

The roles of iron, Fur and PerR in regulating gene expression in
Campylobacter jejuni

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in
partial fulfillment of the requirements for the degree of Doctor of Philosophy in
Biochemistry

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Abstract

Campylobacter jejuni is one of the most frequent causes of gastroenteritis in both the developing and the developed world. Understanding *C. jejuni* biology is paramount to reducing the amount of *Campylobacter jejuni* in the food chain, however our understanding of the regulatory networks that enable *Campylobacter jejuni* to successfully colonize various hosts remains incomplete. *Campylobacter jejuni* has an absolute requirement for iron in order to grow as iron catalyzes a wide range of essential biochemical reactions. In contrast to many Gram negative bacteria, the genome of *Campylobacter jejuni* contains two iron activated Fur-family transcriptional regulators, Fur and PerR, which are primarily responsible for regulating iron homeostasis and oxidative stress respectively. We have used an integrated approach that combines genome wide technologies (ChIP-chip, RNA-seq) and structural studies to define the role of iron, Fur and PerR in *Campylobacter jejuni*. These studies have demonstrated that apo-Fur directly regulates gene transcription in *Campylobacter jejuni*, identified novel ncRNAs that are Fur and/or iron responsive, and revealed that the Fur and PerR regulons are more extensive than previously characterized. These results provide further insight into the surprisingly complex regulatory networks that allow *Campylobacter jejuni* to be a successful gut pathogen.

Acknowledgments

I would first like to thank my supervisor Dr. Alain Stintzi for providing me with the opportunity to conduct my PhD research project in his laboratory, for his continuous support/mentoring throughout my PhD project and for the freedom to pursue the scientific projects that I found interesting.

I would like thank all of my friends and colleagues, both past and present with whom I have worked (although not always very industriously) alongside during my PhD project. In particular, I would like to thank Anne Reid, Martin Stahl, Nabil Hayek, Hai Nguyen, Walid Mottawea, Turki Abujamel, Momen Askora, Virginie Dufour, Karine Gauthier, Jennifer Li, Kelly Grzywacz and Sophie Bérubé for their encouragement and support. Casey Carswell and Rebecca Hood also provided useful and stimulating conversations about life in general and science in particular. I must also gratefully acknowledge the staff at the General Hospital, Tim Horton's and Second Cup for fueling my work with much needed coffee.

I would like to thank my fellow scientific collaborators for their contributions. In particular, Sabina Sarvan, Jean-François Couture, Rebecca Handley and Arnoud van Vliet each played a key role in shaping the scientific work that ultimately resulted in my thesis.

I would especially like to thank Annika Flint and owe her a debt of gratitude for the countless hours she spent carefully proofreading my manuscripts (removing many an errant semicolon!); and for our innumerable discussions during our daily coffee pilgrimages.

Finally, I would like to thank my family for their support and understanding. I would like to give special mention to my nieces, Leah and Clara Robertson, who routinely distracted their Uncle Me from his work long enough to keep his sanity intact.

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List of Abbreviations

BjFur	<i>Bradyrhizobium japonicum</i> ferric uptake regulator
BsFur	<i>Bacillus subtilis</i> ferric uptake regulator
BsPerR	<i>Bacillus subtilis</i> peroxide response regulator
ChIP	Chromatin immunoprecipitation
CjFur	<i>Campylobacter jejuni</i> ferric uptake regulator
CjPerR	<i>Campylobacter jejuni</i> peroxide response regulator
COG	Clusters of Orthologous Groups
CRISPR	Clustered regularly interspaced short palindromic repeats
crRNA	CRISPR RNA
DBD	DNA binding domain
DD	Dimerization domain
DIG	Digoxigenin
dRNA-seq	Differential RNA-seq
EcFur	<i>Escherichia coli</i> ferric uptake regulator
EMSA	Electromobility shift assay
Fur	Ferric uptake regulator
HpFur	<i>Helicobacter pylori</i> ferric uptake regulator
HTS	High throughput sequencing
ICPMS	Inductively coupled plasma mass spectrometry

KEGG	Kyoto Encyclopedia of Genes and Genomes
MEM α	Minimal essential media alpha
MH	Mueller-Hinton
MtZur	<i>Mycobacterium tuberculosis</i> zinc uptake regulator
ncRNA	Non-coding RNA
ORF	Open reading frame
PaFur	<i>Pseudomonas aeruginosa</i> ferric uptake regulator
PCA	Principal component analysis
PerR	Peroxide response regulator
qPCR	Quantitative polymerase chain reaction
r.m.s.d.	Root-mean-square deviation
RPKM	Reads per kilobase of gene per million of total reads sequenced
rRNA	Ribosomal RNA
RT-qPCR	Reverse transcriptase quantitative polymerase chain reaction
ScNur	<i>Streptomyces coelicor</i> nickel uptake regulator
sRNA	Small RNA
TSS	Transcription start site
UTR	Untranslated region
VcFur	<i>Vibrio cholerae</i> ferric uptake regulator

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Chapter 1: General Introduction

Preface

The following chapter consists of a general introduction to *Campylobacter jejuni*, iron metabolism and the Fur/PerR proteins. Portions of this chapter have been previously published as a book chapter (Butcher J, Flint A, Stahl M, Stintzi A. [2010] “Campylobacter Fur and PerR Regulons” in Iron Uptake and Homeostasis in Microorganisms) and as a review article (Stahl M, Butcher J, Stintzi A. [2012] “Nutrient acquisition and metabolism by *Campylobacter jejuni*” [PMID: 22919597](#)) with modifications as deemed appropriate.

The specific contributions of each author for the published works are listed below.

For Butcher J, Flint A, Stahl M, Stintzi A. (2010) “Campylobacter Fur and PerR Regulons”:

Butcher J: Wrote the sections on the Fur/PerR proteins. Constructed all the chapter figures, edited other sections of the manuscript and responded to reviewers comments.

Flint A: Wrote the section on the oxidant stimulons of *C. jejuni*

Stahl M: Wrote the section on energy metabolism and general protein glycosylation

Stintzi A: Wrote the general introduction to *C. jejuni* as well as the sections on the iron stimulon, and iron homeostasis. Also contributed to all other sections in the manuscript.

For Stahl M, Butcher J, Stintzi A. (2012) “Nutrient Acquisition and Metabolism by *Campylobacter jejuni*”:

Stahl M: Wrote the section on carbon sources used by *C. jejuni* and responded to reviewers

Butcher J: Wrote the section on metal acquisition and constructed the all the review figures.

Stintzi A: Contributed to writing/editing the entire manuscript

***Campylobacter jejuni* and its pathogenesis**

Campylobacter jejuni is regarded as the most common cause of bacterial gastroenteritis, threatening human health worldwide (Butzler, 2004; Mead *et al.*, 1999; Moore *et al.*, 2005). *Campylobacter* infection is an acute diarrhoeal disease which ranges from a day of mild watery or bloody diarrhea to severe abdominal pain that can last for several weeks (Zilbauer *et al.*, 2008). This pathogen is also involved in the development of Guillain-Barré syndrome, reactive arthritis and is suspected to be one of the possible etiological factors in inflammatory bowel diseases (Blaser and Engberg, 2008; Boyanova *et al.*, 2004; Moore *et al.*, 2005). While most *Campylobacter* infections are self-limiting, antimicrobial therapies are recommended to treat severe cases and immuno-compromised patients.

Campylobacter is intrinsically resistant to a wide variety of antimicrobials including bacitracin, rifampicin, and vancomycin (Corry *et al.*, 1995; Taylor and Courvalin, 1988). This intrinsic resistance is believed to be conferred by the low permeability of the outer membrane and the endogenous expression of efflux pumps (Zhang and Plummer, 2008). *Campylobacters* are also resistant to β -lactams due to the presence of a chromosomal β -lactamase (Li *et al.*, 2007; Parkhill *et al.*, 2000). Thus, macrolides (especially erythromycin) and fluoroquinolones are the drugs of choice for treatment of campylobacterosis (Bos *et al.*, 2006). However, resistance to these classes of antibiotics has drastically increased during the last decade, compromising future treatments (Alfredson and Korolik, 2007).

The mechanisms surrounding *Campylobacter* resistance to both fluoroquinolones and macrolides have been extensively studied. In contrast with other enteric pathogens, *Campylobacter* resistance to fluoroquinolones (FQ) is almost exclusively mediated through

the acquisition of mutations in *gyrA* (Payot *et al.*, 2006; Yan *et al.*, 2006), although there is some contribution to FQ resistance by the CmeABC efflux pump system (Luo *et al.*, 2003). Indeed, only a single mutation in *gyrA* is required for high level fluoroquinolone resistance in *Campylobacter*. Mutants in *gyrA* arise at high frequency (10^{-6} - 10^{-8} /cell/generation) upon exposure to FQ (Han *et al.*, 2008; Yan *et al.*, 2006). In addition, *C. jejuni* fluoroquinolone resistant strains show increased fitness in the absence of antibiotic as compared to fluoroquinolone sensitive strains (Luo *et al.*, 2005). This suggests that once fluoroquinolone resistance is achieved in a *C. jejuni* population it will be relatively difficult to eliminate.

In contrast with fluoroquinolones, achieving macrolide resistance is much more challenging for *C. jejuni* (Luangtongkum *et al.*, 2009; Payot *et al.*, 2006). Most macrolide resistant strains of *C. jejuni* must acquire several stepwise mutations in the 23S rRNA gene to become resistant to clinically relevant levels of antibiotic. Moreover, these mutations appear to occur infrequently ($\sim 10^{-10}$ /cell/generation) and are thus favoured when *C. jejuni* is exposed to sub-lethal levels of macrolides over extended periods of time (Caldwell *et al.*, 2008; Lin *et al.*, 2007; Payot *et al.*, 2006). Mutations bestowing low levels of macrolide resistance are not stable in *C. jejuni*, in contrast with those that result in high level macrolide resistance (Caldwell *et al.*, 2008; Ladely *et al.*, 2007). However, *C. jejuni* with these high level mutations do suffer a significant fitness loss as compared to wild-type *C. jejuni* (Luangtongkum *et al.*, 2009). Thus macrolide resistance in *C. jejuni* is not expected to persist once the antibiotic pressure is removed.

C. jejuni commensally colonizes the intestine of all avians, including chickens, turkeys, ducks, and wild birds (Lee and Newell, 2006). Although *C. jejuni* is also found in other livestock animals, 50 to 70% of *C. jejuni* illness is due to contaminated poultry and

poultry products (Friedman *et al.*, 2000). *C. jejuni*-positive chickens are typically colonized at up to 10^9 colony-forming-organisms per gram of intestinal content (Lee and Newell, 2006). During the slaughtering process, cecal contents can contaminate the carcass and the processing environment (Keener *et al.*, 2004). Given that 500 to 800 organisms are sufficient to induce disease in susceptible humans, even 1 μg of such material, an invisible amount, could be more than sufficient to pose a risk to the consumer (Black *et al.*, 1988; Robinson, 1981). This constitutes a substantial public health hazard that can be exacerbated by subsequently handling products inappropriately. Various types of poultry products have been implicated with human infections, including poultry or poultry liver, raw or rare chicken, cooked chicken, processed chicken, and prepared chicken meals (Humphrey *et al.*, 2007).

During its infection cycle, *C. jejuni* transits from contaminated food to the stomach, through the intestinal tract and finally to the feces; allowing the transmission to a new host. During this stressful journey, it encounters life-threatening environmental conditions such as the acidic pH of the stomach (Merrell and Camilli, 2002), sudden temperature fluctuations (Konkel *et al.*, 1998; Konkel and Tilly, 2000), the high osmolarity of the gastrointestinal tract (Bremer and Kramer, 2000), intestinal gases (including hydrogen, methane and carbon dioxide) (Tannock, 2000), reactive oxygen and nitrogen compounds (Storz and Zheng, 2000), changes in nutrient availability (Hooper *et al.*, 1998; Hooper *et al.*, 2002), and low inorganic ion concentration (particularly iron starvation) (Hantke and Braun, 2000). In addition, most of these stresses vary between the external environment (e.g. food, water, and milk), different hosts and the different locations within the host. Undoubtedly, stress responses enable the bacterium to surmount these sudden environmental changes and successfully adapt to its new environment. Ultimately, a successful pathogenic colonization

will lead to the establishment of infection and eventually the generation of a stress-free environment. These chemical and physical threats constitute key signals for pathogens to sense that they have invaded the host and also allows for discrimination between different locations.

Transcriptional regulators orchestrate cellular responses to environmental stresses. Analysis of *C. jejuni* genome sequences has revealed the absence of many known key regulators of the stress defence systems found in other foodborne pathogens, such as *Salmonella* sp. and *Escherichia coli* (Parkhill *et al.*, 2000). Unlike most other Gram-negative bacteria, the well-characterized superoxide and peroxide sensing regulators, SoxRS and OxyR, are absent from the *C. jejuni* genomes. Interestingly, *C. jejuni* solely carries the peroxide-sensing regulator PerR, which typically regulates peroxide stress responses in Gram-positive bacteria (Mongkolsuk and Helmann, 2002). In addition to PerR, *C. jejuni* also contains a classical Fur (Ferric Uptake Regulator) protein, which typically regulates iron-uptake genes (Lee and Helmann, 2007). The presence of these two Fur-family orthologs, Fur and PerR, raises fundamental questions regarding the regulation of genes involved in iron metabolism and oxidative stress responses in *C. jejuni*.

Moreover the importance of iron acquisition to the cell cannot be understated. In the absence of sufficient iron sources, the growth of *C. jejuni* is significantly reduced, if not completely eliminated (Palyada *et al.*, 2004). It is this importance that has led *C. jejuni* and other bacteria to develop a wide variety of mechanisms for iron acquisition. One of the key roles for iron within *C. jejuni* is as part of iron sulfur complexes. The presence of enzymes containing iron sulfur complexes at key points in *C. jejuni*'s metabolic pathways also contributes to *C. jejuni* sensitivity to high levels of oxygen, since the iron-sulfur complexes

are sensitive to damage by both molecular oxygen, and reactive oxygen species (Flint *et al.*, 1993).

Iron sources available to *Campylobacter jejuni*

Campylobacter jejuni primarily colonizes the gastrointestinal tract of warm blooded animals, including avians and mammals (Lee and Newell, 2006). While the exact composition of the various iron sources available in the intestine remains incompletely described, heme, soluble complexes and salt forms of iron will be present (Conrad and Umbreit, 2000). Most of the iron originates from the diet, where it largely occurs as a ferric or heme species (Carpenter and Mahoney, 1992). Heme will be primarily derived from the breakdown of meat hemoglobin and myoglobin by pancreatic enzymes (Conrad *et al.*, 1967). Heme iron represents about one third of dietary iron in meat-eating Western countries, with the remainder consisting of ferric ions (Carpenter and Mahoney, 1992). While heme-iron remains soluble throughout the gastrointestinal tract, ferric ions are insoluble under normal physiological conditions. However, they are solubilized during its transit through the low pH environment of the stomach (pH ~3) and then chelated as they enter the less acidic duodenum. This chelation helps keep ferric ions in solution and is accomplished by intestinal mucins, organic acids (particularly, lactic, citric, succinic, malic, and pyruvic acids), bile acids, amino acids (particularly asparagine, glycine and histidine), membrane lipids (particularly fatty acids), and siderophores produced by the intestinal microbiota (Conrad and Schade, 1968; Conrad and Umbreit, 2000; Conrad *et al.*, 1991; Ratledge, 2007; Ratledge and Dover, 2000; Weinberg, 2009). In contrast, certain food constituents (carbonates, phosphates, oxalates, phylates, and tannates) may precipitate ferric ions and form macromolecule complexes, sequestering ferric ions from endogenous or incoming microbes

(Conrad and Umbreit, 2000). The stress hormone norepinephrine, which is secreted in the intestinal lumen, may also chelate iron and constitute an additional iron source for incoming microbes. Indeed, norepinephrine has been shown to stimulate *Campylobacter* growth under iron restricted conditions by acting in a siderophore-like manner (Cogan *et al.*, 2007). Importantly, a proportion of the dietary ferric ions might also be reduced to ferrous ions and maintained in that redox state by intestinal secretions or food constituents such as bile salts and ascorbic acid (Conrad and Schade, 1968). Other possible sources of iron could originate from the sloughing epithelial cells that are extruded from the mucosa into the intestinal lumen during normal mucosal cell proliferation, and from blood cells during inflammation. Finally, another potential source of iron is the non-heme iron binding protein lactoferrin that is present in the gastrointestinal fluid (Gonzalez-Chavez *et al.*, 2009). While lactoferrin tightly sequesters ferric ions from incoming pathogens, thus protecting the host against microbial infections, microbes have evolved specialized system(s) for iron acquisition from ferric-lactoferrin (Ratledge and Dover, 2000; Wandersman and Delepelaire, 2004).

Iron Acquisition Pathways in *Campylobacter jejuni*

Ferrous/Ferric ion acquisition

The *C. jejuni* FeoB specifically imports ferrous ions across the inner membrane of the cell. Ferrous ion uptake is also an important factor for colonization of the chick gastrointestinal tract. In fact, a $\Delta feoB$ mutant in the NCTC11168 strain showed more than a 10^4 fold reduction in chick colonization as compared to wild-type *C. jejuni* (Naikare *et al.*, 2006). *C. jejuni* also contains a FeoA homolog (Cj1397), but its role in ferrous ion import is currently undefined (van Vliet *et al.*, 2002).

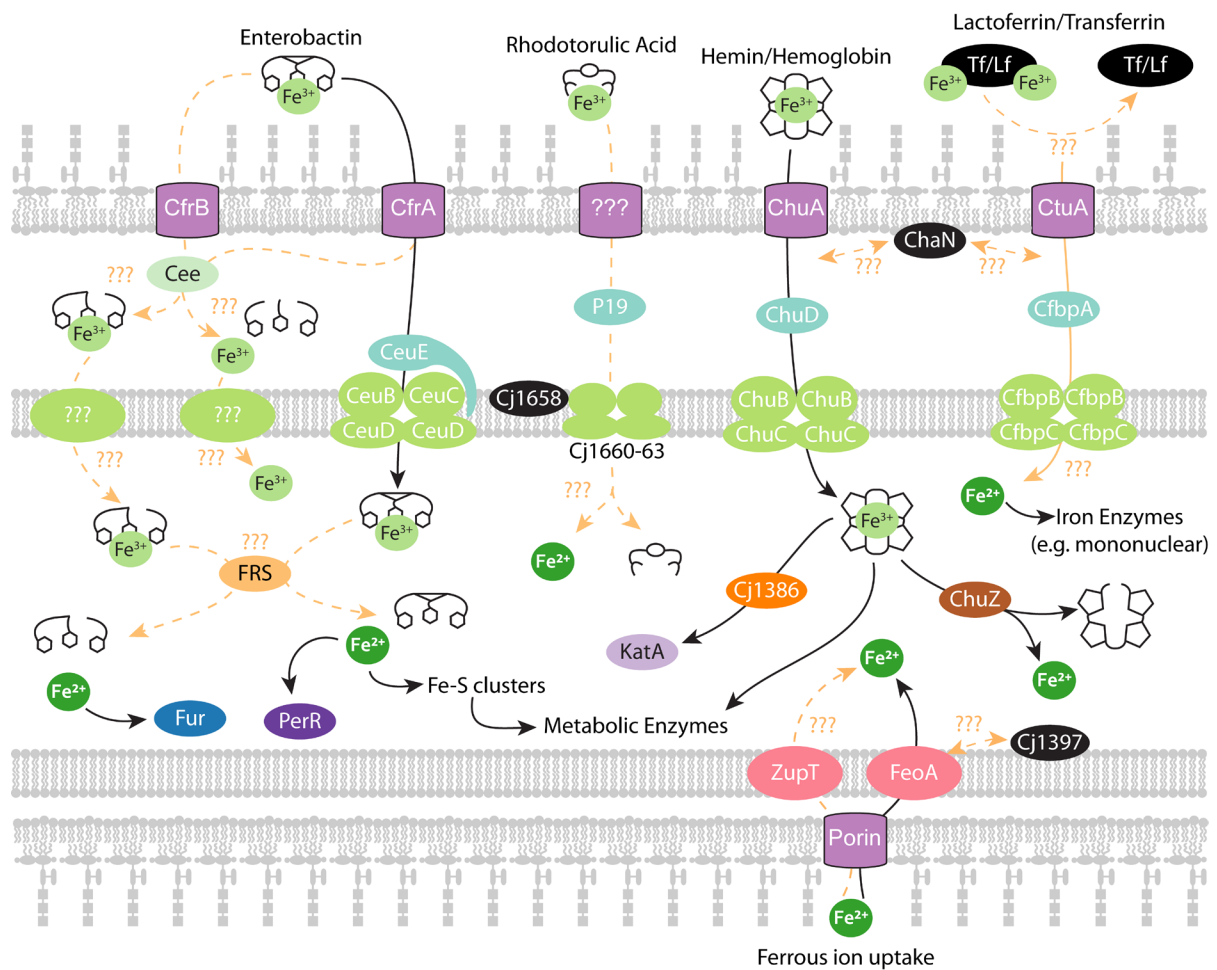


Figure 1.1: Overview of iron uptake systems in *Campylobacter jejuni*

Various sources of iron can be used to support *Campylobacter jejuni* growth although the details of several pathways remain unclear. See text for further discussion on each pathway (FRS = ferric reductase system, incompletely characterized pathways are in light orange).

A small fraction of ferrous ion import could also traffic through ZupT, a transporter primarily responsible for zinc uptake (Figure 1.1). The *E. coli* ZupT homologue has been shown to possess a broad substrate affinity for divalent cations including ferrous ions in *E. coli*, however, it has yet to be determined if this remains true in the case of *C. jejuni* (Rensing *et al.*, 2005).

Iron acquisition through ferric-siderophores

Analysis of *Campylobacter jejuni* genomes reveals an absence of genes encoding enzymes involved in siderophore biosynthesis, suggesting the inability of *Campylobacter* to secrete these powerful iron chelators (Parkhill *et al.*, 2000). Although an early report suggested the presence of siderophore-like activity by some *Campylobacter* strains (Field *et al.*, 1986), further characterization of these strains failed to confirm this observation (A. Stintzi, personal communication). Moreover, the lack of siderophore production by *Campylobacter* is supported by other studies (Baig *et al.*, 1986; Pickett *et al.*, 1992). Nevertheless, *C. jejuni* is able to acquire iron from a multitude of exogenous siderophores, however only the ferric-enterobactin transport system has been fully characterized (Field *et al.*, 1986; Palyada *et al.*, 2004; Zeng *et al.*, 2013). CfrA and CfrB are the two characterized enterobactin transporters in *Campylobacter* (Carswell *et al.*, 2008; Palyada *et al.*, 2004; Xu *et al.*, 2010; Zeng *et al.*, 2013; Zeng *et al.*, 2009). Curiously many *C. jejuni* strains that express CfrB are not able to utilize ferric-enterobactin as a sole iron source; however CfrB is an important colonization factor for those strains that do possess a functional ferric-enterobactin pathway (Xu *et al.*, 2010). In contrast, almost all CfrA expressing *C. jejuni* strains can utilize ferric-enterobactin as a sole iron source and CfrA has been demonstrated to be essential for chick colonization in these strains (Palyada *et al.*, 2004; Xu *et al.*, 2010). This discrepancy

has recently been suggested to be due to the absence or presence of the *C. jejuni* Cee enterobactin esterase present in the periplasm (Zeng *et al.*, 2013) (Figure 1.1). CfrB has been suggested to not interact with the CeuBCDE ABC transporter cassette and thus CfrB mediated enterobactin uptake is completely reliant on Cee to release the ferric ion from enterobactin (Zeng *et al.*, 2013). However the exact details of how the iron is released from the cleaved enterobactin are currently unclear. Deletion of FeoB does not appear to inhibit CfrB-mediated enterobactin use, suggesting that the ferric ion is not reduced in the periplasm for import (although the possibility still exists that ZupT may import ferrous ions as mentioned above) (Zeng *et al.*, 2013). This suggests that either the ferric ion disassociates from the cleaved enterobactin and is taken up by an uncharacterized ferric ion uptake system or that *C. jejuni* possesses a separate uptake system for linearized enterobactin. Additionally, the lack of Cee in the cytoplasm (Zeng *et al.*, 2013) suggests that *C. jejuni* possesses a ferric reductase system that can reduce the ferric-enterobactin complex as recently demonstrated in *E. coli* (Miethke *et al.*, 2011). In contrast with CfrB, CfrA can use either the Cee or CeuBCDE pathways to deliver the iron to the cytoplasm (Palyada *et al.*, 2004; Zeng *et al.*, 2009). In addition, *C. jejuni* has also been shown to be able to use a variety of structurally different catecholate ferric-siderophores in a CfrA dependant manner (Naikare *et al.*, 2013).

Beside catecholate siderophores, *C. jejuni* NCTC11168 is thought to be able to acquire iron from the hydroxamate siderophore rhodotorulic acid via the P19 system (Stintzi *et al.*, 2008). Ferric-rhodotorulic acid is imported into the periplasm through an unidentified outer membrane transporter. Subsequent interactions with P19, Cj1658 and Cj1660-1663 and other unidentified proteins are required for import into the cytosol and release of the bound iron (Stintzi *et al.*, 2008). It is unclear whether the entire ferric-rhodotorulic acid complex

enters the cytosol or if iron release occurs in the periplasm. In contrast with ferric-enterobactin, the abrogation of the ferric-rhodotorulic acid uptake pathways has no effect on *C. jejuni* chick colonization.

Heme iron acquisition

C. jejuni can use either free heme or hemoglobin as an iron source (Naikare *et al.*, 2013; Ridley *et al.*, 2006), although the mechanism for heme removal from hemoglobin is unclear. Heme is imported through the outer membrane by ChuA and transported into the cytosol via the ChuBCD system where ChuZ hydrolyzes the porphyrin ring to release the bound iron (Ridley *et al.*, 2006). The imported heme can also presumably be directly inserted into *C. jejuni* proteins. For example, recent work has shown that Cj1386 is required for proper heme insertion into KatA via an unidentified mechanism (Figure 1.1) (Flint *et al.*, 2012). Curiously, deleting the sole transporter for heme (ChuA) had no effect on *C. jejuni* chick colonization, indicating that heme uptake is not required in a commensal animal model (Naikare *et al.*, 2013).

Iron acquisition through Transferrin/Lactoferrin

Lactoferrin/Transferrin iron uptake is accomplished through CtuA via an unidentified mechanism (Miller *et al.*, 2008). Released iron is then transported through the CfbpABC system into the cytosol. It is currently unclear whether ChaN functions in transferrin/lactoferrin or heme transport. Previous work has shown that ChaN forms dimers that bind heme, but the *chaN* gene is located directly upstream of the lactoferrin/transferrin transporter (CtuA) (Chan *et al.*, 2006). The Δ *ctuA* mutant is also defective in colonizing the pathogenic colostrum-deprived piglet animal model (Stintzi *et al.*, 2005).

Chemistry of iron and reactive oxygen species

Iron is a transition metal absolutely required for most living organisms. In the cell, iron plays either a structural role by stabilizing the protein's tertiary or quaternary structure, or a functional role by contributing to the catalytic activity of enzymes or proteins involved in electron transfer (Harris, 2002). In biological systems, iron exists in three oxidation states: the reduced Fe^{2+} ferrous form, the oxidized Fe^{3+} ferric form, and the very reactive Fe^{4+} ferryl form (Harris, 2002). The ferryl species are formed during the catalytic cycle of various enzymes, including monooxygenase, cytochrome P450, and ribonucleotide reductase (Groves, 2006). Although this high-valent form of iron is essential for the function of these enzymes, there is no evidence that this highly reactive species participates in iron transport or storage as compared to the more stable ferrous and ferric forms of iron.

The redox reactivity of the ferric/ferrous couple has been harnessed for catalysis by a wide range of proteins, enabling many major biological processes, such as nitrogen fixation, photosynthesis, oxygen transport, respiration and DNA synthesis. Iron's value relies almost entirely on its incorporation into enzymes and metalloproteins, either as a porphyrin or iron-sulfur cluster, or as a mononuclear, dinuclear or polynuclear complex (Andrews *et al.*, 2003). Paradoxically, while the physiochemical reactivity of iron is the *raison d'être* for its role as a catalytic or structural cofactor, it is also responsible for the generation of highly reactive hydroxyl radicals (Galaris and Pantopoulos, 2008; Imlay, 2003; Merkofer *et al.*, 2006). Hydroxyl radicals are extremely reactive oxidants capable of damaging DNA, proteins and lipids (Imlay, 2003). Iron reactivity with dioxygen species has received considerable attention and is described by the Fenton reaction (Reaction 1). In this reaction, ferrous ion reacts with hydrogen peroxide and produces hydroxyl radicals and ferric iron (Galaris and

Pantopoulos, 2008). The ferric ion is reduced back to ferrous ion by superoxide anions, thus allowing iron to re-catalyze the Fenton reaction (Reaction 2). In biological systems, superoxide anions can be substituted to some extent by other reducing agents such as ascorbate.

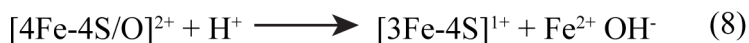
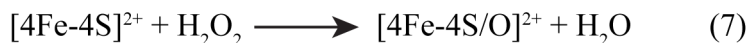
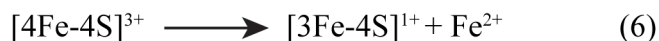


Hydrogen peroxide and superoxides are generated during aerobic bacterial metabolism by the auto-oxidation of the flavin cofactor of redox enzymes (Galaris and Pantopoulos, 2008). Under normal conditions, superoxide anions are removed by superoxide dismutase, which catalyses the dismutation of superoxides to hydrogen peroxide and oxygen (Reaction 3) (Imlay, 2008). Subsequently, hydrogen peroxide is converted to oxygen and water by peroxidases such as catalase (Reaction 4) (Imlay, 2008).



Superoxides and hydrogen peroxide are moderately reactive (Winterbourn, 2008). Both oxidants interact with protein iron-sulfur clusters by binding to the iron atom and oxidizing the cluster (Winterbourn, 2008). The mechanism of protein inactivation by superoxide anions involves a one-electron oxidation of the iron-sulfur clusters and the release of hydrogen peroxide and ferrous ion (Reactions 5 and 6), which in turn could generate highly reactive hydroxyl radicals by Fenton chemistry (Reaction 1). Besides its role in the Fenton reaction, hydrogen peroxide also catalyzes a two electron oxidation of iron-sulfur clusters, via the generation of a ferryl-like species, releasing ferric ion and the

damaged $[3\text{Fe-4S}]^{1+}$ species (Reactions 7 and 8). Thus exposure to hydrogen peroxide inactivates proteins that contain iron-sulfur clusters.



Apart from the above described reactivity, hydrogen peroxide can also interact with the heme-iron cluster of heme-containing proteins (Galaris and Pantopoulos, 2008). In this case, hydrogen peroxide catalyses the oxidation of Fe^{3+} -heme to a reactive oxo- Fe^{4+} -heme cation radical intermediate (Galaris and Korantzopoulos, 1997). Heme containing proteins are reduced back to their initial form in the presence of antioxidants. In turn, this cycle contributes to the elimination of hydrogen peroxide (Galaris and Korantzopoulos, 1997). However, in the absence of adequate amounts of antioxidants, the oxo- Fe^{4+} -heme containing proteins are further oxidized, leading to the liberation of the heme from the active site of the protein and the release of iron, which could then catalyze the Fenton reaction. Clearly, while the reactivity of superoxide anions and hydrogen peroxide is moderate, their main damaging effects are due to the secondary formation of hydroxyl radicals via the Fenton reaction (Merkofer *et al.*, 2006).

It is obvious that perturbation of the delicate cell balance between oxidants, antioxidants, and defence mechanisms will inevitably lead to deleterious effects, and thus create oxidative stress (Green and Paget, 2004; Jones, 2006). Moreover, the extent of cell damage is directly linked to the intracellular level of labile iron. Undoubtedly, the cellular iron status is closely linked to formation of oxidative stress via the catalysis of the Fenton

reaction. It is therefore not surprising that iron homeostasis and oxidative stress defence systems are tightly regulated at both the transcriptional and post-transcriptional levels.

Iron homeostasis and oxidative stress in *Campylobacter jejuni*

In order to avoid iron toxicity and the production of harmful hydroxyl radicals, microorganisms must achieve an effective iron homeostasis by tightly regulating the expression of genes encoding proteins involved in iron acquisition and metabolism in response to iron availability. In Gram-negative bacteria, including *C. jejuni*, the ferric uptake regulator Fur regulates the transcription of these genes. Fur was first described in 1981 as a repressor of iron acquisition systems in *E. coli* (Hantke, 1981). *C. jejuni* also carries another member of the Fur family of metalloregulators, PerR, in addition to the *fur* gene. PerR is a peroxide-sensing regulator and typically regulates peroxide stress responses in Gram-positive bacteria (Mongkolsuk and Helmann, 2002). Its presence in *C. jejuni* might compensate for the lack of the well-characterized superoxide and peroxide sensing regulators of Gram-negative bacteria, SoxRS and OxyR (Imlay, 2008) and thus far, *Campylobacters* are the only Gram-negative bacteria that carry a PerR homolog. Deletion of *perR* in *C. jejuni* results in constitutive expression of KatA and AhpC, and enhanced resistance to peroxide stress (van Vliet *et al.*, 1999). Interestingly, both PerR and Fur appear to regulate the expression of catalase in an iron-dependent manner since both genes must be mutated to fully abolish *kata* iron regulation (van Vliet *et al.*, 1999; van Vliet *et al.*, 1998). This observation clearly indicates a close link between the oxidative stress and iron regulons in *C. jejuni* and suggests an overlap between the Fur and PerR regulons. The function of Fur and PerR and their regulons will be discussed in greater detail below.

The Fur protein and mechanisms of Fur regulation

In most bacteria, including *C. jejuni*, the primary regulator of iron levels is the ferric uptake repressor (Fur) protein (Lee and Helmann, 2007). Although Fur is one of the most extensively studied metalloregulators, there is still considerable controversy over Fur's mechanisms of binding DNA and how Fur influences gene transcription. Fur is a homodimer that binds upstream in the promoter region of iron acquisition genes and blocks transcription during iron replete conditions (Figure 1.2). Fur requires a metal co-factor to bind DNA and this cofactor can be one of several divalent metals, including Fe^{2+} , Mn^{2+} , Zn^{2+} , or Co^{2+} (Mills and Marletta, 2005). However, only iron is believed to be physiologically present at levels sufficient to cause gene repression (Waldron and Robinson, 2009; Waldron *et al.*, 2009). Many Fur proteins (such as *E. coli* Fur or EcFur) also contain a high affinity zinc binding site that is believed to be important for protein folding and stability (Lee and Helmann, 2007). The crystal structure of *P. aeruginosa* Fur (PaFur) has been solved and contains two metal binding sites that are both occupied by Zn^{2+} (Pohl *et al.*, 2003). Only one site was able to exchange the zinc ion with iron, causing this site to be designated as the metal binding site and the other as the structural zinc site. However mutational studies have suggested that the designated zinc binding site may actually be the regulatory metal site and that the PaFur may not contain a structural zinc ion (Lewin *et al.*, 2002). This is contrary to the situation in *E. coli*, where all the residues involved in zinc binding were found to be essential for EcFur function (Coy *et al.*, 1994).

The binding sequence and specificity of Fur has also been subject to controversy. The most compelling model for Fur binding comes from studies in *E. coli* and *B. subtilis*. Initial studies in *E. coli* identified a 19 bp consensus sequence (GATAATGATAATCATTAC) for

Fur binding (Lavrrar and McIntosh, 2003). However, this sequence appeared to be too large for a small dimeric repressor such as Fur. Additional studies with synthetic DNA oligomers suggested that Fur may recognize an array of three hexameric repeats, with the minimal recognition site being GATAAT (Escolar *et al.*, 1998). This model postulates that a minimum of three Fur monomers bind to this site, with one Fur monomer each interacting with one hexameric repeat. Further studies in *B. subtilis* refined the 19 bp consensus to be two overlapping 7-1-7 binding motifs (Baichoo and Helmann, 2002). In this model each 7-1-7 sequence would bind one dimer of Fur and the 19 bp consensus would have two Fur dimers bound. A recent bioinformatic study in *E. coli* has lent support to this model, although it proposes that the flanking two nucleotides are also important for EcFur binding (Chen *et al.*, 2007). However another bioinformatic study in *P. aeruginosa* found that the PaFur dimer could be modelled to bind a 9-1-9 consensus sequence and that the PaFur dimers may bind co-operatively downstream or upstream from the initial binding site (van Oeffelen *et al.*, 2008). In *C. jejuni*, a CjFur consensus sequence has been proposed and was found to be quite different from that of *E. coli* (Palyada *et al.*, 2004). This was somewhat surprising as CjFur has been shown to partially complement a Δfur mutant in *E. coli* (Wooldridge *et al.*, 1994).

Fur is also known to form oligomers on DNA and protect DNA sequences from degradation well beyond its classical binding sites. This oligomerization has been shown to be both Fur concentration and pH dependant (Figure 1.2) (D'Autreaux *et al.*, 2007). These features suggest that Fur may be capable of regulating gene expression beyond its traditional role in metal regulation. Indeed, Fur has been implicated in the pH stress response of *H. pylori* (Gancz *et al.*, 2006) and *Salmonella typhimurium* (Foster and Hall, 1992). This regulation does not require the presence of an activating metal and can be genetically

separated from its metal sensing function (Hall and Foster, 1996). Recent work in *H. pylori* suggests that HpFur may form more stable dimers than EcFur and contains additional residues that may contribute to oligomerization (Vitale *et al.*, 2009). In addition, apo-HpFur has been shown to directly bind and repress gene expression, most notably in the case of *H. pylori* *sodB* (Figure 1.2) (Carpenter *et al.*, 2009a; Ernst *et al.*, 2005). These reports provide additional support that apo-Fur participates in gene regulation, although most studies cannot definitively distinguish between apo-Fur and holo-Fur binding. It should be noted however that in *Bradyrhizobium japonicum*, the Fur-like protein Irr has been shown to bind DNA without the presence of an activating metal and both repress and activate gene transcription (Lee and Helmann, 2007).

Δfur mutants in many bacteria commonly show several genes that are repressed rather than activated (Baichoo *et al.*, 2002; Danielli *et al.*, 2006; Gao *et al.*, 2008; Holmes *et al.*, 2005; Palyada *et al.*, 2004). This suggests that Fur is able to act as an activator of gene transcription as well as a repressor (Figure 1.2) (Lee and Helmann, 2007). It was later discovered that much of this gene activation was an indirect consequence of *fur* deletion. Fur was found to repress various small regulator RNAs (e.g. RyhB in *E. coli*, prrF1/prrF2 in *P. aeruginosa*) (Figure 1.2) (Masse and Gottesman, 2002; Masse *et al.*, 2007; Wilderman *et al.*, 2004). These RNAs post-transcriptionally repress their target genes by binding their mRNAs and enhancing degradation. This degradation coordinates the iron sparing response and decreases the translation of iron-containing proteins during times of iron starvation. RyhB also regulates acid resistance genes in *Shigella flexneri* (Oglesby *et al.*, 2005).

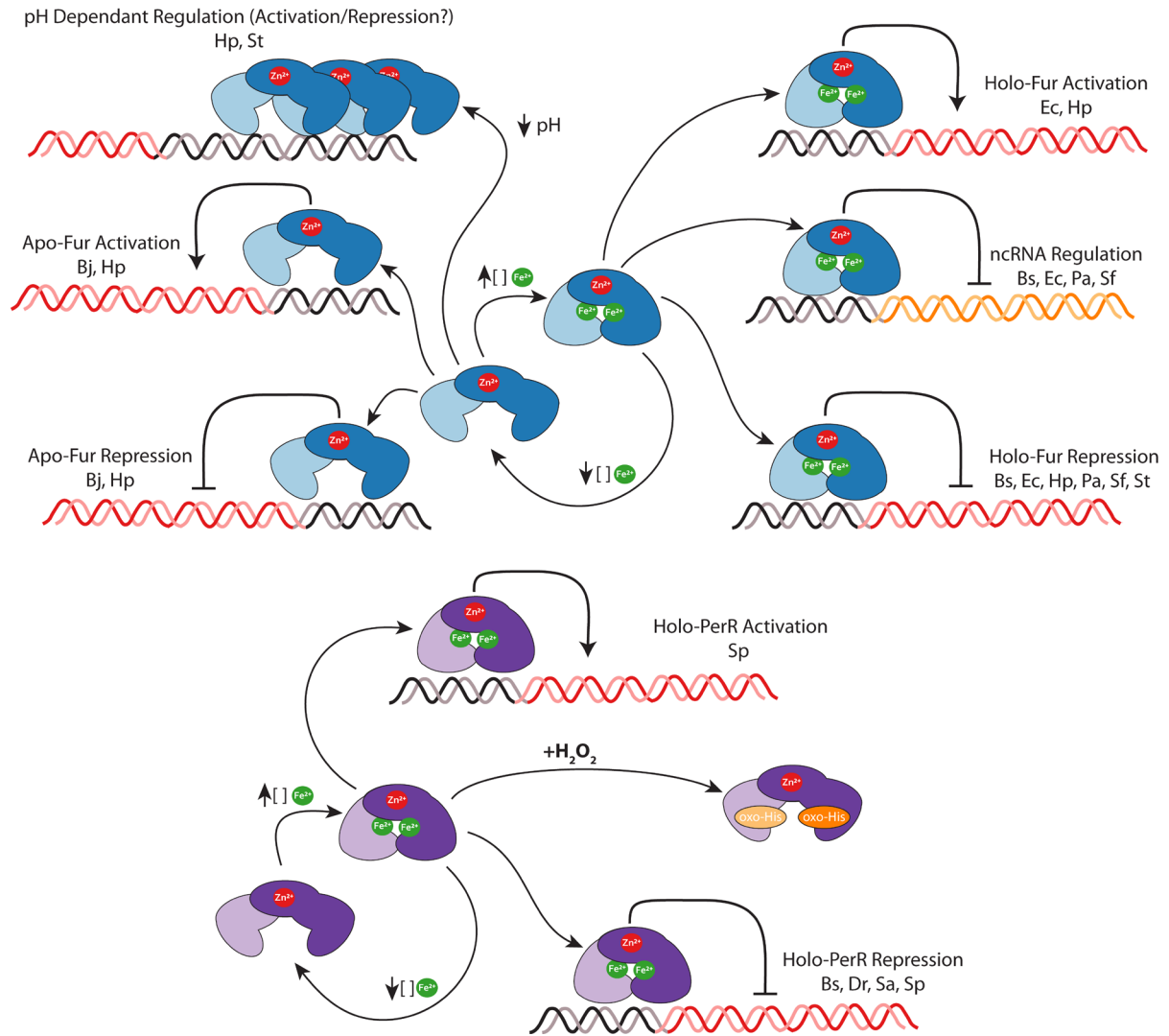


Figure 1.2: Model of gene regulation by the Fur and PerR proteins

The modes of Fur (Top) and PerR (Bottom) regulation are shown along with bacterial species where this type of regulation has been reported. Ec *Escherichia coli*, Bs *Bacillus subtilis*, Dr *Deinococcus radiodurans*, Pa *Pseudomonas aeruginosa*, St *Salmonella typhimurium*, Hp *Helicobacter pylori*, Bj *Bradyrhizobium japonicum*, Sp *Streptococcus pyogenes*, Sa *Staphylococcus aureus*, Sf *Shigella flexneri*. Figure adapted from (Butcher *et al.*, 2010). See Figure 1.3 for *C. jejuni* specific details.

While there has been no *ryhB* homolog identified in *C. jejuni* to date its presence should not be ruled out *a priori* and there may be uncharacterized non-coding RNAs that are involved in iron/Fur dependant gene regulation.

The *Campylobacter jejuni* Fur regulon

The *C. jejuni* Fur regulon has been characterized by two independent studies (Holmes *et al.*, 2005; Palyada *et al.*, 2004). Both studies compared the transcriptome profile of the wild-type strain with a *fur* deletion mutant grown in iron-rich and iron-limited conditions. While the majority of the CjFur regulated genes appear to be holo-CjFur repressed, analysis of the transcriptome data suggests other types of CjFur regulation, including apo-CjFur repression, and both apo-CjFur and holo-CjFur activation. Importantly, these studies cannot distinguish direct from indirect regulation. Consequently, not all of these modes of Fur regulation might truly take place (Figure 1.3). Interestingly, unlike the *fur* gene from other bacteria, the expression of the *fur* gene from *C. jejuni* is not auto-regulated (Palyada *et al.*, 2004).

In agreement with the classical Fur regulation paradigm, all the genes encoding *C. jejuni* iron acquisition systems were found to be holo-CjFur repressed (Holmes *et al.*, 2005; Palyada *et al.*, 2004). These genes encode the ferric-enterobactin transport system (*cfrA-ceuBCE*), the heme transporter complex (*chuABCD*), the ferric-transferrin/ferric-lactoferrin iron acquisition system (*ctuA-cfbpABC*), the ferric-rhodotorulic acid system (*cj1658-p19,cj1660-1663*), and several components of the three TonB-ExbB-ExbD complexes (*tonB1/B3 exbB1/B2, exbD1/D2*) (Figure 1.1).

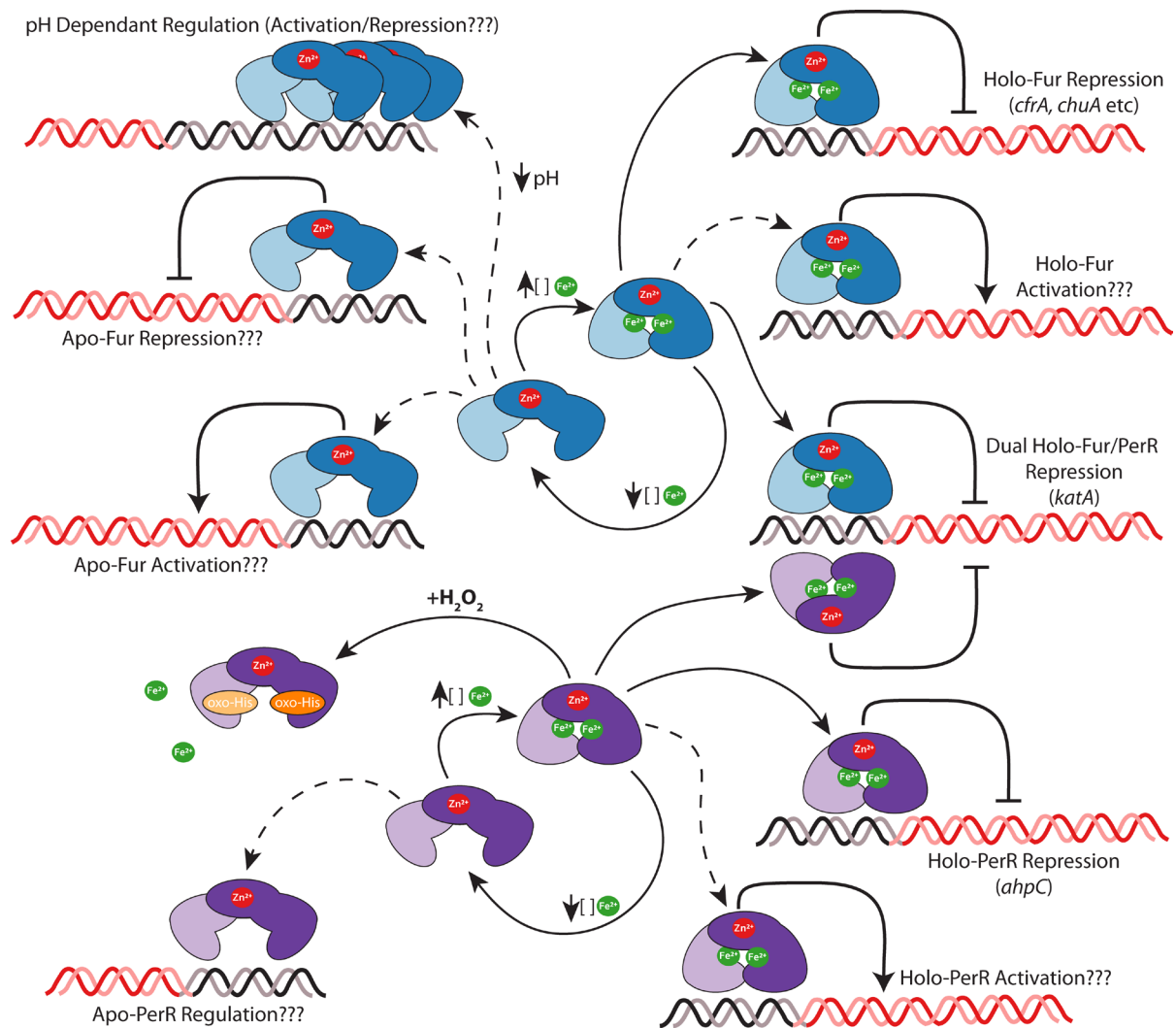


Figure 1.3: Model of Fur and PerR regulation in *Campylobacter jejuni*

The reported modes of regulation by CjFur and CjPerR are highlighted. Established modes of regulation are shown as solid arrows whereas suspected modes are displayed as dashed arrows.

Another category of genes whose expression is holo-CjFur repressed are involved in oxidative stress defence. These genes include catalase (*katA*), alkyl-hydroperoxidase reductase (*ahpC*), thioredoxin reductase (*trxB*), an ankyrin-repeat containing protein (*cj1386*), and a probable oxidoreductase (*cj0559*). These studies have identified the ferric uptake regulator Fur as a master regulator in *C. jejuni*. Besides genes encoding proteins with a clear role in iron acquisition or oxidant detoxification, Fur directly or indirectly regulates genes involved in flagella biogenesis, energy metabolism, fatty acid biosynthesis, other non-iron transporters, and numerous hypothetical proteins (Holmes *et al.*, 2005; Palyada *et al.*, 2004). While the expression of all Fur regulated genes identified by Palyada *et al.* were iron responsive, not all iron responsive genes were found to be Fur regulated. This observation suggests the presence of additional levels of gene regulation in response to iron availability. The expression of these genes is likely under the control of an additional transcriptional regulator and probably not directly regulated by Fur.

The Fur regulon and its effect on colonization and virulence

It has long been established that microorganisms must be able to acquire iron and to tightly regulate iron homeostasis in order to establish and maintain successful host colonization and infection (Ratledge, 2007; Weinberg, 2009). *C. jejuni* is no exception to the rule. Deletion mutants in various iron acquisition systems and the *fur* regulator were shown to be significantly affected in their ability to colonize the host (Palyada *et al.*, 2004). A Δ *cfrA* mutant, which is defective in enterobactin-mediated iron acquisition, was found to be unable to colonize the gastrointestinal tract of chickens (Palyada *et al.*, 2004). Given that enterobactin is not synthesized by *C. jejuni*, this observation suggests that *C. jejuni* gut colonization relies on the production of enterobactin or enterobactin-like molecules by the

gut microbiota. Deletion mutants in the *ctuA* and *ceuE* genes, shown to be implicated in ferric-transferrin or ferric-lactoferrin mediated iron acquisition (Miller *et al.*, 2008) and ferric-enterobactin transport respectively, were also significantly affected in chick colonization (Palyada *et al.*, 2004). Moreover, the Δ *ctuA* mutant was also shown to be attenuated in its ability to survive in the rabbit ileal loop (Stintzi *et al.*, 2005). Other members of the Fur regulon shown to be required for chick gut colonization include *tonB1* and *tonB3* (Naikare *et al.*, 2013), the oxidative stress defence genes *ahpC* and *kataA* (Palyada *et al.*, 2009), flagellum genes (Hendrixson and DiRita, 2004), the NADH dehydrogenase genes (*nuo*) (Weerakoon *et al.*, 2009), and the gluconate dehydrogenase Cj0415 (Pajaniappan *et al.*, 2008; Stintzi *et al.*, 2005). In addition, mutagenesis of the *fur* gene also significantly affected the organism's ability to colonize the gastrointestinal tract of chicks, demonstrating the importance of iron homeostasis *in vivo* (Palyada *et al.*, 2004).

The PerR protein and mechanisms of PerR regulation

Most Gram-negative bacteria sense and respond to reactive oxygen species (ROS) by the OxyR and SoxRS systems, which recognize hydrogen peroxide and superoxide stresses respectively (Mongkolsuk and Helmann, 2002). *C. jejuni* does not contain homolog of either of these proteins and it solely carries the peroxide-sensing regulator PerR. As a member of the Fur family of transcriptional repressors, PerR is thought to be a zinc-containing metalloprotein that utilizes either iron or manganese as a regulatory metal ion. PerR regulates the oxidative stress response in several bacteria, including *Clostridia*, *Streptococcus*, *Bacillus*, *Deinococcus* and *Staphylococcus* (Gryllos *et al.*, 2008; Helmann *et al.*, 2003; Hillmann *et al.*, 2008; Horsburgh *et al.*, 2001; Liu *et al.*, 2014). The primary role of PerR is to sense H₂O₂ and most of its functional characterization has been carried out in *B. subtilis* using both

crystallographic and biochemical approaches (Lee and Helmann, 2006a, b; Traore *et al.*, 2006; Traore *et al.*, 2009). OxyR and PerR use two different mechanisms to sense H₂O₂. OxyR senses the presence of H₂O₂ by the oxidation of two cysteines (Imlay, 2008). These oxidized cysteines subsequently form an intra-molecular disulphide bond that causes an alteration in its regulatory domain, triggering the transcription of OxyR's target genes. In contrast, PerR senses hydrogen peroxide by Fe²⁺-catalyzed oxidation of its regulatory metal binding site, which in turn leads to gene derepression (Lee and Helmann, 2006b). Crystal structure studies have revealed that PerR binds its regulatory iron metal through a coordination complex that includes three histidines (H37, H91, H93) and two aspartates (D85, D104) (Traore *et al.*, 2006; Traore *et al.*, 2009). When Fe²⁺ is bound to its regulatory metal site, the PerR dimer is able to bind DNA and repress gene transcription (Figure 1.3). The presence of H₂O₂ causes the oxidation of either H37 or H91; presumably via the Fenton reaction between H₂O₂ and the bound iron (Lee and Helmann, 2006b). This metal-catalyzed oxidation results in the formation of either oxo-H37 or oxo-H91 PerR species; weakening the affinity of the regulatory metal binding site for iron and eliciting gene derepression (Figure 1.3) (Traore *et al.*, 2009). There is currently no known mechanism by which the oxidized histidine could be reduced to enable PerR recycling (Lee and Helmann, 2006b). Manganese is also able to compete with iron for binding to the regulatory metal site in PerR (Herbig and Helmann, 2001). However, when bound to Mn²⁺, the Mn²⁺-PerR form is insensitive to oxidative inactivation (at physiological levels) and remains associated with DNA even in the presence of H₂O₂ (Lee and Helmann, 2006a).

PerR is believed to repress its target transcripts by binding upstream of the transcriptional start site to DNA sequences termed PerR-boxes. The PerR-boxes from *B.*

subtilis and *Streptococcus pyogenes* have been thoroughly characterized and are believed to be composed of a 7-1-7 inverted repeat comparable to Fur boxes (Baichoo and Helmann, 2002; Gryllos *et al.*, 2008). The *B. subtilis* Fur and PerR boxes are extremely similar (1 bp difference). However despite this similarity, BsFur and BsPerR do not recognize each other's consensus sequences, although artificial sequences recognized by both proteins can be synthesized (Baichoo and Helmann, 2002). Interestingly, in *C. jejuni* the expression of *katA* is regulated by both CjFur and CjPerR (Figure 1.3), suggesting the presence of both Fur and PerR boxes or of a composite Fur/PerR box recognized by both regulators (van Vliet *et al.*, 1999; van Vliet *et al.*, 2002). Bioinformatic analysis of known CjPerR targets have identified a potential PerR consensus sequence that appears to be a 7-1-7 palindrome similar to what has been seen for other Fur family proteins (Kim *et al.*, 2011; van Vliet *et al.*, 2002). However, it is currently unclear whether these putative binding sites are bound by either Fur and/or PerR, and whether CjFur and CjPerR are able to recognize each other's binding sequence.

PerR has not yet been demonstrated to exploit the vast repertoire of mechanisms employed by Fur to regulate gene expression (Figure 1.2). Currently, repression of genes by holo-PerR is the only experimentally confirmed mode of direct regulation (Lee and Helmann, 2007). While there are several reports of PerR activating genes in various bacteria, these studies were not able to differentiate between indirect or direct regulation (Gryllos *et al.*, 2008; Helmann *et al.*, 2003; Palyada *et al.*, 2009).

The PerR regulon

PerR has previously been shown to regulate the expression of two genes; *katA* and *ahpC* (van Vliet *et al.*, 1999) and the global transcriptome has been characterized using

microarray profiling (Palyada *et al.*, 2009). A *C. jejuni* NCTC11168 isogenic deletion mutant of *perR* was constructed and its transcriptome was compared to the wild type strain in the presence and absence of both iron and H₂O₂. The PerR regulon consists of at least 66 genes and these genes appear to be regulated by PerR both dependently and independently of iron and H₂O₂.

Notably, all the genes belonging to the hydrogen peroxide stimulon that were more than 2 fold up-regulated in response to H₂O₂ exposure were found to be PerR regulated (Palyada *et al.*, 2009). This observation indicates that PerR is the primary regulator of H₂O₂ stress in *C. jejuni*. As expected, many of the genes found in the PerR regulon have been previously shown or are believed to be implicated in oxidative stress defence. These genes include *katA* (catalase), *ahpC* (alkyl hydroxyperoxide reductase), *cft* (ferritin), and *trxB* (thioredoxin). Interestingly, PerR appears to directly or indirectly activate its own transcription in the presence of iron and H₂O₂. This auto-activation of PerR is paradoxical as it would consequently result in the down-regulation of oxidative stress defences at a time when they are the most needed. In fact, the transcript levels of *katA* and *ahpC* were found to be lower in the presence of both iron and hydrogen peroxide than their levels in the presence of either hydrogen peroxide or iron alone (Palyada *et al.*, 2009). While the rationale for this mode of regulation is unclear, it might be a direct consequence of the overlap between the PerR and Fur regulons.

The PerR regulon and its effect on virulence

In *C. jejuni*, a Δ *perR* deletion mutant displayed reduced colonization levels (10³ fold reduction) in the chick cecal model of colonization, despite exhibiting extreme resistance to H₂O₂ (Palyada *et al.*, 2009). In the same study, a Δ *katA* deletion mutant was completely

unable to colonize the chick cecum, while a $\Delta ahpC$ deletion mutant was severely attenuated in colonization (10^4 fold reduction). The *in vivo* attenuation of the $\Delta perR$ mutant suggests that CjPerR's role in colonization may also extend beyond the classical oxidative stress defence systems. One possible role for CjPerR in colonization could be in modulating flagella biogenesis, as the $\Delta perR$ deletion mutant was also affected in its motility (Palyada *et al.*, 2009), and proper flagella function has been shown to be essential for *C. jejuni* colonization (Zilbauer *et al.*, 2008).

Rationale for Study

As *Campylobacter jejuni* is one of the most frequent causes of gastroenteritis in both the developing and the developed world, understanding *C. jejuni* biology is paramount to reducing the amount of *C. jejuni* in the food chain. *C. jejuni* has an absolute requirement for iron in order to grow as iron catalyzes a wide range of essential biochemical reactions. Therefore understanding the regulatory networks that underlie iron metabolism should lead to greater insight into how *C. jejuni* is able to be a successful pathogen. *C. jejuni* is fairly unique amongst other gastrointestinal pathogens in possessing two iron-dependant Fur family regulators (CjFur/CjPerR) that are known to co-regulate gene transcription. In addition, there is the potential for both holo- and apo- regulation by CjFur/CjPer, which would add additional layers of complexity to their regulatory pathways. As such, this study aimed to further characterize the roles of iron, Fur and PerR in regulating gene expression in *Campylobacter jejuni*.

Chapter 2: The structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation.

Preface

The following chapter consists of data previously published in the Proceedings of the National Academy of Science under the title “The structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation” [PMID:22665794](https://pubmed.ncbi.nlm.nih.gov/22665794/) by Butcher J*, Sarvan S*, Brunzelle J, Couture JF, Stintzi A. (* each author contributed equally).

The specific contributions of each author to this paper are listed below along with the author contribution section published in the paper.

Butcher J: Designed, preformed and analyzed data from ChIP-on-chip experiments and qPCR/RT-qPCR experiments. Constructed figures for ChIP section of the paper. Wrote the first draft of the introduction, ChIP-on-chip sections of the paper, merged sections from different authors and responded to reviewer’s comments.

Sarvan S: Designed and performed experiments relating to apo-Fur crystallization experiments, gel mobility assays and prepared protein samples for IPCMS analysis. Also constructed several figures relating to the crystal structure of apo-Fur and responded to reviewer’s comments.

Brunzelle J: Collected crystallographic information on apo-Fur

Couture JF: Designed experiments relating to the apo-Fur crystallography and gel mobility assays. Analyzed crystallographic information on apo-Fur to construct the apo-Fur crystal structure. Wrote the apo-Fur crystal structure section of paper, contributed to editing the entire manuscript and also responded to reviewers comments.

Stintzi A: Designed ChIP-on-chip experiments, contributed to writing/editing the entire manuscript and responded to reviewer’s comments.

PNAS Author contribution statement:

Author contributions: J.B., S.S., J.-F.C., and A.S. designed research; J.B. and S.S. performed research; J.S.B. contributed new reagents/analytic tools; J.B., S.S., J.-F.C., and A.S. analyzed data; and J.B., J.-F.C., and A.S. wrote the paper.

Abstract

The full regulatory potential of the ferric uptake regulator (Fur) family of proteins remains undefined despite over 20 years of study. We report herein an integrated approach that combines both genome wide technologies and structural studies to define the role of Fur in *Campylobacter jejuni*. CjFur ChIP-chip assays identified 95 genomic loci bound by CjFur associated with functions as diverse as iron acquisition, flagellar biogenesis and non-iron ion transport. Comparative analysis with transcriptomic data revealed that CjFur regulation extends beyond solely repression and also includes both gene activation and iron independent regulation. Computational analysis revealed the presence of an elongated holo-Fur repression motif along with a divergent holo-Fur activation motif. This diversity of CjFur DNA binding elements is supported by the crystal structure of CjFur, which revealed a novel conformation of its DNA binding domain and absence of metal in the regulatory site. Strikingly, our results indicate that the apo-Fur structure retains the canonical V shaped dimer reminiscent of previously characterized holo-Fur proteins enabling DNA interaction. This conformation stems from a structurally unique hinge domain that is poised to further contribute to CjFur's regulatory functions by modulating the orientation of the DBD upon binding of iron. The unique features of the CjFur crystal structure rationalize the binding sequence diversity that was uncovered during ChIP-chip analysis and defines apo-Fur regulation.

Introduction

Iron is critical to many fundamental biological processes, including DNA synthesis, respiration, and the tricarboxylic acid (TCA) cycle. Unfortunately, while iron is essential for life, it can also be toxic under physiological conditions. Its ability to undergo facile oxidation-reduction can catalyze the production of noxious radical species by Fenton or Haber-Weiss chemistry (Butcher *et al.*, 2010). Most Gram-negative bacteria maintain a remarkably precise control over cytoplasmic iron levels through the transcriptional regulator Fur (ferric uptake regulator) (Butcher *et al.*, 2010; Lee and Helmann, 2007). In the classical Fur regulation paradigm, Fur binds ferrous ions and the dimeric Fe^{2+} -Fur complex (holo-Fur) recognizes target sequences upstream of iron-regulated genes and represses their transcription (Lee and Helmann, 2007). In addition to regulating genes involved in iron homeostasis, Fur has been shown to play an important role in the modulation of bacterial virulence, acid, nitrosative and oxidative resistances, and redox metabolism (da Silva Neto *et al.*, 2009; Gancz *et al.*, 2006; Jackson *et al.*, 2010; Ledala *et al.*, 2010; Lee and Helmann, 2007; McHugh *et al.*, 2003; Palyada *et al.*, 2004; Torres *et al.*, 2010).

In some bacterial species, Fur has been reported to directly activate gene expression, establishing a significant departure from the classical model of Fur-regulation (Carpenter *et al.*, 2009a; da Silva Neto *et al.*, 2009; Jackson *et al.*, 2010). In *H. pylori*, apo-Fur represses transcription of the bacterioferritin-like encoding gene *pfr* and the superoxide dismutase gene *sodB*, resulting in transcriptional activation of these genes in presence of iron (Carpenter *et al.*, 2009a). Also, work on *B. japonicum* Fur protein (BjFur) and the Fur homologue BosR in *B. burgdorferi* have demonstrated that certain Fur family proteins can recognize multiple consensus binding sequences (Friedman and O'Brian, 2003; Ouyang *et al.*, 2011). Recent

transcriptomic analyses have revealed that *Campylobacter jejuni* Fur (CjFur) regulates, either positively or negatively, more than 60 genes encoding proteins involved in iron acquisition, oxidative stress defence, flagella biogenesis, and energy metabolism (Holmes *et al.*, 2005; Palyada *et al.*, 2004). However, these studies failed to discriminate direct from indirect gene regulatory mechanisms.

The structural basis underlying the regulation of gene expression by Fur proteins has been extensively documented (An *et al.*, 2009; Dian *et al.*, 2011; Jacquamet *et al.*, 2009; Lucarelli *et al.*, 2007; Pecqueur *et al.*, 2006; Pohl *et al.*, 2003; Sheikh and Taylor, 2009; Traore *et al.*, 2006; Traore *et al.*, 2009). Fur proteins consistently fold into two domains consisting of an N-terminal DNA-binding domain (DBD) linked by a hinge region to a C-terminal dimerization domain (DD). In contrast to the common fold of these proteins, the coordination, number and geometry of the metal binding sites within Fur family proteins diverge (Dian *et al.*, 2011; Lee and Helmann, 2007; Lewin *et al.*, 2002; Pecqueur *et al.*, 2006; Pohl *et al.*, 2003; Sheikh and Taylor, 2009). Despite these studies, the structural determinants controlling the divergent mode of regulation of gene expression by this family of proteins have remained unresolved.

We herein provide the full extent of the *C. jejuni* Fur regulon using a chromatin immunoprecipitation and microarray analysis approach (ChIP-chip). Our results establish that CjFur regulates 95 transcriptional units and report the presence of apo- and holo-CjFur gene repression and activation in *C. jejuni*. Correspondingly, crystallographic studies reveal that apo-CjFur adopts the canonical V-shaped dimer characteristic of holo-Fur proteins, with two zinc ions per protomer. However, comparative analysis of apo-CjFur with other known Fur proteins reveals that apo-CjFur's DBD is rotated by 180° compared to other known Fur

structures, a structural rearrangement stemming from a reorientation of apo-CjFur hinge region. Overall, our results highlight the structural diversity of the Fur family of proteins and rationalize the consensus binding sequences revealed by our ChIP-chip and transcriptomics data.

Results

Identification of *in vivo* genome-wide CjFur regulatory targets

Transcriptomic analysis of a Δfur mutant in the presence or absence of iron established that CjFur may activate and repress gene expression in both holo- and apo- forms (Palyada *et al.*, 2004). Given that transcriptomic approaches cannot distinguish direct from indirect regulation, we sought to identify CjFur-binding regions on a genomic scale using ChIP-chip experiments. We identified 95 high confidence CjFur-regulated transcriptional units (Table S2.1), however due to the high gene density in the *C. jejuni* genome, we could not distinguish between intergenic and intragenic binding. Figure 2.1A shows a schematic representation of the CjFur binding sites along the *C. jejuni* chromosome together with *C. jejuni*'s response to iron limitation at mid-log phase. Our results are in agreement with previously known CjFur-regulated genes and ChIP-chip results were validated by qPCR analysis for several identified Fur-binding regions (Figure S2.1) (Holmes *et al.*, 2005; Palyada *et al.*, 2004). Fur ChIP enriched genes were categorized into COG groups according to their functional annotation (Figure 2.1B). As expected the COG category for inorganic ion transport and metabolism was found to be statistically over-represented. This functional category comprises genes encoding iron transporters, including the enterobactin (*ceuB*), heme (*chuAB*), and ferric-lactoferrin (*ctuA/chaN*) transporters.

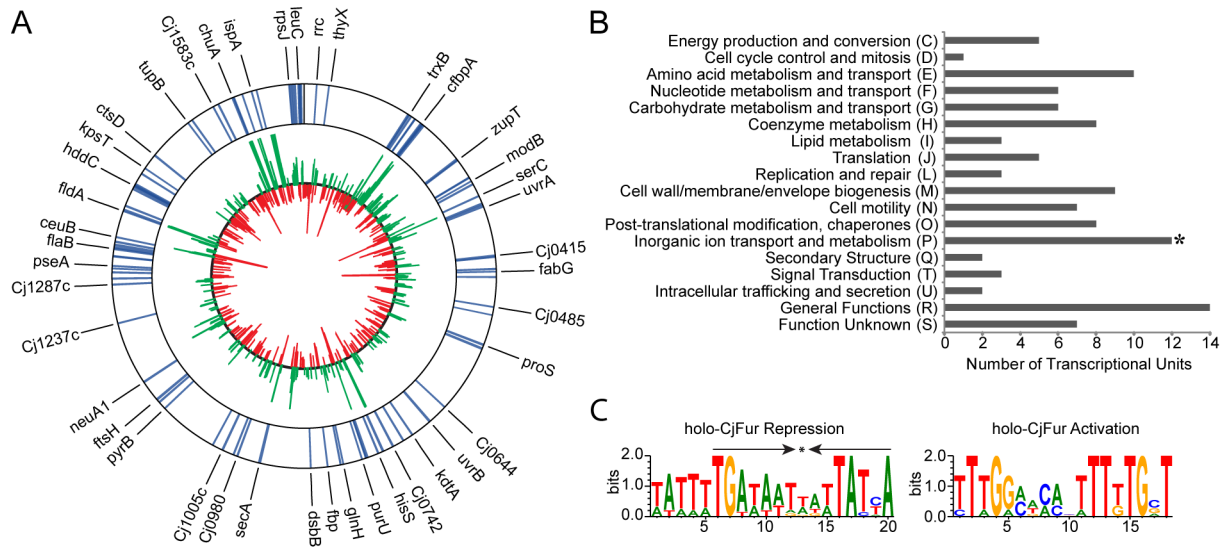


Figure 2.1: Genome Wide ChIP-on-chip of CjFur

A) Genomic Map of CjFur enriched transcriptional units overlaid with *C. jejuni*'s transcriptional response to iron limitation. The outer ring lists all the transcriptional units that were enriched under Fur ChIP (≥ 1.5 enrichment, $p \leq 10^{-4}$). Blue denotes the gene that was found to be enriched under Fur ChIP. The inner ring displays the transcriptional response of each gene to iron limitation with green denoting iron repressed genes and red denoting iron activated genes (≥ 1.5 fold change, $p \leq 10^{-4}$). Figure was made using Circos v0.54 (Krzywinski *et al.*, 2009). **B)** COG Functional Groups present in CjFur ChIP enriched transcriptional units. CjFur ChIP enriched genes encompass a diverse range of COG functional categories indicating that CjFur plays regulatory roles beyond iron metabolism. The COG functional category inorganic ion transport and metabolism was found to be statistically over-represented (*). **C)** Consensus sequences for CjFur binding sites. Left: holo-CjFur repression consensus sequence with its palindromic sequence highlighted with arrows. Right: holo-CjFur activation consensus sequence. Consensus sequences were made using Weblogo 3.

Identified CjFur enriched genes were also compared to the CjFur regulon and iron stimulon that were previously obtained by comparing the transcriptional profiles of the wild-type with a *fur* deletion mutant and by studying the transcriptional response of *C. jejuni* to iron limitation (Holmes *et al.*, 2005; Palyada *et al.*, 2004) (Table S2.1). The CjFur regulon identified using ChIP-chip is significantly different from those previously proposed using transcriptomic approaches (Holmes *et al.*, 2005; Palyada *et al.*, 2004). This is most likely due to the fact that transcriptional approaches cannot differentiate between direct and indirect regulation (and will identify both), cannot identify genes transcriptionally silent under the tested conditions and would not detect genes exhibiting small change in expression. In contrast, ChIP-chip will identify all direct targets that are bound by Fur under the tested conditions independently of fold change. Only 17 of our identified 95 ChIP targets were previously found to be members of the CjFur regulon. While the majority of these genes were found to be under holo-CjFur repression (~53%); our analysis also revealed holo-CjFur activation (4 genes), apo-CjFur repression (4 genes), and apo-CjFur activation (2 genes). It should be noted that in some cases our transcriptomic data may not be able to definitively differentiate between some of the different forms of CjFur regulation as several genes display both apo- and holo- forms of CjFur regulation. Therefore we used RT-qPCR and gel shift assays with apo-CjFur protein to confirm the CjFur dependant regulation of several CjFur ChIP targets (Figure 2.2).

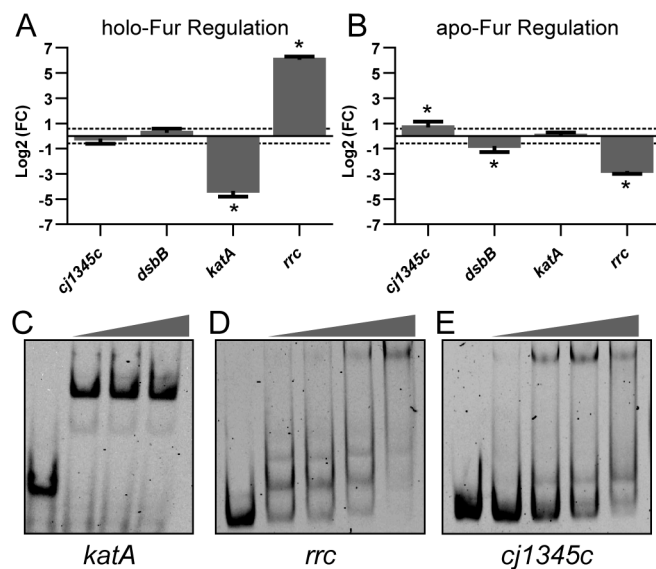


Figure 2.2: Genes directly regulated by either holo- or apo-CjFur

(A, B) Genes differentially expressed under iron replete (A) or iron limiting (B) conditions in a Δfur mutant. Positive fold changes indicate activation while negative fold changes indicate repression. The dotted line represents the 1.5 fold cut-off value and * denotes p values <0.05 . (C, D, E) Gel shift assays of holo-CjFur (C) and apo-CjFur (D, E) demonstrating direct CjFur binding to the promoter regions of genes found to be differentially expressed by RT-qPCR. Holo-Fur concentrations were 0, 5, 50 and 100nM while apo-CjFur concentrations were 0, 50, 100, 200 and 1000 nM. The promoter region for *dsbB* has been previously shown to be bound by apo-CjFur *in vitro* (Grabowska *et al.*, 2011)

Importantly, the apo-CjFur protein extract was confirmed to be free of iron by ICPMS analysis (Table S2.2). As shown Figure 2.2, apo-CjFur exhibits a strong DNA-binding activity with fragments derived from the up-stream regions of the genes *rrc* and *cj1345c*. In addition, it has recently been reported that *dsbB* is apo-Fur repressed in *C. jejuni* (Grabowska *et al.*, 2011) and this gene was both identified as a target in our ChIP results and also found to be apo-CjFur regulated by RT-qPCR (Figure 2.2).

There were 78 genes that were identified as Fur targets in our ChIP assay that were not previously thought to be Fur regulated. Only 30 of these genes are iron regulated (Table S2.1). The fact that ~50% of the identified CjFur genes are not iron regulated indicates that many of CjFur's regulatory roles are iron independent. Moreover, as the transcript level of most CjFur ChIP targets (~82%) was not significantly affected in a Δfur mutant, this indicates the presence of complex regulatory circuits comprising additional transcription factors.

Bioinformatic analysis of potential Fur binding sites

The 5' non-coding regions of the CjFur targets were further analyzed for the presence of conserved sequences that would represent CjFur binding sites. The data set comprised all CjFur targets and sub-sets of transcriptional units categorized based on the modes of CjFur regulation (holo/apo, activation/repression). Despite our extensive testing of different subgroups and alternate transcriptional start sites, we failed to identify a universal consensus sequence for all the CjFur targets and, similarly, we were unable to identify a motif for genes regulated by apo-CjFur. After these analyses, we then sought to identify enriched motifs in the genes regulated by CjFur in presence of iron. In sharp contrast with apo-CjFur regulated genes, we identified consensus motifs for holo-CjFur activated and repressed genes. The

consensus sequence of the holo-Fur repressed CjFur targets (Figure 2.1C) is very similar to the CjFur consensus sequence previously reported (Palyada *et al.*, 2004). This consensus sequence is typical of classical Fur-boxes and contains an internal palindromic 7-1-7 sequence. Interestingly, our analysis also detected the presence a consensus sequence for holo-Fur activated genes (Figure 2.1C). The identified consensus sequence is significantly different from previously identified Fur-boxes. It is non-palindromic and contains two direct repeat sequences of TTTGG that differs markedly from the two inverted repeats in the Fur box (TGATAAT).

Crystal structure of CjFur

In order to understand the biochemical determinants underlying the different modes of Fur regulation, the structure of CjFur was determined at 2.1Å resolution using single wavelength anomalous dispersion experiments (Table S2.3). The structure consists of two protomers (A and B) that form the asymmetric unit and the functional dimer (Figure 2.3). Protomer A consists of residues 4-83 and 90-149 while protomer B comprises residues 2-16 and 26-154.

CjFur contains two modular domains forming a N-terminal DNA binding domain (DBD) and a C-terminal dimerization domain (DD). The DBD of CjFur is composed of 5 consecutives α -helices ($\alpha 1, \alpha 2, \alpha 3, \alpha 3-2$ and $\alpha 4$) followed by a two-stranded antiparallel β -sheet ($\beta 1-\beta 2$). The tip of $\beta 2$ is connected to the DD by an 8-residue hinge region. The DD folds as a mixed α/β domain in which $\beta 3-\beta 4-\beta 5$ form a twisted β -sheet intersected, between $\beta 4$ and $\beta 5$, by the $\alpha 5$ -helix. The structure ends by a short α -helix ($\alpha 6$) that coordinates one zinc ion

Figure 2.3: Crystal Structure of CjFur

A) Crystal Structure of CjFur. Orthogonal views of the CjFur crystal structure in which protomers A and B are rendered in orange and blue respectively. α -sheets and β -helices are labeled accordingly and zinc atoms are depicted as grey spheres. **B)** Electrostatic surface potential of the CjFur crystal structure. Electrostatic potentials are contoured from $+10k_bTe^{-1}$ (blue) to $-10k_bTe^{-1}$ (red). **C)** Sequence alignment of Fur and Fur-like proteins. Sequence alignment of Fur proteins (*C. jejuni* (Cj), *H. pylori* (Hp), *V. cholerae* (Vc), *P. aeruginosa* (Pa), *E. coli* (Ec)) and of the Fur-like Zur from *M. tuberculosis* (MtZur), Nur from *S. coelicolor* (ScNur) and PerR from *B. subtilis* (BsPerR). Sequences were aligned using the ClustalW option in MEGA5 (Tamura *et al.*, 2011). CjFur secondary structure elements are shown above the alignment. Stars indicate the residues involved in the predicted CjFur S2 site. S1 residues are shaded in orange, predicted S2 in yellow and S3 in blue. Positions with 100% amino acid conservation are indicated by dark grey, 100%-80% by medium grey and 80%-60% by light grey.

After elucidating the CjFur structure, we sought to analyze the structural differences between CjFur and other structurally characterized Fur and Fur-like homologs. Using lsqkab, the HpFur, VcFur, PaFur and CjFur structures were overlaid and the r.m.s.d. calculated (Kabsch *et al.*, 1976). We noted that, with the exception of CjFur, all Fur structures aligned reasonably well with r.m.s.d of $\sim 1.8\text{\AA}$ for all atoms (Figure S2.3). Other Fur proteins consistently orient their $\alpha 1$ -helix outside of the V-shaped cleft and their two-stranded anti-parallel β -sheet inside the dimer. In contrast, alignment of CjFur with any Fur proteins (r.m.s.d of $\sim 15\text{\AA}$ for all atoms) revealed drastic differences in the position of the DBD secondary structure elements. These differences include a 180° rotation of CjFur's DBD which positions the $\beta 1$ – $\beta 2$ β -sheet on the exterior of the structure and the $\alpha 1$ -helix within the V-shaped dimer (Figure 2.3A). Close inspection of the overlay of CjFur with HpFur (CjFur's closest homolog) revealed that CjFur's DBD repositioning stems from the conformation of the CjFur hinge region. Indeed, in CjFur, the hinge region is elongated while the structurally equivalent region of HpFur is bent in such a way that it adopts a loose turn. Overall, these observations suggest that the CjFur hinge region plays an important role in controlling the orientation of the DBD.

The metal binding sites of CjFur

After establishing that CjFur adopts a peculiar structural conformation, we hypothesized that metal coordination would also diverge from other Fur homologues. To confirm this hypothesis, anomalous Fourier difference maps were calculated and the resulting electron density analyzed. For consistency, we have utilized the nomenclature recently employed for designating metal binding sites in HpFur (Dian *et al.*, 2011). The

CjFur structure contains two occupied Zn^{2+} binding sites (referred therein as S1 and S3) per protomer. The S1 site contains a Zn^{2+} ion that is tetra-co-ordinated by two pairs of cysteine residues (C105/108 and C145/148) (Figure S2.4A) and is found in the DD of CjFur. This zinc-binding site is known to be important for maintaining the structural integrity of the protein and dimerization in HpFur (Vitale *et al.*, 2009). Given that the S1 site is also found in HpFur and BsPerR and that both proteins exhibit an additional C-terminal α -helix (Figure 3C, S4A), these results suggest that Fur proteins harbouring an additional C-terminal α -helix coordinate a structural Zn^{2+} ion in the S1 site.

The second metal binding site, S3, lies between $\beta 5$ and $\alpha 5$ and is in close proximity to the C-terminal end of the hinge that links the DBD to the DD. In the S3 site, the Zn^{2+} ion is hexa-co-ordinated by residues D101, E120, H137 and two water molecules. The first water molecule (referred therein as W1) is located 2.2Å from the Zn^{2+} ion and makes a 2.6Å hydrogen bond with the side chain amide group of N123. The second water molecule (W2) engages in two hydrogen bonds with the carbonyl group and carboxylate moiety of H99 and E115, respectively (Figure S2.4C). This type of coordination is drastically different from the HpFur and VcFur S3 sites which tetra-co-ordinate the Zn^{2+} ion and lack metal-coordinating water molecules. While the number of coordination in CjFur is analogous to PaFur, there are differences in the mode of coordination. In PaFur, W2 is absent and replaced by the imidazole side chain of H86. In contrast, CjFur is able to coordinate W2 due to the aforementioned 180° rotation of CjFur's DBD, which places the backbone carbonyl group of H99 in an orientation permissive for a W2-mediated hydrogen bond with the Zn^{2+} ion. In addition, E115, which engages in the second W2-mediated hydrogen bond, is unique to CjFur.

Recent reports have suggested that the S2 metal binding site is the iron-sensing site in several Fur proteins (Lee and Helmann, 2007). The crystal structures of VcFur, HpFur and PaFur revealed that all these proteins coordinate metal ions at S2 using different geometries and modes of coordination (Dian *et al.*, 2011; Pohl *et al.*, 2003; Sheikh and Taylor, 2009). Close analysis of the calculated Fourier maps in proximity to the CjFur S2 site failed to detect electron density; suggesting that CjFur S2 site is unoccupied. Consistent with this observation, only three metal coordinating residues (H100, H102, and E113) could be located at the putative iron-binding S2 site (Figure S2.4B). Moreover, ICPMS analysis confirmed the absence of contaminating iron in our protein preparation (Table S2.2). The absence of metal in the S2 site can be explained by the rotation of CjFur's DBD which positions two of the putative Fe²⁺ coordinating residues, namely E93 and H99, in a non-permissive orientation for engaging in metal coordination. Since there is a lack of metal in the S2 regulatory site, the CjFur crystal structure is in the apo- form. However, our apo-CjFur crystal structure still adopts the canonical V shaped conformation that is characteristic of other holo-Fur crystal structures.

Discussion

Our study defines for the first time the apo-Fur structure and the Fur regulon in *C. jejuni*. The structural analysis also reveals a possible mechanism for apo-CjFur regulation, while our ChIP-chip results significantly expand known CjFur target gene loci unveiling regulatory roles beyond iron homeostasis. The central regulatory role of CjFur is highlighted by the identification of over 95 Fur binding sites in proximity to genes encoding proteins involved in diverse biological pathways ranging from metal homeostasis (including iron, zinc, tungsten, and molybdenum) to flagellar and membrane biogenesis, energy production and conversion, and stress responses.

Consistent with the classical role of Fur as a holo-Fur repressor of iron acquisition genes (Lee and Helmann, 2007), the CjFur targets comprise all the genes known to be involved in iron acquisition (e.g. ferric-enterobactin, heme and lactoferrin transporters). Remarkably, while half of the CjFur binding sites are associated with iron regulation, only a fourth of the CjFur targets were previously reported to be deregulated in a *fur* mutant. This pattern is not unprecedented and was also observed for the HpFur and PaFur regulons (Butcher *et al.*, 2011; Danielli *et al.*, 2006).

Genes that were found to be CjFur targets but not differentially expressed in a Δfur mutant include genes involved in the flagella biogenesis and genes involved in zinc and molybdate/tungsten transport. Members involved in flagella biogenesis include the major *C. jejuni* flagellins (*flaAB*) and numerous glycosylation proteins (e.g. *pseA* and *pseF*). By analogy, the HpFur regulon also contains several genes involved in flagellar biogenesis (Danielli *et al.*, 2006). This absence of differential expression of the flagellum genes in the *fur* mutant likely reflects the complex regulatory transcriptional cascade for flagellar genes in

C. jejuni. Indeed, transcriptional control over flagellar biogenesis is quite extensive in *C. jejuni*, involving two σ factors (σ^{28} and σ^{54}), the FlgSR two-component system, and the FlhF regulator (Hendrixson, 2008). The CjFur regulon also includes the zinc, molybdate and tungstate transporters ZupT, ModB and TupB respectively (Rensing *et al.*, 2005; Smart *et al.*, 2009). The direct regulation of these transporters by CjFur reveals additional roles for CjFur in transition metal homeostasis beyond iron regulation.

Interestingly, while we identified four modes of CjFur regulation, holo- and apo-CjFur repression and activation, only two distinct CjFur DNA binding motifs could be identified. The motif identified for holo-CjFur repressed genes matches the consensus sequence previously identified, while the motif identified in holo-CjFur activated genes bears little similarity to the holo-Fur repressed motif (Palyada *et al.*, 2004). The fact that CjFur recognizes two distinct consensus sequences is not unprecedented as recent work on the Fur homologue BosR in *B. burgdorferi* demonstrated that this Fur-like protein could recognize three distinct consensus sequences (Ouyang *et al.*, 2011). Similarly, multiple unique DNA recognition sites were also reported for the *B. japonicum* Fur protein (BjFur) (Friedman and O'Brian, 2003). Overall, our observations highlight the expanding repertoire of DNA sequences recognized by the Fur family of proteins.

Although CjFur should share similar structural features with Fur proteins from other bacteria, none of the previously crystalized proteins has been demonstrated to bind multiple consensus sequences and only HpFur is known to regulate gene expression in its apo-form. Moreover, the crystal structure of HpFur failed to provide structural insights into apo-HpFur function and previous work has suggested that the structure of CjFur differed from other Fur family proteins (Miles *et al.*, 2010a). Since our results indicate that CjFur recognizes

divergent DNA consensus sequences and regulates gene expression independently of iron, we reasoned that CjFur must possess unique structural features that have not yet been described for the Fur family of proteins. Accordingly, comparative analyses of our CjFur crystal structure with other Fur and Fur-like proteins reveal several similarities and differences. Comparable to all Fur proteins, CjFur contains two protomers which form the canonical V-shaped dimer characteristic of holo-Fur proteins. Similarly, analysis of CjFur metal binding sites reveals that the S1 site in CjFur contains a zinc ion that is tetra-coordinated by two pairs of cysteine residues, forming a C4 zinc finger motif, an important structural determinant for dimerization in HpFur and also found in BsPerR (Vitale *et al.*, 2009). The S3 site is also occupied by a zinc ion hexa-coordinated by residues including E120, D101, and H137 and two water molecules. Although the position of the S3 site is analogous to other Fur proteins, it differs significantly from the tetra-coordination state observed in HpFur. The final metal binding site is known as the S2 site and is the regulatory metal binding site in HpFur, VcFur and PaFur. Strikingly, this S2 site is unoccupied in CjFur indicating that the structure of CjFur represents the apo form of the protein. However unlike the previously obtained apo-BsPerR structure (Traore *et al.*, 2009), which has lost its V shaped conformation upon reorientation of its DBD in a near planar arrangement (Figure S4), apo-CjFur maintains the canonical V-shaped conformation reminiscent of other holo-Fur proteins. These observations likely stem from several inter-domain contacts between CjFur DBD and DD. Firstly, residues located at the N-terminal end of $\alpha 1$, which include N5, V6 and GE7 make several van der Waals contacts, hydrophobic interactions and hydrogen bonds with the CjFur DD. Secondly, residues found in the C-terminal end of $\alpha 2$, which include Y38 and H39, make extensive interactions with residues encompassing the $\beta 3$ – $\beta 4$

hairpin of the CjFur DD. Thirdly, the residues succeeding the C4 zinc finger of the DD fold back onto the DBD and engage in several hydrophobic contacts with a loop connecting $\alpha 2$ and $\alpha 3$ of the DBD. Altogether, this extensive network of interactions is strikingly different than apo-BsPerR in which no contacts between the DBD and DD domains are observed. Altogether, these interactions provide a rationale underlying the formation of apo-CjFur's V-shaped conformation. Finally, this conformation places several basic residues on the tip of the V-shaped structure (Figure 2.3B) in a position amenable for engaging in electrostatic interactions with DNA. Thus our structure provides, for the first time, a snapshot view of an apo-Fur protein.

In addition of adopting the V-shaped conformation, further comparative analyses of apo-CjFur with known holo-Fur structures identified notable differences in the orientation of its DNA binding domain (An *et al.*, 2009; Dian *et al.*, 2011; Jacquamet *et al.*, 2009; Lucarelli *et al.*, 2007; Pohl *et al.*, 2003; Sheikh and Taylor, 2009). Indeed, while all holo-Fur proteins align relatively well with each other (r.m.s.d. of ~ 1.8 Å), apo-CjFur displays striking structural differences in the orientation of its DBD. This conformational difference stems from a notable rearrangement of CjFur hinge region which positions the $\alpha 1$ helices inside the V-shaped dimer. Thus, the CjFur hinge region is poised to play an important role in modulating the orientation of the DBD and likely contributes to the rotation of the DBD upon iron binding. These results are in line with a recent study showing that metallation of HpFur S2 triggers a conformation change, which results in the formation of an active holo-Fur protein (Dian *et al.*, 2011). These observations may also suggest that residues composing CjFur hinge region allow for an increased degree of freedom and thereby the positioning of the DBD in multiple orientations. However, given that the CjFur S2 site lacks a metal ion but

the apo-CjFur structure maintains a V-shaped conformation, we postulate that additional mechanisms confer to CjFur the ability to bind to divergent DNA sequences and maintain a V-shape conformation in absence of metal in the S2 site. This added complexity would give CjFur the ability to selectively regulate gene expression depending on other environmental factors along with iron.

In conclusion, our results imply that the DNA binding properties of CjFur will diverge depending both on the occupancy of its regulatory S2 site and the orientation of its unconventional hinge region. In a broader context, these results support the observed four modes of Fur regulation, apo- and holo-Fur activation and repression. Finally, our study has not only provided a genome wide view of Fur binding in *C. jejuni*, but also provides the first view of an apo-Fur structure.

Materials and Methods

C. jejuni and *E. coli* strains were grown under standard conditions with antibiotic supplementation as needed. CjFur was purified and used to generate anti-CjFur antibodies and for crystallization trials. ChIP-chip experiments were completed and the results analyzed as previously described (Danielli and Scarlato, 2010; Palyada *et al.*, 2004). CjFur ChIP enrichment was confirmed using qPCR of known Fur targets. Consensus sequence analysis of CjFur ChIP targets using MEME was done as described previously (Palyada *et al.*, 2004). The CjFur crystal structure was solved using single wavelength anomalous diffraction datasets generated from the Life-Science Access Team beamline at the Advance Photon Source in Chicago. For detailed Materials and Methods, please see SI Materials and Methods.

Acknowledgments

This work was supported by CIHR to A.S. and J.-F.C., and a CIHR-Banting graduate scholarship to J.B. J-F C. acknowledges a Canada Research Chair in structural biology and epigenetics. An Early Research Award by the Ministry of Research and Innovation of Ontario supported this research (J.-F.C). We would also like to acknowledge Fredric Poly for the construction of the pASK-Fur expression vector. Finally, we would like to thank the Quantitative Bioelemental Imaging Center (QBIC) (Northwestern University) for inductively coupled plasma mass spectrometry (ICPMS) analysis.

Chapter 3: The transcriptional landscape of *Campylobacter jejuni* under iron replete and iron limited growth conditions

Preface

The following chapter consists of data previously published in the Public Library of Science ONE under the title “The Transcriptional Landscape of *Campylobacter jejuni* under Iron Replete and Iron Limited Growth Conditions” ([PMID 24223952](#)) by Butcher J and Stintzi A.

For the sake of brevity, not all the supplemental files submitted with this manuscript are reproduced here due to the length and breadth of the genome wide analysis performed. Specifically, Tables S3.3, S3.4, S3.5, S3.6, S3.7, S3.8, and S3.9 are excluded. These tables can be downloaded free of charge at the publisher’s website: [PMID 24223952](#)

The specific contributions of each author to this paper are listed below along with the author contribution section published in the paper.

Butcher J: Designed, performed and analyzed data from all experiments. Wrote manuscript, constructed all the figures, and responded to reviewers comments.

Stintzi A: Designed RNA-seq experiments, contributed to writing/editing the entire manuscript.

PLoS ONE Author contribution statement:

Conceived and designed the experiments: JB AS. Performed the experiments: JB. Analyzed the data: JB AS. Contributed reagents/materials/analysis tools: AS. Wrote the manuscript: JB AS.

Abstract

The genome-wide *Campylobacter jejuni* transcriptional response under iron replete and iron limited conditions was characterized using RNA-seq. We have identified 111 novel *C. jejuni* 5'UTRs and mapped 377 co-transcribed genes into 230 transcriptional operons. In contrast to previous microarray results, the *C. jejuni* iron stimulon is less extensive than previously believed and consists of 77 iron activated genes and 50 iron repressed genes. As anticipated, the iron repressed genes are primarily those involved in iron acquisition or oxidative stress defense. Interestingly, these experiments have revealed that iron is an important modulator of flagellar biogenesis with almost all the components of the flagella found to be iron activated. Given that motility is a well-known *C. jejuni* colonization factor, this suggests that there is an important regulatory coupling of flagellar biogenesis and iron level in *C. jejuni*. In addition we have identified several consensus mutations in the *C. jejuni* NCTC11168 strain that are widespread in the *Campylobacter* research community and which may explain conflicting phenotypic reports for this strain. Comparative analysis of iron responsive genes with the known Fur regulon indicates that many iron responsive genes are not Fur responsive; suggesting that additional iron regulatory factors remain to be characterized in *C. jejuni*. Further analysis of the RNA-seq data identified multiple novel transcripts including 19 potential ncRNAs. The expression of selected ncRNAs was confirmed and quantified with qRT-PCR. The qRT-PCR results indicate that several of these novel transcripts are either Fur and/or iron responsive. The fact that several of these ncRNAs are iron responsive or Fur regulated suggests that they may perform regulatory roles in iron homeostasis.

Introduction

Iron is crucial for microbial growth as it forms the active center of a variety of diverse enzymes involved in essential cellular processes such as energy metabolism and redox reactions (Andrews *et al.*, 2003). While iron is one of the most abundant metals on earth, it is mostly present in the ferric form which is insoluble under neutral pH and aerobic conditions. Microorganisms have thus evolved elaborate strategies to acquire iron from their environment. Pathogens such as *Campylobacter jejuni* face additional challenges in acquiring sufficient iron as their hosts typically restrict free iron to prevent unwanted microbial growth (Butcher *et al.*, 2010). Pathogens subvert this defense system by expressing multiple iron acquisition pathways under conditions of iron limitation. On the other hand, acquiring excess amounts of iron poses its own metabolic risks. Excess iron levels are toxic to most microorganisms by triggering oxidative stress through Fenton chemistry (Andrews *et al.*, 2003; Butcher *et al.*, 2010). Thus microorganisms must carefully regulate iron metabolism to ensure that they acquire sufficient iron to grow yet avoid iron toxicity (Andrews *et al.*, 2003; Butcher *et al.*, 2010).

The classical iron regulatory protein in most Gram-negative bacteria is the ferric uptake regulator (Fur) protein (Andrews *et al.*, 2003; Butcher *et al.*, 2010). Fur senses intracellular iron and represses genes involved in iron acquisition when cellular iron levels rise. In addition, many microorganisms also contain small regulatory RNAs that are involved in regulating iron metabolism. The most studied of these sRNAs is *E. coli* RyhB (Masse *et al.*, 2007). This small regulatory RNA is repressed by Fur under iron-replete conditions. However under iron limited conditions, Fur repression is relieved and RyhB can be transcribed, and together with the Hfq RNA binding protein, target its downstream effectors

(Masse *et al.*, 2007). RyhB targets the mRNA transcripts of several non-essential, iron-binding proteins and promotes their degradation. This iron sparing response ensures that iron is preferentially used for essential cellular reactions (Masse *et al.*, 2007). The combination of these two systems ensures that iron levels are appropriately controlled and that iron is preferentially used for essential pathways. Indeed, pathogens that are missing these regulators, including *C. jejuni*, *H. pylori*, *V. cholerae* and *S. flexneri*, are defective in controlling iron metabolism and show decreased virulence and colonization *in vivo* (Fantappie *et al.*, 2009; Mey *et al.*, 2005; Miles *et al.*, 2010b; Oglesby *et al.*, 2005; Palyada *et al.*, 2004; Simonsen *et al.*, 2011).

Previous work has characterized the transcriptomic response of *C. jejuni* to iron limitation using genome wide microarray analysis (Holmes *et al.*, 2005; Palyada *et al.*, 2004). These microarray studies found extensive remodelling of the *C. jejuni* transcriptome under iron limitation and greatly contributed to our current understanding of *C. jejuni* iron homeostasis. However while microarray studies are instructive, they are being increasingly eclipsed by the utility of next generation high throughput sequencing (HTS) technologies (van Vliet, 2010). Several studies have indicated that HTS technologies can determine gene expression level more accurately than microarrays (Mooney *et al.*, 2013; Sirbu *et al.*, 2012), and also offer exciting insight into the transcript structures present under each condition studied (Mutz *et al.*, 2013; van Vliet, 2010; Wang *et al.*, 2009). The RpoN regulon of *C. jejuni* has been characterized by profiling the transcriptome of an *rpoN* mutant using HTS and compared to results previously obtained using microarray technologies (Chaudhuri *et al.*, 2011). Bioinformatic analysis of the transcriptomic data revealed numerous potential novel transcripts and detected possible transcriptional activity originating from these genomic

regions. More recently, the transcriptomes of several *C. jejuni* strains have been profiled using a differential RNA-sequencing (dRNA-seq) technology that allows for the enrichment of unprocessed RNA species (Dugar *et al.*, 2013). This approach has greatly improved our understanding of transcriptional start sites (TSS) in *C. jejuni* and also provided experimental evidence for several potential ncRNAs.

We report herein the first HTS characterization of *C. jejuni*'s transcriptomic response to iron limitation. Moreover, we have used both mRNA and sRNA specific HTS libraries to fully capture the fully transcriptomic potential of *C. jejuni*.

Methods

Bacterial Strains and growth

C. jejuni NCTC11168 and the Δfur isogenic mutant were routinely cultured on Mueller-Hinton (MH) agar plates under microaerophilic conditions at 37°C in a MACS-VA500 workstation (Don Whitley, West Yorkshire, England). To note, our *C. jejuni* NCTC11168 strain is motile and capable of colonizing chicks in contrast to the genome sequenced strain (Gaynor *et al.*, 2004; Palyada *et al.*, 2004). Colonies were picked and grown overnight in MH agar overlaid with MH broth (biphasic cultures). Overnight cultures were washed in minimal essential media (MEM α) (Invitrogen) and used to inoculate 50 mL MEM α supplemented with 10 mM pyruvate. Freshly prepared FeSO₄ was added at a final concentration of 40 μ M as needed to generate iron replete growth conditions.

Total RNA Isolation

C. jejuni was grown either in iron replete (+40 μ M FeSO₄) or iron limited (-40 μ M FeSO₄) MEM α . *C. jejuni* was inoculated at an initial OD₆₀₀ of 0.05 and grown until midlog phase was reached (OD₆₀₀ 0.2) which normally took ~5-6 hours. Total RNA was then isolated as described previously (Palyada *et al.*, 2004). Briefly, 10% cold RNA stop solution was added to the cultures (10% buffer saturated phenol in absolute ethanol) and cells were pelleted by centrifugation at 6000 x g. Cells were resuspended in TE buffer and RNA extracted using a hot-phenol chloroform method. RNA was precipitated overnight and the RNA pellet was washed five times in 80% ethanol. RNA pellets were resuspended in TE buffer and treated with DNase I (Epicentre) to remove contaminating genomic DNA. Treated samples were purified using the RNeasy kit (Qiagen) and PCR was performed to confirm the

absence of genomic DNA. RNA purity and quality was assessed using a Nanodrop and the Experion RNA StdSens analysis kit (Bio-Rad). Samples with high quality 260/280 and 260/230 ratios and an 18S/16S ratio greater than 1.4 were selected for Illumina sequencing library construction.

Illumina library construction and sequencing

Library construction, cluster generation and sequencing were performed at the Beijing Genomics Institute. In addition, ribosomal RNAs were depleted using the RiboMinusTM transcriptome isolation kit from Life Technologies. Each library consisted of a 1:1 mixture of RNA extracted from two biological replicates grown and isolated separately. Library construction and cluster generation was done using standard Illumina protocols with sequencing performed on an Illumina HiSeq 2000 sequencer. The mRNA libraries were subjected to paired-end sequencing with a target read length of 100 nucleotides. Approximately ~6.5 million high quality reads were obtained per library (Table S3.1), with two sequencing reactions for iron replete conditions and three for iron limited conditions. The miRNA libraries were constructed using the standard protocol for Illumina miRNA sequencing libraries. This included size fractioning the Total RNA and selecting for fragments of ~20-30 nt. The miRNA libraries were subjected to single end sequencing with a target read length of 75 nucleotides and resulted in ~20 million reads per library (Table S3.1) with one sequencing reaction each for iron replete/limited conditions. The Illumina reads were submitted to the Sequence Read Archive at NCBI with the accession numbers SRX327750 and SRX327869 for the mRNA and miRNA experiments respectively.

Read alignment to the *C. jejuni* genome

Paired end reads were aligned to the *C. jejuni* NCTC11168 genome (NC002163) using Bowtie v0.12.7 (Langmead *et al.*, 2009) with the best flag enabled and with 4 bp trimming on each read's 3' end to remove error prone bases. Approximately 90% of reads aligned for each sequencing run. Aligned reads were visualized using Artemis with the Bamview plugin (Carver *et al.*, 2012). Reads corresponding to the miRNA libraries were first trimmed to remove barcode and primer sequences and then aligned with Bowtie in single read alignment mode. Two previously published Illumina sequencing datasets were also aligned to the *C. jejuni* NCTC11168 genome for subsequent analysis and comparison with our dataset (Chaudhuri *et al.*, 2011; Jerome *et al.*, 2011).

RNA-seq fold change analysis

SAMMate with the edgeR statistical package was used to determine gene expression fold changes using the current Genbank protein annotation information for *C. jejuni* NCTC11168. (Robinson *et al.*, 2010; Xu *et al.*, 2011). In addition, we used SAMMate to calculate the RPKM values for all the known *C. jejuni* NCTC11168 ORFs. RPKM values normalize the number of reads aligning to a particular gene by taking into account the gene length and total number of reads that align to the genome. RPKM values can thus be compared across sequencing reactions and between genes to determine the relative expression of genes. Predicted pseudogenes and non-protein coding RNA elements were excluded from the fold change analysis to avoid introducing artifacts. Transcripts showing a fold change ≥ 1.5 with a p value ≤ 0.01 were considered to be significantly differentially expressed.

Absolute Transcript Profiling

The absolute transcript level of each gene in the *C. jejuni* genome was estimated using the calculated RPKM values (Table S3.4). Moreover the RPKM values of each gene were summed by on their Clusters of Orthologous Groups (COG) annotations. Genes that contain multiple COG annotations were included in each category and genes with no COG annotation were included in the “S – Function Unknown” category (Table S3.5). The overall transcriptomic contribution of each COG category under iron replete or iron limited conditions was compared using a two-way ANOVA with a Bonferroni post-test. Overall transcriptional differences of ≥ 1.5 fold with a p value < 0.05 were considered significant.

***C. jejuni* NCTC11168 variant analysis**

Aligned reads were used to determine if our NCTC11168 strain (hereafter denoted as WT^{AS}) differed from the reference sequence (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000). Potential variant sites were called from the aligned reads using the mpileup and bcftools functions in Samtools (Li *et al.*, 2009). It should be noted that Bowtie v0.12.7 does not support gapped alignment and thus potential indels were not identified using this approach. Sites varying from the reference sequence were then manually screened taking into account read depth and read qualities at each site. Consensus mutations were called if present in 4/5 sequencing runs with at least 10 aligned reads at the mutant location in each run and a mpileup consensus quality score > 100 . This analysis was also repeated using previously published *C. jejuni* NCTC11168 RNA-seq results (Chaudhuri *et al.*, 2011). The Chaudhuri *et al.* datasets consisted of a *C. jejuni* NCTC11168 strain and its isogenic *rpoN* deletion mutant and are denoted as WT^{AG} and $\Delta rpoN^{AG}$ (Chaudhuri *et al.*, 2011). These results were compared with resequencing studies to identify common substitution mutations in

NCTC11168 strains used in various different laboratories (Table 3.1) (Cooper *et al.*, 2012; Jerome *et al.*, 2011). Cooper *et al.* have recently re-sequenced the original NCTC11168 strain (NCTC11168-O or WT^{Orig}) and compared this strain to the genome sequenced NCTC11168 (NCTC11168-GS or WT^{Seq}) (Cooper *et al.*, 2012). Jerome *et al.* have also resequenced a NCTC11168 isolate prior to mouse adaptation (Jerome *et al.*, 2011). Only the pre-mouse adapted strain was considered (WT^{LMp}) to avoid conflating mouse specific mutants. We also performed this analysis on the *C. jejuni* NCTC dRNA-seq datasets from the Dugar *et al.* study (Dugar *et al.*, 2013), however no consensus mutations from this dataset passed our cut-off values.

5'UTR Identification

Aligned reads for all sequencing runs were visualized using Artemis (Carver *et al.*, 2012). Potential 5'UTRs were identified by manually inspecting reads that extended beyond the *C. jejuni* NCTC11168 predicted start codon (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000). 5'UTR coordinates and lengths were empirically assigned based on the presence of >3 reads with no potential overlap from reads extending from other ORFs or potential ncRNAs. Care was taken not to over infer the length/presence of 5'UTRs given the relative paucity of *C. jejuni* intergenic regions, along with the presence of many operonic transcripts. A similar 5'UTR screening was also done on the Chaudhuri *et al.* dataset (Chaudhuri *et al.*, 2011). This manual annotation was merged with the automated 5'UTR identification reported by Dugar *et al.* (Dugar *et al.*, 2013).

***C. jejuni* genes in operonic structure**

The average insert size of the various paired-end sequencing reaction was found to be ~200 bp (Figure S3.4). Aligned paired-end reads were used to infer the operonic structure of *C. jejuni* transcripts. Notably, this analysis relies on the active transcription of these operons at high enough levels to be detected accurately. As such, this analysis will undoubtedly underestimate the true number of co-transcribed genes and operonic transcripts present in *C. jejuni* and does not rule out the presence of alternative transcriptional start sites. The aligned reads were parsed using Bedtools (Quinlan and Hall, 2010) to identify reads that overlapped two sequential transcripts coded on the same strand by at least 50 bp into each gene. Genes were selected as potentially being in an operonic structure if they contained >20 reads aligning on both genes in at least two different sequencing samples under the same experimental conditions (iron replete/iron limited) (Table S3.8). Genes that were identified as co-transcribed were also compared with previously predicted operons using a bioinformatic approach (Price *et al.*, 2005). Sequential genes that are present in operonic structure were merged together to form potential operonic transcripts (Table S3.9).

Novel transcript Identification

Potential novel transcripts and antisense sRNAs were identified from the miRNA sequencing libraries. This approach would identify transcripts that would not be seen under normal Illumina library conditions due to the need for ~200 bp library fragment sizes. In addition, the use of strand specific sequencing in the miRNA library approach allowed for the identification of the actual transcript produced (as opposed to its complement). Both the positive and negative strand of the *C. jejuni* genome were screened for regions that contained ≥10 reads aligning to either strand and that were at least 10 bp in length using BEDtools

(Quinlan and Hall, 2010). These regions were further refined by selecting sites that displayed a strand bias in their aligned reads (either >2 fold enrichment or no reads aligning to opposite strand). This analysis was done separately for both the iron replete and iron limited miRNA libraries and potential novel transcripts identified in each condition were subsequently merged together. Potential novel transcripts that were separated by <5 bp were considered to be contiguous transcripts and concatenated together. Regions were classified as potential antisense sRNAs if they were present within and transcribed antisense to known ORFs. Regions were classified as potential ncRNAs if they were present within intergenic regions and not within 50 bp of an ORF transcribed on the same strand. Several putative ncRNAs were identified adjacent (~300bp) to *C. jejuni* rRNA sites and were thus annotated as fragments from rRNA processing. In addition, reads emanating from the *C. jejuni* CRISPR region were annotated as CRISPR processed elements. The intergenic regions found to contain potential novel transcripts were also examined in the Chaudhuri *et al.* dataset to confirm their transcriptional presence and also compared to the list of potential novel transcripts previously bioinformatically identified in this study (Chaudhuri *et al.*, 2011). It is important to note that the transcript sizes identified in this analysis may be underestimates of true transcript sizes since the miRNA library construction has a bias towards smaller RNA fragments and our bioinformatic approach is stringent with regards to the number of reads required to identify sRNAs.

Novel transcript RT-PCR and qRT-PCR

Validation of several putative novel transcripts was done using RT-PCR. Briefly, 5 µg of total RNA purified from iron limited conditions was subjected to reverse transcription using Superscript II (Invitrogen) using the manufacturers recommended protocol with the

optional RNaseH treatment. A control reaction without the addition of Superscript II was also performed. PCR was then done using 2 µl of the first strand cDNA mixture and primers specific to each potential transcript. Primer sets known to amplify mRNA transcripts or genomic regions were also included to ensure the absence of contaminating genomic DNA.

The selected ncRNA regions were also tested by qRT-PCR to determine if they were differentially expressed under iron limitation as described previously (Butcher *et al.*, 2012). In addition, these ncRNA transcripts were also tested to see if they were differentially expressed in a Δfur mutant as compared to wildtype under iron replete conditions. Briefly, total RNA was purified from *C. jejuni* cultures grown under iron limited or iron replete conditions as described above. qRT-PCR was performed on an Applied Biosystems 7300 thermocycler using the QuantiTect SYBR Green RT-PCR Kit from Qiagen with the same primers as in the RT-PCR. Fold changes were determined according to the $\Delta\Delta C_T$ method using *slyD* as an endogenous control (Ritz *et al.*, 2009). qRT-PCR was done on three independent RNA extractions with technical triplicates for each ncRNA tested. ncRNAs were considered differentially expressed if their expression was altered by >1.5-fold with $p < 0.05$.

Northern Blotting

Northern blotting was also used to validate the presence of one of our unique ncRNAs (ncRNA) using the DIG-Northern Starter kit from Roche and following the manufacturer's instructions. Briefly, 1 µg of total RNA purified from *C. jejuni* NCTC 11168 grown under iron replete/limited conditions and the Δfur mutant grown under iron replete conditions was loaded onto a 10% TBE-urea gel and ran for at 100V for 5 hours at 4°C. The RNA was then transferred to a nylon membrane overnight at 30mA at 4°C. DIG labelled

probes for ncRNA_Z and *C. jejuni* 5S RNA were transcribed following the manufacturer's instructions and using the indicated primer sets (5S_F acagatgtggaaacgccttg, 5S_R
TAATACGACTCACTATAGGGGttaacaagtccgcaatgagc, ncRNA_Z_F
TAATACGACTCACTATAGGGGAGGGGTTTTACCCCAAAG, ncRNA_Z_R
TGGGCAAGTAGGACAAAAGG). Probe hybridization was done using the manufacturer's recommended temperatures and incubation times.

Results

Identification of differentially expressed genes under conditions of iron limitation

In order to identify iron regulated genes, the number of reads aligning to each protein coding gene and the corresponding RPKM value was determined using SAMMate (Xu *et al.*, 2011). The different sequencing runs were found to be highly reproducible, with R^2 values ranging from 0.88-0.95 (Figure S3.1). The presence of significantly differentially expressed genes was determined using the edgeR statistical package (Robinson *et al.*, 2010). This analysis determined that 127 genes were differentially expressed under iron replete/limited conditions with 77 iron activated genes and 50 iron repressed genes (Figure 3.1A, Figure 3.2 and Tables S3.2, S3.3).

Iron repressed genes include almost all the known iron acquisition pathways in *C. jejuni* including the enterobactin (*cfrA*, *ceuBDE*) (Palyada *et al.*, 2004), hemin (*chuABCD*, *chuZ*) (Ridley *et al.*, 2006), lactoferrin/transferrin (*chaN/ctuA*, *cfbpABC*) (Miller *et al.*, 2008) and rhodotorulic acid (*cj1658/p19*, *cj1660-1663*) transporters (Butcher *et al.*, 2010). Genes with well-known roles in defense against oxidative stress were also iron repressed and include *kata*, *cj1386*, *ahpC*, *trxB* and *tpx*. In addition, most of the members of *C. jejuni*'s three TonB energy transduction systems (*exbB1/B2*, *exbD1/2*, *tonB1/B2/B3*) were iron repressed. Both the *cj1383c* and *cj1384c* genes located upstream from *kata* were highly differentially expressed under iron limitation.

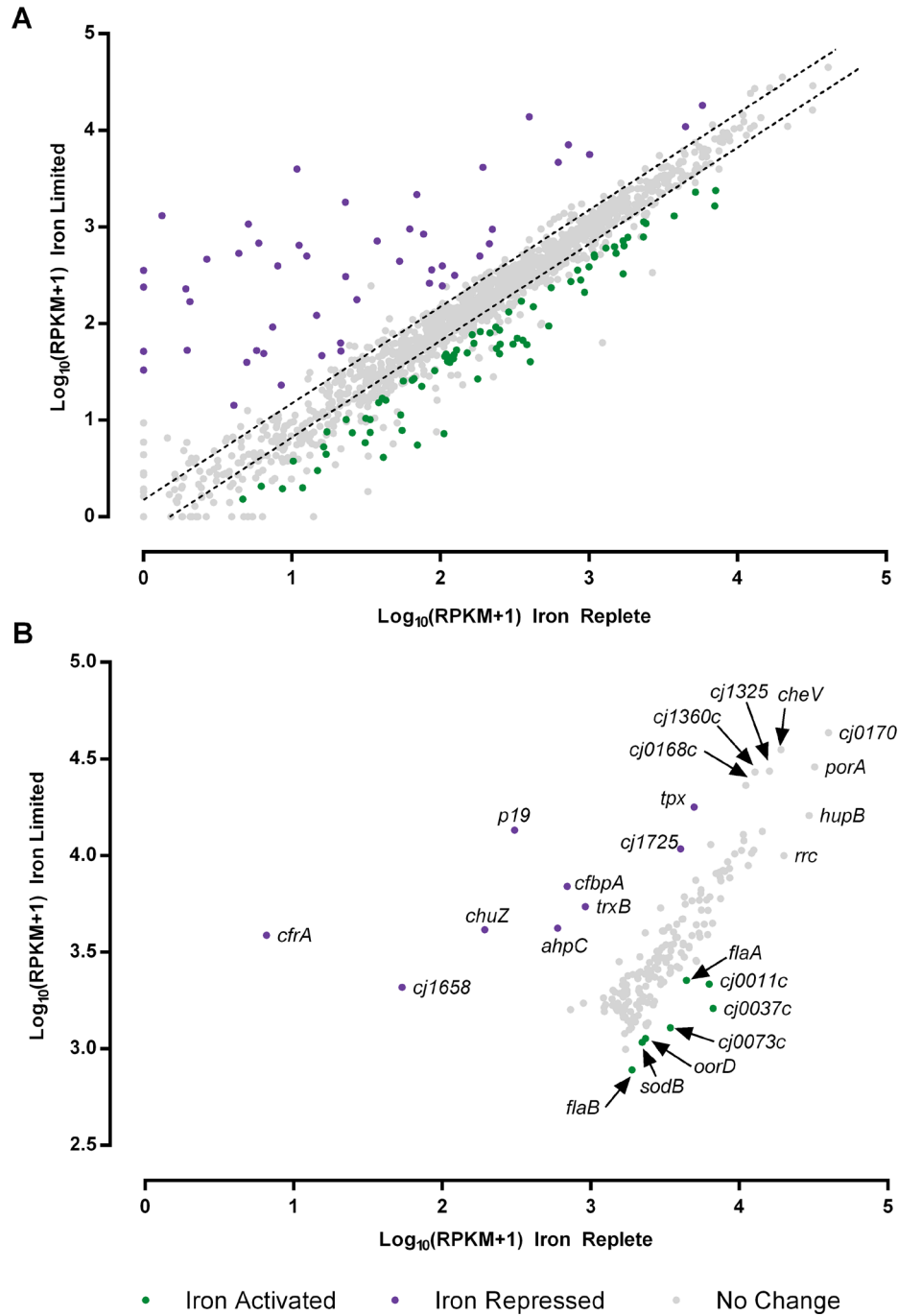


Figure 3.1: Differentially expressed genes under iron limitation

The average $\text{Log}(\text{RPKM}+1)$ value for each gene was calculated under iron limited and iron replete conditions and plotted above. Significantly differentially expressed genes ($p < 0.01$) are highlighted in green (iron activated) or purple (iron repressed) (Panel A). The hashed bars represent 1.5 fold differences in gene expression under each condition. The 90th percentile of the most highly expressed genes were plotted separately to compare the most highly expressed genes under iron limited and iron replete conditions (Panel B)

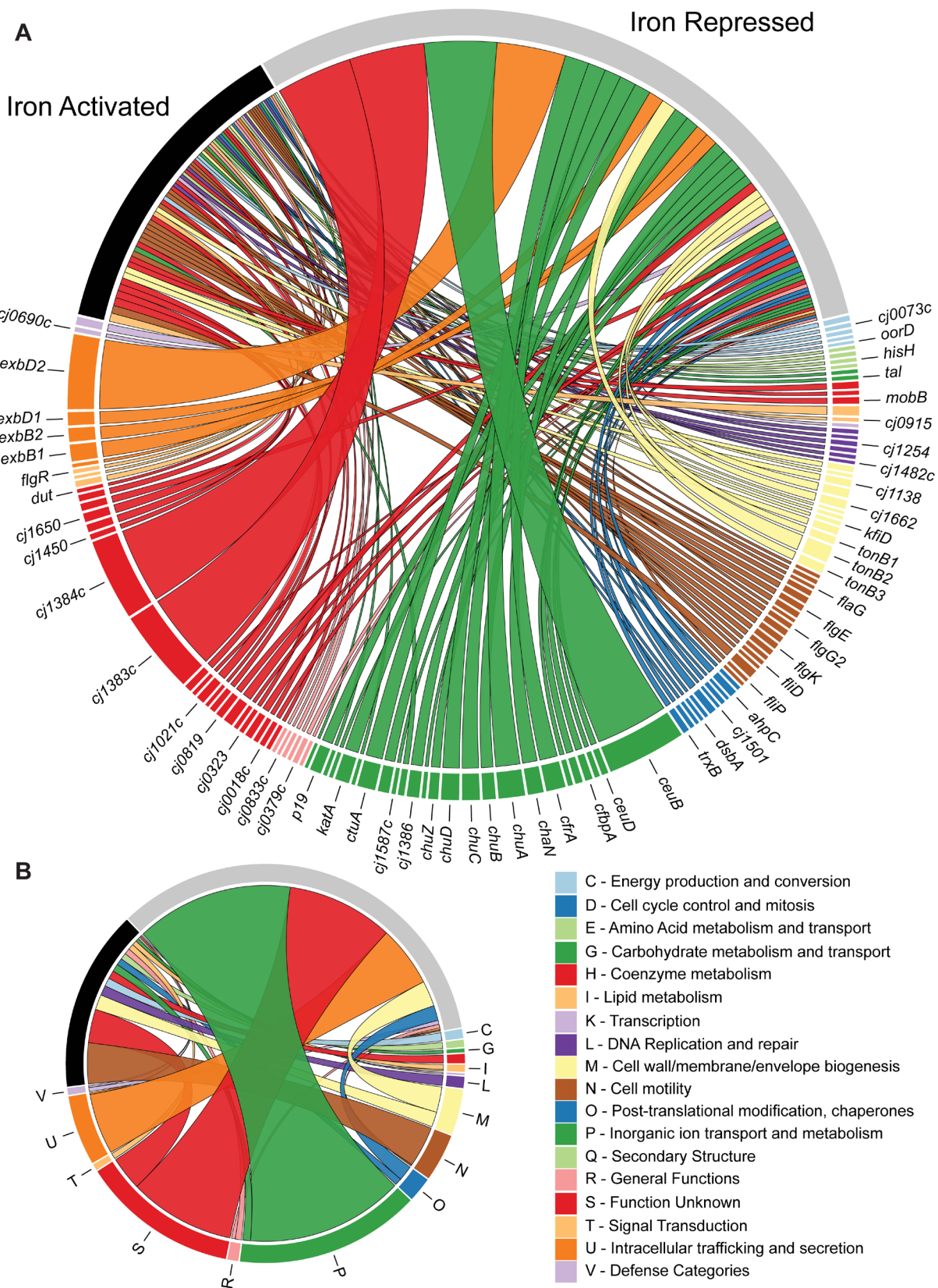


Figure 3.2: Iron activated and repressed genes by functional category

Individual genes that were found to be differentially expressed between iron limiting and iron replete conditions were sorted by COG category and plotted based on their degree of differential expression (Panel A). Genes that were differentially expressed were grouped by COG category and the overall COG category was plotted based on its degree of differential expression (Panel B). The size of the ribbon reflects the degree of activation/repression seen for each gene/COG category. Figure made using Circos v0.54 (Krzywinski *et al.*, 2009)

Many of the iron activated genes are involved in flagellar biogenesis. These include almost all the components of the filament (*flaA*, *flaB*, *flaG*, and *fliD*), hook (*flgE* and *flgE2*) and rod (*flgG*, *flgG2*, *flgH*, *flgI*, and *fliE*) flagellar structures. In addition, several flagellar chaperones (*flgD* and *fliK*) and regulatory proteins (*fliS*, *flgM*, and *flgR*) are also iron activated (Table S3.2). Finally, the transcript levels of the regulatory proteins Fur and CmeR are also increased under iron replete conditions, along with the expression of numerous genes involved in energy metabolism (such as *ccoQ*, *nuoM* and *cj0073c*).

The results from this analysis were compared to those previously obtained using microarray approaches under similar conditions (Palyada *et al.*, 2004). While most of the RNA-seq iron repressed genes were identified under both experimental methods (37/50), a much smaller number of the RNA-seq identified iron activated genes were also found to be significantly differentially expressed by microarray (24/77) (Figure S3.2, Table S3.3). These novel iron activated genes include *fliS*, *flgB*, *flgD*, and *flgG*. Some genes were found to be iron activated in the RNA-seq but iron repressed in the microarray such as *sodB*, *modA*, *mobB* and *dsbA* (Table S3.3). In addition, most of the iron responsive genes have not been identified as being transcriptionally regulated (either directly or indirectly) by the Fur iron regulatory protein in *C. jejuni* (Figure S3.3) (Butcher *et al.*, 2012; Holmes *et al.*, 2005; Palyada *et al.*, 2004).

Profiling absolute transcript levels under iron limitation

To estimate the absolute transcript abundance, the RPKM value for each gene was calculated and used to determine the percent contribution of each gene to the total transcriptional profile of the cell (Table S3.4) and to compute the 90th percentile of expressed genes in either condition (Figure 3.1B). The most abundant transcripts in either iron state are

very similar with *Cj0170*, *porA*, *cheV*, *cj1325*, *cj1360c*, *cj0168c* and *hupB* representing the most highly expressed genes in either condition (Figure 3.1B). As expected, the iron limited state is also characterized by relatively high expression of many iron acquisition genes including *p19*, *cfbpA*, *chuZ* and *cfrA*, while the iron replete state is characterized by the higher expression of flagellar (*flaA*, *flaB*) and energy metabolism (*cj0073c*, *oorD*) genes. The contribution of each COG category to the overall transcriptome was determined by grouping genes by COG function and summing their relative contributions (Table S3.5). As shown Figure 3.3 the overall COG transcriptional profile is quite similar between iron replete and limited conditions, with the exception of the COG categories for inorganic ion transport and metabolism, and DNA replication and repair, which were enriched in iron limited and iron replete conditions respectively ($p < 0.05$)

Analysis of *C. jejuni* NCTC11168 point mutations

Previous studies have highlighted the clonal complexity of *C. jejuni* and shown the contribution of point mutations to the physiology of *C. jejuni* NCTC11168. In order to define the isolate used in this study, we analyzed the RNA-seq data for the presence of point mutations present in our NCTC11168 strain as compared to the published genome-sequenced isolate. Importantly, the use of RNA-seq data to identify mutations is limited to the identification of nucleotide substitutions within protein coding regions in genes expressed at high enough levels to confidently call variants. Moreover, mutations that affect transcript abundance (transcription level or stability) may not be identified using this approach.

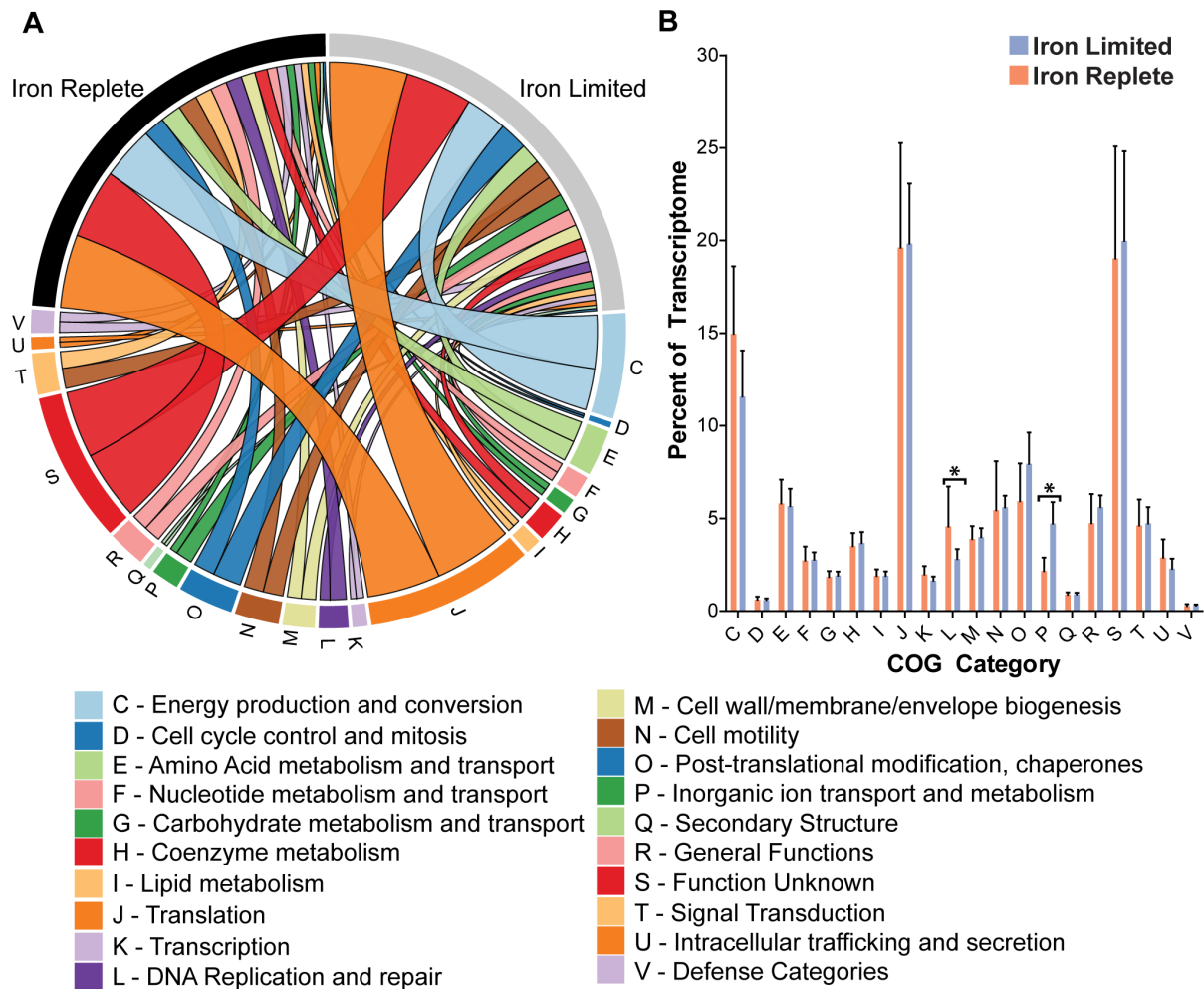


Figure 3.3: The overall transcriptome of *C. jejuni* is relatively unchanged under iron limitation

The absolute abundance of each gene in the *C. jejuni* genome was calculated and used to determine its contribution to the total transcriptome. These contributions were summed and grouped by COG category to determine the overall contribution of each COG category to the *C. jejuni* transcriptome under each condition (Panel A). The only statistically significant differences are enrichment for the COG category for inorganic ion transport and metabolism under iron limited conditions and enrichment for DNA replication and repair under iron replete conditions (Panel B) (* $p < 0.05$). Figure made using Circos v0.54.

Consensus mutations were also called in a conservative manner (see Methods) given the inherent increased error rate expected with this approach considering that the original genomic sequence was transcribed to mRNA, reverse transcribed to cDNA and subsequently PCR amplified. Nonetheless we were able to identify several consensus mutations present in our strain (WT^{AS}) that differed from the reference NCTC11168 genome. These mutations were located in *mreB*, *cheA*, *cheV* and *cj0807* and all resulted in substitution mutations in their respective proteins. We also carried out a similar analysis of a previously published RNA-seq dataset (WT^{AG} and $\Delta rpoN^{AG}$) (Chaudhuri *et al.*, 2011). These mutations were also compared with consensus mutations formerly found in NCTC11168 strains subjected to genome resequencing (WT^{Orig}, WT^{LMp}) (Cooper *et al.*, 2012; Jerome *et al.*, 2011). Interestingly, analysis of WT^{Orig}, WT^{LMp}, WT^{AG} and $\Delta rpoN^{AG}$ strains identified many of the same mutations in *mreB*, *cheA*, and *cj0807* that are present in the WT^{AS} strain, although the WT^{AS} strain appears to contain a unique mutation at base 26831 in *cheV* (Table 3.1).

Several additional mutations originally identified in the WT^{Orig} and WT^{LMp} strains also appear to be present in the WT^{AS} strain but did not pass the stringent cutoffs we employed to avoid calling false positives. Our analysis also identified two additional unique mutations at bases 253436 and 1536694 in the WT^{AG} and $\Delta rpoN^{AG}$ strains as compared to the other NCTC11168 strains (Table 3.1).

Genome wide identification of 5'UTRs

Given the potential role for 5'UTR in gene regulation, the presence and length of *C. jejuni* 5'UTRs were determined by inspecting the reads that aligned to the genome using the Artemis interface. This analysis was performed by combining the datasets generated in this study with the previously published *C. jejuni* RNA-seq dataset (Chaudhuri *et al.*, 2011).

Table 3.1: Common substitution mutations present in *C. jejuni* NCTC 11168 isolates from different laboratories

Location¹	WT^{Seq}	Mutation	Strain(s)	Gene	Consequence
253191	A	G	All*	<i>mreB</i>	Ile to Val
253436	G	A	WT ^{AG}	<i>mreB</i>	Ala to Thr
262345	A	G	All*	<i>cheA</i>	Ile to Thr
263831	G	A	WT ^{AS}	<i>cheV</i>	Arg to Ser
393542	T	A	WT ^{Orig} , WT ^{LMp}	<i>cj0431</i>	Stop to Lys
420550	A	G	WT ^{Orig} , WT ^{LMp}	<i>cj0455c</i>	Stop to Glu
760188	A	G	All*	<i>cj0807</i>	Lys to Glu
1189659	A	G	WT ^{LMp}	<i>porA</i>	Glu to Gly
1536694	A	G	WT ^{AG}	<i>cj1608</i>	Glu to Gly

¹ All locations are based on the WT^{Seq} sequence

* WT^{AS}, WT^{Orig}, WT^{LMp}, WT^{AG} and $\Delta rpoN^{AG}$

The results were then compared to the primary transcriptional start sites (TSS) identified by a recent dRNA-seq study (Dugar *et al.*, 2013). Approximately ~400 putative 5'UTRs were identified from the two RNA-seq datasets (Table S3.6) as compared to the ~625 identified by dRNA-seq. The median 5'UTR length was 31 bases for both datasets, despite individual differences in the computed 5'UTR lengths of specific genes. Nearly 6.9% of the TSS identified in this study could be considered leaderless transcripts with a 5' UTR shorter than 10 nucleotides as compared to 3.5% identified in the Dugar *et al.* study (Dugar *et al.*, 2013).

Genome wide identification of *C. jejuni* genes in operonic structure

In addition to identify gene boundaries, we have drawn on the paired-end sequencing information to identify the *C. jejuni* global operon structure. In total, 377 genes were determined to be in operonic structure using this analysis constituting 230 operons (Table S3.9). Some of these transcripts have been previously described, such as the *mfrABC* (formally *sdhABC*) (Guccione *et al.*, 2010), *cj1658-p19* (Palyada *et al.*, 2004) and *cj0399-fur-lysS-glyA-cj0403* (van Vliet *et al.*, 2000) operonic transcripts. However, comparison of the identified transcripts with a previously published bioinformatic approach revealed the presence of numerous unpredicted operonic structures such as the co-transcription of *cj1420c*, *cj1421c*, *cj1422c* and *hddC*, *sodB* to *cj0170* and *cj0864* to *dsbB* (Price *et al.*, 2005).

Identification and validation of novel transcripts

The presence of novel transcripts and putative sRNAs was determined using the Illumina miRNA sequencing protocol to identify intergenic regions that displayed transcriptional activity. Analysis of the miRNA reads revealed the presence of widespread antisense RNA transcription throughout the *C. jejuni* genome with approximately 190 genes

containing an antisense transcript (Table S3.7). The strand specificity and sensitivity of this approach was confirmed by analyzing the *C. jejuni* CRISPR region. Our analysis identified transcriptional activity from all five CRISPR elements present in the *C. jejuni* NCTC11168 genome in a strand specific manner (Figure 3.4). Moreover in most cases we could identify the individual processed CRISPR elements.

Analysis of the miRNA reads also revealed the presence of 19 novel intergenic transcripts with several of these transcripts also detectable (albeit at very low levels) in the mRNA transcriptional analysis (Table S3.7). Selected novel transcripts were validated using RT-PCR confirming that these regions are transcriptionally active (Figure 3.5A). Several of these transcripts were also identified and validated using Northern blotting in a previous study (Dugar *et al.*, 2013). Similarly, we used Northern blotting to confirm the expression of one of our unique ncRNAs (Figure S3.5A). In addition, the expression of these confirmed transcripts was quantified with qRT-PCR in both the wild-type and Δfur mutant strains under iron replete and iron depleted conditions. The qRT-PCR results indicate that several of these novel transcripts are either Fur and/or iron responsive (Figure 3.5B). Of the five transcripts tested, three were found to be differentially expressed under iron limitation with ncRNA_P and ncRNA_Z showing iron repression and ncRNA_AA showing iron activation (Figure 3.5B). In addition ncRNA_P was found to be Fur repressed while ncRNA_GG was Fur activated (Figure 3.5C). The selected ncRNAs were found to be differentially expressed across varying concentrations of template RNA further validating our results (Figure S3.5B, 3.S5C, 3.S5D).

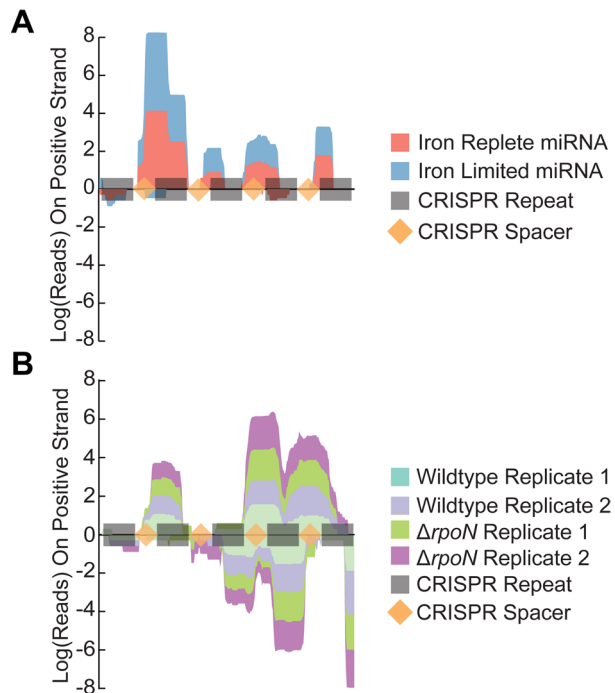


Figure 3.4: The CRISPR region is defined and strand specific in the miRNA sequencing library

Representation of the total number of reads mapping to the CRISPR region in either miRNA (Panel A) or standard (Panel B) Illumina sequencing libraries. Negative values refer to reads aligning on the negative strand. CRISPR repeat elements are highlighted in grey while the CRISPR spacers are in light orange.

Discussion

Although iron homeostasis is now well established in *Campylobacter jejuni*, much remains to be understood about the regulatory mechanisms of the iron regulated genes. In this study, we combined RNA-seq and miRNA-seq approaches to catalogue all expressed transcripts from *Campylobacter jejuni* regardless of their structure or function under both iron replete and iron limited conditions. A total of 409 transcripts were characterized including 19 ncRNA. As compared to the previous transcriptomic studies in *C. jejuni*, our work has revealed 12 new ncRNAs and characterized the structure of 111 additional mRNA transcripts. These differences almost certainly arise from the differential expression of transcripts under different growth conditions emphasizing the regulation of sRNA expression in *C. jejuni*. Thus, our results further underscore the importance of cataloging bacterial transcripts under various conditions for a better understanding of the transcriptome of *C. jejuni* and its physiology.

RNA sequencing of *C. jejuni* under iron limited conditions has underlined the importance of iron in modulating *C. jejuni*'s transcriptome. Iron limitation results in dramatic up regulation of almost all of *C. jejuni*'s iron acquisition systems including those for enterobactin, hemin and lactoferrin/transferrin (Miller *et al.*, 2008; Palyada *et al.*, 2004; Ridley *et al.*, 2006). Moreover, the vast majority of these genes are completely silenced under iron replete condition reflecting the tight regulation of these genes. This pattern of gene expression likely prevents iron overload and enables the expression of the right genes under the appropriate growth condition.

Interestingly, the most obvious feature of the genes that are iron activated is the preponderance of flagellar biogenesis genes. With the exception of the type III secretion

system, almost all components of the *C. jejuni* flagella were found to be iron activated. This suggests that *C. jejuni* only up-regulates non-essential, but energetically costly processes such as flagellar biogenesis under periods of iron sufficiency. It is unclear exactly how *C. jejuni* couples flagellar gene expression with iron levels; however, previous studies have shown that several of these genes (*flaA*, *flaB*, *fliD*) appear to be directly regulated by the ferric uptake regulator Fur protein (Butcher *et al.*, 2012). Moreover, several flagellar regulatory proteins (*fliS*, *flgR*, *flgM*) were also found to be iron activated. The activation of subsequent downstream effectors of *flgR* could thus be an indirect effect of high levels of iron in the cell. Given that motility is a well-known *C. jejuni* colonization factor, this suggests that there is an important regulatory coupling of flagellar biogenesis and iron levels in *C. jejuni*.

This study also provides new insight into *C. jejuni fur* regulation. Unlike other bacterial species, *C. jejuni fur* does not have its own promoter and also does not appear to autoregulate its own expression (van Vliet *et al.*, 2000). *C. jejuni fur* has been reported to be transcribed from two separate promoters that result in different polycistronic transcripts (with and without *gatC*) (van Vliet *et al.*, 2000). We were unable to detect the presence of a *gatC* containing *fur* polycistronic transcript by RNA-seq analysis and propose that the smaller transcript is the predominant form under our experimental conditions. Previous transcriptomic studies have reported that *C. jejuni fur* expression is not affected by iron levels (Holmes *et al.*, 2005; Palyada *et al.*, 2004), however in contrast to previous transcriptomic studies, our RNA-seq analysis revealed *fur* expression was found to be iron activated within *C. jejuni*. Intriguingly, while we identified *fur* to be iron responsive, none of the other members of its operon were found to be differentially expressed. This could be due

to the fact that some of these genes are also transcribed as monocistronic transcripts in addition to their polycistronic forms (Chan and Bingham, 1991).

While the set of iron-regulated genes we identified largely overlap with the set of genes previously identified by microarray analysis, the total number of differentially expressed genes is significantly different. Previous microarray results under similar growth conditions identified approximately 262 genes as being differentially expressed (≥ 2 fold change, $p < 10^{-4}$) (Palyada *et al.*, 2004), while RNA-seq only identified 127 genes as being differentially expressed (Figure S3.2). Moreover, there was a marked bias in the number of genes that were identified as being either iron activated or iron repressed by either technique. Most of the iron repressed genes were identified using both techniques. However, many of the genes identified as being iron activated in the microarray were not differentially expressed in the RNA-seq. In addition, certain genes showed iron activation in the microarray while the RNA-seq found iron repression (Figure S3.2, Table S3.3). Some of these differences may be due to the slightly different growth conditions used in these two studies. These include the addition of 10 mM pyruvate to the media and the presence of 5% hydrogen in the atmospheric conditions for the RNA-seq study (Palyada *et al.*, 2004). Previous reports have found that the addition of hydrogen changes the transcriptome of *C. jejuni* as compared to controls (John *et al.*, 2011). However, it is unclear why the addition of either pyruvate or hydrogen would preferentially alter gene expression in iron replete conditions as compared to iron limited conditions. One possibility is that pyruvate would act as a radical scavenger and reduce overall oxidative stress levels (Verhoeff-Bakkenes *et al.*, 2008). This could explain the differential expression seen for *sodB*, which was identified as being iron activated in the microarray analysis but iron repressed in the RNA-seq. Pyruvate

and hydrogen could also alter the expression of metabolic genes (John *et al.*, 2011); indeed the most highly iron activated genes in the microarray are metabolic genes that were not found to be differentially expressed in the RNA-seq analysis. Another explanation for the discrepancy between the microarray results and the RNA-seq is that the additional replicates present in the microarray study provide more statistical power to identify differentially expressed genes.

Notably, a recently published RNA-seq analysis in *C. jejuni* has found similar results with regard to the concordance between microarrays and RNA-seq (Chaudhuri *et al.*, 2011). This study re-analyzed the transcriptional profile of wild-type NCTC11168 as compared to its isogenic *rpoN* deletion mutant. This study also had to contend with transcriptional differences that could be due to slightly different growth conditions. Nonetheless, their analysis found that the previous microarray results (Kamal *et al.*, 2007) identified significantly more differentially expressed genes as compared to RNA-seq analysis. Intriguingly there was also a bias in the genes that were identified as being differentially expressed in the microarray but not in the RNA-seq. Most of the microarray identified *rpoN* activated genes were not identified in the RNA-seq data, while almost all of the *rpoN* repressed genes were present (Chaudhuri *et al.*, 2011). It remains to be seen whether this is a general phenomenon or specific to the conditions studied in these analyses.

RNA-seq data can also be analyzed with regards to absolute transcript abundance as well as calculating fold changes. RPKM values were used to calculate the most abundant transcripts present under both iron replete and iron limiting conditions and also to determine the relative distribution of transcripts based on COG annotation (Figure 3.3, Tables S3.4, S3.5). The most abundant transcripts in either iron state are very similar with *cj0170*, *porA*,

hupB, *rrc* and *cheV* being amongst the most highly transcribed genes in the *Campylobacter* genome. It is somewhat humbling to note that there is almost nothing known about *cj0170*, despite its place as the most highly expressed gene in the *C. jejuni* genome under both conditions. The high expression of *porA* is unsurprising given its abundance on *C. jejuni*'s surface. *HupB* encodes for DNA binding proteins and its high expression is probably related to this role (Konkel *et al.*, 1994). *Rrc* encodes for an antioxidant protein and its high transcription probably points to its importance in oxidative stress defense (Yamasaki *et al.*, 2004). Serine metabolism is critical for *C. jejuni* colonization (Velayudhan and Kelly, 2002) and *cheV*'s important role in chemotaxis towards serine is highlighted by its high expression level in both conditions (Hartley-Tassell *et al.*, 2010). The overall contribution of different COG categories to *C. jejuni*'s transcriptional profile remains relatively unchanged between iron replete and iron limited states (Figure 3.3). The sole exceptions to this are the category for inorganic ion transport and the category for DNA replication and repair. The overabundance of genes for inorganic ion transport in iron limited conditions is undoubtedly linked to the overexpression of iron acquisition genes. In particular, the periplasmic iron binding protein P19 becomes one of the most highly expressed transcripts under iron limitation. The overabundance of transcripts for DNA replication and repair could reflect the increased growth capacity of *C. jejuni* under iron replete conditions or decreased capacity under iron limited conditions. Interestingly, other categories that contain a large number of differentially expressed genes (i.e. motility genes) do not show an overall change in their transcriptome contribution. This outcome is primarily due to non-statistically significant transcript level changes in other members of the same category. In particular, some of the most highly transcribed genes are related to motility (such as *cheV*) and show non-statistically significant transcriptional decreases under iron rich conditions. These decreases

in transcription by genes such as *cheV* cancel out the increases seen in many over expressed flagellar genes and result in no overall change in the COG category for motility.

C. jejuni has long been known to rapidly mutate due to its lack of several DNA repair systems (Gaasbeek *et al.*, 2009; Han *et al.*, 2008). This raises the possibility that different labs working on the same strain could in fact be using variants that contain mutations in key genes. The presence of these variants could result in laboratories observing different phenotypes when working with the same strain. This has already been seen for the NCTC11168 strain with some laboratories reporting motility and the ability to colonize chicks and others reporting conflicting results (Ahmed *et al.*, 2002; Gaynor *et al.*, 2004; Jones *et al.*, 2004; Palyada *et al.*, 2004). We therefore conducted an analysis of potential point mutations present in both our laboratory NCTC11168 strain and the strain used for the *rpoN* RNA-seq profiling (Chaudhuri *et al.*, 2011). For simplicity we will refer to our NCTC11168 strain as WT^{AS} and the strains used in the *rpoN* RNA-seq study as WT^{AG} and $\Delta rpoN^{AG}$. We also compared our results to NCTC11168 strains that had undergone resequencing studies (WT^{Orig}, WT^{LMp}). Our analysis found that all the RNA-seq sequenced strains (WT^{AS}, WT^{AG}, $\Delta rpoN^{AG}$) contained the same point mutations as compared to the WT^{Orig} and WT^{LMp} strains (Table 3.1). Given the presence of these mutations in multiple independently sourced NCTC11168 strains, this suggests that these mutations are widespread within the *C. jejuni* research community. Further studies would be required to determine whether these mutations could result in functional differences between strains. This is especially true in the case of the *cheA* mutation given *cheA*'s known role in *C. jejuni* colonization (Chang and Miller, 2006). Also, both the WT^{AS} and WT^{AG}/ $\Delta rpoN^{AG}$ strains contain additional unique mutations as compared to the reference sequence. It should be

noted that there may be additional mutations present within non-transcribed regions (i.e. promoter regions) in these strains which were not identifiable by RNA-seq.

We also used our RNA-seq data along with the *rpoN* RNA-seq dataset to systematically identify 5'UTRs in *C. jejuni*. Potential 5'UTRs were manually annotated independently in each RNA-seq dataset and merged to provide a global map of *C. jejuni* 5'UTRs. When merging the datasets the longest 5'UTR identified was taken as the true 5'UTR. The merged dataset contains 420 identified 5'UTRs with a median length of 31 bases (Table S3.6). A recent study has documented *C. jejuni* NCTC 5'UTRs using dRNA-seq and as expected, identified additional and longer 5'UTRs due to dRNA-seq's ability to enrich for unprocessed RNAs (Dugar *et al.*, 2013). However, given that our dataset includes sequencing data from iron limited conditions, we were able to identify the 5'UTRs for many additional genes that are iron repressed and thus not expressed under the dRNA-seq study's growth conditions. These include genes such as *cfrA*, *ctuA*, *chuA* and *tonB3*.

The reads generated in our paired end RNA-seq study were relatively long in size with the average insert size for our libraries at approximately 200 bp in length (Figure S3.4). This allowed us to generate an operon map for the majority of *C. jejuni* genes. Adjacent genes that were identified as being in operonic structure were catalogued and compared to a previous genome wide bioinformatic approach for predicting operon structures (Price *et al.*, 2005). Approximately 380 genes were identified as being co-transcribed. These operonic partners could be further stitched together to postulate the presence of 230 operonic transcripts. Overall there was high concordance between these two approaches with ~90% of the co-transcribed genes identified in this study correctly predicted using the bioinformatic approach. Finally, our data provides experimental evidence for multiple suspected operons

including *sodB-cj0170*, *rrc-cj0011c* and *cj0864-dsbB*. Recent work using β -galactosidase assays has suggested that *cj0864-dsbB* share a common promoter but a direct test for operonic structure was not conducted (Grabowska *et al.*, 2011).

We also used an alternate Illumina sequencing protocol to further depict the complete pool of novel transcripts present within *C. jejuni*. This approach allowed for the strand specific sequencing of small RNA fragments that would otherwise not be detected using conventional approaches. This can be demonstrated by comparing the read profiles over the CRISPR region in *C. jejuni*. CRISPR elements (crRNAs) are transcribed as one long unit that is then processed into the individual RNA fragments (Marraffini and Sontheimer, 2010). Standard Illumina library preparations can capture this preprocessed RNA fragment as seen in the Chaudhuri *et al.* results (our sequencing results showed minimal reads in this region) (Figure 3.4). In contrast, our miRNA sequencing protocol results in clear resolution of each of the individual processed CRISPR elements with minimal overlap. Our results indicate that the crRNAs each appear to be expressed at different levels. These differences could be due to certain crRNAs being more stable than others or point to the existence of alternate crRNA transcriptional start sites. Recent work by Dugar *et al.* has also found that the crRNAs are expressed at different levels and have proposed that each rRNA could be independently transcribed from their own promoter (Dugar *et al.*, 2013). Our results agree with this model however the abundance of the individual crRNAs differs in our study as compared to Dugar *et al.*. Under our experimental conditions the most abundant crRNA is crRNA2, while it is crRNA4 in the dRNA-seq study (Dugar *et al.*, 2013). This difference suggests that *C. jejuni* may differentially regulate the expression of its crRNA's based on environmental conditions.

Further analysis of the miRNA dataset revealed the presence of widespread anti-sense transcription in the *C. jejuni* genome. These antisense RNA species may prove to play important roles with regards to regulating gene expression in *C. jejuni*. In addition, our analysis identified numerous novel transcripts that were produced from intergenic regions. Several of these transcripts had been bioinformatically identified in the Chaudhuri *et al.* RNA-seq study (Chaudhuri *et al.*, 2011) and were also identified by Dugar *et al.* (Dugar *et al.*, 2013) using dRNA-seq (Table S3.7). Our experimental results also demonstrated that several of these ncRNAs are differentially expressed in response to iron and also appear to be regulated by the Fur transcriptional regulator (Figure 3.5B, 3.5C). These findings suggest that these putative ncRNAs may perform regulatory roles in iron homeostasis. Previous work by our lab has determined the CjFur regulon by both microarray and ChIP-chip analyses (Butcher *et al.*, 2012; Palyada *et al.*, 2004). Comparing the RNA-seq iron responsive genes with the CjFur direct and indirect regulons reveals that most iron responsive genes in *C. jejuni* are not directly or indirectly regulated by CjFur (Figure S3.3)(Butcher *et al.*, 2010; Butcher *et al.*, 2012; Palyada *et al.*, 2004). Specifically, the iron responsive ncRNA may be the regulatory link between genes that are iron responsive but not regulated by the Fur protein, while the Fur responsive ncRNAs may explain why numerous Fur regulated genes are indirectly, rather than directly, regulated by the Fur protein.

Searches in sRNA databases (such as Rfam) failed to identify homologous ncRNAs matching the novel sRNAs identified in this study. However, many bacterial ncRNAs are dependent on the Hfq RNA binding protein for full activity (Masse *et al.*, 2007). Since *C. jejuni* does not contain a homologue of Hfq, this could explain why *C. jejuni* sRNAs do not share structural/sequence homology to previously characterized sRNAs in other bacteria. A

similar observation has been reported for *H. pylori*, with many identified sRNAs not matching known sRNA families (Sharma *et al.*, 2010). It should also be noted that *C. jejuni* and *H. pylori* have exceptionally AT rich genomes that could also confound efforts to identify regions of similarity between sRNAs. It is also conceivable that additional novel RNAs remain to be identified in *C. jejuni* as our analysis did not detect some of the ncRNAs previously identified by dRNA-seq (Dugar *et al.*, 2013). This failure to identify all potential ncRNAs may be due to our rigorous screening protocol (see methods). Indeed, our bioinformatic analysis excludes the *C. jejuni* 6S RNA transcript due to its proximity to known ORFs encoded on the same genomic strand (Dugar *et al.*, 2013; Sharma *et al.*, 2010).

In summary, our combined mRNA and miRNA Illumina library approaches have further defined our knowledge of *C. jejuni*'s transcriptomic response to iron limitation. Our RNA-seq study also provides a comprehensive operon map and a genome wide picture of the 5'UTR composition in *C. jejuni*. Finally, our miRNA sequencing approach has identified numerous uncharacterized novel RNAs within *C. jejuni* that are iron responsive and could thus play a role in iron homeostasis. Our results provide substantial novel insights into the transcriptional landscape of *C. jejuni* and thus constitute a valuable reference for *C. jejuni* researchers seeking to understand the transcriptional network of this model organism.

Acknowledgments

The authors thank the Beijing Genomics Institute for performing the Illumina library generation and sequencing reactions. We also thank Annika Flint for her constructive comments on this manuscript.

Chapter 4: Unveiling the iron-dependent/independent Fur- and PerR-regulons of *Campylobacter jejuni*

Preface

The following chapter consists of data that has been submitted for publication in BMC Genomics under the title “Unveiling the iron-dependent/independent Fur- and PerR-regulons of *Campylobacter jejuni*” by Butcher J, Handley R, van Vliet A and Stintzi A.

For the sake of brevity, not all the supplemental files submitted with this manuscript are reproduced here due to the length and breadth of the genome wide analysis performed. Specifically, Tables S4.2, S4.3, S4.4, S4.7, S4.8, S4.9 and S4.10 are excluded. Figures S4.4 and S4.5 are also excluded due to their size (note that miniature versions of these figures are included in the main figures).

The specific contributions of each author to this paper are listed below along with the author contribution section published in the paper.

Butcher J: Designed experiments and performed certain RNA extractions as required. Analyzed data from all experiments, wrote manuscript, constructed the figures, and responded to reviewers comments.

Handley R: Performed most of the RNA extractions and contributed to revising the manuscript for publication.

Van Vliet A: Contributed to revising the manuscript for publication

Stintzi A: Designed RNA-seq experiments, contributed to writing/editing the manuscript.

BMC Genomics Author contribution statement:

JB performed certain RNA extractions, analyzed the data and drafted the manuscript. RH carried out most of the RNA extractions and helped draft/revise the manuscript. AV helped

draft and revise the manuscript. AS designed the research project and helped draft and revise the manuscript.

Abstract

Background: The genome of *Campylobacter jejuni* contains two iron activated Fur-family transcriptional regulators, CjFur and CjPerR, which are primarily responsible for regulating iron homeostasis and oxidative stress respectively. Both transcriptional regulators have been previously implicated in regulating diverse functions beyond their primary roles in *C. jejuni*. To further characterize their regulatory networks, RNA-seq was used to define the transcriptional profiles of *C. jejuni* NCTC11168 wild type, Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ isogenic deletion mutants under both iron-replete and iron-limited conditions.

Results: It was found that 202 genes were differentially expressed in at least one mutant under iron-replete conditions and 331 genes were differentially expressed in at least one mutant under iron-limited conditions. The CjFur and CjPerR regulons characterized in this study were compared to those previously identified using microarray profiling and found to be more extensive than previously understood. Interestingly, our results indicate that apo-Fur/apo-PerR appear to co-regulate flagellar biogenesis in an opposing fashion. Moreover the $\Delta fur\Delta perR$ isogenic deletion mutant revealed that CjFur and CjPerR can compensate for each other in certain cases, suggesting that both regulators may compete for binding to specific promoters.

Conclusions: The CjFur and CjPerR regulons are larger than previously reported. In particular, deletion of *perR* results in the differential expression of a large group of genes in the absence of iron, suggesting that CjPerR may also regulate genes in an apo- manner, similar to what has already been demonstrated with CjFur. Moreover, subsets of genes were found which are only differentially expressed when both CjFur and CjPerR are deleted and includes genes that appear to be simultaneously activated by CjFur and repressed by CjPerR.

In particular the co-regulation of flagellar biogenesis by apo-CjFur/CjPerR represents a potentially novel regulatory function for these proteins. These findings represent additional modes of co-regulation by these two transcriptional regulators in *C. jejuni*.

Keywords

Fur, PerR, co-regulation, iron-independent regulation

Introduction

The microaerophilic bacterial pathogen *Campylobacter jejuni* can colonize and thrive in the gut of many different mammalian and avian hosts (humans, bovines, avians, porcines etc.) (Palyada *et al.*, 2004; Young *et al.*, 2007), and *C. jejuni* is also known to persist in the environment during its transit between different hosts (Bronowski *et al.*, 2014). In all these environments, *C. jejuni* must regulate several functions to be able to cope with these variables and often stressful conditions. Previous work has demonstrated that genes relating to iron metabolism and oxidative stress defense are key factors for *C. jejuni*'s survival *in vivo* (Flint *et al.*, 2014; Palyada *et al.*, 2009; Palyada *et al.*, 2004). The classical iron-regulatory protein in most Gram-negative bacteria, including *C. jejuni*, is the ferric uptake regulator (CjFur) protein, which senses intracellular iron and represses genes involved in iron acquisition accordingly (Butcher *et al.*, 2012; Butcher and Stintzi, 2013; Holmes *et al.*, 2005; Palyada *et al.*, 2004). Interestingly *C. jejuni* also possesses a second iron responsive transcriptional regulator, the peroxide responsive regulator PerR that is responsible for regulating *C. jejuni*'s response to peroxide stress (Palyada *et al.*, 2009; van Vliet *et al.*, 1999). This is in contrast with other gram negative bacteria whose peroxide sensors (e.g. OxyR, OhR) do not utilize iron as a cofactor to sense oxidative stress (Dubbs and Mongkolsuk, 2012).

It has long been known that the members of the CjFur and CjPerR regulons overlap (van Vliet *et al.*, 1999). The best example of this is the *kata* gene which encodes for catalase. Both CjFur and CjPerR must be deleted for this gene to be fully derepressed and unresponsive to iron repression (van Vliet *et al.*, 1999). In contrast, there are other genes which appear to be solely regulated by either CjFur (e.g. *cfrA*, *chuA*) or CjPerR (e.g. *ahpC*)

(Holmes *et al.*, 2005; Palyada *et al.*, 2009; Palyada *et al.*, 2004). At times it has been difficult to clearly define whether a gene is regulated by CjFur and/or CjPerR due to the functional similarity of these two transcriptional regulators. Specifically as CjPerR can use iron and manganese as co-factor, deletion of *fur* may indirectly influence CjPerR activity by altering iron levels in the cell. Moreover, the CjFur and CjPerR binding sequences are likely to be extremely similar based on studies of other organisms harboring multiple Fur family members (Fuangthong and Helmann, 2003).

There have been several studies that characterized the CjFur and CjPerR regulons using microarray transcriptomic profiling of either Δfur or $\Delta perR$ isogenic mutant strains (Holmes *et al.*, 2005; Palyada *et al.*, 2009; Palyada *et al.*, 2004). However directly comparing the profiles obtained in these studies can be difficult due to confounding factors that may alter gene expression independently from transcriptional regulator deletion. In *C. jejuni* these can include differences in growth conditions including the gas content used (e.g. gas packs vs. defined gas mixes) and the composition of the growth media (John *et al.*, 2011; Verhoeff-Bakkenes *et al.*, 2008). In addition, given that CjFur and CjPerR are known to co-regulate genes, there may be members of the CjFur regulon whose differences in expression are being masked by CjPerR and *vice versa*. We have therefore characterized the transcriptomes of our *C. jejuni* NCTC11168 wild-type strain and its corresponding Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ isogenic deletion mutants using RNA-seq. These strains were grown following a standardized protocol to improve the reproducibility of the transcriptomic results. While there is increasing evidence that CjFur also regulates genes *in vivo* in the absence of a metal cofactor (apo- form) (Butcher *et al.*, 2012), there has been little investigation as to whether apo-CjPerR may also be able to regulate gene transcription. Thus the transcriptomes of *C.*

jejuni NCTC11168 wild-type strain and its corresponding Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ were characterized under iron-replete and iron-limited conditions to define the both holo-/apo-regulons of CjFur and CjPerR.

Our results indicate that the CjFur and CjPerR regulons are larger than those previously reported in *C. jejuni* using microarray profiling. This is especially true with regards to apo-regulated genes for both CjFur and CjPerR. In addition we have found subsets of genes which are only differentially expressed when both CjFur and CjPerR are deleted and genes which appear to be simultaneously activated by CjFur and repressed by CjPerR.

Methods

Bacterial Strains and growth

The *C. jejuni* NCTC11168 wild-type, Δfur (Palyada *et al.*, 2004), $\Delta perR$ (Palyada *et al.*, 2009) and $\Delta fur\Delta perR$ (Palyada *et al.*, 2009) strains were routinely cultured on Mueller-Hinton (MH) agar plates under microaerophilic conditions (83% N₂, 4% H₂, 8% O₂ and 5% CO₂) at 37°C in a MACS-VA500 workstation (Don Whitley, West Yorkshire, England). *C. jejuni* strains were grown overnight in MH agar overlaid with MH broth (biphasic cultures). Prior to inoculation, overnight cultures were washed in minimal essential media alpha (MEM α) (Invitrogen) to remove excess iron sources. Glass flasks used for growing strains for RNA extraction were soaked with 1M HCl to solubilize bound metal ions and rinsed twice with ddH₂O prior to sterilization. Strains were grown in a final volume of 50 mL MEM α supplemented with 10 mM pyruvate with freshly prepared FeSO₄ added at a final concentration of 40 μ M as needed to generate iron-replete growth conditions.

Total RNA Isolation

C. jejuni was grown in MEM α under either iron-replete (+40 μ M FeSO₄) or iron-limited (no added iron) conditions until midlog phase was reached (OD₆₀₀ ~0.2). Total RNA was then isolated as described previously (Butcher and Stintzi, 2013; Palyada *et al.*, 2004). Briefly, a 10% cold RNA stop solution used to preserve RNA integrity (10% buffer saturated phenol pH 4.3 in absolute ethanol) was added to the media and cells were pelleted by centrifugation at 6000xg. Pellets were resuspended in TE buffer pH 8.0 and RNA was extracted using a hot-phenol chloroform method (Palyada *et al.*, 2004). RNA was precipitated overnight at -80°C and the RNA pellet was washed five times in 80% ethanol.

RNA pellets were resuspended in TE buffer and treated with DNase I (Epicentre) to remove contaminating genomic DNA. Treated samples were purified using the RNeasy kit (Qiagen) and PCR was performed to confirm the absence of genomic DNA. RNA purity and quality was assessed using a Nanodrop and the Experion RNA StdSens analysis kit (Bio-Rad). Samples with high quality RNA were selected for Illumina sequencing library construction. Total RNA was isolated from three independent biological replicates of wild-type, Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ strains under either iron-replete or iron-limited conditions (24 RNA extractions in total).

Illumina library construction and sequencing

Library construction, cluster generation and sequencing were performed at McGill University and G  nome Qu  bec Innovation (Montreal, Canada). Ribosomal RNAs were depleted using the RiboZero Meta-Bacteria kit (Epicentre) and strand-specific sequencing libraries were constructed using TruSeq kits (Epicentre). Cluster generation was done using standard Illumina protocols with sequencing performed on an Illumina HiSeq 2500 sequencer. The libraries were subjected to paired-end sequencing with a target read length of 100 nucleotides and the samples for iron-replete and iron-limited conditions were run on separate lanes (12 samples/lane). At least 12 million reads were obtained per library (Table S4.1). The Illumina reads have been submitted to the Sequence Read Archive at NCBI with the accession numbers SRP044881 and SRP044881 for the iron-replete and iron-limited conditions respectively.

Read alignment to the *C. jejuni* genome and bioinformatic analysis

Paired end reads were aligned to the *C. jejuni* NCTC11168 genome (NC002163) using Bowtie V2 on the Biolinux platform (Field *et al.*, 2006; Langmead *et al.*, 2009). Approximately 97% of reads aligned for each sequencing run (Table S4.1). Aligned reads were visualized using Artemis with the Bamview plugin (Carver *et al.*, 2012). Raw read counts for each gene were tabulated (Table S4.2) using Bedtools and also used to calculate a reads per kilobase coding sequence per million sequenced reads (RPKM) value for each gene (Table S4.3). The calculated RPKM values were used to test the homogeneity of each strain's biological replicates by performing PCA analysis in R using prcomp (R Core Team, 2014). Samples which appeared to cluster differently from their mates were excluded from the downstream differential expression analyses (Figures 4.1, S4.1,S4.2,S4.3). The edgeR statistical package was used to determine gene expression fold changes using the current Genbank gene annotation information for *C. jejuni* NCTC11168 (Quinlan and Hall, 2010; Robinson *et al.*, 2010). Transcripts showing a fold change ≥ 1.5 with a FDR value ≤ 0.05 were considered to be significantly differentially expressed (Tables S4.4, S4.5, S4.6) (Butcher and Stintzi, 2013). Differentially expressed genes were subsequently merged based on growth condition and clustered (using average linkage Euclidean distance) based on their expression profile using Genesis (Figures 4.2, 4.3, 4.S4, 4.S5) (Sturn *et al.*, 2002). Differentially expressed genes for each condition were also subjected to gene set enrichment analysis (GSEA) on annotated KEGG pathways using GAGE with a FDR cutoff of <0.1 (Luo *et al.*, 2009). Selected significantly enriched pathways were visualized using Pathview (Luo and Brouwer, 2013).

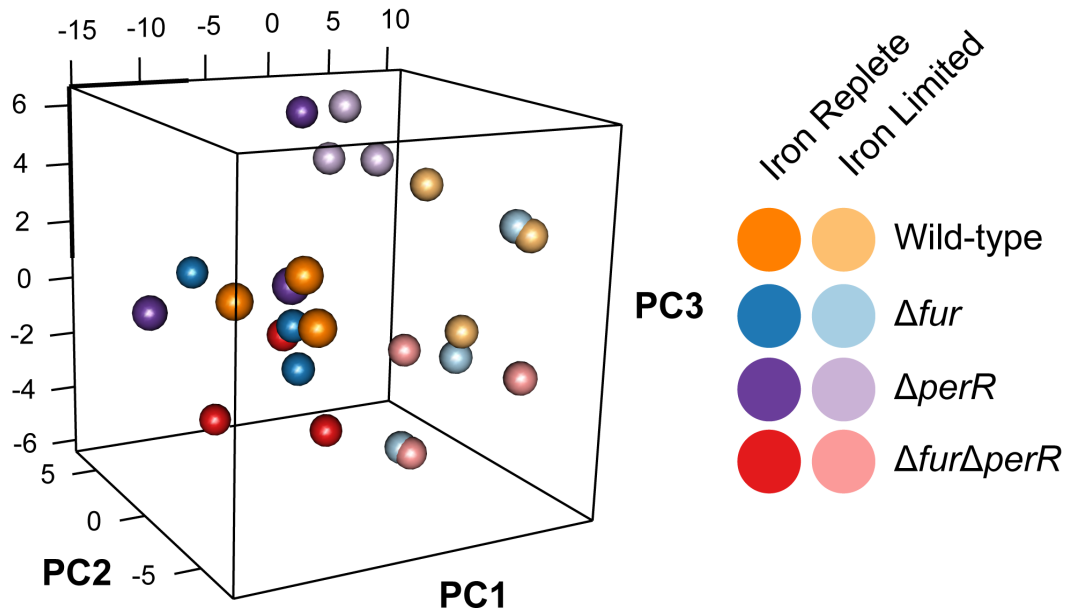


Figure 4.1: PCA analysis of *C. jejuni* transcriptomic samples

The Log(RPKM+1) values for each gene in the *C. jejuni* NCTC11168 genome were used for PCA analysis to show the overall structure of the transcriptomic data and to identify sample outliers. Iron replete samples are in dark colors and iron limited samples in light colors. The PCA showed distinct sample separation based on the iron status of the growth medium highlighting the key influence of iron on the *C. jejuni* transcriptome. The samples for each strain tend to cluster together with two noticeable exceptions in the $\Delta perR$ iron replete and Δfur limited groups.

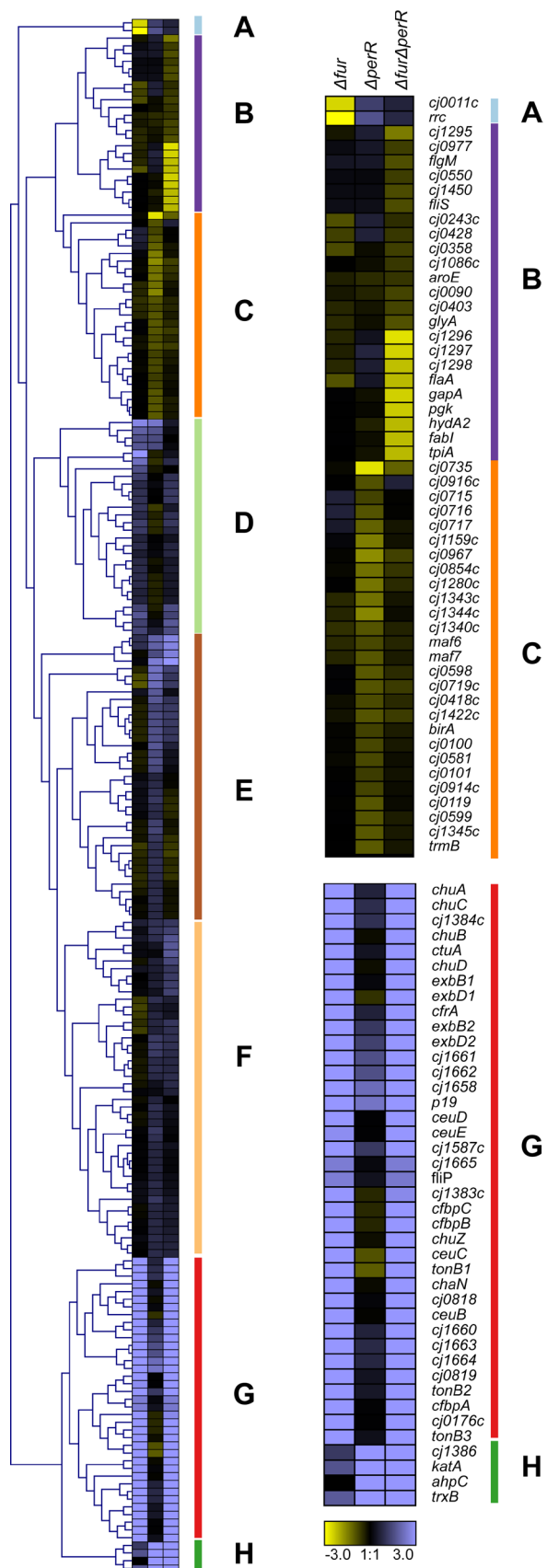


Figure 4.2: Hierarchical clustering of genes differentially expressed under iron replete conditions

Genes found to be differentially expressed in at least one strain under iron replete conditions were subjected to hierarchical clustering in Genesis to identify corresponding genes with similar expression profiles. The columns each represent one strain (Δfur , $\Delta perR$, $\Delta fur\Delta perR$) and relative fold changes in expression are presented in a Log_2 scale with upregulated genes in blue and downregulated genes in yellow. The clustering resulted in 8 main clusters (A-H). Clusters A, B, C, G and H are expanded to highlight the genes present in each cluster. See Table S4.5 for further details and Figure S4.2 for full histogram.

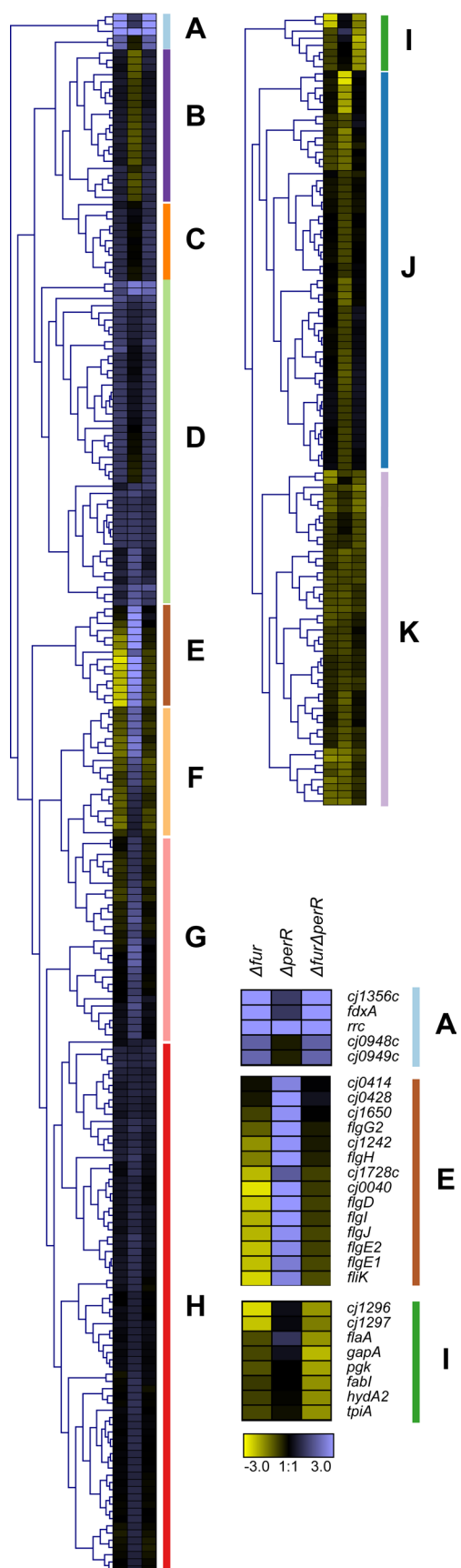


Figure 4.3: Hierarchical clustering of genes differentially expressed under iron limited conditions

Genes found to be differentially expressed in at least one strain under iron limited conditions were subjected to hierarchical clustering in Genesis to identify corresponding genes with similar expression profiles. Clusters I-K were split from the original clustering figure for ease of viewing. The columns each represent one strain (Δfur , $\Delta perR$, $\Delta fur\Delta perR$) and relative fold changes in expression are presented in a Log2 scale with upregulated genes in blue and downregulated genes in yellow. The clustering resulted in 11 main clusters (A-K). Clusters A, E and H are expanded to highlight the genes present in each cluster. See Table S4.6 for further details and Figure S4.3 for full histogram.

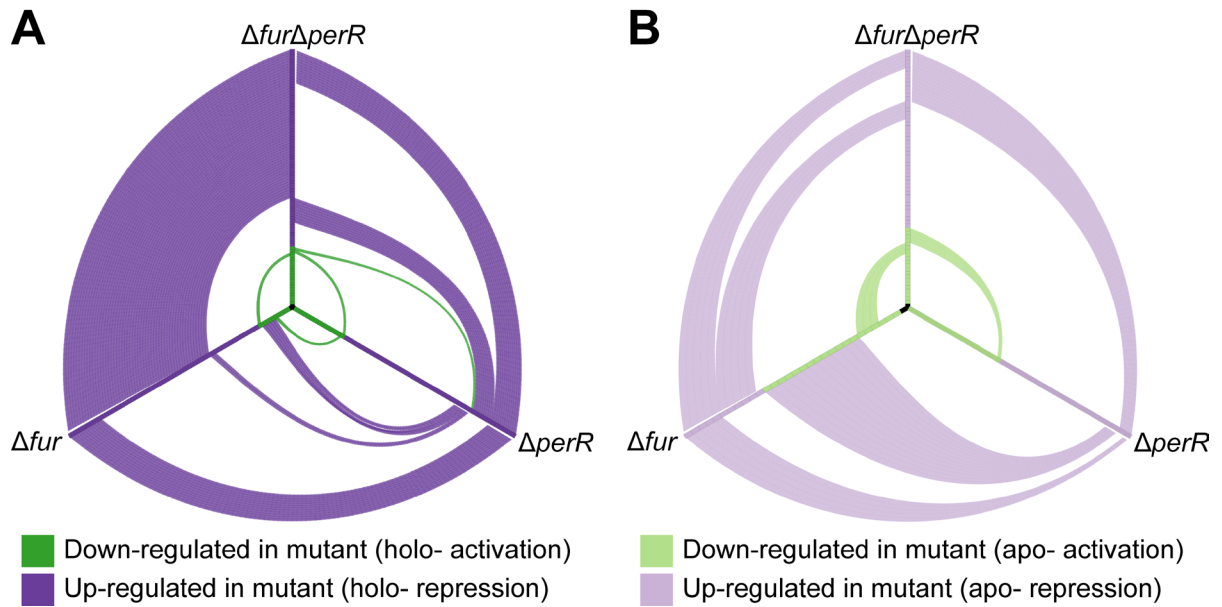


Figure 4.4: Overlap between the genes differentially expressed in the Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ strains

Genes differentially expressed in each strain are ordered along each axis under either iron replete (A) or iron limited (B) conditions. Genes present in multiple strains are connected by ribbons and coloured based on their mode of regulation (green for activation, purple for repression).

The similarities and differences between the genes found to be differentially expressed in various strains were visualized using hive plots with differentially expressed genes as nodes and genes present in multiple strains connected with ribbons (Krzywinski *et al.*, 2012).

In addition, genes identified as being part of the Δfur and $\Delta perR$ regulons via RNA-seq were compared to the Δfur and $\Delta perR$ regulons previously characterized using microarrays (Palyada *et al.*, 2009; Palyada *et al.*, 2004). Genes were considered significantly expressed in the microarrays using the same parameters as in the original papers (>2 fold change, $p < 0.001$). Iron responsive genes were also determined by comparing the wild-type grown under iron-replete and iron-limited conditions (Table S4.10) to the iron responsive genes previously identified via microarray profiling or RNA-seq (Butcher and Stintzi, 2013; Palyada *et al.*, 2004).

Bioinformatic analysis of potential Fur and PerR consensus binding sites

The promoter regions of Fur/PerR regulated genes under either iron-limited or replete conditions units were analyzed for the existence of potential binding sites. Genes were analyzed as a whole and also as subgroups based on their potential mode of regulation (apo/holo and activation/repression). Regions consisting of 300 bp upstream the predicted start codon of each gene were retrieved from the RSAT server (Thomas-Chollier *et al.*, 2011) (<http://rsat.ulb.ac.be/>) and analyzed for potential regulatory motifs by the MEME suite of analysis tools (Bailey *et al.*, 2009) (<http://meme.nbcr.net>). The genes containing the holo-Fur repression motif consisted of *ceuB*, *cfbpA*, *cfrA*, *chan*, *chuA*, *chuZ*, *cj0176c*, *cj0818*, *cj1384c*, *cj1587c*, *cj1658*, *dsbI*, *exbB2*, *katA* and *tonB3*.

Results

Confirming sample consistency via PCA analysis

Principal component analysis (PCA) was performed to show the overall structure of the transcriptomic data and identify sample outliers based on the growth media (iron-replete vs. deplete) or genetic background (wild-type vs. Δfur vs. $\Delta perR$ vs. $\Delta fur\Delta perR$). The PCA showed distinct sample separation based on the iron status of the growth medium highlighting the key influence of iron on the *C. jejuni* transcriptome (Figure 4.1). Moreover the samples for each strain tended to cluster together with two noticeable exceptions (Figure 4.1). One of the $\Delta perR$ samples (perr_plusIron_3) grown in the presence of iron did not group with other iron-replete samples and instead clustered with iron-limited samples. In addition one of the Δfur samples (fur_minusIron_2) grown in iron-limited conditions was almost indistinguishable via PCA from the wild-type samples. Therefore both of these outlier samples were removed from downstream differential expression analysis, to avoid introducing artifacts or confounding the identification of differentially expressed genes. To note, including these outlier samples resulted in a significantly lower number of differentially expressed genes with a high number of known false negative results (data not shown).

Previous work in our laboratory has also used RNA-seq to identify iron responsive genes in wild-type *C. jejuni* NCTC11168 (Butcher and Stintzi, 2013). These previous sequencing results were compared to the current wild-type results to determine the similarity of the runs and whether the results from both studies could be merged for meta-analysis. PCA clearly separated the transcriptomes based on the sequencing runs (Figure S4.6,S4.7). In fact, the sequencing results for these two studies could be separated by two axes

corresponding to either growth condition (iron-replete vs iron-limited) or study (current vs previous). Therefore to avoid biased results, the iron regulon was reanalyzed using only the data from this study and compared to the iron regulon that was determined previously.

Transcriptome signatures of the Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ mutants under iron-replete conditions

The major objective of this work was to define the Fur and PerR regulatory networks. Because Fur and PerR regulated genes are known to be iron-repressed we first determined the Fur and PerR regulon under iron-replete conditions as these genes would be differentially expressed between the wild-type and mutant strains. Globally, 86 genes were found to be differentially expressed in the Δfur mutant (73 up-regulated and 13 down-regulated), 111 genes in the $\Delta perR$ mutant (85 up-regulated and 26 down-regulated) and 95 genes in the $\Delta fur\Delta perR$ mutant (72 up-regulated and 23 down-regulated) (Table S4.5). Genes found to be differentially expressed in at least one strain were subjected to hierarchical clustering analysis (using average linkage Euclidean distance). The analysis generated 8 main clusters, with clusters A-C representing down-regulated genes and clusters D-H representing up-regulated genes (Table S4.5, Figure 4.2). Clusters A, B and C contain genes that were primarily down-regulated in the Δfur , $\Delta fur\Delta perR$ or $\Delta perR$ strains respectively. The most down-regulated Δfur genes (Cluster A) consisted of the *rrc-cj0011c* operon (29.2 and 5.8 fold activation). Cluster B primarily consists of genes highly down-regulated (~1.5-8 fold) in the $\Delta fur\Delta perR$ strain, including *cj1295-1298* and several flagellar genes such as *flgM*, *fliS* and *flaA*. Cluster C contains genes primarily down-regulated in the $\Delta perR$ strain (~2 fold) and includes several motility associated genes (*cj1340c-cj1345c*, *maf6*, *maf7*). Interestingly, no genes were found to be down-regulated in all three strains and only *cj1340c* (Δfur ,

ΔperR), *flaA* (*Δfur*, *ΔfurΔperR*) and *cj1442c* (*ΔperR*, *ΔfurΔperR*) were down-regulated in two of the three strains (Figure 4.4).

Clusters D-F consist of genes that were slightly up regulated in at least one of the three strains (~2 fold). In contrast with the down regulated genes, many up regulated genes were up-regulated in multiple strains. Clusters G and H contain genes that were highly up-regulated in at least two strains (~6-120 fold). Genes in cluster G are greatly up-regulated in the *Δfur* and *ΔfurΔperR* strains. Most of these genes are directly involved in iron acquisition (e.g. hemin: *chuABCDZ*; enterobactin: *cfrA*, *ceuBCDE*; lactoferrin/transferrin: *cfbpABC*, *ctuA*, *chan*; rhodotorulic acid: *cj1658-p19*, *cj1660-1665*; ferrous ions: *feoB*, *cj1397*) or supplying the energy required for iron uptake across the outer membrane (e.g. *tonB1/B2/B3*, *exbB1/B2*, *exbD1/D2*). Many of these genes have been previously been shown to be directly regulated by Fur (Holmes *et al.*, 2005; Miller *et al.*, 2008; Ridley *et al.*, 2006). Cluster H consists of four genes that are highly up-regulated in all three strains and are involved in oxidative stress defense (*kata*, *cj1386*, *ahpC* and *trxB*). The *C. jejuni* alkyl hydroperoxidase (*ahpC*) has been previously shown to be directly regulated by PerR (Kim *et al.*, 2011), while the catalase (*kata*), thioredoxin *trxB* and the heme trafficking protein *cj1386* have been suggested to be PerR regulated (Palyada *et al.*, 2009; van Vliet *et al.*, 1999). In addition *kata*, *trxB* and *cj1386* have been proposed to be Fur regulated (Holmes *et al.*, 2005; Palyada *et al.*, 2004), although Fur has only been shown to bind the *kata* promoter (Butcher and Stintzi, 2013) (note that *kata* and *cj1386* are not co-transcribed (Flint *et al.*, 2012)). Most of the genes in both clusters G and H have also been previously found to be iron responsive in *C. jejuni* (Butcher and Stintzi, 2013; Holmes *et al.*, 2005; Palyada *et al.*, 2004).

The promoter regions of differentially expressed genes in each strain (Δfur , $\Delta perR$ and $\Delta fur\Delta perR$) under each condition (iron-replete and iron-limited) were analyzed by MEME to identify potential binding motifs. However we were only able to identify a consensus binding motif for Fur holo-repressed targets (Figure S4.8) that was essentially identical to those already published (Butcher *et al.*, 2012; Palyada *et al.*, 2004).

Transcriptome signatures of the Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ mutants under iron-limited conditions

In order to identify apo-Fur and apo-PerR regulated genes we also characterized the Fur and PerR regulons under iron-limited conditions. Globally, 31 genes were found to be differentially expressed in the Δfur mutant (11 up-regulated and 20 down-regulated genes), 373 genes in the $\Delta perR$ mutant (160 up-regulated and 113 down-regulated genes) and 72 genes in the $\Delta fur\Delta perR$ mutant (49 up-regulated and 23 down-regulated genes) (Table S4.6). Hierarchical clustering analysis of the differentially expressed genes revealed 11 main clusters (A-K) of expression profiles with clusters I-J being primarily composed of genes that were down-regulated in at least one strain and clusters C, D, G and H containing genes that were up-regulated in at least one strain (Figure 4.3; ~2-4 fold). Cluster A is composed of genes that were strongly up-regulated in the Δfur and $\Delta fur\Delta perR$ strains such as *cj1356c*, *fdxA*, *rrc* and *cj0948c-0949c* (~4-8 fold). Cluster I contains genes that were strongly down-regulated in the Δfur and $\Delta fur\Delta perR$ strains such as *flaA*, and the flagellar modification island genes *cj1296-97* (~3-4 fold).

Cluster B contains genes up-regulated in the $\Delta perR$ mutant while being down-regulated in the Δfur and $\Delta fur\Delta perR$ mutants, while cluster F consists of genes down-regulated in the $\Delta perR$ mutant while being up-regulated in the Δfur and $\Delta fur\Delta perR$ mutants.

Interestingly Cluster E is primarily composed of genes that were strongly down-regulated in the Δfur mutant (~3-4 fold down-regulation) and strongly up-regulated in the $\Delta perR$ mutant (~4-6 fold up-regulation) (also see Figure 4.4). These include many different flagellar biogenesis genes such as *flgE1*, *flgE2*, *flgG*, *flgH*, *flgI*, *flgJ*, and *fliK* (to note, most of these genes have been previously shown to be required for *C. jejuni* motility (Flint *et al.*, 2014; Kamal *et al.*, 2007)). In addition, none of the genes present in Cluster E were found to be differentially expressed in the $\Delta fur\Delta perR$ mutant (Table S4.6).

Gene-set enrichment analysis of Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ under both iron-rich and iron-limited conditions

Gene-set enrichment analysis was used to determine if there were significantly enriched KEGG pathways present in the differentially expressed genes found in each strain under each growth condition. This analysis identified five KEGG categories that were enriched under at least one condition (Table S4.7, S4.8). Interestingly, the KEGG category for “Flagellar Assembly” was enriched under both the Δfur and $\Delta perR$ iron-limited conditions but not in the $\Delta fur\Delta perR$ deletion mutant (Figure 4.5).

Cross platform correlation of the Δfur and $\Delta perR$ regulons

To validate the results from the RNA-seq analysis we compared the obtained Fur and PerR regulons to the regulons previously defined using a microarray platform under similar growth conditions (Palyada *et al.*, 2009; Palyada *et al.*, 2004). The correlation between the two platforms was assessed by plotting the log-transformed values of the fold change obtained by RNA-seq against the log-transformed values of the fold change obtained by microarrays (only the differentially expressed genes were included).

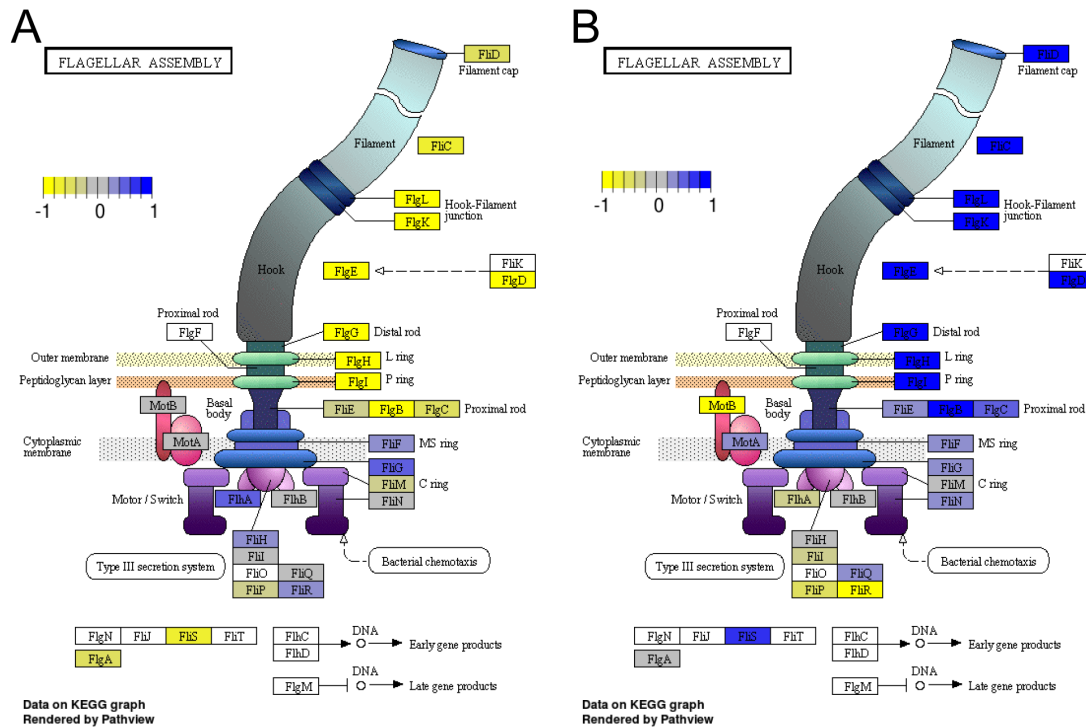


Figure 4.5: Genes enriched in KEGG’s “Flagellar Assembly” category under iron limitation

Genes that belong to the KEGG’s “Flagellar Assembly” category under iron-limited conditions in the Δfur mutant (A) and $\Delta perR$ mutant (B) are shown. Genes absent from *C. jejuni* are denoted in white, unchanged genes in grey, up-regulated genes in blue and down-regulated genes in yellow. To note, the scale of differential expression is normalized from the highest to lowest expressed gene. Most of the genes down-regulated in the Δfur mutant are conversely up-regulated in the $\Delta perR$ mutant.

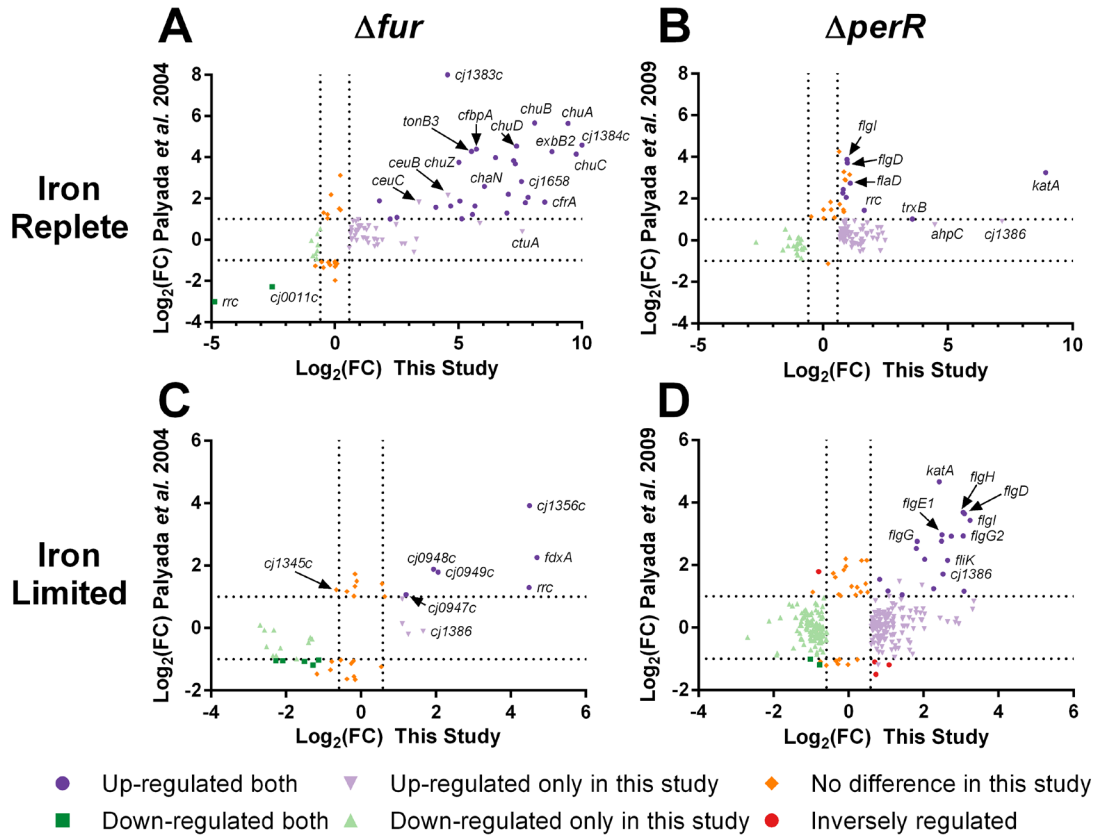


Figure 4.6: Comparison between CjFur and CjPerR regulons as determined by RNA-seq and microarray profiling

The results of the RNA-seq in this study and previous CjFur (A,C) or CjPerR (B,D) microarray profiling results were plotted according to Log₂(fold change). Only genes found to be significantly expressed in either the RNA-seq or microarrays are shown with the dashed lines representing the Log₂(FC) cut-offs. Up-regulated genes are in purple and down-regulated genes in green. Genes not found to be differentially expressed in the present study are in orange and genes showing opposite regulation are in red. Genes mentioned in the text are highlighted. See Table S4.7 for details for all differentially expressed genes.

These two independent measures of differential gene expression showed poor correlation with correlation coefficients of 0.57/0.43 for Δfur (iron-replete/limited) and 0.08/0.13 for $\Delta perR$ (iron-replete/limited). However, for both the Δfur and $\Delta perR$ mutants there was a greater agreement between the genes identified as up-regulated as compared to down-regulated in each condition (Figure 4.6, Table S4.7).

In addition, the RNA-seq analysis identified more genes as being differentially expressed as compared to the microarrays: 86/31 vs 49/30 for Δfur (RNA-seq vs microarray, iron-replete/limited) and 111/272 vs 23/49 for $\Delta perR$ (RNA-seq vs microarray, iron-replete/limited). For the Δfur mutant under iron-replete conditions, genes commonly identified as up-regulated include most of the known iron acquisition pathways (*ceuBCE*, *chuABCDZ*, *cfrA*, *cfbpABC*, *chaN/ctuA*, *cj1658/p19*, *cj1660-1665*, *exbB1/D1*, *exbB2/D2*, *feoB*, *tonB3*), as well as the highly iron responsive *cj1383c-cj1384c* genes located upstream from *katA*. The only genes found to be activated in the Δfur mutant by both platforms were *rrc-cj0011c*. Genes identified in the RNA-seq but not the microarray profiling include the iron acquisition proteins *ctuA/cj0178* (transferrin/lactoferrin) and *ceuBC* (enterobactin). The commonly identified up-regulated genes for the Δfur mutant under iron-limited conditions include *rrc*, *fdxA*, *cj1386* and *cj0948c-0949c*.

The commonly identified up-regulated $\Delta perR$ genes under iron-replete conditions include the antioxidant genes *katA*, *trxB* and *rrc* as well as a variety of genes involved in flagellar biogenesis (e.g. *flaD*, *flgD*, *fgl*). In contrast with the microarray profiling, the RNA-seq analysis also identified the antioxidant genes *ahpC* and *cj1386* as being CjPerR regulated. There were no CjPerR activated genes in common between the RNA-seq and microarray profiling under iron-replete conditions. The RNA-seq analysis for the $\Delta perR$

mutant under iron-limited conditions identified many differentially expressed genes that were not present in the microarray profiling. Many of the commonly identified up-regulated genes are also differentially expressed under iron-replete conditions (e.g. *kata*, *cj1386*, *flgI*, *flgD*). The $\Delta perR$ mutant under iron-limited conditions was also the only condition where there was a disagreement between the RNA-seq and microarray data for whether a gene was significantly up-regulated or down-regulated upon *perR* deletion (Figure 4.6).

Comparison of the iron regulon from two independent RNA-seq experiments and microarray profiling

Two previous studies from our laboratory have used similar growth conditions to determine iron-responsive genes in *C. jejuni* using both microarray profiling and RNA-seq (Butcher and Stintzi, 2013; Palyada *et al.*, 2004). Iron-responsive genes were determined by comparing the wild-type transcriptomes in the presence and absence of iron. This analysis found 686 genes differentially expressed in response to iron as compared to 270 genes identified using microarray profiling and 127 genes identified in an earlier RNA-seq study (Figure 4.7, Table S4.10).

Overall, the RNA-seq analysis identified more differentially expressed genes as compared to the microarray analysis. Approximately 26% of the genes identified in the RNA-seq analysis were also found to be differentially expressed in the microarray profiling experiments (180/686). In addition, there were 75 genes identified in the microarray analysis that were not found to be differentially expressed via RNA-seq (Table S4.10).

In contrast, all of the genes identified as being iron regulated in a previous RNA-seq study were also found to be differentially expressed in this work (Butcher and Stintzi, 2013). However, the fold changes determined in this previous RNA-seq study appear to be greater

as compared to this work. In particular, the $\text{Log}_2(\text{FC})$'s in the previous study exceeded >25 in some cases as compared to a maximum of $\text{Log}_2(\text{FC})$ of ~ 10 in this work (Figure 4.7). Indeed, most of these extremely differentially expressed genes (e.g. *cj0243c*, *cj0878*, *cj0466*, *cj0730*, *cj1722*) were not identified as significant in the previous RNA-seq study due to high variability in the number of reads across biological replicates (Butcher and Stintzi, 2013). However, all these genes were found to be significantly differentially expressed in this dataset.

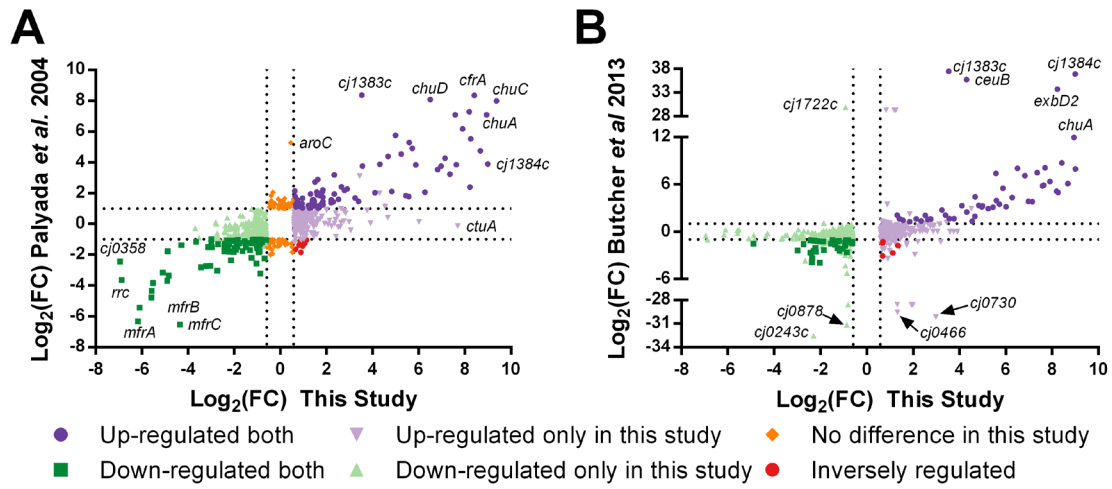


Figure 4.7: Comparison between iron regulons determined in this work and previous studies

The results of the iron regulons as determined in in this study and previous microarray (A) and RNA-seq (B) results were plotted according to Log₂(fold change). Only genes found to be significantly expressed in each study are shown with the dashed lines representing the Log₂(FC) cut-offs. Up regulated genes are in purple with down regulated genes in green. Genes not found to be differentially expressed in the current study are in orange and genes showing inverse regulation are in red. See Table S4.8 for details for all differentially expressed genes.

Discussion

Although the importance of the CjFur and CjPerR transcriptional regulators on *C. jejuni*'s *in vivo* fitness is now well established, there are still gaps in our understanding of their regulatory networks. Importantly, and in contrast to many other bacteria, CjFur regulates gene expression in an iron independent manner. However it is unknown whether CjPerR is also able to regulate genes independently from iron. As well, the degree to which the CjFur/CjPerR regulons overlap is unclear as the transcriptome of a double deletion of *fur* and *perR* has not been described. In this study, we have used RNA-seq to profile the transcriptomes of Δfur , $\Delta perR$ and $\Delta fur\Delta perR$ isogenic deletion mutants in order to characterize the CjFur and CjPerR regulons under both iron-replete and iron-limited conditions. In contrast to previously reported results, we have found that the regulons for both of these metalloregulators are more extensive than anticipated. We have also found that these regulons appear to highlight roles for both proteins in metal dependant (holo-) and metal independent (apo-) regulatory functions. Our work also highlights the importance of having multiple biological replicates when conducting genome wide analyses to identify and remove outlier samples that may mask expression differences.

Unsurprisingly, the most up-regulated genes under iron-replete conditions in the isogenic deletion mutants are those previously characterized as being involved in iron acquisition (Figure 4.2 [Cluster G]) or in oxidative stress defense (Figure 4.2 [Cluster H]). These two functions were clearly separated with the iron acquisition genes up-regulated in the Δfur mutant and the oxidative stress defense genes up-regulated in the $\Delta perR$ mutant. The Δfur mutant had relatively few genes down-regulated under iron-replete conditions with only *rrc* and *cj0011c* forming a distinct CjFur activated cluster. In contrast, there were many

more genes down-regulated in the $\Delta perR$ mutant and in the $\Delta fur\Delta perR$ mutant (Figure 4.2 [Clusters BC]), suggesting that PerR may also activate gene expression in a holo-form.

Interestingly there were a large number of genes differentially expressed in the isogenic deletion mutants under iron-limited conditions, which was unexpected as both CjFur and CjPerR are metalloregulators. In particular, there were 31 and 272 genes differentially expressed in the Δfur and $\Delta perR$ mutants respectively (Figure 4.3). While apo-CjFur has been previously shown to regulate gene expression, this result suggests that CjPerR may also regulate genes independently from iron. However, additional studies using purified apo-CjPerR protein would be required to demonstrate this specific mode of regulation as transcriptomic studies cannot distinguish between direct and indirect regulation.

Under iron-limited conditions the Δfur mutant also showed relatively few down-regulated genes as compared to up-regulated genes. The oxidative stress defense genes *cj1386*, *fdxA* and *rrc* were found to be regulated in an apo-CjFur like manner. *Rrc* has been previously shown to be differentially expressed in a Δfur mutant under iron-limited conditions by qRT-PCR and directly bound by apo-Fur (Butcher *et al.*, 2012). Previous work has also shown *cj1345c* to be differentially expressed in Δfur mutant under iron-limited conditions using qRT-PCR and also shown to be directly bound by apo-CjFur via EMSA (Butcher *et al.*, 2012). However in this study, *cj1345c*'s change in expression level was not statistically significant. There was also a cluster of genes that appear to be up-regulated in the Δfur mutant and down-regulated in the $\Delta perR$ mutant under iron-limited conditions (Figure 4.3 [Cluster E], Figure 4.4). Interestingly these genes are primarily composed of flagellar biogenesis genes (*flgD*, *flgE1*, *flgE2*, *flgG2*, *flgH*, *flgI*, *flgJ*, *fliK*). Indeed, GSEA found that the KEGG category for flagellar assembly was enriched in both the Δfur and

$\Delta perR$ iron-limited conditions, with most of the flagellar assembly apparatus under opposing regulation in these two mutants (Table S4.7, S4.8, Figure 4.5). Further supporting the fact that these genes appear to be differentially regulated by CjFur/CjPerR is the fact that these genes were not differentially expressed in the $\Delta fur\Delta perR$ mutant (Figure 4.4), suggesting that each protein exerts its influence independently and GSEA did not identify flagellar assembly as being significantly enriched in the $\Delta fur\Delta perR$ mutant (Table S4.7, S4.8). This represents another manner by which the CjFur and CjPerR regulons may interact with each other. In the other cases where CjFur and CjPerR are known to co-regulate the same gene (e.g. *kata* repression), both transcription factors have the same effect on transcription. These interactions between the CjFur and CjPerR regulons further contrast with other bacteria, where their corresponding Fur and PerR regulons are separate from each other (Fuangthong and Helmann, 2003).

When comparing the regulons identified in this study using RNA-seq (CjFur, CjPerR and iron) to those identified using microarray profiling the most obvious observation is that the RNA-seq identified substantially more differentially expressed genes as compared to the microarray profiling (Figure 4.2,4.3,4.6). This is likely due to several factors. Firstly, the greater dynamic range offered by RNA-seq allows for the assignment of genes being differentially expressed at a lower fold change as compared to microarrays (1.5 fold as compared to 2 fold) which naturally leads to more genes being assigned as differentially expressed. Indeed, most of the genes identified using both techniques were those that showed the greatest level of differential expression. In addition, microarrays often have difficulties identifying differentially expressed genes which are expressed at high levels in both the test and control conditions due to signal saturation. For example, previous microarray profiling

did not detect any significant change in *ceuBC/ctuA* in the Δfur mutant and *ahpC* in the $\Delta perR$ mutant (Palyada *et al.*, 2009; Palyada *et al.*, 2004). This is despite the presence of Fur/PerR boxes in each promoter region and gel shift assays showing that each promoter was directly bound by either CjFur/CjPerR (Holmes *et al.*, 2005; Kim *et al.*, 2011; Miller *et al.*, 2008). As RNA-seq works by directly counting the reads aligning to each gene it does not suffer from this same limitation and correctly identified these genes as being differentially expressed in the CjFur/CjPerR mutants respectively.

In this study, PCA analysis was used to determine the relative similarity of each strain's biological replicates. This analysis identified two samples that appeared to be substantially different from their mates and that were subsequently discarded in the downstream analyses. Including these samples in the differential expression analysis leads to markedly different results depending on each strain. Including the erroneous $\Delta perR$ iron-replete sample in the differential analysis leads to an iron-limited gene profile (i.e. all iron acquisition genes become significantly up-regulated) whereas including the erroneous Δfur iron-limited sample leads to essentially no genes being identified as differentially expressed (data not shown). The potential for variability between samples influencing results is highlighted when comparing the iron regulon determined in this study with a previously published dataset (Butcher and Stintzi, 2013). In the previous study, the iron-replete samples showed a greater level of variability (Figure S4.4) that resulted in many genes not being identified as significantly differentially expressed. However, it should be noted that all the genes actually identified as being iron regulated were confirmed in this work. Moreover, while some of the variability present between these aforementioned samples could be deduced by plotting sample RPKMs and determining the R^2 value for each pair of replicates,

some samples identified as outliers in the PCA analysis could not be confidently distinguished from their mates based on R^2 values alone (data not shown). As such, higher order analyses for determining sample similarity (PCA vs R^2 values etc.) and multiple (>2) replicates for different samples are highly recommended for future studies employing RNA-seq for differential gene expression analysis. Another factor that could influence the effectiveness of RNA-seq analyses is the number of reads aligning to coding regions (i.e. sequencing depth). While sequencing depth is a difficult concept to define in transcriptomic studies (due to variations in the complexity of each unique transcriptome), increasing the number of reads aligning to each gene will likely increase the confidence in the downstream analyses (Haas *et al.*, 2012). The number of reads aligning to genes is 10x greater in this current study as compared to the previous RNA-seq study identifying iron regulated genes in *C. jejuni*. This greater sequencing depth appears to have more successfully captured the distribution of transcripts expressed at low levels in either iron-replete/iron-limited conditions and resulted in attenuated fold changes as compared to previous work (Figure 4.7, Panel B).

Conclusions

In conclusion, our results indicate that both the CjFur and CjPerR regulons are larger than previously believed. In particular, deletion of *perR* appears to result in the differential expression of a large group of genes in the absence of iron, suggesting that CjPerR may also regulate genes in an apo- manner; similar to what has already been demonstrated with CjFur. We have also found subsets of genes which are only differentially expressed when both CjFur and CjPerR are deleted. Finally, we have genes which appear to be simultaneously

activated by CjFur and repressed by CjPerR, which represents another manner in which these two regulons interact with each other.

Authors' contributions

JB performed certain RNA extractions, analyzed the data and drafted the manuscript. RH carried out most of the RNA extractions and helped draft/revise the manuscript. AV helped draft and revise the manuscript. AS designed the research project and helped draft and revise the manuscript.

Acknowledgments

This work was supported CIHR (MOP#84224) to AS and an Ontario Graduate Scholarship to JB. This work was also supported in part by a Norwich Research Park PhD studentship to RH, and the Biotechnology and Biological Sciences Research Council Institute Strategic Programme Grant BB/J004529/1. The authors thank also thank Annika Flint for her constructive comments on this manuscript.

Chapter 5: General Discussion

CjFur regulates a diverse group of functionally distinct pathways in *C. jejuni*

While transcriptional profiling can be a powerful tool for dissecting regulatory pathways, one of its major drawbacks is its inability to distinguish indirect from direct regulation. Thus the genes seen to be differentially expressed upon deletion of a transcriptional regulator are not always those that are directly regulated by this protein, but instead may be differentially expressed due to a myriad of unrelated factors. Moreover, as gene expression is often controlled by several different transcriptional regulators, deletion of only one may not result in a large difference in expression levels that can be easily detected. To overcome these issues with regards to CjFur we therefore applied ChIP-chip methodology (Chapter 2) to further probe the regulatory networks that this protein controls in *C. jejuni*.

We found that CjFur is involved in regulating a wide variety of functionally distinct pathways in *C. jejuni* (Figure 5.1). In particular we have identified CjFur as being involved in regulating various aspects of flagellar/capsule biogenesis, various oxidoreductases and non-iron metal acquisition systems. The fact that flagellar/capsule biogenesis appears to be directly regulated by CjFur provides a possible explanation as to why the Δfur mutant is defective in the commensal chick colonization model (Palyada *et al.*, 2004) as the flagella/capsule are well known *C. jejuni* colonization factors (Gilbreath *et al.*, 2011). The fact that CjFur also regulates various oxidoreductases can also be rationalized by CjFur's known role in oxidative stress defense (via *katA*) and may be related to the absence in *C. jejuni* (with the exception of CjPerR) of the regulator proteins that are typically involved in controlling these processes (e.g. OxyR, SoxRS) in other bacteria (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000).

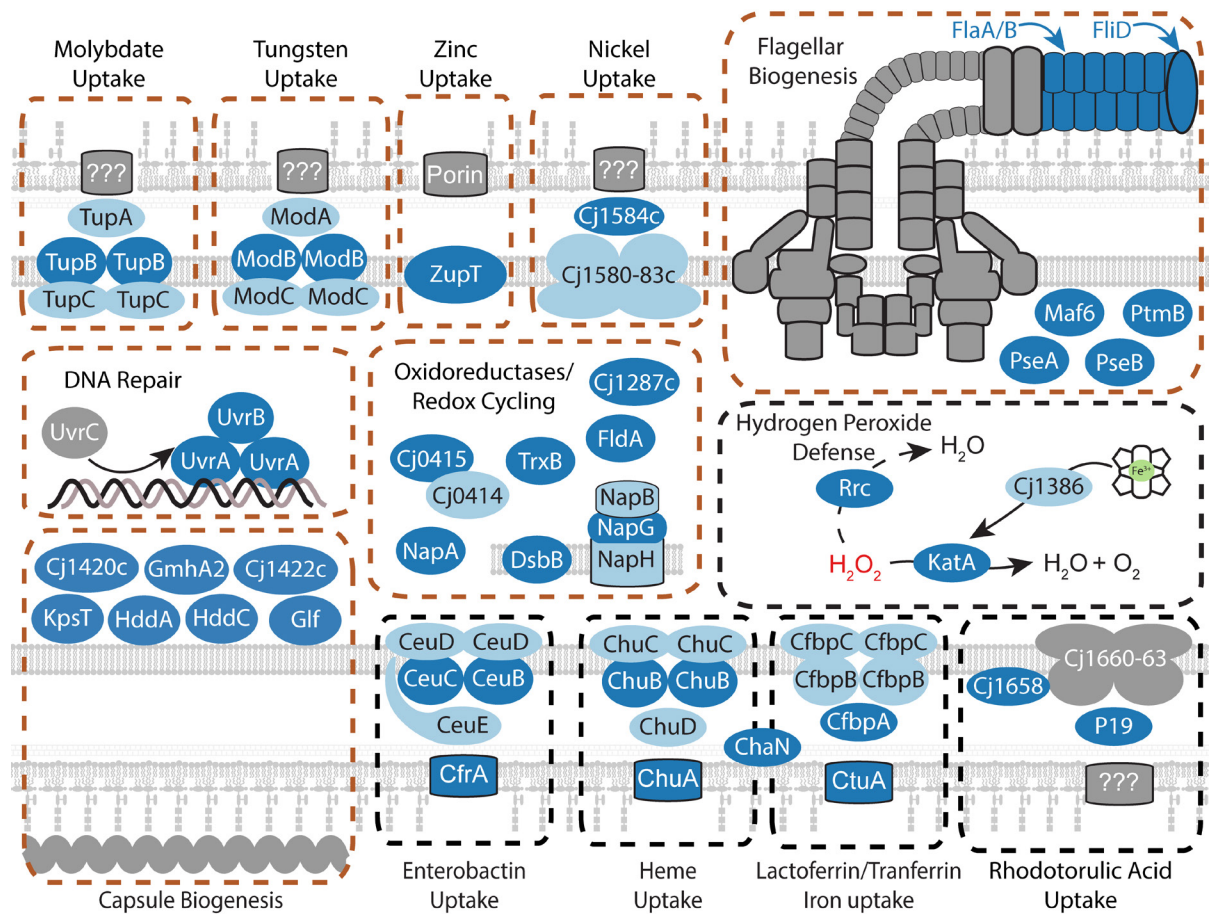


Figure 5.1 ChIP-chip reveals novel pathways directly regulated by CjFur

Selected pathways regulated by CjFur. Brown boxes highlight novel pathways identified in this work while black boxes are previously known pathways. Dark blue=Direct targets of CjFur, Light Blue=operon members of direct targets, Grey=genes not found to be directly regulated by CjFur.

Excess iron has been shown to cause DNA damage *in vivo* and also exacerbate UV-induced DNA damage (Chao and Aust, 1993). This could explain the reason why UvrAB are regulated by iron as these are the proteins involved in detecting DNA damage and recruiting UvrC for DNA repair (Truglio *et al.*, 2006).

Unexpectedly, CjFur was also found to directly regulate with genes that are involved in the influx of other metal ions, including those for molybdate/tungsten (*modB*, *tupB*), nickel (*cj1584c*) and zinc (*zupT*) (Howlett *et al.*, 2012; Smart *et al.*, 2009; Taveirne *et al.*, 2009). Intriguingly, the *C. jejuni* genome does not appear to contain annotated transcriptional regulators for either nickel or zinc (Gundogdu *et al.*, 2007; Parkhill *et al.*, 2000) and thus it is unclear how these systems are regulated in response to metal ion concentrations. *C. jejuni* does contain a transcriptional regulator for molybdate/tungsten, ModE. However ModE does not have an identified DNA binding motif so it is unclear how this protein actually binds to DNA in response to ligand binding (Smart *et al.*, 2009; Taveirne *et al.*, 2009). Thus *C. jejuni* may have adapted to the lack of classical transcriptional regulators for these processes by co-opting the CjFur protein for their proper regulation. Indeed, many molybdate/tungsten co-factored proteins also require iron for proper function (Hille, 2002) and the sole confirmed nickel dependant enzyme in *C. jejuni* is a NiFe dehydrogenase (Howlett *et al.*, 2012). The ZupT protein may also be involved in ferrous ion acquisition (in addition to FeoB), which would explain its Fur regulation (Rensing *et al.*, 2005). It is unclear whether the holo- or apo-versions of Fur would be involved in regulating these metal acquisition pathways. Curiously, it has been suggested that the exact metal ion bound by Fur family proteins may influence its binding affinity for DNA (Mills and Marletta, 2005). It is thus tempting to speculate that CjFur may be able to respond differently to various divalent cations and thus regulate

specific pathways based on the metal present in its regulatory site (Figure 5.3). While this would potentially represent a novel regulatory mechanism for Fur family regulators, it remains to be seen whether it exists *in vivo*.

Iron, CjFur and CjPerR all contribute to regulating *C. jejuni* flagellar biogenesis

The flagellar apparatus has long been established as an important colonization and virulence factor in *C. jejuni*. Accordingly, there has been significant research focused on how the flagellar structure is organized genetically and how the various components are regulated at the transcriptional level (Balaban and Hendrixson, 2011; Bingham-Ramos and Hendrixson, 2008; Chaudhuri *et al.*, 2011; Gilbreath *et al.*, 2011; Hendrixson, 2008; Hendrixson and DiRita, 2003, 2004; Joslin and Hendrixson, 2008; Kamal *et al.*, 2007; Lertsethtakarn *et al.*, 2011). Numerous studies have characterized the regulatory cascade that governs flagellar biogenesis: starting from the assembly of the type 3 secretion system (T3SS) to the efflux of the anti- σ^{28} factor FlgM (Figure 5.2). This work has revealed that iron is an important modulator of flagellar biogenesis and that many of the flagellar structural and regulatory elements are responsive to iron (Chapter 3). Moreover, this work has highlighted the role of CjFur and CjPerR in regulating flagellar biogenesis. CjFur was shown to directly regulate several structural components of the flagella (Chapter 2) and many components of the flagella are dysregulated in Δfur and $\Delta perR$ deletion mutants (Chapter 4). Interestingly, many of the flagellar genes which are differentially expressed in response to iron appear to be regulated by the apo- forms of CjFur/CjPerR rather than the holo-forms (Figure 5.2).

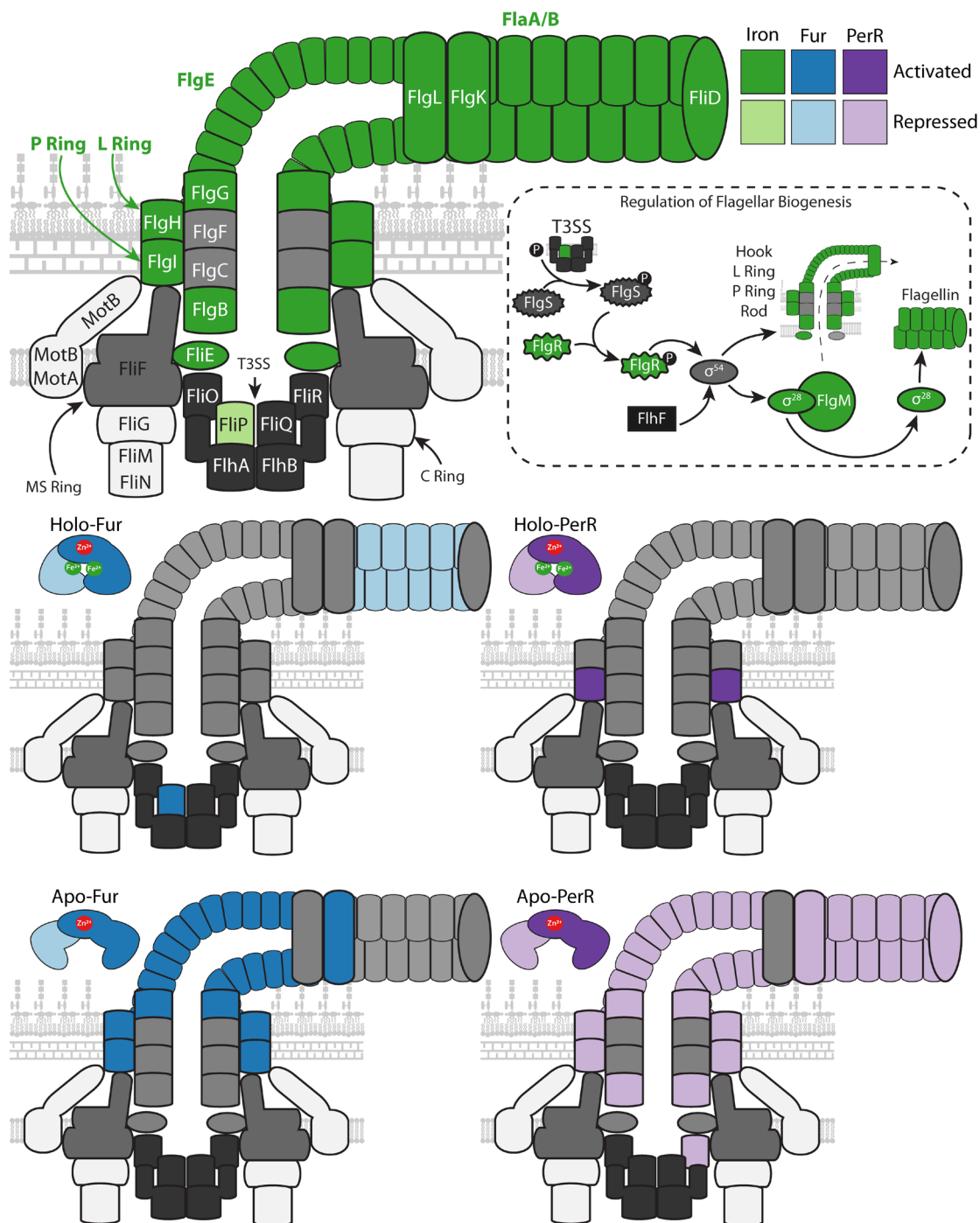


Figure 5.2: Iron, CjFur and CjPerR regulated components of the *C. jejuni* flagella

Structure of the *C. jejuni* flagella along with the regulatory cascade that controls the assembly of flagellar subcomponents is detailed above. Iron, CjFur and CjPerR activated genes are highlighted in green, blue and purple respectively based on data from Chapter 3 and 4. Figure inspired by (Gilbreath *et al.*, 2011; Minamino *et al.*, 2008; Neal-McKinney and Konkel, 2012).

Thus, the expression of flagella genes may be tuned by the overall level of iron available to the cell as measured by the ratio of the apo/holo forms of CjFur/CjPerR as opposed to the simple on/off system of gene expression normally associated with Fur family regulators. This would allow for a more controlled expression of flagellar genes, with a gradual increase in flagellar gene expression as internal iron levels rise. Whether this is truly the case *in vivo* remains to be fully explored, as well as how this potentially tunable expression system would interact with the known flagellar regulatory cascade.

ncRNAs in *Campylobacter jejuni*

One of the interesting findings of this work was the identification of multiple ncRNAs in *C. jejuni*. Moreover several of these identified ncRNAs have also been identified in various *C. jejuni* strains independently (Dugar *et al.*, 2013). RT-qPCR targeting some of the more promising ncRNA candidates revealed that several ncRNAs appear to be both Fur and/or iron responsive (Figure 3.5). This suggests that they may play roles in regulating iron metabolism in *C. jejuni* as has been described in other human pathogens such as *E. coli* and *P. aeruginosa*. Both *E. coli* RyhB and *P. aeruginosa* PrrF1/PrrF2 are regulated by EcFur and PaFur respectively (Masse and Gottesman, 2002; Masse *et al.*, 2007; Wilderman *et al.*, 2004), suggesting that this mode of Fur regulation may be more widespread than previously believed. However, in contrast to EcFur/PaFur, CjFur also regulates genes in an apo-dependant manner. It is therefore likely that apo-CjFur may also regulate ncRNAs and would thus be involved in regulating ncRNA functions that extend beyond iron metabolism. This may explain why many CjFur regulated genes are not responsive to iron conditions (Figure S3.3) and help rationalize the variety of functionally distinct pathways that were seen to be directly regulated by CjFur in the ChIP-chip study (Figure S2.1).

Novel regulatory paradigms for CjFur and CjPerR

The studies described in this thesis have greatly expanded our knowledge surrounding the regulatory mechanisms employed by both CjFur and CjPerR to perform their regulatory roles (Figure 5.3). We have shown that CjFur binds to regions that are transcriptionally repressed in a Δfur deletion mutant suggesting that they are directly activated by CjFur. It is still somewhat unclear how this activation occurs mechanistically as Fur is classically seen as a repressor that functions by blocking RNA polymerase access to promoters (Lee and Helmann, 2007). In *E. coli* holo-EcFur has been shown to activate ferritin gene expression by blocking H-NS mediated gene silencing (Nandal *et al.*, 2010). It remains to be seen whether CjFur also employs this strategy for activating gene expression. We have also demonstrated that CjFur is able to bind to genes that are differentially expressed in a Δfur deletion mutant independently from iron. Precisely how holo-Fur and apo-Fur can both regulate genes independently from each other remains unclear. With the exception of our work, there are only two confirmed genes that are regulated by apo-Fur (*H. pylori* *sodB* and *prf*) (Carpenter *et al.*, 2009a; Carpenter *et al.*, 2009b; Miles *et al.*, 2010a) which has hindered efforts to understand this regulation mechanistically. Recent work has suggested that HpFur binds to different grooves of the dsDNA strand and this is how the holo/apo forms can regulate different promoter regions (Agriesti *et al.*, 2014). As discussed above, CjFur also appears to regulate ncRNAs in *C. jejuni* in an iron dependent and independent manner. Currently it is unknown whether this regulation is direct or indirect as our ChIP-chip work was performed before these ncRNAs were identified.

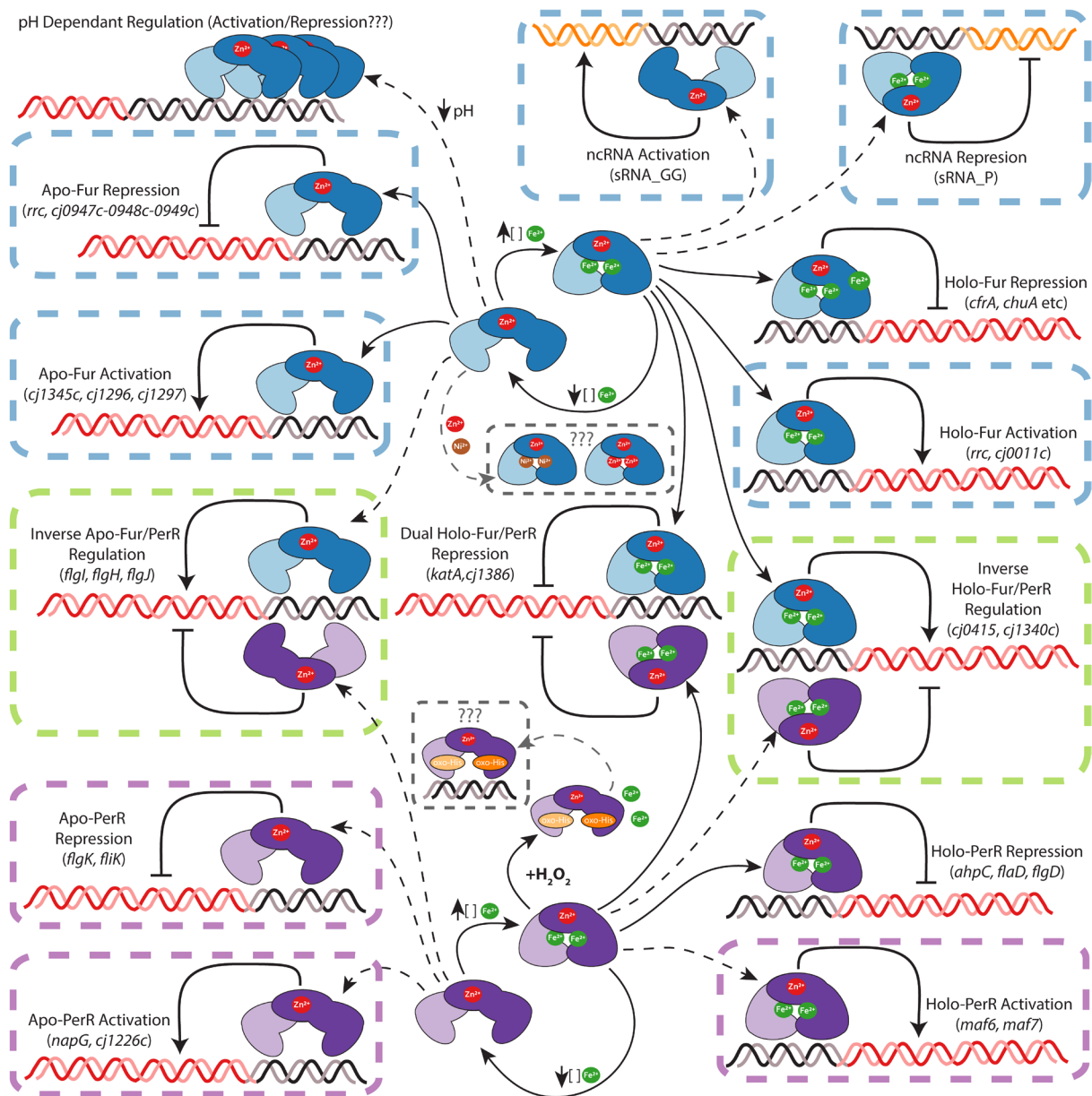


Figure 5.3: Updated model of Fur and PerR regulation in *Campylobacter jejuni*

Model for CjFur and CjPerR regulatory network incorporating the results obtained from this thesis work. Newly identified modes of regulation are enclosed with dashed boxes (blue=CjFur, purple=CjPerR, green=co-regulation). Regulatory mechanisms awaiting direct binding confirmation are represented by dashed arrows. Speculative modes of regulation raised by this work are shaded in grey.

Curiously this work revealed that CjPerR also appears to activate gene transcription and regulate genes in an iron independent manner. In fact, there were more genes differentially expressed in a $\Delta perR$ mutant under iron limited conditions than in the Δfur mutant, suggesting that a major facet of CjPerR's biological function has been thus far unappreciated. There are limited studies that have focused on direct CjPerR binding to promoter regions (Kim *et al.*, 2011) so some caution is required before definitively assigning these genes as being directly regulated by CjPerR. However, given the fact that CjFur regulates genes in an apo- manner it would not be unexpected if CjPerR was also able to perform this feat. An interesting corollary of potential apo-CjPerR regulation would be whether the oxo-CjPerR form of the protein would still be able to bind DNA. The current PerR regulatory paradigm is that oxidation of the PerR protein causes release of its regulatory ferrous ions and results in the PerR protein forming a planer structure that is unable to bind DNA properly. If apo-CjPerR is able to bind to DNA and regulate gene transcription, this would suggest that the oxo-CjPerR may still have the ability to influence gene expression (Figure 5.3).

This work has also identified novel co-regulation that appears to exist between the CjFur and CjPerR regulons. While it has long been known that both proteins co-repress *kata* transcription (van Vliet *et al.*, 1999), we have identified groups of genes that are simultaneously repressed by CjPerR and activated by CjFur. Moreover, this repression and activation appears to occur in both iron dependant and independent states. These additional co-regulated genes have further highlighted the complexity of these two interconnected regulons in *C. jejuni* as in other bacteria, Fur and PerR do not appear to co-regulate gene transcription. In particular, the inverse co-regulation of flagellar genes by apo-CjFur and

apo-CjPerR (as discussed previously) suggests that the CjFur/CjPerR regulons are also intertwined with a multitude of other regulatory pathways.

Future Directions

The research described in this thesis opens up several different avenues for further study. While it is now clear that apo-CjFur (and possibly CjPerR) possesses regulatory functions, it is unclear exactly how these regulatory functions are accomplished and where the proteins themselves bind (Figure 5.3). Detailed analyses of the binding regions of apo-CjFur/CjPerR (e.g. EMSA, ChIP-Seq) would reveal the motifs that the apo- proteins bind. These binding motifs could be compared to those identified for the holo- versions of these proteins to determine what allows the proteins to differentiate between an apo-/holo- binding site. Specific point mutants in both CjFur/CjPerR could be constructed to further characterize the mechanisms that differentiate apo vs holo binding.

Moreover, similar experiments could be conducted with different activating metals (e.g. nickel, zinc) to determine if the activating metal modulates the sequence specificity of CjFur/CjPerR and whether that has a biological consequence for *C. jejuni* (Figures 5.1, 5.3). It would be fascinating to determine whether *C. jejuni* has evolved to use a single metal sensor to regulate the uptake pathways for multiple different metals as *C. jejuni* is missing the obvious candidates for nickel or zinc regulation. The fact that CjFur/CjPerR also appear to regulate flagellar biogenesis (Figure 5.2) could also be further probed by taking advantage of the multiple reporter systems/constructs that have already been developed in *C. jejuni* for deciphering flagellar regulatory cascades (Hendrixson and DiRita, 2003; Joslin and Hendrixson, 2008).

Another noteworthy avenue of research would be to determine whether oxo-CjPerR is capable of performing regulatory functions (Figure 5.3). The classical PerR paradigm is that oxo-PerR is unable to bind DNA, cannot be repaired and is presumably degraded by the cell. However as apo-CjPerR does appear to have regulatory functions it would be interesting to see if oxo-CjPerR is able to replicate apo-CjPerR binding. An even more curious result would be if oxo-CjPerR had its own unique regulatory targets. This would transform CjPerR regulation from being more “passive” (e.g. simply blocking transcription until oxidized) to more “active” (e.g. turning on transcription in response to oxidation) in the mold of OxyR.

Further work could also be focused on characterizing the type of regulation by CjFur on these ncRNAs (Figure 5.3). The regulatory targets of these ncRNAs could be identified through the use of either deletion and/or inducible constructs coupled to genome wide profiling technologies (RNA-seq, mass spectrometry). How these ncRNAs influence gene expression could then be determined by various molecular biology techniques (pulse chase experiments to measure transcript stability, fusing target 5'/3' UTRs to reporter gene constructs etc.). As *C. jejuni* does not have an identified Hfq protein for facilitating RNA-RNA interactions, the mechanisms underlining the action of *C. jejuni* ncRNAs may be novel and reveal currently unappreciated means of bacterial gene regulation that have not yet been identified.

Overall conclusions

In conclusion, these results have revealed the breadth and complexity of the role of iron, Fur and PerR in regulating gene expression in *C. jejuni*. In particular, we have demonstrated a direct role for apo-CjFur in regulating gene expression in *C. jejuni* and provided a structural rational for this regulation. We have also proposed a role for CjFur in

regulating a diverse group of functions including DNA repair, capsule biogenesis and non-iron cation transport. Our work has further characterized the transcriptome of *C. jejuni* and identified potential ncRNAs that may perform unappreciated regulatory functions controlling gene expression in response to iron limitation. We have revealed that CjFur/CjPerR (and thus iron as well) are involved in the regulation of flagellar biogenesis. Finally, these results provide further evidence indicate that both the CjFur and CjPerR regulons are larger than previously believed and provide evidence for the multiple modes of regulation that each protein appears to use to properly regulate gene function.

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For Butcher J*, Sarvan S*, Brunzelle J, Couture JF, Stintzi A. (2012) “The structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation” PNAS 109(25):10047-52 [PMID:22665794](#):

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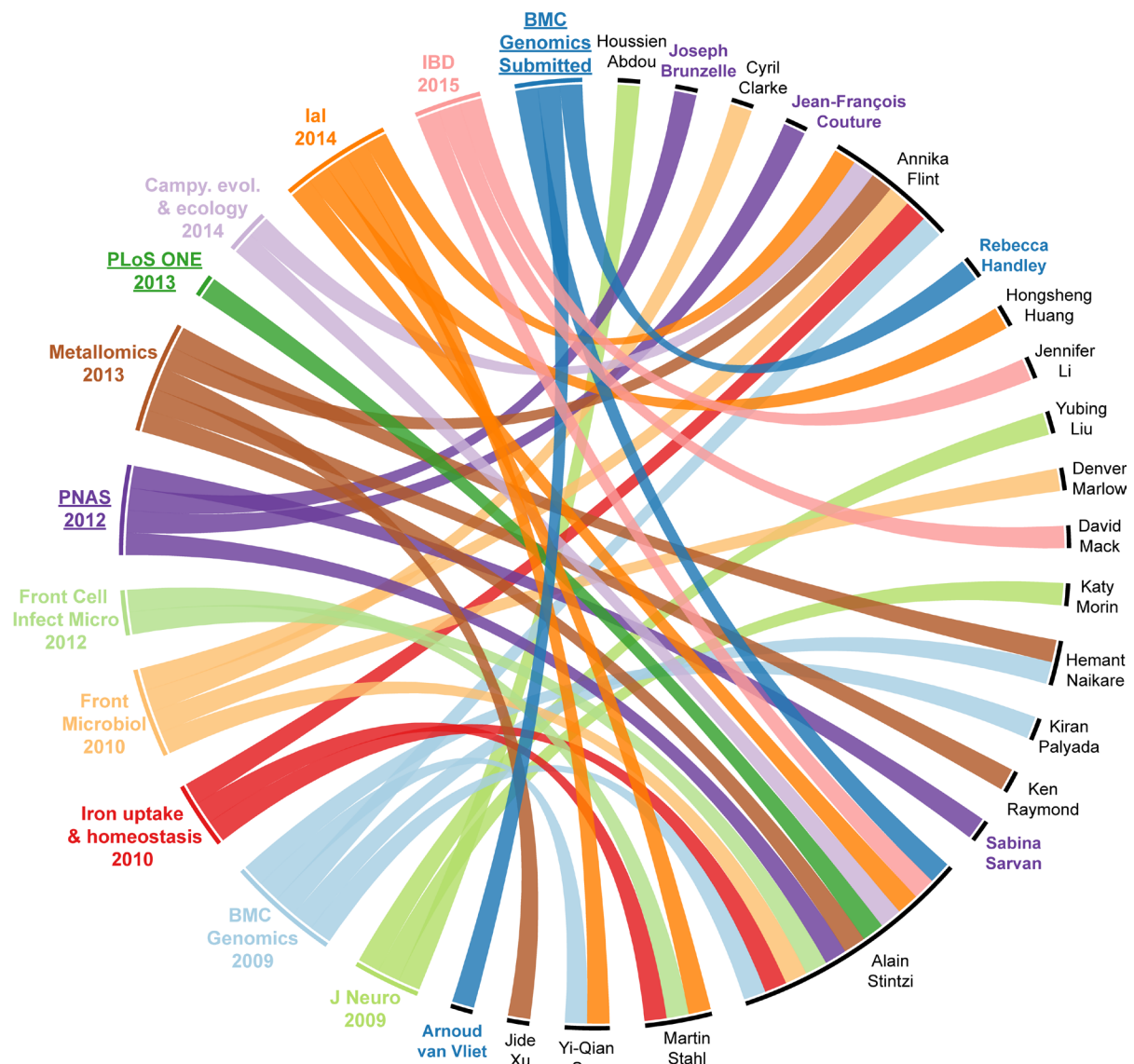


Figure 6: Coauthors during the course of PhD research

Publications completed during the course of the candidate's PhD research are presented along with the co-authors for each publication. Co-authors are linked to their papers with ribbons. Publications serving as sections of this thesis are underlined. See Curriculum Vitae for full citation details for each publication.

Appendices

Supplemental Information for Chapter 2

Supplemental Materials and Methods for Chapter 2

Bacterial Strains and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table S2.4. All *C. jejuni* strains were grown at 37°C under microaerophilic conditions (83% N₂, 4% H₂, 8% O₂ and 5% CO₂) in Mueller-Hinton (MH) broth, MEM α medium supplemented with 10 mM pyruvate and 40 μ M FeSO₄, or on MH-agar plates. Media were supplemented as required with chloramphenicol (20 μ g/mL) or kanamycin (10 μ g/mL). Plasmids were constructed and propagated in *E. coli* DH5 α . All *E. coli* strains were grown at 37°C in Luria-Bertani (LB) broth or on LB-agar plates. When relevant, ampicillin, chloramphenicol or kanamycin was added to the *E. coli* LB medium at a concentration of 100 μ g/mL, 10 μ g/mL or 50 μ g/mL, respectively.

Construction of *C. jejuni* Fur expression vectors

The *fur* gene was PCR amplified from *Campylobacter jejuni* TGH9011 with Pfx (Invitrogen) using primers furSE and furAS which contain a BsaI restriction site on their ends (Table S2.2). The TGH9011 Fur contains a single amino acid substitution (T42I) as compared to NCTC11168 Fur. This site is not conserved across Fur proteins (Figure 2) and would not be expected to influence Fur binding. The PCR products were purified using a Qiagen PCR purification kit and was then cut with BsaI and ligated into a pASK-IBA7 vector to create pASK-Fur. This vector was used to transform *E. coli* DH5 α by electroporation. Transformed cells were selected by plating on LB agar plates supplemented with 100 μ g/mL ampicillin. Selected colonies were grown in LB broth supplemented with

100 µg/ml ampicillin to mid-log phase and aliquoted for storage at -80°C. The resulting plasmids were sequenced to ensure the presence of unaltered *C. jejuni fur* sequence. A strain containing the correct Fur sequence was designated as AS1130 and subsequently used to produce CjFur protein for antibody production. A different construct was used to purify the Fur protein for crystallographic studies to avoid the presence of purification tags (see below). Briefly, the *C. jejuni fur* sequence from pASK-Fur was PCR amplified and cloned in-frame with a PCR sequence containing a Strep-Tag followed by the sequence for SMT3 (SUMO). The resulting Strep-SMT3-CjFur cassette was then digested NdeI and XhoI, cloned into pET3d and subsequently transformed into Rosetta cells.

Expression and Purification of *C. jejuni* Fur protein for antibody production

CjFur protein was expressed and purified from AS1130 according to the manufacturer's instructions (IBA, *Strep*-tag). Briefly, cells were grown to mid-log phase in LB broth at 37°C with shaking at 200 rpm and protein expression was then induced by adding anhydrotetracycline to a final concentration of 200 ng/mL. The cells were incubated for 3 hours at 37°C with shaking and then pelleted by centrifugation. The cells were resuspended in 100 mM Tris-HCl pH 8.0 and 150 mM NaCl buffer (Wash Buffer) with the addition of Complete Mini protease inhibitor (Roche), incubated with 5 µg/mL DnaseI and 1 mg/mL lysozyme for 30 min at 4°C and subsequently lysed by sonication. The lysates were applied to a Strep-Tactin Sepharose column, washed 5 times with wash buffer and eluted with wash buffer supplemented with 2.5 mM desthiobiotin. Fractions were analyzed by SDS-PAGE to determine the presence of purified protein. CjFur eluted as a doublet on SDS-PAGE gels as has been previously observed for Fur family proteins (Bsat and Helmann, 1999; Tiss *et al.*, 2005). Both bands were excised from SDS-PAGE gels and sequenced using

LC-MS/MS (Ottawa Institute of Systems Biology) and found to be *C. jejuni* Fur. The obtained protein was determined to be ~95% pure and was used to generate polyclonal rabbit antibodies against *C. jejuni* Fur.

Anti-CjFur Antibody Production

Anti-CjFur antibodies were produced by Invitrogen Corporation by inoculating rabbits with 1 mg of purified Fur protein. Bleeds were taken at 4, 8 and 10 weeks after inoculation. The bleeds were pooled and further purified with an affinity column constructed from purified Fur protein. An ELISA assay was used to confirm a specific response to *C. jejuni* Fur and also indicated that there was no pre-existing reactivity to Fur protein before inoculation.

Western Blot Analysis

C. jejuni NCTC 11168 strain and Δfur mutant were grown to mid-log phase in biphasic MH cultures. The cells were pelleted by centrifugation, resuspended in PBS buffer with Complete Mini Protease Inhibitor (Roche) and lysed by sonication. Protein content was quantified using a Quant-It protein assay kit (Invitrogen). Ten μ g of each cell lysate along with 100 ng of purified CjFur protein was run on a 12.5% SDS-PAGE gel for 60 min at 120V and subsequently transferred to a PVDF membrane at 100V for 60 min. The membranes were blocked overnight at 4°C in 5% skim milk powder. The membranes were then probed with 1:1000 anti-CjFur antibody for one hour followed by washing and incubation with 1:10,000 anti-Rabbit-IgG antibody conjugated with HRP. Blots were incubated with Pierce ECL (Fisher) chemiluminescent substrate according to the manufacturer's instructions and visualized using x-ray film.

Immunoprecipitation of DNA-Fur complexes

C. jejuni NCTC 11168 and Δfur strains were grown to mid-log phase in MEM- α medium supplemented with 10 mM sodium pyruvate and 40 μ M FeSO₄ under microaerophilic conditions. Previous work had demonstrated that these iron replete conditions would result in repression of known Fur regulated targets and thus ensure that Fur protein would be bound to its target sequences and allow for comparison with our previously obtained transcriptomic data. However, as cells were harvested during midlog phase, it is unlikely that all Fur proteins would be metallated and a fraction of the Fur would be present in the apo- form. All cross linking steps were performed under the same microaerophilic conditions. Briefly, the cells were treated with 1.1% v/v formaldehyde for 10 min to crosslink protein-DNA complexes. The cross linking was stopped with the addition of 0.125 M glycine for 15 min. The cells were then pelleted and washed with cold RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.1 % Na deoxycholate and 0.1% NP-40). Bacterial pellets were resuspended in 2 mL RIPA buffer and 200 μ L of a 40 mg/mL bacterial protease inhibitor was added (Sigma). Cells were lysed and DNA fragmented by sonication. Cell debris was removed by centrifugation for 7 min at 12 000 rpm and samples were run on agarose gels to determine degree of DNA fragmentation. Sonication times were determined empirically to generate an average DNA fragment size of ~500 bp. Fragmented DNA lysates were preincubated with Protein G agarose (Sigma) for 3 hours at 4°C. The agarose was removed by centrifugation and the cleared lysates incubated with 10 μ g of anti-Fur antibody overnight at 4°C. Protein G agarose was added for 4 hours at 4°C, after which the agarose-antibody-chromatine complex was harvested by centrifugation and washed five times with cold RIPA buffer, followed by resuspension in 500 μ L of 50 mM

TrisHCl pH 7.5, 10 mM EDTA, 1% SDS. Elution of DNA was performed by incubating this suspension for 20 min at 65°C, followed by centrifugation at 13 000 rpm to remove the agarose and cross-linking was reverted by incubation of the supernatant for 10 hours at 65°C. Finally, the DNA was purified and washed five times with 450 µl ddH₂O using a Micron-YM30 filter (Millipore), and then dried with a SpeedVac vacufuge and resuspended in 55 µl H₂O. Chromatin immunoprecipitation with the Δfur mutant constituted our negative control.

Amplification and dUTP labelling of immunoprecipitated DNA

Immunoprecipitated DNA was amplified following the protocol from Agilent (Agilent Mammalian ChIp-on-chip V10.0). Briefly, the DNA ends were blunted with T4 DNA polymerase (Invitrogen) and linkers (Table S2.2) were ligated on the ends by T4 DNA ligase (NEB). The resulting DNA pool was PCR amplified using JW102 as a primer. An additional round of PCR amplification was used to generate sufficient quantities of DNA for microarray analysis. Once sufficient quantities of DNA had been generated, the DNA was labelled with aminoallyl-dUTP with the Bioprime labelling system (Invitrogen) for 3 h at 37°C and subsequently purified using a Qiagen PCR purification kit as per the manufacturer's instructions.

Probe labelling, microarray hybridization and analysis

The aminoallyl-labelled DNA was concentrated under vacuum using a SpeedVac and resuspended into 10 µL with 0.1 M sodium carbonate buffer (pH 9). The aminoallyl-labeled cDNA was coupled to indocarbocyanine or indodicarbocyanine dye (GE Healthcare) as previously described. The fluorescently labelled cDNA was purified using Qiaquick PCR spin columns according to the manufacturer's instructions (Qiagen). Differentially labelled

immunoprecipitated DNA derived from *C. jejuni* NCTC11168 or Δfur were pooled, dried under vacuum and dissolved in 36 μ L of hybridization buffer (5x SSC buffer (1x SSC is 0.15 M NaCl and 0.015 M sodium citrate at pH 7), 0.1% SDS, 25% formamide, and 25 μ g salmon sperm DNA). The labelled cDNA pool was hybridized to a *C. jejuni* NCTC 11168 microarray. The construction and validation of the *C. jejuni* microarrays has been previously described (Stintzi, 2003). The hybridization was performed under a cover slip using a pre-hybridized microarray slide (slides which have been incubated at 42°C for 45 min in 5x SSC buffer, 0.1% SDS, 25% formamide, and 1% bovine serum albumin). Following an 18 hour incubation at 42°C in a humidified chamber (Arrayit, Sunnyville, Calif.), the microarray slides were washed consecutively in 2x SSC – 0.1% SDS for 5 min, 0.1x SSC – 0.1% SDS for 10 min at room temperature, and four times in 0.1x SSC for 1 min at room temperature. Finally, the slides were washed with distilled water, dried by centrifugation and scanned with a laser-activated confocal scanner (ScanArray Gx, Perkin Elmer) at a 10 μ m resolution.

ChIP Microarray Data Analysis

The signal intensities for each spot were collected using ScanArray Express software (PerkinElmer Life Sciences). Spots within regions of printing or hybridization anomalies were excluded from the analysis. The raw fluorescence intensity values were background subtracted. Spots with background-subtracted values below 3 times the standard deviation of the local background in both channels were also excluded from further analysis. Subsequently, the background-subtracted fluorescent intensity in each wavelength (channel 1 for indocarbocyanine (Cy3) and channel 2 for indodicarbocyanine (Cy5)) was normalized using the MIDAS software (available from TIGR, <http://www.tigr.org/software/>) and by applying an iterative log mean centering algorithm. Microarray data from four independent

biological experiments were collected, yielding 21 measurements per gene (since the probe for each gene is printed three times on each microarray). Genes with less than four non-zero measurements were excluded from further analysis. Finally, the ratio of the channels 2 to 1 was converted to $\log_2$, and the data was statistically analyzed with the empirical Bayes method as previously described (Long *et al.*, 2001; Palyada *et al.*, 2009; Palyada *et al.*, 2004). Chromosomal regions exhibiting reproducible and statistically significant enrichment were classified as CjFur binding sites. The locations of the Fur-binding regions were manually refined by taking into consideration gene annotation, operonic structure and transcriptional direction. Given that our ChIP protocol generated fragments of 0.5-1.0 KB in size and ~62% of *C. jejuni* genes are < 1 KB in length, we were not able confidently distinguish between intergenic and intragenic binding. Genes were considered to be enriched if they had a fold enrichment (ratio (Fur-IP from *C. jejuni* wild-type)/(Fur-IP from Δfur) ≥ 1.5 (NCTC/ Δfur)) with a Bayesian p value ≤ 0.001 .

qPCR confirmation of ChIP-on-chip targets.

Selected genes were confirmed for enrichment using quantitative PCR. DNA template was prepared as described for the microarray analysis except that the immunoprecipitated DNA was not amplified or labelled. Both immunoprecipitated and input genomic DNA from NCTC 11168 and Δfur cultures were analyzed to ensure equal loading and template quality. qPCR reactions were done using Taq polymerase in a final volume of 50 μ L. The reaction conditions were as follows: 1x PCR reaction buffer, 11.4 mM $MgCl_2$, 0.06% TritonX-100 (Fisher), 4 nM ROX reference dye, 0.16x SYBR green, 8 U/mL Taq, 0.2 μ M of each primer and either 1 ng immunoprecipitated DNA or 100 ng input genomic DNA. All reagents were obtained from Invitrogen unless otherwise noted. Fold enrichment

was calculated as previously described (Palyada *et al.*, 2009) using the $\Delta\Delta C_T$ method, with primers for *ilvC* gene acting as an endogenous control. Similar fold changes were obtained when using *ahpC* as an alternate endogenous control (data not shown).

COG Functional Enrichment.

CjFur ChIP enriched genes were categorized according to their annotated COG function. COG groups that contained at least 3 members were selected for further analysis to determine if any groups were overrepresented within the Fur regulon. Functional enrichment of COG categories in Fur regulated genes was determined by performing a one-tailed Fisher's Exact test with a Bonferroni correction and a $p < 0.05$ was considered significant.

Determining Fur Transcriptional Regulation

Previous work has used transcriptional approaches to define the Fur regulon by comparing the transcriptome of wildtype NCTC11168 to an isogenic *fur* deletion mutant in the presence and absence of iron. This allowed for the identification of genes that were differentially expressed in a Fur and iron dependant manner (holo-Fur regulation) and a Fur and iron independent manner (apo-Fur regulation). The analysis of this data was repeated as previously described (Palyada *et al.*, 2004) and used to assign identified Fur ChIP targets as holo-Fur activated/repressed, apo-Fur activated/repressed and unknown (Supplemental Table 1). The regulation of several genes was also assayed using RT-qPCR (see below for details).

Bioinformatic Analysis of Potential Fur Binding Sites

The promoter regions of CjFur ChIP enriched transcriptional units were analyzed for the existence of potential CjFur binding sites by the MEME suite of analysis tools

(<http://meme.nbcr.net>). Transcriptional units were analyzed as a whole and also as subgroups based on their potential mode of CjFur regulation (apo-CjFur activation/repression, holo-CjFur activation/repression). Regions consisting of 250 bp upstream the predicted start codon (both with/without gene overlap) of each transcriptional unit were retrieved from the RSAT sever (<http://rsat.ulb.ac.be/>) and analyzed for potential regulatory motifs using MEME. In the case of transcriptional units in operonic structure, we also tested for consensus sequences present in the promoter region of the predicted lead transcript. Consensus sequence logos from identified motifs were constructed using WebLogo (<http://weblogo.berkeley.edu/>). The transcriptional units that were used to generate the holo-CjFur repression motif consisted of *kataA*, *chuA*, *cj1613c*, *trxB*, *cj0177*, *cj0176c*, *ceuB*, *cj1384c*, *cj1627c*, *cj0263*, *cj0262c*. The holo-CjFur activation motif was generated using *cj0145*, *cj1097*, *cj1349c*, *cj1476c*, *cj1588c*, *cj1650*, *flaB*, *flaG*, *napA*, *rrc*. (Palyada *et al.*, 2009; Palyada *et al.*, 2004)

RT-qPCR analysis of Fur ChIP Targets

Total RNA was extracted from *C. jejuni* NCTC11168 and Δfur cultures as previously described (Palyada *et al.*, 2004). Briefly, cells were grown to mid-log phase in MEM- α medium with 10 mM sodium pyruvate and either with or without 40 μ M FeSO₄ supplementation under microaerophilic conditions. Cells were harvested by adding 10% cold RNA stop solution was added to the cultures (10% buffer saturated phenol in absolute ethanol) and cells were pelleted by centrifugation at 6000g. Cells were resuspended in TE buffer and RNA extracted using a hot-phenol chloroform protocol. The extracts were washed five times in 80% ethanol after overnight RNA precipitation and RNA pellets were resuspended in TE buffer. RNA extracts were treated with DNase I to remove contaminating genomic DNA. Treated samples were purified using the RNeasy kit from Qiagen and tested

for the absence of DNA using PCR. RT-qPCR was performed on an Applied Biosystems 7300 thermocycler using the QuantiTect SYBR green RT-PCR kit from Qiagen as described previously (Palyada *et al.*, 2009). Gene fold changes were determined according to the $\Delta\Delta C_T$ method using *slyD* as an endogenous control. The primer sets used for *dsbB*, *cj1345c* and *rrc* are listed in Table S2; the primers for *katA* and *slyD* have been described elsewhere (Palyada *et al.*, 2009; Ritz *et al.*, 2009). Genes were considered differentially expressed if their expression was altered by >1.5 fold with a *p* value <0.05. Genes that had positive fold changes in NCTC11168 as compared to the Δfur mutant are activated; negative fold changes are indicative of repression. Genes differentially expressed under iron limited conditions were considered as being apo-Fur regulated and genes differentially expressed under iron replete conditions were considered as being holo-Fur regulated.

CjFur purification and crystallization.

CjFur was overexpressed in Rosetta cells, as a Strep-SUMO fusion protein following induction with 0.1 mM IPTG for 3 hours at 37°C. Rosetta cells were centrifuged at 3000 rpm for 30 minutes and resuspended in PBS buffer (pH 7.5). Cells were lysed by sonication and centrifuged at 16000 rpm for 30 minutes and the supernatant was clarified by filtration. The filtered solution was applied onto Strep-TACTIN (Novagen) beads and the column was washed with 15-column volumes (CV) of PBS. The fusion protein was eluted according to manufacturer's instructions and the Strep-SUMO tag was removed using the SUMO specific protease ULP1 by a 16 hour incubation at 4°C. CjFur was further purified by applying the proteins onto S-sepharose (GE Healthcare) equilibrated in 20 mM Citrate pH 6.0 and 5 mM beta-mercaptoethanol (Buffer A). CjFur was eluted with 10 CV of buffer A supplemented with 300mM NaCl and further purified by size-exclusion chromatography (Superdex 75).

The protein eluted as a single peak at an apparent molecular weight of ~34 kD, corresponding to a dimer. CjFur fractions were pooled and concentrated to 10 mg x ml⁻¹. Plate-shaped crystals were grown in 200 mM MnSO₄ and 10-20% PEG3350 at 4°C. Crystals were harvested in the mother liquor supplemented with 20% ethylene glycol and flash-frozen in liquid nitrogen.

ICPMS Metal Analysis

Inductively coupled plasma mass spectrometry (ICPMS) was performed at the Quantitative Bioelemental Imaging Center (QBIC) using a ThermoFisher X Series II ICP-MS. Each MS spectra was recorded in triplicate using two serial dilutions (1/20 and 1/100) of purified CjFur. Table S2.2 shows that the CjFur preparation is predominantly found in complex with Zn²⁺ with trace amounts of Cu²⁺.

Data collection and crystal structure determination.

The datasets were collected at the Life-Science Collaborative Access Team beamline at the Advance Photon Source, Chicago. CjFur's structure was determined by single wavelength anomalous diffraction at the zinc peak wavelength (Table S3). The reflections were processed and scaled using HKL2000 (Otwinowski and Minor, 1997) and four zinc atoms were identified and refined using the SHELX C/D programs (Sheldrick, 2010). Phases were calculated using SHELX-E and the Arp/Warp program was used to generate the initial model. Two chains (referred therein as protomer A and B), were traced to near completion (140 amino acids for chain A and 120 residues for chain B). This initial protein structure was then used as a search model for molecular replacement using Phaser. The missing residues were modeled in the calculated phases using COOT (Emsley and Cowtan, 2004) and the

structure was further refined using Refmac (Winn, 2003). The quality of the model was assessed using Molprobity (Chen *et al.*, 2010; Davis *et al.*, 2007) and analysis of protein backbone phi/psi angles, calculated using Molprobity indicates that 97.8% of non-glycine residues are found in the favoured regions of the ramachandran plot.

Gel mobility assays

Holo-Fur: Forward and reverse Cy5 labelled primers corresponding to a 40 bp DNA fragment of the *katA* promoter region were purchased from Eurofins MWG Operon. Oligonucleotides were annealed by incubation at 95°C for 10 minutes and slowly cooled down to room temperature. For the gel shift assays, 0, 5, 50 and 100 nM of purified recombinant CjFur were incubated with 50 µM of MnCl₂ for 30 minutes on ice in binding buffer (20 mM Bis-Tris Borate pH 7.4, 50 mM KCl, 3 mM MgCl₂, 5% glycerol, 0.1% Triton X-100 and 50 µM MnCl₂). 1nM of Cy5 labelled DNA fragment and 1 µg of poly (dI-dC) were then added to the protein and incubated for 30 minutes on ice. Samples were run for 50 minutes at 100V on a 6% non-denaturing polyacrylamide gel (19:1) at 4°C. Gels were freshly prepared with 100 mM Bis-Tris Borate pH 7.4 and 100 µM MnCl₂ (buffer A) and pre-run for 30 minutes at 150V at 4°C using buffer A as running buffer.

Apo-Fur: The promoter regions upstream of the *rrc* and *Cj1345c* operons (~200bp) were PCR-amplified from *C. jejuni* using the following primer pairs with the forward primers labelled with Cy5: Cjrrc_For/Cjrrc_Rev and Cj1345c_For/Cj1345c_Rev (Table S2). (Eurofins MWG Operon). Approximately 2.5 nM of Cy5 labelled DNA fragment was incubated with increasing amount of purified recombinant CjFur (0, 50, 100, 200 and 1000 nM) for 40 minutes on ice in a binding buffer devoid of MnCl₂. Samples were run for 60 minutes at 100V on a 5% non-denaturing polyacrylamide gel (19:1) at 4°C.

All gel shift assays were scanned using Typhoon Scanner (GE Healthcare Typhoon Trio) and analyzed using Image Quant TL software.

Supplemental Results for Chapter 2

Purification of *C. jejuni* Fur protein and generation of polyclonal anti-Fur antibodies suitable for ChIP experiments.

We expressed and purified the *C. jejuni* Fur protein in order to generate specific anti-CjFur antibodies. The obtained protein was determined to be ~95% pure and was used to generate polyclonal rabbit antibodies against *C. jejuni* Fur. Western blotting demonstrated that the anti-Fur antibodies were specifically targeted against the Fur protein, with a complete loss in reactivity in the Δfur deletion mutant (Figure S5). Additional testing demonstrated that these antibodies do not react with the Fur homolog PerR that is also present in *C. jejuni*.

Supplemental Figures for Chapter 2

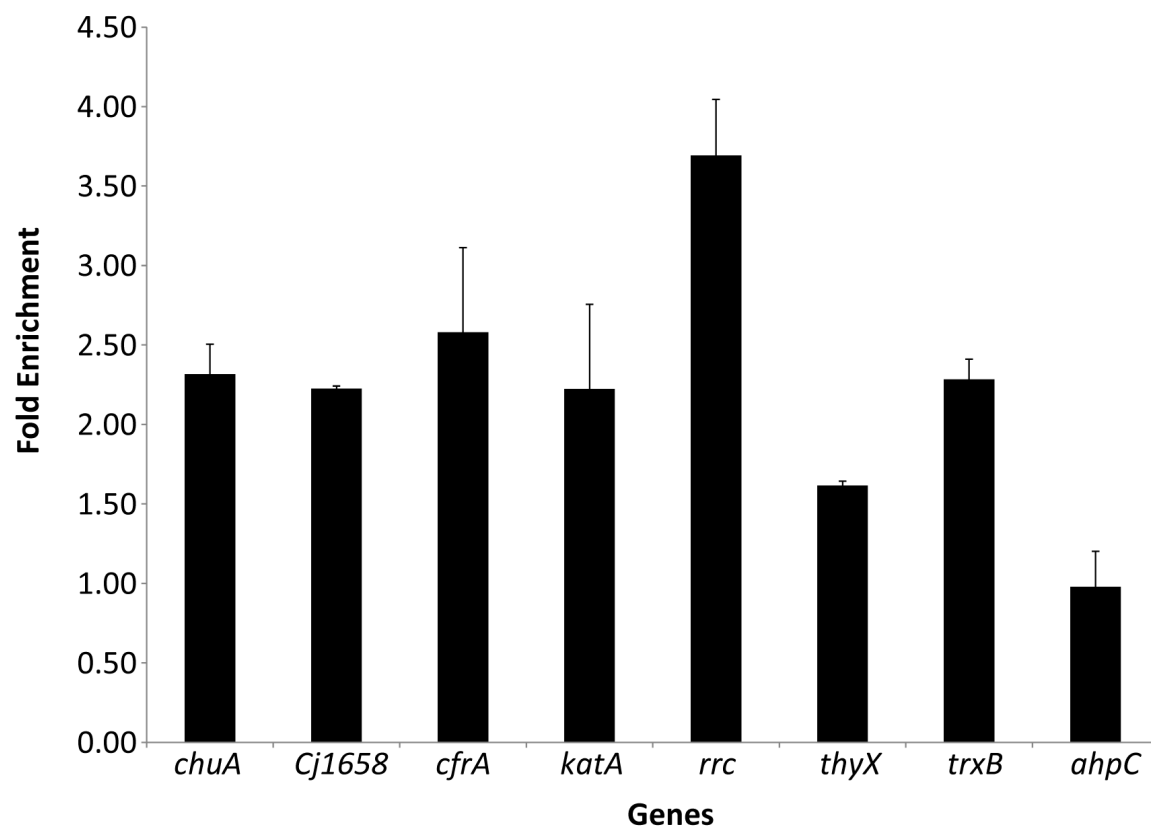


Figure S2.1: qPCR validation of selected Fur-ChIP targets

Selected genes were chosen for qPCR validation of CjFur enrichment. The fold enrichment was determined by the $\Delta\Delta C_T$ method as compared to a negative control (*ilvC*). The CjFur enriched genes were also seen to be enriched by qPCR analysis (*chuA*, *Cj1658*, *katA*, *rrc*, *thyX*, *trxB*) while an additional negative control (*ahpC*) did not show enrichment.

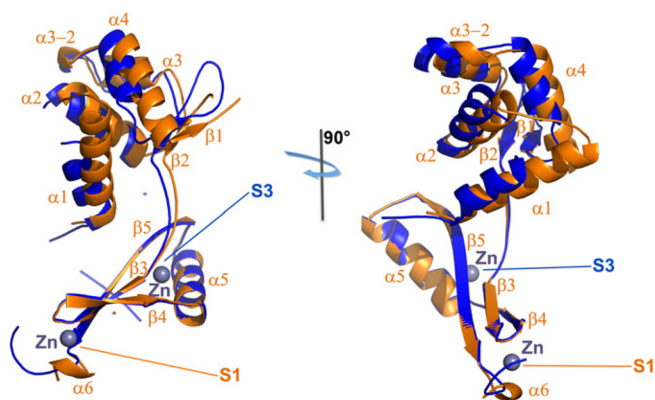


Figure S2.2: CjFur dimer is asymmetric

Shown are two orthogonal views of CjFur aligned protomers in which β -strands, α -helices and metal binding sites are rendered as in Supplemental Figure 2.3.

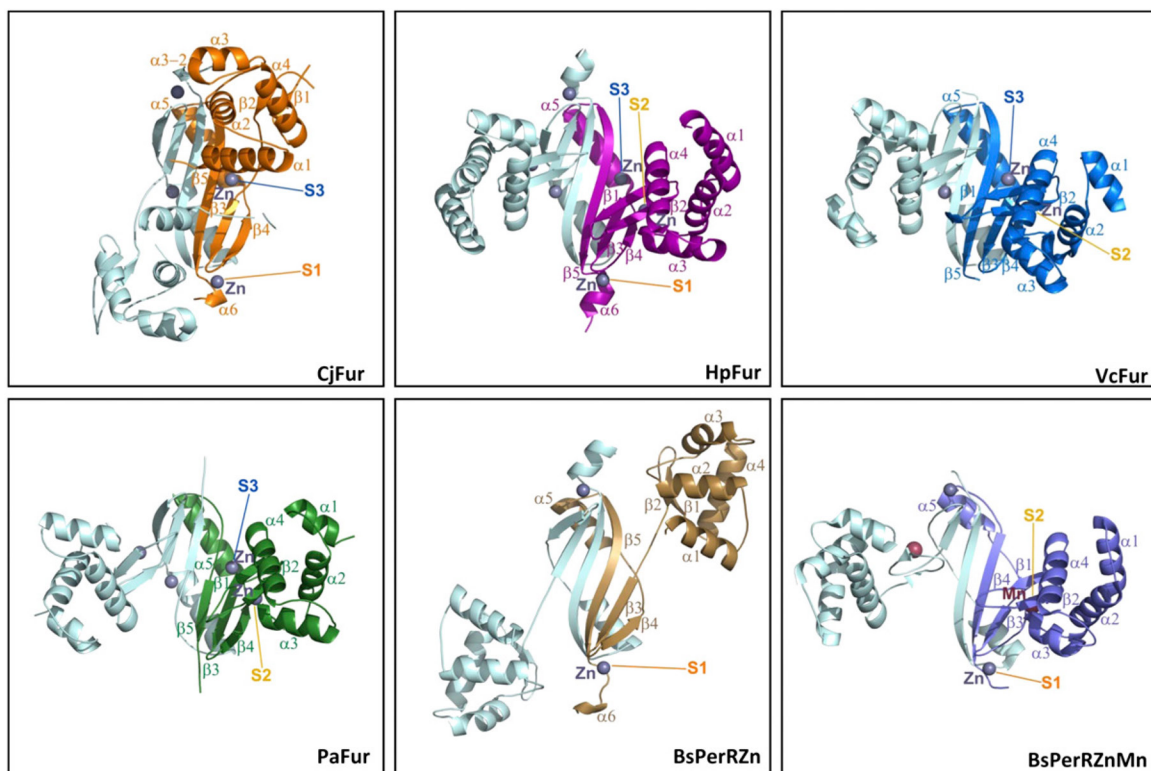
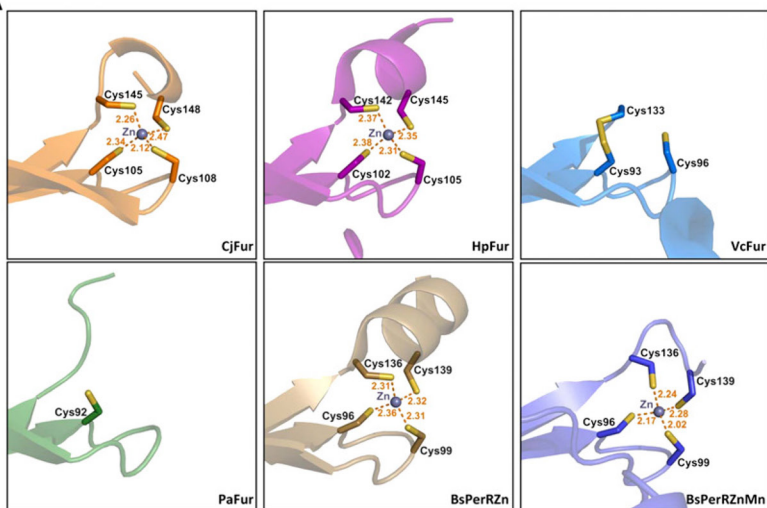


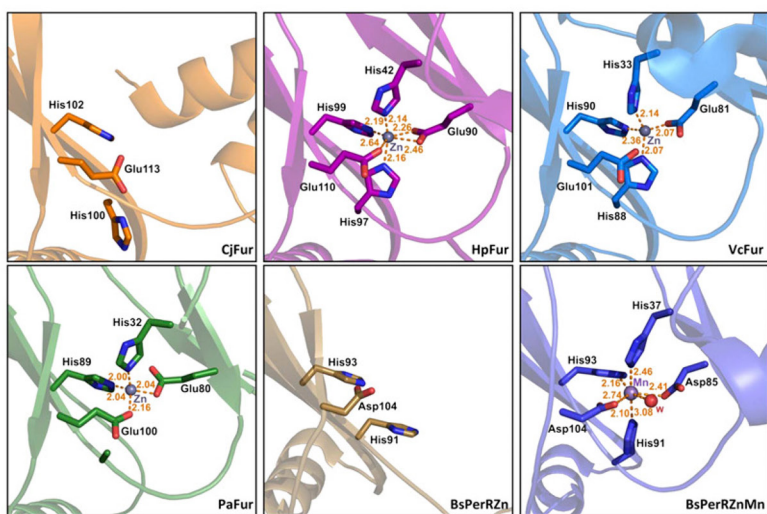
Figure S2.3: CjFur DBD adopts an atypical conformation

Overall structure of Fur and Fur-like proteins in which protomer A is highlighted in orange (CjFur), purple (HpFur, 2XIG), blue (VcFur, 2W57), green (PaCjFur, 1MZB), brown (BsPerRZn, 2FE3) and violet (BsPerRZnMn 3F8N). β-sheets, α-helices and metal binding sites of each protomer A are labeled accordingly. To facilitate the analysis, the dimerization domains are shown in the same orientation.

A



B



C

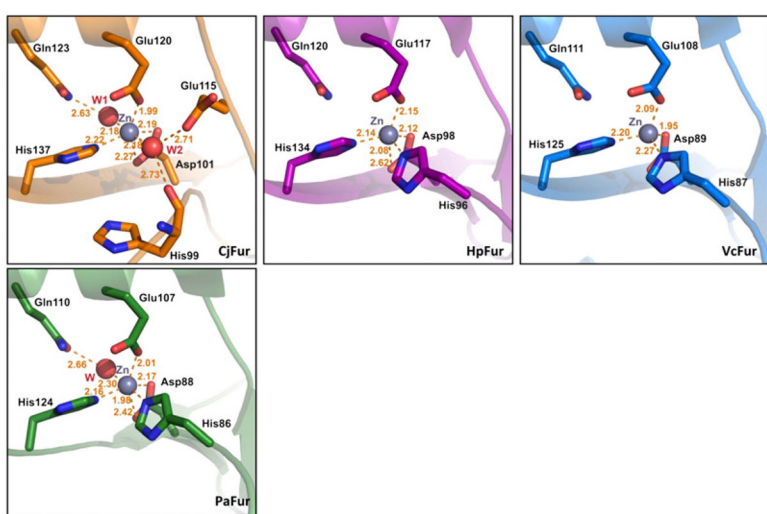


Figure S2.4: CjFur metal coordination diverges from other Fur and Fur-like proteins.

S1 (A), S2 (B) and S3 (C) metal binding sites of CjFur, HpFur (2XIG), VcFur (2W57), PaFur (1MZB), BsPerRZn (2FE3) and manganese bound BsPerRZn (3F8N). Carbon atoms are rendered as in Figure S2.3 while zinc and manganese atoms are highlighted in grey and purple respectively. Hydrogen bonds and water molecules are depicted as orange dash lines and red sphere respectively.

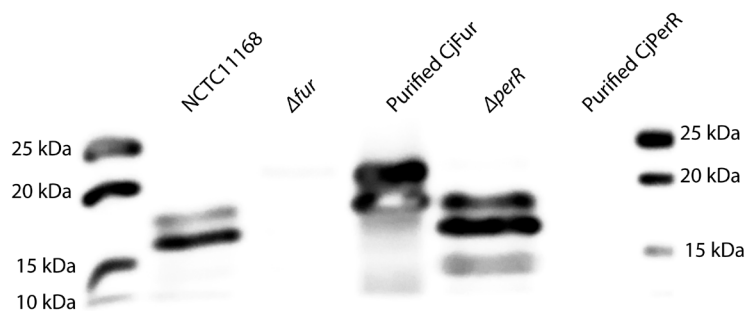


Figure S2.5: Confirmation of Anti-CjFur antibody specificity.

The specificity of the anti-CjFur antibody was determined by western blot analysis. Either 10 μ g of protein lysate or 100 ng of purified protein was separated by SDS-PAGE. Anti-Fur antibodies reacted with protein in the NCTC 11168 and $\Delta perR$ mutant *C. jejuni* strains, as well as the purified CjFur protein. This reactivity was lost in the Δfur mutant and the anti-Fur antibodies also did not react with purified CjPerR.

Supplemental Tables for Chapter 2

Gene Name	Annotation	Pathway	Iron	apo-Fur	holo-Fur	Fur ChIP Target
accA	acetyl-CoA carboxylase carboxyltransferase subunit α	Fatty acid biosynthesis				TRUE
acs	acetyl-coenzyme A synthetase	Fatty acid biosynthesis	Repressed		Repressed	
aroC	chorismate synthase	Amino acid biosynthesis	Repressed		Repressed	
arsB	putative arsenical pump membrane protein	Drug/analogue sensitivity and antibiotic resistance		Activated		
atpG	ATP synthase F1 sector gamma subunit	Energy metabolism			Repressed	
bioA	adenosylmethionine-8-amino-7-oxononanoate transferase	Biosynthesis of cofactors, and prosthetic groups	Repressed			TRUE
bioB	putative biotin synthase	Biosynthesis of cofactors, and prosthetic groups			Activated	
ceuB	enterochelin uptake permease	Cations transport/binding proteins	Repressed		Repressed*	TRUE
ceuC	enterochelin uptake permease	Cations transport/binding proteins	Repressed		Repressed	
ctbA	putative iron-uptake ABC transport periplasmic protein	Cations transport/binding proteins	Repressed		Repressed*	TRUE
ctbB	putative iron-uptake ABC transport permease protein	Cations transport/binding proteins	Repressed		Repressed*	TRUE
ctbC	putative iron-uptake ABC transport system ATP-binding protein	Cations transport/binding proteins	Repressed		Activated	
ctrA	enterobactin outer membrane receptor	Cations transport/binding proteins	Repressed		Repressed	
chaN	putative iron transport protein	Cations transport/binding proteins	Repressed		Repressed*	TRUE
chuA	haemin uptake system outer membrane receptor	Cations transport/binding proteins	Repressed		Repressed*	TRUE
chuB	putative haemin uptake system permease protein	Cations transport/binding proteins	Repressed		Repressed*	TRUE
chuC	putative haemin uptake system ATP-binding protein	Cations transport/binding proteins	Repressed		Repressed	
chuD	putative haemin uptake system periplasmic haemin-binding protein	Cations transport/binding proteins	Repressed		Repressed	
chuZ	haemin oxygenase	Biosynthesis of cofactors, and prosthetic groups			Repressed	
Cj0011c	putative non-specific DNA binding protein	DNA replication, restriction/modification, and repair	Activated	Activated	Repressed	
Cj0018c	small hydrophobic protein	Membranes, lipoproteins and porins	Repressed		Activated	
Cj0025c	putative transmembrane symporter	Other transport/binding proteins			Activated	
Cj0034c	hypothetical protein	Hypothetical unknown proteins		Repressed	Activated	
Cj0037c	putative cytochrome c	Energy metabolism	Activated	Repressed	Repressed	
Cj0055c	hypothetical protein	Hypothetical unknown proteins			Activated	
Cj0057	putative periplasmic protein	Hypothetical unknown proteins	Repressed	Repressed	Activated	
Cj0072c	putative iron binding protein	Cations transport/binding proteins		Repressed		
Cj0073c	non-flavin iron-sulfur oxidoreductase complex member	Energy metabolism	Activated		Activated	
Cj0123c	putative transcriptional regulator	Broad regulatory functions			Activated	
Cj0129c	outer membrane protein	Membranes, lipoproteins and porins	Activated		Activated	
Cj0139	putative endonuclease	DNA replication, restriction/modification, and repair	Activated		Activated	
Cj0145	putative TAT pathway signal sequence domain protein	Hypothetical unknown proteins	Activated			TRUE
Cj0150c	aminotransferase	Miscellaneous				TRUE
Cj0153c	putative rRNA methylase	RNA synthesis, RNA modification and DNA transcription			Activated	
Cj0156c	conserved hypothetical protein	Hypothetical unknown proteins			Repressed	
Cj0159c	putative 6-pyruvoyl tetrahydropterin synthase	Miscellaneous				TRUE
Cj0160c	putative radical SAM domain protein	Miscellaneous				TRUE
Cj0171	hypothetical protein	Hypothetical unknown proteins		Repressed	Repressed	
Cj0184c	possible serine/threonine protein phosphatase	Signal transduction	Activated	Repressed		
Cj0239c	nifU protein homolog	Conserved hypothetical proteins	Repressed	Repressed		
Cj0243c	hypothetical protein	Hypothetical unknown proteins		Activated		
Cj0251c	highly acidic protein	Conserved hypothetical proteins			Repressed	
Cj0261c	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		
Cj0262c	putative methyl-accepting chemotaxis protein	Chemotaxis and mobility	Repressed			TRUE
Cj0264c	molybdopterin-containing oxidoreductase	Miscellaneous		Repressed	Repressed	
Cj0265c	putative cytochrome C-type haem-binding protein	Energy metabolism	Repressed	Repressed	Repressed	
Cj0290c	pseudogene (partial glpT)	Carbohydrates and organic acid proteins			Repressed	
Cj0294	moeB/thiF family protein	Miscellaneous			Repressed	TRUE
Cj0302c	putative molybdenum-pterin binding protein	Miscellaneous		Repressed		
Cj0310c	putative efflux protein	Drug/analogue sensitivity and antibiotic resistance	Repressed			
Cj0339	putative transmembrane transport protein	Other transport/binding proteins	Repressed			TRUE
Cj0358	putative cytochrome C551 peroxidase	Detoxification	Activated	Repressed	Activated	
Cj0362	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		
Cj0364	hypothetical protein	Hypothetical unknown proteins		Repressed		
Cj0399	hypothetical protein	Hypothetical unknown proteins	Repressed	Activated		
Cj0403	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed	Repressed	
Cj0404	putative 1-acyl-SN-glycerol-3-phosphate acyltransferase	Phospholipids			Repressed	
Cj0414	putative oxidoreductase subunit	Miscellaneous	Repressed	Repressed	Repressed	
Cj0415	putative oxidoreductase subunit	Miscellaneous	Repressed	Activated	Activated	TRUE
Cj0418c	hypothetical protein	Hypothetical unknown proteins				TRUE
Cj0420	hypothetical protein	Hypothetical unknown proteins	Repressed		Repressed	
Cj0424	putative acidic periplasmic protein	Miscellaneous		Activated		
Cj0428	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed	Repressed	
Cj0481	putative lyase	Miscellaneous	Activated		Activated	
Cj0485	putative oxidoreductase	Miscellaneous				TRUE
Cj0499	putative histidine triad (HIT) family protein	Miscellaneous				TRUE
Cj0529c	hypothetical protein	Hypothetical unknown proteins	Activated		Repressed	
Cj0559	oxidoreductase	Miscellaneous	Repressed		Repressed	
Cj0569	hypothetical protein	Hypothetical unknown proteins		Activated	Repressed	
Cj0571	putative transcriptional regulator	Broad regulatory functions	Activated	Activated		
Cj0629	hypothetical protein	Hypothetical unknown proteins			Repressed	
Cj0633	hypothetical protein	Hypothetical unknown proteins	Activated		Activated	
Cj0644	putative TatD-related deoxyribonuclease protein	Miscellaneous		Repressed		TRUE
Cj0645	putative secreted transglycosylase	Murein sacculus and peptidoglycan		Activated		
Cj0723c	putative integral membrane zinc-metalloprotease	Degradation of macromolecules	Repressed			TRUE
Cj0742	pseudogene (putative outer membrane protein)	Membranes, lipoproteins and porins				TRUE
Cj0770c	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		
Cj0771c	putative periplasmic protein	Hypothetical unknown proteins		Repressed		
Cj0818	hypothetical protein	Hypothetical unknown proteins	Repressed		Activated	
Cj0844c	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		

Cj0859c	hypothetical protein	Hypothetical unknown proteins		Repressed		
Cj0889c	putative sensory transduction histidine kinase	Signal transduction		Repressed		
Cj0892c	hypothetical protein	Hypothetical unknown proteins	Activated	Activated		
Cj0947c	putative hydrolase	Miscellaneous		Repressed		
Cj0948c	putative transmembrane transport protein	Other transport/binding proteins	Activated	Repressed	Repressed	
Cj0949c	hypothetical protein	Hypothetical unknown proteins	Activated	Activated	Activated	
Cj0950c	hypothetical protein	Hypothetical unknown proteins		Activated	Repressed	
Cj0980	putative peptidase	Degradation of macromolecules				TRUE
Cj0983	hypothetical protein	Hypothetical unknown proteins	Repressed	Activated		
Cj1005c	putative membrane bound ATPase	Miscellaneous				TRUE
Cj1025c	hypothetical protein	Hypothetical unknown proteins		Activated		
Cj1026c	hypothetical protein	Hypothetical unknown proteins		Repressed		
Cj1089c	hypothetical protein	Hypothetical unknown proteins	Repressed	Repressed		
Cj1095	hypothetical protein	Hypothetical unknown proteins	Repressed		Repressed	
Cj1110c	putative MCP-type signal transduction protein	Signal transduction				TRUE
Cj1144c	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		
Cj1168c	putative integral membrane protein (dedA homolog)	Membranes, lipoproteins and porins	Activated	Activated		
Cj1169c	hypothetical protein	Hypothetical unknown proteins	Repressed		Repressed	
Cj1222c	putative two-component sensor	Signal transduction			Activated	
Cj1224	putative iron-binding protein	Cations transport/binding proteins		Activated	Activated	
Cj1237c	putative phosphatase	Miscellaneous	Repressed			TRUE
Cj1241	putative transmembrane transport protein	Other transport/binding proteins			Activated	
Cj1242	hypothetical protein	Hypothetical unknown proteins	Activated	Activated		
Cj1251	hypothetical protein	Hypothetical unknown proteins		Activated		
Cj1252	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		
Cj1279c	putative fibronectin domain-containing lipoprotein	Pathogenicity		Repressed		
Cj1287c	malate oxidoreductase	General intermediary metabolism	Repressed			TRUE
Cj1295	conserved hypothetical protein	Hypothetical unknown proteins	Activated			TRUE
Cj1306c	hypothetical protein	Hypothetical unknown proteins				TRUE
Cj1324	hypothetical protein	Surface polysaccharides, lipopolysaccharides/antigens		Activated		
Cj1330	hypothetical protein	Hypothetical unknown proteins				TRUE
Cj1340c	hypothetical protein	Hypothetical unknown proteins	Activated	Activated		
Cj1343c	putative periplasmic protein	Hypothetical unknown proteins		Repressed	Activated	
Cj1345c	putative periplasmic protein	Hypothetical unknown proteins		Activated		TRUE
Cj1356c	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed	Activated	
Cj1370	putative nucleotide phosphoribosyltransferase	Purines, pyrimidines, nucleosides and nucleotides		Activated	Repressed	
Cj1372	putative periplasmic protein (vacJ homolog)	Pathogenicity			Repressed	
Cj1377c	putative ferridoxin	Energy metabolism	Repressed		Activated	
Cj1381	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		
Cj1383c	hypothetical protein	Hypothetical unknown proteins	Repressed		Activated	
Cj1384c	hypothetical protein	Hypothetical unknown proteins	Repressed		Activated	
Cj1387c	helix-turn-helix protein	Broad regulatory functions			Activated	
Cj1394	putative fumarate lyase	Miscellaneous			Repressed ^d	TRUE
Cj1406c	hypothetical protein	Hypothetical unknown proteins			Repressed	
Cj1410c	hypothetical protein	Hypothetical unknown proteins	Activated	Repressed		
Cj1418c	putative transferase	Miscellaneous			Activated	
Cj1420c	putative methyltransferase	Hypothetical unknown proteins				TRUE
Cj1421c	putative methyltransferase	Hypothetical unknown proteins		Repressed		
Cj1422c	possible sugar transferase	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
Cj1427c	putative sugar-nucleotide epimerase/dehydratase	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
Cj1463	hypothetical protein	Hypothetical unknown proteins	Activated	Activated		
Cj1465	hypothetical protein	Hypothetical unknown proteins	Activated	Activated		
Cj1496c	hypothetical protein	Hypothetical unknown proteins			Repressed	
Cj1497c	hypothetical protein	Hypothetical unknown proteins		Activated		
Cj1500	hypothetical protein	Hypothetical unknown proteins	Repressed		Repressed	
Cj1501	hypothetical protein	Hypothetical unknown proteins	Activated		Repressed	
Cj1548c	putative NADP-dependent alcohol dehydrogenase	Miscellaneous				TRUE
Cj1582c	putative peptide ABC-transport system permease protein	Other transport/binding proteins	Activated	Activated	Repressed	
Cj1583c	putative peptide ABC-transport system permease protein	Other transport/binding proteins	Activated			TRUE
Cj1588c	putative MFS transport protein	Other transport/binding proteins				TRUE
Cj1627c	hypothetical protein	Hypothetical unknown proteins			s	TRUE
Cj1650	hypothetical protein	Hypothetical unknown proteins		Activated		
Cj1658	hypothetical protein	Hypothetical unknown proteins	Repressed	Repressed	Repressed	
Cj1660	hypothetical protein	Hypothetical unknown proteins	Repressed		Repressed	
Cj1661	possible ABC transport system permease protein	Other transport/binding proteins	Repressed		Activated	
Cj1662	putative integral membrane protein	Other transport/binding proteins	Repressed		Repressed	
Cj1663	putative ABC transport system ATP-binding protein	Other transport/binding proteins	Repressed		Repressed	
Cj1664	possible periplasmic thiredoxin	Energy metabolism	Repressed		Repressed	
Cj1665	possible lipoprotein thiredoxin	Energy metabolism	Repressed		Repressed	
Cj1677	hypothetical protein	Hypothetical unknown proteins		Repressed	Activated	
Cj1678	hypothetical protein	Hypothetical unknown proteins	Repressed		Activated	
Cj1710c	putative metallo-beta-lactamase family protein	Miscellaneous	Repressed			TRUE
Cj1713	putative radical SAM domain protein	Hypothetical unknown proteins	Activated			TRUE
Cj1721c	possible outer membrane protein	Membranes, lipoproteins and porins				TRUE
Cj1724c	putative GTP cyclohydrolase I	Biosynthesis of cofactors, and prosthetic groups				TRUE
Cj1725	putative periplasmic protein	Hypothetical unknown proteins	Repressed			TRUE
<i>cmeG</i>	putative multidrug efflux transporter	Miscellaneous	Repressed	Repressed		
<i>ctsD</i>	putative type II protein secretion system D protein	Protein and peptide secretion	Activated			TRUE
<i>ctuA</i>	putative tonB dependant outer membrane receptor	Cations transport/binding proteins			Repressed ^d	TRUE
<i>cydB</i>	cytochrome bd oxidase subunit II	Energy metabolism			Activated	
<i>dfp</i>	DNA /pantothenate metabolism flavoprotein	DNA replication, restriction/modification, and repair				TRUE
<i>dnaK</i>	heat shock protein dnaK	Chaperones, chaperonins, heat shock				TRUE
<i>dnaQ</i>	exonuclease, possibly dna polymerase III epsilon subunit	DNA replication, restriction/modification, and repair		Repressed		

<i>dprA</i>	SMF family protein	Hypothetical unknown proteins	Repressed	Repressed	Activated	
<i>dsbB</i>	putative disulfide oxidoreductase	Protein translation and modification		Repressed		TRUE
<i>exbB1</i>	biopolymer transport protein	Other transport/binding proteins	Repressed		Activated	
<i>exbB2</i>	putative exbB/tolQ family transport protein	Other transport/binding proteins	Repressed		Activated	
<i>exbD1</i>	biopolymer transport protein	Other transport/binding proteins	Repressed	Activated	Repressed	
<i>exbD2</i>	putative exbD/tolR family transport protein	Other transport/binding proteins	Repressed		Repressed	
<i>fabG</i>	3-oxoacyl-[acyl-carrier protein] reductase	Fatty acid biosynthesis	Repressed			TRUE
<i>fabZ</i>	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase	Fatty acid biosynthesis		Repressed		
<i>fbp</i>	putative fructose-1,6-bisphosphatase	General intermediary metabolism				TRUE
<i>fdxA</i>	ferridoxin	Energy metabolism	Activated	Activated	Activated	
<i>flaA</i>	flagellin	Surface structures	Activated			TRUE
<i>flaB</i>	flagellin	Surface structures		Repressed		TRUE
<i>flaD</i>	flavodoxin	Energy metabolism	Repressed		Repressed	TRUE
<i>flgB</i>	putative flagellar basal-body rod protein	Surface structures		Repressed		
<i>flgD</i>	putative flagellar hook assembly protein	Surface structures		Activated		
<i>flgE</i>	flagellar hook protein	Surface structures	Activated	Activated	Activated	
<i>flgE2</i>	flagellar hook subunit protein	Surface structures	Activated	Repressed	Activated	
<i>flgG</i>	flagellar basal-body rod protein	Surface structures		Repressed		
<i>flgG2</i>	putative flagellar basal-body rod protein	Surface structures	Activated	Repressed		
<i>flgH</i>	putative flagellar L-ring protein precursor	Surface structures	Activated	Repressed		
<i>flgL</i>	flagellar P-ring protein	Surface structures	Activated	Repressed		
<i>flgL</i>	putative flagellin	Surface structures	Activated	Repressed	Activated	
<i>flgR</i>	signal-transduction regulatory protein	Signal transduction		Repressed		
<i>flhD</i>	putative flagellar hook-associated protein	Surface structures	Activated			TRUE
<i>flhK</i>	flagellar hook-length control protein	Surface structures	Activated	Activated		
<i>flhP</i>	flagellar biosynthesis protein	Surface structures			Repressed	
<i>ftsH</i>	membrane bound zinc metalloprotease	Degradation of macromolecules				TRUE
<i>fur</i>	ferric uptake regulator	Broad regulatory functions		Activated	Repressed	
<i>glf</i>	UDP-galactopyranose mutase	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
<i>glhH</i>	glutamine-binding periplasmic protein	Amino acids and amines transport/binding proteins				TRUE
<i>glyA</i>	serine hydroxymethyltransferase	Amino acid biosynthesis		Activated	Repressed	
<i>gmhA2</i>	putative phosphoheptose isomerase	Surface polysaccharides, lipopolysaccharides/antigens	Activated			TRUE
<i>groEL</i>	60 kD chaperonin (cpn60)	Chaperones, chaperonins, heat shock		Activated	Repressed	
<i>hddA</i>	putative sugar kinase	Surface polysaccharides, lipopolysaccharides/antigens	Activated	Repressed		TRUE
<i>hddG</i>	putative sugar-phosphate nucleotidyltransferase	Surface polysaccharides, lipopolysaccharides/antigens	Activated			TRUE
<i>hemD</i>	uroporphyrinogen-III synthase	Biosynthesis of cofactors, and prosthetic groups	Activated	Activated		
<i>hipO</i>	hippurate hydrolase	Miscellaneous	Repressed			TRUE
<i>hisS</i>	histidyl-tRNA synthetase	Aminoacyl tRNA synthetases and their modification				TRUE
<i>hupB</i>	DNA-binding protein HU homolog	Nucleoproteins	Activated		Repressed	
<i>infC</i>	translation initiation factor IF-3	Protein translation and modification	Repressed		Activated	
<i>iscS</i>	putative aminotransferase (nifS protein homolog)	Miscellaneous	Repressed	Repressed		
<i>ispA</i>	geranyltransferase	Biosynthesis of cofactors, and prosthetic groups	Activated			TRUE
<i>ispE</i>	putative 4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	Miscellaneous			Repressed	
<i>katA</i>	catalase	Detoxification	Repressed			TRUE
<i>kdtA</i>	3-deoxy-D-manno-octulosonic-acid transferase	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
<i>kpsT</i>	capsule polysaccharide export ATP-binding protein	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
<i>ktrA</i>	putative K ⁺ uptake protein	Cations transport/binding proteins			Repressed	
<i>leuC</i>	3-isopropylmalate dehydratase large subunit	Amino acid biosynthesis	Activated			TRUE
<i>leuC</i>	3-isopropylmalate dehydratase large subunit	Amino acid biosynthesis	Activated			
<i>livH</i>	branched-chain amino-acid ABC transport system permease protein	Amino acid biosynthesis				
<i>livH</i>	amino-acid ABC transport system permease	Amino acids and amines transport/binding proteins				TRUE
<i>lspA</i>	lipoprotein signal peptidase	Protein and peptide secretion		Activated		
<i>maf6</i>	motility accessory factor	Surface structures	Activated	Repressed	Repressed	TRUE
<i>maf7</i>	motility accessory factor (function unknown)	Surface structures	Activated	Activated	Activated	
<i>map</i>	methionine aminopeptidase	Protein translation and modification				TRUE
<i>metA</i>	putative homoserine O-succinyltransferase	Amino acid biosynthesis	Repressed			TRUE
<i>metB</i>	putative O-acetylhomoserine (thio)-lyase	Amino acid biosynthesis	Repressed		Activated	TRUE
<i>mfd</i>	transcription-repair coupling factor	DNA replication, restriction/modification, and repair	Activated	Activated		
<i>mmnC</i>	5-methylaminomethyl-2-thiouridine methyltransferase	Miscellaneous	Repressed	Repressed		
<i>modA</i>	putative molybdate-binding lipoprotein	Anions transport/binding proteins	Repressed			
<i>modB</i>	putative molybdenum transport system permease protein	Anions transport/binding proteins	Repressed	Repressed	Repressed	TRUE
<i>modC</i>	putative molybdenum transport ATP-binding protein	Anions transport/binding proteins		Activated	Activated	
<i>mogA</i>	molybdopterin biosynthesis protein	Biosynthesis of cofactors, and prosthetic groups		Activated		
<i>murD</i>	UDP-N-acetylmuramoylalanine-D-glutamate ligase	Murein sacculus and peptidoglycan				TRUE
<i>murE</i>	UDP-N-acetylmuramoylalanine-D-glutamate-2,6-diaminylase	Murein sacculus and peptidoglycan	Repressed		Activated	
<i>napA</i>	periplasmic nitrate reductase	Energy metabolism	Activated			TRUE
<i>napG</i>	putative ferredoxin	Energy metabolism	Activated			TRUE
<i>neuA1</i>	acetylneuraminate cytidyltransferase	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
<i>neuB1</i>	N-acetylneuraminic acid synthetase	Surface polysaccharides, lipopolysaccharides/antigens		Repressed		
<i>nusA</i>	transcription termination factor	RNA synthesis, RNA modification and DNA transcription		Activated	Activated	
<i>omp50</i>	outer membrane protein	Membranes, lipoproteins and porins	Repressed		Activated	
<i>p19</i>	periplasmic protein	Miscellaneous	Repressed		Activated	
<i>peb2</i>	major antigenic peptide PEB2	Miscellaneous	Repressed	Activated		
<i>petA</i>	putative ubiquinol-cytochrome C reductase iron-sulfur subunit	Energy metabolism	Activated	Activated	Activated	
<i>petB</i>	putative ubiquinol-cytochrome C reductase cytochrome B subunit	Energy metabolism	Activated	Repressed	Activated	
<i>petC</i>	ATP synthase F0 sector B' subunit	Energy metabolism	Activated		Activated	
<i>pgsA</i>	CDP-diacylglycerol-glycerol-3-phosphate-3-phosphatidyltransferase	Phospholipids			Activated	
<i>prfA</i>	peptide chain release factor 1	Protein translation and modification			Activated	
<i>proS</i>	prolyl-tRNA synthetase	Aminoacyl tRNA synthetases and their modification				TRUE
<i>pseA</i>	hypothetical protein Cj1316c	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
<i>pseB</i>	possible sugar nucleotide epimerase/dehydratase	Surface structures		Activated		
<i>pseF</i>	acetylneuraminate cytidyltransferase	Surface polysaccharides, lipopolysaccharides/antigens				TRUE
<i>pssA</i>	CDP-diacylglycerol-serine O-phosphatidyltransferase	Phospholipids			Activated	

<i>ptmB</i>	acyneuramate cytidyltransferase	Surface structures	Activated			TRUE
<i>purA</i>	adenylosuccinate synthetase	Purines, pyrimidines, nucleosides and nucleotides		Repressed		
<i>purU</i>	formyltetrahydrofolate deformylase	Purines, pyrimidines, nucleosides and nucleotides	Activated			TRUE
<i>pyrB</i>	aspartate carbamoyltransferase	Purines, pyrimidines, nucleosides and nucleotides	Activated			TRUE
<i>rdxA</i>	nitroreductase	Miscellaneous		Repressed		
<i>ribD</i>	putative riboflavin-specific deaminase	Biosynthesis of cofactors, and prosthetic groups		Repressed		
<i>rimM</i>	putative 16S rRNA processing protein	Ribosome maturation and modification	Activated	Repressed		
<i>rmhB</i>	ribonuclease HII	Degradation of macromolecules	Activated		Activated	
<i>rplF</i>	50S ribosomal protein L6	Ribosomal protein synthesis and modification	Repressed		Activated	
<i>rplO</i>	50S ribosomal protein L15	Ribosomal protein synthesis and modification	Repressed		Activated	
<i>rplP</i>	50S ribosomal protein L16	Ribosomal protein synthesis and modification			Activated	
<i>rplQ</i>	50S ribosomal protein L17	Ribosomal protein synthesis and modification	Activated		Activated	
<i>rpoA</i>	DNA-directed RNA polymerase alpha chain	RNA synthesis, RNA modification and DNA transcription			Activated	
<i>rpsI</i>	30S ribosomal protein S9	Ribosomal protein synthesis and modification			Activated	
<i>rpsJ</i>	30S ribosomal protein S10	Ribosomal protein synthesis and modification				TRUE
<i>rpsR</i>	30S ribosomal protein S18	Ribosomal protein synthesis and modification		Repressed		
<i>rpsS</i>	30S ribosomal protein S19	Ribosomal protein synthesis and modification			Activated	
<i>rrc</i>	non-haem iron protein	Cations transport/binding proteins	Activated	Repressed	Activated	TRUE
<i>ruvC</i>	crossover junction endodeoxyribonuclease	DNA replication, restriction/modification, and repair	Activated	Repressed		
<i>mfrA</i>	methylmenaquinol:fumarate reductase member (<i>sdhA</i>)	Energy metabolism	Activated	Activated		
<i>mfrB</i>	methylmenaquinol:fumarate reductase member (<i>sdhB</i>)	Energy metabolism	Activated	Activated		
<i>mfrE</i>	methylmenaquinol:fumarate reductase member (<i>sdhC</i>)	Energy metabolism	Activated	Repressed	Repressed	
<i>secA</i>	preprotein translocase SECA subunit	Protein and peptide secretion				TRUE
<i>secE</i>	preprotein translocase SecE subunit	Protein and peptide secretion	Repressed	Repressed	Repressed	
<i>serC</i>	phosphoserine aminotransferase	Amino acid biosynthesis	Activated			TRUE
<i>surE</i>	surE protein homolog	Drug/analogue sensitivity and antibiotic resistance				TRUE
<i>thyX</i>	thymidylate synthase	Miscellaneous	Activated			TRUE
<i>tonB1</i>	possible tonB transport protein	Other transport/binding proteins	Repressed		Repressed	
<i>tonB2</i>	putative tonB transport protein	Other transport/binding proteins	Repressed		Repressed	
<i>tonB3</i>	tonB transport protein	Other transport/binding proteins	Repressed		Repressed	
<i>topA</i>	DNA topoisomerase I	DNA replication, restriction/modification, and repair			Repressed	
<i>trmD</i>	tRNA (guanine-N1)-methyltransferase	Aminoacyl tRNA synthetases and their modification		Repressed		
<i>trxB</i>	thioredoxin reductase	Biosynthesis of cofactors, and prosthetic groups	Repressed		Repressed*	TRUE
<i>tupA</i>	putative tungsten transport protein	Anions transport/binding proteins		Repressed	Repressed	
<i>tupB</i>	putative anion-uptake ABC-transport system permease	Anions transport/binding proteins				TRUE
<i>tupC</i>	putative anion-uptake ABC-transport system ATP-binding protein	Anions transport/binding proteins		Repressed	Repressed	
<i>uvrA</i>	excinuclease ABC subunit A	DNA replication, restriction/modification, and repair	Repressed			TRUE
<i>uvrB</i>	excinuclease ABC subunit B	DNA replication, restriction/modification, and repair				TRUE
<i>uvrC</i>	excinuclease ABC subunit C	DNA replication, restriction/modification, and repair			Repressed	
<i>zupT</i>	putative zinc transporter	Hypothetical unknown proteins	Repressed			TRUE

* All regulation refers to Palyada *et al* unless otherwise noted

* Also identified in Holmes *et al*

^b Identified in Holmes *et al* but not in Palydata *et al*

Genes that do not have a holo/apo Fur regulation designation were not differentially expressed in a Δfur mutant, so their mode of regulation is unknown.

Table S2.1: List of Fur ChIP enriched genes along with their transcriptional regulation by iron or apo/olo-Fur

Listed are the genes identified as being directly bound by CjFur in the ChIP-on-chip analysis along with their presumed mode of regulation.

Table S2.2: ICPMS metal analysis of recombinant CjFur protein

Metal	Metal content (%)*
Mn ²⁺	< 0.2
Fe ²⁺	< 0.2
Co ²⁺	< 0.1
Ni ²⁺	< 0.1
Cu ²⁺	1.55
Zn ²⁺	260.45

*The percent metal content is shown as a molar ratio of metal/CjFur concentration as determined by ICPMS.

Listed are the results for ICPMS metal analysis for the purified recombinant CjFur protein used for the crystallization trials and for the gel mobility shift assays.

Data collection		Zn SAD	Native
	Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
	Cell dimensions		
	a, b, c (Å)	35.8, 84.5, 128.2	35.9, 85.3, 128.4
	Resolution	30-2.1 (2.2-2.1) [!]	30-2.1 (2.2-2.1)
	R _{sym}	6.0 (50.8)	4.5 (47.2)
	I / σ I	22.3 (3.0)	22.3 (3.1)
	Completeness	99.9 (99.0)	99.9 (99.9)
	Redundancy	4.7 (4.5)	4.7 (4.5)
Phasing			
	atom found	4	
	CC _{all} (%)	45.3	
	CC _{weak} (%)	28.4	
	PATFOM	49.8	
Refinement			
	Resolution		27.8 – 2.1
	Reflections		22655
	R _{work} /R _{free}		23.7 / 26.7
	No. atoms		
	Protein		2353
	Ligands		4
	Water		61
	B-factors (Å ²)		
	Protein		38.0
	Ligands		32.7
	Water		41.1
	R.m.s. deviations		
	Bond lengths (Å)		0.019
	Bond angles (°)		1.783
	Molprobity score		1.82
	Ramachandran favored (%) [*]		97.78

[!] Highest resolution shell.

^{*}There are no Ramachandran outliers

Table S2.3: Data collection, phasing and refinement statistics for the crystal structure of CjFur.

Primers	Primer Sequence	Function
furSE	ATGGAGGTCTCAGCGATGCTGATAGAAAATGGAATATGA	forward primer for cloning fur gene
furAS	ATGGTAGGTCTCATATCATATTTTACCTTTGCTTTTGATTATTA	reverse primer for cloning fur gene
JW102	GCGGTGACCCGGGAGATCTGAATTC	adapter oligo for ChIP DNA amplification
JW103	GAATTCAGATC	adapter oligo for ChIP DNA amplification
JBCHUA1F	TGCTTTCAAATTCATTTTATCC	forward primer for chuA qPCR analysis
JBCHUA1R	TTGGGTGCAAATTTTACTCCTT	forward primer for chuA qPCR analysis
JBCJ16581F	GCTTAGAAGAAGACCTTGCGATA	reverse primer for Cj1658 qPCR analysis
JBCJ16581R	TTCATTATCATTATCCTTGATTTTTGA	forward primer for Cj1658 qPCR analysis
JBCJ01771F	TTTATGTTGAGCGCTTGTGC	forward primer for Cj0177 qPCR analysis
JBCJ01771R	GTGTTTTTCGCCAAGCAGTA	reverse primer for Cj0177 qPCR analysis
JBKATA1F	TGCACCAATAACCAAACTGA	forward primer for katA qPCR analysis
JBKATA1R	TCAGCTATAATGTTTCCAAAATCG	reverse primer for katA qPCR analysis
JBRR1F	CGCATCGCTTCTCATTGTAA	forward primer for rrc qPCR analysis
JBRR1R	TGCCACATTTTGGCATTTA	reverse primer for rrc qPCR analysis
JBTHYX1F	AGCAGCGCTATCCCTTTAC	forward primer for thyX qPCR analysis
JBTHYX1R	TTGTTGCATGAGAACAAACAGA	reverse primer for thyX qPCR analysis
JBTRXB1F	CGTTGGTGCACAATCTAAACA	forward primer for trxB qPCR analysis
JBTRXB1R	AAAGTCGGCACTAAGTCCA	reverse primer for trxB qPCR analysis
RTAHPCL	TTCTTGCTTGATGCTGATGG	forward primer for ahpC qPCR analysis
RTAHPCR	CTGCAGGACAACTTACCA	reverse primer for ahpC qPCR analysis
RTILVCL	TCTAGGTGGTGGCACTCCTT	forward primer for ilvC qPCR analysis
RTILVCR	CACAGCTTGCTCACCAAAAA	reverse primer for ilvC qPCR analysis
F primer sumo	AGATTGGTGGTGCTACGTATATGCTGATAGAAAATGTGGA	forward primer to place Fur in frame with the 3' end of SUMO
R primer sumo	CTATCTGCTCGAGTTATATTTTACCTTTGCTTTTGA	reverse primer for cloning SUMO Fur in pET3d
JBRT_1345CF1	GCGTAGGAGAAAAATGGAAGAA	forward primer for cj1345c RT-qPCR analysis
JBRT_1345CR1	AAAAGCTAAATTTGGAGCCACT	reverse primer for cj1345c RT-qPCR analysis
JBRT_RRCF1	TGCAAAAATCGCTGAAGATG	forward primer for rrc RT-qPCR analysis
JBRT_RRCR1	ATAAAGGACAGGCTGCTGGA	reverse primer for rrc RT-qPCR analysis
JBRT_DSBBF1	TTCCAAATGGCAAGATACGC	forward primer for dsbB RT-qPCR analysis
JBRT_DSBBR1	CCACCTATAGCCATAACAAGCA	reverse primer for dsbB RT-qPCR analysis
CjkatA_For	5'Cy5-tgcattttatgataataaattcaaaataaatttagttt	forward primer for katA gel shift analysis
CjkatA_Rev	5'Cy5-aaactaaatttttgaattttatcaataaaatgca	reverse primer for katA gel shift analysis
Cjrrc_For	5'Cy5-gggGGTGCTCTTAAATGTCCTTTTTTATCGCA	forward primer for rrc gel shift analysis
Cjrrc_Rev	TTTGACTCCTTTTTGATTATCAAAAAGTA	reverse primer for rrc gel shift analysis
Cj1345c_For	5'Cy5-gggAAAAGAGCCAAATTTAGGTGTTATCATCAA	forward primer for cj1345c gel shift analysis
Cj1345c_Rev	TTTAACTCCTTAAATACTCTCTTGTITTA	reverse primer for cj1345c gel shift analysis
Strains	Strain Description	Source
DH5a	<i>endA1 hsdR17(r_K⁺ m_K⁻) supE44 thi-1 recA1 thi-1 relA1 gyrA96 Δ(lacZYA-argF) U169 deoR [f80dlacΔ(lacZ ΔM15)]</i>	Invitrogen
AS1130	DH5a with pASK-Fur	This study
sumo-Fur strain	Rosetta (Novagen)	This study
AS144	<i>C. jejuni</i> NCTC11168	Palyada <i>et al</i> 2004
AS230	<i>C. jejuni</i> NCTC11168 Δ <i>fur</i> ::Cm ^R	Palyada <i>et al</i> 2004
Plasmids	Plasmid Description	Source
pASK-IBA7	Expression vector, amp ^R , N-terminal Strep-Tag II epitope	IBA (Germany)
pASK-Fur	pASK-IBA7 with <i>C. jejuni</i> fur gene cloned into BsaI site	This study
sumo+Fur plasmid	expression vector, amp ^R , N-terminal Strep-SUMO-tag epitope, Strep-SUMO-F	This study

Table S2.4: List of Strains, Plasmids and Primers

Supplemental Information for Chapter 3

Supplemental Figures for Chapter 3

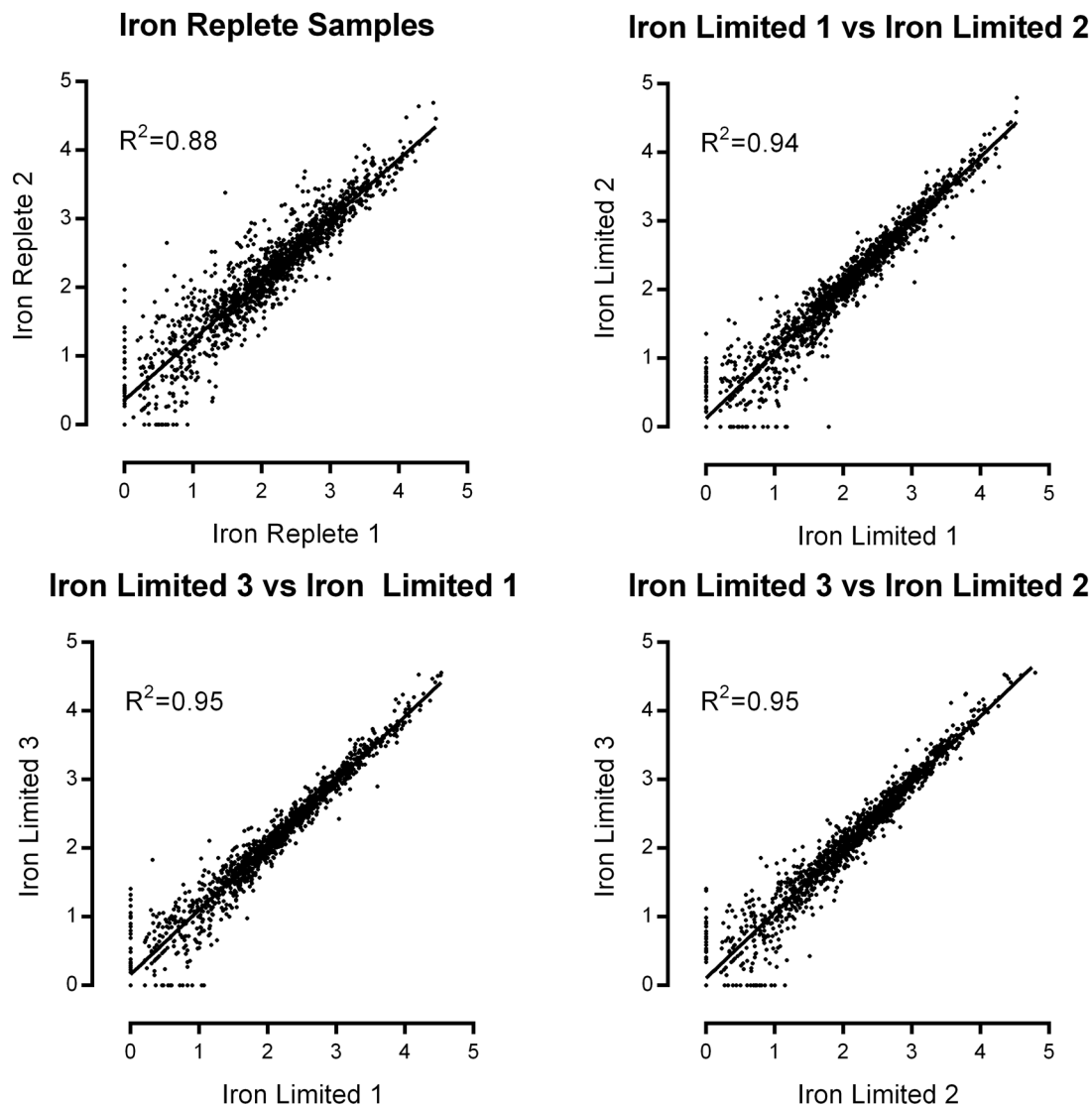


Figure S3.1: Sequencing runs are highly similar

The Log₁₀(RPKM+1) values obtained for each gene were compared across sequencing reactions from different biological replicates with the same growth condition. There is a high degree of similarity between different sequencing runs indicating low biological variability between samples.

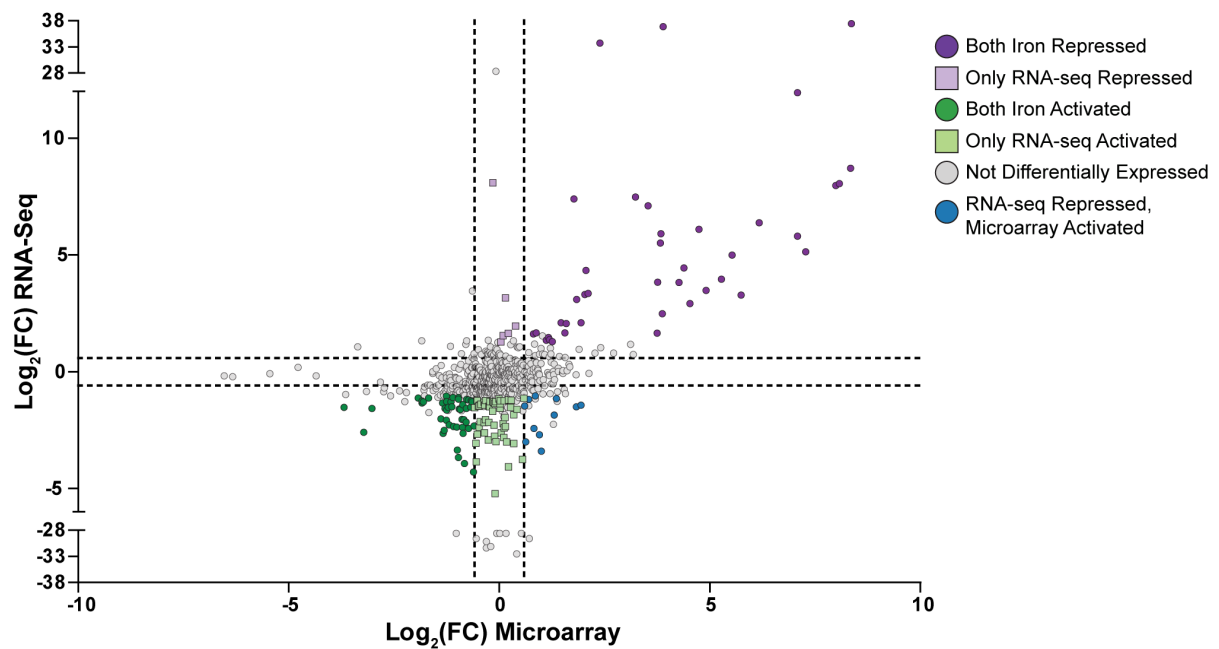


Figure S3.2: Plot of $\text{Log}_2(\text{Fold Change})$ RNA-seq and microarray results

The results of the RNA-seq in this study and previous microarray results (Palyada *et al.*, 2004) were plotted according to $\text{Log}_2(\text{fold change})$. Genes identified as being iron repressed by both techniques are either dark purple (iron repressed) or dark green (iron activated). Genes only identified in the iron seq analysis are shown as light purple or green squares (repressed and activated respectively). Genes in blue were iron activated in the RNA-seq and iron repressed in the microarray. Genes in grey were not significantly differentially expressed in the RNA-seq analysis. See Table S3.2 for specific gene details.

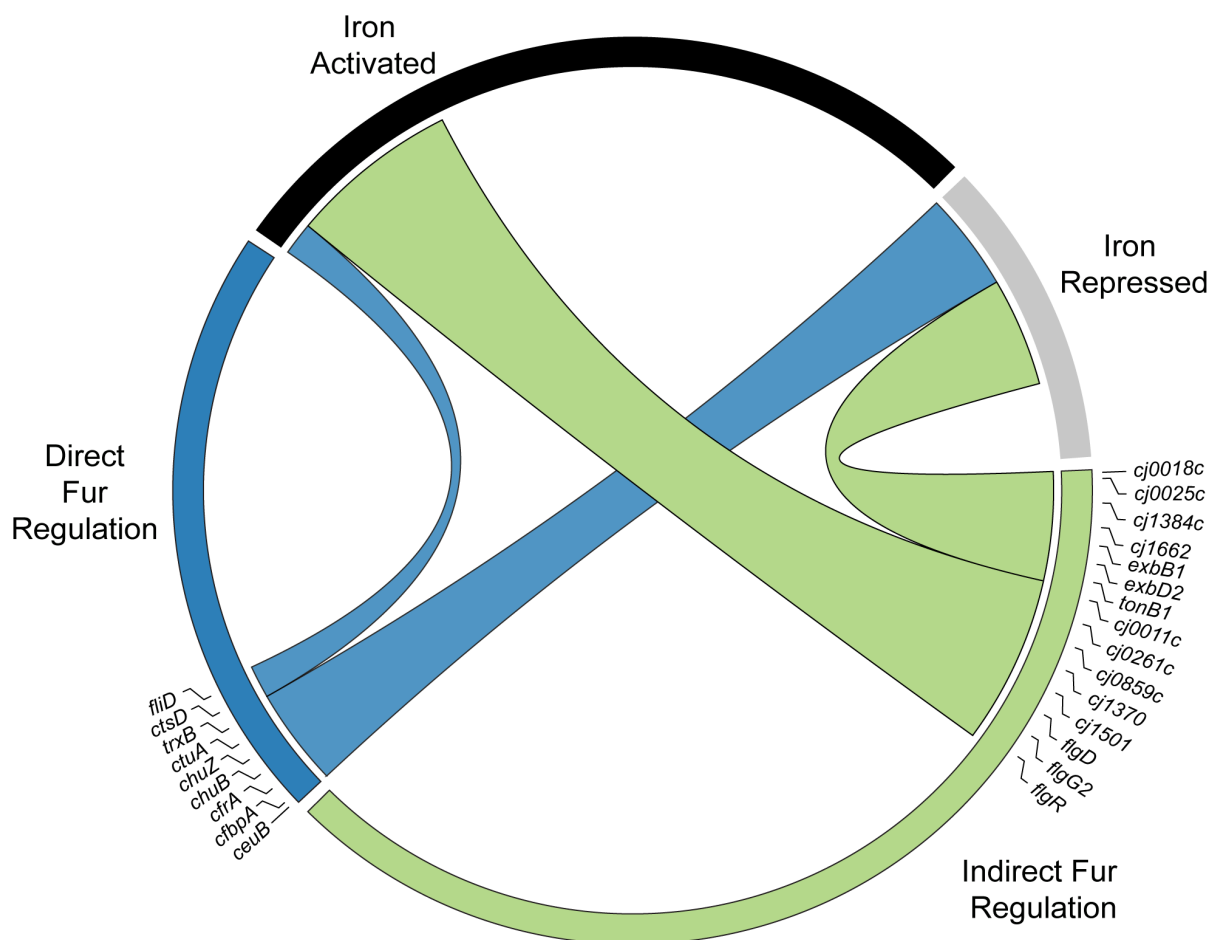


Figure S3.3: Comparison between Fur regulation and iron responsive genes

The RNA-seq iron regulon was compared to the direct and indirect CjFur regulons determined using ChIP-chip and microarrays respectively (Butcher *et al.*, 2012; Palyada *et al.*, 2004). While the majority of the iron repressed genes were regulated by CjFur, most iron activated genes were not CjFur responsive. This suggests the presence of additional iron responsive regulatory elements in *C. jejuni*. The length of the bars/ribbons is proportional to the number of genes in each group. Figure made using Circos v0.54 (Krzywinski *et al.*, 2009).

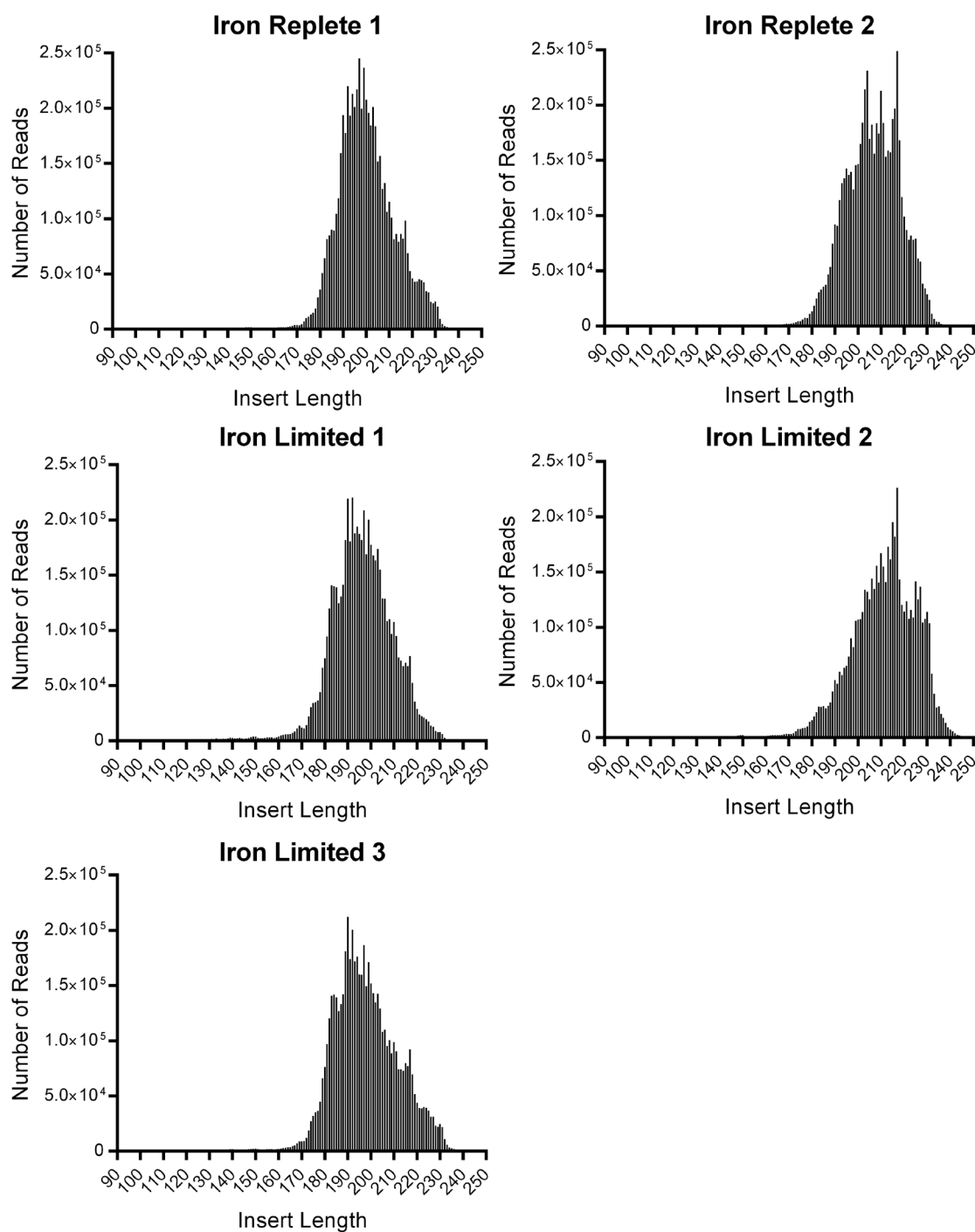


Figure S3.4: Average insert size of Illumina sequencing libraries

The average insert size for each of the Illumina sequencing libraries constructed was calculated based on the paired-read alignment to the *C. jejuni* NCTC 11168 genome

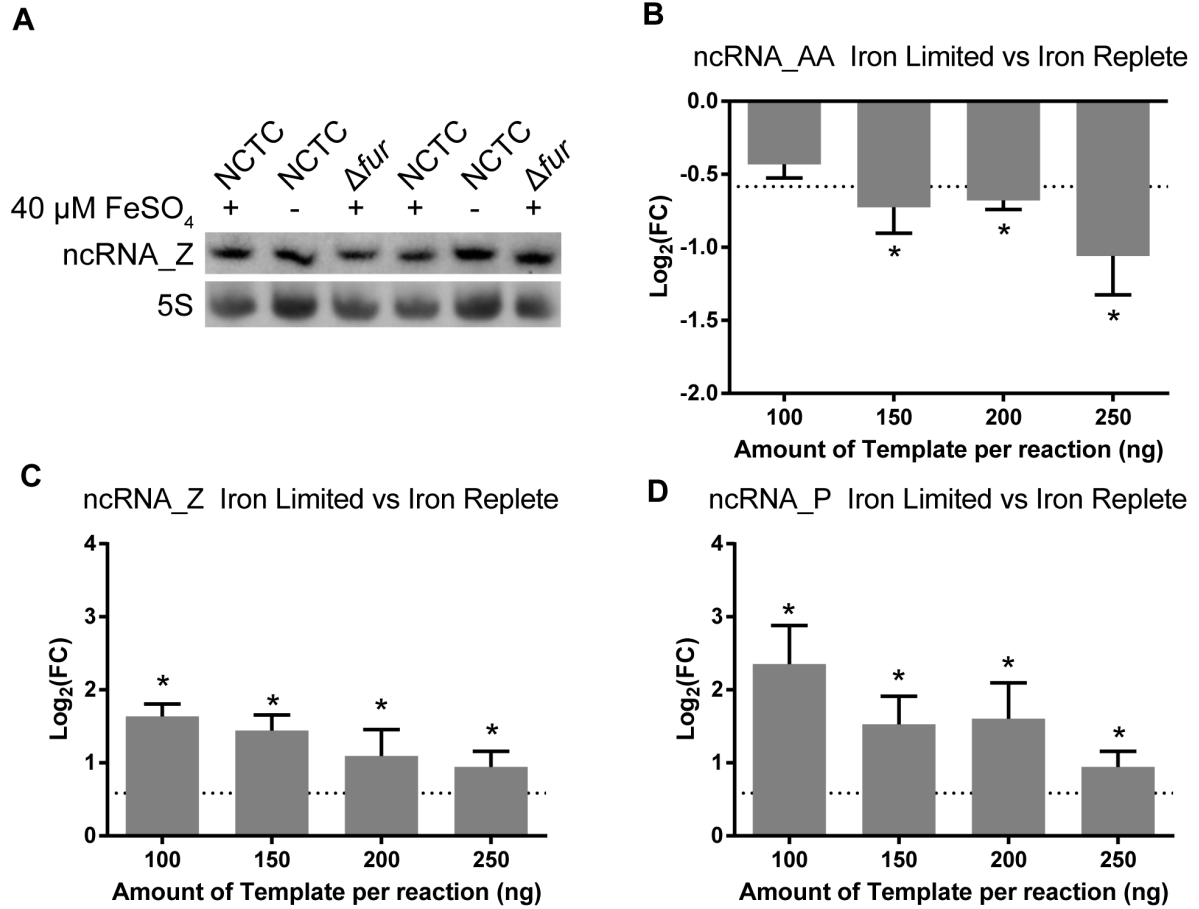


Figure S3.5: Additional ncRNA validation experiments

Panel A: Northern blot showing expression of ncRNA_Z across different conditions. The 5S RNA is also shown as a loading control. Densitometry analysis did not reveal differential expression of ncRNA_Z when normalized to the loading control. Panels BCD: qRT-PCR experiments of ncRNAs AA, Z and P with varying levels of template RNA for each RT reaction. Differentially expressed conditions (>1.5 fold change, $p < 0.05$) are indicated with an asterisk.

Supplemental Tables for Chapter 3

For the sake of brevity, not all the supplemental files submitted with this manuscript are reproduced here due to the length and breadth of the genome wide analysis performed for this manuscript. Specifically, Tables S3.3, S3.4, S3.5, S3.6, S3.7, S3.8, and S3.9 are excluded. These tables can be accessed from the publisher's website: [PMID 24223952](https://pubmed.ncbi.nlm.nih.gov/24223952/)

Sample	Source	Type of Illumina Library	Total Reads	Reads successfully aligned	Reads on ORFs	Reads on rRNA/tRNA	Reads on intergenic sRNAs ¹
Iron_Replete_1	This Study	Transcriptomic*	13,477,780	12,226,912	1,248,705	10,859,250	159
Iron_Replete_2	This Study	Transcriptomic*	12,802,068	12,183,390	1,578,262	10,458,736	357
Iron_Limited1	This Study	Transcriptomic*	13,477,780	12,119,494	1,207,255	10,706,883	123
Iron_Limited2	This Study	Transcriptomic*	12,844,446	11,485,048	1,029,493	10,419,780	67
Iron_Limited3	This Study	Transcriptomic*	13,111,112	11,732,464	1,166,186	10,455,484	99
Iron_Replete	This Study	miRNA	21,524,201	20,093,158	5,510,241	11,763,562	65,708
Iron_Limited	This Study	miRNA	20,101,098	17,533,109	3,891,008	11,514,107	96,839
ERR036497	Chaudhuri et al	Transcriptomic*	7,255,306	5,657,771	3,014,490	2,531,074	1,405
ERR036498	Chaudhuri et al	Transcriptomic*	7,007,005	5,583,296	2,484,312	3,013,665	1,089
ERR036499	Chaudhuri et al	Transcriptomic*	7,169,195	4,726,040	3,271,651	1,327,299	1,227
ERR036500	Chaudhuri et al	Transcriptomic*	7,064,429	4,621,146	3,197,000	1,296,955	1,590
SRR064701	Jerome et al	Genomic DNA	27,900,999	21,547,712	NA [#]	NA [#]	NA [#]
SRR064702	Jerome et al	Genomic DNA	19,012,732	12,514,228	NA [#]	NA [#]	NA [#]

* mRNA library

[#] Not applicable under genomic sequencing

¹ sRNAs A to II

Table S3.1: Statistics for high throughput sequencing runs

Listed are the read statistics for the high throughput sequencing runs performed in this study as well as runs that were reanalyzed from other studies (Chaudhuri *et al.*, 2011; Jerome *et al.*, 2011).

Gene	Gene Annotation	Log ₂ (FC)	p value
<i>aat</i>	leucyl/phenylalanyl-tRNA protein transferase	-1.38	1.40E-03
<i>acpP2</i>	putative acyl carrier protein	-4.08	8.25E-10
<i>acpP3</i>	putative acyl carrier protein	-1.58	5.13E-04
<i>ahpC</i>	alkyl hydroperoxide reductase	2.92	6.19E-10
<i>asd</i>	aspartate-semialdehyde dehydrogenase	-1.29	2.16E-03
<i>ccoQ</i>	cb-type cytochrome C oxidase subunit IV	-1.10	6.32E-03
<i>ceuB</i>	enterobactin permease	35.70	1.18E-15
<i>ceuD</i>	enterobactin permease	2.75	3.46E-08
<i>ceuE</i>	enterobactin uptake periplasmic binding protein	2.06	7.42E-06
<i>cfbpA</i>	iron-uptake ABC transport system periplasmic protein	3.29	7.19E-12
<i>cfbpB</i>	iron-uptake ABC transport system permease protein	3.31	3.81E-09
<i>cfbpC</i>	iron-uptake ABC transport system ATP-binding protein	2.10	4.25E-03
<i>cfrA</i>	enterobactin uptake outer membrane protein	8.71	2.84E-42
<i>chaN</i>	putative iron transport protein	7.10	1.04E-28
<i>chuA</i>	heme uptake system outer membrane receptor	11.94	7.42E-49
<i>chuB</i>	heme uptake system permease protein	5.80	6.74E-23
<i>chuC</i>	heme uptake system ATP-binding protein	7.97	1.17E-24
<i>chuD</i>	heme uptake system periplasmic heme-binding protein	8.05	4.32E-29
<i>chuZ</i>	heme oxygenase	4.44	4.61E-18
<i>cj0011c</i>	putative DNA binding protein	-1.57	8.72E-05
<i>cj0017c</i>	putative ATP/GTP binding protein	1.66	2.03E-04
<i>cj0018c</i>	putative hydrophobic protein	2.10	1.01E-05
<i>cj0025c</i>	putative transmembrane symporter	1.34	5.10E-03
<i>cj0037c</i>	putative cytochrome C	-2.08	2.50E-07
<i>cj0073c</i>	non-flavin iron-sulfur containing oxidoreductase	-1.52	1.44E-04
<i>cj0122</i>		-1.96	6.31E-04
<i>cj0128c</i>		-1.36	1.77E-03
<i>cj0159c</i>	putative 6-pyruvoyl tetrahydropterin synthase	-2.77	8.84E-10
<i>cj0176c</i>	hypothetical lipoprotein	3.14	4.73E-03
<i>cj0208</i>	adenine-specific methyltransferase	-1.16	8.51E-03
<i>cj0261c</i>		-1.60	6.47E-03
<i>cj0323</i>		-1.75	5.36E-03
<i>cj0378c</i>	putative ferric reductase-like transmembrane protein	1.66	3.74E-04
<i>cj0379c</i>	putative sulfite oxidase subunit YedY	1.96	2.05E-05
<i>cj0412</i>	putative ATP/GTP-binding protein	-1.29	5.50E-03
<i>cj0608</i>	putative outer membrane protein	-2.34	2.71E-03
<i>cj0690c</i>	possible restriction /modification enzyme	-1.84	6.37E-05
<i>cj0727</i>	putative solute binding protein	1.64	1.30E-03
<i>cj0809c</i>	putative hydrolase	-1.53	5.21E-04
<i>cj0818</i>	putative lipoprotein	4.34	1.34E-14
<i>cj0819</i>		2.93	1.82E-07
<i>cj0833c</i>	putative oxidoreductase	-1.12	6.08E-03
<i>cj0843c</i>	putative secreted transglycosylase	-1.24	2.68E-03
<i>cj0900c</i>	putative hydrophobic protein	-2.39	8.26E-03
<i>cj0915</i>	putative hydrolase	1.36	3.19E-03
<i>cj0916c</i>		-1.43	2.62E-03
<i>cj1020c</i>	putative cytochrome C	-1.52	1.13E-03
<i>cj1021c</i>		-3.75	1.60E-05
<i>cj1138</i>	cell division/peptidoglycan biosynthesis protein	-2.93	4.01E-03
<i>cj1169c</i>	putative hydrophobic protein	1.46	1.73E-03
<i>cj1191c</i>	putative PAS domain signal-transduction sensor	-2.40	6.51E-06
<i>cj1254</i>	hypothetical protein	-1.80	2.21E-04
<i>cj1321</i>	transferase	-1.34	4.46E-03
<i>cj1349c</i>	possible fibronectin/ fibrinogen-binding protein	-3.07	9.35E-05
<i>cj1383c</i>	hypothetical protein	37.46	1.86E-25
<i>cj1384c</i>	hypothetical protein	36.89	2.71E-22
<i>cj1386</i>	ankyrin-repeat protein involved in heme trafficking	5.51	8.43E-20
<i>cj1450</i>	putative ATP/GTP-binding protein	-2.15	1.65E-07
<i>cj1461</i>	hypothetical protein	-1.70	1.11E-03
<i>cj1463</i>		-3.35	4.29E-13
<i>cj1481c</i>	putative helicase	-1.40	9.69E-04
<i>cj1482c</i>	hypothetical protein	-1.13	8.13E-03
<i>cj1501</i>		-1.07	8.83E-03

<i>cj1542</i>		-1.63	1.48E-04
<i>cj1564</i>	putative anaerobic C4-dicarboxylate transporter	-1.14	4.03E-03
<i>cj1582c</i>		-2.59	2.49E-03
<i>cj1587c</i>	putative ABC transporter membrane region	1.62	5.81E-04
<i>cj1650</i>	putative periplasmic protien	-3.86	6.53E-12
<i>cj1658</i>	putative integral membrane protein	4.99	3.08E-21
<i>cj1660</i>	putative integral membrane protein	3.17	6.47E-10
<i>cj1661</i>	possible ABC transport system permease protein	3.82	8.86E-14
<i>cj1662</i>	putative integral membrane protein	3.84	7.31E-12
<i>cj1663</i>	putative ABC transport system ATP binding protein	3.48	2.12E-12
<i>cj1664</i>	possible periplasmic thiredoxin	3.10	6.06E-10
<i>cj1680c</i>		-2.64	5.91E-04
<i>cj1725</i>	hypothetical protein	1.29	3.14E-03
<i>cmeA</i>	membrane fusion component of efflux system	-1.04	9.71E-03
<i>cmeR</i>	bile efflux repressor	-1.17	5.17E-03
<i>ctsD</i>	putative type II protein secretion system D protein	-1.34	1.60E-03
<i>ctuA</i>	putative transferrin/lactoferrin uptake receptor	8.09	5.44E-37
<i>ddlA</i>	putative D-alanine--D-alanine ligase	-1.32	4.20E-03
<i>dsbA</i>	putative disulphide isomerase	-1.19	6.00E-03
<i>dsbD</i>	putative thiol:disulfide interchange protein	-1.30	7.96E-03
<i>dut</i>	putative dUTPase	-1.44	6.05E-04
<i>exbB1</i>	exbB/tolQ family transport protein	7.48	5.74E-21
<i>exbB2</i>	exbB/tolQ family transport protein	6.09	2.30E-22
<i>exbD1</i>	exbB/tolQ family transport protein	5.91	4.24E-09
<i>exbD2</i>	exbB/tolQ family transport protein	33.73	1.74E-07
<i>flaA</i>	flagellin	-1.19	2.83E-03
<i>flaB</i>	flagellin	-1.56	1.01E-04
<i>flaG</i>	flagellin	-1.24	2.00E-03
<i>flgB</i>	putative flagellar basal-body rod protein	-2.13	1.38E-06
<i>flgD</i>	putative flagellar hook assembly protein	-2.67	2.76E-10
<i>flgE</i>	flagellar hook protein flgE	-2.38	1.11E-08
<i>flgE2</i>	flagellar hook subunit protein	-2.38	5.32E-09
<i>flgG</i>	flagellar basal-body rod protein	-1.23	2.23E-03
<i>flgG2</i>	putative flagellar basal-body rod protein	-2.05	1.07E-06
<i>flgH</i>	putative flagellar L-ring protein precursor	-2.51	2.19E-09
<i>flgI</i>	flagellar P-ring protein	-2.44	5.82E-09
<i>flgK</i>	putative flagellar hook-associated protein	-2.35	1.27E-08
<i>flgL</i>	flagellar hook-associated protein	-2.23	5.29E-08
<i>flgM</i>	anti- σ factor	-1.64	9.15E-05
<i>flgR</i>	σ^{54} associated transcriptional activator	-1.50	2.10E-04
<i>fliD</i>	putative flagellar hook-associated protein	-1.51	1.55E-04
<i>fliE</i>	putative flagellar hook-basal body complex protein	-1.24	2.06E-03
<i>fliK</i>	putative flagellar hook-length control protein	-3.93	6.84E-16
<i>fliP</i>	flagellar biogenesis protein	1.27	5.03E-03
<i>fliS</i>	flagellar protein	-1.22	2.40E-03
<i>fur</i>	ferric uptake repressor	-1.37	7.28E-04
<i>hisH</i>	amidotransferase HisH	-1.37	1.02E-03
<i>katA</i>	catalase	6.38	9.45E-29
<i>kdpB</i>	potassium transporting ATPase B chain	1.54	1.85E-03
<i>kfiD</i>	putative UDP-glucose 6-dehydrogenase	-1.47	1.39E-03
<i>lolA</i>	outer-membrane lipoprotein carrier protein	-3.00	6.75E-08
<i>mobB</i>	putative molybdopterin-guanine biosynthesis protein	-2.70	2.39E-05
<i>modA</i>	putative molybdate-binding lipoprotein	-1.47	2.75E-04
<i>nuoM</i>	NADH dehydrogenase I chain M	-1.10	6.83E-03
<i>oorD</i>	subunit of 2-oxoglutarate:acceptor oxidoreductase	-1.04	9.08E-03
<i>p19</i>	periplasmic iron binding protein	5.14	3.38E-22
<i>pebC</i>	ABC amino-acid transporter ATP-binding protein	-1.23	2.23E-03
<i>sodB</i>	superoxide dismutase	-1.15	3.78E-03
<i>tal</i>	putative transaldolase	-1.48	4.80E-04
<i>tonB1</i>	tonB transport protein	3.35	2.38E-08
<i>tonB2</i>	tonB transport protein	7.40	1.78E-27
<i>tonB3</i>	tonB transport protein	3.97	6.46E-15
<i>tpx</i>	thiol peroxidase	1.65	1.93E-04
<i>trxB</i>	thioredoxin reductase	2.49	6.66E-08

Table S3.2: Genes differentially expressed between iron limited and iron replete conditions

Listed are the genes that were found to be significantly differentially expressed under iron limitation (≥ 1.5 with a p value ≤ 0.01)

COG Letter	COG Annotation	% Total Transcriptome IronReplete	% Total Transcriptome IronLimited
C	Energy production and conversion	14.96%	11.57%
D	Cell cycle control and mitosis	0.61%	0.60%
E	Amino Acid metabolism and transport	5.81%	5.67%
F	Nucleotide metabolism and transport	2.73%	2.78%
G	Carbohydrate metabolism and transport	1.84%	1.92%
H	Coenzyme metabolism	3.51%	3.68%
I	Lipid metabolism	1.90%	1.91%
J	Translation	19.62%	19.83%
K	Transcription	1.98%	1.64%
L	Replication and repair	4.57%	2.80%
M	Cell wall/membrane/envelope biogenesis	3.89%	3.99%
N	Cell motility	5.45%	5.60%
O	Post-translational modification, protein turnover, chaperone functions	5.93%	7.94%
P	Inorganic ion transport and metabolism	2.16%	4.72%
Q	Secondary Structure	0.88%	0.91%
R	General Functional Prediction only	4.75%	5.61%
S	Function Unknown	1.47%	1.35%
T	Signal Transduction	4.62%	4.73%
U	Intracellular trafficking and secretion	2.89%	2.29%
V	Defense Categories	0.28%	0.30%

Table S3.5: Percent contribution of COG categories to the total transcriptome of *C. jejuni*

Listed are the overall contributions of each COG category to the total transcriptome

Supplemental Information for Chapter 4

Supplemental Figures for Chapter 4

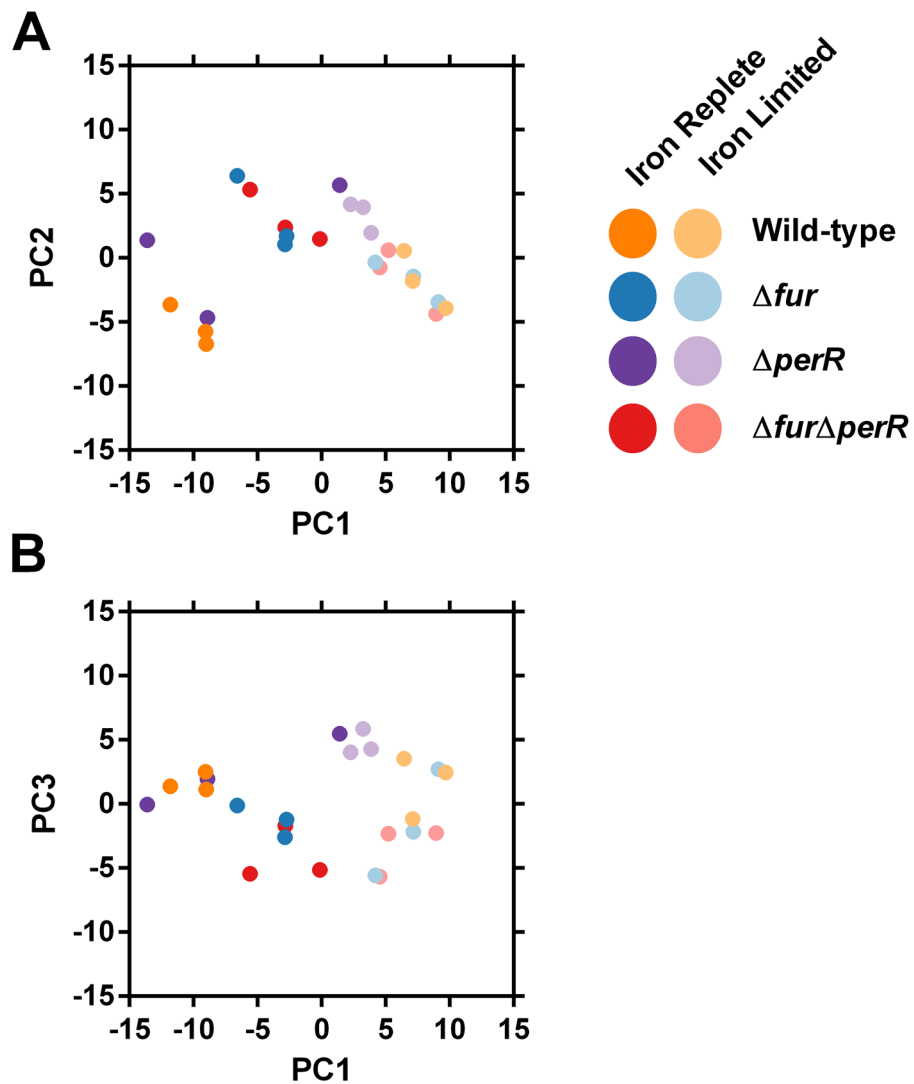


Figure S4.1: Biplots on PCA analysis of *C. jejuni* transcriptomic samples

Biplots of the PCA analysis (Figure 4.1) from the *C. jejuni* transcriptomic samples in this study. The PC1 vs PC2 (A) and PC1 vs PC3 (C) are plotted.

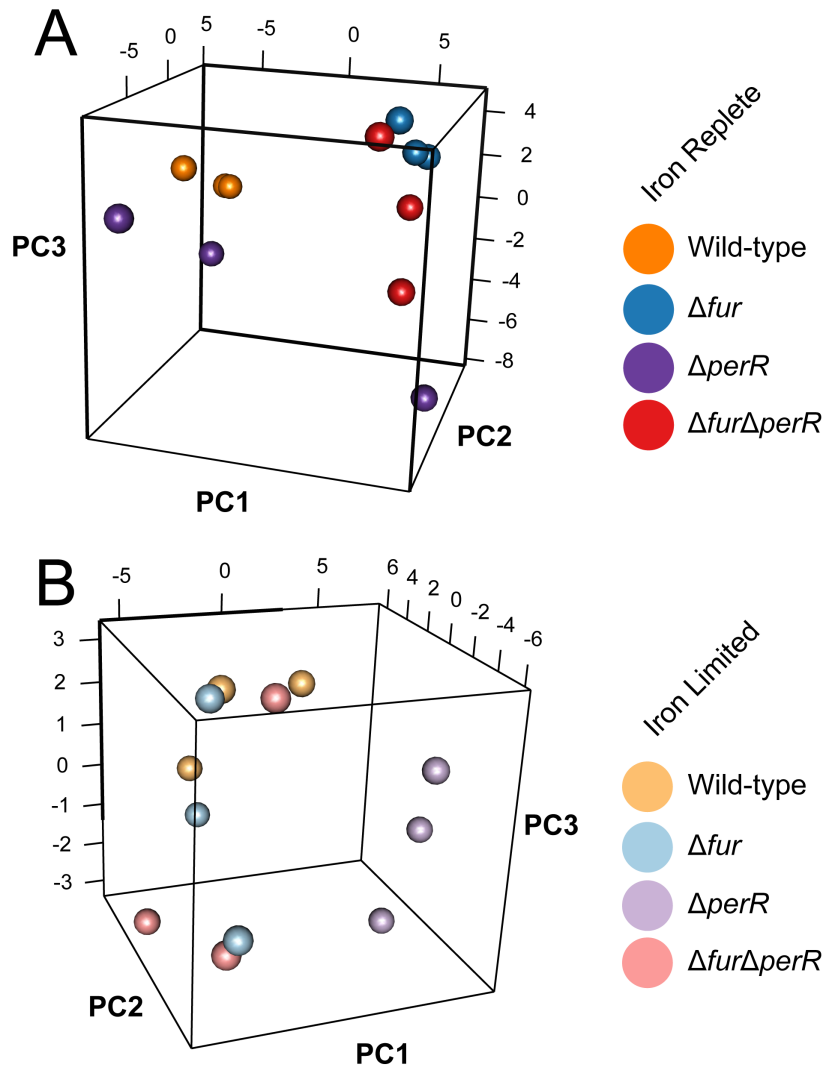


Figure S4.2: PCA analysis of *C. jejuni* transcriptomic samples by growth condition

The Log(RPKM+1) values for each gene in the *C. jejuni* NCTC11168 genome were used for PCA analysis under iron replete (A) or iron limited (B) conditions to show the overall structure of the transcriptomic data and to identify sample outliers. Under iron replete conditions different strains cluster together with the exception of one of the $\Delta perR$ samples (this sample clusters with iron limited samples in the expanded PCA analysis). The samples under iron limited conditions do not cluster as tightly as the iron replete conditions indicating greater variability in their transcriptomes.

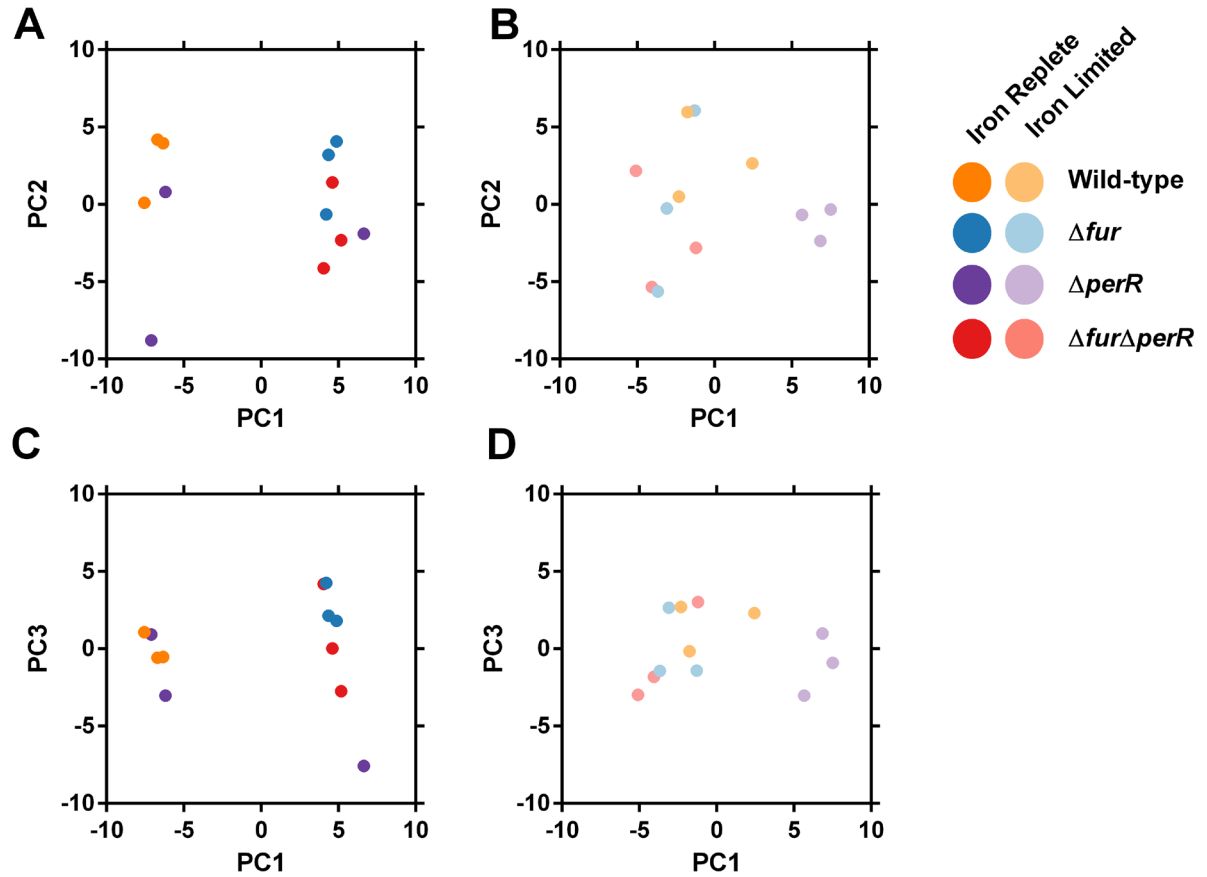


Figure S4.3: Biplots on PCA analysis of *C. jejuni* transcriptomic samples by growth condition

Biplots of the PCA analysis (Figure 4.1) from the *C. jejuni* transcriptomic samples in this study. The PC1 vs PC2 (A,B) and PC1 vs PC3 (C,D) for iron replete conditions (A,C) and iron limited conditions (C,D) are plotted.

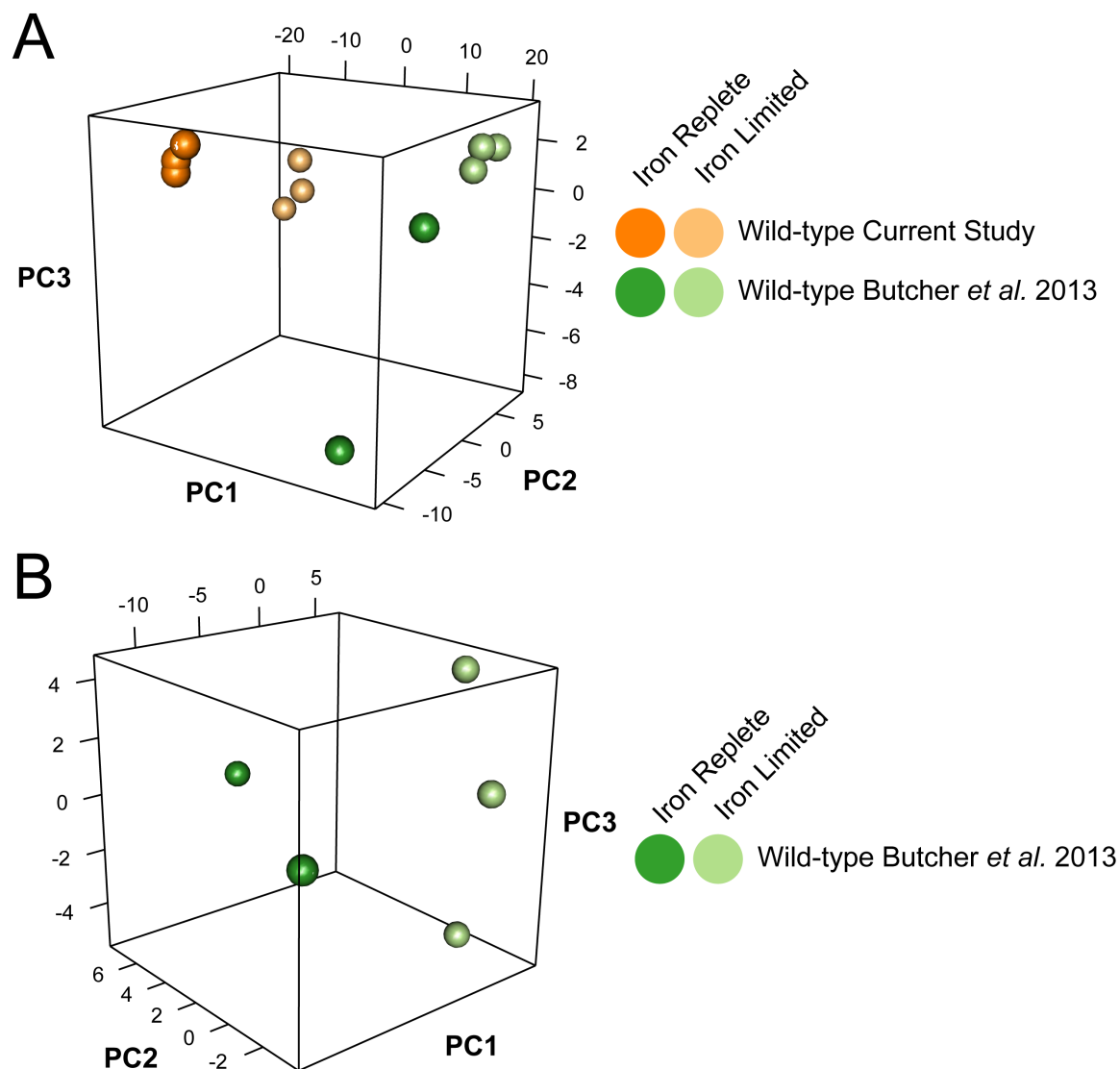


Figure S4.6: PCA analysis of previous RNA-seq samples

The Log(RPKM+1) values for each gene in the *C. jejuni* NCTC11168 genome were used for PCA analysis from the wild-type samples in this study and Butcher *et al.* 2013 (A) or in Butcher *et al.* alone (B). When comparing both studies samples can be differentiated based on two main axes that correspond to growth condition (iron replete vs iron limited) and study (current study vs previous study). With the exception of the iron replete samples from Butcher *et al.* 2013, all the samples cluster closely with their biological replicates. When the wild-type samples from Butcher *et al.* are analyzed separately (B) they separate based on growth condition, with no obvious biases between biological replicates.

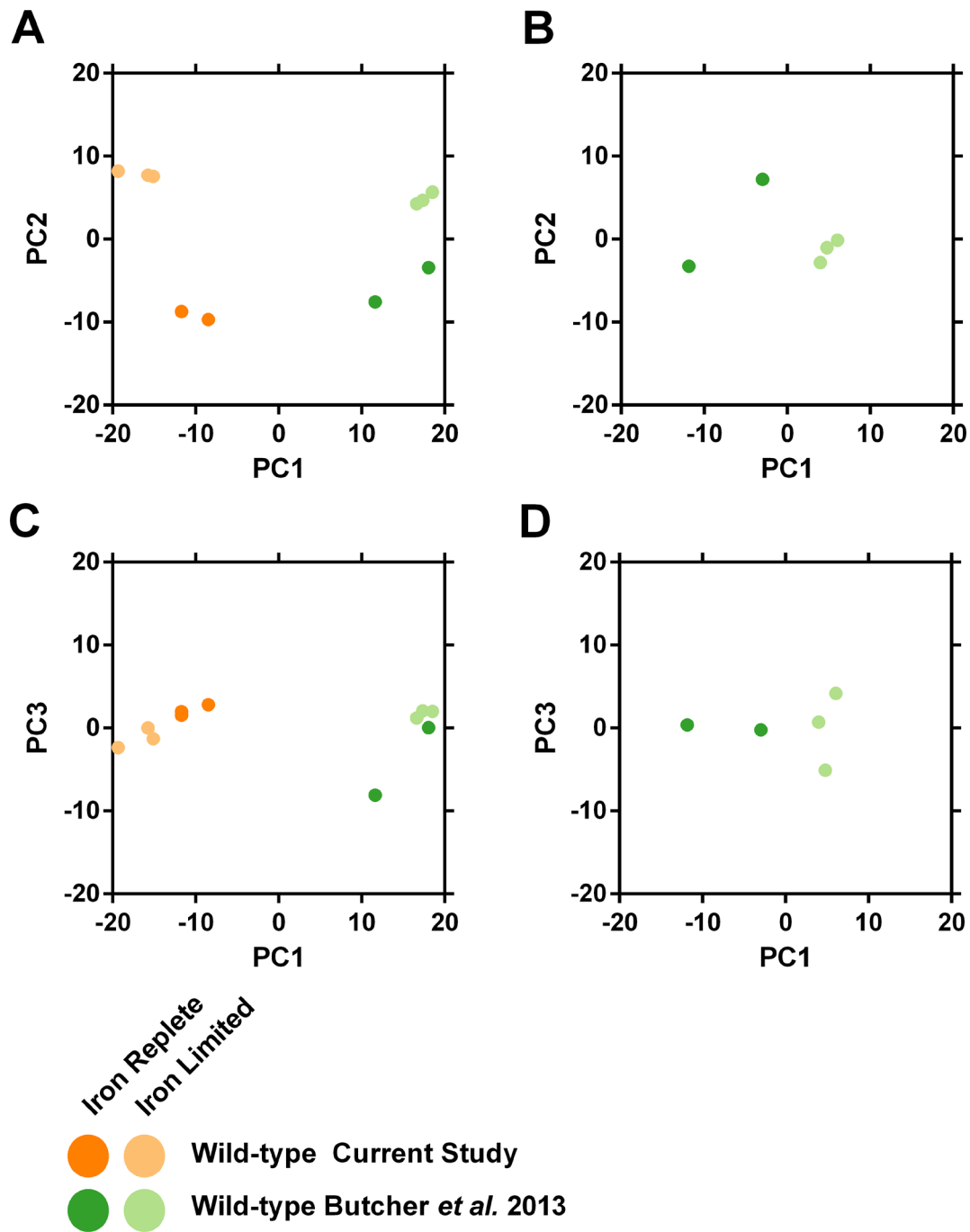


Figure S4.7: Biplots of PCA analysis on previous RNA-seq samples

Biplots of the PCA analysis (Figure S4.4) from the wild-type samples in this study and Butcher *et al.* 2013 (A,C) or in Butcher *et al.* alone (B,D). The PC1 vs PC2 (A,B) and PC1 vs PC3 (C,D) of each comparison is plotted.

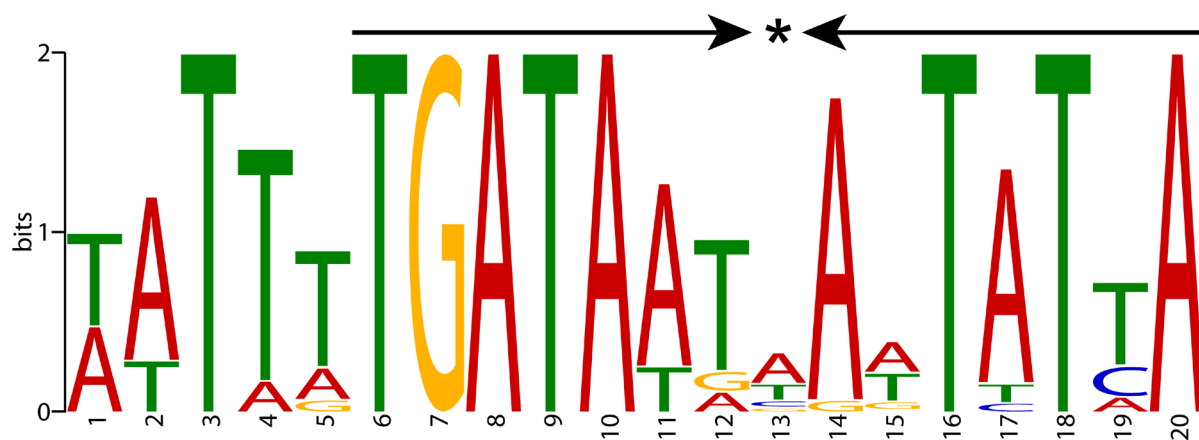


Figure S4.8: holo-CjFur repression binding motif

The holo-CjFur repression motif contains an inverted palindrome (marked by arrows and centered at the asterisk) and is quite similar to those previously reported for holo-CjFur repression (Butcher *et al.*, 2012; Palyada *et al.*, 2004).

Supplemental Tables for Chapter 4

For the sake of brevity, not all the supplemental files submitted with this manuscript are reproduced here due to the length and breadth of the genome wide analysis performed. Specifically, Tables S4.2, S4.3, S4.4, S4.7, S4.8, S4.9 and S4.10 are excluded.

Sample Name	Strain	Growth Media	Total Reads	% Read successfully aligned	% Reads on rRNA	General Comments
wt_plusIron_1	Wild-type NCTC11168	MEM α +40 μ M FeSO ₄	12,179,920	97.85%	26.11%	excluded from analysis based on PCA
wt_plusIron_2	Wild-type NCTC11168	MEM α +40 μ M FeSO ₄	17,221,523	97.97%	15.98%	
wt_plusIron_3	Wild-type NCTC11168	MEM α +40 μ M FeSO ₄	14,897,575	97.33%	25.21%	
fur_plusIron_1	Δfur isogenic deletion mutant	MEM α +40 μ M FeSO ₄	14,070,975	97.24%	38.06%	
fur_plusIron_2	Δfur isogenic deletion mutant	MEM α +40 μ M FeSO ₄	16,652,575	97.15%	35.95%	
fur_plusIron_3	Δfur isogenic deletion mutant	MEM α +40 μ M FeSO ₄	15,196,127	97.03%	23.09%	
perr_plusIron_1	$\Delta perR$ isogenic deletion mutant	MEM α +40 μ M FeSO ₄	15,044,531	96.81%	33.10%	
perr_plusIron_2	$\Delta perR$ isogenic deletion mutant	MEM α +40 μ M FeSO ₄	16,605,911	97.52%	16.17%	
perr_plusIron_3	$\Delta perR$ isogenic deletion mutant	MEM α +40 μ M FeSO ₄	18,401,208	96.93%	24.01%	
furperr_plusIron_1	$\Delta fur\Delta perR$ isogenic deletion mutant	MEM α +40 μ M FeSO ₄	17,209,636	97.57%	24.81%	
furperr_plusIron_2	$\Delta fur\Delta perR$ isogenic deletion mutant	MEM α +40 μ M FeSO ₄	15,124,826	96.95%	21.82%	
furperr_plusIron_3	$\Delta fur\Delta perR$ isogenic deletion mutant	MEM α +40 μ M FeSO ₄	17,264,924	97.10%	18.21%	
wt_minusIron_1	Wild-type NCTC11168	MEM α	14,874,933	96.94%	21.57%	excluded from analysis based on PCA
wt_minusIron_2	Wild-type NCTC11168	MEM α	15,199,776	97.62%	13.73%	
wt_minusIron_3	Wild-type NCTC11168	MEM α	17,041,456	97.77%	19.89%	
fur_minusIron_1	Δfur isogenic deletion mutant	MEM α	15,179,630	97.12%	25.72%	
fur_minusIron_2	Δfur isogenic deletion mutant	MEM α	16,443,703	97.11%	17.72%	
fur_minusIron_3	Δfur isogenic deletion mutant	MEM α	16,054,849	97.03%	23.13%	
perr_minusIron_1	$\Delta perR$ isogenic deletion mutant	MEM α	14,764,852	96.92%	32.72%	
perr_minusIron_2	$\Delta perR$ isogenic deletion mutant	MEM α	15,826,029	96.32%	23.22%	
perr_minusIron_3	$\Delta fur\Delta perR$ isogenic deletion mutant	MEM α	16,204,925	97.21%	21.14%	
furperr_minusIron_1	$\Delta fur\Delta perR$ isogenic deletion mutant	MEM α	15,134,905	97.21%	33.72%	
furperr_minusIron_2	$\Delta fur\Delta perR$ isogenic deletion mutant	MEM α	17,344,844	97.47%	16.16%	
furperr_minusIron_3	$\Delta fur\Delta perR$ isogenic deletion mutant	MEM α	15,284,667	97.27%	19.48%	

Table S4.1: Statistics for high throughput sequencing runs

Listed are the read statistics for the high throughput sequencing runs performed in this study.

Gene List	Δfur	$\Delta perR$	$\Delta fur \Delta perR$	Cluster	Gene List	Δfur	$\Delta perR$	$\Delta fur \Delta perR$	Cluster	Gene List	Δfur	$\Delta perR$	$\Delta fur \Delta perR$	Cluster
<i>acs</i>	1.14			F	<i>cj0560</i>	1.24		1.11	D	<i>cj1662</i>	6.96	1.58	6.93	G
<i>ahpC</i>	4.48	4.39		H	<i>cj0561c</i>			1.10	F	<i>cj1663</i>	5.67		5.63	G
<i>argB</i>	1.52	1.22		E	<i>cj0581</i>		-0.87		C	<i>cj1664</i>	5.57		5.57	G
<i>argC</i>		0.85		F	<i>cj0598</i>		-1.05		C	<i>cj1665</i>	2.48		2.49	G
<i>argD</i>	1.21			F	<i>cj0599</i>		-1.00		C	<i>cj1722c</i>	-0.80			F
<i>aroE</i>		-0.64		B	<i>cj0715</i>	0.67			C	<i>cj1723c</i>	-0.70			F
<i>bioB</i>	0.78			F	<i>cj0716</i>	0.67			C	<i>cstA</i>			0.65	F
<i>birA</i>	-0.78			C	<i>cj0717</i>		-1.17		C	<i>ctuA</i>	7.58		7.36	G
<i>ceuB</i>	4.58	5.72		G	<i>cj0719c</i>		-1.01		C	<i>cydA</i>		1.56		E
<i>ceuC</i>	3.40	4.58		G	<i>cj0735</i>		-2.68		C	<i>cydB</i>		1.62		E
<i>ceuD</i>	3.32	4.50		G	<i>cj0771c</i>		0.71		F	<i>dnaE</i>	0.68			D
<i>ceuE</i>	2.84	3.55		G	<i>cj0818</i>	6.50		6.31	G	<i>dnaJ</i>		1.17	0.86	F
<i>cfbpA</i>	5.73	5.00		G	<i>cj0819</i>	4.87		4.76	G	<i>exbB1</i>	7.24		7.02	G
<i>cfbpB</i>	5.06	4.42		G	<i>cj0854c</i>		-1.34		C	<i>exbB2</i>	8.79		8.54	G
<i>cfbpC</i>	4.68	4.19		G	<i>cj0859c</i>		1.12		E	<i>exbD1</i>	7.30		7.27	G
<i>cfrA</i>	8.49	8.68		G	<i>cj0914c</i>		-0.81		C	<i>exbD2</i>	7.82	1.30	8.19	G
<i>cgb</i>	1.72	1.77		D	<i>cj0915</i>			1.46	F	<i>fabi</i>			-2.23	B
<i>chaN</i>	6.05	5.89		G	<i>cj0916c</i>		-0.97		C	<i>feoB</i>	0.88			D
<i>chuA</i>	9.44	9.11		G	<i>cj0947c</i>	0.86		1.08	D	<i>flaA</i>	-0.98		-2.20	B
<i>chuB</i>	8.09	7.94		G	<i>cj0948c</i>	0.96		1.17	D	<i>flaD</i>		1.10		E
<i>chuC</i>	9.77	9.62		G	<i>cj0949c</i>	1.01		1.14	D	<i>fldA</i>	2.24			D
<i>chuD</i>	7.35	6.72		G	<i>cj0967</i>		-1.68		C	<i>flgD</i>		0.98		E
<i>chuZ</i>	5.02	4.49		G	<i>cj0977</i>			-0.75	B	<i>flgI</i>		0.97		E
<i>cj0004c</i>	0.72			F	<i>cj0982c</i>		0.97		F	<i>flgJ</i>		0.92		E
<i>cj0005c</i>	1.12			F	<i>cj0987c</i>		1.78		E	<i>flgM</i>			-0.94	B
<i>cj0008</i>	1.61			D	<i>cj1025c</i>		0.81		E	<i>flgS</i>		0.90		E
<i>cj0011c</i>	-2.54	1.27		A	<i>cj1068</i>	0.82			D	<i>fliK</i>		0.93		E
<i>cj0017c</i>	0.95	2.00	2.66	E	<i>cj1069</i>	0.88			D	<i>fliP</i>	2.52		2.42	G
<i>cj0018c</i>	0.95	1.95	2.65	E	<i>cj1086c</i>			-0.66	B	<i>fliS</i>			-0.91	B
<i>cj0021c</i>	0.72			E	<i>cj1159c</i>		-1.50		C	<i>fur*</i>	-2.08		-2.44	NA*
<i>cj0034c</i>	0.96			F	<i>cj1169c</i>		2.22		E	<i>gapA</i>			-2.47	B
<i>cj0035c</i>	1.15			F	<i>cj1199</i>			1.77	F	<i>glnA</i>	2.71	2.39		D
<i>cj0044c</i>	0.88			E	<i>cj1200</i>			1.78	F	<i>gltB</i>	1.92			D
<i>cj0045c</i>	1.01			E	<i>cj1241</i>		1.61		E	<i>gltD</i>	1.31	1.51		D
<i>cj0090</i>		-0.81		B	<i>cj1280c</i>		-1.40		C	<i>glyA</i>			-0.97	B
<i>cj0100</i>	-0.86			C	<i>cj1295</i>			-1.47	B	<i>hisH1</i>		0.82		E
<i>cj0101</i>	-0.73			C	<i>cj1296</i>			-2.66	B	<i>hrcA</i>		1.76		E
<i>cj0119</i>	-1.03			C	<i>cj1297</i>		0.73		B	<i>hydA2</i>			-2.20	B
<i>cj0123c</i>	0.79			F	<i>cj1298</i>			-2.26	B	<i>katA</i>	1.67	8.92	9.44	H
<i>cj0124c</i>	0.82			F	<i>cj1326</i>	1.35		1.54	D	<i>ktrA</i>		0.93		F
<i>cj0125c</i>	0.86			F	<i>cj1340c</i>	-0.59	-1.01		C	<i>leuA</i>		1.49		E
<i>cj0145</i>		1.17		F	<i>cj1343c</i>		-1.35		C	<i>leuB</i>		1.61		E
<i>cj0170</i>	1.60			D	<i>cj1344c</i>		-1.61		C	<i>leuC</i>		1.43		E
<i>cj0171</i>	3.20			D	<i>cj1345c</i>		-1.04		C	<i>maf6</i>		-0.87		C
<i>cj0172c</i>	1.28	0.97		D	<i>cj1372</i>		0.84		F	<i>maf7</i>		-1.03		C
<i>cj0176c</i>	5.57	4.90		G	<i>cj1383c</i>	4.57		2.76	G	<i>metE</i>			1.33	F
<i>cj0182</i>	0.88	0.98		D	<i>cj1384c</i>	10.26		8.23	G	<i>neuB2</i>	0.59		0.75	D
<i>cj0186c</i>		0.85		F	<i>cj1386</i>	1.14	7.17	7.74	H	<i>omp50</i>		2.17		E
<i>cj0200c</i>	-0.69	0.94		F	<i>cj1387c</i>		1.05	1.31	F	<i>p19</i>	7.71	2.32	7.47	G
<i>cj0225</i>	1.36			E	<i>cj1388</i>		0.93	1.70	F	<i>perR*</i>		-2.48	-3.20	NA*
<i>cj0243c</i>	-0.96			B	<i>cj1397</i>	1.18			D	<i>pgk</i>			-2.40	B
<i>cj0250c</i>		0.78		F	<i>cj1406c</i>		0.81		F	<i>pgsA</i>	0.74			D
<i>cj0251c</i>		0.75		F	<i>cj1422c</i>		-1.00	-0.73	C	<i>prfA</i>	1.37		1.52	D
<i>cj0255c</i>		0.75		F	<i>cj1450</i>			-0.90	B	<i>pseB</i>		0.80		E
<i>cj0358</i>	-0.89			B	<i>cj1500</i>		2.30		E	<i>pseC</i>		0.81		E
<i>cj0378c</i>		1.51	2.58	E	<i>cj1501</i>		1.75	1.03	E	<i>putP</i>		0.76		F
<i>cj0379c</i>		2.49	3.44	E	<i>cj1533c</i>			1.24	F	<i>rnhB</i>		0.83	0.90	F
<i>cj0403</i>		-0.82		B	<i>cj1542</i>		0.77		F	<i>rrc</i>	-4.87	1.65		A
<i>cj0414</i>		0.76		F	<i>cj1555c</i>		0.68		F	<i>tonB1</i>	4.08		4.08	G
<i>cj0415</i>	-0.73	0.68		F	<i>cj1582c</i>		0.87		E	<i>tonB2</i>	5.14		5.01	G
<i>cj0418c</i>		-0.90		C	<i>cj1583c</i>		1.29		E	<i>tonB3</i>	5.53		5.08	G
<i>cj0420</i>	1.05			D	<i>cj1584c</i>		1.38		E	<i>tpiA</i>			-2.19	B
<i>cj0424</i>		0.86		F	<i>cj1587c</i>	3.30	1.15	3.35	G	<i>trmB</i>		-1.07		C
<i>cj0428</i>	-0.78			B	<i>cj1618c</i>	1.73		1.36	D	<i>trpD</i>		0.74		F
<i>cj0454c</i>	0.71			D	<i>cj1627c</i>	0.88			D	<i>trpE</i>		0.87		F
<i>cj0550</i>		-0.74		B	<i>cj1650</i>	-0.71	0.95		E	<i>trxB</i>	1.80	3.58	4.27	H
<i>cj0553</i>		0.86		E	<i>cj1658</i>	7.56	2.19	7.55	G	Only significant Log2 fold changes are shown on this table for simplicity				
<i>cj0555</i>	0.63	0.98	1.11	F	<i>cj1660</i>	5.87		5.66	G	*expected to be differentially expressed due to gene deletion, not included in clustering				
<i>cj0559</i>	0.85			D	<i>cj1661</i>	7.02	1.60	6.89	G					

Table S4.5: Differentially expressed genes under iron replete conditions

Gene List	Δfur	$\Delta perR$	$\Delta fur \Delta perR$	Cluster	Gene List	Δfur	$\Delta perR$	$\Delta fur \Delta perR$	Cluster	Gene List	Δfur	$\Delta perR$	$\Delta fur \Delta perR$	Cluster
<i>acnB</i>	-0.71			H	<i>cj0947c</i>	1.20		1.11	D	<i>flaD</i>	-1.28	2.48		F
<i>ald2</i>	-0.80			K	<i>cj0948c</i>	1.94		1.94	A	<i>flaG</i>		1.45		G
<i>amiA</i>	-0.72			J	<i>cj0949c</i>	2.06		2.01	A	<i>flgB</i>		2.03		F
<i>argC</i>			1.18	C	<i>cj0959c</i>			1.11	C	<i>flgD</i>	-2.35	3.09		E
<i>aroE</i>	-0.89	-0.75		K	<i>cj0967</i>		1.06		K	<i>flgE1</i>	-2.26	2.49		E
<i>arsB</i>	-0.96			J	<i>cj0970</i>	-1.71			K	<i>flgE2</i>	-2.28	2.74		E
<i>aspA</i>			-1.35	K	<i>cj0973</i>		-1.50		K	<i>flgG</i>		1.83		F
<i>cadF</i>	1.20			H	<i>cj0977</i>		-0.72		F	<i>flgG2</i>	-1.13	3.06		E
<i>cbpA</i>	0.77			H	<i>cj0982c</i>		-0.69		H	<i>flgH</i>	-1.51	3.06		E
<i>cdtA</i>	0.74			H	<i>cj1025c</i>		1.93		G	<i>flgI</i>	-2.09	3.24		E
<i>cdtB</i>	0.84			H	<i>cj1026c</i>		1.96		G	<i>flgK</i>	-1.35	2.27		F
<i>cdtC</i>	0.62			H	<i>cj1028c</i>		0.63		H	<i>flgM</i>		-1.23	-0.84	F
<i>ceuB</i>			1.54	D	<i>cj1034c</i>		0.99		G	<i>flgS</i>		1.08		G
<i>ceuC</i>			1.19	C	<i>cj1040c</i>		-0.67		K	<i>fliD</i>		1.19		G
<i>ceuD</i>			1.19	D	<i>cj1062</i>		0.86		H	<i>fliK</i>	-2.52	2.64		E
<i>ceuE</i>			0.84	C	<i>cj1068</i>		0.76		J	<i>fliL</i>		0.98		H
<i>cfa</i>	-1.31			F	<i>cj1069</i>		-0.87		J	<i>fliR</i>		-1.10		B
<i>cj0004c</i>		0.82		H	<i>cj1079</i>		-0.98		J	<i>flis</i>		0.67		F
<i>cj0005c</i>		1.07		H	<i>cj1084c</i>		-0.89		K	<i>frdC</i>		0.97		H
<i>cj0011c</i>	1.10	0.70	0.96	D	<i>cj1110c</i>		0.81		H	<i>fur*</i>	-2.50		-2.53	NA*
<i>cj0014c</i>		0.85		H	<i>cj1112c</i>		0.73		H	<i>gapA</i>			-2.18	I
<i>cj0015c</i>		0.72		H	<i>cj1153</i>		1.59		D	<i>glnP</i>		1.13		J
<i>cj0034c</i>		1.05		G	<i>cj1158c</i>		-1.16		K	<i>glpT</i>		-1.05		K
<i>cj0035c</i>		-0.77		H	<i>cj1159c</i>		-1.37		J	<i>glta</i>		1.20		D
<i>cj0037c</i>		1.71		G	<i>cj1168c</i>		-0.70		J	<i>gpsA</i>		-0.68		J
<i>cj0040</i>	-2.69	3.34		E	<i>cj1174</i>		-0.72		J	<i>hddC</i>		-0.85		J
<i>cj0044c</i>		-1.18		F	<i>cj1224</i>		1.57		G	<i>hemD</i>		-0.95		J
<i>cj0045c</i>		1.43		G	<i>cj1225</i>		1.38		G	<i>hemH</i>		-0.73		B
<i>cj0073c</i>		1.08		D	<i>cj1226c</i>		-1.02		K	<i>hspR</i>		0.69		H
<i>cj0074c</i>		1.23		D	<i>cj1240c</i>		0.92		H	<i>htrA</i>			-1.36	K
<i>cj0075c</i>		1.25		D	<i>cj1242</i>	-1.72	2.94		E	<i>hydA</i>		1.16		G
<i>cj0080</i>		1.09	1.51	D	<i>cj1245c</i>		-0.90		K	<i>hydA2</i>			-1.69	I
<i>cj0091</i>			-0.92	K	<i>cj1251</i>		-0.84		J	<i>hydB</i>		0.84		H
<i>cj0101</i>		0.61		K	<i>cj1252</i>		-1.24		B	<i>iamB</i>			0.98	B
<i>cj0119</i>		-0.97		B	<i>cj1254</i>		-1.01		B	<i>ilvD</i>		1.49		D
<i>cj0120</i>		-1.14		B	<i>cj1258</i>		0.82		H	<i>infA</i>		0.81		H
<i>cj0121</i>		-0.68		B	<i>cj1275c</i>		-0.84		J	<i>katA</i>		2.42	2.06	D
<i>cj0122</i>		-0.66		H	<i>cj1276c</i>		0.66		J	<i>kdpB</i>		-1.13		K
<i>cj0123c</i>			1.09	D	<i>cj1279c</i>		-0.84		K	<i>kdpD</i>		0.61		K
<i>cj0124c</i>			1.00	D	<i>cj1280c</i>		-1.00		K	<i>ktrA</i>			1.11	C
<i>cj0133</i>	-0.77			J	<i>cj1285c</i>		-0.71		J	<i>ldh</i>		0.81		H
<i>cj0141c</i>	-0.69			B	<i>cj1295</i>	-1.39		-0.96	F	<i>leuA</i>		1.63		D
<i>cj0154c</i>			1.28	D	<i>cj1296</i>	-2.58		-1.78	I	<i>leuB</i>		1.33		G
<i>cj0182</i>	-0.87			J	<i>cj1297</i>	-2.32		-1.48	I	<i>leuC</i>		0.89		H
<i>cj0186c</i>			1.16	D	<i>cj1298</i>	-1.60			K	<i>livF</i>		-0.80		J
<i>cj0190c</i>	0.79			B	<i>cj1325</i>			0.93	C	<i>livG</i>		-0.74		J
<i>cj0200c</i>	1.01	0.87		D	<i>cj1326</i>	1.11		1.35	D	<i>livM</i>		-0.71		J
<i>cj0230c</i>	-0.75			J	<i>cj1340c</i>		-1.90		J	<i>lpxA</i>		0.77		B
<i>cj0238</i>	0.64			H	<i>cj1343c</i>		-2.53		J	<i>lpxD</i>		-0.75		K
<i>cj0239c</i>	0.86			H	<i>cj1344c</i>		-2.69		J	<i>maf6</i>		-1.92		J
<i>cj0243c</i>	2.44			F	<i>cj1345c</i>		-1.47		J	<i>maf7</i>		-2.12		J
<i>cj0250c</i>	0.80			H	<i>cj1356c</i>	4.50	1.18	4.43	A	<i>mapA</i>		0.81		H
<i>cj0251c</i>	1.17			H	<i>cj1386</i>	1.66	2.52	2.19	D	<i>metA</i>		1.02		H
<i>cj0253</i>	0.72			H	<i>cj1387c</i>		1.80	1.13	D	<i>metC_1</i>		-0.69		J
<i>cj0260c</i>	1.40			G	<i>cj1388</i>		1.17	1.31	D	<i>metC_2</i>		-1.26		J
<i>cj0265c</i>	1.72			F	<i>cj1422c</i>		-0.95		K	<i>metY</i>		1.62		G
<i>cj0266c</i>	0.97			J	<i>cj1450</i>		1.46		F	<i>mfd</i>		-0.95		K
<i>cj0270</i>	0.97			H	<i>cj1459</i>		0.77		H	<i>mnmC</i>		-1.02		B
<i>cj0277</i>	-1.00			B	<i>flgl</i>	-2.15	2.85		E	<i>moaC</i>		-0.91		H
<i>cj0323</i>			-1.46	K	<i>cj1465</i>		1.44		F	<i>moeB</i>		0.68		H
<i>cj0331c</i>			1.18	D	<i>cj1467</i>		0.93		H	<i>mrp</i>		0.63		H
<i>cj0353c</i>			0.99	C	<i>cj1477c</i>		0.67		H	<i>mutS</i>		-0.61		J
<i>cj0358</i>	0.91			H	<i>cj1493c</i>		-0.73		J	<i>napB</i>		-0.79		H
<i>cj0364</i>	1.02			H	<i>cj1500</i>		0.79	1.26	D	<i>napG</i>		-0.77		H
<i>cj0391c</i>	1.73			G	<i>cj1501</i>		0.64	1.10	D	<i>napH</i>		0.73		H
<i>cj0403</i>			-0.84	K	<i>cj1506c</i>		1.21		G	<i>ndk</i>			1.21	D
<i>cj0414</i>	2.64			E	<i>cj1513c</i>		1.05		H	<i>nrfA</i>		1.25		G
<i>cj0415</i>	2.02			G	<i>cj1514c</i>		0.93		H	<i>nrfH</i>		1.35		G
<i>cj0416</i>	0.83			F	<i>cj1520</i>		-1.25		K	<i>oorA</i>		0.90		H
<i>cj0417</i>	0.64			F	<i>cj1541</i>		0.95		H	<i>oorB</i>		0.81		H
<i>cj0418c</i>	0.79			K	<i>cj1542</i>		0.72		H	<i>oorC</i>		0.75		H

cj0423	0.76	H	cj1546	0.67	H	oorD	0.87	H		
cj0428	3.08	E	cj1555c	0.64	H	panD	0.87	K		
cj0449c	0.90	H	cj1584c	0.99	H	perR*	-5.12	-6.04	NA*	
cj0495	0.70	J	cj1585c	-0.88	H	petA	1.45		D	
cj0500	-0.84	J	cj1587c	0.79	H	petB	1.24		D	
cj0522	-1.26	J	cj1588c	1.01	H	petC	1.15		D	
cj0523	-1.58	J	cj1608	0.75	H	pgk		-1.94	I	
cj0524	-1.11	K	cj1621	-0.80	H	pnp	-0.59		B	
cj0552	-1.01	H	cj1631c	1.43	G	porA	0.96		H	
cj0560	-1.27	J	cj1632c	1.99	F	pseB	1.81		G	
cj0584	-0.81	J	cj1633		1.10	pseC	1.36		G	
cj0590	-0.77	J	cj1650	2.88	E	pth		1.10	C	
cj0598	-1.01	K	cj1656c	1.65	G	purH		1.20	D	
cj0599	-1.29	J	cj1668c	0.82	H	purL		1.22	D	
cj0600	-1.82	J	cj1714	1.62	D	ribF	-1.02		J	
cj0602c	-1.18	K	cj1721c	0.68	H	rnpA		0.94	C	
cj0628	1.64	D	cj1728c	-2.20	1.96	rplM		1.19	D	
cj0635	0.86	B	clpX	-0.75	B	rpmB	1.26	1.22	D	
cj0636	-1.11	J	cmeC	-0.61	J	rpmE		1.22	D	
cj0647	-0.86	B	crcB	-1.12	J	rpmF		1.12	D	
cj0660c	-0.98	B	ctsE	-0.88	B	rpmH		0.95	C	
cj0717	-0.58	J	cydA		1.22	rpmJ		1.08	D	
cj0719c		-1.10	cydB		1.09	rrc	4.49	3.31	4.38	A
cj0728	-0.74	K	cynT	0.90	H	smpB		-0.84		K
cj0729	-1.30	K	dcuB		-0.91	sodB		0.96		H
cj0730	-1.37	K	def	-0.76	J	tal			0.86	C
cj0736	-1.04	K	dprA	-1.02	B	thiE	-0.89			K
cj0741	-1.09	K	dsbD	-0.96	-0.80	thiG	-0.83			H
cj0770c	1.15	G	exbB3	-0.84	B	tlyA	-0.95			J
cj0771c	1.17	G	exbD1	-0.80	J	tonB1	1.48			J
cj0772c	1.24	G	exbD3	-1.08	B	tonB3		-1.01		K
cj0777	-0.77	J	fabI		-1.73	tpiA		-1.72		I
cj0814	1.05	G	fdhA	1.07	H	trmB	-1.19			K
cj0834c	0.70	H	fdhB	0.96	H	trmE		1.20		D
cj0848c	-0.88	K	fdhC	1.01	H	trpD	1.22			D
cj0849c	-0.70	K	fdxA	4.70	1.11	4.51	trpE	1.92	1.15	D
cj0859c	2.03	F	feoB		-0.88	K	wlaI	-0.85		J
cj0890c	0.64	J	flaA	-1.11	-1.76	I	zupT	-1.41		K
cj0904c	-0.86	J	flaB	1.80	1.76	D	Only significant Log2 fold changes are shown on this table for			

Only significant Log2 fold changes are shown on this table for simplicity

*expected to be differentially expressed due to gene deletion, not included in clustering

Table S4.6: Differentially expressed genes under iron limited conditions

Curriculum Vitae

Publications

- **Butcher J**, Handley R, van Vliet A and Stintzi A. (Submitted to BMC Genomics) “Unveiling the iron-dependent/independent Fur- and PerR-regulons of *Campylobacter jejuni*”
- Li J, **Butcher J**, Mack D, Stintzi A. (2015) “Influences of the Microbiome in the pathogenesis of Inflammatory bowel disease” *Inflammatory Bowel Diseases* 21(1):139-53 [PMID: 25248007](#)
- Flint A*, Sun YQ*, **Butcher J**, Stahl M, Huang H, Stintzi A. (2014) “Phenotypic screening of a targeted mutant library reveals *Campylobacter jejuni* defenses against oxidative stress” *Infect. Immun*, 82, 2266-2275. [PMID: 24643543](#)
- Flint A, **Butcher J**, Stintzi A. (2014) “Campylobacter strategies for colonizing the host gastrointestinal tract” In Sheppard S and Méric G (Eds.) “Campylobacter Ecology and Evolution”
- **Butcher J**, Stintzi A. (2013) “The transcriptional landscape of *Campylobacter jejuni* under iron replete and iron limited growth conditions” *PlosONE* 8(11):p.e79475 [PMID: 24223952](#)
- Naikare H*, **Butcher J***, Flint A, Xu J, Raymond KN, Stintzi A. (2013) “*Campylobacter jejuni* ferric-enterobactin receptor CfrA is TonB3 dependent and mediates iron acquisition from structurally different catechol siderophores” *Metallomics* DOI: 10.1039/c3mt20254b [PMID: 23702883](#)

- **Butcher J***, Sarvan S*, Brunzelle J, Couture JF, Stintzi A. (2012) “The structure and regulon of *Campylobacter jejuni* ferric uptake regulator Fur define apo-Fur regulation” *PNAS* 109(25):10047-52 [PMID: 22665794](#)
- Stahl M, **Butcher J**, Stintzi A. (2012) “Nutrient Acquisition and Metabolism by *Campylobacter jejuni*” *Frontiers in Cellular and Infection Microbiology* 2:5 [PMID: 22919597](#)
- Flint A, **Butcher J**, Clarke C, Marlow D, Stintzi A. (2010) “Use of a rabbit soft tissue chamber model to investigate *Campylobacter jejuni*-host interactions” *Frontiers in Microbiology*. 1:126 [PMID: 21687769](#)
- **Butcher J**, Flint A, Stahl M, Stintzi A. (2010) “Campylobacter Fur and PerR Regulons” In Cornelis P and Andrews SC (Eds.) “Iron Uptake and Homeostasis in Microorganisms.”
- Palyada K, Sun YQ, Flint A, **Butcher J**, Naikare H, Stintzi A. (2009) “Characterization of the oxidative stress regulon of *Campylobacter jejuni*.” *BMC Genomics* 10 p.481 [PMID: 19835633](#)
- **Butcher J**, Abdou H, Morin K, Liu Y. (2009) “Micromanaging oligodendrocyte differentiation by noncoding RNA: toward a better understanding of the lineage commitment process” *J Neuroscience* 29, 5365-5366 [PMID: 19403803](#)

Scholarships

- Ontario Graduate Student Scholarship, May 2012 – May 2013 (\$15,000)

- CIHR Fredrick Banting and Charles Best Doctoral Award, May 2009 – May 2012 (\$105,000)
- NSERC Industrial Undergraduate Student Research Award, Fiscal Year 2005 (\$4,500)
- NSERC Industrial Undergraduate Student Research Award, Fiscal Year 2004 (\$4,500)
- NSERC Industrial Undergraduate Student Research Award, Fiscal Year 2003 (\$4,500)

Awards

- Training Assistant of the Year (Biochemistry) 2013-14, University of Ottawa, November 2014
- Syed Sattar Student Award (PhD student), University of Ottawa, March 2014
- Award of Excellence in Graduate Studies for the Ph.D. in Biochemistry, University of Ottawa, December 2013
- CSM Cederlane Student Symposium Award, 63rd Annual Canadian Society of Microbiologists Conference, Ottawa, Canada, June 2013
- Student Presentation Award, 8th International Biometals Symposium, Brussels, Belgium, July 2012
- 3rd Best Poster, Biochemistry Graduate Student Poster Day, Ottawa, Canada, May 2010
- 2nd Best Poster, Systems Biology Symposium, Ottawa, Canada, April 2009
- Best Poster Award, 6th International Biometals Symposium, Santiago de Compostela, Spain, July 2008

Oral Presentations

- **Butcher J**, Stintzi A. “What is the role of the gut microbiota in Inflammatory Bowel Diseases?” 3rd China-Canada Symposium on Systems Biology and Personalized Medicine, Shanghai, China, May 2014
- **Butcher J**, Stintzi A. “Ironing out the details: Characterizing transcriptome structure and identifying novel transcripts in *Campylobacter jejuni* under iron limitation” 63rd Annual Canadian Society of Microbiologists Conference, Ottawa, Canada, June 2013
- **Butcher J**, Stintzi A. “The full repertoire of iron regulated transcripts and sRNA in *C. jejuni*” Canadian *Campylobacter* Workshop 2012, Ottawa, Canada, September 2012
- **Butcher J**, Stintzi A. “RNA-seq profiling reveals unanticipated complexity in *C. jejuni*’s transcriptional repertoire” 8th International Biometals Symposium, Brussels, Belgium, July 2012
- **Butcher J**, Sun YQ, Flint A, Palyada K, Naikare H, Stintzi A. “Characterization of the regulatory networks controlling iron metabolism in *C. jejuni*” Canadian *Campylobacter* Conference, Montreal, Canada, September 2008
- **Butcher J**, Sun YQ, Flint A, Palyada K, Naikare H, Stintzi A. “The Regulatory network controlling iron metabolism in *Campylobacter jejuni*” 6th International Biometals Symposium, Santiago de Compostela, Spain, July 2008

Poster Presentations

- Grzywacz K, **Butcher J**, Mohamed I, Barrington K, Mack D, Stintzi A. “The effects of lactoferrin supplementation on the intestinal microbiota of premature infants receiving

probiotics” North American Society for Pediatric Gastroenterology, Hepatology and Nutrition, Atlanta, Georgia, USA, October 2014

- **Butcher J**, Handley R, van Vliet A, Stintzi A. “Unveiling the iron dependent/independent Fur and PerR regulons in *Campylobacter jejuni*” 9th International Biometals Symposium, Durham, North Carolina USA, July 2014
- **Butcher J**, Grzywacz K, Mack D, Stintzi A. “Defining the specific and synergistic effects of probiotic and/or lactoferrin supplementation on the gut microbiota composition of newborn piglets” 3rd China-Canada Symposium on Systems Biology and Personalized Medicine, Shanghai, China, May 2014
- **Butcher J**, Handley R, Reuter M, Stintzi A, van Vliet A. “Characterisation of the *Campylobacter jejuni* transcriptome using complementary RNA-seq technologies” 17th International Workshop on Campylobacter, Helicobacter and related organisms, Aberdeen, United Kingdom, September 2013
- **Butcher J**, Stintzi A. “Ironing out the details: Characterizing transcriptome structure and identifying novel transcripts in *C. jejuni* under iron limitation” Ottawa Institute for Systems Biology Symposium, Mont-Tremblant, Canada, June 2013
- **Butcher J**, Stintzi A. “The full repertoire of iron regulated transcripts and sRNA in *C. jejuni*” Canadian Campylobacter Workshop 2012, Ottawa, Canada, September 2012
- **Butcher J**, Stintzi A. “RNA-seq profiling reveals unanticipated complexity in *C. jejuni*’s transcriptional repertoire” 8th International Biometals Symposium, Brussels, Belgium, July 2012

- **Butcher J**, Stintzi A. “RNA-Seq reveals unique insights into *C. jejuni* transcript structure under iron limitation” Second China-Canada Systems Biology Conference, Ottawa, Canada, June 2012
- **Butcher J**, Stintzi A. “ChIP-chip characterization reveals novel regulatory roles for Fur in *C. jejuni*” 16th International Workshop on Campylobacter, Helicobacter and related organisms, Vancouver, Canada, September 2011
- **Butcher J**, Stintzi A. “Global Characterization of the Fur regulatory network in *C. jejuni* using ChIP-on-chip analysis” 7th International Biometals Symposium, Tucson, Arizona, USA, July 2010
- **Butcher J**, Stintzi A. “ChIP-on-chip analysis reveals diverse roles for Fur and PerR in *C. jejuni*” 59th Annual Conference of the Canadian Society of Microbiologists, Montreal, Canada, June 2009
- **Butcher J**, Stintzi A. “Global Characterization of the Fur and PerR regulons in *C. jejuni* by ChIP-on-chip analysis.” Systems Biology Symposium, Ottawa, Canada, April 2009
- **Butcher J**, Sun YQ, Flint A, Palyada K, Naikare H, Stintzi A. “The regulatory network controlling iron metabolism in *C. jejuni*” 6th International Biometals Symposium, Santiago de Compostela, Spain, July 2008

Education

- Doctorate in Biochemistry (In Progress) - University of Ottawa, Ottawa, Canada
- Baccalaureate in Science, Honours Biochemistry (COOP) - 2006, University of Ottawa, Ottawa Canada