grown (data not shown). As alpha hemolysis is due to the formation of 
methemoglobin in the presence of hydrogen peroxide, we hypothesized that the 
heuR mutant may degrade hydrogen peroxide more readily than wild-type C. 
jejuni. This was tested directly by incubating the heuR mutant in the presence of 
hydrogen peroxide, which revealed that the mutant is resistant. Growth of wild-
type C. jejuni was generally limited by hydrogen peroxide at concentrations 
above 0.15 mM and exhibited an IC50 value of 0.233 mM (Figure 6A). In
contrast, the *heuR* mutant was unaffected by hydrogen peroxide with significantly increased growth compared to wild-type *C. jejuni* at concentrations above 0.15 mM. As a result, the IC50 value was not experimentally determined and by extrapolation was found to be 5.8 mM. The *chuA* mutant exhibited an intermediate phenotype in the presence of hydrogen peroxide where growth remained relatively unaffected until concentrations reached 0.60 mM, which was significantly more growth than that of wild-type *C. jejuni*. As such, the *chuA* mutant exhibited an IC50 of 0.526 mM, a more than two-fold increase compared to wild-type *C. jejuni*, but approximately 10-fold lower than the *heuR* mutant.

Complementation of the *heuR* mutant with cloned *heuR* resulted in a slight hypersensitivity compared to wild-type *C. jejuni* with an IC50 value of 0.203 mM (Figure 6B).

**Figure 6.** Dose-response analysis of *C. jejuni* mutants grown in presence of hydrogen peroxide. (A) Growth of wild-type *C. jejuni*, the *heuR* mutant, and the *chuA* mutant in response to increasing concentrations of hydrogen peroxide. (B) Complementation analysis of hydrogen peroxide resistance conferred by the *heuR* mutation. Wild-type with empty vector, the *heuR* mutant with empty vector, or the *heuR* mutant with cloned *heuR* were grown in the presence of increasing hydrogen peroxide concentrations. All growth is expressed as a percentage of that observed for each strain in media alone. Statistical analysis performed using a Student’s t test; *: p < 0.01.
Resistance of the heuR mutant to hydrogen peroxide is correlated with increased catalase activity. We hypothesized that the elevated resistance of the heuR mutant to hydrogen peroxide may result from increased catalase activity. To determine whether this was the case, suspensions of strains were normalized and added to 30% H$_2$O$_2$, revealing that wild-type C. jejuni carrying empty vector was normally weakly positive (Table 3). In contrast, the heuR mutant transformed with empty vector was strongly positive for catalase activity. When heuR was re-introduced into the heuR background, catalase activity decreased and cells resembled wild-type activity. Also, similar to wild-type C. jejuni, the chuA mutant remained weakly positive, though the chuA mutant did appear to exhibit more catalase activity than wild-type C. jejuni.

Table 3. Catalase activity of C. jejuni strains.

| Strain (plasmid)          | Catalase activity |
|---------------------------|-------------------|
| Wild-type C. jejuni heuR  | +/-               |
| chuA                      | +                 |
| Wild-type C. jejuni (pECO102) heuR | +/-         |
| (pECO102)                 | ++++              |
| heuR (pECOheuR)           | +/-               |

Discussion

Using a TnSeq approach, we identified several determinants required by C. jejuni for efficient colonization of the chicken gastrointestinal tract (4). One determinant was a component of the Campylobacter heme utilization gene cluster, chuB. In this work, we describe a regulator, which we have named HeuR, which is also required for wild-type levels of colonization of the chicken.
gastrointestinal tract. Additionally, because HeuR is predicted to contain a PAS
domain - a protein signature often associated with gas molecule and redox
potential sensing – and that it and chu are both required for chicken colonization,
we hypothesized that HeuR regulates heme uptake through chu gene
expression.

Recently, HeuR was also found to control expression of Cjj81176_1390,
as the gene product was overexpressed in a heuR mutant (13). The group that
identified this phenotype named HeuR the _Campylobacter_ flagella interaction
regulator (CfiR) due to flagellar interactions that occurred in response to gene
disruption. This group further found that the gene product of Cjj81176_1390
forms dimers linked by a disulfide bridge that may be responsive to the redox
status of the cell (13). Our observations, recounted below, show that HeuR
controls more genes than just those that promote flagellar interactions, including
many that may be involved in combating oxidative stress (Figure 7). As such, we
suggest that the designation CfiR does not accurately represent the full
regulatory role in the bacterium and it is our recommendation that the name be
changed to HeuR.

Figure 7. Model of HeuR regulation of the chuABCDZ loci and heme
homeostasis in _C. jejuni_. Within the vertebrate host, heme is liberated from
hemoglobin in an undefined manner. HeuR binds the intergenic region between
chuZ and chuABCD to promote expression of heme acquisition functions. ChuA
is the outer membrane heme receptor. ChuBCD transport heme to the cytoplasm
of the cell where iron is liberated from by the heme oxygenase ChuZ. The *heuR*
mutant exhibits decreased expression of the chuABCDZ loci, decreased ability to
use heme and hemoglobin as a source of nutrient iron, and decreased cell-
associated iron levels as a consequence. Additionally, the *heuR* mutant exhibits
decreased alpha hemolysis and increased catalase production.

As mentioned above, by RNAseq analysis of a *heuR* mutant strain, we
identified several genes whose mRNA products were significantly less abundant
in this background. Several of these were found to be involved in inorganic ion
transport and metabolism, including all the genes in the previously identified
*Campylobacter* heme utilization (*chu*) gene cluster. Transcript levels of several
genes involved in enterochelin transport were decreased in the mutant strain, but
were not identified by COG analysis as being involved in ion transport. As a
result, this COG analysis is not comprehensive as the number of genes in the
HeuR regulon that are involved in iron transport and metabolism is likely
underreported. Nevertheless, based on these results, it becomes apparent that
HeuR is primarily involved in maintaining sufficient levels of intracellular iron by
upregulating systems that can scavenge for iron from diverse sources, which is
supported by our ICP-MS data.

In addition to those genes that were underexpressed, transcripts of
several were more abundant in the *heuR* mutant. Most of these genes are in two
gene clusters: Cjj81176_1390-1394 and Cjj81176_1214-1217. The former,
Cjj81176_1390-1394, includes the overexpressed gene product (Cjj81176_1390)
noted above that promotes flagellar interactions, supporting both the previous study and our work (13). The latter gene cluster that is upregulated includes one gene that encodes an additional ion transporter and multiple genes predicted to be involved in methionine biosynthesis. The methionine biosynthetic enzymes encoded by these genes have been shown in other organisms to be sensitive to oxidation/oxidative stress (14). In an oxidative environment, these enzymes may need to be replenished, and their inverse expression with that of iron scavenging systems may be common. Whether upregulation of these genes is a direct or indirect effect of HeuR is currently unknown.

Standard growth assays using the iron chelator, desferrioxamine, found that both the heuR and chuA mutants are reduced for growth in iron-chelated conditions. As this was observed in media without the addition of hemoglobin, it may indicate that the Chu system is also partially responsible for the transport of free iron into the cell. In this case, it would likely be an accessory system since growth was completely restored in these mutants when iron was supplemented back, indicating the presence of a robust, primary iron transport system. When heme and hemoglobin were added to these mutants, growth was not restored, supporting the conclusion that both are required for using heme as an iron source. Re-introduction of heuR resulted in the ability to use heme as an iron source, likely through the Chu system, as overexpressing heuR in the chuA mutant did not enable the strain to use hemoglobin.

Analysis of cell-associated levels of iron showed that the heuR mutant is significantly decreased for iron and that these levels resemble those of a chuA
mutant. Most of the iron reduction observed in these experiments is likely due to decreased heme utilization, as cultures were obtained from media containing sheep’s blood. This is further supported by the observation that complementation with heuR restores iron levels in a heuR mutant background, but was unable to do so in a chuA mutant background. As our data indicates a minor role for HeuR and the Chu system in acquiring iron independently of heme, we are currently determining whether decreased iron is apparent in the heuR and chuA mutants grown in media without heme or hemoglobin. This will help us determine to what extent other iron sources are impacting cellular iron levels in these mutants.

When the heuR mutant was grown on blood plates, differences in the hemolytic pattern relative to wild-type C. jejuni became apparent. Typically, C. jejuni exhibits alpha hemolysis on blood plates, likely due to the production of hydrogen peroxide that oxidizes the hemoglobin in the blood to methemoglobin (15). Alpha hemolysis was not observed in the heuR mutant, indicating that this mutant either produces less hydrogen peroxide or exhibits increased catalase activity. The mutant was highly resistant to hydrogen peroxide, which was associated with elevated levels of catalase activity; re-introduction of heuR led to both decreased resistance to hydrogen peroxide and catalase activity. Of the genes known to regulate catalase activity in C. jejuni - including ahpC, fur, katA and perR - none were found to be significantly up- or down-regulated in the heuR mutant (data not shown). Also, since iron levels are similar in the chuA mutant, it is unlikely that iron levels are responsible for the elevated catalase activity. Thus
we hypothesize that HeuR regulates the production of an unknown factor that perhaps post-transcriptionally regulates the activity of *C. jejuni* catalase. We are currently working to determine what this factor is.

The *heuR* mutant exhibited significant decreases in colonization of the chicken cecum. This leads to two questions: i) is this system mainly required for combating oxidative stress in the cecum or is it used for other cellular processes and ii) is the system acquiring iron from a heme source or does it serve the dual purpose of acquiring free iron from the environment.

Oxidative stress in the cecum may come from a variety of sources, like metabolism by the intestinal microflora or from the generation of reactive oxygen species due to intestinal inflammation. While the *Campylobacter*-chicken relationship is often considered to be commensal in nature, *C. jejuni* colonization of the chicken gastrointestinal tract may result in low levels of transient inflammation (Davis and DiRita, unpublished) (16-18). Also, while not mutually exclusive, iron acquisition systems like Chu may be more important for acquiring iron that can be used in other cellular processes including energy generation and DNA replication (6). We are currently working to determine which of the processes during chicken colonization iron is most important for.

Second, the question arises as to whether *C. jejuni* uses the Chu system to acquire iron from either/or a heme or non-heme source. It is currently unknown how much heme chickens consume in a commercial diet and we could find no data on heme availability in their natural diet. With the exception of higher plants that can produce heme in the plastids, the absence of heme...
molecules in most grains, plants, and insects make it less obvious how chickens
would have access to heme-containing molecules (19). The likelihood then
becomes that *C. jejuni* uses this system to either scavenges heme from a
microbial source or from the host. The *C. jejuni* 81-176 genome encodes all
heme biosynthetic enzymes with the exception of homologs for
protoporphyrinogen oxidase (*hemY* or *hemG*). As such, in an iron rich
environment, *C. jejuni* can likely synthesize its own heme. It could then use the
heme uptake system to recover heme from other *Campylobacter* or from heme
producing residents of the chicken microbiota. Lastly, it remains possible that
this system is able to transport free iron. While the heme receptor and heme
oxygenase (ChuA and ChuZ, respectively) would likely not be needed for free
iron transport, the accompanying ABC transport system (ChuBCD) may be
promiscuous and function similar to other iron transporters. We are currently
investigating whether the ChuBCD transporter can translocate non-heme iron
into *C. jejuni*.

**Materials and Methods**

**Day-of-hatch chick colonization.** Day-of-hatch white leghorn chickens were
inoculated by oral gavage with 100 µl of an approximately $1 \times 10^9$ cfu/ml bacterial
suspension in sterile PBS ($10^8$ cfu). Colonized chickens were housed for 7 days,
euthanized, and cecal contents were collected. Cecal contents were
homogenized in sterile PBS and serial dilutions were plated on *Campylobacter*
selective media: MH agar containing 10% sheep’s blood, cefoperazone (40
µg/ml), cycloheximide (100 µg/ml), trimethoprim (10 µg/ml), and vancomycin (100
µg/ml). Plates were grown for 48h under microaerobic conditions and colonies
enumerated. Statistical analysis of the medians was performed using a Mann-
Whitney test.

For the competition experiment, equal numbers of wild-type C. jejuni and
the heuR mutant were mixed and approximately 1x10^8 of each were used to
inoculate day-of-hatch chicks by oral gavage. Harvest of cecal contents was
done the same as above except serial dilutions were plated on Campylobacter
selective media with and without kanamycin (100 µg/ml). Competitive index
values were calculated following correction for inoculum amounts by dividing the
number of heuR mutants by the number of wild-type bacteria. Statistical analysis
was done using a one-sample t-test against a hypothetical value of 1.

All chicken experiments and protocols were approved by the Institutional
Animal Care and Use Committee in the Animal Care Program at Michigan State
University.

**RNAseq analysis.** Triplicate cultures of wild-type DRH212 and the heuR mutant
were grown on MH agar supplemented with 10% whole sheep’s blood and 10
µg/ml trimethoprim overnight at 37°C under microaerobic conditions. Cells were
harvested and grown on new media for a second night using the same
conditions. Following growth, cells were harvested from each individual plate
and RNA was extracted using a previously described protocol (20). DNA was
degraded using Turbo DNA-free (Thermo) according to the manufacturer’s
instructions and removal was confirmed by conventional PCR. RNA quality was
assessed using an Agilent 2100 Bioanalyzer and libraries were generated using
the TruSeq RNA Library Preparation Kit (Illumina). Libraries were sequenced
using an Illumina HiSeq 2000 formulated for single-end, 50-nucleotide reads.

Analysis of sequence data was performed using SPARTA: Simple
Program for Automated reference-based bacterial RNAseq Transcriptome
Analysis (21). Briefly, raw reads were trimmed and assessed for quality using
Trimmomatic and FastQC, respectively (22). Trimmed reads were mapped to
the C. jejuni 81-176 reference genome using Bowtie and expression levels
determined by HTSeq (23, 24). Lastly, differential expression analysis was
performed using edgeR (25). Cluster of orthologous group (COG) identification
of selected genes was performed using Microbial Genomic context Viewer
(MGcV).

Protein purification. The gene heuR was amplified by PCR from C. jejuni 81-
176 genomic DNA using primers 5’Cjj1389 BamHI and 3’Cjj1389 SalI. The PCR
fragment was subcloned into pGEM-T Easy using the manufacturer’s protocol
and sequenced using primers T7 and SP6. Fragments that were confirmed to
have maintained the full nucleotide sequence of heuR were excised using BamHI
and SalI and ligated into the expression vector, pQE-30 (Qiagen). Ligation
products were transformed into E. coli NEB C3013 and insertion was confirmed
using restriction digest of purified plasmids.
Induction of 6xHis-HeuR using 1 mM IPTG was confirmed for six constructs by SDS-PAGE and Coomassie staining. One strain was used for protein purification where a 1L culture was grown to mid-log and induced for 3h at 37°C using 1mM IPTG. Cells were pelleted at 8,000 rpm for 20 min at 4°C and re-suspended in 20 ml lysis buffer (pH 8.0) (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole). The cell suspension was passed three times through a french press and cell debris was removed by centrifugation at 8,000 rpm for 20 min at 4°C. The cleared lysate was added to 5 ml of NiNTA agarose that was pre-equilibrated with lysis buffer and rocked overnight at 4°C. This resin-protein mixture was packed by gravity into a chromatography column and lysate was passed through and collected. Non-specific proteins were removed by washing the resin bed twice with 5 column volumes (25 ml) of wash buffer (pH 8.0) (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole). Bound protein was eluted four times using 1 ml of elution buffer (pH 8.0) (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole). Successful purification of 6xHis-HeuR was verified by SDS-PAGE and Coomassie staining. Fractions containing the highest amount of HeuR were pooled and dialyzed against storage buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5 mM EDTA, 0.02% Triton X-100, 2 mM DTT, 50% glycerol).

Concentrations of 6xHis-HeuR were determined using a BCA Protein Assay (Pierce) and aliquots of protein were stored at -80°C.

**Electrophoretic mobility shift assays.** A specific DNA probe was generated from the *chuZA* promoter region and a non-specific probe was generated from...
the mapA-ctsW intergenic region by PCR of C. jejuni 81-176 genomic DNA. Amplicons were purified using a PCR Purification Kit (Qiagen) and subsequently end-labeled with [γ-32P] ATP using T4 polynucleotide kinase (PNK) (New England Biolabs). DNA binding reactions containing radiolabeled specific (chuZA promoter) and non-specific probes (mapA-ctsW intergenic) at approximately 0.25 nM, DNA binding buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5% glycerol), 25 ng/µl poly(dA-dT), and 100 µg/ml BSA were incubated for 5 min at room temperature. Purified 6xHis-HeuR was diluted two-fold in binding buffer and 1 µl containing 500, 250, 125, 60, 30, 15, 7.5, or 3.75 µg of protein was added to individual 19 µl binding reactions (20 µl total). These reactions – and a control reaction without 6xHis-HeuR – were incubated for 15 min at room temperature and subsequently resolved on a 5% polyacrylamide native Tris-glycine gel at 4°C. Gels were imaged using a Typhoon phosphoimager.

Hemoglobin and heme growth assays. For bacterial growth assays, C. jejuni strains were grown overnight on tryptic soy agar plates supplemented with 5% sheep blood and appropriate antibiotics (chloramphenicol and/or kanamycin). The following day, plate cultures were utilized to inoculate 5 mL of Mueller-Hinton broth supplemented with appropriate antibiotics. These liquid cultures were incubated under shaking conditions at 37°C overnight. The following day, cultures were diluted 1:10 in fresh Mueller-Hinton broth (26) supplemented with appropriate antibiotics (chloramphenicol and/or kanamycin) alone (Medium
Alone) or supplemented with 40, 80, 160 µM of the iron chelator desferrioxamine mesylate (Sigma Aldrich) alone or in combination with 0.1 µg/mL of purified human hemoglobin (1) or 0.1 µg/mL of hemin at 37°C in room air supplemented with 5% CO₂. Bacterial growth was measured using a spectrophotometric reading at 600 nm (OD₆₀₀). Statistical analysis was performed using a Student’s t-test.

ICP-MS analysis. Cultures of wild-type C. jejuni DRH212, the heuR mutant, and a chuA mutant, were grown overnight at 37°C under microaerobic conditions on MH agar with 10% whole sheep’s blood containing either 10 µg/ml trimethoprim or 100 µg/ml kanamycin – for mutant strains. For complementation analysis, wild-type with pECO102, the heuR mutant with pECO102, the heuR mutant with pECOheuR, the chuA mutant with pECO102, and the chuA mutant with pECOheuR were grown on MH agar with 10% sheep’s blood containing either chloramphenicol (30 µg/ml) alone – for wild-type – or chloramphenicol and kanamycin (100 µg/ml) - for the plasmid-containing mutants. Cultures were grown overnight under microaerobic conditions at 37°C. Strains were passed for a second night on fresh media and harvested for iron quantification. Approximately 10¹⁰ cells were washed three times with 5 ml and re-suspended in 500 µl of molecular-grade, distilled water. Cells were solubilized overnight at 50°C in 1ml 50% nitric acid, followed by the addition of 9 ml of water. Extracts were analyzed for ⁵⁷Fe using A Thermo Scientific ICAP Q quadrupole inductively coupled plasma mass spectrometer in kinetic energy
dispersion mode. Instrument calibration was done with aliquots of a Fe synthetic standard solution diluted at 2.5, 10, and 100 ppb and natural water standards NIST SRM 1640 and 1643e. A solution of 100 ppb Fe was analyzed every 5 - 6 samples to monitor instrumental drift; all samples were corrected for linear drift. Oxides formation rate was determined to be < 2% by measuring CeO/Ce and double-charged cation formation rate was found to be < 3% by measuring 137Ba²⁺/137Ba from a solution with approximately 1 ppb Ce and 1 ppb Ba. Parts-per-billion levels for each strain were normalized to those observed for the wild-type equivalent in each experiment and the standard deviation was plotted. Statistical analysis was performed using a Student’s t-test.

**Hydrogen peroxide dose-response assays.** Cultures of wild-type *C. jejuni*, the *heuR* mutant, and the *chuA* mutant were grown overnight at 37°C under microaerobic conditions on MH agar with 10% sheep’s blood. Typically, *C. jejuni* exhibits alpha hemolysis on blood plates, likely due to the production of hydrogen peroxide that oxidizes the hemoglobin in the blood to methemoglobin. Strains were passed for a second night on new media under the same conditions. Cells were harvested into MH broth and normalized to an OD A₆₀₀ of 0.05. These suspensions were added to equal volumes (100 µl:100 µl) of 2-fold dilutions of MH broth with hydrogen peroxide in a 96-well plate. Hydrogen peroxide concentrations ranged from 10 mM to 0.16 mM. This dilution resulted in bacterial concentrations of 0.025 OD and hydrogen peroxide concentrations ranging from 5 mM to 0.08 mM. These cultures were grown statically for 48h at 37°C under
microaerobic conditions followed by re-suspension of bacteria and recording of terminal OD $A_{600}$ values. IC50 values were calculated using non-linear regression analysis of normalized responses with variable slopes in GraphPad Prism 6. Statistical analysis of differences between strains at each concentration of hydrogen peroxide was performed using a Student’s t-test.

For complementation analysis of the $heuR$ phenotype, the complemented strains used for ICP-MS analysis were subjected to the same hydrogen peroxide dose-response assay as those strains above. Statistical analysis was also performed using a Student’s t-test.

Catalase assays. Cultures of wild-type $C. jejuni$, the $heuR$ mutant, and the $chuA$ mutant were grown overnight at 37°C on MH agar with 10% sheep’s blood under microaerobic conditions. These strains were passed for a second night on new media under the same conditions and cells were harvested into sterile PBS. Care was given to avoid contaminating the suspensions with media since blood exhibits catalase activity. Suspensions were normalized to an OD $A_{600}$ of 1.0 and 10 µl of suspension was added to 10 µl of 30% hydrogen peroxide (J.T. Baker) on a glass slide. Reactions were performed in triplicate and qualitatively noted.

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S1. Dose-response analysis of desferrioxamine. *C. jejuni* strains grown in the presence of increasing amounts of desferrioxamine (DFOM). Growth at each concentration of DFOM is expressed as percent growth of that strain in media alone.
**Figure 1.**

A. 

![Graph showing microbial content](image)

- **p < 0.01**
- **NS**

B. 

![Graph showing competitive index](image)

- **p < 0.0001**
Figure 2.

A. 

- Amino acid transport and metabolism
- Cell cycle control
- Cell motility
- Energy production and metabolism
- Function unknown
- General function
- Inorganic ion transport and metabolism

Total = 19

B. 

- Amino acid transport and metabolism
- Energy production and conversion
- Function unknown
- General function
- Inorganic ion transport and metabolism
- Nucleotide transport
- Ribosomal structure and biogenesis

Total = 10
**Figure 3.**

![Graph showing 6xHis-HeuR (µg) and corresponding bands](image-url)
Figure 4.

A. 0.1 μg/ml heme

B. 0.1 μg/ml hemoglobin

C. 100 μM ferric chloride
Figure 5.

A.

\[ \frac{p < 0.01}{\text{NS}} \]

\[ \frac{p < 0.01}{\text{NS}} \]

\[ \frac{p < 0.01}{\text{NS}} \]

% WT Fe\(^{2+}\)

WT \quad \text{heuR} \quad \text{chuA}

B.

\[ \frac{p < 0.001}{\text{NS}} \]

\[ \frac{p < 0.01}{p < 0.01} \]

\[ \frac{p < 0.01}{p < 0.01} \]

\[ \frac{p < 0.001}{p < 0.001} \]

% WT Fe\(^{2+}\)

WT + pECO102 \quad \text{heuR} + pECO102 \quad \text{heuR} + pECOheuR \quad \text{chuA} + pECO102 \quad \text{chuA} + pECOheuR
Figure 6.

A. 

B. 

[Graphs showing the effect of H$_2$O$_2$ on growth]
Figure 6.
Supplemental Figure.