# RESPONSE OF *CAMPYLOBACTER JEJUNI* TO IRON AND HYDROGEN PEROXIDE

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# CHAPTER I

## INTRODUCTION

The microaerophilic enteric pathogen *Campylobacter jejuni* is one of the most frequently isolated causes of bacterial diarrhea worldwide. Around 2.5 million episodes of *Campylobacter* infection occur in the US annually with more than 13,000 cases requiring hospitalization (1). Although substantial insight has been gained about the ecology, epidemiology and pathogenicity of Campylobacters, the mechanisms involved in pathogenesis are not well characterized.

To colonize the gastrointestinal tract *Campylobacter* must be able to compete successfully in a complex and dynamic environment for nutrients and to tolerate a series of environmental insults like acid stress in the stomach, low or high osmolarity, oxidative stress, fluctuation in the temperature and pH etc (2). The ability of the Campylobacters to initiate adaptive survival response to their diverse range of environments is cardinal to the infective cycle of these pathogens (2). Regulation of virulence gene expression is a common theme shared among pathogenic organisms enabling them to conserve resources by expressing only the appropriate subset of genes in a specific environment (3). To characterize these genes, it is necessary to identify the shifts in gene expression by monitoring the transcriptome profile level or by assessing changes in the level of protein synthesis (2). This study of campylobacter stress response will utilize the enormous gene

and protein database generated by the sequencing project of *C. jejuni* NCTC 11168 genome (4). Among various environmental stimuli iron and oxidative stress might play major role in the pathogenesis of Campylobacters. Iron is the key environmental signal for the pathogenesis of many human pathogens (5, 6, 7). In the mammalian host, most iron is sequestered intracellularly, or bound by the extracellular glycoprotein transferrin (in the blood and lymph), or by lactoferrin, in exocrine secretions (8, 9, 10). As a consequence, iron bioavailability within the mammalian host is extremely limited in contrast to the external environment (6) and has been shown to be a key signal for pathogens such as *Campylobacter* to sense that they have invaded a mammalian host and trigger the expression of virulence determinants (6,7,11). Hence, virulence factors in a variety of microbial pathogens are observed to be iron-regulated (7, 12). Consequently, it is clear that the inhibition of iron acquisition would be at least bacteriostatic, if not eventually bactericidal. A good understanding of the mechanism(s) employed by the bacteria to acquire iron is a prerequisite to any vaccine or drug design. However, to date, very little is known about iron acquisition in *Campylobacter* species. To gain a complete picture of *Campylobacter* responses to the iron availability in its environment, I propose to investigate the iron regulon of *C. jejuni* using DNA microarray analysis.

As a microaerophilic organism, *C.jejuni* must resist oxidative stress either during transmission or infection. Oxidative stress is caused by increased levels of reactive oxygen species (ROS) like hydrogen peroxide ($H_2O_2$), superoxide anion or hydroxyl radical. Increased levels of ROS are caused by the exposure to radiation, metals, and redox drugs. These ROS are deleterious to cells as they cause damage to membrane proteins and DNA. (13). Few genes that are known to combat oxidative stress in *C. jejuni*

have been identified. They are catalase (*katA*) (14,15), alkyl hydroperoxidase (*ahpC*) (16) and superoxide dismutase (*sodB*) (17,18). However, gene expression profiling at the whole genome level has not yet been done. Hence, we propose to investigate gene expression profile of *C. jejuni* in response to oxidative stress inducing agents.

There is a clear connection between iron and oxidative stress in the cell. Iron that enters the macrophage/phagosome stimulates the formation of reactive oxygen species namely hydroxyl radical and superoxide ion through the involvement of the hydrogen peroxide, superoxide and perhaps even nitric oxide via Fenton reaction (19). It has been shown that interaction of $H_2O_2$ with myeloperoxidase, reduced iron or products of nitric oxide synthase may lead to formation of more toxic intermediates such as hypochlorous anion, hydroxyl radicals, hydroxide anions and peroxynitrite (20). Also, the expression of oxidative stress defense genes, *ahpC* and *katA* genes is transcriptionally repressed in response to increasing concentration of iron (16). Hence, the response of *C.jejuni* to oxidative stress in iron rich and iron deficient conditions might differ. With the above information in the background, I propose the following objectives.

1. Investigation of the *Campylobacter jejuni* iron regulon
2. Investigation of the*Campylobacter jejuni* response to oxidative stress.

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# CHAPTER II

## REVIEW OF LITERATURE

### 1. *Campylobacter jejuni*

*Campylobacter jejuni* are small, motile, spirally curved, Gram-negative rods with polar flagellum at one or both the ends of the cell (67). They are catalase and oxidase positive and urease negative (151). They are fastidious organisms and require complex growth media and are incapable of fermenting carbohydrates (67). Campylobacters are microaerophilic and require oxygen concentration of 3-15% and carbon dioxide concentration of 3-5 % (67). The temperature range for the growth of *C. jejuni* is 34-44°C with an optimum of 42°C which reflects the body temperature of the domestic poultry of which it is a natural commensal (100).

*C. jejuni* is a normal intestinal microflora of all avians including wild birds, chickens, turkeys, ducks and quails (101). However they are considered pathogenic to human beings. As low as 500-800 organisms could result in campylobacter gastroenteritis (3, 35). Contamination of meat often occurs from the intestinal contents during the slaughter or meat processing. *Campylobacters* enter the human food chain by subsequent consumption of contaminated undercooked meat and other cross-contaminated food products (17, 36). Outbreaks associated with contaminated milk, water and other cross contaminated foods like lettuce and tuna salad, have been reported (4, 36, 151). According to the 1996 report by the Foodborne Diseases Active Surveillance Network

(FoodNet), which is the principal foodborne disease component of the centers for Disease control and Prevention's (CDC), *Campylobacter* accounted for 46% of laboratory-confirmed cases of bacterial gastroenteritis. This number was more than the cases reported for *Salmonella* and *Shigella* put together (28% and 17%, respectively). In the recent 2003 report, FoodNet reported the number at 33.4%, next only to *Salmonella* at 38.5%. The annual incidence rates for *Campylobacter* among different populations also decreased from 21.7 to 13.3 per 100,000 from 1996 to 2002 (1). However, keeping in mind the population coverage under the CDC's FoodNet program is only 14% of the US population (1), the actual numbers of incidence could be higher for *Campylobacter* which necessitates the study of the pathogenesis of this pathogen.

## 2. Campylobacteriosis

The incidence and clinical spectrum of the disease are different in developed and developing countries. In developing countries, *Campylobacter* is the most commonly isolated bacterial pathogen from diarrheic stools of children that are less than two-year-old and the disease does not appear to be important in adults (17). In developed countries, however, infection occurs in both adults and children. Forty to sixty percent of children under five years of age show the incidence in developing countries (17) while only 0.3% children show the incident of *Campylobacter* in developed countries.

In developing countries, campylobacteriosis is characterized by watery diarrhoea, fever, abdominal pain, vomiting and dehydration (17). In developed countries, campylobacteriosis is characterized by bloody stool, fever and abdominal pain that is often more severe than that observed for *Shigella* and *Salmonella* infections (17). *C.*

*jejuni* infection is now recognized as the most common infection preceding Guillain-Barre syndrome (GBS) which is an acute, post-infectious immune mediated disorder affecting the peripheral nervous system leading to acute flaccid paralysis (96). It has been reported that 30-40% of the patients with GBS have evidence of previous *Campylobacter* infection. While some serotypes of *C. jejuni* are more likely to cause GBS than others, host factors are not ruled out (96). Its been recently reported that the carbohydrate mimicry between human ganglioside GM1 and *Campylobacter jejuni* lipooligosaccharide causes GBS (174).

In contrast to *Salmonella* and other enteric pathogens, the pathophysiology of the *C. jejuni* is poorly understood. The lack of genetic and pathophysiologic information on this major pathogen lead the Sanger Center (UK), to take an initiative to sequence the genome of *C. jejuni* NCTC 11168, the strain used in our present study (107). *C. jejuni* has a circular chromosome of 1,641,481 base pairs with approximately 30.6% G+C content and is predicted to encode 1654 proteins and 54 stable RNA species (107). The genome presents no insertion sequences or phage-associated sequences. Short but highly variable numbers of homopolymeric tracks of Gs and Cs were present in genes encoding the biosynthesis or modification of surface structures of *C. jejuni* (107). This could help *C. jejuni* in adapting to different environmental niches by modifying its surface structures.

### 3. *Campylobacter* virulence and colonization factors

Colonization of the intestine by *C. jejuni* requires expression of several putative virulence factors. These are motility and chemotaxis, adhesion and invasion, toxins, iron

acquisition, surface polysaccharide structures, oxidative stress defence and heat shock response (151).

### *3.1 Motility and chemotaxis*

The flagellum of *C. jejuni* is composed of a basal body, hook, and filament. The flagellar filament is comprised of two proteins, FlaA and FlaB, although it appears that FlaA is the predominant subunit (3). The high level of motility conferred by polar flagella helps *C. jejuni* to move through the viscous environments and to adhere and invade Caco-2 intestinal epithelial cells (138). Flagella and motility have also been implicated in adhesion and invasion of *C. jejuni* and are required for intestinal colonization in chicken colonization models (13, 98, 163, 164). Although more than 36 ORFs are annotated to function in the biosynthesis, export or assembly of flagella (107), (See Table 1) relatively few have been investigated. In recent studies using random transposon mutagenesis or site-directed mutagenesis, it was shown that mutations in at least 19 genes led to altered or absent motility of *C. jejuni* (13, 18, 51, 57). The *C. jejuni* flagella has been shown to be post-translationally modified through glycosylation (25) and flagella has been successfully tested as part of a subunit vaccine in mice indicating its importance in the pathogenesis (80).

The chemotactic ability to detect and respond to chemical gradients is essential for the lifestyle of mucosal pathogens (2). *C. jejuni* genome reveals the presence of orthologues of the chemotaxis genes *cheA*, *cheW*, *cheV*, *cheY*, *cheR* and *cheB*, ten chemoreceptor genes and two aerotaxis genes (87). Chemotactic ability is an important mechanism involved in the colonization of *C. jejuni* as the three non-chemotactic mutants isolated

from wild type *C. jejuni* failed to colonize the intestine of suckling mice (141). A mutant in *cheY*, which encodes a chemotaxis regulatory protein, showed non-adherent and non-invasive phenotypes with INT407 cells and was attenuated in ferret model of the disease (173).

Table 1. List of genes associated with flagellar biosynthesis, export and assembly in *Campylobacter jejuni.*

| Name of the Gene | Function |
|---|---|
| *flaD* | Putative flagellin |
| *cheA* | Chemotaxis histidine kinase |
| Cj1318 | Unknown |
| Cj0390 | Putative transmembrane protein |
| *fliP* | Flagellar biosynthesis protein |
| *fliY* | Putative flagellar motor switch protein |
| *Cj1293* (*flaA1*) | Possible sugar nucleotide epimerase |
| *rpoN* | RNA polymerase sigma 54 |
| *flgE* | Central flagellar-hook protein |
| *flaA* | Major flagellin subunit |
| *flhA,flhB* | Probable flagellar biosynthetic protein |
| *fliI* | Flagellum-specific ATP synthase |
| *fliR* | Probable flagellar biosynthesis protein |
| *flgD* | Possible hook-associated assembly protein |
| *flgH* | Probable flagellar L-ring precursor protein |
| *motA* | Probable flagellar motor proton channel |
| *motB* | Possible flagellar motor protein |
| *fliM* | Probable flagellar motor switch |
| *cheA* | Probable chemotaxis histidine kinase |
| *cheV* | Probable chemotaxis protein |
| *cheY* | Chemotaxis regulatory protein |
| *fliA* | Probable RNA polymerase |
| Cj1026c | Probable lipoprotein |
| Cj1189c,Cj1190c | Possible signal transduction protein |
| Cj0062c | Probable integral membrane protein |
| Cj0248, Cj0883c | Unknown/no identity |
| *flgR* | Sigma-54 associated transcriptional activator |

### *3.2 Adhesin and Invasion*

Invasion of epithelial cells by *C. jejuni* results in cellular injury and consequent loss of cellular function and diarrhea. As a result, invasion has been proposed as an important pathogenic mechanism for *C. jejuni* (162). Electron microscopic observations indicated that cell invasion is the primary mechanism of colon damage and diarrheal disease caused by *C. jejuni* in experimentally infected monkeys (126). Flagellum was the first *C. jejuni* determinant found to be involved in adherence and invasion (41, 163). Other adhesins in *C. jejuni* are surface exposed lipoprotein (JlpA), common antigen (PEB1) and *Campylobacter* adhesion fibronectin (CadF) protein (60, 71, 108). PEB1 and PEB3 are common antigens recognized by convalescent-phase sera from nearly 80% of *C. jejuni*-infected patients (109). A *C. jejuni* peb1A mutant showed 50- to 100-fold less adherence to HeLa cells and 15-fold less invasion of INT 407 intestinal epithelial cell culture and also was affected in intestinal colonization in the BALB/c mouse colonization model following oral challenge (108). The binding of *C. jejuni* to fibronectin (Fn), a component of the extracellular matrix, is mediated by an outer-membrane protein termed CadF (*Campylobacter* adhesin to fibronectin). It was shown that *C. jejuni* 81-176 utilizes CadF as an adhesin for host cells by competitive binding assays using wild-type and *cadF* mutant of *C. jejuni* isolate (93). A *cadF* mutant did not colonize newly hatched chickens (180). In *C. jejuni* TGH 9011 strain, a *jlpA* mutant is affected in its ability to adhere to the HEp-2 human epithelial cells. This adherence of *C. jejuni* TGH 9011 strain to HEp-2 cells was inhibited in dose-dependent manner when HEp-2 cells were preincubated with recombinant JlpA indicating an essential role of JlpA in adhesion (60). Interestingly, JlpA interacts with the HEp-2 cell surface heat shock protein (Hsp) 90α and initiates signaling

pathways that lead to activation of NF-κB and p38MAP kinase in the epithelial cells (61). NF-κB plays a central role in induction of innate immune response in infection of epithelial cells by bacterial enteropathogens (142). P38 MAP kinases play an important role in regulating the pro-inflammatory responses (61).

*C. jejuni* synthesizes a set of proteins collectively referred to as *Campylobacter* invasion antigens (Cia proteins) during coculture with epithelial cells that help *C. jejuni* to internalize into the mammalian cells (73). It has been recently shown that the secretion of Cia proteins requires a functional flagellar export apparatus as the Cia proteins were not detected in the supernatant fluids from any of the following mutants: Δ*flgB* and Δ*flgC* (which form the basal body of the flagella), Δ*flgE2* (which form the hook) or Δ*flaA flaB* (which form the filament structure) (74). In another report, the FlaC protein of *Campylobacter jejuni* TGH9011 was shown to mediate the binding of HEp-2 epithelial cells and influence the invasion (130). FlaC is homologous to the N- and C-terminus of the *C. jejuni* flagellin proteins, FlaA and FlaB, but lacks the central portion of these proteins. The *flaC* null mutant was normal in adherence to HEp-2 cells but was defective in cellular invasion (130). Interestingly, FlaC was also transported through the flagellar apparatus as the mutants in the flagellar basal rod gene (*flgF*) and hook gene (*flgE*) did not secrete FlaC. A *waaF* mutant of *C. jejuni* showed decreased invasion of INT407 cells. The waaF enzyme catalyzes the addition of the second heptose to the core oligosaccharide of LPS and LOS.

### *3.3 Toxins*

*Campylobacter jejuni* genome contains genes encoding the cytolethal distending toxin (CDT) (161). The CDT toxin is encoded by three adjacent genes *cdtA, cdtB* and *cdtC*. CDT is a tripartite toxin composed of three subunits, CdtA, Cdt B and CdtC. CdtB carries out the enzymatic activity while CdtA and CdtC serve as heteromeric B subunit required for the delivery of CdtB (76). CdtB has been shown to exhibit the features of a type I deoxyribonuclease (75). This causes direct DNA damage leading to cell cycle arrest at G2/M phase, cytoplasm distention, chromatin fragmentation and eventually cell death (49, 75, 166). The crystal structure of the holotoxin has been elucidated for *Haemophilus ducreyi* and putative DNA binding residues in CdtB that are essential for toxin activity have been identified (99). The live cells of the wild type *C. jejuni* 81-176 strain have been shown to induce the release of IL-8 from INT 407 cells during adhesion or invasion (53). Similarly, the membrane fractions of the *C. jejuni* could also induce the release of IL-8 from INT407 cells. However, the membrane fractions from the mutants defective in any of the three *cdt* genes were unable to induce the secretion IL-8. Surprisingly, live cells of mutants of *C. jejuni* 81-176 defective in any of the *cdt* genes were not affected in inducing IL-8 release from INT407 during adherence to or invasion (53). This suggests that the *C. jejuni* can induce IL-8 release by two different mechanisms: one that requires the live bacterial cells to adhere or invade intestinal epithelial cells (INT407) and another that require the binding of the membrane preparations to the epithelial cells (53).

Enterotoxin activity was first documented in *C. jejuni* in 1983 in which culture supernatants of *C. jejuni* caused intraluminal fluid secretion in the rat RILT model (125).

While this was confirmed by some groups (40, 62, 90) many other groups have been unable to detect any enterotoxin activity in *C. jejuni* strains (92, 110, 158). Hence it remains controversial (161). Moreover, the genome of *C. jejuni* NCTC11168 does not contain a cholera-like toxin gene.

### *3.4 Surface polysaccharide structures*

Lipo-oligosaccharide (LOS) and capsular polysaccharide structures form the major component of *C. jejuni* outer membrane. The surface polysaccharide structures and the flagella have been shown to be sialylated (97). This sialylation of the core oligosaccharides (LOS) of many *C. jejuni* strains results in high similarity to the carbohydrate moieties of gangliosides, which are sialic acid containing glycolipids expressed in high abundance in the nervous system. Because of the molecular mimicry of terminal sugar residues of LOS with the human gangliosides found on the peripheral nerves, antibodies produced against the *C. jejuni* LOS would react with human gangliosides resulting in peripheral paralysis called Guillain-Barre syndrome (95, 97). Monoclonal antibodies raised against *C. jejuni* LOS were recently shown to be cross reactive with neuronal gangliosides and caused neurotransmission block in mice thus proving the molecular mimicry hypothesis (38).

Most of the *C. jejuni* strains tested produced LOS while about one-third of strains also produced high-molecular weight LPS-type molecule referred as O-antigen (94). Genome sequence of *C. jejuni* NCTC 11168 has revealed gene cluster with significant similarity to *E.coli kps* genes which are involved in the synthesis of capsular polysaccharides (CPS) (107). This high molecular weight LPS-type molecule has been shown to be

biochemically and genetically unrelated to LOS and to be similar to capsular polysaccharides (66). In fact, mutational analysis of *kps*-like genes in *C. jejuni* demonstrated their involvement in the production of capsular polysaccharide. Furthermore, phospholipase treatment of extracts containing this molecule revealed that the lipid group is a phospholipid (a feature of CPS), not lipid A (a feature of LPS) (66).

A gene cluster in *C. jejuni* comprising 11 genes related to sugar biosynthesis and transport designated *wlaB* through *wlaM* was suggested to be involved in both LOS and CPS biosynthesis (169) since inactivation of the genes *wlaFGHIJK* resulted in an altered LOS profile. However, mutations in the genes *wlaFGHIJKL* had no effect on the LOS production in *C. jejuni* 81176 but resulted in reduced levels of protein glycosylation (139). Therefore, this locus was named *pgl* locus after protein glycosylation locus. The genes *wlaFGHIJKL* were alternatively named as *pglBACDEF*, respectively. Expression of this *pgl* cluster in *E. coli* resulted in the glycosylation of AcrA protein (157). The biological significance of protein glycosylation in *C. jejuni* remains unclear. It is hypothesized that the bacterial glycoproteins may interact with host cell receptors to mediate adherence. Indeed, a *C. jejuni* mutant in *pglH* gene which is involved in glycosylation was significantly affected in both adhesion and invasion of human colon cancer cells (Caco-2) and showed substantially reduced colonization of one day-old chicks (65). *C. jejuni* was also shown to glycosylate flagella (25). Interestingly, structural analysis of the flagellin from *C. jejuni* showed that they are not modified with sialic acid but with pseudaminic acid (143), which is synthesized via the Cj1293 gene (39). Cj1293 catalyzes the first step in the biosynthesis of bacillosamine, a sugar found in *C. jejuni* protein glycosylation motifs (20).

### *3.5 Response to temperature change*

The *Campylobacter* during its life cycle encounters various levels of temperatures ranging from 4°C to 44°C such as water at 25°C to30°C, refrigerated food at 4°C, chicken intestine at 42°C and human intestine at 37°C (134). In response to high temperatures, bacteria produce heat shock proteins which act either as chaperones to promote the folding of cellular proteins or as proteins to degrade the potentially deleterious mis-folded proteins (151). *C. jejuni* genome reveals up to 17 such proteins, many of which have already been characterized, including GroEL, GroES, DnaJ, DnaK, GrpE, HrcA, and Lon (72, 105, 133, 134). A *C. jejuni dnaJ* mutant is impaired in its ability to colonize chicken (72, 171) and GroEL and GroES have been shown to be immunogenic in experimentally infected rabbits (171). The bacteria senses, adapts and responds to temperature stress by regulating gene expression and this response of *C. jejuni* to both heat-shock (133) and cold-shock (134) has been studied in our laboratory using DNA microarray technology. Up to 336 genes were identified to be differentially expressed when the temperature was changed from 37°C to 42°C (133). Genes encoding the chaperones, chaperonins and heat shock proteins displayed the most dramatic and rapid upregulation immediately after the temperature change. These proteins act either by repairing and preventing the damages caused by an accumulation of unfolded proteins or by assisting in proper folding of newly synthesized proteins (175). Genes encoding proteins involved in membrane structure modification were differentially expressed suggesting a different protein membrane make up at the two different growth temperatures (37° C and 42° C) which could help *C. jejuni* to better adapt to the intestinal tract of chicken and man (133). In another report, *C. jejuni* cold shock response was

studied where the cold-shock was induced 10 minutes after a sudden temperature drop from the optimal growth temperature of 42°C to 37, 32,10 or 4°C (134). Overall, 218 genes (13% of bacterial genome) were found to be differentially expressed in at least one of the four cold-shock experiments (134). The genes superoxide dismutase (*sodB*) and cytochrome C551 peroxidase gene (Cj0358) were upregulated upon cold shock indicating that oxidative stress defense is a component of cold shock. In addition *cft* (ferritin) and Cj1224 (putative iron binding protein) which encode proteins with potential functions in oxidative stress defense were also increased upon temperature drop from 42°C to 10°C and 4°C (134).

### *3.6 Iron acquisition*

Iron is the first row transition metal and the fourth most abundant element in the earth's crust. Iron exists in one of the two readily interconvertible redox states: the reduced ferrous form ($Fe^{+2}$) and the oxidized ferric form ($Fe^{+3}$). The redox potential of the iron ranges widely from -300 to +700mV and iron also adopts different spin states (high or low) in both ferric and ferrous form, depending on its ligand environment (27, 121). Both these properties make iron an extremely versatile prosthetic component for incorporation into enzymes/proteins as a biocatalyst or electron carrier in the electron transport chain. These proteins participate in functions such as photosynthesis, respiration, oxygen transport, gene regulation and biosynthesis of DNA and proteins.

*3.6.1 Availability of iron*

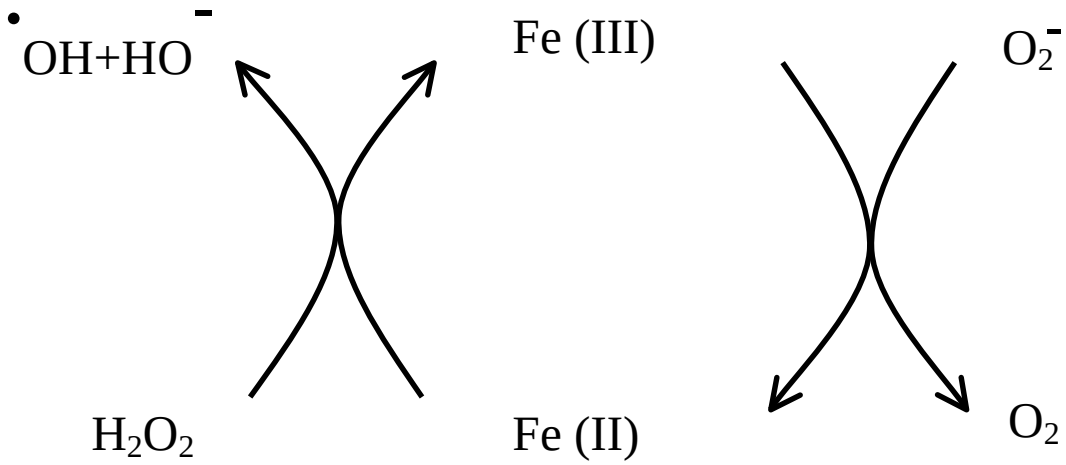
In *mammalian hosts*, most of body's iron is found intracellularly in ferritin, heme compounds (hemin and hemoglobin) and iron-sulfur clusters in various proteins, and extracellularly in host iron binding proteins like transferrin or lactoferrin in body fluids (30). Thus, although the iron is present in the body fluids, the amount of free iron in equilibrium with iron-binding proteins can be calculated to be of the order of $10^{-18}$ M (44). In the environment, the soluble ferrous form (0.1M at pH 7.0) is oxidized to extremely insoluble ferric form ($10^{-18}$ M at pH 7.0) by oxygen, thus, severely limiting the amount of free iron (6). In spite of abundance of iron both in environment and in mammalian host tissues, the available free iron is extremely limited for bacteria. Hence bacteria have evolved various mechanisms to scavenge iron from the environment under iron-restricted conditions. Bacteria express specific iron uptake systems and/or secrete 'siderophores' in response to low iron conditions. These siderophores are low molecular weight compounds (<1000Da) and are characterized by their high specificity and affinity towards ferric iron (12, 168). Siderophores scavenge ferric iron from the extra cellular milieu and make iron available to bacteria. These ferric-siderophore complexes are taken up through specific outer membrane receptors. Although the iron is essential for bacterial physiology, excess of iron could prove toxic to bacteria under aerobic conditions. In the presence of oxygen iron is not only poorly available but also potentially toxic. Hydrogen peroxide that results from aerobic metabolism reacts with the ferrous form of iron to result in highly reactive hydroxyl radicals and hydroxyl anions through the Fenton reaction (85). In another reaction, ferric form is reduced by superoxide anion to yield ferrous form of iron and molecular oxygen. These two reactions are together called the

Haber-Weiss cycle (113) (see Figure 1). The hydroxyl radical, hydroxyl anion, superoxide anion and hydrogen peroxide are together termed as reactive oxygen species (ROS). These ROSs will cause damage to biomolecules like proteins, lipids and other cell components that could result in cell death. Hence, bacteria have to tightly regulate the uptake, storage and use of iron within the cells.

Figure 1

Formation of reactive oxygen species is explained by Haber –Weiss cycle. Hydrogen peroxide that results from aerobic metabolism reacts with the ferrous form of iron to result in highly reactive hydroxyl radicals and hydroxyl anions through the Fenton reaction (Left-hand side of the cycle). Ferric iron is reduced by superoxide anion to yield ferrous form of iron and molecular oxygen (right-hand side of the cycle). These two reactions are together called the Haber-Weiss cycle.

(Source: Iron metabolism in pathogenic bacteria. Ratledge, C and Dover, L.G. Annual Review of Microbiology 2000 Vol. 54. Page 885)



*3.6.2 Ferric uptake regulator*

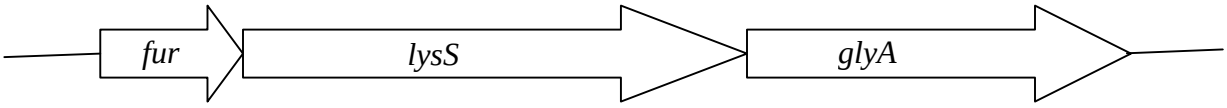
The Fur protein is a global regulator of gene expression in Gram-negative bacteria in response to intracellular iron concentration. Fur is a homodimer composed of 17-KDa subunits with an N-terminal domain that binds to DNA and a C-terminal domain that binds to the ferrous iron and mediates dimerization (19, 137). In addition, Fur is able to multimerize through protein-protein interactions, but the protein domains involved are likely to be different from those for dimerization (79). Under iron rich conditions, Fur binds the divalent ion ($Fe^{+2}$) and acquires a configuration to be able to bind to target DNA sequences. These sequences are known as 'Fur boxes' or 'iron boxes' and are located in the promoter region of iron regulated genes. Binding of the $Fe^{+2}$–Fur complex to the Fur box blocks the transcription of the downstream gene by inhibiting the RNA polymerase enzyme. When the cellular iron levels fall below a critical threshold, the equilibrium is displaced to release the ferrous iron from the Fur protein and the RNA polymerase is now able to transcribe the genes downstream (29). Classically, Fur has been known to be a repressor with ferrous iron as a co-repressor (7, 28, 29, 48). Recently, Fur has also been shown to positively regulate the transcription of several genes: superoxide dismutase (*sodB*) gene in *E.coli* ( 26, 55) and catalase (*KatA*) gene in *Staphylococcus aureus* (55) and the non-heme containing ferritin (*Pfr*) gene in *Helicobacter pylori* (24). Recently, it has also been shown to positively regulate *pan1*, *norB* and *nuoABCDE* genes that code for iron containing metalloprotein complexes involved in anaerobic and aerobic respiration (23). Fur is also shown to positively regulate the expression of a set of genes indirectly at post-transcriptional level through the repression of another repressor, RyhB, a small regulatory RNA (sRNA) (89). Fur

represses the synthesis of the sRNA *ryhB*, and RyhB in turn negatively regulates synthesis of at least six proteins that bind iron in the cell. Thus, Fur indirectly regulates intracellular iron storage and utilization as well as iron uptake (89). In *Pseudomonas aeruginosa* a similar mechanism has been reported where in Fur positively regulates the set of genes through two tandem small RNAs, PrrF1 and PrrF2, which are functional homologues of RyhB (167).

In addition to participating in the iron homeostasis, Fur protein also controls other cellular and metabolic processes thatare not related to iron metabolism. These include acid shock response (10, 46, 179), chemotaxis (64), bioluminescence (86), swarming (91), production of toxins (117) and other virulence factors (83). Homologues of Fur have been described in many Gram-negative bacteria, e.g. *Vibrio, Yersinia, Salmonella, Pseudomonas, Helicobacter, Bordetella, Legionella Neisseria* and *Haemophilus* are among few (29). Fur homologues are also found in some of the Gram-positive bacteria like *Bacillus, Staphylococcus* and also in *Cyanobacteria* (29). Many of these are able to complement an *E.coli fur* mutant indicating that the mode of action at the transcriptional level is shared by these microorganisms. *C. jejuni* genome also contains a Fur homologue, Cj0400 (107). The Fur protein was previously described in two independent studies (14, 170). Chan *et al* (1995) identified the *fur* gene in the TGH strain of *Campylobacter* by independently sequencing the upstream region of the *lysS* gene (14). Wooldridge *et al* (1994) identified the *fur* gene using chloramphenicol acetyl transferase reporter gene assay and by partial complementation of an *E.coli fur* mutant (170). In *C. jejuni* the *fur* gene is located in an operon that includes two downstream housekeeping genes *lysS* and *glyA* , leading to a multi-cystronic mRNA ( see Figure 2) (153). The *lysS*

Figure 2

Schematic diagram showing the chromosomal organization of the *fur* gene along with the downstream *lysS* and *glyA* genes, which encode the housekeeping enzymes lysyl-tRNA synthetase and serine hydroxymethyltransferase, respectively. The genes are drawn approximately to scale.



and *glyA* , leading to a multi-cystronic mRNA ( see Figure 2) (153). The *lysS* and *glyA* genes encode for the housekeeping enzymes lysyl-tRNA synthetase and serine hydroxy methyltransferase, respectively (107). The *C. jejuni fur* gene is unique as it does not have its own promoter and is expressed from two promoters located in front of the first and second open reading frames upstream of the *fur* (153). A *C. jejuni fur* mutant was generated by insertional mutagenesis using a kanamycin antibiotic resistance cassette (154). Insertional mutagenesis of the *fur* gene has been possible in only a few bacterial species like *E.coli* (146), *Vibrio cholerae* (82) and *Yersinia pestis* (131). In *Pseudomonas aeruginosa* (156) , *Neisseria gonorrhoeae* (144), and *Vibrio anguillarum* (165) only *fur* mutants with point mutations could be obtained.

*3.6.3 Furregulon*

The Fur regulon consists of group of genes whose transcription is regulated by the Fur protein. There are several techniques used to identify Fur-regulated genes.

A Fur titration assay (FURTA) was originally developed for *E.coli*(135) and has been later used in *Salmonella typhimurium* to identify Fur regulated genes (148). In FURTA, an *E.coli* recipient strain is used which harbors an iron regulated chromosomal *fhuF:lacZ* fusion. The expression of this fusion is very sensitive for small changes in iron concentration due to a weak affinity of Fur-$Fe^{+2}$ complex to the *fhuF* promoter. Upon introduction of a multicopy plasmid containing a Fur regulated gene or just the nucleotide sequence of potential Fur-box, the available Fur-$Fe^{+2}$ complexes would be titrated off from the promoter of *fhuF:lacZ* fusion on the chromosome allowing the expression of lacZ and the colonies would be lac+ on McConkey plate supplemented with iron and

IPTG. Adopting FURTA has been shown to be unsuccessful with *C. jejuni* (154) and a related gastric pathogen, *Helicobacter pylori*, possibly due to the fact that *E.coli* Fur is not properly interacting with the *C. jejuni* Fur boxes (32).

A SELEX-like (Systematic Evolution of Ligands by Exponential Enrichment) cycle selection procedure was adopted in *P.aeruginosa* (see Figure 3) (103) to identify the Fur regulated genes. In brief, SELEX consists of *in vitro* binding of chromosomal DNA fragments with recombinant-Fur. The bound complexes would be selected by using the anti-Fur antibody. This tripartite complex would be purified on a protein G sepharose column to get rid of unbound DNA fragments. The remaining DNA fragments would be eluted and enriched by PCR to start another cycle of selection. After 3-5 selection cycles, the enriched DNA pool was cloned and sequenced to locate them on the genome (103). This lead to the identification of 20 putative Fur regulated genes in *P. aeruginosa*.

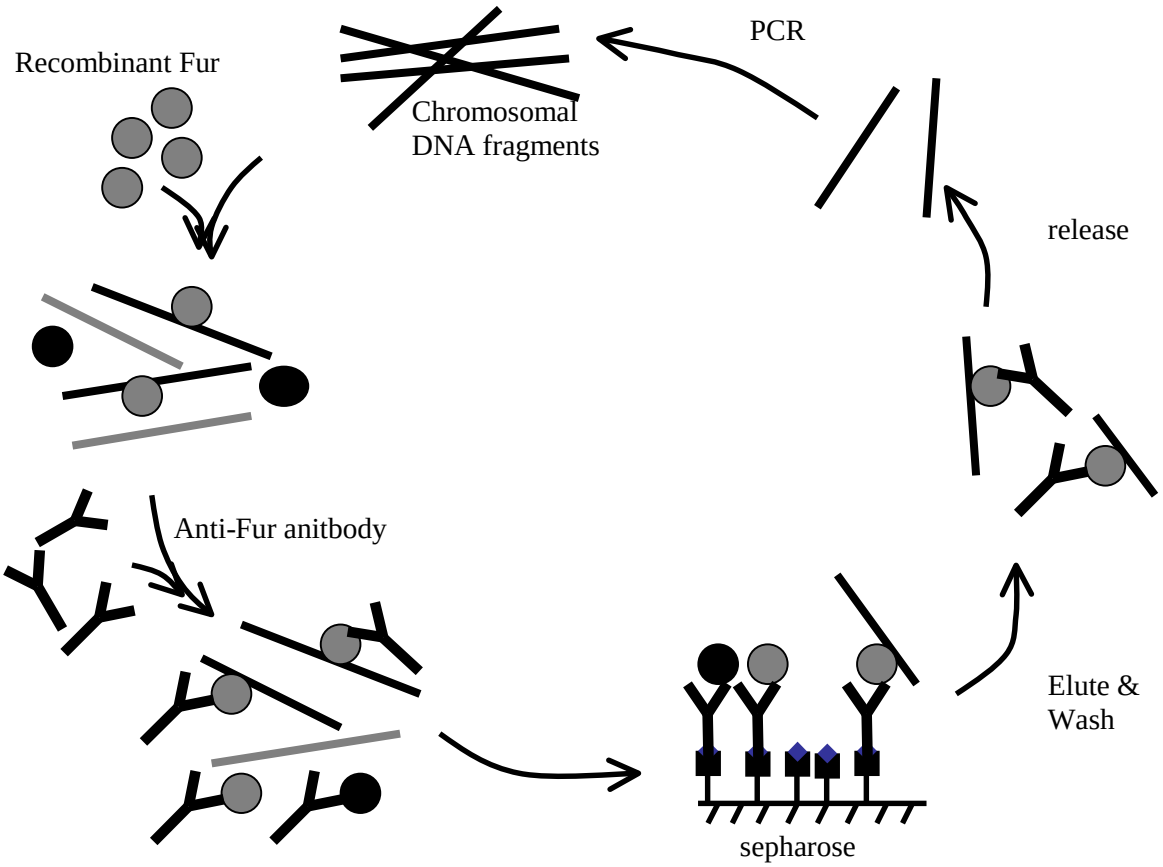
An Electrophoretic mobility shift assay (EMSA) was used to identify Fur regulated genes in *Neisseria gonorrhoeae.* In this assay, the purified gonococcal Fur was allowed to bind with the different promoter fragments that contained putative Fur boxes (128).

Finally, another method consists of constructing a *fur* mutant. This *fur* mutant is then used to identify either the genes or proteins that are constitutively expressed in the *fur* mutant when compared to the wild type strain. Protein profiles of *C. jejuni* wild type and the *fur* mutant were compared using SDS-PAGE and 2D electrophoresis to identify iron-regulated proteins whose expression is derepressed in the *fur* mutant (154). Seven such proteins were identified whose expression is iron-regulated in the wild type strain but not

**Figure 3**

SELEX- like procedure adopted to identify the Fur-regulated genes in *P. aeruginosa.* Chromosomal fragments were allowed to bind with recombindant-Fur protein. Anti-Fur antibody was used to select the bound complexes. This tripartite complex was purified on sepharose column. After eluting and washing, the DNA fragments would be released from the complex for further enrichment by PCR. Normally, 3-5 selection cycles were performed before cloning the enriched DNA pool for sequencing.

(Source: Ochsner, UA and Vasil, ML.1996. PNAS, Vol.93. Page 4409-4414)



in the *fur* mutant. They were 19Kda periplasmic protein (p19), putative hemin uptake system periplasmic hemin-binding protein (ChuD), enterobactin uptake periplasmic binding protein (CeuE), hemin uptake system outer membrane receptor (ChuA), putative iron uptake protein (CfrA) and two other uncharacterized proteins Irp32 and Iro 80. This comparison of the 2D gel electrophoresis patterns of the wild-type strain versus the *fur* mutant has been successfully used to identify Fur regulated proteins in *Vibrio cholerae* (82), *Yersinia pestis* (131), *Neisseria gonorrhoeae (34, 144)* and *S. typhimurium* (34). Analysis of the Fur regulon at the whole genome level has also been possible with the use of microarray technology (127). The wild type and the *fur* mutant were compared to identify the genes which were constitutively expressed in the *fur* mutant in *Bacillus subtilis* (8), *Neisseria meningitidis B* (43) and *Shewanella oneidensis* (43).

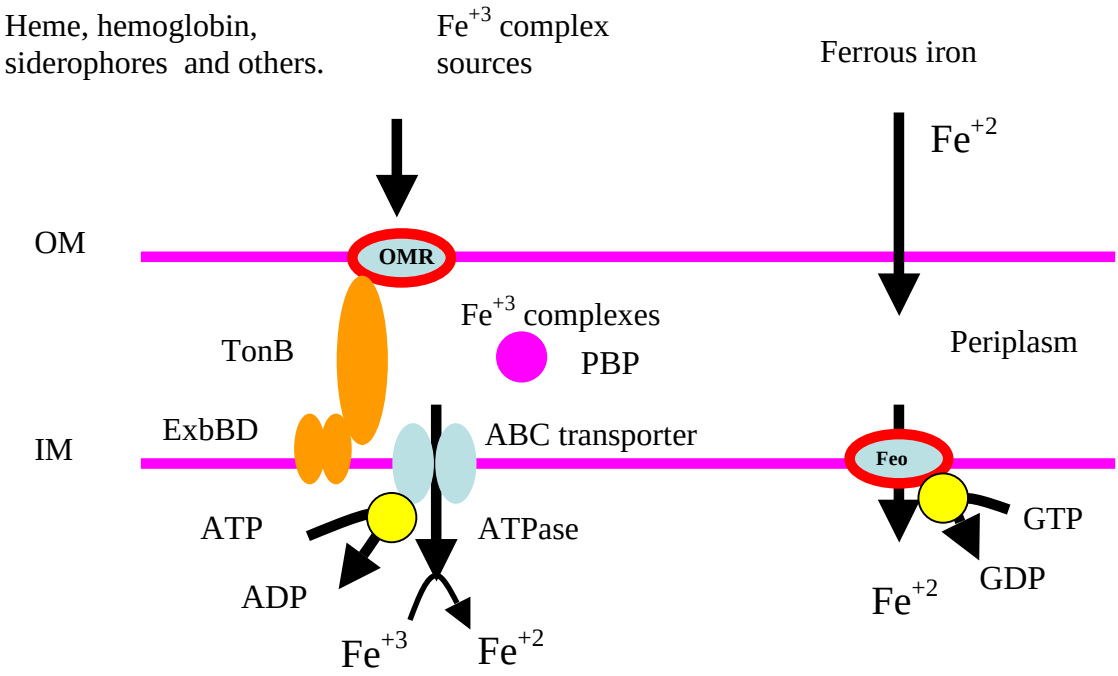
*3.6.4 Iron acquisition systems in C. jejuni*

In Gram-negative bacteria iron transport could be divided into two parts: ferrous iron and ferric iron acquisition (Figure 4). The ferrous iron being soluble is readily available for bacteria and passes freely through the outer membrane porins and requires active transport only through the inner membrane through a specific receptor named FeoB. The ferric iron is insoluble under physiological conditions and is mostly complexed with siderophores or host iron binding proteins like transferrin, lactoferrin, hemoglobin, and ferritin. This complexed ferric iron is transported across the outer membrane either independently or along with the complexed protein. This complex being too large to pass through the porins and hence requires substrate specific, high-affinity outer membrane

receptors. Driven by the proton motive force at the cytoplasmic membrane (CM), TonB-exbB-exbD system of proteins delivers energy from CM to OM receptors.

**Figure 4**

Iron uptake across the Gram-negative cell membrane. Ferrous iron freely passes through the outer membrane (OM) but requires FeoB receptor at the inner membrane (IM). Ferric iron is usually in complex with host iron binding proteins. Ferric iron in complex with iron binding compounds is transported through the specific outer membrane receptors (OMR) into periplasm where it binds to periplasmic binding proteins (PBP). ABC transporters mediate ferric iron transport across the IM with the help of an ATPase.



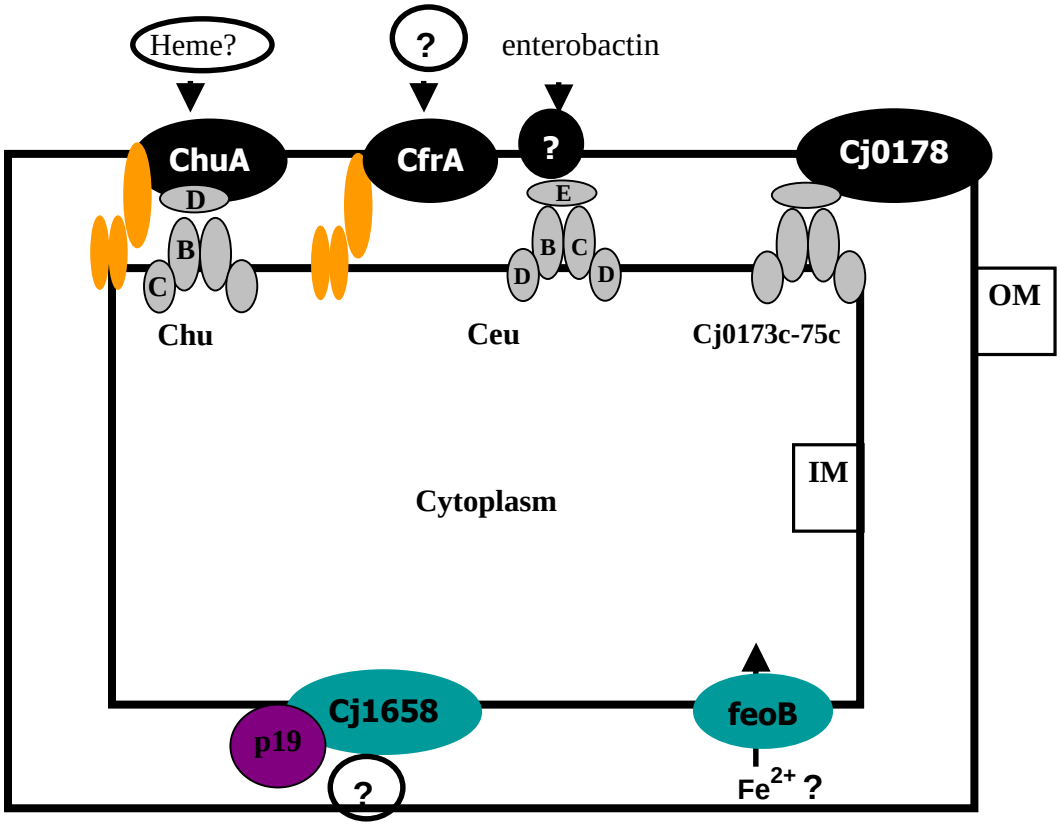
The genome of *C. jejuni* encodes mulitple iron acquisition systems which are mostly organized in operonic structures (Figure 5)**:** the enterobactin uptake system *CeuEBCD* the siderophores receptor orthologue *CfrA*, a predicted hemin uptake operon *ChuABCD*, a putative outer membrane siderophores receptor Cj0178, a putative periplasmic iron uptake ABC transport system Cj0173c to Cj0175c, a ferrous ion transporter *feoB* and Cj1658-p19 genes which encode a periplasmic protein and putative membrane protein (107, 152). There are three copies each of the accessory *tonB, exbB* and *exbD* genes which form the energy transducing machinery. The detailed description of each of these systems follows:

*3.6.5 Ferrous iron acquisition*

The first bacterial ferrous iron specific transport system was identified in *E.coli* which consists of *feoAB* operon (63). The *feoA* gene encodes a protein of unknown function containing 75 amino acids while *feoB* encodes the transporter protein of 773 amino acids. FeoB protein had an apparent molecular mass of 70KDa and was localized in the cytoplasmic membrane (63). A recent report suggests that the N-terminus of the FeoB protein is similar to G protein with GTPase activity (47, 88). *C. jejuni* genome contains a gene that encodes a FeoB homolog (Cj1398) (107). FeoB appears to be physiologically important because an *E.coli feoB* mutant failed to colonize the mouse intestine (136) and a *H. pylori feoB* mutant was unable to colonize the stomach of infected mice (155). In *S. typhimurium, feoB* mutants were out competed by the wild type strain during mixed

Figure 5

Model showing the iron transport systems in *Campylobacter jejuni*. *CeuEBCD* enterobactin uptake system, CfrA- siderophore receptor orthologue, *ChuABCD*-predicted hemin uptake operon, Cj0178-putative outer membrane siderophores receptor, Cj0173c-75c-putative periplasmic iron uptake ABC transport system, FeoB-ferrous ion transporter. Cj1658-putative membrane protein, p19-periplasmic protein. Question marks indicate that either the substrate or the protein is unknown. OM-outer membrane, IM-inner membrane. Source: Modified from (152)




TonB-ExbB-ExbD complexes form the energy transducing machinary

colonization of the mouse intestine. In contrast, *feoB* mutation did not attenuate *S. typhimurium* for oral or intraperitoneal infection of mice (147). It appears from these studies that the predominant form of iron in the low oxygen environment of the intestine could be ferrous iron. However, in the case of *C. jejuni* a *feoB* mutant in the strain *C. jejuni* M129 was not affected in the ferrous iron uptake (120). Further investigation on the function of this gene in *C. jejuni* is currently under investigation in our lab.

*3.6.6 Ferric iron uptake systems*

1. *ceuBCDE*

Genes in the *CeuBCDE* operon have been annotated to encode components of the enterochelin (enterobactin) uptake system consisting of an ABC transport system (107), which has been shown to mediate enterobactin transport in the related pathogen *Campylobacter coli* (123). *CeuE* was the first gene to be characterized. This gene encodes a lipoprotein, homolog to siderophore-specific periplasmic binding proteins (106). Further characterization of this region in *C. coli* showed that the *CeuE* gene is downstream of *ceuB, ceuC,* and *ceuD* genes *(123).* The CeuB and CeuC proteins are cell membrane permeases and CeuD is likely to be the ATPase protein energizing the transport process. A *ceuD* single mutant and a *ceuB-ceuD* mutant were unable to use enterobactin as sole iron source, while a *ceuE* mutant showed significantly reduced growth with ferric-enterobactin (123) demonstrating their role in enterobactin-mediated iron transport. However, the outer membrane receptor for ferric enterobactin has so far not been identified.

*2. CfrA*

*CfrA* gene was originally identified in *C. coli* (45) and this gene showed significant sequence identity to siderophore receptors of several bacteria. But, a *C. coli CfrA* mutant was still able to use hemin, enterobactin and ferrichrome as sole iron sources suggesting that CfrA is a component of a transport system for yet another unidentified iron source (45). CfrA was shown to be both iron- and Fur-regulated protein in *C. jejuni* using 2D electrophoresis (154).

*3. chuABCD*

*C. jejuni* genome contains a putative hemin uptake system encoded by *chuABCD* genes (107). Originally, Pickett *et al* (1992) isolated *C. jejuni* mutants deficient in heme utilization and all of these mutants lacked a 70 KDa OM protein (112). However, this protein was not further characterized. Later a protein with similar molecular mass was shown to be both iron- and Fur-regulated and was identified by N-terminal protein sequencing to be encoded by the c*huA* gene (154). The *chuA* gene is in operonic structure with *chuB, chuC* and *chuD* genes which encode the predicted ABC transporter system consisting of a permease, an ATPase and periplasmic binding protein (124). A *C. jejuni chuA* mutant was unable to grow on hemin as the sole iron source but mutants in *chuB, chuC* and *chuD* were not affected in hemin utilization indicating that some other transport system might complement the transport of heme or heme iron into the cytoplasm (124) or that heme could be directly acquired from the periplasmic space.

*4. Other iron uptake systems*

An iron- and Fur- regulated protein, Iro80, was identified in the analysis of OM profiles of *C. jejuni fur* mutant (154) . While this protein was not further characterized, it is probably encoded by Cj0178 gene (152). This gene exhibits significant homology with PhuR hemin receptor (102) but its function in *C. jejuni* is currently unknown. Similarly, the Cj0173c-Cj0175c genes encode putative iron uptake ABC transporter system components (107), but their role in *C. jejuni* iron acquisition still needs to be elucidated. The protein p19 was originally identified as a prominent component of *C. jejuni* proteins obtained by glycine extraction (58) and was later shown to be iron and Fur-regulated (154). Cj1658 gene which is located upstream of *p19* encodes a putative outer membrane protein. This observation together with the fact that *p19* is iron and Fur regulated suggests a possible role for these proteins in iron acquisition in *C. jejuni* (152). However, this has not been experimentally proven.

*5. TonB-ExbB-ExbD system*

OM receptors use the energy transduced by the TonB-ExbB-ExbD system proteins to transport ferric iron complexes into the cell. The ExbB and ExbD proteins are integral CM proteins. The TonB protein acts as an energy transducing protein and interacts with high-affinity outer membrane receptors at specific sites called ‘TonB boxes’ (69). The CM proton motive force is used by ExbB/D to convert TonB to an energized conformation by interacting at the N-terminal domain of TonB (78). Once the energized TonB contacts the OM receptors, TonB N-terminus is released from the ExbB/D complex and would associate with the OM (77, 81, 115). The crystal structure of the TonB C-

terminal domain showed that the last 77 residues formed an intertwined dimer (15). In a recent study, however, when truncated C-terminal fragments of different lengths were tested, it was found that dimerization is not essential for TonB to bind to the *E. coli* OM siderophore receptor FhuA (70). TonB has been shown to be essential for virulence in *E. coli* (145) *Bordetella pertussis* (116), *Shigella dysenteriae* (122) and *P. aeruginosa* (140). A *C. coli tonB* mutant lost the ability to utilize hemin, ferrichrome and enterchelin as iron sources (45). The number of TonBs present in different organisms varies. *E.coli* has a single *tonB,* gene (115). *Vibrio cholerae* (129) and *Pseudomonas* (177) have two *tonB* genes, *tonB1* and *tonB2. C. jejuni* NCTC 11168 has three sets of *tonB-exbB-exbD* genes. Although this could indicate some functional redundancy, there could be some unique functions for each of these TonBs. As *Campylobacter* grows in different environmental niches ranging from chicken gut, refrigerated foods to human intestine, the presence of multiple TonB systems might help *C. jejuni* to acquire iron in these different conditions. In addition, one of these TonBs might be involved in the uptake of Vitamin B12 as it is the case in *E. coli* (16).

### *3.6.7 Iron storage proteins*

There are three classes of iron storage proteins in bacteria, the ferritins, bacterioferritins and the Dps protein (5, 6). The genome of *C. jejuni* NCTC 11168 contains the ferritin which is encoded by Cj0612c and a putative bacterioferritin which is encoded by Cj1534c (107). Another strain of *C. jejuni* 81-176 has been shown to possess the Dps iron binding protein (56). Ferritins and bacterioferritins have 24 identical subunits while Dps proteins have 12 subunits. These subunits assemble to form a spherical protein shell

surrounding a central cavity that acts as an iron storage reservoir. Each ferritin or bacterioferritin can accommodate at least 2000-3000 iron atoms per 24-mer, whereas the Dps proteins accommodate approximately 500 iron atoms per 12-mer (6). *C. jejuni cft* mutant was shown to be affected in growth under iron restriction suggesting that it acts as an intracellular iron depot (159). Bacterioferritins are heme containing proteins and share significant homology with DNA binding proteins of the Dps family (5). Recently, an iron binding protein, Dps, was identified and characterized in the strain of *C. jejuni* 81-176. It was shown to bind 40 iron atoms per monomer and conferred hydrogen peroxide stress resistance as evidenced by the sensitivity of the *dps* mutant to $H_2O_2$ (56). However the role for Cj1534c which encodes the putative bacterioferritin is yet to be determined in *C. jejuni*.

*3.6.8 Siderophore uptake and production*

Not all strains of *C. jejuni* are able to synthesize siderophores (33). Only 7 of the 26 strains tested appeared to produce siderophore like molecules (33). However the chemical structure of these siderophores has not yet been determined. While *C. jejuni* NCTC 11168 does not contain any homologues for siderophore biosynthesis (107), *C. jejuni* strains are known to use exogenous siderophores synthesized by other microorganisms. Bioassays have shown that *C. jejuni* can use enterobactin and ferrichrome but not aerobactin, rhodotorulic acid and desferrioxamine B (33). *C. jejuni* is relatively limited in the host-iron compounds it can utilize. While hemin and hemoglobin support the growth of *C. jejuni*, ferritin, lactoferrin and transferrin do not (112, 152).

### *3.7 Oxidative stress defense*

Being a facultative intracellular bacterium, *C. jejuni* is exposed to various oxygen species generated by its own respiratory metabolism and by the respiratory burst oxidase as the bacterium remains bound within an endosome (22, 68). These products include superoxide, hydrogen peroxide and halogenated oxygen molecules. In addition, interaction of hydrogen peroxide with myeloperoxidase, reduced iron or products of nitric oxide synthase may lead to formation of more toxic intermediates such as hypochlorous anion, hydroxyl radicals, hydroxide anions, nitrogen dioxide and peroxynitrite (31). All these are collectively called reactive oxygen species (ROS). ROS cause damage to biomolecules like proteins, lipids and other cell components resulting in the death of the cell. Therefore, bacteria would try to remove superoxides and peroxides before they cause significant damage to biomolecules. *C. jejuni* defenses against these oxygen species which are discussed below.

#### *3.7.1 Defenses against superoxide stress*

Superoxide is converted into hydrogen peroxide and oxygen by an enzyme called superoxide dismutase (SOD). SODs need metal cofactors for their activity and these metals can be iron, manganese and copper-zinc. *E. coli* has all the three types of SODs (85). *C. jejuni* encoded superoxide dismutase is cofactored by iron and the protein is located in the cytoplasmic space (111, 119). A *SodB* mutant of a *C. jejuni* strain 81-176 showed a significant decrease in intracellular survival in INT407 intestinal epithelial cell culture implying a possible role for SodB in the intracellular survival (111). Furthermore, this mutant was significantly less efficient at colonizing the one-day old chicks than the

parental strain (118). It was shown that freezing and thawing results in generation of superoxide radicals and SodB deficient mutants were sensitive to freezing and thawing (132).

*3.7.2 Defenses against peroxide stress*

Hydrogen peroxide is generated as a by-product of aerobic metabolism and by the dismutation of superoxides. Peroxidases convert hydrogen peroxide to water and oxygen. *C. jejuni* has catalase, alkylhydroperoxide reductase, thiol peroxidases and cytochrome c peroxidases thatlikely help this pathogen to overcome the peroxide stress (152).

Catalase is produced by most aerobic as well as microaerophilic bacteria. Catalase causes the breakdown of hydrogen peroxide to water and oxygen. Catalase is encoded by the gene *KatA* in *C. jejuni* and *KatA* mutants have been obtained in *C. coli* (42) and *C. jejuni* (21, 150). The *C. coli KatA* mutant was more susceptible to killing by $H_2O_2$ (42). However, its survival on the chicken skin or in the milk or its resistance to freeze-thaw stress was not affected (132). In *C. jejuni* M129 strain catalase does not contribute to *in vitro* survival within HEp-2 human epithelial cells but does play a role in the survival within both murine and porcine peritoneal macrophages (21). This catalase converts the hydrogen peroxide to oxygen and water. The expression of KatA was found to be iron dependent but Fur independent (154) and to be regulated through the repressor PerR (150).

Alkyl hydroperoxide reductase (AhpC) converts reactive hydroperoxides to the corresponding alcohols. In *E. coli* AhpC catalytic subunit is recycled by AhpF

flavoprotein unit (114). Surprisingly, a homologue of AhpF was not identified in the genome of *C. jejuni* NCTC 11168 (107). The absence of AhpF homologue raises questions on the molecular mechanisms of AhpC activity in *C. jejuni*. A *C. jejuni ahpC* mutant was found to be more sensitive to exposure to atmospheric oxygen and cumene hydroperoxide (9), indicating that AhpC protein may play an important role in protecting *C. jejuni* from oxidative stress damages. However, this *ahpC* mutant was not affected in its resistance to hydrogen peroxide (9). The expression of AhpC was shown to be iron repressed and not regulated by Fur protein (154). Its repression has been shown to be mediated by the PerR repressor (150).

Tpx protein belongs to family of Thiol peroxidases and protects enzymes like glutamine synthetase against inactivation by oxidative stress (160). Bacterioferritin comigratory protein, Bcp, is another member of the thiol peroxidase family (59). Although *C. jejuni* has both a Tpx homolog (Cj0779) and a Bcp ortholog (Cj0271), their role in oxidative stress has not been studied (152). *C. jejuni* also has two periplasmic cytochrome peroxidases, Cj0020c and Cj0358, which likely afford protection for the periplasmic cytochromes (37, 152). Similarly, their role in *C. jejuni* has not yet been studied.

FdxA was reported as a novel component of the oxidative stress response in *C. jejuni* (149). FdxA encodes ferredoxins which are small iron-sulfer proteins and function as electron carriers in metabolic reactions. Aerotolerance of the *fdxA* mutant was clearly affected when compared to the wild type but there was no reduction in peroxide stress resistance (149).

*C. jejuni* ferritin, Cft, has been shown to help in the oxidative stress as *cft*mutant showed increased sensitivity to both superoxide and peroxide stress inducers (159). This could be due to the fact that *cft* mutant cannot sequester the free iron present in the cytoplasm. This unsequestered free iron results in the generation of more ROS that damage biomolecules.

Recently, a novel oxidative stress-sensitive protein of 27 KDa was found to be decreased as a result of either exogenous hydrogen peroxide stress or endogenous oxidative stresses in aerobic conditions (172). This protein was identical to Cj0012c and has similarity to two non-heme iron containing proteins rubredoxin oxidoreductase (Rbo) and rubrerythrin (Rbr). These proteins are considered to be involved in oxidative stress protection in anaerobic microorganisms (84).

*3.7.3 Peroxide sensing by perR protein*

When the total protein profiles of *C. jejuni* grown in iron-rich and iron-restricted medium were compared, KatA and AhpC were found to be iron repressed and this repression was still observed in the *fur* mutant background (154). This suggested the presence of another regulatory system, other than Fur. In *Bacillus subtilis*, *ahpC* and *katA* were shown to be regulated by one of the Fur homolog called PerR (11). Genome wide search in *C. jejuni* has identified a PerR homologue (Cj0322) and the PerR protein of the *C. jejuni* is 32% and 37% identical to the *B.subtilis* PerR protein and *C. jejuni* Fur protein, respectively (150). In the *C. jejuni perR* mutant, *ahpC* and *KatA* are expressed at high levels making the *perR* mutant resistant to the effect of oxidative stress inducers cumene hydroperoxide and hydrogen peroxide (150). *C. jejuni perR* mutant showed

growth characteristics similar to the parental strain indicating that PerR may not be a global gene regulator. In addition, the Fur regulated proteins were still iron repressed in the *perR* mutant indicating that the *perR* mutation does not affect the Fur regulated genes (150).

*3.7.4 PerR regulon*

In *S. aureus*, PerR acts as an iron- and manganese-dependent repressor of oxidative stress components (*KatA, ahpC, trxB*), heme biosynthesis operon (*hemA),* iron storage protein (*cft*) and the ferritin like Dps homologue (*MrgA*) (54). PerR is also shown to be involved in virulence in *S. aureus* as the *perR* mutant is significantly attenuated in the murine subcutaneous skin model of infection (54). In *Bacillus subtilis,* PerR regulates peroxide stress response in response to iron, manganese and peroxide stress (11). But in Mycobacterium species, iron-independent regulation of KatG (hydroperoxidase I) is observed by the PerR homologue FurA (176).

*PerR* mutants have been used to study the PerR regulated genes and PerR binding site (5'-TTANAATNATTAT-3') has been identified in *B.subtilis* (52). A consensus PerR binding sequence has been proposed for *S. aureus* as 5'-atTAtAATTATTATaAt-3' (Lowercase letters indicate less conserved residues) (54). Similarly, analysis of promoters of genes functioning in the oxidative stress defense resulted in a consensus sequence for the PerR-box as 5'-TTANAATNATTAT 3' for *C. jejuni* (152). To note, these *C. jejuni* and *S. aureus* consensus PerR-boxes have not been experimentally confirmed. PerR regulation in *C. jejuni* has been only demonstrated for *ahpC* and *KatA* genes (150).

DNA microarray technology has been used to study the global transcriptional response of bacteria to oxidative stress agents as well to describe the PerR regulon. In *Bacillus subtilis,* PerR regulated genes were strongly induced by the both low and intermediate levels (8 μM and 58 μM) of $H_2O_2$ and were derepressed in the *perR* mutant (50). Around 8% of the genes were significantly altered in the *perR* mutant and the magnitude of the peroxide induction of the PerR regulated genes corresponded well with the extent of derepression in the perR mutant strain with a correlation coefficient of 0.9 (50). In *E. coli*, similar observation is reported where in the peroxide response regulator OxyR activates most of the hydrogen peroxide inducible genes (178). In this report the authors have confirmed the induction of many oxidative stress genes that were identified over the last couple of decades in addition to identifying many new genes such as *hemH* and the *SufABCDSE* operon (178). *HemH*encodes the ferrochelatase that catalyzes the conversion of protoporphyrin IX to protoheme, the final step of protoheme biosynthesis and *SufA-E* encode proteins involved in iron-sulfur cluster metabolism. In *Pseudomonas aeruginosa*, transcriptome analysis of the response to hydrogen peroxide at 1mM showed an upregulation of protective mechanisms including production of cytotoxins that could impair immune cell functioning and a downregulation of primary metabolism (104).

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## CHAPTER III

# IRON ACQUISTION AND REGULATION IN *CAMPYLOBACTER JEJUNI*

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**Abstract.**

Iron affects the physiology of bacteria in two different ways: as a micronutrient for bacterial growth and as a catalyst for the formation of hydroxyl radicals. In this study, we used DNA microarrays to identify the *C. jejuni* genes that have their transcript abundance affected by iron availability. The transcript levels of 647 genes were affected following the addition of iron to iron-limited *C. jejuni* cells. Over a 15 minute time period, several classes of affected genes were revealed, including immediate-early response genes as well as those specific to iron acquisition and metabolism. In contrast, only 208 genes were differentially expressed during steady state experiments comparing iron-rich and iron-limited growth conditions. As expected, genes annotated as being involved in either iron acquisition or oxidative stress defense were down-regulated during both time course and steady state experiments, while genes encoding proteins involved in energy metabolism were up-regulated. Because the level of protein glycosylation increased with iron limitation, iron may modulate the level of *C. jejuni* virulence by affecting the degree of protein glycosylation. Since iron homeostasis has been shown to be Fur regulated in *C. jejuni*, an isogenic *fur* mutant was used to define the Fur regulon by transcriptome profiling. A total of 53 genes were Fur regulated, including many genes not previously associated with Fur regulation. A putative Fur binding consensus sequence was identified in the promoter region of most iron-repressed and Fur-regulated genes. Interestingly, a *fur* mutant was found to be significantly affected in its ability to colonize the gastrointestinal tract of chicks, highlighting the importance of iron homeostasis *in vivo*. Directed mutagenesis of other genes identified by the microarray analyses allowed the characterization of the ferric-enterobactin receptor, previously named CfrA. Chick

colonization assays indicated that mutants defective in enterobactin mediated iron acquisition were unable to colonize the gastrointestinal tract. In addition, a mutation in a receptor (Cj0178) for a yet uncharacterized iron source also resulted in reduced colonization potential. Overall, this work documents the complex response of *C. jejuni* to iron availability, describes the genetic network between the Fur and iron regulons, and provides insight regarding the role of iron in *C. jejuni* colonization *in vivo*.

## Introduction

Iron is known to catalyze a wide range of biochemical reactions essential for most living organisms (1). For example, it plays a crucial role as a cofactor in DNA synthesis, as well as in electron transfer reactions. The broad use of this transition metal is due to its physicochemical properties (16). Iron readily forms complexes with common biological donor ligands such as oxygen, nitrogen and sulfur. This allows its insertion into the active sites of many metabolic proteins. Iron's value resides in the reactivity of the $Fe^{3+}/Fe^{2+}$ redox couple, which enables it to catalyze enzymatic reactions (16). Paradoxically, this iron reactivity is also responsible for the generation of hydroxyl radical (·OH), which is particularly biotoxic (30). The production of this highly reactive radical is a component of oxidative stress and can damage all biological macromolecules (18, 30).

Iron bioavailability in an aerobic neutral pH environment or in the mammalian host is limited to $10^{-18}$ M and $10^{-24}$ M respectively. This level is far below the minimum requirement for bacterial growth ($10^{-7}$ M) (8). Consequently, microorganisms have evolved complex systems to efficiently capture iron, regulate its acquisition, and detoxify its excess. Many bacteria acquire iron by synthesizing and exporting powerful ferric ion chelators called siderophores (8). Together with cell surface receptors specific for the iron-siderophore complexes, they provide iron to the organisms under the most nutritionally depleted conditions. Other bacterial iron transporter mechanisms include reduction via a surface reductase and subsequent transport of the ferrous iron across the membrane (39), proteolytic degradation of the host iron-binding proteins (10), and surface receptors for mammalian iron carriers such as transferrin, lactoferrin and heme (1).

The significance of iron acquisition and metabolism for successful microbial proliferation is displayed by numerous examples from medical and environmental biology (34). One example is *Pseudomonas aeruginosa*, an opportunistic human pathogen. Siderophore deficient mutants of *P. aeruginosa* show no virulence when injected into burned mice (the infection model) (25). Remarkably, wild-type virulence is restored by co-injection with a purified siderophore (25). Siderophore production has also been shown to be a virulence-associated factor for several other pathogens, including *Escherichia coli* (20, 26), *Yersinia pestis* (5), and *Aeromonas hydrophila* (23). Undoubtedly, iron is a key environmental signal for the pathogenesis of many, if not all, human pathogens.

Members of the bacterial genus *Campylobacter* are responsible for an outstanding number of food-borne infections (24). Upon entrance into the mammalian host, *Campylobacter* must colonize, survive and replicate in the gastrointestinal tract, and should consequently be able to efficiently acquire the essential iron nutrient. Only a few strains of *C. jejuni* have been shown to produce siderophores (seven strains of 26 tested), and these siderophores are uncharacterized (12). It is not known yet if *C. jejuni* NCTC 11168 (the strain used in our study) produces a siderophore. However, all strains of *Campylobacter* tested were able to acquire iron from enterobactin, a siderophore produced by *Escherichia coli* and other enteric bacteria (12). Analysis of the annotated genome sequence of *C. jejuni* NCTC 11168 predicts the lack of genes required for enterobactin biosynthesis, sugggesting that *Campylobacter* may acquire iron in the gastrointestinal tract via siderophores (such as enterobactin) produced by the indigenous microflora, even if it does not synthesize its own siderophore.

In order to avoid iron toxicity, microorganisms must achieve an effective iron homeostasis by tightly regulating the expression of genes encoding the proteins involved in iron acquisition and metabolism in response to iron availability (34). In gram-negative bacteria, Fur is the ferric uptake regulator for the transcription of these genes (1, 7). The Fur protein is a homodimer which, upon binding its co-repressor $Fe^{2+}$, binds to a consensus sequence (named Fur-box) at the promoter of Fur-regulated genes, repressing their transcription (1, 7). The ferric uptake regulator gene (*fur*) from *C. jejuni* NCTC 11168 was previously characterized (51, 56). The *in vitro* protein profile of a *fur* null mutant was studied and revealed at least three iron regulated proteins from the outer-membrane, four from the periplasm and periplasmic membrane, and two from the cytoplasm (51). The N-terminal amino-acid sequences of two of the outer membrane proteins, CfrA and ChuA, have high homology with ferric-siderophore receptors. A *C. jejuni* strain lacking ChuA was unable to use hemoglobin or hemin as an iron source; thus ChuA is likely the receptor for these compounds (55). The two iron regulated cytoplasmic proteins, catalase (KatA) and alkyl hydroperoxide reductase (AhpC), were identified as oxidative stress defense proteins. The other proteins were not further characterized. Interestingly, in contrast to other gram-negative bacteria, oxidative stress and iron acquisition are separately regulated in *C. jejuni* (55). While genes encoding proteins involved in iron uptake have been shown to be Fur regulated, genes encoding proteins involved in the oxidative stress defense have been shown to be PerR regulated (55). In fact, previous studies have shown that the expression of *katA* and *ahpC* is iron-repressed via the PerR regulator (52).

The complete genome sequence of *C. jejuni* NCTC 11168 suggests the presence of several other potential iron assimilation mechanisms (29). These consist of at least five putative iron-binding proteins, two putative outer-membrane ferric-siderophore receptors, two putative iron uptake ABC transporter systems (permeases and ATP transporters), a complete putative hemin uptake transporter system, a ferrous iron transporter protein, and three putative TonB/ExbB/ExbD complexes. In addition to its nutritional role, iron availability has also been shown to be a key signal for pathogens to sense that they have invaded the host. Indeed, virulence factors other than iron transport systems are often iron regulated (34). However, to date little is known about the iron regulon in *Campylobacter* species, and additionally whether other *Campylobacter* virulence determinants are regulated by iron availability. Here, we present a genome wide picture of the *Campylobacter jejuni* NCTC 11168 response to iron availability using DNA microarray technology. Moreover this study further defines the importance of iron homeostasis and acquisition *in vivo*.

## Materials and Methods

*Bacterial strain and growth conditions*

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α strain was routinely cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar plates. Plasmid-containing strains were grown in medium supplemented with chloramphenicol 20 μg/ml. *Campylobacter jejuni* NCTC 11168 was obtained from the National Collection of Type Culture (NCTC, England) and routinely maintained at 37°C in a microaerophilic chamber (Don Whitley, West Yorkshire, England) containing 83% $N_2$, 4% $H_2$, 5% $O_2$ and 8% $CO_2$ on Mueller-Hinton (MH) agar plates, MH medium or MEM α medium (Invitrogen) with chloramphenicol added as required at a concentration of 20 μg/ml.

*Microarray construction*

The *C. jejuni* NCTC 11168 microarray was constructed as previously described (41, 43). Briefly, a set of 3,308 oligonucleotides were designed using PRIMER 3 (Code available at: http://www.genomewi.mit.edu/genome_software/other/primer3.htm) to amplify, via polymerase chain reaction (PCR), an internal fragment from each of the 1,654 predicted open reading frames (ORFs) identified in the annotated genomic sequence of *C. jejuni* NCTC 11168. Genomic DNA (20 ng) was used as a template in the first round of PCR amplifications using standard methods in a 96-well plate format. The PCR products were analyzed by agarose gel electrophoresis. Successful PCR products were re-amplified to reduce the amount of residual genomic DNA carried-over from the first PCR. PCR reactions without product or with incorrectly sized products were performed again by modifying the reaction conditions or by designing new primers. DNA fragments were

obtained for approximately 98% of the ORFs. PCR products were purified using the Millipore $PCR_{96}$ cleanup kit and quantified using the PicoGreen dsDNA quantitation reagent from Molecular Probes. They were diluted in a 50% DMSO solution at a concentration of 75 ng/μl, and re-arrayed into a 384-well format. They were then printed on aminosilane-coated glass microscope slides (CMT GAPS-II from Corning Inc., Corning, N.Y.) using an arrayer robot (Molecular Dynamic) in a repeating 22x7 spot pattern. Each block was printed in duplicate. Finally, the DNA fragments were immobilized onto the slides by baking at 80ºC for 4 hours. The quality of the microarray printing, the efficiency of the DNA binding to the slide and the spot morphology were assessed by direct labeling of the spotted DNA with a fluorescent nucleic acid stain (POPO-3 iodide from Molecular Probes). In addition, the hybridization capacity of the bound DNA was confirmed by using fluorescently labeled genomic DNA.

*Sampling and isolation of total RNA*

The iron-restricted *Campylobacter* cells were grown in 250 ml of MEM α medium (Invitrogen) microaerobically at 37°C. At early mid log phase (OD600 of approximately 0.1), a 50 ml sample was removed (time 0 minute) and immediately mixed with 5 ml of a cold RNA degradation stop solution (10% buffer-saturated phenol in ethanol), which has been previously shown to keep the bacterial transcriptome intact (6, 43). Ferrous sulfate was added to the remaining 200 ml of broth culture to a concentration of 40 μM. Then, samples of 25 ml were collected at times 1 min, 3 min, 5 min, 7 min, 9 min and 15 min after the addition of ferrous ion, rapidly mixed with 2.5 ml of the RNA degradation stop solution, and placed on ice. Cells were immediately collected by centrifugation at 4°C

(10 min, 8000 x g) and resuspended in lysozyme-TE buffer (50 mM Tris-Cl pH 8, 1 mM EDTA, 0.5 mg/ml lysozyme). Total RNA was isolated using a hot phenol-chloroform protocol (48). After ethanol precipitation, the RNA was resuspended in RNase free water and the remaining traces of genomic DNA were removed by two consecutive treatments with DNase I (Invitrogen) and the RNA was further purified with a RNeasy kit (Qiagen, Valentia, CA). The absence of genomic DNA was confirmed by PCR, using several sets of primers previously used for the microarray construction. The RNA concentration was determined using the RiboGreen RNA quantitation reagent from Molecular Probes following the manufacturer's protocol. The RNA integrity was evaluated by agarose gel electrophoresis. Purified total RNA was stored at -80ºC.

*Probe labeling and slide hybridization*

Total RNA from each growth condition was converted to cDNAs in the presence of aminoallyl-dUTP using Superscript-II (Invitrogen) at 42°C. The reverse transcription reaction was performed as follows: 16 μg of total RNA was combined with 10 μg of random hexamers in a 34.35 μl reaction mixture containing 8 μl of 5 x Superscript II reverse transcriptase buffer and 2 μl of 0.1 M DTT. After a 5 min incubation at 65°C, the reaction mixture was brought to a final volume of 40 μl by adding (final concentrations): 0.5 mM of each deoxy GTP, ATP and CTP; 0.16 mM of deoxy TTP; 0.34 mM of aminoallyl-deoxy UTP (aa-dUTP); and 2 μl of Superscript-II; and incubated at 42°C for 120 min. At the end of the first strand synthesis, the reaction was stopped and the RNA hydrolyzed by adding 4 μl of 50 mM EDTA, 2 μl of 10 N NaOH, and incubating at 65°C for 20 min. This reaction was neutralized by adding 4 μl of 5 M acetic acid. The

aminoallyl-labeled cDNA was purified from free amines and unincorporated aa-dUTP by adding 450 µl of water and spinning through a Microcon YM-30 filter (Millipore) for 8 min at 8000 x g. This washing step was repeated three times. After the last wash, the aminoallyl-labeled probes were concentrated to less than 8 µl under vacuum in a SpeedVac and adjusted to a final volume of 10 µl by adding 1 µl of 1 M Na-carbonate pH 9.0 and water. The resulting aminoallyl-labeled cDNA was coupled to monoreactive fluors (Amersham) by adding 10 µl of DMSO containing 1/6 of one reaction vial FluoroLink™ Cy3 or Cy5 dye and incubating for 45 min at room temperature in the dark. This reaction was quenched by adding 4.5 µl of 4 M hydroxylamine and incubating 15 min at room temperature in the dark. Fluorescent Cy3 and Cy5 labeled cDNAs were combined and purified using Qiaquick PCR spin columns according to the manufacturer's instructions (Qiagen, Valencia, CA). The fluor-labeled cDNA mix was dried under vacuum using a SpeedVac and resuspended in 15.14 µl water, to which was added 2.5 µl of salmon sperm DNA (10 mg/ml), 9 µl of 20 x SSC buffer (3 M NaCl, 0.3 M sodium citrate, pH 7), 0.36 µl SDS 10% and 9 µl formamide. Prior to hybridization, microarray slides were prehybridized at 42°C for 45 min in prehybridization buffer (25% formamide, 5x SSC buffer, 0.1% SDS and 1% BSA), rinsed with water, and dried by spinning. The probe was denatured by boiling for 2 min followed by cooling to 42°C. The probe was then applied to the microarray slide under a coverslip (Grace Bio-labs), placed in a humidified chamber (ArrayIt™), and incubated at 42°C overnight. Following hybridization, slides were washed in 2x SSC, 0.1% SDS for 5 min at 42°C, 0.1x SSC, 0.1% SDS for 10 min at room temperature, and 4 times in 0.1x SSC for 1 min at room temperature. Slides were then rinsed with distilled water and dried by centrifugation.

*Data collection and analysis*

Microarrays were scanned with a ScanArray 3000 confocal scanner (Perkin Elmer) at 10 μm resolution and analyzed with GenePix Pro 4 software (Axon Instruments, Foster City, Calif.). Spots were removed from further analysis if any of the 2 following criteria was met: (1) the spots were localized within regions of hybridization or slide abnormalities, (2) the fluorescent mean intensities in both channels, 1 (Cy5) and 2 (Cy3), were below three times the standard deviation of the local background. By using the second criteria, all 192 negative controls were uniformly excluded from the microarray data. Then, the fluorescence intensity in each wavelength was normalized by applying a locally weighted linear regression (Lowess) using the MIDAS software (available from TIGR; http://www.tigr.org/software/) (36). Following normalization, the ratio of channels 2 to 1 were $\log_2$ transformed, and the data were statistically analyzed using the empirical Bayes method (22). The time course experiment was repeated twice (biological replicate), and at least two measurements were generated per experiment (technical replicate). For the comparison of the wild-type and Fur mutant transcriptomes, the microarray experiment was repeated three times (biological replicate). Genes were selected as being differentially expressed if their *p*-value was equal or below $10^{-6}$ and their fold change in transcript abundance was above 2. Finally, genes were grouped by hierarchical clustering analysis using the Genesis software available from Graz University of Technology (http://genome.tugraz.at) (45).

*Real time quantitative RT-PCR*

Real-time quantitative RT-PCR was performed by using the ABI Prism 7700 DNA analyzer (Applied Biosystems, Foster City, CA) and the QuantiTect™ SYBR® Green RT-PCR kit (Qiagen, Valencia, CA) according to the following protocol: 250 ng of total RNA (DNase-I treated) was mixed with 25 µl of 2x QuantiTect SYBR Green RT-PCR Master Mix (containing HotStarTaq DNA polymerase, RT-PCR buffer, dNTP mix, SYBR Green I, the passive reference dye ROX, and 5 mM $MgCl_2$), 0.5 µM of gene specific primers, and 0.5 µl of QuantiTect RT mix (containing Omniscript and Sensiscript reverse transcriptases) in a 50 µl final volume. The cDNA synthesis was performed by incubating this reaction mixture at 50°C for 30 min. This reverse transcription step was immediately followed by concomitant activation of the HotStarTaq DNA polymerase and deactivation of the reverse transcriptases by heating the reaction at 95°C for 15 min. PCR amplification was composed of 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 45 s. In order to confirm the generation of specific PCR products, the PCR reaction was immediately followed by melting curve analysis of the RT-PCR product accordingly to the manufacturer's recommendations (Applied Biosystems, Foster City, CA). The microarray data of 22 genes was confirmed by real time RT-PCR analysis (*chuC*, *chuD*, Cj1658, *exbB2*, *flaA*, *flgE2*, *fliD*, *fliS*, *ilvC*, *kpsE*, *kpsM*, *lpxK*, *p19*, *peb2*, *peb3*, *slyD*, *tonB2*, *waaD*, *waaE*, *pglE* (also named *wlaK)*, *pglF* (also named *wlaL*) and *pglH* (also named *wlaC*)). The primers used are the same as those utilized for the construction of the microarray. The sequences of these primers are available upon request. The relative expression level of each gene was normalized to either *slyD* (encoding the peptidyl-prolyl cis-trans isomerase) or *ilvC* (encoding the ketol-

acid reductoisomerase). The expression of both *slyD* and *ilvC* was found to be invariant under different growth conditions, allowing the use of their expression level as a reference value for quantification. Quantitative values were obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, as recommended by Applied Biosystems. The $C_T$ value corresponds to the PCR cycle at which there is the first detectable increase in fluorescence associated with the exponential growth of the PCR products. The transcript level from each RNA sample was assayed six times and the mean $C_T$ value was used for further analysis. For data analysis, the fold induction of the specific genes was calculated as follows:

Fold induction = $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T,\ tmin} - \Delta C_{T,\ 0min}$, and $\Delta C_{T,\ tmin\ and\ 0min}$ are obtained by subtracting the mean $C_T$ value of the specific gene from the mean $C_T$ value of the reference gene at time t (tmin) or time zero (0min).

*Protein electrophoresis and glycoprotein analysis*

*C. jejuni* NCTC 11168 grown with and without iron was harvested by centrifugation to yield individual bacterial pellets from each time point equal to an optical density of 0.3 at 600nm. The harvested pellets were immediately frozen at –70°C. For glycoprotein analysis, frozen bacterial pellets were lysed with modified SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, no glycerol, bromophenol blue or β-mercaptoethanol), and protein levels were assessed by Micro BCA (PIERCE). Based on the measured protein levels, dilutions of the samples were prepared in SDS sample buffer (modified sample buffer + 25% glycerol, 720mM β-mercaptoethanol and 0.01% bromophenol blue) to contain 4.5 μg protein /10 μl. Ten and 12% SDS-PAGE gels were

loaded with 4.5 μg total protein per lane and electrophoresed until the bromophenol blue was at the bottom of the gel. Initially, gels were silver stained (Bio-Rad Silver Stain Plus) to verify that the protein measurements from Micro BCA were accurate. If necessary, samples were adjusted to give more uniform silver staining, through the use of gel imaging equipment and software (UVP Epi Chem II Darkroom and UVP labworks Image Acquisition and Analysis Software). Once the samples were diluted to provide equal protein loading, duplicate gels were either silver stained or electrotransferred to PVDF membranes (Millipore Immobilon-P). The protein blots were blocked with 1% BSA in PBS (0.01 sodium phosphate, 0.15 M sodium chloride, pH 7.2) containing 0.05% Tween 20 for 30 min, washed briefly in PBS with 0.05% Tween 20, and incubated with 5 μg/ml horseradish peroxidase labeled *Wisteria floribunda* lectin (E-YLabs) in PBS without Tween 20 for 1 hr. After lectin incubation, the blots were washed 2X for 10 min with PBS containing 0.05% Tween 20. Excess Tween 20 was removed by brief rinsing in Tris-HCl pH 7.6. Blots were then reacted with Tris-HCl pH 7.6, 0.6 mg/ ml 3,3'-diaminobenzidine (DAB) and 0.03% hydrogen peroxide. Data resulting from blots and silver stained gels were digitally recorded (UVP Biodocit System).

*Operon mapping*.

Total RNA was extracted from exponentially-growing *C. jejuni* cells after growth in MEM-α as described above. First strand cDNA synthesis and subsequent PCR amplifications were performed using the Qiagen One-Step RT-PCR system (Qiagen, Valencia, CA), according to the manufacturer's recommendations and using the primer pairs listed in Table 2 and represented in Figure 2. One hundred to 1000 ng of total RNA

was used for each reaction. The co-transcription of *exbB1, exbD1 and tonB1* was assayed with a combination of 4 primers: *exbB1-F, exbD1-R , exbD1 F,* and *tonB1-R*. The co-transcription of *exbB2, exbD2* and *tonB2* was assayed with primers *exbB2-F, exbD2-R, exbD2-F,* and *tonB2-R*. The co-transcription of *exbB3, exbD3* and *Cj0111* was assayed with primers *exbB3-F, exbD3-R, exbD3F* and *Cj0111-R*. The co-transcription of *fldA*, Cj1383c, and Cj1384c was assayed with primers *fldA-R, Cj1383c-F* and *Cj1383c-R*, and *Cj1384c-F*. Finally, the co-transcription of Cj1658 and *p19* was assayed with primers *Cj1658-F* and *p19-R*. As a positive control, each operon was also amplified with the same primers, using chromosomal DNA as a template. As a negative control, PCR reactions (without previous reverse transcription) were performed on the same RNA templates to confirm the absence of contaminating genomic DNA. RT-PCR products were electrophoresed through 1% agarose gels containing ethidium bromide and visualized with UV light. One Kb and 100 bp DNA ladders (Bayou Biolabs, Harahan, LA) served as size markers.

*Construction of the C. jejuni mutants*

*fur*, *cfrA*, *ceuE*, and Cj0178 mutants of *C. jejuni* NCTC 11168 were constructed using the same inactivation strategy. *C. jejuni* NCTC 11168 chromosomal DNA was prepared using the Wizard Genomic DNA purification kit (Promega) and *fur* gene was PCR amplified using the primers *fur-01* and *fur-02* (which both contain a *Bgl*II site). The PCR reaction was catalyzed with Pfx DNA polymerase (Invitrogen), and was hot-started to ensure high specificity of the products being synthesized. The resulting 1479 bp fragment was digested with *Bgl*II, and ligated to the *Bam*HI restricted pUC19 vector (yielding the

plasmid pAS226). A deletion of 390 bp was generated within the *fur* gene by inverse PCR using the primers *fur-03* and *fur-04*, which both contain a *Bam*HI restriction site. The PCR product was cut with *Bam*HI, self-ligated, and introduced into *E. coli* DH5α, yielding the plasmid pAS227. A *Bam*HI restricted $Cm^r$ cassette from the plasmid pRY111 (58) was then cloned into pAS227 (previously digested with *Bam*HI), yielding pAS229. The orientation of the $Cm^r$ cassette was determined by DNA sequencing. The plasmid construct containing the $Cm^r$ cassette in the same orientation as the gene of interest was used to transform *C. jejuni* NCTC 11168 using a standard protocol (58) to generate the *fur* mutant by allelic exchange. Transformants were selected on MH agar plates containing 20 μg/ml of chloramphenicol. Finally, the double homologous recombination event was confirmed by analyzing the $Cm^r$ clones using PCR with the corresponding gene primer sets. The primers and plasmids used for the mutants construction are listed in Table 2.

The *cfrA* mutant (AS269), the *ceuE* mutant (AS265) and the *Cj0178* mutant (AS211) were constructed in a similar fashion to the *fur* mutant. Briefly, the *cfrA* mutant, AS269, was constructed by amplification of a 1903 bp DNA fragment containing the *cfrA* gene using the primers *cfrA-01* and *cfrA-02* which both contain *Bgl*II site. The product was digested with *Bgl*II and cloned into the *Bam*HI restricted pUC19 vector yielding the plasmid pAS266. A 556 bp deletion was created within the *cfrA* gene by inverse PCR using the primers *cfrA-03* and *cfrA-04* (which both contain a *Bam*HI site). The $Cm^r$ cassette was introduced into the created *Bam*HI site of the *cfrA* gene, yielding the final construction pAS268.

The *ceuE* mutant, AS265, was constructed following PCR amplification of a 1743 bp DNA fragment containing the *ceuE* gene using the primers *ceuE-01* (which contains an *Eco*RI restriction site), and *ceuE-02* (which contains a *Sph*I restriction site). The product was digested with *Eco*RI and *Sph*I, and cloned into the *Eco*RI/*Sph*I restricted pUC19 vector, yielding pAS261. A 522 bp deletion was created within the *ceuE* gene by inverse PCR using the primers *ceuE-03* and *ceuE-04* (which both contain a *Bam*HI site). The $Cm^r$ cassette was introduced into the created *Bam*HI site of the *ceuE* gene, yielding the final construction pAS263.

The Cj0178 mutant, AS211, was constructed as followed: a 1991 bp DNA fragment (harboring the Cj0178 gene) was PCR amplified using the primers *Cj0178-01* and *Cj0178-02,* which both contain an *Eco*RI site. The product was digested with *Eco*RI, and cloned into an *Eco*RI restricted pUC19 vector yielding the plasmid pAS207. A deletion of 511 bp was generated within the Cj0178 gene by inverse PCR using the primers Cj0178-*03* and Cj0178-*04*, which both contain a *Bgl*II restriction site. The $Cm^r$ cassette was introduced into the created *Bgl*II site of the Cj0178 gene, yielding the final construction pAS209.

*Growth promotion assays.*

The ability of iron sources to promote the growth of *C. jejuni* NCTC 11168 wild-type and mutant strains was assessed on standard iron-limited assay plates as described by others (2, 12, 14). Briefly, the bacterial strains to be tested were grown to mid-log phase in MH medium, harvested by centrifugation, and resuspended in MH medium to an OD of 1.0 at 600 nm. One ml of this bacterial suspension was added to 24 ml of melted MH agar

containing 40 μM of desferriferrioxamine mesylate salt (DFO), poured into Petri dishes, and allowed to solidify. Sterile disks containing 10 μl of enterobactin (at a concentration of 10 mM) were laid upon the surface and growth zones were measured after 24 hours of incubation at 37°C under microaerophilic conditions. Enterobactin was isolated from bacterial growth supernatants using standard protocols as previously described (44).

*Chick colonization assays*.

One day old specific-pathogen-free broiler chicks were obtained from Tyson Farms, Arizona. Chicks were housed in a room maintained at 22ºC, equipped with a brooder maintained at 33-35ºC. Chicks were checked upon arrival, by taking cloacal swabs for culture, to verify they did not carry *Campylobacter*. Chicks were provided water and a commercial chicken starter diet *ad libidum*. Food and water were withheld for 2 to 3 hours prior to the challenge. For this challenge, *C. jejuni* wild-type and mutant strains were cultured in an MH broth biphasic culture medium at 37°C under microaerophilic conditions. At mid-log phase, the bacterial strains were harvested and resuspended in PBS buffer. Each chick (three to four days old) was inoculated orally with 1 ml of a bacterial suspension containing between $10^5$ and $3.10^5$ viable *C. jejuni*. A control group of uninfected birds was also included. Each strain of *C. jejuni* was inoculated into 5 birds. The ability of each *C. jejuni* strain to colonize the chick ceca was evaluated four days post challenge by determining viable counts as described by others (13, 31). Briefly, the chicks were humanely euthanized and their ceca were collected. Then, the cecal contents were homogenized, serially diluted in phosphate-buffered saline and plated onto *Campylobacter* agar base (Oxoid CM935) containing the *Campylobacter* selective

karmali supplements (Oxoid SR167E). Viable counts were expressed as logarithms of cfu per gram of ceca. The data was statistically analyzed using a non-Parametric Mann-Whitney Rank Sum test. *P* values below 0.05 were regarded as statistically significant.

*Computational analysis of the Fur promoter*.

Fur regulated genes were selected according to the transcriptional profiling experiments. Intergenic regions and 500 bp regions extending upstream from the start codon of every gene were retrieved using the regulatory sequence analysis tools at http://rsat.ulb.ac.be/rsat/ (50). These upstream regions were analyzed for the presence of a potential promoter motif using the MEME algorithm at http://meme.sdsc.edu/meme/website/intro.html (3). Finally, a consensus sequence logo was built by compilation of the potential motif sequences of each regulated genes using WebLogo (http://weblogo.berkeley.edu/logo.cgi) (38).

## Results and Discussion

### Experimental design, statistical analysis and validation of the microarray data

Genes encoding proteins involved in iron metabolism are commonly known to be iron repressed (1, 7, 34). Therefore, in order to determine the global change in gene expression profile elicited in *C. jejuni* in response to iron availability, we performed two sets of experiments.

The first set of experiments addressed the immediate response of *C. jejuni* gene expression to the addition of ferrous sulfate. The changes in transcript levels were determined as a function of time after the addition of iron. Briefly, cultures were grown to mid-log phase in the iron-limited medium MEMα in which the genes encoding proteins involved in iron metabolism are highly expressed. This medium contains a trace amount of iron and has been previously used to study *C. jejuni* iron metabolism (51). At the initial time point (0 min), a sample of the growth culture was removed and the total RNA extracted. Then, ferrous sulfate was added to the remaining bacterial culture at a concentration of 40 μM. Under this condition, the genes encoding proteins involved in iron metabolism are repressed. At 1, 3, 5, 7, 9 and 15 min, samples from the growth culture were removed and the total RNA purified. All total RNA samples were reverse transcribed and fluorescently labeled as previously described (41, 43). Finally, the relative abundance of gene transcripts at each time point after the addition of ferrous sulfate was compared with the level of transcripts at the 0 min time point (corresponding to the transcriptome of *C. jejuni* grown in iron limited condition) by using the *C. jejuni* NCTC 11168 microarray. Each hybridization was repeated twice, yielding two technical replicates for each time point. In addition, two independent time course experiments were

carried out, constituting two biological replicates. A total of 24 measurements per gene were performed. The data were normalized, merged, and reported as the $\log_2$ ratios of the transcript abundance of *C. jejuni* grown in iron-starved conditions (time point 0) to that of *C. jejuni* after the addition of ferrous sulfate to the growth medium. The significance of the differential abundance of transcripts was analyzed using a regularized t-test based on a Bayesian statistical analysis of variance (22). This statistical method has been shown to be more reliable than a simple t-test by reducing the false positive rate. Genes were selected as being differentially expressed using a *p* value below $10^{-6}$ (which corresponds to a significance level below 0.01 after Bonferroni correction) and a minimum of 2-fold change in transcript abundance in at least one of the 6 time points.

The bacterial response to the sudden addition of a nutrient is not necessarily identical to the transcriptome profile during balanced growth. Indeed, the physiological response of *E. coli* to the sudden addition of acetate significantly differs from the transcriptome profile of *E. coli* during balanced growth in an acetate-containing medium (28). Therefore, the second set of experiments served to compare the transcriptome profiles of *C. jejuni* grown to mid-log phase in both iron-limited (MEMα medium) and iron-rich medium (MEMα medium containing 40 μM of ferrous sulfate). This experiment was repeated three times (biological replicates) and analyzed similarly to the time course experiment.

To support the reliability of our microarray data, the expression of 22 genes was confirmed by real time RT-PCR (data not shown). A high level of concordance was observed between the microarray results and the real time RT-PCR data (with a correlation coefficient of 0.98). However, like our previous gene expression study (in

which we used the same microarray platform), microarray data analysis under-estimates fold changes compared to real time RT-PCR measurements (41). This fold change difference between these two technologies has been previously reported by others and suggests a smaller dynamic range for microarray analysis (57). Nevertheless, the high level of correlation between the two technologies confirms the reliability of the data produced in this study. Moreover, our microarray data are in agreement with previous results from others who studied iron regulation in *C. jejuni* at the level of one or a few genes (51).

**Global analysis and kinetics of *C. jejuni* gene expression in response to iron availability**

Table 3 highlights the number of up- or down-regulated genes grouped by hierarchical clusters and functional categories, respectively. The complete statistical analysis can be found online at http ://www.cvm.okstate.edu/research/Facilities/ CampyLab/Index.htm. Three main points can be extracted from these data: (1) up to 500 genes are transiently differentially expressed in the time course experiment; (2) differentially expressed genes are equally distributed between up- and down-regulated genes; and (3) the number of genes differentially expressed during balanced growth (mid-log phase experiment; 208 genes) is considerably smaller than the number of genes differentially expressed at 5, 7, 9 and 15 min after the addition of iron (278, 353, 462, and 370 respectively). It is apparent from these data that the immediate response of *C. jejuni* to the sudden addition of iron differs significantly from the bacterial transcriptome during steady-state growth in the presence of iron at mid-log phase. Consequently, the observed transcriptome profile of *C.*

*jejuni* at 1, 3, 5, 7, 9 and 15 min after the addition of ferrous sulfate likely represents the bacterial adaptation to the new growth medium, while the transcriptome profile at mid-log phase represents the steady-state transcriptome.

Notably, groups of transcripts whose abundance was affected in the time course and not in the steady-state experiment encode ribosomal proteins, surface structures, and proteins of unknown function (Table 3).

**Transcript profiling of *C. jejuni* at mid-log phase in iron depleted or iron rich growth conditions**

The major objective of this work was to identify the genes associated with the mechanism of iron acquisition and metabolism. Because these genes should be differentially expressed at mid-log phase in response to iron availability, we further analyzed the genes which satisfy this criterion. Globally, the transcript abundance of 208 genes was found to be significantly altered between iron-limited and iron-rich growth conditions during steady state. In order to visualize the temporal expression of these genes in response to the addition of iron, we subjected our microarray data to hierarchical clustering analysis (11, 45). This cluster analysis identified six major clusters, named A, B, C, D, E, and F (Figure 1).

Clusters A and E are composed of transcripts that have their level decreased in response to the addition of iron. Genes from cluster A are down-regulated earlier than genes from cluster E. Genes from clusters A and E are down-regulated at the steady state between 4- to 256- fold and 2- to 13- fold, respectively. As expected, genes that have been reported or annotated as playing a role in iron acquisition or detoxification belong to these two

clusters (Table 4). With the exception of ferritin, which is involved in iron storage, the other proteins are likely involved in the transport of iron or iron-complexes through the bacterial membrane. Our microarray data confirm the previous findings showing that the expression of *chuA*, *cfrA* and *p19* are iron regulated (51), and also identify new members of the iron regulon. While ChuA appears to be required for hemin transport in *C. jejuni* (35, 55), the functional annotations of the other iron uptake systems are strictly based on homologies with known proteins from other bacteria. Interestingly, genes from the third ExbB-ExbD energy transducing complex (*exbB3* and *exbD3*) were found to be iron induced, suggesting this set of genes is unlikely to be involved in iron acquisition.

Both clusters, A and E, contain genes encoding proteins involved in oxidative stress defense. These proteins are the peroxide stress regulator (PerR), the catalase (KatA), the alkyl hydroperoxide reductase (AhpC), a probable thiol peroxidase (Tpx) and the iron-containing superoxide dismutase (SodB) (Table 4). SodB mediates the dismutation of superoxide anion into water and hydrogen peroxide, which is subsequently converted to water and oxygen by KatA (30). The AhpC protein catalyzes the reduction of alkyl hydroperoxide to alcohols (30). Previous reports have shown that AhpC and SodB are required for *C. jejuni* survival in aerobic conditions, and that a *C. jejuni katA* mutant is affected in its survival ability within macrophages (4, 9, 31, 40). The Tpx protein exhibits high homology with antioxidant enzymes from other bacteria, but its role in oxidative stress defense requires further investigation in *C. jejuni*.

Interestingly, several genes from the A and E clusters encode enzymes that do not require iron as a cofactor and are not expected to play a role in iron acquisition or detoxification,

and thus may play an essential physiological role under iron limited conditions (Table 4). It will be interesting to determine what role, if any, these enzymes play *in vivo*.

Finally, it should be noticed that the expression of the gene annotated as the ferrous iron uptake protein, *feoB*, was not identified as being significantly repressed by the addition of ferrous iron by using the gene selection algorithm described in the Materials and Methods section. Recently, FeoB has been proposed to be not required for ferrous iron uptake in *C. jejuni* (33). Thus, although based on sequence annotation we expected an immediate repression of the *feoB* gene after ferrous iron addition, recent findings suggest that this gene is not likely to be critical for iron uptake, and thus not necessarily iron-regulated. While *feoB*'s expression was not affected during the time course experiment, 1.9 fold repression of *feoB* expression ($p=1.2x10^{-5}$) was seen at mid-log phase. Our findings suggest that if ferrous iron does regulate the expression of *feoB*, the effect is long-term rather than immediate.

Clusters B and F contain genes that have their transcript abundance increased in response to the addition of ferrous sulfate. Genes from cluster B are more rapidly and highly induced than genes from cluster F. Fold changes at mid-log phase vary from 2.6- to 92-fold, and from to 2- to 13-fold for clusters B and F, respectively. These two clusters are dominated by genes encoding proteins involved in energy metabolism. Interestingly, many of these genes encode enzymes that require iron for their function. Consequently, these enzymes may provide the most efficient cellular functions in the presence of iron (Table 5). Of interest, the oxidoreductase OorABCD may have a complementary function to several of the putative iron-independent oxidoreductase enzymes listed in Table 4 (Cj0414, Cj0559, and Cj1287c) under iron-rich growth condition. From cluster B, the

ferredoxin FdxA (with 14 fold induction at mid-log phase), is worth noting since these results confirm the previously reported induction of *fdxA* expression by iron (54). A *fdxA* mutant has been shown to be affected in its aerotolerance ability, suggesting a role for FdxA in oxidative stress defense (54).

Clusters C and D contain genes that are antagonistically expressed between the two sets of experiments (the time course and the mid-log experiment). Genes from cluster C have their transcript abundance increased during the first 15 min, and decreased at mid-log phase. Genes from this cluster encode essentially proteins involved in macromolecule biosynthesis and modification (ribosomal proteins, proteins involved in ribosome maturation and modification, DNA replication, restriction and modification, and RNA synthesis). Genes from cluster D have their transcript abundance decreased during the first 15 min, and increased at mid-log phase. These encode proteins involved in energy metabolism, surface structures, amino-acid transport, as well as many proteins of unknown function. The significance of the expression profile of the genes from these two clusters is unclear and requires further investigation. However, these results clearly highlight the impact of microarray experimental design on gene expression analysis, and demonstrate that time course experiments yield data that are significantly different from steady state experiments.

**Characterization of several iron regulated operons**

The gene clustering analysis suggested that a number of differentially expressed genes might be co-transcribed. Therefore, we used RT-PCR to assess the co-transcription of

several sets of genes that were found to be either iron-repressed (*exbB1-exbD1-tonB1*, *exbB2-exbD2-tonB2*, *fldA*-Cj1383c-Cj1384c, and Cj1658-p19), or iron-induced (*exbB3-exbD3*-Cj0111) by microarray analysis. None of these genes have been previously described to be co-transcribed. The transcripts were mapped from *C. jejuni* cells grown to mid-log phase in an iron limited or iron rich medium. The RT-PCR reactions were performed using primers that anneal across pairs of genes (Figure 2 and Table 2). Each RT-PCR reaction gave a product of the expected size, as shown in Figure 2. These results confirm the transcriptional organization of these genes in operons. The operon *fldA*-Cj1383c-Cj1384c is divergently transcribed from *katA* and in the opposite direction from its downstream gene, and thus likely constitutes a single transcriptional unit. While Cj1383c and Cj1384c encode proteins of unknown function, *fldA* codes for a flavodoxin. The expression of these three genes was found to be repressed upon addition of iron, similarly to the expression of the catalase gene *katA*, indicating these other genes might also play a role in oxidative stress defense. The operon *exbB2-exbD2 tonB2* is divergently transcribed from Cj1627c and is in the opposite orientation from its downstream gene Cj1631c. Therefore, this set of genes likely constitutes an independent transcriptional unit. Finally, Cj1658-p19, *ExbB1- exbD1-tonB1* and *exbB3-exbD3*-Cj0111 appear to be transcribed together based on our RT-PCR data.

**Effect of iron on *Campylobacter* glycosylation profile**

Recently, *C. jejuni* has been shown to possess a system of "general" protein glycosylation encoded by the *pgl* gene cluster (these same genes were annotated *wla* in the genome of *C. jejuni* NCTC 11168) (47). In *C. jejuni* NCTC 11168, this cluster contains 12 genes

named consecutively as *wlaB*, *pglH* (*wlaC*), *pglI* (*wlaD*), *pglJ* (*wlaE*), *pglB* (*wlaF*), *pglA* (*wlaG*), *pglC* (*wlaH*), *pglD* (*wlaI*), *wlaJ*, *pglE* (*wlaK*), *pglF* (*wlaL*), and *pglG* (*wlaM*). The mutation of several genes from this locus demonstrated the participation of their products in the general glycosylation pathway (21, 47, 59).

Up to 30 genes have been identified to encode potential glycoproteins in *C. jejuni* (59), among which 15 were found to be iron regulated at mid-log phase by our microarray data analysis (*trxA*, *sodB*, Cj0175c, Cj0238, *ahpC*, Cj0276, Cj0415, Cj0420, *tpx*, Cj0906c, Cj0998c, Cj1032, *tsf*, *p19*, and *cgpA*). Interestingly, our microarray data suggested that the transcript abundance of the *pgl* genes is affected by the iron availability. Only *wlaB*, *pglH*, *pglA* and *pglC* were selected as significantly up-regulated (up to 3 fold) at the 7 and 9 minute time points. In addition, the induction of *pglH* (*wlaC*) was confirmed by real time RT-PCR. While *pglE* and *pglF* were not selected as differentially expressed using our selection algorithm, RT-PCR analysis revealed a 2 fold repression (3 min after the addition of ferrous ion). The observed differential expression between the *pgl* genes which participate in the same pathway for protein glycosylation is unclear and requires further investigation. Although these genes are located in a cluster within the *C. jejuni* genome, no current evidence about the operonic structure(s) for this region has (have) been reported. Despite the complexity of the gene expression data for genes related to protein glycosylation and glycoprotein production, the apparent differential abundance of the transcripts encoding some of the glycoproteins and some of the Wla-Pgl proteins during the time course experiments predicted variation in the glycosylation level and therefore in the lectin reactivity. In order to test this hypothesis, the lectin binding properties of whole cell lysates were analyzed by SDS polyacrylamide gel

electrophoresis (Figure 3, panel A), and revealed (following electrotransfer of the SDS-PAGE gel to PVDF membrane) with horseradish peroxidase labeled *Wisteria floribunda* lectin (Figure 3, panel B), which interacts with oligosaccharides terminating with N-acetylgalactosamine (37). The whole cell lysates were obtained from the same *C. jejuni* growth cultures as those used to purify the total RNA for the microarray analysis. The lectin blot analysis revealed a reduced lectin reactivity with proteins extracted from *C. jejuni* grown in iron rich medium (Figure 3, lanes 5, 9, and 15 min, and lane mid-log (+Fe)) compared to iron limited medium (Figure 3, lane labeled reference). Given the lack of data on the functional role of each *pgl* protein, the correlation between the decreased lectin reactivity and the gene expression is difficult to address. Nevertheless, our data clearly demonstrate that proteins from *C. jejuni* grown in iron-limited medium are hyper-glycosylated compared to proteins from *C. jejuni* grown in iron-rich medium. Additional supporting evidence regarding the importance of iron as a potential regulator of protein glycosylation comes from ongoing studies involving *C. jejuni* strains grown on different media types and under different temperatures to determine the effects on protein glycosylation. Consistently, strains grown on MH medium (lower iron) showed greater lectin reactivity per equal amount of proteins analyzed, than those grown on 5% sheep blood plates (higher iron), this was consistent whether the base agar was tryptic soy agar or MH agar (DS Threadgill, unpublished data). Although the glycosylation profiles cannot be directly compared because of the use of solid media vs. liquid, these results further support the potential role of iron in the regulation of protein glycosylation in *C. jejuni*. Recently, Szymanski and colleagues demonstrated that a *C. jejuni pgl* mutant is affected in its ability to adhere to and invade INT407 cells, as well as to colonize the

mouse gastrointestinal tract (46). Because invasion has been shown to be an important component of *Campylobacter* pathogenesis, the degree of glycosylation may play a role in modulating the level of *C. jejuni* virulence. This hypothesis is supported by the hyper-glycosylated phenotype of *C. jejuni* grown in iron-limited medium, a growth condition which mimics the iron availability within the host gastrointestinal tract.

**The Fur regulon**

The ferric uptake regulator, Fur, has been previously shown to repress the transcription of at least 7 iron regulated proteins in *C. jejuni*. Five were characterized and identified as being CeuE, ChuD, p19, ChuA and CfrA (51). The other two were not further characterized. In order to identify other genes transcribed under the control of Fur, the *C. jejuni fur* gene was disrupted and two complementary genome wide expression profiling experiments were performed.

In the first experiment, RNAs were extracted from *C. jejuni* wild type stain and its *fur* mutant at mid-log phase in iron limited condition. Both RNA pools were reverse transcribed to cDNA, fluorescently labeled, and co-hybridized to the microarray slides. Under this growth condition, all the genes that are Fur regulated in the absence of its coeffector $Fe^{2+}$ (apoFur) will be identified. It should be noticed that the transcript level of *lysS*, which is located downstream of *fur*, was not affected by the *fur* mutation as shown by our microarray data, indicating that the constructed mutation is non-polar.

In the second experiment, RNAs were extracted from *C. jejuni* wild type strain and its *fur* mutant 15 min following the addition of ferrous sulfate (at a concentration of 40 μM) to an iron-limited mid-log phase bacterial culture. The total RNAs were processed as

described earlier. Under this growth condition, the genes that are Fur regulated in the presence of $Fe^{2+}$ will be identified. The microarray data are presented as the ratio of the transcript level of the wild type strain to that of the *fur* mutant. Both microarray experiments were repeated three times (biological replicates) with two technical replicates each, yielding six measurements per gene. The data were statistically analyzed as described in the Materials and Methods section, and merged to the microarray data from the time course and mid-log experiments. Figure 4 represents the hierarchical clustering analysis of the Fur regulated genes (using a Pearson correlation distance). In total, 53 genes were found to be Fur regulated and can be grouped into 4 major hierarchical clusters, named A, B, C and D (Figure 4). Of note, unlike other bacterial *fur* genes, *C. jejuni fur* expression is characterized by the absence of iron-responsive auto-regulation (53). Indeed, *fur* was not selected as an iron regulated gene by our microarray analysis of time course and mid-log phase experiments (Figure 1).

Cluster A contains 8 genes that are iron-induced and apo-Fur-repressed. Interestingly, Fur repression decreased between 1.2 to 2.1-fold upon the addition of iron but is not completely abolished. This decrease in Fur repression could only partially explain the increase in transcript level of these 8 genes following the addition of iron. Indeed, the genes from this cluster displayed an iron induction in their mRNA levels up to 4 fold. Consequently, the expression of these genes is likely under the control of an additional regulator. In addition, it should be noted that these microarray experiments cannot distinguish between a direct or indirect effect of Fur on gene expression. The role of these 8 genes and why they are regulated by iron could not be deduced from their annotation and thus remains to be fully determined.

Cluster B contains 6 genes that have their transcript level increased in response to the addition of iron (in the time course experiment) and are either Fur-activated and/or Fur-repressed. Of note, the expression of *fdxA* (encoding a ferredoxin) is apo-Fur repressed. As discussed above, FdxA is probably involved in oxidative stress defense. This protein has been proposed to reduce the oxidized state of the alkyl hydroxyperoxide reductase (AhpC) in a similar fashion as AhpF in other gram-negative bacterium (54, 55).

Cluster C contains 10 genes that are iron-repressed during the time course experiment and are apo-Fur-activated. Of interest among these are the 5 proteins involved in flagella biogenesis (FlaD, FlgE2, FlgG2, FlgH, and FlgI). While the reduction of the flagellar related transcripts upon the addition of iron and formation of $Fe^{2+}$-Fur complex might explain their iron repression during the time course experiment, it is inconsistent with their over-expression at mid-log phase in iron-rich medium. Consequently, the expression of these genes is undoubtedly under the control of other regulators and/or indirectly controlled by Fur. Indeed, three regulators, RpoN, FlgR and FliA have been previously shown to regulate flagellar expression (19).

Cluster D contains 29 genes that have their expression repressed by iron and $Fe^{2+}$-Fur. The expression profile of these genes is in agreement with the well established model of Fur as a repressor of genes encoding proteins involved in iron acquisition and assimilation. Indeed, 17 genes from this cluster encode proteins involved in heme transport (ChuABCD), iron transporter systems (CfrA, CeuBC, Cj0173c-175c, p19, and Cj1658), and several components of the energy transducing TonB-ExbB-ExbD complex. Interestingly, another gene from this cluster encodes a thioredoxin (TrxB), which has been recently shown to be PerR regulated and involved in oxidative stress defense in *S.*

*aureus* (17). As an efficient thiol donor, thioredoxin likely reduces the oxidized cellular proteins and therefore contributes to the maintenance of the intracellular redox status. The expression profile of the other genes from this cluster and the function of their products in iron metabolism are unclear and require further investigation.

**A *fur* mutant is affected in chick colonization**.

It is important to mention that the NCTC 11168 strain used in our study is phenotypically different from the sequenced *Campylobacter jejuni* NCTC 11168 (11168-GC) strain described by Gaynor *et al.* (13). This strain of NCTC 11168-GS was shown to be rod-shaped, non-motile, and unable to colonize the gastrointestinal tract of chicks. In constrast,the *C. jejuni* NCTC 11168 strain used in our study is helically-shaped, fully motile and able to colonize chicks at a level of $\geq 8.10^7$ cfu per gram of cecal content with an inoculum of $10^3$ cfu (A. Stintzi, unpublished data). Consequently, it is a different variant from the genome-sequenced *C. jejuni* NCTC 11168 strain, and was therefore effectively used to investigate the mechanism of chick colonization. Of note, the 11168 strain used in our study, although clearly distinct from NCTC 11168-GC, appears to be similarly poorly invasive into human intestinal INT407 cells (Stintzi, unpublished data), suggesting the invasion defect noted by Gaynor et al. may exist separately from the other defects.

The *C. jejuni* NCTC 11168 and the derivative *fur* mutant (AS230) were orally inoculated into groups of 2-day-old chicks. The ability of both stains to colonize the chicks was assessed by enumeration of bacteria in the ceca 4 days post-inoculation. As shown in Figure 5, the *fur* mutant exhibited a significant reduction in chick ceca colonization as

compared to the wild type strain ($P<0.05$, using a non-Parametric Mann-Whitney Rank Sum test). This data clearly demonstrates the importance of iron homeostasis *in vivo*. The effect of the *fur* mutation on the ability of *C. jejuni* to colonize chicks is likely multi-factorial. The *fur* mutation may increase oxidative stress due to the over-accumulation of iron. Additionally, several of the Fur-activated genes might be required for chick colonization. For example, several genes encoding proteins involved in flagella biosynthesis, which is known to be required for chick colonization, were found to be Fur-activated (Figure 4). Alternatively, the constitutive expression of iron-regulated proteins in the *fur* mutant could provide attractive targets for chick antibodies, thus reducing the colonization level of this mutant through enhanced immune clearance.

**Computational analysis of a putative Fur binding sequence**

In *E. coli*, upon association with $Fe^{2+}$, the Fur protein binds a 19-bp consensus site (named Fur box) with the sequence 5'-GATAATGATAATCATTATC-3', and represses the transcription of the downstream gene. This Fur box was determined by DNase I protection assays and footprinting experiments (1). Three successive interpretations of the functional pattern of Fur binding to this sequence have been proposed (1): (1) originally, Fur was assumed to recognize two 9 bp inverted repeats in the palindromic sequence, (2) this initial assumption was then challenged by studies suggesting that Fur recognizes three adjacent hexameric repeats in a head-to-head-to-tail orientation, (3) and finally, a third interpretation proposed that the 19-bp consensus sequence represents overlapping heptamer inverted repeats for the binding of two Fur dimers. Despite the apparent discrepancy of the Fur box sequence interpretation, the consensus sequence is

conserved and has been identified in many bacterial species. *C. jejuni* Fur was shown to recognize the *E. coli* consensus Fur box, suggesting the presence of a similar sequence upstream of Fur regulated genes in *C. jejuni* (56).

Since this Fur box has been shown to be present upstream of iron and Fur repressed genes, we searched for the presence of a conserved sequence element upstream of the initiation codon of the genes from cluster D. As a first step, genes were grouped into potential operonic structures based on our operon mapping experiment and physical distances between genes. Genes that were separated by less than 15 bp and putatively transcribed in the same orientation were assumed to lie on the same operon. By using this strategy, 16 probable operons were identified. As a second step, the intergenic DNA regions upstream of the initiation codon of the first gene from each operon were searched for conserved sequence elements conforming to a potential Fur box. Specifically, the upstream sequences of the following genes were analyzed: *cfrA*, Cj1658, *chuA*, *ceuB*, Cj0177, Cj0176c, Cj1384c, Cj1613c, Cj1377c, *exbB2*, *tonB3*, *trxB*, *aroC*, *murE*, Cj1375, and *acs* using MEME algorithm (3). A 19-bp conserved element was identified upstream of 11 of the genes (*cfrA*, Cj1658, *ceuB*, Cj0177, Cj0176c, *chuA*, Cj1384c, Cj1613c, *exbB2*, *tonB3*, and Cj0818). The consensus sequence logo obtained from this search is shown in Figure 6. The absence of the element upstream of the other genes suggests that they are indirectly regulated by Fur. Interestingly, the predicted *C. jejuni* consensus sequence poorly matches the *E. coli* consensus Fur box (1). Confirmation of this putative *C. jejuni* Fur box will require additional experimentation, including DNA footprinting.

**Identification of the ferric-enterobactin receptor**.

In gram-negative bacteria, the translocation of the ferric-siderophore complex through the bacterial membranes is energy dependent and usually requires a specific outer-membrane receptor, a periplasmic binding protein, and an inner membrane ABC transport system. While the analysis of the *C. jejuni* NCTC 11168 genome reveals the presence of a periplasmic binding protein (CeuE) and an ABC-complex (CeuBCD) with high identity to the *Campylobacter coli* ferric-enterobactin transport system, the specific outer-membrane receptor has not been identified yet. In agreement with their role in ferric-enterobactin uptake, the *ceuBCDE* genes were found to be iron and Fur regulated by our microarray analysis. In an effort to characterize the ferric-enterobactin receptor in *C. jejuni* NCTC 11168, we confirmed the ability of ferric-enterobactin to promote *C. jejuni* growth in iron limited medium, and constructed mutations into genes identified by our microarray analysis which might fulfill this function.

First, the ability of *C. jejuni* NCTC 11168 to utilize ferric-enterobactin as a sole iron source was confirmed by standard growth promotion assay (2, 12, 14). This assay consists of analyzing the ability of ferric-enterobactin to support the growth of *C. jejuni* on iron-restricted medium. This is done by supplementing the growth medium with a sufficient amount of the iron chelator desferrioxamine mesylate salt (DFO) to completely inhibit *Campylobacter* growth in the absence of iron. As shown in Figure 7, enterobactin mediates iron acquisition in *C. jejuni* NCTC 11168. Next, a *ceuE* mutant was constructed in order to confirm the role of the periplasmic binding protein CeuE in ferric-enterobactin transport (Figure 8). The *ceuE* mutant was slightly impaired (zone of growth 2.8$\pm$0.9 cm versus 3.5±1.4 cm for the wild-type), and not fully abolished, in its ability to acquire iron

from ferric-enterobactin (Figure 7). This observation, while consistent with the previous results in *C. coli*, suggests that the CeuE protein is not strictly essential for the utilization of iron from ferric-enterobactin.

Based on our microarray data and the genome annotation, *C. jejuni* NCTC 11168 possesses only 3 iron-regulated outer-membrane proteins with homology to high-affinity outer-membrane receptors, CfrA, ChuA and Cj0178. Since ChuA has been proposed to be required for hemin transport (55) and a *C. coli cfrA* mutant appeared to be able to acquire iron from ferric-enterobactin (14), Cj0178 was the best candidate for the ferric-enterobactin outer-membrane receptor. However, as shown in Figure 7 a *C. jejuni* Cj0178 mutant (Figure 8) was not affected in its ability to utilize iron from ferric-enterobactin, suggesting that Cj0178 is the outer-membrane receptor of a yet unidentified iron source. Given that our microarray analysis did not identify any other candidate for the ferric-enterobactin receptor, we re-evaluated the function of CfrA in enterobactin mediated iron transport. In contrast to the previous study with *C. coli*, the *C. jejuni cfrA* mutant (Figure 8) was fully abolished in its ability to acquire iron from ferric-enterobactin (Figure 7), demonstrating the role of CfrA as the ferric-enterobactin receptor in *C. jejuni* NCTC 11168. Because the *C. jejuni* and *C. coli* CfrA proteins are almost identical (98.7% identity in 696 aa overlap), suggesting conserved functions, *C.coli* may possess an additional receptor able to mediate iron acquisition from enterobactin. If so, enterobactin would still be able to support the growth of a *C. coli cfrA* mutant in an iron restricted medium. Similar findings have been observed with *Salmonella enterica* which has two receptors, FepA and IroN, for ferric-enterobactin (32). Alternatively, it should be noted that the growth promotion assay in the previous *C. coli* study was performed by using

EDDHA (ethylenediamine bis(2-hydroxyphenyl) acetic acid) as an iron chelator instead of DFO. These two chelators differ significantly in their effectiveness at binding iron, with DFO being the most powerful iron chelator (42, 60). In aqueous solution, enterobactin could spontaneously breakdown into the trimers, dimers and monomers of dihydroxybenzoyl serine (DHBS) (15, 27). As a result, the growth promotion assays may have been performed with a mixture of enterobactin and its breakdown products. Since DHBS is a weaker iron chelator than enterobactin, it will compete less efficiently for iron with DFO than with EDDHA (42). In other words, the breakdown products of enterobactin will be more efficient in delivering iron to the bacterium in a medium containing EDDHA than DFO. Consequently, the growth promotion observed in the *C. coli cfrA* mutant might have been DHBS mediated. Similarly to *E. coli*, *C. coli* may possess an additional outer-membrane receptor specific for the DHBS iron complex. In addition, because DHBS mediated iron acquisition would likely not be observed on a DFO-containing medium, it is possible that *C. jejuni* also possesses a DHBS specific receptor. We are currently investigating this possibility.

**Ferric-siderophore transporters are required for *C. jejuni* NCTC 11168 chick colonization**.

The role of CfrA, CeuE and Cj0178 in colonization was investigated in the chick model. Groups of 5 chicks were orally inoculated with the wild-type strain and the mutants at a dose between $10^5$ and $3.10^5$ cfu. As shown in Figure 5, all three mutants, *cfrA*, *ceuE* and Cj0178, were significantly affected in their ability to colonize the gastrointestinal tract of chicks ($P<0.05$, using a non-Parametric Mann-Whitney Rank Sum test). At 4 days post-

infection, the number of *cfrA* and *ceuE* mutant per gram of ceca was below the detection limit of our assay (500 cfu/g of ceca). This result clearly demonstrates the requirement of the two outer-membrane receptors (CfrA and Cj0178) as well as of the ferric-enterobactin periplasmic binding protein (CeuE) in chick gut colonization. Overall, these experiments highlight for the first time the importance of iron acquisition in the gut colonization process by *C. jejuni*. Moreover, the inability of the *ceuE* mutant to colonize the ceca indicates an essential role for enterobactin in gut colonization. In addition, we can not exclude a possible role for CfrA and Cj0178 in adhesion. In support of this possibility, CfrA exhibits significant identity with the *E. coli* O157 Iha adhesin protein (34.2% of identity) (49).

## Conclusion.

The transcriptional response of *C. jejuni* to iron availability is pleiotropic, involving the differential expression of genes belonging to several functional groups, ranging from energy metabolism, to cell surface structures, to iron acquisition, and to oxidative stress defense. Several of these genes encode proteins that have been previously shown to be involved in the mechanism of *Campylobacter* pathogenesis. Interestingly, the level of *Campylobacter* glycoproteins and/or the efficiency of glycan addition to those proteins was found to increase with iron limitation. While the biological function of glycosylation remains to be established, a mutant deficient in the glycosylation process has been shown to be affected in its ability to bind and invade INT407 cells, as well as to colonize the mouse gastrointestinal tract (46). Consequently, iron availability likely modulates the abilities of *Campylobacter* to adhere, invade and colonize the host gastrointestinal tract. In support to this hypothesis, a *fur* mutant showed significantly reduced chick ceca colonization, highlighting the importance of iron homeostatis *in vivo*. Thus, as in other pathogens, iron might constitute a key environmental signal for controlling *Campylobacter* pathogenesis.

In addition, this work demonstrated the requirement of iron acquisition in the colonization process and/or survival of *C. jejuni in vivo*. The ferric-enterobactin receptor, not previously annotated in the genome sequence, was identified as being the CfrA protein. Finally, chick colonization assays with strains mutated in genes encoding components of ferric-siderophore transport systems show the importance of enterobactin in gut colonization.

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studies of the free ligands and their $Mg^{2+}$, $Ca^{2+}$, $Cu^{2+}$, and $Fe^{3+}$ chelates. Inorg Chem **42:**5412-21.

**Table 1**: Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| DH5α | *endA1 hsdR17* ($r_k^-m_k^-$) *supE44 thi-1 recA1 gyrA relA1 Δ(lacZYAargF U169deoR [f80dlacΔ(lacZ0 M15]* | Invitrogen |
| ***C. jejuni*** | | |
| AS144 | *C. jejuni* NCTC 11168 | NCTC |
| AS 230 | AS144Δ*fur* | This study |
| AS 269 | AS144Δ*cfrA* | This study |
| AS 265 | AS144Δ*ceuE* | This study |
| AS 211 | AS144ΔCj0178 | This study |
| **Plasmids** | | |
| pUC19 | Cloning and suicide vector, $Amp^r$ | Biolabs |
| pRY111 | $cm^r$ resistance gene | (58) |
| pAS 226 | pUC19 carrying *fur* | This study |
| pAS 227 | pUC19 carrying Δ*fur* | This study |
| pAS 229 | pUC19 carrying Δ*fur::cm*$^r$ | This study |
| pAS 266 | pUC19 carrying *cfrA* | This study |
| pAS 267 | pUC19 carrying Δ*cfrA* | This study |
| pAS 268 | pUC19 carrying Δ*cfrA::cm*$^r$ | This study |
| pAS 261 | pUC19 carrying *ceuE* | This study |
| pAS 262 | pUC19 carrying Δ*ceuE* | This study |
| pAS 263 | pUC19 carrying Δ*ceuE::cm*$^r$ | This study |
| pAS 207 | pUC19 carrying Cj0178 | This study |
| pAS 208 | pUC19 carrying ΔCj0178 | This study |
| pAS 209 | pUC19 carrying ΔCj0178::*cm*$^r$ | This study |

[a]$Cm^r$, chloramphenicol resistance gene;
$Amp^r$, ampicillin resistant

**Table 2**: Primers used in this study

| Primer | DNA sequence (5'-3') |
|---|---|
| fur-01 | GGAAGATCTGGCTTAAGGGTATTATCAATG |
| fur-02 | GGAAGATCTCATATCAACATAACGTTTACG |
| fur-03 | CGCGGATCCAAGTAAAACATCATATTCCAC |
| fur-04 | CGCGGATCCGGTGTTTGTGGTGATTGTAAT |
| cfrA-01 | ATGCAGATCTGCTTCTGGCTTTACCCAAG |
| cfrA-02 | ATGCAGATCTTCCCAGCCATTTGTAAAGC |
| cfrA-03 | ATGCGGATCCTGCCTTTGTAGGACTTTGAGC |
| cfrA-04 | ATGCGGATCCAACAATGTTTCGCCAAGAGC |
| ceuE-01 | ATGCGAATTCGATACATTAAGCGGGGGACA |
| ceuE-02 | ATGCGCATGCTGTGCTTAGCGTAGGTTTTGG |
| ceuE-03 | ATGCGGATCCTTTTTAGCAGGAACGCCAAC |
| ceuE-04 | ATGCGGATCCGCAACAAAGAACGTGCTCAA |
| Cj0178-01 | ATGCGAATTCTTTGTAACTCCTGGGGCAAC |
| Cj0178-02 | ATGCGAATTCTTGCACCTCTGCTCTCATTG |
| Cj0178-03 | ATGCAGATCTTTTTTCTACCGGCAAGTTCG |
| Cj0178-04 | ATGCAGATCTTTTCAGCTGCCATACACGAG |
| exbB1-F | AATGCAGAAGCAAATGCAAC |
| exbD1-R | GCCATTCTCTTTTAATTTTTGCAT |
| exbD1-F | AATGGCTCACAAAGAAGAGGAG |
| tonB1-R | ATTTCTTTAGGCGGTTTTGG |
| exbB2-F | GGCATTTATAGCTTTTTGGTGTG |
| exbD2-R | AAATTTTCATGTTCTTTGGCTTT |
| exbD2-F | CATTATGCTTGTTTTGCTTGC |
| tonB2-R | TCCATTATAGTGTGGAAAATTTAAAGA |
| exbB3-F | TGGCTTTCTTTATATTTTATTTTAGCA |
| exbD3-R | TTCTTAAGACTGAAATAACATCGTCA |
| exbD3-F | TGCTTGTATTGCTTGCGATT |
| Cj0111-R | TTTTTATTTTGCGGAGGATAAGT |
| fldA-R | CATTGTCAAGAGCAAGTCCAA |
| Cj1383c-F | CCGCAAATTTATCGCTATTT |
| Cj1383c-R | AATTTGATTTGATATTTTATACGGAAC |
| Cj1384c-F | GGTGCAGATGAAATCACTCCT |
| Cj1658-F | ATGCAGATCTGCTCAGCTTTTGCTAGGGTAGA |
| p19-R | ATCAACATGGCGTCCAAAAC |

**Table 3**: Number of genes up-regulated (columns labeled "+") or down-regulated columns labeled "-") at different time points following the addition of ferrous sulfate to a mid-log phase iron-limited *C. jejuni* culture. Genes are grouped by functional categories according to the Sanger Center annotation.

| | Number of genes | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 min | | 3 min | | 5 min | | 7 min | | 9 min | | 15 min | | Mid-log | |
| Functional category | + | - | + | - | + | - | + | - | + | - | + | - | + | - |
| Small molecule degradation | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 3 | 0 | 3 | 1 | 0 |
| Energy metabolism | 0 | 0 | 6 | 2 | 14 | 9 | 14 | 17 | 18 | 22 | 11 | 17 | 23 | 2 |
| General intermediary metabolism | 0 | 0 | 0 | 0 | 0 | 4 | 0 | 3 | 1 | 4 | 0 | 5 | 1 | 1 |
| Amino acid biosynthesis | 1 | 0 | 2 | 1 | 3 | 3 | 1 | 3 | 4 | 5 | 10 | 5 | 2 | 5 |
| Polyamine synthesis | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Purines, pyrimidines, nucleosides and nucleotides | 0 | 0 | 1 | 1 | 2 | 1 | 4 | 2 | 5 | 2 | 7 | 2 | 3 | 0 |
| Biosynthesis of cofactors, prosthetic groups and carriers | 0 | 0 | 2 | 1 | 3 | 4 | 2 | 6 | 2 | 9 | 2 | 9 | 1 | 8 |
| Fatty acid biosynthesis | 0 | 0 | 3 | 1 | 5 | 2 | 5 | 3 | 5 | 3 | 5 | 0 | 1 | 0 |
| Broad regulatory functions | 0 | 1 | 0 | 1 | 0 | 2 | 1 | 2 | 1 | 3 | 0 | 3 | 0 | 1 |
| Signal transduction | 0 | 0 | 2 | 2 | 2 | 6 | 2 | 7 | 4 | 5 | 1 | 5 | 0 | 2 |
| Ribosomal protein synthesis and modification | 0 | 0 | 9 | 0 | 15 | 0 | 31 | 0 | 39 | 0 | 43 | 1 | 1 | 8 |
| Ribosome maturation and modification | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 2 | 0 | 2 | 0 | 0 | 1 |
| Aminoacyl tRNA synthetases and their modification | 0 | 0 | 1 | 1 | 1 | 2 | 3 | 2 | 4 | 3 | 3 | 1 | 1 | 1 |
| Nucleoproteins | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| DNA replication, restriction/modification, repair | 0 | 0 | 0 | 0 | 2 | 1 | 3 | 2 | 8 | 3 | 5 | 1 | 2 | 2 |
| Protein translation and modification | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 1 | 7 | 1 | 6 | 1 | 0 | 0 |
| RNA synthesis, RNA modification and DNA transcription | 0 | 0 | 1 | 0 | 2 | 1 | 4 | 1 | 4 | 1 | 5 | 1 | 1 | 1 |
| Phospholipids | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Degradation of macromolecules | 0 | 0 | 0 | 0 | 2 | 0 | 3 | 0 | 2 | 0 | 1 | 2 | 1 | 2 |
| Membranes, lipoproteins and porins | 0 | 0 | 1 | 0 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 0 | 2 |
| Surface polysaccharides, lipopolysaccharides and antigens | 0 | 0 | 0 | 1 | 2 | 1 | 6 | 1 | 8 | 3 | 3 | 1 | 4 | 0 |
| Surface structures | 0 | 0 | 2 | 6 | 2 | 9 | 2 | 12 | 2 | 12 | 1 | 7 | 5 | 0 |
| Murein sacculus and peptidoglycan | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 1 | 2 | 1 | 3 | 1 | 1 | 1 |
| Miscellaneous periplasmic proteins | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 1 | 0 | 2 | 1 | 2 |
| Amino acids and amines transport/binding proteins | 0 | 0 | 0 | 0 | 3 | 1 | 3 | 0 | 6 | 5 | 2 | 5 | 4 | 0 |
| Cations transport/binding proteins | 0 | 1 | 1 | 5 | 2 | 10 | 2 | 14 | 1 | 13 | 1 | 11 | 1 | 14 |
| Carbohydrates and organic acids transport/binding proteins | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 3 | 1 | 4 | 1 | 5 | 0 | 0 |
| Anions transport/binding proteins | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 2 | 0 | 2 | 0 | 2 | 0 | 0 |
| Other transport/binding proteins | 0 | 0 | 4 | 5 | 7 | 10 | 6 | 11 | 6 | 14 | 4 | 12 | 6 | 13 |
| Chaperones, chaperonines, heat shock | 0 | 0 | 0 | 1 | 3 | 3 | 3 | 1 | 3 | 1 | 2 | 2 | 0 | 2 |
| Cell division | 0 | 0 | 1 | 0 | 2 | 0 | 1 | 0 | 2 | 0 | 1 | 0 | 0 | 0 |
| Chemotaxis and mobility | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 | 1 | 1 | 2 | 0 | 0 | 0 |
| Protein and peptide secretion | 1 | 0 | 3 | 0 | 5 | 0 | 6 | 0 | 8 | 0 | 5 | 0 | 1 | 1 |
| Detoxification | 0 | 0 | 1 | 2 | 1 | 2 | 1 | 2 | 1 | 3 | 1 | 2 | 1 | 4 |
| Pathogenicity | 0 | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 | 0 |
| IS, plasmid related function | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Drug/analogue sensitivity and antibiotic resistance | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 3 | 1 | 3 | 0 | 2 | 1 |
| Conserved hypothetical proteins | 0 | 0 | 1 | 2 | 4 | 2 | 4 | 3 | 4 | 2 | 1 | 0 | 1 | 2 |
| Unknown | 0 | 0 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Miscellaneous | 1 | 1 | 6 | 5 | 9 | 9 | 10 | 9 | 12 | 13 | 7 | 11 | 8 | 6 |
| Hypothetical unknown proteins | 4 | 2 | 27 | 23 | 40 | 49 | 58 | 55 | 84 | 65 | 61 | 49 | 33 | 20 |
| **Total** | 8 | 5 | 79 | 62 | 139 | 139 | 186 | 167 | 254 | 208 | 202 | 168 | 106 | 102 |

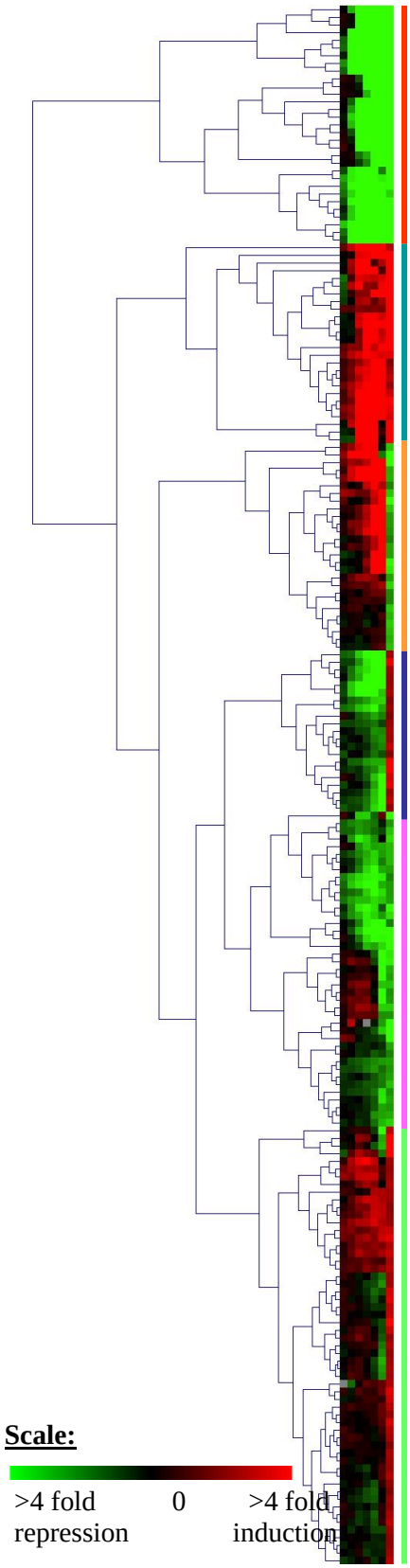
**Table 4:** Some of the genes from Cluster A and E that show substantial reduction in expression after the addition of ferrous sulfate.

| Protein ID | Proposed functions |
|---|---|
| **Involved in iron transport and storage** | |
| ChuABCD | Putative hemin uptake system |
| CfrA | Putative ferric-siderophore receptor protein |
| Cj1661-1663 and Cj0173c-0175c | Putative ABC transporter systems |
| TonB3, ExbB2, ExbD2, TonB1, ExbB1, and ExbD1 | Members of the three putative TonB-ExbB-ExbD energy transducing complexes |
| p19 and Cj1658 | Iron-regulated proteins with identity to components of iron transporters |
| Cj0177 | Putative lipoprotein sharing homology with a protein involved in iron acquisition |
| CeuC | Putative enterobactin uptake permease |
| Cft | Ferritin |
| Cj0203, Cj0891c, Cj1163c, and Cj0339 | Putative transmembrane tranporter proteins |
| **Involved with oxidative stress responses** | |
| PerR | Peroxide stress regulator |
| KatA | Catalase |
| AhpC | Alkyl hydroperoxide reductase |
| Tpx | Thiol peroxidase |
| SodB | Iron-containing superoxide dismutase |
| **Iron not required as co-factor, physiologically important under iron-limited conditions?** | |
| Cj0414 | Putative oxidoreductase |
| Cj0723c | Zinc metalloprotease, involved in macromolecule degradation |
| PanC, PanB, BioC, MoaE, ThiC, NadE, TrxA, and TrxB | Co-factor biosynthesis |
| FldA | Flavodoxin |
| Cj0559 | Oxidoreductase |
| Cj1287c | Malate oxidoreductase |

**Table 5:** Some of the genes from Clusters B and F that are substantially upregulated in response to iron addition.

| Protein ID | Proposed functions related to energy metabolism |
|---|---|
| SdhABC | Succinate dehydrogenase complex |
| NapABGH | Periplasmic nitrate reductase |
| Cj1357c, Cj1358c | Putative periplasmic cytochrome c's |
| NrdB | Ribonucleoside-diphosphate reductase |
| OorABCD | 2-oxoglutarate:acceptor oxidoreductase |
| HydC | Ni/Fe-hydrogenase B-type cytochrome subunit |
| Cj0074c | Putative iron-sulfur protein |
| Cj0358 | Putative cytochrome C551 peroxidase |
| Cj0012c | Non-haem iron protein |

**Figure 1:** Hierarchical cluster analysis of genes found to be significantly up- or down-regulated at mid-log phase. Going from left to right, the columns represent the transcriptome change at 1 min, 3 min, 5 min, 7 min, 9 min, and 15 min after the addition of ferrous sulfate, and at mid-log phase. The intensity of the color is proportional to the fold change as represented by the scale at the bottom. Detail gene names are shown for each cluster.




**Cluster A**: *chuB*, *chuC*, *chuD*, Cj1383c, Cj1384c, *exbB2*, *katA*, *chuA*, *tonB3*, Cj1661, Cj1662, Cj1663, Cj0175c, *ahpC*, Cj1658, Cj1613c, *exbB1*, *aroC*, *exbD1*, *cfrA*, p19, Cj0240c, *perR*, Cj0818, Cj0203, Cj0343c, Cj1377c, *exbD2*, Cj0177, Cj1386, *trxB*

**Cluster B**: Cj0119, Cj1357c, *fdxA*, Cj0948c, Cj0012c, Cj1356c, Cj1358c, Cj0011c, Cj1582c, *napA*, *napG*, *napB*, *napH*, Cj1475c, Cj0633, Cj1501, Cj1341c, Cj1436c, *pfs*, *nrdB*, Cj1080c, Cj1474c, *plsX*, *sdhA*, *sdhB*, *sdhC*

**Cluster C:** Cj0722c,Cj0946, Cj1170c, Cj1500, *rplB*, Cj1089c, Cj1710c, *ksgA*, Cj0017c, *nusG*, *rplT*, *rpsN*, *ffh*, *rpmI*, *rpsF*, Cj0018c, *ssb*, *htpG*, *rpsC*, Cj0906c, *ilvE*, *ceuE*, *dapD*, *mrp*, *rpmA*, *rplU*, *thrS*

**Cluster D:** Cj0021c, Cj0909, Cj0484, Cj1242, Cj0977, *flgH*, Cj0919c, Cj1541, *flgE2*, Cj1585c, Cj1656c, *cynT*, *frdB*, *hydA*, Cj0735, Cj1482c, Cj0910, Cj0075c, *sdaA*, Cj0920c, *sdaC*

**Cluster E**: Cj1189c, *cbpA*, Cj0962, Cj1190c, Cj0173c, Cj0555, Cj1375, Cj1725, Cj0339, Cj0414, Cj0981c, Cj1163c, Cj0239c, *metY*, *cft*, Cj0174c, *murE*, *tpx*, Cj0420, *panB*, Cj0998c, *htrA*, Cj0723c, *moaE*, *panC*, Cj0236c, *peb2*, *ceuC*, *cysM*, *tonB1*, Cj0983, *fldA*, *bioC*, *nadE*, Cj0999c, *thiC*, Cj0559, Cj0982c, Cj1287c, *sodB*, *trxA*

**Cluster F**: Cj0256, Cj0073c, Cj0074c, Cj0358, Cj0362, Cj0892c, *era*, *pyrE*, *flhB*, Cj0949c, Cj1425c, Cj1430c, *tkt*, Cj1477c, Cj1484c, Cj1389, *rnhB*, *proC*, *trmA*, Cj0428, Cj0488, Cj0833c, Cj0834c, Cj0832c, Cj1295, Cj1583c, *oorB*, *oorC* , Cj1069, *nuoN*, *birA*, *oorA*, *oorD*, Cj0784, *hydD*, *queA*, Cj1032, Cj0719c, Cj1423c, Cj1581c, Cj1297, *fliG*, Cj1321, *ptmA*, Cj1086c, *pyrB*, Cj0903c, *ccoQ*, Cj1165c, Cj1485c, Cj0911, *rpsU*, *hydC*, Cj0412, Cj1468, *murB*, *tyrA*

**Figure 2:**

Operon mapping by RT-PCR analysis of iron- and Fur- regulated genes. The template RNA was purified from mid-log phase bacteria grown in iron-rich or iron-limited medium MEMα for the iron-induced (*exbB3, exbD3*Cj0111) and iron -repressed genes, respectively. Predicted RT-PCR fragments with gene names are shown at the bottom. The gel lanes match the RT-PCR fragment labels. Lanes M1 and M2 correspond to the 1 kb and 100 bp DNA ladders, respectively.

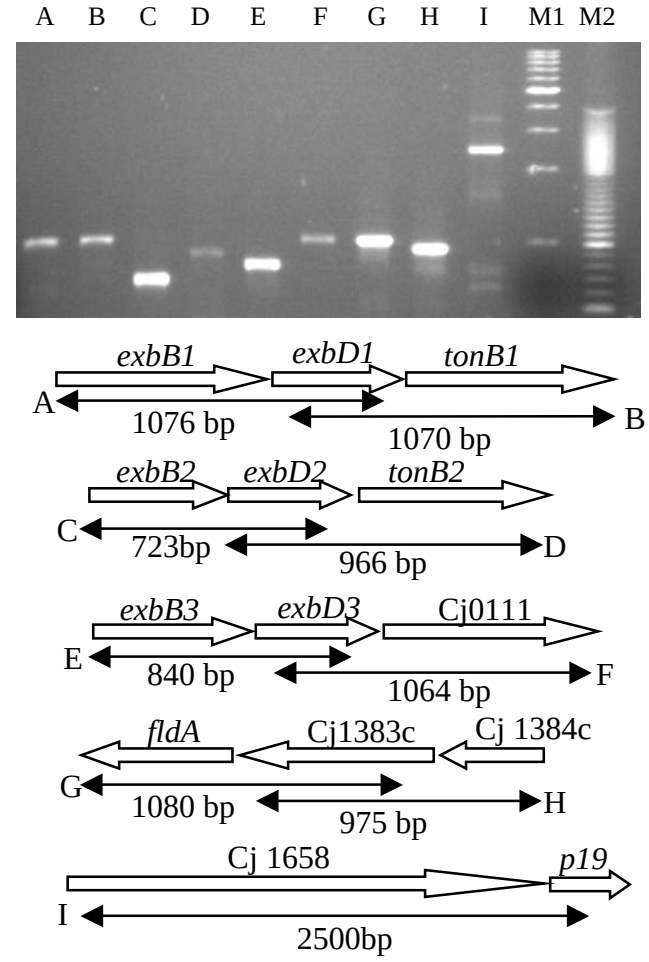

A B C D E F G H I M1 M2
exbB1
exbD1
tonB1
A
1076 bp
1070 bp
B
exbB2
exbD2
tonB2
C
723bp
966 bp
D
exbB3
exbD3
Cj0111
E
840 bp
1064 bp
F
fldA
Cj1383c
Cj 1384c
G
1080 bp
975 bp
H
Cj 1658
p19
I
2500bp

**Figure 3:**

Whole-cell lysates of *C. jejuni* proteins analyzed on 12.5% SDS-PAGE. Panel A corresponds to silver staining, and panel B corresponds to lectin blotting. Total proteins were prepared from *C. jejuni* grown to mid-log phase in iron-limited medium (MEMα; lanes labeled 0 min and mid-log) or iron-rich medium (MEMα + 40 μM $FeSO_4$). Lanes labeled 5 min, 9 min, and 15 min correspond to the protein profiles of *C. jejuni* grown in iron limited medium at 5, 9, and 15 min, after the addition of ferrous sulfate. The lane labeled mid-log (+Fe) corresponds to the protein profile of *C. jejuni* grown to mid log phase in the iron rich medium.

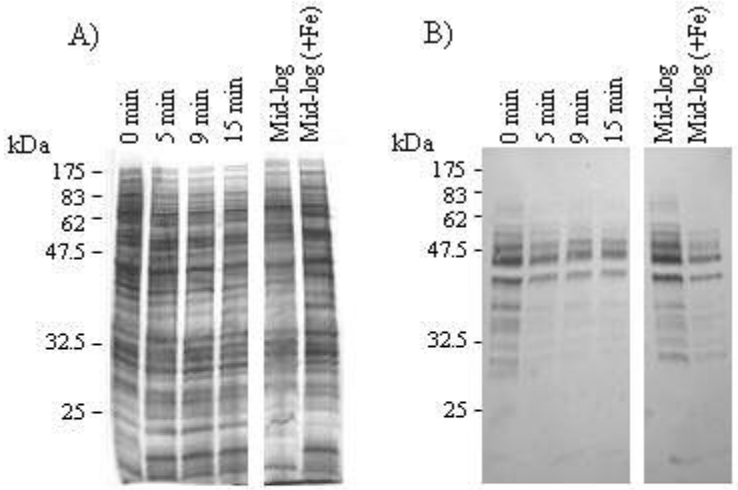

A)
0 min
5 min
9 min
15 min
Mid-log
Mid-log (+Fe)
kDa
175
83
62
47.5
32.5
25
B)
0 min
5 min
9 min
15 min
Mid-log
Mid-log (+Fe)
kDa
175
83
62
47.5
32.5
25

**Figure 4:**

Hierarchical cluster analysis of Fur regulated genes. Columns 1 to 7 correspond to *C. jejuni* gene expression changes in response to the addition of iron to an iron-limited medium at the time points 1, 3, 5, 7, 9, and 15 min and at the mid-log phase, respectively. Columns 8 and 9 represent the change in transcript level of the wild-type *C. jejuni* strain as compared to the *fur* mutant grown to mid-log phase in iron-limited medium and 15 min after the addition of $FeSO_4$ respectively. The shade of red and green indicates the level of fold change. Genes are sub-grouped into 4 clusters, named A, B, C and D.

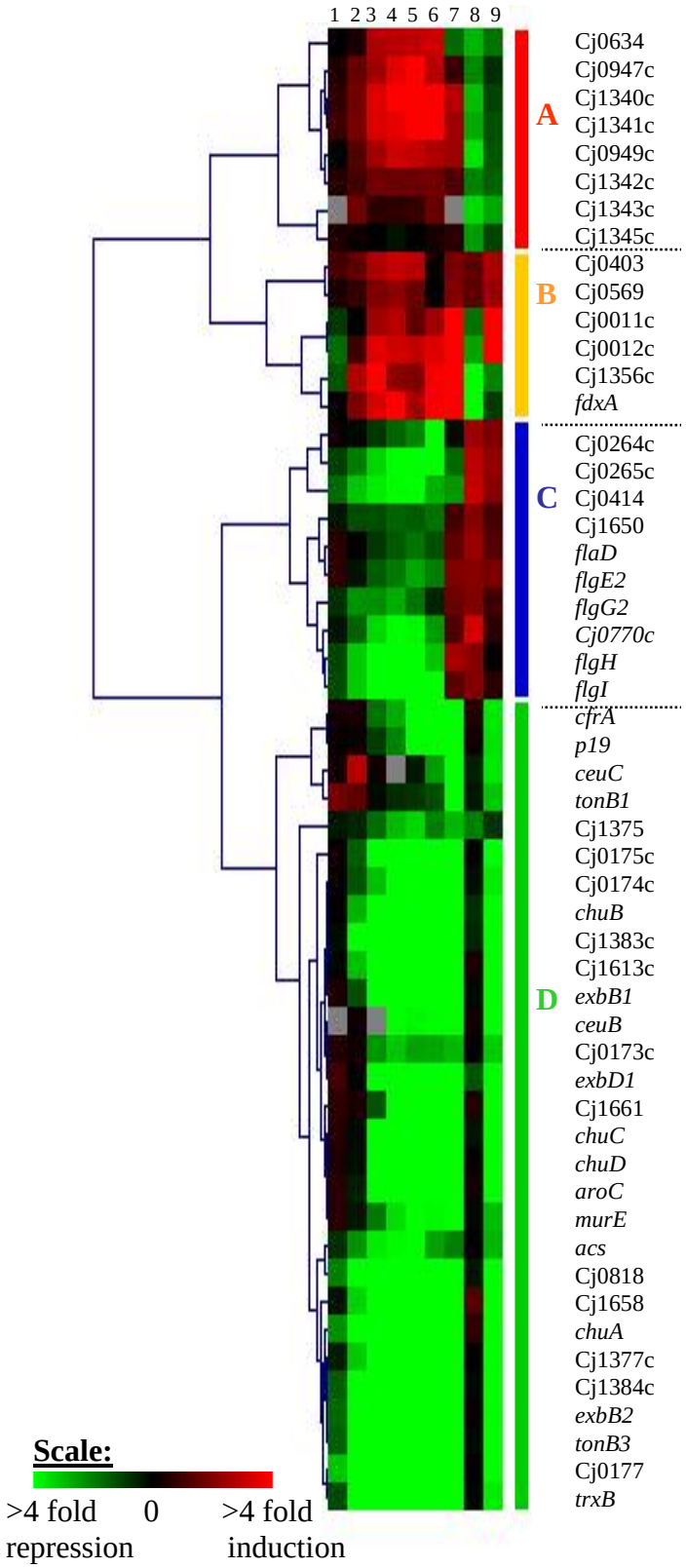

1 2 3 4 5 6 7 8 9
A
B
C
D
Cj0634
Cj0947c
Cj1340c
Cj1341c
Cj0949c
Cj1342c
Cj1343c
Cj1345c
Cj0403
Cj0569
Cj0011c
Cj0012c
Cj1356c
fdxA
Cj0264c
Cj0265c
Cj0414
Cj1650
flaD
flgE2
flgG2
Cj0770c
flgH
flgI
cfrA
p19
ceuC
tonB1
Cj1375
Cj0175c
Cj0174c
chuB
Cj1383c
Cj1613c
exbB1
ceuB
Cj0173c
exbD1
Cj1661
chuC
chuD
aroC
murE
acs
Cj0818
Cj1658
chuA
Cj1377c
Cj1384c
exbB2
tonB3
Cj0177
trxB
Scale:
>4 fold repression
0
>4 fold induction

**Figure 5:**

Colonization properties of the *C. jejuni* mutant strains in the chick model. Groups of 4 chicks were inoculated with the *C. jejuni* wild-type (wt) strain NCTC 11168, or with the *fur*, *cfrA*, *ceuE*, and Cj0178 mutants (as indicated) at a dose of $10^5$ to $3.10^5$ cfu. The columns represent the means and the error bars indicate the standard deviations.

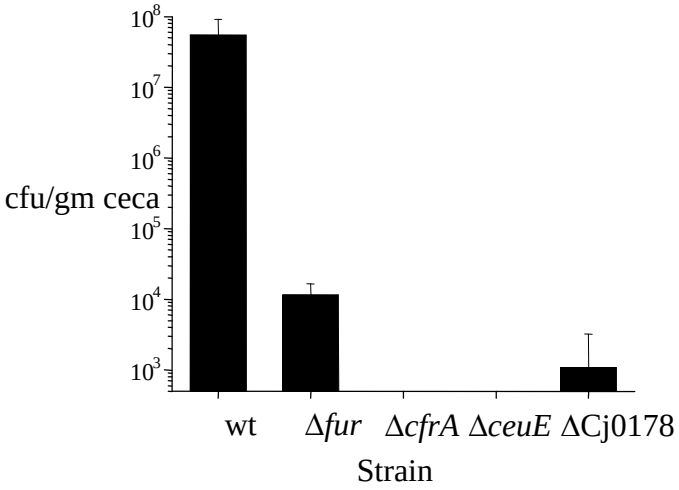

$10^8$
$10^7$
$10^6$
cfu/gm ceca
$10^5$
$10^4$
$10^3$
wt
Δ*fur*
Δ*cfrA*
Δ*ceuE*
ΔCj0178
Strain

**Figure 6:**

Sequence logo of the potential Fur binding site. The height of each letter indicates the relative frequency of that base at that position. The height of each stack of letters corresponds to the sequence conservation at that position.

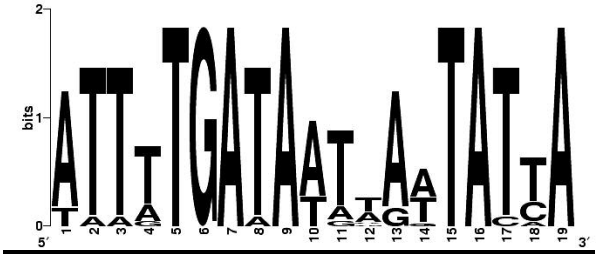
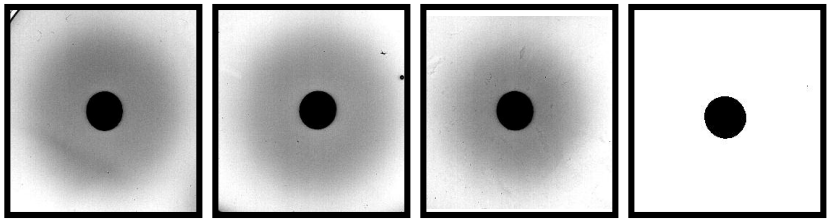

bits
2
1
0
5′
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
3′

**Figure 7:**

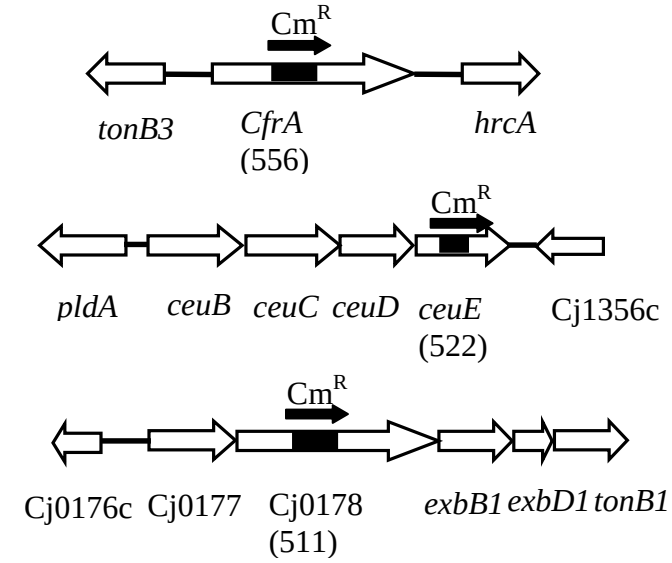
Enterobactin growth promotion tests of the wild type strain *C. jejuni* NCTC 11168, and the *C. jejuni* mutants ΔCj0178, Δ*ceuE*, and Δ*cfrA*. A halo of growth around the filter paper disk containing 10 µl of enterobactin (10 mM) indicates the utilization of the siderophore by the tested strain. The diameters±SD of growth promotion zones are shown in parenthesis (in cm).



| NCTC | ΔCj0178 | Δ*ceuE* | Δ*cfrA* |
|---|---|---|---|
| (3.5±1.4 cm) | (3.5±1.3 cm) | (2.8±0.9 cm) | (no growth) |

**Figure 8:**

Diagram of the genetic organization of the mutants *cfrA*, *ceuE* and Cj0178 described in this study. Each mutant was constructed by site directed deletion and insertional mutagenesis with the chloramphenicol resistant marker ($Cm^R$). The length of each deletion is shown in parenthesis (in base pairs). The solid black arrow represents the position and orientation of the inserted chloramphenicol antibiotic resistance cassette in each gene. Given the absence of a transcriptional terminator downstream of the $Cm^R$ gene and the orientation of this gene with respect to the mutated gene, the constructed mutations are likely non-polar. All genes are drawn approximately to scale.


Cm^R
tonB3
CfrA
(556)
hrcA
Cm^R
pldA
ceuB
ceuC
ceuD
ceuE
(522)
Cj1356c
Cm^R
Cj0176c
Cj0177
Cj0178
(511)
exbB1
exbD1
tonB1

# CHAPTER IV

# RESPONSE OF *CAMPYLOBACTER JEJUNI* TO HYDROGEN PEROXIDE

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## Abstract

*Campylobacter jejuni* is exposed to various harsh environments during the course of its infection cycle. Being microaerophilic, oxidative stress is one of the important stresses that *C. jejuni* encounter. Oxidative stress is imposed by highly reactive oxygen species (ROS) like hydrogen peroxide, hydroxyl radical, superoxide anion and singlet oxygen among others. The ROS cause damage to biomolecules like DNA, proteins and lipids resulting in cell death. Most microorganisms including *campylobacter* mount a response to the ROS by differentially expressing genes to counter the oxidative stress. In this study we have used DNA microarrays to explore the reponse of *C. jejuni* to oxidative stress. Transciptome profile of *C. jejuni* to oxidative stress inducers hydrogen peroxide (HP), cumene hydroperoxide (CHP) and menadione was measured. In contrast to the HP and CHP, menadione induced a high number of genes in the DNA replication category suggesting that it is more damaging to the bacterial cell than the two other oxidants at the 1 mM concentration used. We also measured *C. jejuni* response to HP in presence and absence of iron. Both catalase (*katA*) and alkyl hydroxyperoxidase (*ahpC*) were induced in cells in presence of iron while only the catalase was induced in cells in the absence of iron suggesting an additive effect of iron over oxidative stress. Response to oxidative stress is regulated by the PerR regulator in *C. jejuni*. Therefore, we compared the transcriptome profile of *C. jejuni* wld type strain with the *perR* mutant. As expected the *katA* was found to be regulated by PerR However, the *ahpC* gene, previously shown to be PerR regulated was not found to be under control of PerR. Genes involved in flagellar biogenesis and motility were found to be PerR regulated. The *perR* mutant was unable to

colonize chicken ceca indicating an important role for this transcriptional regulator in in vivo growth and survival.

## Introduction

*Campylobacter jejuni* is a Gram-negative, microaerophilic, facultative intracellular bacterium and is one of the most common causes of bacterial gastroenteritis worldwide both in developing and developed countries. According to the 2003 report of the Food-borne Diseases Active Surveillance Network (FoodNet), which is the principal food-borne disease component of the Centers for Disease Control and Prevention (CDC's), 33.4% of the laboratory diagnosed cases of infection were due to *Campylobacter* (1). This was second only to *Salmonella* infections that were reported at 38.5%. *Campylobacter*iosis is an acute illness resulting in gastrointestinal disturbances (33). The symptoms vary depending on socioeconomic conditions. In the developing countries *Campylobacter* mainly affects children under two years resulting in watery diarrhea, vomiting and dehydration (12). However, in developed countries, *campylobacter*iosis is characterized by bloody stools, acute abdominal pain that is often more severe than that observed for *Salmonella* and *Shigella* infections (12).

*Campylobacter jejuni* cells are exposed to various harsh environments during the course of the infection cycle. Oxidative stress is imposed by reactive oxygen species (ROS) that have an unpaired electron in their outer orbit that makes them more reactive towards organic molecules. These ROS include hydrogen peroxide, hydroxyl radical, superoxide anion, singlet oxygen among others. The microaerophilic *Campylobacter* is exposed to atmospheric oxygen during its transmission from host to host in either food or water. This exposure could potentially result in the production of reactive oxygen species. In addition, *C. jejuni* has been shown to reside within intestinal epithelial cells as well as in granulocytes bound within an endosome (4, 7, 27). During phagocytosis *Campylobacter*

is exposed to oxygen radicals generated by the respiratory burst. Incompletely reduced oxygen molecules during the aerobic metabolism also result in the generation of these reactive species. Overall, these ROS attack DNA resulting in strand breaks, cause lipid peroxidation and damage other biomolecules resulting in the death of cell. In order to survive this oxidative insult, bacteria respond by production of proteins to counteract, sustain and repair the damage caused by these ROS. Relatively few proteins have so far been identified to be involved in oxidative stress defense in *C. jejuni*. They include superoxide dismutase (SodB) (38, 42), catalase (KatA) (14), alkyl hydroperoxide reductase (ahpC) (5), ferredoxin (FdxA) (59) and the protein encoded by the gene Cj0012c (64).

Iron containing SodB was characterized in both *C. jejuni* and *C. coli* (38, 41). The *C. coli sodB* mutant was affected in its ability to colonize chicken ceca (41). A *SodB* mutant of *C. jejuni* 81176 was significantly affected in its intracellular survival in INT-407 epithelial cells (38). Both KatA and AhpC were shown to be peroxide stress defense proteins repressed by iron through the peroxide stress regulator PerR in *C. jejuni* (62, 63). It has been shown that *C. jejuni* catalase provides resistance to the hydrogen peroxide (HP) *in vitro* but does not play a role in intraepithelial cell survival *in vivo* (14). However, catalase is essential for the *C. jejuni* intra-macrophage persistence (14). FdxA was the first iron induced protein of *C. jejuni* which encodes ferredoxin that confers aerotolerance for *C. jejuni* when exposed to atmospheric oxygen. (59). In *C. jejuni* the gene Cj0012c encodes an oxidative stress sensitive protein of 27KDa whose levels were decreased after 15 minutes of exposure to HP (64). This was similar to rubredoxin

oxidoreductase (Rbo) and rubrerythrin (Rbr) found in other bacteria to be involved in oxidative stress protection in anaerobic microorganisms (31).

OxyR regulates oxidative stress genes including catalase and alkyl hydroxyperoxide reductase in *Salmonella* (11) and other Gram-negative bacteria (52). In *C. jejuni* PerR is the functional analog of the OxyR and has been shown to repress *katA* and *ahpC* in response to iron (60). A *C. jejuni perR* mutant is hyper resistant to effects of HP due to high amounts of constitutively expressed KatA and AhpC (60). The bacterial response to oxidative stress at the whole genome level has been studied in *E. coli* (2), *Bacillus subtilis* (19, 32), *Pseudomonas aeruginosa* (35) and *Synechocystis* species (29) using DNA microarray technology. Microarrays have also been used to study the OxyR regulon in *E. coli* (2) and PerR regulon in *Synechocystis* species (29). In *Bacillus subtilis*, it was shown that the peroxide stress response was regulated by a repressor that senses both metal ion levels and HP (10). It was later confirmed that PerR is the metal-binding repressor protein and its sensitivity to HP can be altered by growth conditions, especially the metal ion composition of the medium (8, 20).

When compared to other bacteria, relatively few genes or proteins are known in *C. jejuni* that respond to oxidative stress. In order to gain a full understanding of the *C. jejuni* response to oxidative stress, DNA microarray was used to monitor the transcriptome changes for HP, CHP and menadione. In addition, an isogenic *PerR* mutant was used to identify the PerR regulon in *C. jejuni* by comparing its transcriptome profile with the wild-type strain. The response of *C. jejuni* and *PerR* mutant to HP was also studied when the cells were grown in an iron-replete condition. This is the first report of *C. jejuni*

response to oxidative stress at whole genome level. We also report for the first time the PerR regulon of *C. jejuni* at whole genome level.

## Materials and methods

*Bacterial strains, plasmids and growth conditions*

The bacterial strains used in this study are listed in Table 1. *E. coli* DH5α strain was routinely cultured aerobically at 37°C in Luria-Bertani (LB) broth or on LB agar plates. Plasmid-containing strains were grown in medium supplemented with chloramphenicol 20 μg/ml or kanamycin 30 μg/ml. *Campylobacter jejuni* NCTC 11168 was obtained from the National Collection of Type Culture (NCTC, England) and routinely maintained at 37°C in a microaerophilic chamber (Don Whitley, West Yorkshire, England) containing 83% $N_2$, 4% $H_2$, 8% $O_2$ and 5% $CO_2$ on Mueller-Hinton (MH) agar plates, MH broth medium or MEM α medium (Invitrogen) with chloramphenicol and kanamycin added as required at a concentration of 20 μg/ml and 30 μg/ml, respectively.

*Microarray construction*

The *C. jejuni* NCTC 11168 microarray was constructed as previously described (36, 48, 49). Briefly, an internal fragment was amplified from each of the 1654 open reading frames (ORFs) present in the *C. jejuni* genome sequence. Approximately 98% of the ORFs could be successfully amplified. These PCR products were diluted in a 50% DMSO solution and were printed on aminosilane-coated glass microscope slides. The quality of the microarray printing was analyzed using direct labeling of the spotted DNA with a fluorescent nucleic acid stain POPO-3 iodide (Molecular Probes).

*Sampling and isolation of total RNA*

To study the effect of different oxidative stress inducing agents, the experimental design is given in Figure 1. *C. jejuni* NCTC 11168 was grown in 250 ml of MEM alpha medium

(in 500 ml flask). At mid-log phase the culture was divided into 50 ml each into four 100ml flasks as shown in Figure 1. Ferrous sulphate was added to the two flasks in the group 1 at 40 µM concentration. Fifteen minutes after the addition of iron, one of the flasks in each group was treated with hydrogen peroxide (HP) at 1mM. Then 25 ml samples were taken from each flask and processed for RNA isolation.

To identify the PerR regulon, the wild type strain of *C. jejuni* and the *PerR* mutant were grown in 500 ml flasks in 250 ml MEM alpha medium. At mid log phase, 50 ml cultures were transferred into 100 ml flasks as shown in Figure 2. One set of the wild type and PerR mutant (group 3) was treated with 40 µM ferrous sulphate. While another set (group 4) was not treated. Fifteen minutes after the addition of iron, all the four flasks in groups 3 and 4 were treated with 1mM HP. Twenty five ml samples were collected from all four flasks before and 10 minutes after the addition of HP and were processed to harvest RNA.

The response of *C. jejuni* to three different oxidative stress inducers was investigated as shown in Figure 3. *C. jejuni* was grown microaerobically in 250 ml of MEM α medium (Invitrogen) in 500 ml flask at 37°C using 500 ml flask. At mid-log phase, 50 ml of the culture from the 500 ml flask was transferred into four 100 ml flasks. One of the flasks was kept as control without addition of any oxidative stress inducing agents. One mM hydrogen peroxide (HP), 1mM cumene hydroperoxide (CHP) and 1mM menadione were added to the remaining three flasks. Ten minutes after the addition of the addition of these different agents, 25 ml samples were collected from each flask and processed for RNA isolation.

The culture samples collected in the above experiments were immediately mixed with 0.1 volume of a cold RNA degradation stop solution (10% buffer-saturated phenol in ethanol) and centrifuged at 4°C (10 min, 8000 x g) to collect the bacterial cells. Hot phenol-chloroform protocol was used to harvest the total RNA (54). The traces of remaining genomic DNA were removed by DNase I treatment and the absence of genomic was confirmed by PCR. Quantitation of the RNA was done using Ribogreen RNA quantitation reagent (Molecular Probes).

*Probe labeling and slide hybridization*

The reverse transcription procedures for RNA and the labeling protocols for the cDNA were performed according to the published protocols as previously described (36, 48, 49).

*Data collection and analysis*

The microarray image acquisition and analysis of the data was done as previously described in our earlier study (36). Briefly, microarrays were scanned with a ScanArray 5000 scanner (Perkin Elmer) and images were analyzed with GenePix Pro 4 software (Axon Instruments). Spots were removed from further analysis if they were either localized within regions of hybridization or slide abnormalities, or if the fluorescent mean intensities in both channels, 1 (Cy5) and 2 (Cy3), were below three times the standard deviation of the local background. Then, the fluorescence intensity in each wavelength was normalized by applying a locally weighted linear regression (Lowess) using the MIDAS software (46). Following normalization, the ratio of channels 2 to 1 were $\log_2$ transformed. The data given represents three data points per gene. Genes were selected as being differentially expressed if their fold change in transcript abundance was above 2.

The data presented in this study are preliminary and they need to be repeated for better statistical accuracy.

*Construction of C. jejuni mutants*

Generation of the *fur* mutant (AS 230) has been described in our previous study (36). The *perR* mutant of *C. jejuni* NCTC 11168 was also constructed using the same inactivation strategy as follows. *C. jejuni* NCTC 11168 chromosomal DNA was prepared using the Wizard Genomic DNA purification kit (Promega) and *perR* gene was PCR amplified using the primers perR -01 (5'-ATGCAGATCTGTTATGGACAAGGTGTGGCA-3') and perR -02 (5'-ATGCAGATCTCATTGGAACTATCCAAAGTTGG-3') (which both contain a BglII site). The PCR reaction was catalyzed with Pfx DNA polymerase (Invitrogen), and was hot-started to ensure high specificity of the products being synthesized. The resulting 1275 bp fragment was digested with BglII, and ligated to the *Bam*HI restricted pUC19 vector (yielding the plasmid pAS213). A deletion of 284 bp was generated within the *perR* gene by inverse PCR using the primers perR -03 (5'-ATGCGGATCCCACATAGTCTTTGCGGAGTAGC-3') and perR -04 (5'-ATGCGGATCCGATAGGCAATCTCGTCAATCA-3') which both contain a BamHI restriction site. The PCR product was cut with BamHI and ligated with BamHI restricted chloramphenicol resistance cassette ($Cm^r$) from the plasmid pRY111 (65) yielding the plasmid pAS214. At the same time, the BamHI restricted inverse PCR product was also ligated with a 1.4 Kb kanamycin resistance cassette ($Kam^r$) obtained from pILL600 (28) by BamHI restriction, yielding the plasmid pAS215. The orientation of both the $Cm^r$ and $Kam^r$ cassettes was determined by DNA sequencing. The plasmid construct, pAS214, containing the $Cm^r$ cassette in the same orientation as the gene of interest was used to

transform *C. jejuni* NCTC 11168 using a standard protocol (65) to generate the *perR* mutant (AS216) by allelic exchange. Transformants were selected on MH agar plates containing 20 μg/ml of chloramphenicol. Similarly, the plasmid construct pAS215, that contain $Kam^r$ cassette in the same direction as the gene of interest, was used to transform the *C. jejuni fur* mutant (AS230) in order to construct the Δ*perR*Δ*fur* double mutant, AS232. Transformants were selected on the MH plates containing appropriate antibiotics. Finally, the double homologous recombination events were confirmed by analyzing the $Cm^r$ and $Cm^r$ $Kan^r$ clones by PCR using the corresponding sets of gene primers.

*Disk inhibition assays.*

The sensitivity of *C. jejuni* wild-type and other mutant strains to different oxidative stress inducing agents was evaluated by disk inhibition assays. Disk inhibition assays were performed according to previously published procedures (5, 59) with some modifications. Briefly, the bacterial strains were grown to mid-log phase in MH medium. The cultures were harvested by centrifugation and resuspended in PBS to an $OD_{600}$ of 0.250. One ml of this bacterial suspension was added to 24 ml of melted MH agar, poured into Petri dishes, and allowed to solidify. Sterile filter disks containing 10 μl each of HP (3%), CHP (3%) and menadione (3M) were laid upon the surface of the agar. The zone of inhibition around the filter disks was measured (in cm) after 24 hours of incubation under microaerophilic conditions at 37°C. Comparison of mutants for each of these agents was tested using a single F-test using Analysis of Variance (ANOVA) approach. In order to find the most significant mutant, multiple comparisons were conducted by using pair wise t-tests using Bonferoni correction at a 0.05 level of significance.

*Oxidative stress survival assays*

*C. jejuni* wild-type, Δ*perR*, Δ*fur* and Δ*perR*Δ*fur* strains were grown in MH broth overnight in microaerophilic chamber at 37°C. The cultures were resuspended in PBS buffer to an $OD_{600}$ of 0.25. Two ml of the resuspended cultures were taken in plastic tubes and placed at 37°C in the microaerophilic chamber. A 50 µl sample was taken from each of the cultures for enumeration of the bacterial count before the addition (time zero) of 1mM HP. Fifty µl samples were collected at 15, 30, 45 and 60 minutes after the addition of $H_2O_2$. Collected samples were serially diluted in PBS buffer containing catalase (Sigma) at 10mg/ml, plated in duplicates on MH plates and incubated at 37°C in the microaerophilic chamber. The cells were counted after 48 hours and the percentage of survival was calculated by dividing the number of colony forming units (cfu) at each of the different time points with the cfu at time zero.

*Chick colonization assays.*

Colonization of the chick cecum was studied using one day old specific-pathogen-free broiler chicks as described in our earlier study (36). Briefly, three to four day old chicks were inoculated with 0.25 ml of a bacterial suspension containing between 1.5 to 7 $x10^3$ viable *C. jejuni*. Each strain of the *C. jejuni* was inoculated into group of five chicks. On the fourth day after infection chicks were humanely euthanized and their ceca were collected. The cecal contents were homogenized, serially diluted in PBS buffer and plated onto *Campylobacter* agar base (Oxoid CM935) containing the *Campylobacter* selective karmali supplements (Oxoid SR167E). Viable counts were expressed as logarithms of cfu per gram of ceca. The data was statistically analyzed using a non-Parametric Mann-Whitney Rank Sum test.

## Results and discussion

### Overview of the microarray design

The repertoire of genes induced by the oxidative stress would include genes that encode proteins that would prevent, counteract or repair the damage caused by the ROS which are generated during the process. In this study we investigated the response of *Campylobacter* to three main ROSs namely hydrogen peroxide, hydroperoxide and superoxide. We used HP, CHP and menadione to generate these respective ROS (Figure 3). CHP is an organic hydroperoxide that support xenobiotic oxidation mediated by heme-containing enzymes (13, 24). Menadione, a quinone compound, is a superoxide generator. Menadione is reduced to form semiquinone radicals thatusually have a high affinity for oxygen and reduce oxygen to superoxide (56). During this process, the menadione is regenerated thus allowing the redox cycle to continue resulting in the production of large amount of superoxide (56). The experimental design was as shown in the Figure 3. Mid-log grown *C. jejuni* were treated with HP, CHP or menadione at 1mM concentration for ten minutes. Sample from each of the three flasks were competitively hybridized with the sample from the control to observe the changes in the *C. jejuni* transcriptome profile in each condition. In all the experiments the 10 minute time point was chosen as we wanted to study the immediate response of *C. jejuni.* In addition, it has been shown for *P. aeruginosa* (35), *E. coli* (2) and *B.subtilis* (32) that a 10 minute stress is sufficient enough to detect changes in mRNA profile in response to the addition of HP.

The oxidative damage caused by these ROSs is further potentiated by the presence of iron in the media and/or cell which interacts with these ROS to result in more toxic radicals (25, 52). To evaluate this, we studied the transcriptome of campylobacter to 1mM HP in

iron-limited and iron-replete conditions (Figure 1). The group 1 flasks were supplemented with 40 µM $FeSO_4$ for 15 minutes to create an iron-replete condition. The group 2 flasks were not treated with the iron source. Ferrous sulphate at 40 µM level for fifteen minutes has been shown in our previous study to be sufficient enough to create an iron-replete condition based on the iron repression of iron uptake genes (36). Hence, the response of campylobacter to HP was studied 15 minutes after the addition of iron.

Finally, the *perR* mutant was used along with the wild type strain to identify the PerR regulon. Since the sensitivity of the PerR to HP could be altered by the growth conditions especially the metal ion levels, the comparison of the perR mutant with the wild type strain was done in both iron-limited and iron-rich conditions (Figure 2). Ferrous sulphate was added to both the flasks in group 3 at the concentration of 40 µM. The flasks in the group 4 were not treated with iron source. Fifteen minutes after the addition of iron source, both the flasks in each group were treated with 1mM HP. Samples were collected 10 minutes after the addition of HP and processed for microarray hybridization and analysis.

**Transcriptome profile of *C. jejuni* in response to $H_2O_2$**

*Iron-limited condition: genes induced by addition of $H_2O_2$ (Group 2)*

Table 2 lists the forty genes that were induced in the iron-restricted cells treated with HP. *KatA* gene, encoding the enzyme catalase, was induced 5-fold in this condition as expected. The peroxide stress regulator, *perR*, itself was upregulated by > 2-fold. Induction of *KatA* has been shown to be regulated by PerR in *C. jejuni* in response to iron

(60). The induction of the *perR* gene was observed in *B.subtilis* following a challenge with paraquat and HP (32). Another category of upregulated (from 2.26 to 5.96 fold) genes included genes of the iron regulon. These are *chuABCD, exbB1, exbD1, tonB1 feoB* and Cj0178. All these genes have been shown to be part of the Fur regulon in our earlier study (36). The genes *chuABCD* were iron repressed in our previous study and encode transport proteins of a probable hemin uptake system (36). The genes *exbB1, exbD1* and *tonB1* were shown to be in operonic structure and were iron repressed in *C. jejuni* (36). They constitute the energy transduction system which transduces the energy from the inner membrane to the outer membrane receptor to enable ferric iron uptake. The reason for the induction of iron as well as Fur regulated genes could be due to the fact that the promoters of these genes could possibly be shared between Fur and PerR. Indeed, the proposed consensus sequence for the Fur and PerR shows a significant similarity (Figure 8) (61).

The genes involved in the ribosomal protein synthesis and modification constituted a significant portion (22.5%) of the total genes induced. These were *rpsBEFSPI* and *rplVRM*. This is in contrast to the study on *P.aeruginosa* where genes belonging to this functional category were found to be down regulated (35). The *fur* gene was not induced by the HP in our study. Only the 1mM menadione stress induced the induction of *fur* gene expression (see the comparison section below). This is in contrast to studies in *E. coli* and *B.subtilis.* The expression of the *fur* gene in *E. coli* was shown to increase in response to addition of HP and superoxide. This induction of the *fur* gene expression was demonstrated to be OxyR and SoxRS mediated (66). Similarly, the expression of *B.subtilis fur* was induced more than 4-fold after HP and paraquat challenges (32). This

observation suggests that the mechanism of response to oxidative stress is clearly different in *C. jejuni*.

Several genes were expressed in iron-limited cells due to addition of HP that might be transcribed from divergent promoters. One set of such genes contains CJ1709c, Cj1710c, Cj1711c (*KsgA*) and Cj0712 (*rimM*). The other set of genes includes Cj1383c, Cj1384c, Cj1385 (*katA*) and Cj1386. In both these cases, they appear to be transcribed from a divergent promoter due to the presence of intergenic space between *ksgA* & *rimM* and *katA* & Cj1386. However, this needs to be experimentally proved by RT-PCR using several combinations of primers.

*Iron-rich condition: genes induced by the addition of $H_2O_2$ (Group 1)*

Thirty three genes were found to be upregulated in iron-rich condition by the addition of HP. The list of induced genes is given in Table 3. *KatA* and *ahpC* were the two genes with the highest fold induction, 10.5 and 6.8, respectively. *KatA* was induced more than twice that seen in iron-limited condition. Interestingly, *ahpC* was not induced by HP in the iron-limited cells (group 2). *AhpC* encodes the alkyl hydroperoxide reductase which converts reactive hydroperoxides to corresponding alcohols using NADH as a cofactor. In *E. coli*, AhpC is a catalytic subunit and is recycled by AhpF flavoprotein subunit (39). The reduced form of the AhpC subunit reduces alkyl hydroperoxides to alcohols and in the process it gets oxidized. The AhpF subunit reduces and recycles AhpC by transferring electrons from NAD(P)H to the oxidized form of AhpC (5). However, AhpF homologue is absent in *C. jejuni* (37). It has been shown that in *C. jejuni* the transcription of both *katA* and *ahpC* is repressed by iron (5, 63). However, in *C. jejuni fur* mutant the

expression of these two proteins was still iron responsive indicating the presence of an additional regulator system (63). Later, these two proteins were shown to be iron-repressed via PerR regulator (60). Our microarray data suggests that while *katA* induction by HP appears to be independent of the iron loading status of the cell, *ahpC* induction is seen only in iron-rich cells.

Another important gene that was induced was *trxB* which encodes the thioredoxin reductase. The expression of this gene was more than 4-fold upregulated. In prokaryotes, thioredoxin and glutaredoxin contain a pair of redox active cysteines and play a major role in the protection of cells against ROS by providing reducing power to key reductive enzymes (3, 21). Thioredoxin (TrxA) is maintained in its reduced form by thioredoxin reductase (TrxB) at the expense of NADPH (3). *C. jejuni* has both thioredoxin (encoded by *trxA*) and thioredoxin reductase (encoded by *trxB*). In *S. aureus*, both *trxA* and *trxB* were induced in response to several oxidative stress compounds like diamide, menadione, t-butyl hydroperoxide (58). However, the addition of HP did not induce these genes in *S. aureus* (58). Since *C. jejuni* lacks an AhpF homologue and the *trxA* gene was not significantly upregulated (1.74 fold change) in our experiment, the induction of *trxB* along with the *ahpC* indicates a possible role for TrxB in the recycling of AhpC. This indeed is the case in *Helicobacter pylori*, where AhpC recycling is TrxB mediated (6). Both *ahpC* and *trxB* were not selected as induced by HP in iron-limited *C. jejuni* cells. Interestingly, the members of iron regulon induced by HP in iron-limited cells were conspicuous by their absence in the list of upregulated genes in iron-replete cells. More than one third of the induced genes are annotated to encode hypothetical proteins. Their role in the oxidative stress remains to be elucidated.

*Genes down regulated by addition of $H_2O_2$*

Fourteen genes were down regulated in iron-rich condition due to the addition of 1mM HP (Table 4). Of note, among them is the gene *fdxA* which encodes a putative ferredoxin. *FdxA* is the first known example of an iron-induced gene in *C. jejuni* (59). FdxA has been previously shown to be involved in oxidative stress defense of *C. jejuni*. A *fdxA* mutant was found to be compromised in its aerotolerance (59). However, this mutant was not affected in its resistance to HP (59).

In the iron-limited condition, the expression of 58 genes was found to be down regulated (Table 5). The majority of the genes (18 of the 58) encoded hypothetical proteins of unknown function. The second major category of down regulated genes consisted of genes (17 out of 58) that encode proteins involved in the energy metabolism of the cell. This is probably due to bacteria slowing down its metabolism as a result of oxidative stress induced by the HP by shutting down the cell energy producing machinery. This might help bacteria to stop the internal source of the production of HP from aerobic metabolism. For example, *nuo* genes (*nuoIJH*) which are down-regulated more than 2-fold encode subunits of NADH dehydrogenase enzyme that plays role in respiratory chain. The genes involved in the tri-carboxylic acid cycle, *sucC*(succinyl -coA synthetase beta chain), *gltA* (citrate synthase), *mdh* (malate dehydrogenase), *icd* (isocitrate dehydrogenase) and *frdC* (fumarate reductase cytochrome B subunit) were down regulated at least 2-fold. Four genes, probably operonic, that encode an ABC-type amino acid transport system were down regulated due to HP addition. They include Cj0919c, Cj0920c, Cj0921c (*peb1A*) and Cj0922c (*pebC*).

**Response to CHP and menadione**

Mid-log grown *C. jejuni* were allowed to react with HP, CHP and menadione at 1 mM for 10 minutes. The response was compared to the untreated cells. *C. jejuni* responded with the induction of 44, 98 and 170 genes in case of HP, CHP and menadione, respectively. The Venn diagram (Figure 4) gives the number of induced genes commonly shared by all the three different agents. HP shared 6 and 3 genes between menadione and CHP, respectively, while CHP and menadione shared 40 genes. Seven genes were commonly induced among all the three agents. They were Cj0111, Cj1089c, Cj1252 (all encode hypothetical proteins), *perR* (encoding the peroxidase stress regulator), Cj0139 (encoding a putative endonuclease), *rpsB* (encoding 30S ribosomal protein S2) and *chuC* (encoding putative hemin uptake system ATP-binding protein). The *perR* was induced 2.12-, 3.2- and 2.6- fold in HP, CHP and menadione, respectively. The genes *uvrC* (encodes excinuclease ABC subunit C), *recR* (encodes recombination protein) and *xerD* (encodes DNA recombinase) were induced 2.15, 2.27 and 2.47 fold by either CHP or menadione. These proteins in addition to the Cj0139 (which was induced in all the three agents) are part of SOS regulon that are involved in DNA repair process. Induction of these genes appears logical since they are needed to repair the DNA damage caused by these agents. Similar increase in mRNA levels of genes relevant to DNA repair or related functions has been noted in *P. aeruginosa* treated with HP (35). *RecA* gene has also been shown to be induced by HP by 3-fold in *P. aeruginosa* (35). In *B.subtilis*, *recA* and *uvrABC* genes were induced by the addition of HP and paraquat (32). The highest number of genes was induced by menadione. This could be explained by the mode of action of menadione. Menadione is a quinone and will be reduced to semiquinone radicals which have high

affinity for oxygen (56). These semiquinone radicals reduce oxygen to superoxide radical and in the process menadione is regenerated leading to the production of more superoxide radicals (40, 56). Superoxide also take part in metal catalyzed reactions to form more toxic species of active oxygen such as hydroxyl radical and singlet oxygen (18). Redox cycling nature of menadione and the subsequent production of ROS might have resulted in the higher number of genes being induced in response to menadione. Induction of a relatively higher number of genes induced in the DNA replication/repair category in CHP and menadione is probably a reflection of extensive DNA damage caused by these oxygen species. There were 6 and 11 genes induced by CHP and menadione, respectively. This indicates that the CHP and menadione are probably more damaging to the bacterial cell than HP at 1mM concentration.

In addition, the higher number of genes differentially expressed in menadione and CHP could probably be due to induction of several global regulators. Three (PerR, Cj1000 and Cj1036c) and four global regulators (Fur, PerR, LytB and Cj1036c) were induced in response to CHP and menadione, respectively. PerR was the only global regulator induced byHP. The subsequent induction or repression of genes by these regulat ors might have further contributed to the large number of genes differentially expressed in menadione and CHP.

The *fur* gene was not induced in response to the addition of HP and CHP. However, the expression of this gene was induced upon addition of menadione. The induction of the *fur* genes probably serves two purposes. First, Fur sequesters the free iron in the cell.

Second, it acts as a repressor of iron transport systems to prevent further iron uptake which could potentiate the damage already caused by the free radicals.

Fifty eight, 109 and 170 genes were down regulated in HP, CHP and menadione treated cells, respectively. Genes encoding hypothetical proteins constituted the major portion among the down-regulated genes by all the three agents. The second major category of down-regulated genes encoded proteins involved in energy metabolism. Downregulation of a large number of genes involved in energy metabolism might probably reflect a temporary growth arrest in response to the oxidative stress induced by these agents. Genes involved in energy metabolism could have been repressed temporarily in order to reduce the aerobic respiration which is the main source of the endogenously produced ROS. The genes down regulated *fdhB* (encoding putative formate dehydrogenase iron-sulfur subunit), *frdB* (fumarate reductase cytochrome B subunit), *sdhABC* (encoding succinate dehydrogenase complex), *napGH* (encoding putative ferredoxin) contain iron sulfur in their active centers. The ROS attack these iron-sulfur centers and convert ferrous iron to ferric iron thus inactivating these proteins. Hence, *C. jejuni* repress the production of these proteins which appear to use iron-sulfur in their active centers.

Some interesting findings were noted with regard to the genes *ahpC, sodB* and *trxB* in the *C. jejuni* response to the three different agents. We found no induction of *trxB* in CHP and menadione treated cells. Though a little induction was seen in HP (1.8 fold), it was not enough to be selected as significantly induced. However, *trxB* was significantly induced (4.18 fold) due to HP in the *C. jejuni* in iron-rich medium and was found to be PerR regulated in our study. In *S. aureus*, TrxB is involved in the oxidative stress

resistance and was found to be induced on exposure to menadione but not to HP (58). Tryptic soy broth that was used to grow *S. aureus* is an essentially iron-rich medium (58).

AhpC, which acts on hydroperoxides, is not induced in the CHP treated cells in our study. An *ahpC* mutant of *C. jejuni* was reported to be hypersensitive to CHP with greater zone of killing using disk inhibition assay (5). This means that the *ahpC* gene should be induced in response to addition of CHP. However, contrary to our expectation, our microarray results show that the *ahpC* gene was not induced in cells treated with CHP. This could be due to the strain differences and/or experimental protocol. Another possibility is that the basal level of the AhpC expression might have been sufficient to deal with CHP stress induced in our study. We might need to use higher concentration of the CHP to induce the expression of AhpC. We tested the sensitivity of *C. jejuni* NCTC 11168 strain using 10 µl of the 3% CHP, while the other study was done with the strain *C. jejuni* 81116 with 3 µl of 3% CHP (5). There appears to be different mechanisms of dealing with CHP stress in *C. jejuni* NCTC 11168. Constructing an *ahpC* mutant in NCTC 11168 and studying its sensitivity to CHP would help to answer this question. In other organisms like *E. coli* and *S. typhimurium*, mutations in *ahpC* conferred increased sensitivity to killing by CHP (53).

The gene *sodB* encodes a superoxide dismutase which inactivates the superoxide to hydrogen peroxide and water. Menadione being a superoxide inducer, we expected the induction of *sodB* gene in cells treated with menadione. However, our microarray results suggest that this was not the case. This non-induction of *sodB* indicates that *C. jejuni* might have an alternate mechanism to deal with the superoxide stress. It is quite possible

that one of the hypothetical proteins that are induced in menadione treated cells might serve to inactivate the superoxide generated in the medium. Alternatively, the menadione stress could not have been sufficient enough to induce the *sodB* expression. In *C. jejuni* it has been shown that SodB provides protection against oxidative stress during survival in food (41), colonization of chicken intestinal tract (41), freezing and thawing (47) and survival in the macrophages (38).

**PerR regulon**

To identify the members of the PerR regulon we compared the transcriptional profiles of the wild type strain and the *perR* mutant in response to the addition of 1mM HP. The response of both strains was compared in both iron-limited and iron-replete conditions in order to investigate the role of iron in PerR regulation (Figure 2). Similar to the Fur protein, repression by PerR requires a divalent metal ion. The addition of either Mn (II) or Fe(II) was sufficient enough for PerR to repress *mrgA* and *katA* genes in case of *B.subtilis* (9, 10). In *B.subtilis,* PerR-$Fe^{+2}$ dissociates more readily than PerR-$Mn^{+2}$ from the target sequences upon exposure to HP (20).

Overall, 53 genes were found to be upregulated in the *perR* mutant in the iron-limited condition (group 4). Some of the important categories of genes and their proposed functions are given in the Table 6. Of the genes upregulated in *perR* mutant in iron-limited condition, transport and binding proteins (which included iron regulated proteins) constituted the major portion (22.6%) of the PerR regulon. Genes encoding different parts of the flagella were induced 2.2 to 7.4 fold in the *perR* mutant. Of note is gene Cj1293 which encodes a possible sugar nucleotide epimerase/dehydratase. This gene has

been shown to encode an enzyme, UDP-GlcNAc $C_6$-dehydratase/$C_4$-reductase, which is essential for the biosynthesis of pseudaminic acid, the major post-transcriptional modification of *Campylobacter* flagellin (16). *AhpC* gene was not selected as significantly induced in *perR* mutant in iron-limited condition although it was very close to the threshold of 2-fold (1.948 fold). *TrxB* gene was induced 2.68 fold in the *perR* mutant while *trxA* was not. In *S. aureus*, the expression of *trxA* (encoding thioredoxin) and *trxB* genes has been shown to be induced following exposure to oxidative stress inducing agents diamide, menadione and tau-butyl hydroperoxide (58). But, HP did not induce the *trxA* and *trxB* genes in *S. aureus* (58). The HP-induced genes belonging to the functional category of transport or binding proteins *chuABC, CfrA, trxB, exbB2*, Cj0818, Cj1658, Cj0175c, Cj1383c and Cj1613c have been also shown to be regulated by the Fur protein in our previous study (Chapter 3). It appears that Fur and PerR regulate the expression of these genes depending on the iron and oxidative stress status of the cell. The dual regulation of these genes might play a role in fine tuning of the *C. jejuni* response to the environmental conditions it faces during its life cycle. A potential PerR box has been proposed for *C. jejuni*, which is quite similar to that of the putative consensus Fur box of *C. jejuni* (Figure 8) (61). The genes *trxB, katA, ahpC* and the *perR* itself were shown to have the putative PerR box (61).

In the iron-rich condition, 44 genes were induced in the *perR* mutant. Table 7 lists some of the important genes belonging to the PerR regulon in iron-rich condition. Of the 44 genes, the significant majority encoded proteins involved in biogenesis/assembly of flagella (13 of 44, 29.5%). These genes were *flaB, flaD, flaG, flgB, flgD, flgE, flgE2, flgG, flgG2, flgH, flgI and flgK*. Flagellar biogenesis and motility consumes a significant

amount of energy within the cell. This, in turn, requires the cell to increase its energy production. During this process more ROS are generated in the cell. Repression of these genes by PerR would probably reduce the production of ROS. Again, catalase was 3.79 fold induced in the *perR* mutant when compared to the wild type strain. But the *ahpC* gene was not selected as significantly induced by HP in the *perR* mutant in iron-rich condition and in fact it showed a down-regulation trend. Interestingly, PerR was not selected as one of the genes of the PerR regulon. This is similar to the *C. jejuni* Fur gene which is characterized by the absence of iron responsive autoregulation (62). Autoregulation of PerR has been suggested in *B.subtilis* (20) and *S. aureus* (22).

**PerR and Fur regulon**

Bacteria have learned to regulate the uptake of metals and oxidative stress responses (52). The excess free iron inside the cell stimulates the production of ROS by the Fenton reaction which results in oxidative stress. In *E. coli* the *fur* gene is induced by oxidants and is controlled by the OxyR and the SoxR/S systems (66). This upregulation of the *fur* gene helps bacteria to shut down the iron uptake systems that would prevent the oxidative stress on the cell due to extraneous sources of iron. In addition, the Fur protein also sequesters iron present in the cell thus lowering available free iron for ROS production (66). Only the menadione was found to induce the *fur* gene in our study. Fur was also not selected as the member of the PerR regulon in our microarray data. In *B.subtilis*, the PerR has been shown to mediate the repression of its own gene and that of *fur* (15).

A consensus for the *C. jejuni* PerR box has been proposed (61). Since the proposed Fur box in *C. jejuni* is similar to the PerR box (See Figure 8), it is possible that there may be

regulatory cross talk between PerR and Fur. Indeed, a *fur* mutation influences the activity of the PerR regulated *katA* gene (60). Currently it is not known whether the *C. jejuni* PerR and Fur have an absolute requirement for a metal cofactor binding (61).

**Sensitivity to oxidative stress inducing agents**

*C. jejuni fur*, *perR* and *perRfur* mutants along with the wild type strain were compared in their sensitivity to different oxidative stress inducing agents using disk inhibition assay. The three different agents chosen were hydrogen peroxide (HP), cumene hydroperoxide (CHP) and menadione. The results of the assay are given in the Table 8 and graphically represented in the Figure 5. All the three mutants tested (*perR*, *fur* and *perRfur*) were significantly resistant ($P<0.05$) to HP when compared to wild type (i.e. they had smaller zone of inhibition). Among them, the *fur* mutant was more sensitive ($2.27 \pm 0.153$ cm) than either the *perR* or *perRfur* double mutant. For CHP, the *fur* mutant showed significantly higher sensitivity (i.e. larger zone of inhibition) when compared to the wild type, *perR* and *perRfur* double mutants. However, neither the *perR* mutant nor the *perRfur* double mutant was significantly affected ($P<0.05$) when compared to the wild type in its sensitivity to CHP. All the three mutants were significantly ($P<0.05$) more sensitive to menadione as compare to the wild type strain. The *perRfur* double mutant was affected to a greater extent than either the *perR* or *fur* mutant alone in its sensitivity to menadione.

KatA and AhpC are known to detoxify HP and CHP, respectively. Van Vliet *et al*., reported that *ahpC* is regulated by PerR and that a *perR* mutant was resistant to CHP (60). However, our microarray results suggest that PerR is not responsible for the *ahpC*

regulation as the *ahpC* was selected as one of the genes in the PerR regulon. Disk inhibition assay shows that the *perR* mutant is equally sensitive to CHP as the wild type strain. In addition, *ahpC* was not induced in cells treated with CHP. All these above results suggest that the isolate of *C. jejuni* NCTC 11168 used in our study differs in its mechanism to detoxify CHP from the isolate of NCTC 11168 used by Van Vliet *et al* (60). On the contrary, these isolates appear to have similar mechanism to detoxify HP through *katA* since katA was found to be regulated by PerR in both the isolates and *perR* mutants in both the isolates were resistant to HP. In another study with a different strain of campylobacter (i.e.*C. jejuni* 81116) (5), the *ahpC* mutant was found to be sensitive to CHP indicating that AhpC is involved in the clearance of CHP. However, the sensitivity of this *ahpC* mutant was not affected by HP. This difference could be attributed to the strain differences between *C. jejuni* 81116 and *C. jejuni* NCTC 11168. Clearly, construction of an *ahpC* mutant in *C. jejuni* NCTC 11168 and testing its sensitivity to CHP would address this question.

In our study, the *fur* mutant was sensitive to both CHP and menadione but was resistant to HP as compared to the wild type strain. The iron load due to constitutive expression of iron uptake systems in the *fur* mutant acts as a pro-oxidant by increasing the concentration of hydroxyl radicals and hence *fur* mutants are generally more sensitive to oxidative stress (57). This mechanism has been suggested for the increased sensitivity of the *E. coli fur* mutant to HP (57). But in *S. aureus* a different mechanism appeared to be the cause of the sensitivity of the *fur* mutant to HP. In *S. aureus*, Fur positively regulates the catalase gene (*katA*) and hence a *S. aureus fur* mutant is sensitive to HP owing to the

reduced amount of catalase produced in the cell (23). In our study, on the contrary, the *fur* mutant of *C. jejuni* showed reduced killing as evidenced by the smaller zone of inhibition to HP when compared to the wild type strain. This suggests that the *fur* mutant is resistant to HP. It appears that Fur might be negatively regulating some of the genes involved in HP resistance. Therefore in the absence of the Fur protein, those genes are derepressed resulting in the inactivation of HP. Indeed, Fur has been shown to co-regulate *katA* expression along with PerR (60). This would be a novel finding since Fur is classically known to repress the genes involved in iron acquisition. There are few reports that show the role of Fur in regulating the genes involved in the oxidative stress defense (34, 55). In *E. coli* Fur negatively regulates *SodA* gene by the classical iron dependent repression and positively regulates the *sodB* gene by an indirect mechanism (34). Later, *sodA* was also shown to be negatively regulated by another global regulator ArcA (involved in the aerobic respiration) and ArcA can easily displace Fur from the *sodA* promoter (55). A similar finding that Fur also regulates genes in oxidative stress in *C. jejuni* would provide more strength to the idea of Fur as a global regulator in this pathogen. Indeed, in other bacteria Fur has been shown to regulate genes involved in the production of toxins and other virulence factors (30, 50), chemotaxis (26), metabolic pathways (17, 51) and acid shock response (45).

**Oxidative stress survival studies**

The ability of the *perR*, *fur* and *perRfur* mutants and the wild type strain to survive HP stress was further tested by oxidative stress survival assay. Mid-log grown cells were resuspended in PBS and were exposed to 1 mM HP for 60 minutes. The viability of the

cells was measured at 15, 30, 45 and 60 minute intervals. Figure 6 shows the results of the oxidative stress survival assay. *PerR* mutant was significantly resistant with up to 75% of the cells remaining viable at the end of 60 minute time point. The *fur* mutant and the wild type strains showed similar ability to survive the oxidative stress by HP though the *fur* mutant had slightly higher viability than the wild type at all the time points. The *perRfur* double mutant showed an initial drop but recovered later and ended with almost the same number of cells as the wild type strain and the *fur* mutant by the 60 minute time point. These results are in agreement with the disk inhibition assay done with HP except for the behavior of the double mutant. The *perRfur* double mutant was resistant to the HP in the disk assay while it appears to be equally sensitive as the wild type or the fur mutant in the survival assay. This could be due to the differences in the kinetics of HP inhibition in the liquid and solid medium.

**PerR is attenuated in colonization of chicken ceca**

The chicken has been used as a model for colonization in studies with *C. jejuni*. Colonization potential of the *perR* mutant and the *perRfur* double mutant was assessed along with the wild type strain. As shown in the Figure 7 both the mutants were significantly affected ($P<0.05$) in their ability to colonize chicken ceca. The number of *perRfur* double mutants recovered per gram of ceca was below the detection limit of our assay (500 CFU/g of ceca). We have earlier shown that the *fur* mutant is also affected in the colonization of chicken ceca (36). The additive effect of loss of *perR* and *fur* resulted in the *perRfur* double mutant being severely affected in its colonization ability of chicken ceca. This data clearly demonstrates that PerR is required for *in vivo* growth in the chicken colonization model. These results reflect studies in *Staphylococcus aureus* where

both the *fur* and the *perR* mutants were attenuated in their virulence in the murine abscess model of infection (22, 23). Similarly, *Listeria monocytogenes perR* and *fur* mutants failed to replicate in the spleen of infected mice indicating that both these genes are essential for *in vivo* growth of this pathogen (43). *PerR* mutant of *S. pyogenes* was also found to be attenuated in its virulence in a murine air sac model of infection (44).

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**Table 1.** Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| DH5α | *endA1 hsdR17* ($r_k^- m_k^-$) *supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYAargF U169deoR [f80dlac*Δ*(lacZ0 M15]* | Invitrogen |
| ***C. jejuni*** | | |
| AS144 | *C. jejuni* NCTC 11168 | NCTC |
| AS 230 | AS144Δ*fur* | (36) |
| AS 216 | AS144Δ*perR* | This study |
| AS 232 | AS144Δ*perR* Δ*fur* | This study |
| **Plasmids** | | |
| pUC19 | Cloning and suicide vector, $Amp^r$ * | Biolabs |
| pRY111 | Chloramphenicol resistance gene | (65) |
| pILL600 | Kanamycin resistance gene | (28) |
| pAS 213 | pUC19 carrying *perR* | This study |
| pAS 214 | pUC19 carrying Δ *perR::cam* | This study |
| pAS 215 | pUC19 carrying Δ *perR::Kan* | This study |

*$Amp^r$, ampicillin resistant

**Table 2.** Genes induced due to addition of 1mM hydrogen peroxide to *C. jejuni* cells in iron-limited conditions. (Figure 1,Group 2)

| Name | Function | Fold change |
|---|---|---|
| *ksgA* | putative dimethyladenosine transferase | 9.77 |
| Cj1710c | Hypothetical unknown protein | 8.78 |
| Cj1386 | ankyrin-repeat containing protein | 6.68 |
| Cj0177 | Hypothetical unknown protein | 5.97 |
| *exbB1* | biopolymer transport protein | 5.96 |
| *exbD1* | biopolymer transport protein | 5.50 |
| *katA* | catalase | 4.95 |
| *chuC* | Putative hemin uptake system(HAS) ATP-binding protein | 4.37 |
| *feoB* | ferrous iron transport protein | 3.78 |
| *chuD* | Putative HAS periplasmic haemin-binding protein | 3.66 |
| *aroC* | chorismate synthase | 3.57 |
| *chuA* | HAS outer membrane receptor | 3.34 |
| Cj1384c | Hypothetical unknown protein | 3.14 |
| Cj0818 | Hypothetical unknown protein | 2.92 |
| Cj1383c | Hypothetical unknown protein | 2.91 |
| *rpsS* | 30S ribosomal protein S19 | 2.82 |
| *tonB1* | possible tonB transport protein | 2.42 |
| Cj0390 | Hypothetical unknown protein | 2.39 |
| Cj1709c | putative ribosomal pseudouridine synthase | 2.36 |
| *chuB* | putative HAS permease protein | 2.35 |
| *rpsE,I,P* | 30S ribosomal protein S5,S9,S16 | 2.29 |
| Cj0178 | putative outer membrane siderophore receptor | 2.26 |
| *tal* | putative transaldolase | 2.21 |
| Cj0139 | putative endonuclease | 2.21 |
| *rplV,R* | 50S ribosomal protein L22, L18 | 2.19 |
| *rplM* | 50S ribosomal protein L13 | 2.17 |
| *rpsB* | 30S ribosomal protein S2 | 2.14 |
| *rimM* | putative 16S rRNA processing protein | 2.13 |
| *perR* | peroxide stress regulator | 2.11 |
| *trmD* | tRNA (guanine-N1)-methyltransferase | 2.10 |
| Cj0111 | Hypothetical unknown proteins | 2.09 |
| Cj1252 | Hypothetical unknown proteins | 2.08 |
| *rpsF* | 30S ribosomal protein S6 | 2.05 |
| Cj1089c | Hypothetical unknown proteins | 2.04 |
| *purH* | Phosphoribosylaminoimidazolecarboxamide formyltransferase / IMP cyclohydrolase | 2.02 |
| *thiL* | putative thiamin-monophosphate kinase | 2.01 |
| *flhA* | flagellar biosynthesis protein | 2.00 |

**Table 3.** Genes induced due to addition of 1mM hydrogen peroxide to *C. jejuni* cells grown in iron-rich conditions. (Figure 1, Group 1)

| Name | Function | Fold change |
|---|---|---|
| *katA* | catalase | 10.53 |
| *ahpC* | alkyl hydroperoxide reductase | 6.88 |
| Cj0025c | putative transmembrane symporter | 5.51 |
| Cj0378c | Hypothetical unknown protein | 3.61 |
| *trxB* | thioredoxin reductase | 4.18 |
| *flhB* | flagellar biosynthetic protein | 3.41 |
| Cj1211 | Hypothetical unknown protein | 3.00 |
| *ksgA* | putative dimethyladenosine transferase | 2.79 |
| Cj0982c | putative amino-acid transporter periplasmic solute-binding protein | 2.84 |
| Cj0830 | Hypothetical unknown protein | 2.68 |
| Cj0430 | Hypothetical unknown protein | 2.49 |
| Cj0240c | putative aminotransferase | 2.49 |
| Cj0801 | Hypothetical unknown protein | 2.68 |
| *perR* | peroxide stress regulator | 2.42 |
| Cj0239c | nifU protein homolog | 2.45 |
| Cj0343c | Hypothetical unknown protein | 2.25 |
| *p19* | periplasmic protein p19 | 2.23 |
| *atpE* | ATP synthase F0 sector C subunit | 2.40 |
| *fdhB* | putative formate dehydrogenase iron -sulfur subunit | 2.33 |
| Cj0760 | Hypothetical unknown protein | 2.25 |
| Cj0587 | Hypothetical unknown protein | 2.31 |
| *ktrB* | putative K+ uptake protein | 2.23 |
| *cdsA* | phosphatidate cytidylyltransferase | 2.17 |
| Cj1038 | probable cell division/ peptidoglycan biosynthesis protein | 2.35 |
| *dnaK* | heat shock protein dnaK | 2.13 |
| *kpsM* | putative capsule polysaccharide export system inner membrane protein | 2.19 |
| Cj0339 | putative transmembrane transport protein | 2.05 |
| *pssA* | CDP-diacylglycerol--serine O-phosphatidyltransferase | 2.03 |
| Cj1003c | Hypothetical unknown protein | 2.12 |
| *grpE* | heat shock protein grpE | 2.13 |
| *kdtA* | 3-deoxy-D-manno-octulosonic-acid transferase | 2.01 |
| Cj1210 | Hypothetical unknown protein | 2.02 |
| Cj1663 | putative ABC transport system ATP-binding protein | 2.00 |

**Table 4.** Main categories of genes down regulated due to addition of 1mM hydrogen peroxide to *C. jejuni* cells grown in iron-rich conditions.

| Category and name of proteins | Known/proposed function |
|---|---|
| Energy Metabolism | |
| Cj1357c | putative periplasmic cytochrome C |
| Cj1358c | putative periplasmic cytochrome C |
| SdhA | succinate dehydrogenase flavoprotein subunit |
| SdhB | putative succinate dehydrogenase iron-sulfur protein |
| FdxA | ferredoxin |
| NapB | periplasmic nitrate reductase small subunit (cytochrome C-type protein) |
| Other categories | |
| LeuC | 3-isopropylmalate dehydratase large subunit |
| RpsS | 30S ribosomal protein S19 |
| RpoB | DNA-directed RNA polymerase beta chain |
| FlaA | Flagellin |
| FlgE2 | flagellar hook subunit protein |
| Cj0358 | putative cytochrome C551 peroxidase |
| Cj1358c | hypothetical unknown proteins |
| Cj1388 | hypothetical unknown protein |

**Table 5.** Major categories of genes down regulated due to addition of 1 mM hydrogen peroxide to *C. jejuni* cells grown in iron-limited condition.

| Category and name of proteins | Known/proposed function |
|---|---|
| Energy Metabolism | |
| AckA | acetate kinase |
| CcoQ | cb-type cytochrome C oxidase subunit IV |
| Cj0074c | putative iron-sulfur protein |
| Cj0075c | putative oxidoreductase iron-sulfur subunit |
| FdhB | putative formate dehydrogenase iron-sulfur subunit |
| FrdC | fumarate reductase cytochrome B subunit |
| GltA | citrate synthase |
| Icd | isocitrate dehydrogenase |
| Mdh | malate dehydrogenase |
| NapH | putative ferredoxin |
| NuoH, NuoJ, NuoK | NADH dehydrogenase I chain H, J, K |
| OorA, OorA, OorD, SucC | A,D,C subunit of oxoglutarate :acceptoroxidoreductase |
| Amino acids and amines transport/binding proteins | |
| Cj0919c,Cj0920C | putative ABC-type amino-acid transporter permease proteins |
| Peb1A | probable ABC-type amino-acid transporter periplasmic solute-binding protein |
| PebC | ABC-type amino-acid transporter ATP-binding protein |
| PutP | sodium/proline symporter |
| SdaC | serine transporter |
| Hypothetical proteins | |
| Cj0073c, Cj0238, Cj0427, Cj0553 Cj0741, Cj0832c, Cj0851c, Cj0852c, Cj0986c, Cj1452, Cj1484c, Cj1485c, Cj1486c, Cj1486c, Cj1513c, Cj1542, Cj1560, HspR | Hypothetical unknown proteins |

**Table 6.** PerR regulon: Major categories of genes upregulated in the *perR* mutant in iron-limited condition with their proposed function (Figure 2, Group 4)

| Category and name of proteins | Known/proposed function |
|---|---|
| Oxidative stress response | |
| KatA | Catalase |
| TrxB | Thiredoxin reductase |
| Transport/binding proteins | |
| ChuABC, | Putative hemin uptake system |
| CfrA, | Putative ferric siderophore receptor protein |
| Cj0175c, Cj1663, exbB2,exbD2 | (Putative)ABC transporter systems |
| Cj0203, Cj0935c,Cj1588c | Putative transmembrane proteins |
| Surface structures | |
| FlabB | Flagellin |
| FlgD | Putative flagellar hook assembly protein |
| FlgE | Flagellar hook protein flgE |
| FlgE2 | Flagellar hook subunit protein |
| FlgG | Flagellar basal-body rod protein |
| FlhB | Flagellar biosynthetic protein |
| Cj1293 | Possible sugar nucleotide epimerase/dehydratase |
| Hypothetical proteins | |
| Cj0034c,Cj0041,Cj0177,Cj0391c, Cj0420,Cj0423,Cj0428,Cj0520, Cj0552,Cj0772c,Cj0818,Cj1383c, Cj1613c,Cj1658,Cj1660 | Hypothetical unknown proteins |

**Table 7.** PerR regulon: Major categories of genes that were found upregulated in the *perR* mutant in iron-rich condition (Figure 2, Group 3) along with their proposed function.

| Category and name of proteins | Known/proposed function |
|---|---|
| Oxidative stress response | |
| KatA | Catalase |
| Surface structures | |
| FlaB | Flagellin |
| FlaD | Putative flagellin |
| FlaG | Possible flagellar protein |
| FlgB | Putative flagellar basal-body rod protein |
| FlgD | Putative flagellar hook assembly protein |
| FlgE | Flagellar hook protein flgE |
| FlgE2 | Flagellar hook subunit protein |
| FlgG | Flagellar basal-body rod protein |
| FlgG2 | Putative flagellar basal-body rod protein |
| FlgH | Putative flagellar L-ring protein precurso |
| Flgl | Flagellar P-ring protein |
| FlgK | putative flagellar hook-associated protein |
| Cj1293 | Possible sugar nucleotide epimerase/dehydratase |
| Hypothetical proteins | |
| Cj0041,Cj0391c,Cj0428,Cj0428, Cj0553,Cj0772c,Cj0977,Cj0987c, Cj1242,Cj1295,Cj1356c,Cj1388, Cj1464,Cj1631c,Cj1656c,Cj1714 | Hypothetical unknown proteins |

**Table 8.** The sensitivity to oxidative stress was measured using disk inhibition assay. Hydrogen peroxide (HP), cumene hydroperoxide (CHP) and menadione were used to test the sensitivity of four strains. Sterile disks containing 10 µl of 3% HP, 3%CHP and 3M menadione were placed on the agar containing *C. jejuni*. The zone of inhibition was measured in centimeters after 24 hours. Multiple comparisons were made using pair wise t-tests ($P<0.05$). The values given are the means of three independent experiments. Means with the same letter are not significantly different from one another.

| Strains | $H_2O_2$ | CHP | Menadione |
|---|---|---|---|
| NCTC 11168 wt | $2.75 \pm 0.05^{a}$ | $2.97 \pm .057^{a}$ | $4.67 \pm 0.153^{a}$ |
| *perR* | $1.53 \pm 0.057^{c}$ | $2.63 \pm 0.153^{a}$ | $5.53 \pm 0.058^{b}$ |
| *fur* | $2.27 \pm 0.153^{b}$ | $3.4 \pm 0.05^{b}$ | $5.57 \pm 0.058^{b}$ |
| *perRfur* | $1.43 \pm 0.115^{c}$ | $2.9 \pm 0.173^{a}$ | $5.93 \pm 0.208^{c}$ |

**Figure 1**

Experimental design to investigate the *Campylobacter jejuni* response to 1 mM concentration of HP in iron-rich condition (group 1) and iron-limited conditions (group 2). *C. jejuni* in flasks belonging to group 1 were treated with 40 µM of $FeSO_4$ for 15 minutes before the addition of HP. One of the flasks in each group was treated with 1 mM HP for 10 minutes. Samples were harvested and processed for hybridization as described in materials and methods section.

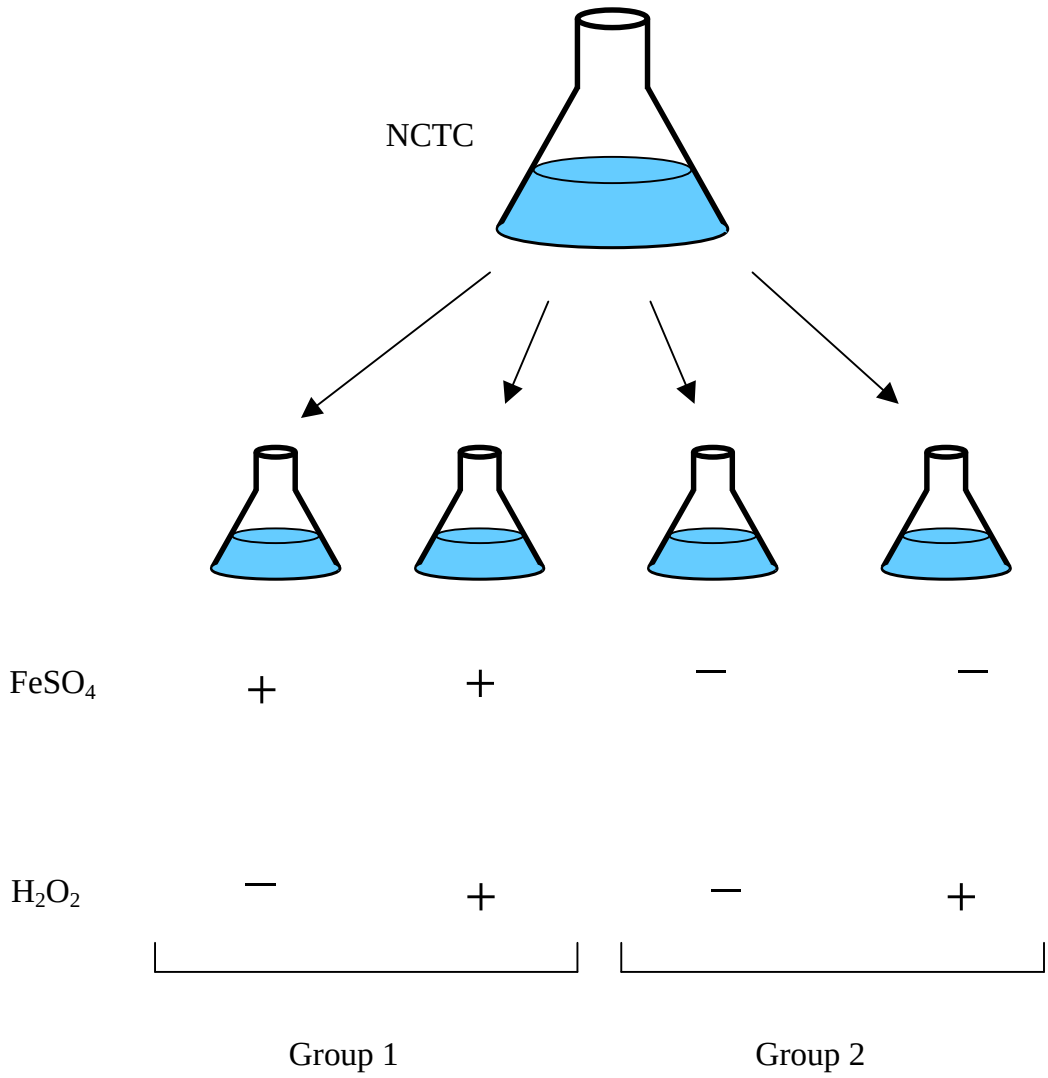

NCTC
FeSO4
+
+
−
−
H2O2
−
+
−
+
Group 1
Group 2

**Figure 2**

Experimental design to identify the PerR regulon in both iron rich condition (group 3) and iron-limited condition (group 4). *C. jejuni* in flasks belonging to group 3 were treated with 40 µM of $FeSO_4$ for 15 minutes before the addition of HP. Flasks in both the groups were treated with 1 mM HP for 10 minutes. Samples were harvested and processed for microarray hybridization (See materials and methods section)

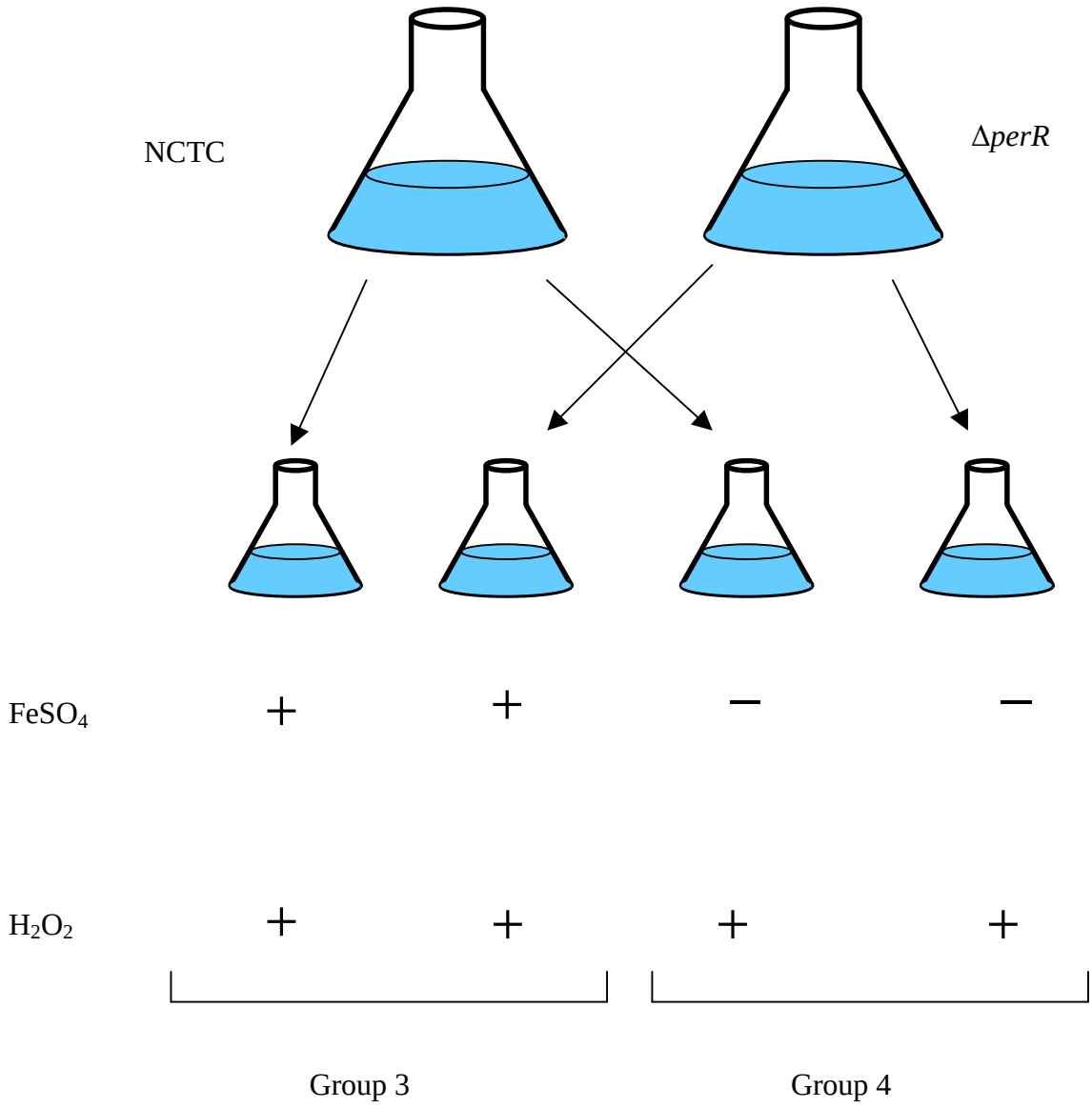

NCTC
ΔperR
FeSO4
+
+
−
−
H2O2
+
+
+
+
Group 3
Group 4

**Figure 3**

Experimental design to compare the response of *C. jejuni* to HP, cumene hydroperoxide (CHP) and menadione. Mid-log grown *C. jejuni* were split into four flasks. Three of the flasks were treated with HP, CHP and menadione each at 1 mM concentration. One flask was left as control. Ten minutes after the addition, samples were harvested and processed for competitive microarray hybridization as described in the materials and methods section.

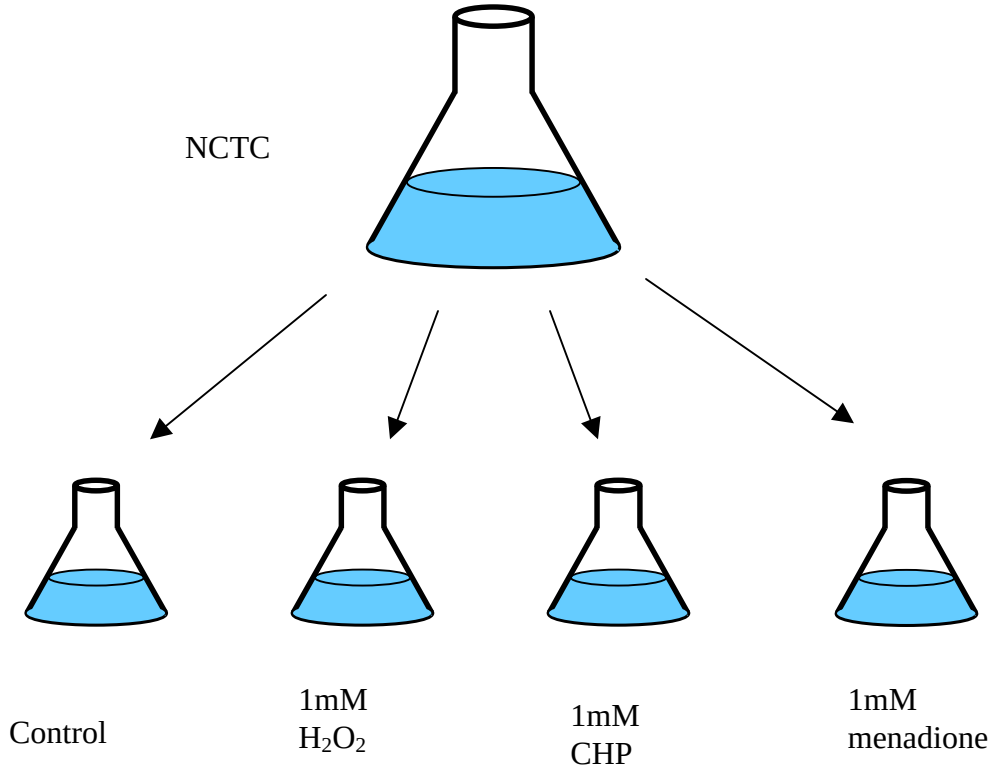

NCTC
Control
1mM
$H_2O_2$
1mM
CHP
1mM
menadione

**Figure 4**

Venn diagram showing the results of the *C. jejuni* response to HP, CHP and menadione.

The experiment was done as described in figure 3. Seven genes were found to be induced in all the three agents while 40 genes were commonly induced between CHP and menadione. Menadione accounted for the largest number of induced genes when compared to HP and CHP.

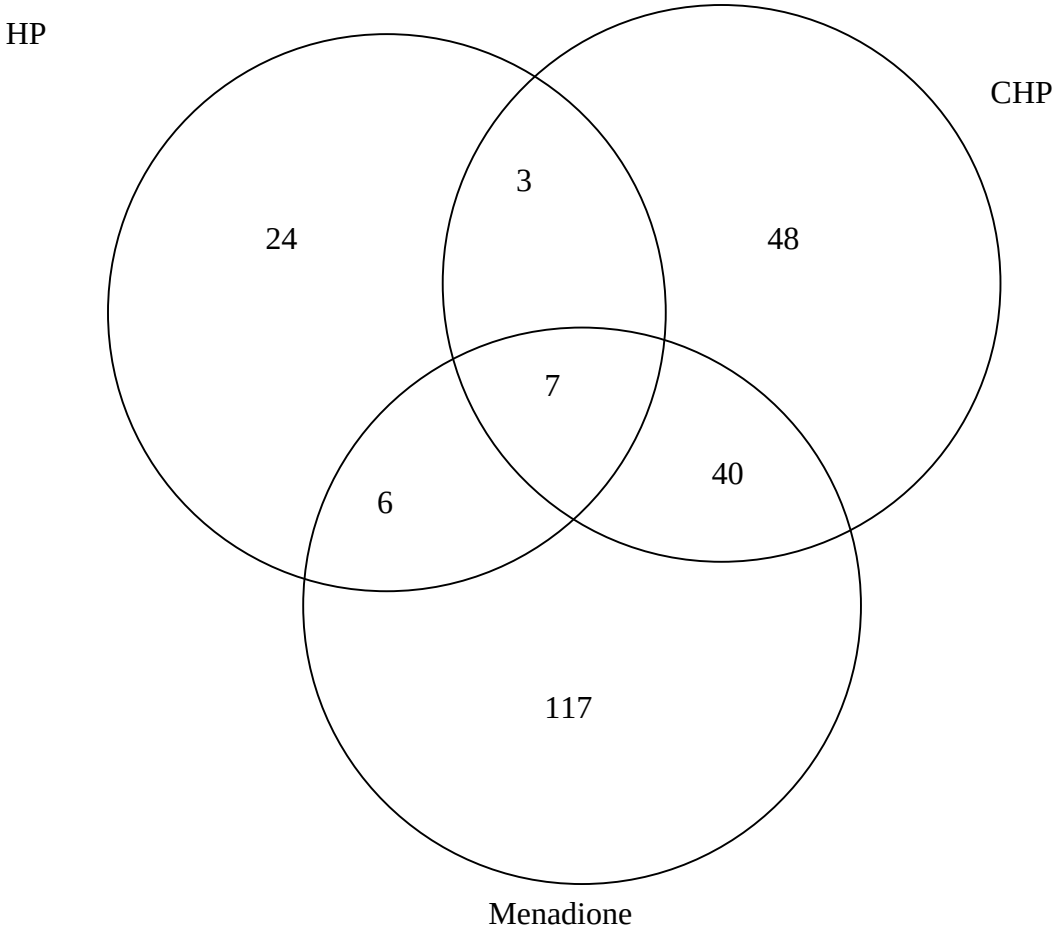

HP
CHP
3
24
48
7
40
6
117
Menadione

**Figure 5**

Bar histogram showing the results of disk inhibition assay. The mutants Δ*perR*, Δ*fur*, Δ*perRfur* along with the wild type *C. jejuni* were tested for their sensitivity to HP, CHP and menadione. Sterile filter disks containing 10 µl of 3% HP, 3% CHP and 3M menadione solution were kept on the agar plate containing *C. jejuni*. The zone of inhibition around the filter disks was measured in centimeters after 24 hours. The numerical values given are given in the table 8.

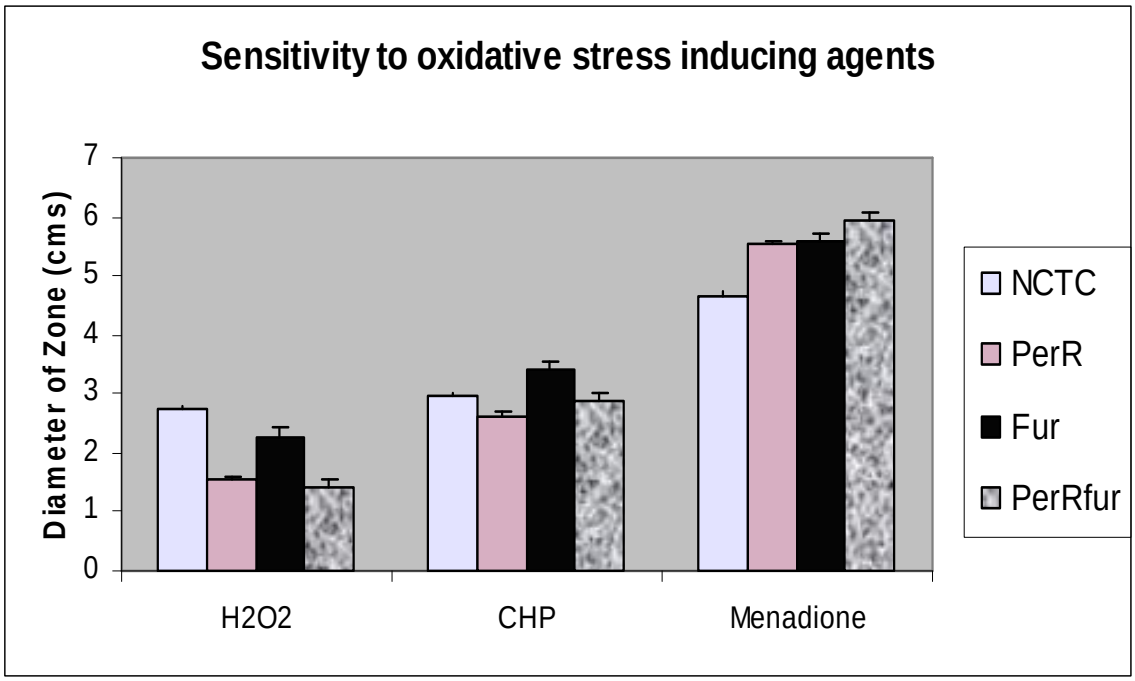

Sensitivity to oxidative stress inducing agents
Diameter of Zone (cms)
0
1
2
3
4
5
6
7
H2O2
CHP
Menadione
NCTC
PerR
Fur
PerRfur

**Figure 6**

Results of the oxidative stress survival assay for *C. jejuni* wild type strain, *fur, perR* and *perRfur* mutants. Overnight grown cultures were resuspended in PBS buffer and exposed to 1 mM concentration of HP. Samples were collected over 60 minutes, serially diluted in PBS containing catalase and plated on MH plates to assess the survival percentage of different strains.

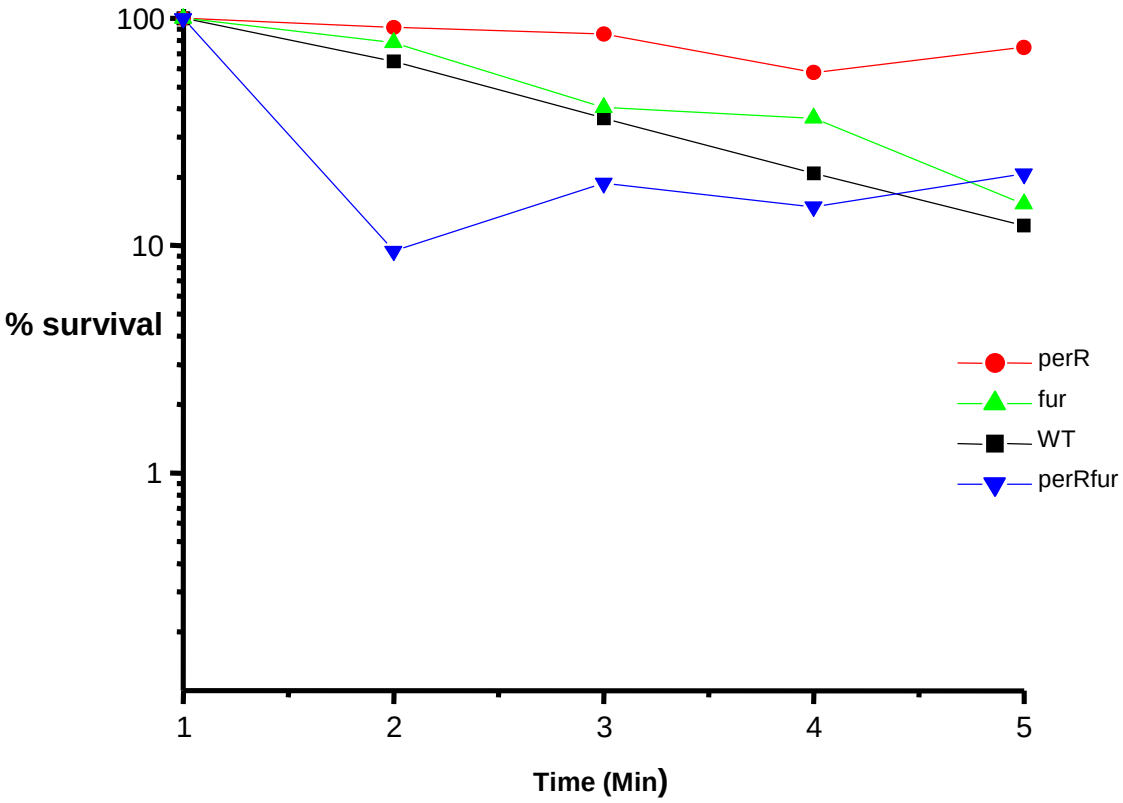

100
10
1
% survival
1
2
3
4
5
Time (Min)
perR
fur
WT
perRfur

**Figure 7**

Results of the chicken colonization assay. The *perR* mutant and the *perRfur* double mutant were tested along with the wild type *C. jejuni* for their ability to colonize chicken ceca. Groups of five chicks were inoculated with each strain containing 1.5 to 7 $x10^3$ viable organisms. The enumeration represents the counts at 4 days post infection. The column represents the means and the error bars indicate the standard deviation.

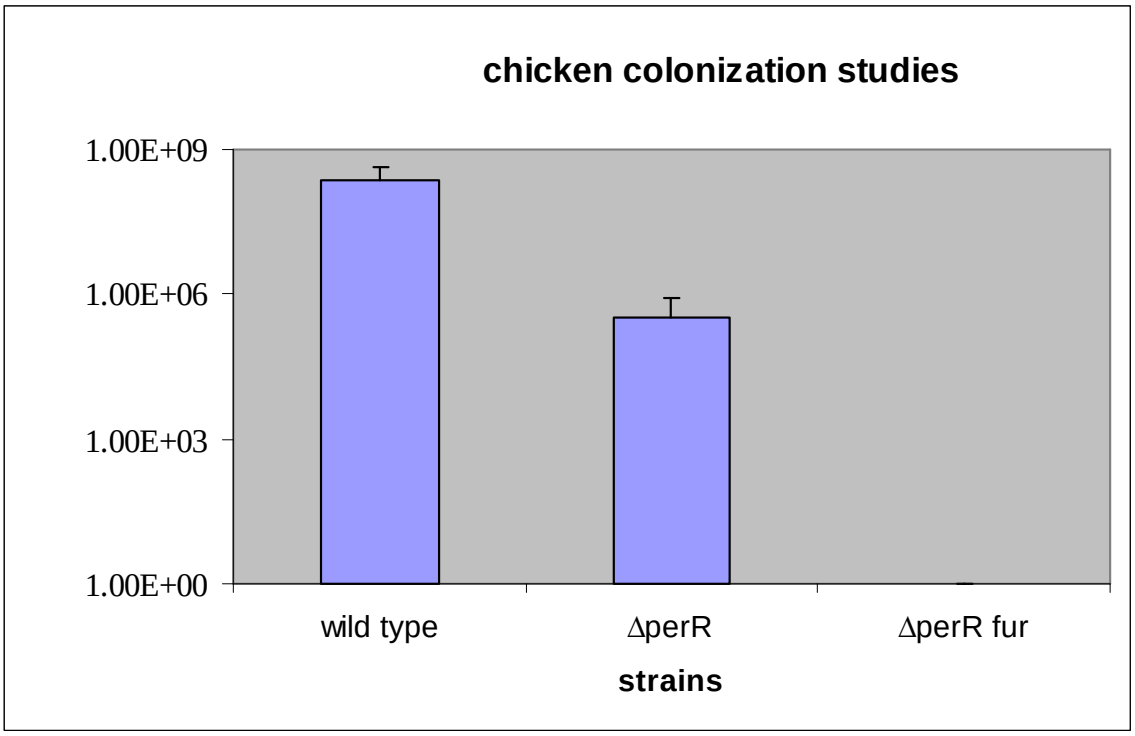
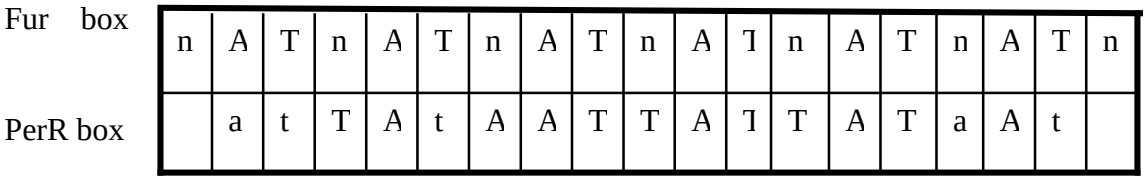

chicken colonization studies
1.00E+09
1.00E+06
1.00E+03
1.00E+00
wild type
ΔperR
ΔperR fur
strains

**Figure 8**

Comparison of proposed consensus sequence for PerR and Fur box as discussed in (61). The lowercase letters indicate less conserved residues. Fur box consensus is from the promoters of genes functioning in iron transport or iron storage. PerR box consensus is from the promoters of genes functioning in oxidative stress defense.


Fur box
PerR box
n A T n A T n A T n A Ɪ n A T n A T n
a t T A t A A T T A Ɪ T A T a A t

# CHAPTER V

# CHARACTERIZATION OF IRON- AND FUR-REGULATED GENES: IDENTIFICATION OF CHUA AS THE HEME RECEPTOR

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## Abstract

*Campylobacter jejuni* is a Gram-negative microaerophilic organism that causes food-borne gastroenteritis in human beings in US. Iron availability is one of the key factors that determine *Campylobacter*'s ability to colonize the intestine of human beings. We have investigated the response of *Campylobacter* to iron using microarrays in our previous study. A *C. jejuni fur* mutant was also used to define the Fur-regulon by transcriptome profiling. We identified numerous genes to be regulated both by iron and Fur. In this study we chose three of such genes- *p19*, Cj1658 and *chuA*. The gene *p19* encodes a periplasmic protein, Cj1658 encodes an integral membrane protein and *chuA* encodes a potential hemin uptake outer membrane receptor. We constructed mutations in these genes and the mutants were characterized by growth promotion assays using iron sources such as hemin, hemoglobin, ferrichrome, cepabactin, alcaligin, coprogen, rhodoturilic acid were used to promote the growth of the mutants and the wild type strain. No phenotype was found with respect to Δp19 and ΔCj1658 mutants while Δ*chuA* mutant was found to be defective in the utilization of hemin and hemoglobin. All three mutants were not affected significantly in their ability to colonize chicken ceca

## Introduction

Iron plays a key role in the bacterial metabolism as a micronutrient and hence bacteria have evolved complex mechanisms to acquire this key nutrient (1). Many bacteria acquire iron by synthesizing ferric iron chelators called siderophores (1, 2). Siderophores are low molecular weight substances that scavenge and chelate the ferric iron and make it available for the bacteria. To get into the bacterial cell, siderophores require specific outer membrane receptors. Other methods of acquiring iron include transport of ferrous iron after the reduction at the bacterial surface (10) and proteolytic degradation of the host iron-binding proteins (4).

In our previous study, we investigated the iron regulon of *C. jejuni*. In addition, a *fur* mutant was constructed and used to identify Fur regulon. Among many genes differentially expressed, we chose three of them for further investigation *CfrA, CeuE,* Cj0178. A mutant in *CfrA* was found to be defective in the utilization of enterobactin as the sole source of iron demonstrating the role of CfrA as the ferric-enterobactin receptor.

In this study, we investigate the function of additional iron- and Fur-regulated membrane proteins, *p19,* Cj1658 and *chuA.* Picket and coworkers were the first to isolate mutants that were defective in heme utilization by chemical mutagenesis in *C. jejuni* 81-176 strain (9). These mutants lacked 70Kda OM protein. Later, van Vliet *et al,* found an iron- and Fur-regulated 70Kda protein called ChuA in the strain *C. jejuni* NCTC 11168 and hypothesized that this protein could be the 70Kda protein that was lacking in mutants of the strain *C. jejuni* 81-176 used by Picket *et al.* (14). Genome sequence of *C. jejuni* NCTC 11168 contains four genes *ChuABCD* (8). *ChuA, B, C, and D* encode outer

membrane protein, cell membrane permease, an ATPase, and a periplasmic binding protein, respectively. The role of ChuA in *C. jejuni* NCTC 11168 has been so far assumed to be involved in the heme uptake. However, it has not been proven conclusively. The purpose of this study was to construct a mutant in *chuA* and test its ability to use the heme as an iron source.

The gene *p19* (Cj1659) is preceded by the Cj1658 gene that encodes a putative membrane protein in *C. jejuni* genome (8). P19 protein was identified originally as a periplasmic protein in the glycine extract of *C. jejuni* strain 81176 (5) and subsequently identified to be Fur and iron regulated in *C. jejuni* NCTC 11168 strain (14). Since the homologues of Cj1658 and p19 proteins were found on the 102-Kb iron uptake pathogenicity island of *Yersinia pestis* (3, 13) and they were both iron- and Fur-regulated in our previous study (7), we investigated further the roles of these two proteins by construction of mutants in these two genes.

In this study we have constructed mutants in the genes *p19*, Cj1658 and *chuA* and tried to characterize them by growth promotion assay and chicken colonization studies.

## Materials and Methods

*Bacterial strains and growth conditions*

The bacterial strains used in this study are given in the Table 1. *E.coli* DH5α and *C. jejuni* were grown as previously described (7, 11).

*Construction of C. jejuni mutants*

Generation of the Δ*chuA* (AS235), Δ*p19* (AS256), *and* Δ1658 (AS260) mutants was done using the same inactivation strategy followed in our earlier studies. Briefly, *C. jejuni* NCTC 11168 chromosomal DNA was used to amplify *chuA* gene by PCR using the primers chuA-01 (which contains a SacI site) and chuA-02 (which contains a PstI site). The sequences of the primers chuA-01 and chuA-02 as well as other primers used in this study are given in the Table 2. The resulting 1895 bp fragment was digested with *SacI* and PstI, and ligated to SacI/PstI restricted pUC19 vector, yielding the plasmid pAS332. A deletion of 969 bp was generated within the *chuA* gene by inverse PCR using the primers chuA-03 and chuA-04 (which both contain a BamHI site). The PCR product was cut with BamHI and self-ligated yielding the plasmid pAS333. This plasmid was cut with BamHI and ligated with a BamHI restricted chloramphenicol resistance cassette ($Cm^r$) from the plasmid pRY111 (15) yielding the final plasmid construct pAS334.

Similarly, Cj1658 gene was amplified using primers Cj1658-01 and Cj1658-02 which both contain a BglII site. The resulting 1965 bp product was digested with BglII enzyme and ligated with BamHI restricted pUC19 to obtain the construct pAS257 (4651 bp). Inverse PCR was done to delete 998 bp of this construct using primers chuA-03 and chuA-04 (which both contain BamHI site). The resulting product was self-ligated and

transformed in *E.coli* to obtain pAS258. This construct was digested with BamHI enzyme and ligated with BamHI restricted chloramphenicol resistance cassette ($Cm^r$) from the plasmid pRY111 (15) yielding the final plasmid plasmid pAS259.

The *P19* gene was amplified from the *C. jejuni* chromosome using primers p19-01 ad p19-02 (which both contain a BglII site). The resulting 1570 bp product was digested with BglII enzyme and ligated with BamHI restricted pUC19 to obtain pAS253 (4256 bp). An internal fragment of *p19* (297 bp) was deleted from the pAS253 using primers p19-03 and p19-04 (which both contain BamHI site) and the product was self-ligated and transformed in *E.coli* DH5α to obtain pAS254. This plasmid pAS254 was cut with BamHI enzyme and ligated with BamHI restricted chloramphenicol resistance cassette ($Cm^r$) from the plasmid pRY111 yielding pAS256.

The final plasmid constructs pAS334, pAS259 and pAS256 which contain the $Cm^r$ cassette in the same direction as that of the gene of interest were used to transform *C. jejuni* NCTC 11168 using a standard protocol (15) to generate the Δ*chuA*(AS235), ΔCj1658(AS260) and Δ*p19*(AS256) mutants, respectively, by allelic exchange. Transformants were selected on MH agar plates containing 20 μg/ml of chloramphenicol. Finally, the double homologous recombination events were confirmed by analyzing the $Cm^r$ clones by PCR using the corresponding sets of gene primers.

*Growth promotion assays*

Dfferent iron sources were tested for their ability to promote the growth of the mutants and wild type strain as described in our previous study (7). Briefly, the bacterial strains were grown to mid-log phase in MH medium and were resuspended to an optical density

of 1.0 at 600nm in a fresh MH medium. One ml of this culture was mixed with 24 ml of the MH agar containing iron chelator DFO at 40 µM concentration and poured into Petri plates and allowed to solidify. Sterile disks containing 10 µl of different iron sources hemin (bovine), hemoglobin (human), ferrichrome, cepabactin, alcaligin and rhodoturilic acid were placed on the surface of the agar. Twenty-four to forty-eight hours after the incubation, the plates were observed for zones of growth around the filter disks.

*Chicken colonization studies*

The three mutants- Δ*p19*, ΔCj1658 and Δ*chuA* were tested for their ability to colonize the chicken ceca. A group of five chicks were inoculated orally with 1.75 to 3.28 X $10^5$ viable *C. jejuni*. After 4 days of infection, chicks were humanely euthanized and their cecal contents were harvested, diluted and plated on the Karmali-agar plates as described in our previous study (7). Viable counts were expressed as the logarithms of CFU per gram of cecum. The data were statistically analyzed using non-parametric Mann-Whitney rank sum test. Values of $P<0.05$ were regarded as statistically significant.

## Results and discussion

### Growth promotion assay

Growth promotion assay was used to test the ability of the mutants Δ*p19*, ΔCj1658 and Δ*ChuA* to use different iron compounds on an iron chelated agar medium. The iron compounds used were hemin (bovine), hemoglobin (human), ferrichrome, cepabactin, alcaligin and rhodoturilicacid .

Δ*P19* and ΔCj1658 mutants were not affected in the uptake of the compounds tested when compared to the wild type strain. However, Δ*ChuA* mutant was affected in its utilization of hemoglobin (human) and hemin (bovine), while the wild type strain showed a small zone of growth around the disk (Figure 1). This data suggests that the ChuA protein is the heme receptor. The *C. jejuni* NCTC 11168 strain used in our study appears to have the same phenotype as that of other strain used in the earlier study.

### All the three mutants are not affected in the chicken colonization

As shown in Figure 2, the colonization level of the three mutants was not significantly different from that of the wild type strain suggesting that Δ*p19*, ΔCj1658 and Δ*ChuA* mutants were not affected in their ability to colonize the chicken ceca. Consequently these genes might not play a significant role in the chicken colonization. In addition, these experiments suggest that heme may not play a major role in deciding the colonization ability of *C. jejuni.* Similar findings were noted for *E.coli* strain that causes neonatal meningitis in neonatal rats (6). In this study, the *E.coli* mutant in the hemin receptor ChuA was not attenuated in producing the bacteremia while the mutant in another siderophore receptor IroN (for the siderophore salmochelin) indicated a major

role of this gene during the bacteremic step (6). Similarly, in *Yersinia pestis* the deletion of the hemin uptake locus (Hmu) system had no effect on virulence in a mouse model of bubonic plague indicating that the *hmu* system is not essential for infection in mice (12).

**Future directions to complement the present work**

Our microarray study has identified several genes that are iron and Fur-regulated. Though we have studied several of those genes in our study, there remains multitude of genes that could be further characterized. For example, the genes Cj1383c and Cj1384c that encode hypothetical proteins were highly repressed in response to the addition of iron suggesting that they might play a role in acquiring yet an unidentified iron source. In addition, Cj0203, Cj0891c, Cj1163c and Cj0339 genes that encode putative transmembrane transport proteins could possibly mediate the transport of an iron source. Direct mutagenesis of these genes as well as additional iron-regulated genes could be carried out by construction of knockouts in these genes. These mutants could be further characterized by growth promotion assay using several potential siderophores and other host iron-binding proteins. They would also be tested for their ability to colonize chicken ceca.

We have identified CfrA as the enterobactin receptor in our study. The mutant in the gene *ceuE* that has been annotated to be putative enterobactin uptake permease was slightly affected in utilization of enterobactin as evident by the results of growth promotion assay. But the mutant in the gene Cj0178 was not affected in enterobactin utilization. Since enterobactin is known to break down into monomers, dimers and trimers of

dihydroxylbenzoylserine (DHBS) in aqueous solution, each of these breakdown products can be used to test whether they promote the growth of the mutants in growth promotion assays. In addition, radiolabelled enterobactin uptake assays would be used to assess the ability of these mutants to use enterobactin.

Consensus sequence for the Fur box has so far been only putative in *C.jejuni.* The identification of the Fur box can be done using recombinant Fur protein by gel shift and DNAase I footprinting assays.

Future experiments on the response of *C. jejuni* to oxidative stress would include studying both the time and dose dependent response to hydrogen peroxide (HP). Several concentrations of HP would be tried, say 2mM, 5mM and 10mM. In addition, response to a particular concentration of HP would be investigated over a period of 30 minutes in a time course dependent study.

Directed mutagenesis of the genes *ahpC, katA, sodB* and *trxB* would help better characterize the *Campylobacter* response to oxidative stress with respect to HP, CHP and menadione. In particular, construction of an *ahpC* mutant and testing its sensitivity to CHP would explain the apparent contradiction we found with respect to the expression of *ahpC*. Since the *fur* mutant was also resistant to the HP based on the disk inhibition assay, it would be interesting to further explore the role of Fur in the oxidative stress in *C. jejuni*. Microarray experiments with the *fur* mutant and the wild type strain to compare their response to oxidative stress would help identify the genes that are possibly regulated by the Fur protein.

Finally, all these mutants in the genes responsible for iron uptake and oxidative stress defense would be tested in a piglet model along with the wild type strain in the in vivo competitive assays. Equal numbers of both the wild type strain and each of the mutants would be used to infect colostrum deprived day-old piglet. At four days post infection, the animals would be sacrificed and the intestinal contents would be harvested, diluted and plated to isolate the mutant or the wild type strains that were able to colonize intestine.

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**Table 1.** List of primers used in the study. The bold letters indicate the restriction enzyme sites.

| Name of the primer | Sequence (from 5' to 3') |
|---|---|
| ChuA-01 | ATGC**GAGCTC**ATGCGGATTCAAATTTACGC |
| ChuA-02 | ATGC**CTGCAG**ATTGCGAATTCCTGCTAAAA |
| ChuA-03-*BamHI* | CGA**GGATCC**TGTTTGGCACCATTGATACCT |
| ChuA-04-BamHI | CGA**GGATCC**GGTGGGATTTTTACGGCTTT |
| P19-01 | ATGC**AGATCT**GGCTACTTTCAAACGCACAA |
| P19-02 | ATGC**AGATCT**AAACAAGCAAGGCTAAAGCAA |
| P19-03 | ATGC**GGATCC**ACCTCTTGGCTCCATTTCAA |
| P19-04 | ATGC**GGATCC**GACGCCATGTTGATGAAGAA |
| Cj1658-01 | ATGC**AGATCT**GCTCAGCTTTTGCTAGGGTAGA |
| Cj1658-02 | ATGC**AGATCT**GAGGGATTAAAGTTTCATAGTAAGGA |
| Cj1658-03 | ATGC**GGATCC**GTTTGGCATTGGCTTCTAGG |
| Cj1658-04 | ATGC**GGATCC**ACCGCTTTTGGAGTTTCTTG |

**Table 2.** List of strains used in this study

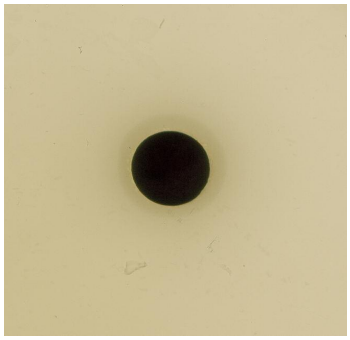
| Strain or plasmid | Relevant characteristics | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| DH5α | *endA1 hsdR17* ($r_k^- m_k^-$) *supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYAargF U169deoR [f80dlac*Δ*(lacZ0 M15]* | Invitrogen |
| ***C. jejuni*** | | |
| AS144 | *C. jejuni* NCTC 11168 | NCTC |
| AS 235 | *C. jejuni* NCTC 11168 Δ *chuA::Cam*$^r$ | This study |
| AS 256 | *C. jejuni* NCTC 11168 Δ *p19::Cam*$^r$ | This study |
| AS 260 | *C. jejuni* NCTC 11168 Δ Cj1658::*Cam*$^r$ | This study |
| **Plasmids** | | |
| pUC19 | Cloning and suicide vector, *Amp*$^r$ * | Biolabs |
| pRY111 | Chloramphenicol resistance gene, *cam*$^r$** | (15) |
| pAS 232 | pUC19 carrying *chuA* | This study |
| pAS 233 | pUC19 carrying Δ *chuA* | This study |
| pAS 234 | pUC19 carrying Δ *chuA::cam*$^r$ | This study |
| pAS 253 | pUC19 carrying *p19* | This study |
| pAS 254 | pUC19 carrying Δ *p19* | This study |
| pAS 255 | pUC19 carrying Δ *p19::cam*$^r$ | This study |
| pAS 257 | pUC19 carrying Cj1658 | This study |
| pAS 258 | pUC19 carrying Δ Cj1658 | This study |
| pAS 259 | pUC19 carrying Δ Cj1658*:: cam*$^r$ | This study |

*Amp$^r$, ampicillin resistant, **cam$^r$, chloramphenicol resistant

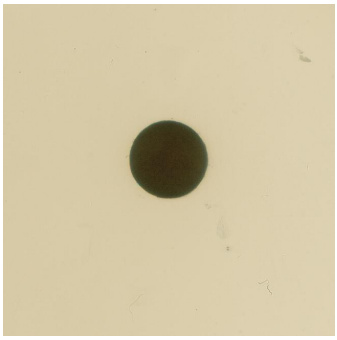
**Figure 1**

Results of growth promotion assay. Both the *C. jejuni* wild type and the chuA mutant were tested for their ability to use hemoglobin and hemin. While we see a zone of growth around the filter disk containing the heme compounds was seen in the case of the wild type strain, no such zone of growth was observed in the *chuA* mutant.

A) Hemoglobin (Human)

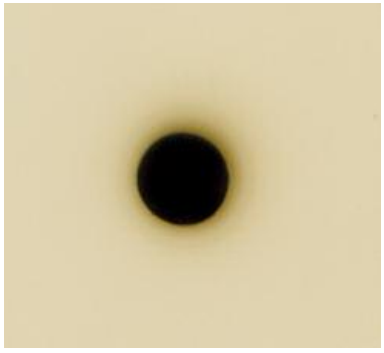


NCTC 11168

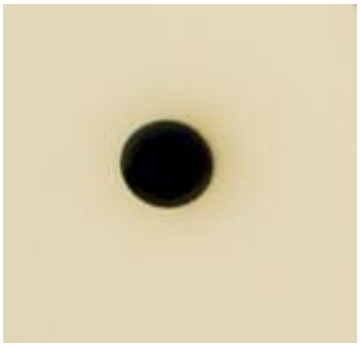


Δ*ChuA*

B) Hemin (Bovine)



NCTC 11168

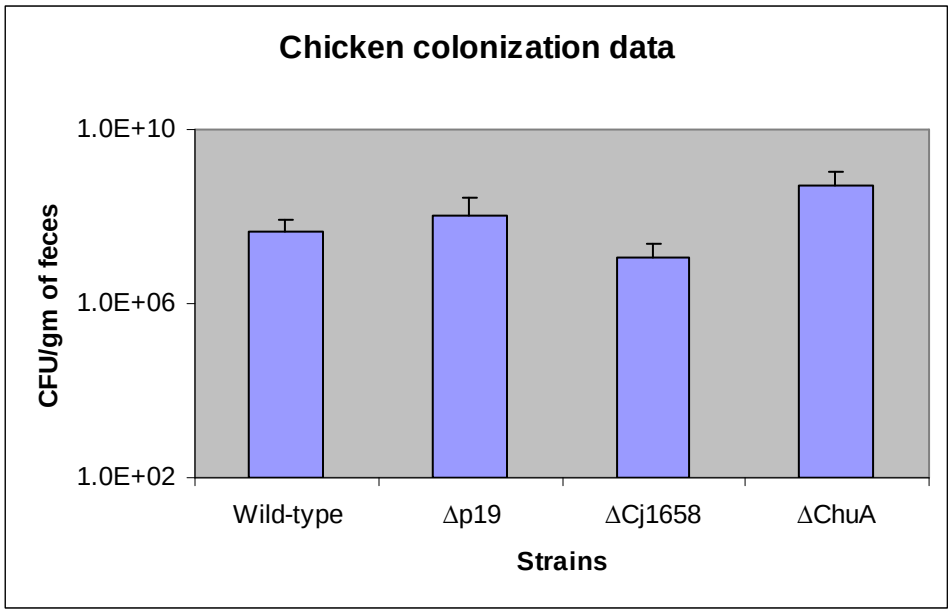


Δ*ChuA*

**Figure 2**

Chicken colonization properties of the strains Δ*p19*, ΔCj1658 and Δ*chuA.*

Groups of five chicks were inoculated orally with *C. jejuni* wild type or mutant strains. The bars represent the means of values from five birds and error bars represent the standard deviation. None of the three mutants were significantly ($P<0.05$) affected when compared to the wild type strain using non parametric Mann-Whitney test.


Chicken colonization data
1.0E+10
1.0E+06
1.0E+02
CFU/gm of feces
Wild-type
Δp19
ΔCj1658
ΔChuA
Strains

# CHAPTER VI

## CONCLUSIONS

The purpose of this research was to evaluate the *Campylobacter jejuni* response to iron and hydrogen peroxide. We used DNA microarrays to achieve our objectives. The transcriptional response of *C. jejuni* to iron availability was found to be pleiotropic involving the differential expression of genes belonging to several functional groups. The transcript levels of around 647 genes were affected following the addition of iron to iron-limited *C. jejuni* cells over a 15 minute time period. In contrast, 208 genes were differentially expressed during balanced growth (mid-log experiment). This clearly highlights the impact of the microarray experimental design on gene expression analysis suggesting that the time course experiments yield data that are significantly different from steady state experiments. Genes involved in iron acquisition were down regulated during both the time course and steady state experiments while genes encoding proteins involved in energy metabolism were up-regulated. We also showed that iron might modulate the level of virulence by affecting the degree of protein glycosylation. Fur regulates the genes involved in the iron metabolism in most of the Gram-negative bacteria. Hence, a mutant in the *fur* gene was constructed in *C. jejuni* to identify the Fur regulon. A total of 53 genes were found to be Fur regulated when the transcriptional profile of the wild type strain and the *fur* mutant was compared. The *fur* mutant was found to be significantly affected in its ability to colonize the chick ceca highlighting the

importance of iron homeostasis *in vivo*. We further extended our study by directed mutagenesis of the genes identified by microarray. These genes were *cfrA, ceuE,* Cj0178, *p19,* Cj1658 and *chuA.* Results of growth promotion assay allowed the characterization of CfrA as the enterobactin receptor and ChuA as the heme receptor. While the *cfrA, ceuE,* Cj0178 mutants were affected in chicken colonization, *p19,* Cj1658 and *chuA* were not.

Since the iron availability and generation of ROS are closely linked through the Fenton reaction, we further investigated the *C. jejuni* response to oxidative stress using microarrays. Transcript level of 40 genes was induced in the iron restricted cells due to the addition of hydrogen peroxide (HP) which included catalase gene (*KatA*) in addition to many iron regulated genes. Thirty genes were induced in iron-loaded cells upon addition of HP. Cells in iron rich condition responded with induction of both *katA* and *ahpC* indicating the possibility of more damage due to the presence of iron. Our study also suggested the possible role of TrxB in the recycling of the AhpC enzyme. An isogenic *perR* mutant was constructed to identify the genes involved in the oxidative stress response by transcriptome profiling with the wild type strain. *KatA* was found to be in the PerR regulon in both iron rich condition and iron limited conditions. Surprisingly, *ahpC* gene which was earlier shown to be under PerR control was not in the PerR regulon suggesting an alternate mode of control in the *C. jejuni* strain used in our study. Significant number of genes involved in flagellar biogenesis and motility were found to be PerR regulated. Repression of these genes by PerR would probably reduce the production of ROS since flagellar biogenesis and motility consumes a significant amount of energy within the cell which in turn results in the production of more ROS. Menadione induced the highest number of genes when compared to HP and CHP owing

to its redox cycling nature. CHP and menadione induced higher number of genes belonging to the DNA replication/repair category suggesting that these agents might be more damaging to the bacterial cell than HP at 1mM concentration tested. Oxidative stress resulted in the repression of a large number of genes involved in the energy metabolism probably reflecting a temporary growth arrest. In addition, this repression could also serve to reduce the aerobic respiration which is the main source of the endogenously produced ROS. Disk inhibition assay demonstrated that the Δ*fur* mutant was resistant to HP suggesting that the Fur protein might be involved in oxidative stress defense. Generally, hydroperoxides are cleared by the enzyme AhpC in bacteria. However, *ahpC* was not induced upon addition of CHP and it was not a selected as part of the PerR regulon in our study. In addition, the Δ*perR* mutant was sensitive to CHP. Taken together these results suggest that the *C. jejuni* strain used in our study uses an alternate mechanism to clear CHP stress. The inability of the *perR* mutant to colonize the chicken ceca demonstrated that the PerR is required of the *in vivo* growth.

Taken together our study clearly suggests that *C. jejuni* has developed two similar and possibly overlapping regulatory mechanisms in the form of Fur and PerR. It appears that Fur and PerR regulate the expression of these genes depending on the iron and oxidative stress status of the cell. Our study supports the fact that the dual regulation of genes by PerR and Fur might play a role in fine tuning of the *C. jejuni* response to the environmental conditions encountered during its life cycle.

**APPENDIX**

# Use of Genome-Wide Expression Profiling and Mutagenesis to Study the Intestinal Lifestyle of *Campylobacter jejuni*

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This paper has been accepted in the Journal -Infection and Immunity

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## Summary of my role in this work

During my PhD program I also co-authored a paper which has been accepted in the Journal 'Infection and Immunity' and will be published in the near future. My role in this publication is described below:

I have constructed three mutants- ΔCj0178, Δ Cj0571 and Δ*spoT* -that have been used in this study. One of the mutants, *C. jejuni* ΔCj0178, also has been previously used in our study and has been described in the chapter III of this thesis. In addition, I was also involved in the *in vivo* competition experiments where different mutant strains were tested for their ability to survive and grow in the rabbit ileal loop.

## Abstract

*Campylobacter jejuni* is the most common bacterial cause of diarrhea worldwide. To colonize the gut and cause infection, *C. jejuni* must successfully compete with endogenous microbes for nutrients, resist host defenses, persist in the intestine, and ultimately infect the host. These challenges require the expression of a battery of colonization and virulence determinants. In this study, the intestinal lifestyle of *C. jejuni* was studied using whole genome microarray, mutagenesis, and a rabbit ileal loop model. Genes associated with a wide range of metabolic, morphological, and pathological processes were expressed in vivo. The in vivo transcriptome of *C. jejuni* reflected its oxygen-limited, nutrient-poor, and hyper-osmotic environment. Strikingly, the expression of several *C. jejuni* genes was found to be highly variable between individual rabbits. In particular, differential gene expression suggested that *C. jejuni* extensively remodels its envelope in vivo by differentially expressing its membrane proteins and by modifying its peptidoglycan, and glycosylation composition. Furthermore, mutational analysis of 7 genes, *hspR*, *hrcA*, *spoT*, Cj0571, Cj0178, Cj0341, and *fliD*, revealed an important role for the stringent and heat shock response in gut colonization. Overall, this study provides new insights on the mechanisms of gut colonization, as well as possible strategies employed by *Campylobacter* to resist or evade the host immune responses.

## Introduction

*Campylobacter* is the most common non-viral etiological agent of infectious enteritis in humans and has been implicated in 14.2% of the 76-million food-borne illnesses reported annually in the United States (21). *Campylobacter* infections vary from mild diarrhea to severe abdominal pain (49). Rarely, they result in the development of Guillain-Barré syndrome, which is the primary cause of acute neuromuscular paralysis in the US (49). Because of the extremely high number of cases of food-borne infections reported yearly worldwide, the development of new strategies to fight these infections is urgently needed, and will depend on developing an understanding of host-pathogen interactions. The complete genomic sequence of *C. jejuni* NCTC 11168 was released in 2000 (27) providing new opportunities for the investigation of *Campylobacter* pathogenesis.

Despite the high incidence of *Campylobacter*-mediated diarrhea, the microbial factors that govern gut colonization and pathogenesis are poorly understood in comparison with other enteric pathogens. In a complete infection cycle, *Campylobacter* cells are transferred from contaminated foods to the stomach, the intestinal tract and, finally, to the feces, allowing their transmission to a new host (6, 13). During this stressful journey through the gastrointestinal tract, *Campylobacter* encounters and must adapt to life-threatening environmental conditions, such as the acidic pH of the stomach, the high osmolarity of the gastrointestinal tract, intestinal gases, reactive oxygen and nitrogen compounds, changes in nutrient availability, and low inorganic ion concentrations (13). For successful colonization, *Campylobacter* cells must survive in the intestinal tract, either as free living microorganisms in the mucus layer, attached to the epithelium, or intracellularly in epithelial cells (6). *Campylobacter* determinants involved

in colonization and pathogenesis include flagella, host cell adherence and invasion, and toxin production (6). Non-motile and aflagellated *Campylobacter* were shown to be affected in their ability to colonize the gastrointestinal tract as well as to invade the epithelial cells (25, 56). Many suspected adhesins have been identified, such as lipo-oligosaccharide (20), flagella (56), or surface exposed proteins (CadF, PEB1) (23, 29). Host cell invasion has been extensively studied and is thought to be an important step in *Campylobacter* infection (6). Indeed, biopsies of humans diagnosed with *C. jejuni* enteritis revealed the presence of intra-cellular *Campylobacter* cells (47). Interestingly, toxin production has recently been proposed to modulate the host immune response, allowing the bacteria to escape the immune surveillance (9).

To identify new potential virulence factors, we analyzed *C. jejuni* lifestyle in the gut using microarray technology. The *C. jejuni* NCTC 11168 genome-wide expression profile was assessed during host colonization and pathogenic development, using a mammalian model of gastroenteritis, the rabbit ileal loop model. In addition, mutants were constructed by deleting genes of interest identified by our microarray analysis, and assessed for their ability to survive in the gastrointestinal tract of rabbits.

## Materials and Methods

*Bacterial strains, plasmids, and preparation of inocula.*

The bacterial strains and plasmids used in this work are listed in Table 1. The *Campylobacter jejuni* NCTC 11168 strain was acquired from the National Collection of Type Culture (NCTC, England) in the spring 2000. *Campylobacter* strains were cultured in Mueller-Hinton (MH) medium or on MH agar plates at 37°C in a microaerophilic chamber (Don Whiteley, West Yorkshire, England). Chloramphenicol resistant mutants were maintained on MH medium supplemented with 20 µg/ml of chloramphenicol.

*C. jejuni* inocula were prepared by microaerobic culture (84% $N_2$, 5% $O_2$ and 11% $CO_2$) in MH medium at 37°C with agitation using a stirrer. The bacterial growth was monitored by measuring the optical density at 600 nm. At early mid-log phase (OD600 of approximately 0.3), the bacterial culture was split in two and one half was used to produce purified total RNA from *C. jejuni* grown in vitro, while the other half was used to inoculate the rabbit ileal loops. Bacteria were collected by centrifugation (10 min, 6000 x g), washed once with sterile PBS buffer, and resuspended in PBS buffer at a concentration of approximately 6.6 x $10^{10}$ cfu/ml.

For the in vitro growth experiments, the *C. jejuni* wild-type and mutant strains were grown in MH biphasic medium at 37°C under microaerophilic conditions (83% $N_2$, 4% $H_2$, 8% $O_2$ and 5% $CO_2$).

*Rabbit ileal loop model (RIL) and isolation of Campylobacter jejuni total RNA.* Rabbits were checked upon arrival to see if they carried *Campylobacter* by taking cloacal swabs for culture. Ileal loops were prepared according to published methods (3, 8). Briefly, New

Zealand White rabbits (<2 kg, female) were anesthetized, a laparotomy was performed, and two 20-cm sections of ileum with intact mesenteric blood supply were ligated per animal. Each loop was inoculated with approximately $10^{11}$ mid-log phase *Campylobacter jejuni* in 1.5 ml PBS buffer. The size of the inoculum was confirmed by bacterial enumeration on MH agar plates. Loops of two rabbits were injected with sterile PBS buffer and served as control animals. After replacing the intestinal loops in their appropriate position in the abdominal cavity, the abdominal wall and skin were closed in standard fashion and the rabbits were allowed to recover from anesthesia. The rabbits were anesthetized again 24 or 48 hours after the inoculation, the intestinal loops were excised intact, and the animals were then euthanized.

RNA turnover in the samples was quickly stopped by submerging the entire loops into 10 ml of RNAlater solution (Ambion, Austin, TX). The loops were first weighed in order to evaluate fluid accumulation and the contents of each loop as well as the mucus layer then were recovered into 20 ml of a 50% solution of RNAlater in PBS buffer and centrifuged at low speed to remove epithelial cells (5 min at 1,000 x g). Thereafter, *Campylobacter* bacteria were separated from the intestinal microflora by filtration through 0.8 µm filters. *Campylobacter* cells were pelleted by centrifugation and total RNA was isolated using a hot phenol-chloroform protocol, as previously described (40). Traces of genomic DNA were removed by two or three consecutive treatments with DnaseI Amp grade enzyme (Invitrogen, Carlsbad, CA). The absence of contaminating genomic DNA was confirmed by polymerase chain reaction (PCR). RNA was further purified 2 to 5 times using a Qiagen RNAeasy mini kit (Qiagen, Valencia, CA) and the concentration of RNA was determined using RiboGreen RNA quantitation reagent (Molecular Probes, Eugene, OR).

*Microarray construction and hybridizations***.**

DNA microarrays were prepared using PCR amplified fragments of each annotated open reading frame from *C. jejuni* NCTC 11168, as previously described (40, 41). Twenty µg of total RNA from each growth condition (in vitro and in vivo) were converted to cDNA using 2 pmol of *C. jejuni* 3' specific primers (set of 1654 3' primers used for the PCR amplification of *C. jejuni* ORFs) and coupled to monoreactive fluors (Cy3 and Cy5), according to previously described procedures (40).

*Data collection and analysis.*

Microarray slides were scanned at 532 nm (Cy3) and 635 nm (Cy5) wavelengths with a laser-activated confocal scanner (ScanArray 3000) at 10 µm resolution, generating two TIFF images. Fluorescence intensities of each spot were collected using the GenePix Pro 3.0.5 software (Axon Instruments, Foster City, CA.) after manual optimization of spot registration, and exported to OriginPro 7 spreadsheets (OriginLab Corporation, Northampton, MA). The analysis of the fluorescence data was conducted as follows: (1) The spots were filtered and excluded based on slide abnormalities or low signal (corresponding to spots flagged bad or not found). (2) After background subtraction, all spots with fluorescent mean intensities below three times the standard deviation of the background in both channels were removed from the final data analysis. (3) The fluorescence intensity in each wavelength was $\log_2$ transformed and normalized using locally weighted linear regression (lowess) performed by the MIDAS software (available from TIGR; http://www.tigr.org/software/).

For the microarray analysis, *Campylobacter* RNA was isolated 48 hours post-infection from five RIL rabbits. Each cDNA sample was individually co-hybridized with cDNA obtained from in vitro growth (mid-log phase bacteria) on microarray slides. The microarray hybridization was repeated up to three times depending on the amount of RNA purified from each rabbit, yielding between 2 and 6 measurements per gene per rabbit (each gene was spotted in duplicate on each slide). The microarray data were statistically analyzed using the "Significant Analysis of Microarray (SAM)" algorithm, which was specifically developed for genomic expression data mining (the Microsoft Excel add-in software is available at http://www-stat.stanford.edu/~tibs/SAM/) (46). Briefly, SAM uses the standard deviation of repeated gene expression measurements to assign a score to each gene. It then estimates, for a particular score, a false discovery rate by permutations of the data. This SAM analysis ascertains that genes identified as "differentially expressed" do not arise from a random fluctuation of the large quantity of data generated (46). To identify genes whose expression differed significantly between in vivo and in vitro growth, we performed a one class response analysis by considering the five rabbits as one class. We applied a false discovery rate of 0.11% and a delta value of 0.9. To identify genes with variable expression between rabbits, we performed a multiple classes analysis by treating each rabbit as one class. We applied a false discovery rate threshold of 1.64% and a delta of 0.19. The microarray data of SAM positive genes were extracted into a text output file using the Samster software (available at http://falkow.stanford.edu/whatwedo/software/software.html). Finally, the ratios of the fluorescence intensities of all replicate spots from the hybridization of RNA derived from

each rabbit were averaged and used for further analysis. The data generated by this study are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab.

*Campylobacter jejuni mutants construction.*

Knockout mutants of *C. jejuni* NCTC 11168 were constructed by independently mutating 6 genes; *hrcA*, Cj0571, *spoT*, *hspR*, Cj0341 and *fliD*. The same inactivation strategy was used for the *hrcA*, Cj0571, *spoT*, and *hspR* mutants. Briefly, the gene to be mutated was amplified by PCR from *C. jejuni* NCTC 11168 chromosomal DNA, which was extracted using a standard protocol (35). The PCR product was cloned into pUC19, using a unique restriction site (Table 2), and deletions of 42 bp, 600 bp, 627 bp, and 72 bp were made by inverse PCR in *hrcA*, Cj0571, *spoT*, and *hspR*, respectively. The chloramphenicol resistance cassette ($Cam^r$) was PCR amplified from pRY111 (55) using primers with appropriate restriction sites and cloned into the deletion site. Specific primers used for the first PCR amplification and the following inverse PCR are listed in Table 2. Recombinant plasmids carrying the $Cam^r$ gene in the same orientation as the genes of interest were selected by DNA sequencing and transformed into *C. jejuni* NCTC 11168 using standard protocols (55). Transformants were identified on MH agar plates containing 20 µg/ml of chloramphenicol. The identity of the mutants was confirmed by PCR analysis using a combination of primer sets annealing within the mutated gene and the $Cam^r$ gene.

The Cj0341 and *fliD* mutants were isolated from a library of random mutants generated using the EZ::TN$^{TM}$ pMOD$^{TM}$-3<R6Kγori/MCS> transposon (Epicentre), in which the $Cam^r$ cassette from pRY111 has been cloned (Stintzi, unpublished data). The mutant library was constructed following the manufacturer's recommendation, and the Cj0341

and *fliD* mutants were identified during the first trial to assess the randomness of the library. The insertion site of the transposon in the Cj0341 and *fliD* mutants was identified using a single primer PCR procedure and DNA sequencing of the resulting amplification product, as described by others (16). The Cj0341 and fliD mutations were confirmed by PCR amplification using a primer that anneals within the mutated gene and another primer that anneals within the $Cam^r$ gene.

*In vivo survival assays using a pool of mutants.*

The ability of the *C. jejuni* mutants to survive within the rabbit ileal loops was assessed. Each mutant was grown individually in MH broth to mid-log phase, harvested by centrifugation (10 min, 6000 x g), washed and resuspended in PBS buffer. The mutants were mixed to constitute the input pool by combining $5 \times 10^9$ cfu of each mutant with $7.5 \times 10^{10}$ cfu of the wild-type strain, *C. jejuni* NCTC 11168, in 5 ml of PBS buffer. One ml of this suspension was used to prepare genomic DNA following standard protocols. The remaining bacterial suspension was used to equally inoculate 4 ileal loops from 2 rabbits (1 ml per loop). At 48 hours post-infection, loop contents were harvested in PBS buffer and centrifuged at low speed to remove debris and epithelial cells. The supernatant was immediately processed to prepare genomic DNA, constituting the recovered pool of genomic DNA. Each loop was processed individually.

The difference in relative abundance of each mutant between the input and recovered pools was evaluated by quantitative PCR, which was performed using an ABI Prism 7700 DNA analyzer (Applied Biosystems, Foster City, CA) and the QuantiTect™ SYBR® Green PCR kit (Qiagen, Valencia, CA), according to the following protocol: 500 ng of

genomic DNA was added to 25 µl of 2xQuantiTect SYBR Green PCR solution and 0.3 µM of each specific primer in a 50 µl final volume. The HotStar Taq DNA polymerase was activated by heating the reaction at 95°C for 15 min. PCR amplification was performed by 40 cycles of denaturation at 94°C for 15 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s. The specificity of the PCR reaction was confirmed by melting curve analysis of the PCR product following the manufacturer's recommendations (Applied Biosystems, Foster City, CA). Genomic DNA of each mutant was specifically amplified from both pools by using a combination of two primers, with one of them annealing within the mutated gene and the other within the chloramphenicol resistant cassette. The sequences of the specific primer sets are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab. The relative abundance of each mutant was then normalized to the DNA pool by using the *cydA* or *argD* genes. The competitive ratio of the relative abundance of each mutant between the input and recovered pools was obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, as recommended by Applied Biosystems. The abundance of each mutant was assessed twice per loop and the mean $C_T$ value for each ileal loop was used for further analysis. The $C_T$ value corresponds to the PCR threshold cycle at which the fluorescence detected is significantly higher than the baseline value. The ratio of the mutant in the input to the recovered pool was calculated as follows: ratio input/recovered = $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T = \Delta C_{T,\ recovered} - \Delta C_{T,\ input}$, and $\Delta C_{T,\ recovered\ or\ input}$ is obtained by subtracting the mean $C_T$ value of the specific gene from the mean $C_T$ value of the reference gene (*cydA* or *argD*) in the genomic DNA from the input or recovered pool. Given that the wild-type strain was inoculated at a higher level than the mutants, the population as a whole (the mutants

plus the wild-type strain) should have a growth similar to the wild-type strain alone. The same assumption is usually made for the analysis of data generated by signature tag mutagenesis (10). Consequently, the normalized competitive ratio input/recovered of *C. jejuni* NCTC 11168 should be approximately equal to 1. A competitive ratio input/recovered above 1 indicated that the mutant was attenuated in vivo, while a competitive ratio below 1 indicated that the mutant survived better in vivo than the wild-type. The data was statistically analyzed using the Student *t*-test and a *P* value below 0.01 was considered significant.

*In vitro and in vivo competition experiments.*

*C. jejuni* wild-type and mutant strains were grown in biphasic MH medium to mid-log phase, centrifuged and resuspended in PBS buffer to $OD_{600} \approx 1.8$ (approximately $10^{10}$ cfu/ml). Two milliliter (each) of the wild-type and the mutant strains were mixed at a 1 to 1 ratio. Four ileal loops (from 2 rabbits) were injected (each) with 1 ml of this suspension. The initial 1:1 mixture of mutant and wild-type strains was confirmed by plating serial dilutions of this mixed culture on MH agar with and without chloramphenicol (20 μg/ml). At 48 hours post-infection, the loops were recovered as described above. Their content as well as the mucus layer were collected and homogenized in 10 ml of PBS buffer. Serial dilutions of bacteria recovered from each rabbit's loop were plated on karmali-agar plates (*Campylobacter* agar base (Oxoid CM935) supplemented with the *Campylobacter* selective karmali supplements (Oxoid SR167E)) and karmali-agar plates containing chloramphenicol (20 μg/ml). Plates were incubated at 37°C for 3 days before the colonies were counted. The titer of the mutant

was obtained from the CFU recovered on karmali-agar plates containing chloramphenicol, and the titer of the wild-type bacteria was calculated by subtracting the number of mutants to the total number of bacteria recovered on karamali-agar plates without antibiotic. Finally, the in vivo competitive index was calculated for each loop and corresponds to the ratio of the mutant to the wild-type strain.

For the in vitro competition assays, overnight cultures of the wild-type and mutant strains were mixed in a 1:1 suspension into fresh MH medium. This suspension was used to inoculate three replicate biphasic MH cultures. The bacterial growth was monitored by measuring the $OD_{600}$ over time. The titer of each strain was determined in the inoculum, and at early stationary phase, by plating on MH-agar and MH-agar containing chloramphenicol (20 μg/ml). The in vitro competitive index was calculated for three independent growth experiments and is defined as the ratio of mutant to wild-type.

A Student's *t* test was used to statistically analyze the data from the in vivo and in vitro competition assays.

*Real-time quantitative RT-PCR analysis.*

The relative expression of 9 genes (*flgE2*, Cj0178, *katA*, *spoT*, *ahpC*, *fliD*, Cj0571, *cydA*, and Cj0366) was confirmed by real-time quantitative RT-PCR, as previously described (40), using the QuantiTectTM SYBR Green RT-PCR kit (Qiagen, Valencia, CA), according to the manufacturer's recommendations. The relative expression of each gene was normalized to either the 16S or 23S RNA, and the extent of its induction was obtained using the comparative threshold cycle ($\Delta\Delta C_T$) method, as described above. The

primers used are available online at http://www.cvm.okstate.edu/research/Facilities/CampyLab.

*Necropsy and histopathology.*

Four rabbits were used to evaluate the pathological changes in the RIL model caused by *C. jejuni* NCTC 11168. The loops were prepared as described above for the transcriptional profiling experiments. The loops from two rabbits were inoculated with 1 x $10^{11}$ cfu of mid-log phase *C. jejuni* NCTC 11168, while the loops of the two other rabbits were injected with sterile PBS buffer. At 48 hours post-infection, the rabbits were anesthetized, the loops were recovered, and the animals were then euthanized with an overdose of Beuthanasia D (>0.25 ml/kg of body weight). A 0.5 cm middle section of each loop was immediately excised, flushed with formalin, linearly opened, placed flat on a card, and fixed in buffered 10% formalin, embedded in paraffin, section at 5 μm and stained with hematoxylin and eosin. Specimens were examined for evidence of inflammation, villus epithelial cell attenuation and crypt dilatation or hyperplasia.

## Results and Discussion

### The rabbit ileal loop (RIL) model for campylobacteriosis.

This animal model was initially chosen because of its documented ability to accurately model the histopathological lesions associated with human *Campylobacter* gastroenteritis (8). In addition, *Campylobacter* cells can be collected in a number sufficient for the investigation of in vivo genome-wide transcripts abundance. The model was created by surgical ligation of 20 cm sections of ileum, resulting in the cessation of the normal peristaltic flux, thereby facilitating *Campylobacter* gut colonization. The strain of *C. jejuni* NCTC 11168 used in our study is helically shaped, fully motile, and colonizes the gastrointestinal tract of chicks (26). Therefore, this strain is phenotypically different from the sequenced *C. jejuni* NCTC 11168 strain recently described by Gaynor et al. (11), which was described to be straight rod-shaped, non-motile, and a poor colonizer of chicks. To explore the feasibility of this model to study *Campylobacter* lifestyle in the gut by transcriptome profiling, we undertook a pilot study involving 4 rabbits: Rabbit intestinal loops were sampled at 24 or 48 hours post-inoculation (using 2 rabbits per time point). To note, the *C. jejuni* strain was passaged three times in vitro before its inoculation in the ileal loops. *C. jejuni* NCTC 11168 colonized the rabbit gut at bacterial concentrations of $10^5$ cfu/loop and $10^7$ - $10^9$ cfu/loop, at 24 and 48 hours post-inoculation, respectively. The initial decrease in bacterial population from $10^{11}$ to $10^5$ cfu per loop during the first 24 hours after inoculation reflects the challenges of surviving in a hostile environment, while the subsequent bacterial growth from $10^5$ to $10^9$ cfu/loop at 48 hours suggests the successful adaptation and colonization of *C.jejuni* in the rabbit intestinal

tract. Considering that $10^5$ cells would not generate sufficient amounts of RNA to perform microarray hybridization, we decided to harvest the bacterial cells at 48 hours post-infection in the present study.

Intestinal distension resulting from accumulation of gas and fluid (the first signs of diarrhea) was qualitatively observed at 48 hours post-infection in all infected rabbits but not in the control animals injected with the PBS buffer. Two infected rabbits and two control animals were used to evaluate pathological changes. Fluid accumulation was quantitatively estimated by weighing the intestinal content of the 8 loops, which indicated an increase of 0.09 ± 0.05 g content/g of ileal tissue in the infected loops compared with control loops. This difference was found to be statistically significant ($P < 0.05$ using a paired *t*-test). In contrast to the observations of Everest *et al*. (8), histopathological analysis of the ileal tissues did not reveal any severe pathology. This lack of damages likely reflects the inability of *C. jejuni* NCTC 11168 to invade epithelial cells or to exert morphologically evident cytotoxic effects on intestinal epithelial cells. In fact, the strain of *C. jejuni* NCTC 11168 used in our study is poorly invasive into human epithelial INT407 cells (30). Consequently, the transcriptome profile presented in this work reflects non-invasive *Campylobacter* lifestyle in the intestine during survival, colonization and the initial stages of pathogenesis.

**In vivo expression profiling validation**.

While microarrays provide a powerful approach for the investigation of gene expression, the performance of these expression studies *in vivo* is technically challenging. To date, expression profiling experiments have been limited mainly to in vitro environments. In

the present study, in vivo colonization of the intestinal tract by *C. jejuni* was investigated by conducting transcriptional expression profiling experiments during growth and survival within the natural gut environment. We utilized microarrays containing spotted PCR products representing approximately 98% of the annotated open reading frames of *C. jejuni* NCTC 11168 (40). The challenge of recovering intact *C. jejuni* mRNA from the intestine to ensure acquisition of an accurate and specific transcriptome profile was addressed by excising the entire intestinal loops and immediately submerging them in RNA stabilization solution to block RNA turnover. In order to minimize RNA degradation and/or changes in the gene expression level, loops were immediately processed for RNA extraction and quantitative histopathological traits were not recorded. The content of each loop, including the mucus layer, was recovered in RNA stabilization solution, and *C. jejuni* was purified by filtration through 0.8 µm filters. This physical separation removed most of the endogenous microflora; more than 80% of the bacterial population was estimated to be constituted of *C. jejuni*. The yield of RNA recovered was between 12 to 55 µg per loop. The total RNA extracted from each rabbit's two loops were combined. Twenty µg of RNA was reverse transcribed using *C. jejuni* specific 3' end primers and fluorescently labeled with the Cy5 dye which fluoresces red. The relative abundance of transcripts was monitored by competitive hybridization with RNA extracted from bacteria grown in vitro to mid-log phase and labeled with the green fluorescent Cy3 dye. To address any potential cross-hybridization with RNA extracted from the remaining natural intestinal microflora, RNA was also purified from ileal loops of rabbits which had been injected with PBS buffer only. The yield of total RNA purified from the uninfected loops was between 1 and 3 µg per loop. The total RNA harvested

from two uninfected loops were combined, reversed transcribed, labeled with Cy5 and hybridized to the *C. jejuni* microarray. As shown in Figure 1, this RNA did not cross-hybridize with genes from *C. jejuni*. In addition to physical enrichment of *C. jejuni* by filtration, the use of 3' specific primers to synthesize cDNA further enhanced the specificity of the assay. A similar approach was employed by Talaalt and coworkers to amplify mycobacterial RNA from a mixture containing mammalian RNA (43).

**Global gene expression analysis and validations**

*C. jejuni* NCTC 11168 was inoculated into 5 rabbits and colonized the loops of these rabbits at a bacterial concentration of $3.10^8$ cfu/loop (rabbit 1), $2.10^8$ cfu/loop (rabbit 2), $5.10^8$ cfu/loop (rabbit 3), $4.10^7$ cfu (rabbit 4), and $4.10^8$ cfu/loop (rabbit 5). *Campylobacter* RNA samples were extracted from each rabbit 48 hours post-infection and individually hybridized to the microarray slides up to three times, depending on the amount of RNA purified from each loop. Specifically, rabbits 1, 4 and 5 yielded two measurements per gene, rabbit 2 yielded six measurements per gene, and rabbit 3 yielded four measurements per gene. The data were quantified, normalized, and reported as the ratio of gene expression of *C. jejuni* grown in the rabbits to that of *C. jejuni* grown in vitro. To limit the number of genes falsely identified as differentially expressed, we performed a statistical procedure. This test consisted in applying the significance analysis of microarray (SAM) algorithm to our microarray data. This statistical method has been shown to be more reliable than a standard *t* test or the use of a fold change threshold, and is relatively conservative in declaring a significant change in gene expression (46). A one class response SAM analysis, using the five rabbits as one group, identified 348 genes as

being differentially expressed between in vivo and in vitro growth with a false discovery rate of 0.11%. All SAM selected genes exhibit expression ratios greater than 1.5. As demonstrated in our previous study using the same microarray platform, a 1.5 fold differential expression is technically and biologically significant (40). This *Campylobacter* microarray platform has previously been shown to generate data with a high level of concordance with quantitative RT-PCR (40). However, in order to address the reliability of the microarray data generated in this study, the change in transcript abundance in rabbit 4 between in vitro and in vivo growth was confirmed for 9 genes (*flgE2*, Cj0178, *katA*, *spoT*, *ahpC*, *fliD*, Cj0571, *cydA*, and Cj0366) by real-time quantitative RT-PCR. The *flgE2* gene was found to be 4 fold up-regulated, Cj0178 was 200 fold up-regulated, *katA* was 130 fold up-regulated, *spoT* was 61 fold up-regulated, *ahpC* was 4 fold up-regulated, *fliD* was 5 fold down-regulated, Cj0571 was 2.5 fold up-regulated, *cydA* was 350 fold up-regulated and Cj0366 was 300 fold up-regulated. Similarly to our previous study, while the quantitative RT-PCR confirmed the trend in differential gene expression observed with the microarray analysis, a quantitative difference in the fold change was observed between these two technologies. This difference reflects a lower dynamic range for the microarray experiments as compared to quantitative real-time RT-PCR, as previously reported by others (53). Notably, very few genes were found to be differentially regulated more than 20 fold by the microarray analysis while the real-time RT-PCR found several genes up-regulated more than 100 fold. This observation highlights the semi-quantitative nature of microarray experiments and the low dynamic range of this technology (4). Furthermore, this technical limitation appears to be amplified in situations where a gene exhibits a very low expression level

under only one of the growth condition, which is the case of in vivo genome-wide expression analysis. As a consequence, the fold change in gene expression presented in this study should be significantly underestimated. Nevertheless, differentially expressed genes were readily identified by statistical analysis. Overall, 185 genes were found to be induced in vivo in all five rabbits. Among them, 177 exhibited more than two-fold differential expression with 91 of them showing more than 4-fold differential expression. Of the 199 genes found to be repressed in vivo, the expression level of 153 genes was reduced 2 fold and the expression level of 32 genes was reduced more than 4 fold.

Multiple class response SAM analysis (considering each rabbit as one group) as well as two class unpaired data SAM analysis (considering each rabbit as one group and comparing each rabbit with each other) revealed some gene expression variability between rabbits. Multiple class response SAM analysis identified 170 genes differentially expressed between rabbits with a false discovery rate of 1.64%. Importantly, very few genes were found to be antagonistically expressed between rabbits. Indeed, the trend of differential expression remained essentially the same, while only the amplitude of change in transcript abundance varied. To confirm the variability of gene expression between rabbits and to rule out the possibility of intrinsic noise, we compared the expression measurements of these 170 genes within each rabbit and between rabbits (using the microarray data from rabbits 2 and 3). A high level of concordance with a correlation coefficient higher than 0.9 was obtained between replicate microarray hybridizations of RNA isolated from the same rabbit (Figure 2, panels A and B), whereas a very weak correlation was observed between hybridizations of RNA samples originating from two different rabbits (Figure 2, panel C). The variability of gene expression between rabbits

was further confirmed by quantitative real-time RT-PCR for *flgE2* and Cj0178, which encode for the flagellar hook subunit protein and a putative outer membrane ferric-siderophore receptor, respectively. These were found to be differentially expressed between rabbits 3 and 4. The microarray analysis indicated that the *flgE2* gene was over-expressed in rabbit 4 and down-regulated in rabbit 3, while the expression of Cj0178 was essentially unaffected in rabbit 3 and up-regulated in rabbit 4. By using the same RNA preparation as the one used for the microarray hybridization, quantitative RT-PCR confirmed the differential expression of both genes. The expression of *flgE2* was found to be down-regulated approximately 70-fold in rabbit 3, and up-regulated 4-fold in rabbit 4, compared with in vitro growth. Cj0178 was found to be equally expressed in rabbit 3 and over-expressed 200-fold in rabbit 4 relative to in vitro growth.

The observed variability in gene expression patterns is unclear but likely reflects both physiological and intrinsic variations in the rabbits. This hypothesis is in agreement with the observed difference in colonization level (1 log) and the variation in the amount of fluid accumulation (±55%) between rabbits. Obviously, the gastrointestinal environment cannot be controlled and is likely to vary from one rabbit to another, leading to variations in *C. jejuni* colonization and gene expression profiles. In addition, it is unknown if the rabbits used in this study had previously encountered *C. jejuni*. If it was the case, an immune response would likely take effect by 48 hours post-infection and might also result in the observed gene expression variability. Recently, Boyce *et al.* reported the genome-wide expression profile of *Pasteurella multocida* recovered from blood of infected chickens 20 hours after inoculation (2). Although blood has questionable pathological relevance because it is not the site of infection of *P. multocida*, similarly to

our study the authors observed a variable bacterial gene expression profile between infected hosts (2). More recently, Xu *et al.* characterized the transcriptome of *Vibrio cholerae* during intestinal growth 8 hours post-infection using the rabbit ileal loop model (54). In contrast to our study, *V. cholerae* gene expression was similar in the three rabbits tested. All together, these data highlight the complexity of studying genome-wide gene expression in vivo.

**_Campylobacter_ lifestyle in the gut.**

Overall, the expression of 482 genes was found to be significantly altered in vivo. Based on their expression profiles, genes can be grouped into two major categories: (1) genes exhibiting similar differential expression in all 5 rabbits tested (348 genes, Figure 3); and (2) genes with variable expression between rabbits (170 genes, Figure 4). It should be noticed that 36 genes belong to both categories. These genes exhibit similar expression alteration in vivo in all five rabbits but different fold change amplitudes between rabbits.

To elucidate further the intestinal lifestyle of *Campylobacter*, we grouped genes by functional annotations and mapped their expression profiles to all known biological processes, thus allowing the investigation of the overall physiological status of *C. jejuni* grown in vivo. This approach revealed the involvement of a wide range of metabolic, morphological and pathological processes (Figures 3 and 4). Figures 3 and 4 list only the genes found to be significantly differentially expressed between in vivo and in vitro growth by SAM analysis. However, a biological process was considered to play a role in *Campylobacter* physiology in the intestine when the constituting genes were found to be either up-regulated or equally expressed in vivo compared to in vitro growth.

**Energy and central intermediary metabolism**

The in vivo transcriptome pattern of *C. jejuni* was consistent with the oxygen-limited environment found in the intestine. The expression of genes encoding for the key enzymes in the oxidative phosphorylation pathway was decreased dramatically in all 5 rabbits. These genes encode for NADH dehydrogenase (*nuoG, nuoL* and *nuoH*), and succinate dehydrogenase (*sdhABC*). Recently, fumarate, nitrate, nitrite, and N- or O-oxides have been shown to constitute alternative terminal electron acceptors, allowing *C. jejuni* to carry out respiration under oxygen-restricted conditions in vitro (36). However, the genes encoding for the reductases involved in this alternative respiratory pathway were all down-regulated in vivo. In contrast, the genes encoding for the cytochrome bd oxidase (*cydAB*) were expressed in vivo but not or only slightly expressed in vitro. The differential expression of the *cydA* gene was confirmed by quantitative real-time PCR. The expression of *cydA* was found to be 350 fold higher in vivo compared to in vitro growth. Although the CydAB oxidase catalyses the oxidation of menaquinone using oxygen as an electron acceptor, this enzymatic complex has been shown in *E. coli* to possess a high affinity for oxygen, allowing the bacterium to carry out respiration under limited oxygen tension (5). In addition, the expression of *E. coli cydAB* is known to be induced under limiting oxygen conditions (5). Similarly, the CydAB complex could facilitate *C. jejuni* respiration in the oxygen-limited environment of the intestine. Interestingly, formate dehydrogenase (encoded by *fdhABCD*) was the only enzyme identified by the microarray analysis to be over or equally expressed in vivo relative to in vitro and capable of transferring electrons to the menaquinone pool. Other genes

encoding enzymes with similar activity were found to be down-regulated in vivo. FdhABCD enzyme participates in the respiratory chain of many bacterial species, enabling these organisms to respire using formate as an alternative terminal electron donor under anaerobic conditions (33). In *C. jejuni*, the formate dehydrogenase, together with the CydAB complex, could allow the bacterium to carry on oxygen respiration even under extreme oxygen limited conditions.

The expression of the genes encoding enzymes involved in gluconeogenesis, the citric acid cycle, and the pentose phosphate pathway, were all down-regulated in vivo, except for fructose bi-phosphate aldolase (*fba*). Down-regulation of these genes is consistent with the oxygen deprived intestinal environment and the up-regulation of the carbon storage regulator, *csrA*. In *E. coli*, CsrA has been shown to repress the expression of genes involved in glycogen catabolism, gluconeogenesis, glycolysis, and motility (34). This enzyme likely performs a similar function in *C. jejuni*.

**Macromolecular synthesis and processing**

Genes encoding proteins involved in the synthesis and modification of macromolecules, in particular the ribosomal proteins (with the exception of the *rpsA* gene) and aminoacyl tRNA synthetases, were among the most highly up-regulated in vivo. The significance of this contradictory expression of *rpsA* (which encodes the ribosomal protein S1) and other genes from the same functional group is puzzling and requires confirmation by an alternative method and further investigation. In *E. coli*, the ribosomal protein S1 has been shown to be essential for cell viability, to promote the efficiency of translation, and to act as a repressor for its own synthesis (37). Depletion of the protein S1 resulted in a

stringent response consistent with amino acid starvation and an increased production of ppGpp (37). Therefore, the down-regulation of the *rpsA* expression would suggest the induction of a stringent response in *C. jejuni* during intestinal colonization.

**Biosynthesis of cofactors**

Another group of genes expressed in vivo encodes proteins involved in the biosynthesis of the cofactors, biotin (*bioABCD*), riboflavin (*ribADFH*), thiamine (*thiCDEGHJL*), pantothenate (*panBC*), coenzyme A (*accB* and *acs*) and folic acid (*folCD*). These genes were found to be either up-regulated or equally expressed in vivo relative to in vitro growth (with the exception of two genes, *thiG* and *D*, from the thiamine biosynthetic pathway, which were found to be down-regulated). The expression of these genes suggests that these cofactors are unavailable in the intestine. As a consequence, and because biotin, riboflavin, thiamine and pantothenate are produced only by microbes and higher plants, these biosynthetic pathways could constitute an ideal target for drug development. This evidence that biotin is unavailable in the intestine is corroborated by the up-regulation of *V. cholerae* biotin biosynthetic genes during intra-intestinal growth (54), and by the inability of a *V. cholerae* biotin biosynthesis mutant to colonize the gastrointestinal tract of mice (22).

**Virulence and colonization determinants**

Suspected virulence and colonization factors of *Campylobacter* include motility and chemotaxis, host cell adherence and invasion, toxin production, lipo-oligosaccharide and surface structure biosynthesis, oxidative stress defense, iron acquisition and heat shock

response (49). In contrast to genes encoding proteins involved in general metabolism or bacterial physiology, the expression of many genes related to virulence and/or colonization factors were highly variable among infected rabbits. The most notable among genes with flexible expression were those coding for proteins involved in flagellum biosynthesis. Motility is known to be an essential requirement for *C. jejuni* to colonize the host gut and ultimately cause disease (49). Considering that the flagellin subunit is the immunodominant antigen recognized during human or animal infection, it is assumed that the gene encoding this protein is expressed in vivo (28). However, our microarray data suggest that there is considerable inter-animal expression variability among genes belonging to the flagellum locus. Most of the genes belonging to the flagellum locus were found to be down-regulated in 4 rabbits (and at a different level), while they were slightly up-regulated in one rabbit. This variability may allow the bacterium to evade the host immune system by shutting down flagellum production once colonization is accomplished. In support of this hypothesis, *C. jejuni* flagella have recently been proposed to be necessary for passage through the gastrointestinal tract of chickens, but not for persistence in the chicken's ceacum (51). A similar effect on the expression of flagellar genes was also recently demonstrated in *Salmonella enterica* during macrophage intracellular growth (7).

Another functional category of genes expressed in vivo relates to iron-responsive genes which encode proteins involved in iron metabolism and oxidative stress defense. Several of the genes encoding iron acquisition systems were found to be either up-regulated or equally expressed between in vivo and in vitro growth. These genes code for a putative ferric-siderophore transporter system (Cj0178 and Cj0173c-Cj0175c), a putative iron

transporter (p19 and Cj1658), and the three TonB-ExbB-ExbD energy transducing complexes. The genes encoding a putative heme outer-membrane transporter (ChuABCD) were found to be only slightly expressed in vivo, suggesting that heme does not constitute the main iron source in the gut. The genes encoding the components of the ferric-enterobactin uptake permease (*ceuBCDE*) were found to be up-regulated in vivo, however the *cfrA* gene encoding the ferric-enterobactin receptor appeared to not be expressed. As a microaerophilic bacterium, *C. jejuni* must deal with free oxygen radicals and other reactive molecules generated by normal aerobic metabolism and host defenses against microbial attack (49). The expression of most genes known to be associated with *C. jejuni* oxidative stress response was found to be increased during gastrointestinal growth (49). To note, the up-regulation of these genes is in agreement with an iron limited environment. Theses genes include *sodB* (superoxide dismutase), *ahpC* (alkyl hydroperoxide reductase), *tpx* (probable thiol peroxidase) and *katA* (catalase). Consistent with the expression of these genes in vivo, a mutation of *C. coli sodB* has been reported to impede colonization of chick gut (31). Furthermore, a mutation in *katA* sensitizes *C. jejuni* to hydrogen peroxide and reduces its intracellular survival in macrophages (49). In *H. pylori*, the catalase KatA has been shown to be required for persistent colonization in the mouse model (14). These results highlight the iron-restricted conditions in the rabbit intestine. The importance of iron metabolism for successful host colonization has been established for most pathogens (32), and should also be an essential factor for *C. jejuni* colonization in the intestine. Indeed, a *fur* mutant of *C. jejuni*, as well as a *cfrA*, *ceuE* and Cj0178 mutants, were recently shown to be significantly affected in their ability to colonize the gastrointestinal tract of chicks (26).

Interestingly, *C. jejuni* possesses a system of general protein glycosylation, which has been proposed recently to play an important role in *C. jejuni* pathogenesis (42). Indeed, mutation of genes encoding the glycosyltransferases, *pglB* (also named *wlaF*) and *pglE* (also named *wlaK*), affected their ability to adhere to and invade human intestinal cells, as well as to colonize the gastrointestinal tract of mice (42). In our study, the expression of three genes, *pglB*, *pglE* and *pglG*, which belong to this functional category, was found to varied between growth conditions. The expression of *pglB* and *pglG* was highly induced in vivo (in 4 rabbits) while the *pglE* expression was either similar or repressed in vivo compared with in vitro growth. The expression of the other genes from the glycosylation cluster (*pglH*, *pglA*, *wlaJ*, and *pglF*) was found to be similar in both growth conditions. It is not clear why expression of the genes belonging to the same biological pathway varied. However, a similar difference in expression between the *pgl* genes was observed previously in *Campylobacter* in response to temperature up-shift (40) and iron starvation (26). Considering the absence of data on the functional role of each *pgl* gene in protein glycosylation, the significance of their expression profiles is difficult to assess.

The genes encoding the recently discovered multidrug efflux pump in *C. jejuni*, *cmeABC*, were found to be highly up-regulated in vivo. Expression of *cmeB* was confirmed by real time RT-PCR to be up-regulated by approximately 300-fold in vivo compared with in vitro growth. This tripartite multidrug efflux transporter is composed of an outer membrane protein, CmeC (Cj0367c), a periplasmic fusion protein, CmeA (Cj0365c), and an inner membrane efflux transporter, CmeB (Cj0366c) (18). Interestingly, this efflux system has been shown to contribute greatly to bile resistance and to be required for the colonization of the chick's gastrointestinal tract (18, 19). Given the presence of a high

concentration of bile salts in the gut, the up-regulation of these genes could contribute significantly to the survival of *Campylobacter* in the host by allowing the bacterium to resist the harmful effects of these salts.

Another important functional set of genes with variable expression between rabbits encodes for proteins involved in peptidoglycan biosynthesis. Specifically, *murB* ( a putative UDP-N-acetylenolpyruvoylglucosaminereductase*)*, *murC (*UDP-N-acetylmuramate-alanine ligase*)*, and *pbpC (*penicillin-binding protein*)* were among the genes from this category that were the most differentially regulated. The differential expression of this category of genes suggests that there may be a modification of the murein sacculus in vivo, probably in response to the high osmolarity of the intestinal environment. Likewise, *Staphylococcus aureus* modifies its peptidoglycan layer under conditions of high osmolarity (50). While the activation of genes involved in peptidoglycan synthesis may constitute a repair mechanism necessary for the bacterial adaptation to environmental stress, the modification of the peptidoglycan structure may have broad implications for the stiffness and elasticity of the cell surface, thereby conditioning the bacterium to its ecological niche.

Among the other genes annotated or previously characterized as virulence- or colonization-associated factors, Cj1279c (putative fibronectin domain-containing lipoprotein) and several genes involved in the heat shock response were found to be significantly up-regulated in vivo. Heat shock proteins are induced in response to various stresses and act by repairing and preventing damage caused by the accumulation of unfolded proteins. The importance of the heat shock response for *Campylobacter* intestinal tract colonization has previously been demonstrated (49). Genes identified as

heat shock proteins (40) and induced in vivo include *clpB* (ATP-dependent CLP protease ATP-binding subunit), *dnaK* (heat shock protein), *grpE* (heat shock protein), *hrcA* (putative heat shock regulator), and *htpG* (hsp90 family heat shock protein). The up-regulation of these genes, together with the previous identification of ClpB as a B-cell antigen in human disease (45), suggests that these proteins play an important role in bacterial growth within the gastrointestinal tract.

**Mutational analysis**.

In order to study further the *C. jejuni* lifestyle in the intestinal tract of rabbits, we constructed knockout mutations of genes identified by our microarray analysis and investigated the ability of these mutants to survive in the rabbit intestinal loop by comparison with the parent strain, *C. jejuni* NCTC 11168. Seven genes were selected and individually mutated. The main goal of this mutational analysis was to disrupt physiological functions that appeared to be important for the colonization of the ileal loop. In particular, the microarray data suggested an important role for the genes involved in the heat shock response, the stringent response, the iron metabolism, and the biogenesis of the flagellum in the intestinal lifestyle of *C. jejuni*. Therefore, the heat shock response was disrupted by mutagenesis of its two transcriptional regulators *hrcA* and *hspR*. The stringent response was disrupted by mutagenesis of the *spoT* gene which encodes the guanosine-3',5'-bis(diphosphate)3'-pyrophosphohydrolase. The iron metabolism was disrupted by mutagenesis of the Cj0178 gene which encodes a ferric-siderophore outer-membrane receptor. This gene has been previously shown to be induced under iron restriction and is highly up-regulated in vivo. The flagellum

biogenesis was disrupted by mutagenesis of the *fliD* gene which encodes for the flagellar hook-associated protein. A mutant in the *fliD* gene has been shown to be non-motile (12). The *fliD* gene was found to be down-regulated in all 5 rabbits. Interestingly, among the genes encoding transcriptional regulators, Cj0571 was the only one from this category found to be significantly up-regulated in all 5 rabbits by the microarray analysis, suggesting an important role for this protein in vivo. Consequently, this gene was also mutated. Finally, a mutant into Cj0341 was chosen to be tested in the ileal model as an experimental control. The expression of this gene was found to be off under in vivo and in vitro growth conditions. Therefore, this mutant should not be affected in its ability to colonize the ileal loop.

The 7 mutants and the parent strain were pooled together and inoculated into 4 ileal loops constructed in 2 different rabbits. After 48 hours post-inoculation, the ileal contents were recovered and directly processed for chromosomal DNA purification. Then, the relative amount of each mutant was evaluated by quantitative real-time PCR, as described in the Materials and Methods section. The competitive ratio of the number of cells at the time of the inoculation (in the input pool) to the number of cells recovered 48 hours post-inoculation was normalized to the entire bacterial population in the pool for each mutant (Figure 5) so that the competitive ratio of the whole population is equal to 1. Considering that the wild-type strain was present in excess, compared with each mutant in the input pool, and assuming that it represents the major proportion of the population in the recovered pool, it should have a competitive ratio input/recovered equal to approximately 1. Therefore, any deviation of the competitive ratio from a value of 1 would indicate an effect of the mutation on the survival ability of *C. jejuni* in the rabbit ileal loop. As

expected, the competitive ratio of the Cj0341 mutant was 0.9, indicating that this strain colonizes the ileal loop as well as the wild type. Of the 6 other mutants, 1 was unaffected (Cj0571), 1 had an advantage over the others with respect to survival in and colonization of the rabbit ileal loop (*fliD*; $P<0.003$), and 4 were significantly attenuated (*hrcA*, Cj0178, *spoT* and *hspR*; $P\leq0.002$). The *spoT* and *hspR* mutated strains were the most affected mutants, while the *hrcA* and Cj0178 mutants were only slightly attenuated. Because mutants are out-competed by many other strains during a mixed infection, the in vivo phenotype of the affected mutants was confirmed in a 1 to 1 competition assay. Each mutant was independently mixed with the wild-type strain in equal numbers and injected into 4 ileal loops (constructed in two rabbits). The 1 to 1 ratio of the inocula was confirmed by cfu determination. Forty-eight hours post-infection, the loop contents were plated on selective medium for bacterial enumeration. Then, the competitive index was calculated as the ratio of the mutant to the wild type strain recovered from each ileal loop. As shown in Figure 6 (panel A), 3 out of the 5 mutants were confirmed to be statistically affected in their colonization ability (with a $p \leq 10^{-4}$). In order to determine whether the colonization phenotype of these mutants was specific for in vivo growth, an in vitro competition assay was performed. An equal amount of each mutant and wild-type strain was mixed in MH broth. The cultures were incubated at 37°C until late log phase (~30 hours), after which serial dilutions were plated on MH-agar with or without chloramphenicol. These experiments were performed in triplicate. The in vitro competitive index was determined as described for the in vivo competition assay (Figure 6, panel B). Four of the 5 mutants were found to be statistically affected in their ability to out-compete the wild-type strain during in vitro growth. Finally, in order to determine

whether the in vitro growth defect was caused by the competition with the wild-type strain, the growth kinetic of each mutant was independently determined (Figure 7). All five mutants were found to have a growth defect in vitro, with the *spoT, hspR,* and *fliD* mutants being the most affected.

The competitive index of the *hspR* mutant in vitro and in vivo was $1.5.10^{-2}$ and $7.10^{-5}$ respectively. While this mutant is affected in vitro, the 200 fold difference between the in vitro and in vivo competitive indexes indicates a significant in vivo specific growth defect. The attenuation of the *hspR* mutant in vivo suggests a role for the heat shock regulatory network in *Campylobacter* gut colonization. In contrast, the *hrcA* mutant was not significantly affected in its in vitro growth neither in its ability to colonize the ileal loop. While the function of *hspR* and *hrcA* in *C. jejuni* is essentially unknown, the products of these genes were recently demonstrated to repress the transcription of the major heat shock proteins in *H. pylori* (*groESL*, *hrcA*, *grpE*, and *dnaK*) (38, 39). In addition, the HrcA-mediated repression was shown to be dependent on the binding of HspR to the promoter region (38). Interestingly, the transcription of the *cbpA-hspR-orf* operon was found to be exclusively regulated by HspR (38). Consequently, it is tempting to propose that the loss of HspR induces an increase in the abundance of heat shock proteins. Given that heat shock proteins are major immuno-dominant antigens, the over-expression of these proteins would likely contribute to host resistance (52) . As a result, the *hspR* mutants should be less capable of colonization and survival in the host. In addition, given the absence of colonization defect for the *hrcA* mutant, the amount of these proteins would not be increased at a sufficient level in this mutant to induce host resistance. In support of this hypothesis, *H. pylori hspR* and *hspR-hrcA* double mutants

were reported to have attenuated colonization efficiency in wild-type mice, while they were unaffected in IL-12-deficient mice (15). Furthermore, *C. jejuni* infected patients have been shown to develop humoral response against the heat shock protein DnaK (44).

In *E. coli*, *spoT* codes for a bi-functional enzyme able to catalyze the biosynthesis and the degradation of hyperphosphorylated guanine (p)ppGpp (1). In most eubacteria, (p)ppGpp has been shown to accumulate in response to stringent conditions, such as amino acid starvation, triggering the down-regulation of genes encoding the transcription and translation apparatus (1). As shown in Figure 6, the *spoT* mutant exhibits a competitive index of $9.10^{-6}$ in vivo. In contrast to this in vivo result, the in vitro competitive index was only 0.06, suggesting a significant in vivo specific defect. The decreased ability of the *C. jejuni spoT* mutant to colonize the host gut suggests an important role for the stringent response in vivo, likely allowing *Campylobacter* to deal with periods of nutrient starvation or other environmental stresses in the intestinal tract.

While the function of Cj0178 has not been characterized, this protein exhibits high homology with ferric-siderophore outer-membrane receptors. In addition, the expression of Cj0178 is Fur regulated and induced in response to iron starvation (26). Consequently, Cj0178 is probably required for the acquisition of iron from an uncharacterized siderophore. The Cj0178 mutant exhibits a competitive index of 0.05 in vivo and 0.37 in vitro. Given that *C. jejuni* NCTC 11168 does not seem to produce any siderophore (48), the growth defect of this mutant in vitro is unclear. The significant attenuation of the Cj0178 mutant in vivo is in agreement with its over-expression in our microarray experiment and might suggest an important role for this iron acquisition system in gut colonization.

Interestingly, while the *fliD* has a significant growth defect in vitro (exhibiting a a competitive index of 0.1), it colonizes the ileal loop as well as the wild-type strain (exhibiting a competitive index of 1.3). The *fliD* gene encodes a putative flagellar hook-associated protein. In *H. pylori*, *fliD* is an essential component in the assembly of a functional flagellum and is required for colonization of the gastric mucosa of mice (17). Similarly to *H. pylori*, the *C. jejuni fliD* mutant is non-motile and aflagellated (12). While the survival of the *fliD* mutant in the gut is in disagreement with the essential role of the flagellum in the colonization of the gastrointestinal tract, it is consistent with the characteristics of the RIL animal model. Indeed, the physical ligation of the rabbit intestinal tract likely favors the survival of mutants affected in their ability to adhere to the mucus or the intestinal epithelial cells. In addition, considering that flagellin is the major immunodominant antigen during infection (24), the loss of the flagellum structure should promote evasion of the immune system. Consequently, a *fliD* mutant will have an advantage over the wild-type in vivo and thus compete better during in vivo than in vitro growth.

**Concluding remarks**.

This genome-wide expression profiling study revealed important elements of the *Campylobacter* lifestyle during host intestinal tract colonization. In addition to the genes discussed above that have known or potential functions, many other genes of unknown function were also found to be differentially expressed between in vivo and in vitro growth, and therefore constitute many new directions for future investigations. The transcriptome pattern of *C. jejuni* in vivo was consistent with that expected in an

environment that is oxygen-limited, hyper-osmotic, nutrient restricted, and containing reactive oxygen compounds. Interestingly, the comparison of the *C. jejuni* transcriptomes between different rabbits revealed gene expression variability during the course of an infection. This flexibility in gene expression is probably essential for *Campylobacter* to adapt to the changing environment of the gut. Furthermore, the genes encoding proteins involved in flagellum biogenesis were found to be differentially expressed between rabbits. They were up-regulated in one rabbit and down-regulated (at a different level) in all others. While the up-regulation of these genes is in agreement with the role of the flagellum in gut colonization, the decreased expression of these genes probably reflects a bacterial strategy to evade the host response. Finally, regulation of both the heat shock response and the stringent response were found to be necessary for efficient colonization of the host gastrointestinal tract.

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**Table 1**: Bacterial strains and plasmids

| Strain or plasmid | Relevant characteristics[a] | Source or reference |
|---|---|---|
| ***E. coli*** | | |
| DH5α | *endA1 hsdR17* ($r_k^-m_k^-$) *supE44 thi-1 recA1 gyrA relA1* Δ*(lacZYAargF U169deoR [f80dlac*Δ*(lacZ0 M15]* | Invitrogen |
| ***C. jejuni*** | | |
| NCTC11168 | *C. jejuni* NCTC 11168 | NCTC |
| AS 283 | NCTC11168ΔCj0571 | This study |
| AS 287 | NCTC11168Δ*spoT* | This study |
| AS272 | NCTC11168Δ*hspR* | This study |
| AS277 | NCTC11168Δ*hrcA* | This study |
| AS211 | NCTC11168ΔCj0178 | (26) |
| AS317 | NCTC11168ΔCj0341 | This study |
| AS318 | NCTC11168Δ*fliD* | This study |
| **Plasmids** | | |
| pUC19 | Cloning and suicide vector, $Amp^r$ | Biolabs |
| pRY111 | *cam* resistance gene | (55) |
| pAS282 | pUC19 carrying ΔCj0571*::cam* | This study |
| pAS271 | pUC19 carrying Δ*hspR::cam* | This study |
| pAS315 | pUC19 carrying Δ*hrcA::cam* | This study |
| pAS286 | pUC19 carrying Δ*spoT::cam* | This study |

[a]cam, chloramphenicol resistance gene;
$Amp^r$, ampicillin resistant

**Table 2**: Primers used in this study.

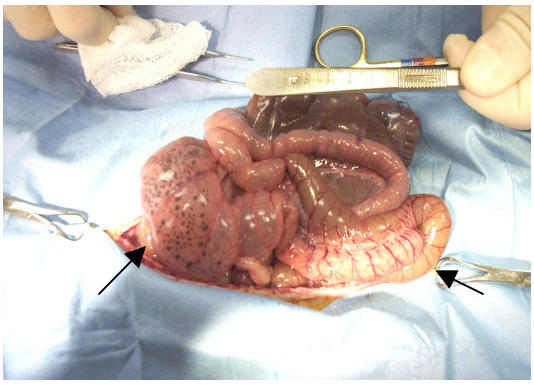
| Primer | DNA sequence from 5' to 3' (restriction site)[a] |
|---|---|
| Primers used for gene cloning | |
| spoT-01 | ATGC**GAATTC**GAGATTTTAACCTATCCTTGACAC (*Eco*RI) |
| spoT-02 | ATGC**CTGCAG**CCATGATGCCATTCTTGAAA (*Pst*I) |
| Cj0571-01 | ATGC**GAATTC**ATGCAAGAAAATTTCATACGC (*Eco*RI) |
| Cj0571-02 | ATGC**CTGCAG**TCCCGTTGTAGCATCTTTTG (*Pst*I) |
| hrcA-01 | ACGT**GGTACC**AATAGAGTGCTAGATATGAAGGAA (*Kpn*I) |
| hrcA-02 | ACGT**GGTACC**GATGGTTTTCACTATCTACATGAA (*Kpn*I) |
| hspR-01 | ACGT**GGTACC**GGAAAACAAAGCAGAAATGGAGC (*Kpn*I) |
| hspR-02 | ACGT**GGTACC**GCACCACTGCGGTTGAAGAAA (*Kpn*I) |
| Primers used for inverse PCR | |
| spoT-03 | ATGC**GGATCC**AAAACAGGAAAAGCAAAAGCA (*Bam*HI) |
| spoT-04 | ATGC**GGATCC**AATGCCTTTTCTTTGCAT (*Bam*HI) |
| Cj0571-03 | ATGC**GGATCC**GCTTAATTTTCCCAAAGCAAA (*Bam*HI) |
| Cj0571-04 | ATGC**GGATCC**AGAACTGAAAATACGGCTAGAAGA (*Bam*HI) |
| hrcA-03 | ACGT**CCATGG**ATTGCTTCTATAGCAAATCAA (*Nco*I) |
| hrcA-04 | ACGT**GCGGCCGC**AAGCTCTTAGATTGTCAAATTCAC (*Not*I) |
| hspR-03 | ACGT**CCATGG**TTGCCATCGGTTCTACTTGG (*Nco*I) |
| hspR-04 | ACGT**GCGGCCGC**GAATCAATCTTGCTGGAGTA (*Not*I) |

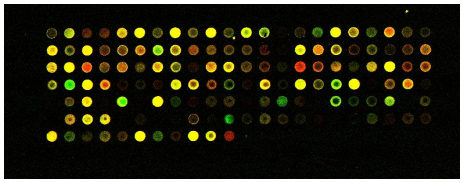
[a]The restriction sites used for cloning are highlighted in bold and indicated in parenthesis.

**Figure 1**

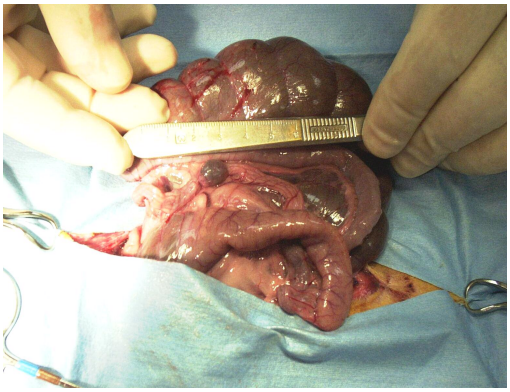
Detection of *C. jejuni* transcriptome in vivo. Panels A and B show the rabbit ileal loops 48 hours post-inoculation with *C. jejuni* or PBS buffer, respectively. The arrows indicate intestine distended with gas and fluid accumulation. Total RNA was extracted from the intestinal contents, reverse transcribed using *C. jejuni* specific 3' primer, and labeled with the Cy5 dye. This labeled cDNA was co-hybridized to the microarray with Cy3 labeled cDNA, obtained from in vitro grown bacterial RNA.

A)





B)



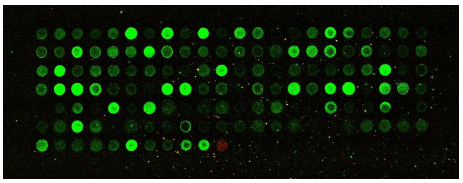
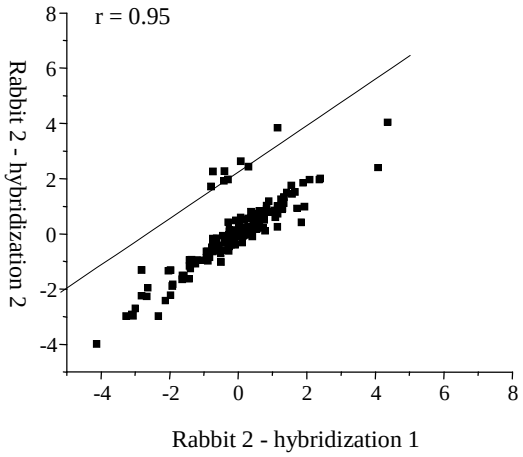


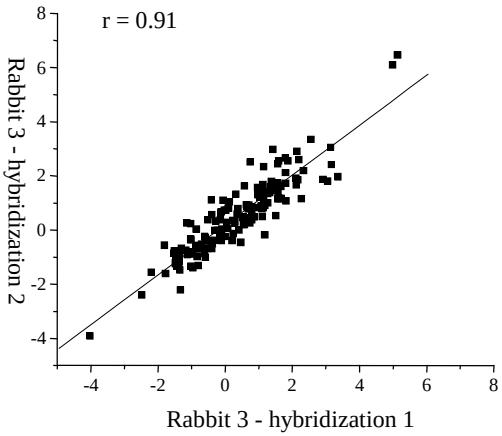
**Figure 2**

Scatter plots showing the relationship between the $\log_2$ value of the gene expression ratio obtained from hybridization experiments with bacterial cDNA derived from the same rabbit (Panels A and B) or from two different rabbits (Panel C). The solid lines represent the linear regression fit of the data.

A)


r = 0.95
Rabbit 2 - hybridization 2
Rabbit 2 - hybridization 1

B)


r = 0.91
Rabbit 3 - hybridization 2
Rabbit 3 - hybridization 1

C)

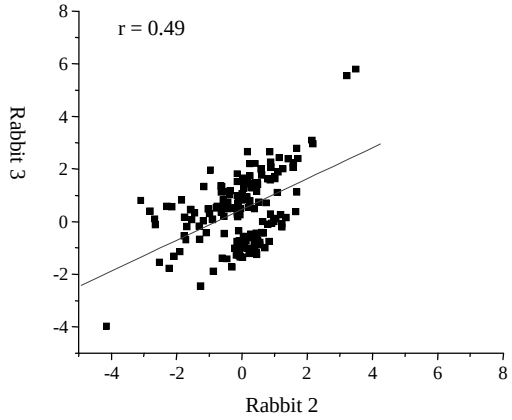
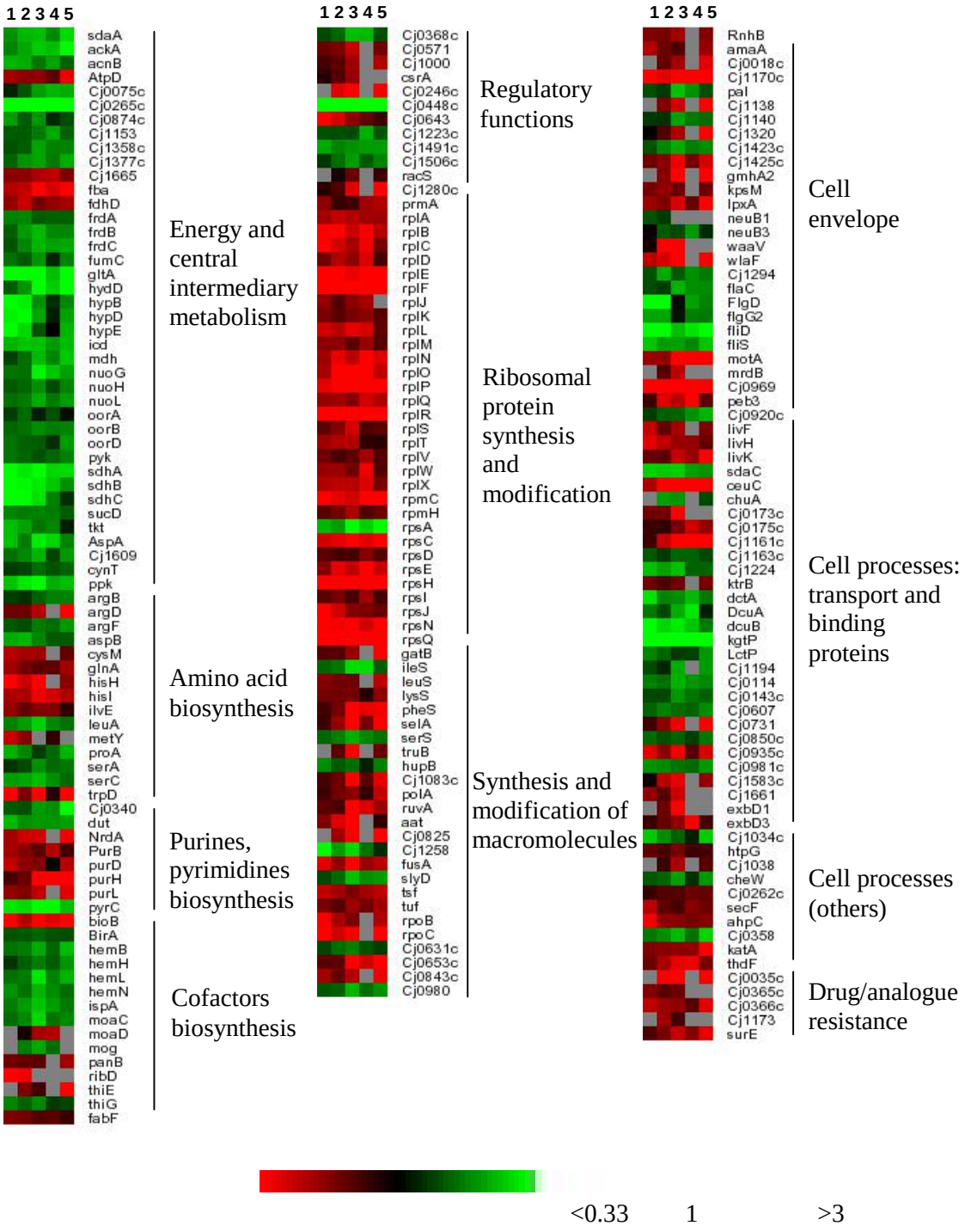

r = 0.49
Rabbit 3
Rabbit 2

**Figure 3**

Global view of genes with similar expression pattern between rabbits grouped by functional categories according to the Sanger Center *C. jejuni* genome database. Each row represents one gene and each column represents the expression profile in one rabbit (the mean of the fold change expression ratio of the technical replicates). The column label corresponds to the rabbit numbering. An increasing red intensity denotes genes that have their expression significantly increased in vivo compared to in vitro growth and an increasing green intensity indicates genes that have their expression significantly decreased in vivo compared to in vitro growth. A gray color indicates missing data. Genes with unknown functions are not represented.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ackA
acnB
AtpD
Cj0075c
Cj0265c
Cj0874c
Cj1153
Cj1358c
Cj1377c
Cj1665
fba
fdhD
frdA
frdB
frdC
fumC
gltA
hydD
hypB
hypD
hypE
icd
mdh
nuoG
nuoH
nuoL
oorA
oorB
oorD
pyk
sdhA
sdhB
sdhC
sucD
tkt
AspA
Cj1609
cynT
ppk
Energy and central intermediary metabolism
argB
argD
argF
aspB
cysM
glnA
hisH
hisI
ilvE
leuA
metY
proA
serA
serC
trpD
Amino acid biosynthesis
Cj0340
dut
NrdA
PurB
purD
purH
purL
pyrC
Purines, pyrimidines biosynthesis
bioB
BirA
hemB
hemH
hemL
hemN
ispA
moaC
moaD
mog
panB
ribD
thiE
thiG
fabF
Cofactors biosynthesis
1 2 3 4 5
Cj0368c
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Cj1000
csrA
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Cj0448c
Cj0643
Cj1223c
Cj1491c
Cj1506c
racS
Regulatory functions
Cj1280c
prmA
rplA
rplB
rplC
rplD
rplE
rplF
rplJ
rplK
rplL
rplM
rplN
rplO
rplP
rplQ
rplR
rplS
rplT
rplV
rplW
rplX
rpmC
rpmH
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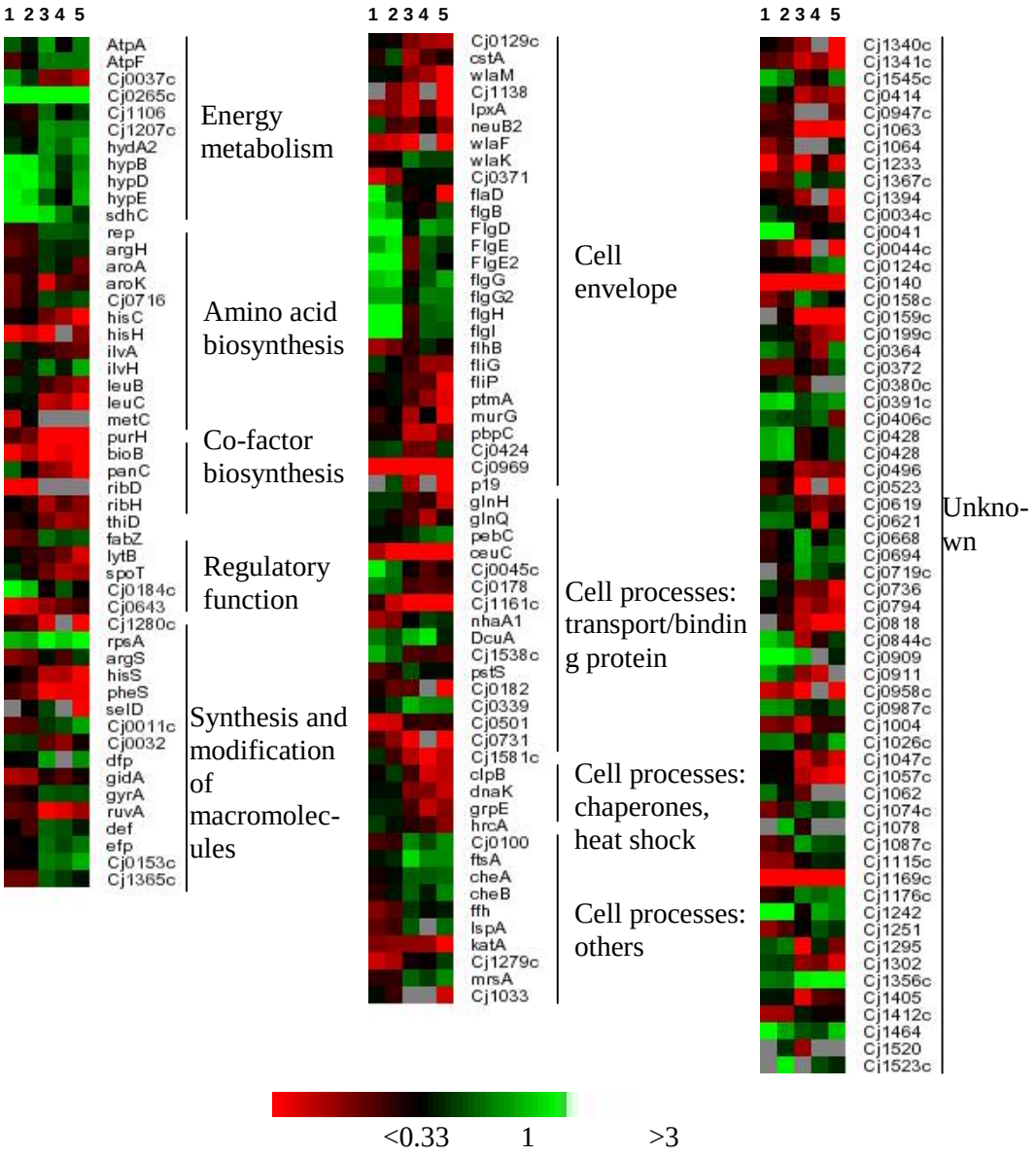
rpsC
rpsD
rpsE
rpsH
rpsI
rpsJ
rpsN
rpsQ
Ribosomal protein synthesis and modification
gatB
ileS
leuS
lysS
pheS
selA
serS
truB
hupB
Cj1083c
polA
ruvA
aat
Cj0825
Cj1258
fusA
slyD
tsf
tuf
rpoB
rpoC
Cj0631c
Cj0653c
Cj0843c
Cj0980
Synthesis and modification of macromolecules
1 2 3 4 5
RnhB
amaA
Cj0018c
Cj1170c
pal
Cj1138
Cj1140
Cj1320
Cj1423c
Cj1425c
gmhA2
kpsM
lpxA
neuB1
neuB3
waaV
wlaF
Cj1294
flaC
FlgD
flgG2
fliD
fliS
motA
mrdB
Cj0969
peb3
Cell envelope
Cj0920c
livF
livH
livK
sdaC
ceuC
chuA
Cj0173c
Cj0175c
Cj1161c
Cj1163c
Cj1224
ktrB
dctA
DcuA
dcuB
kgtP
LctP
Cj1194
Cj0114
Cj0143c
Cj0607
Cj0731
Cj0850c
Cj0935c
Cj0981c
Cj1583c
Cj1661
exbD1
exbD3
Cell processes: transport and binding proteins
Cj1034c
htpG
Cj1038
cheW
Cj0262c
secF
ahpC
Cj0358
katA
thdF
Cell processes (others)
Cj0035c
Cj0365c
Cj0366c
Cj1173
surE
Drug/analogue resistance
<0.33
1
>3

**Figure 4**

Global view of genes with a variable expression pattern between rabbits. Each row represents one gene. Columns 1, 2, 3, 4, and 5 represent the expression profile in rabbit 1, 2, 3, 4, and 5 respectively. For each rabbit, the microarray data correspond to the mean of the fold change expression ratio of the technical replicates. Red and green denotes transcripts that have their abundance increased or decreased in vivo compared to in vitro growth, respectively. The red and green intensities are proportional to the fold increase or decrease with maximal fold changes in transcript abundance of 3 and 0.33, respectively. A gray color denotes missing data.


1 2 3 4 5
AtpA
AtpF
Cj0037c
Cj0265c
Cj1106
Cj1207c
hydA2
hypB
hypD
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sdhC
Energy metabolism
rep
argH
aroA
aroK
Cj0716
hisC
hisH
ilvA
ilvH
leuB
leuC
metC
Amino acid biosynthesis
purH
bioB
panC
ribD
ribH
thiD
Co-factor biosynthesis
fabZ
lytB
spoT
Cj0184c
Cj0643
Regulatory function
Cj1280c
rpsA
argS
hisS
pheS
selD
Cj0011c
Cj0032
dfp
gidA
gyrA
ruvA
def
efp
Cj0153c
Cj1365c
Synthesis and modification of macromolec-ules
1 2 3 4 5
Cj0129c
cstA
wlaM
Cj1138
lpxA
neuB2
wlaF
wlaK
Cj0371
flaD
flgB
FlgD
FlgE
FlgE2
flgG
flgG2
flgH
flgI
flhB
fliG
fliP
ptmA
murG
pbpC
Cj0424
Cj0969
p19
Cell envelope
glnH
glnQ
pebC
ceuC
Cj0045c
Cj0178
Cj1161c
nhaA1
DcuA
Cj1538c
pstS
Cj0182
Cj0339
Cj0501
Cj0731
Cell processes: transport/bindin g protein
Cj1581c
clpB
dnaK
grpE
hrcA
Cell processes: chaperones, heat shock
Cj0100
ftsA
cheA
cheB
ffh
lspA
katA
Cj1279c
mrsA
Cj1033
Cell processes: others
1 2 3 4 5
Cj1340c
Cj1341c
Cj1545c
Cj0414
Cj0947c
Cj1063
Cj1064
Cj1233
Cj1367c
Cj1394
Cj0034c
Cj0041
Cj0044c
Cj0124c
Cj0140
Cj0158c
Cj0159c
Cj0199c
Cj0364
Cj0372
Cj0380c
Cj0391c
Cj0406c
Cj0428
Cj0428
Cj0496
Cj0523
Cj0619
Cj0621
Cj0668
Cj0694
Cj0719c
Cj0736
Cj0794
Cj0818
Cj0844c
Cj0909
Cj0911
Cj0958c
Cj0987c
Cj1004
Cj1026c
Cj1047c
Cj1057c
Cj1062
Cj1074c
Cj1078
Cj1087c
Cj1115c
Cj1169c
Cj1176c
Cj1242
Cj1251
Cj1295
Cj1302
Cj1356c
Cj1405
Cj1412c
Cj1464
Cj1520
Cj1523c
Unkno-wn
<0.33
1
>3

**Figure 5**

Competitive colonization ability of 7 mutants (*hrcA*, Cj0178, Cj0571, *spoT*, Cj0341, *hspR*, and *fliD*). The strains were pooled with the parent strain *C. jejuni* NCTCCC 11168 (constituting the input pool) and inoculated into 4 rabbit ileal loops. Forty-eight hours post-inoculation, the intestinal content was recovered and processed for chromosomal DNA extraction. The number of bacteria was estimated by quantitative real-time PCR for each mutant as described in the Materials and Methods section. The normalized competitive ratio corresponds to the ratio of the number of mutant cells to the total number of bacterial cells in the input pool divided by the ratio of the number of mutants to the total number of bacteria in the recovered pool. The data is the mean of eight determinations (four biological replicates with two technical replicates each), and the error bars represent the standard deviations.

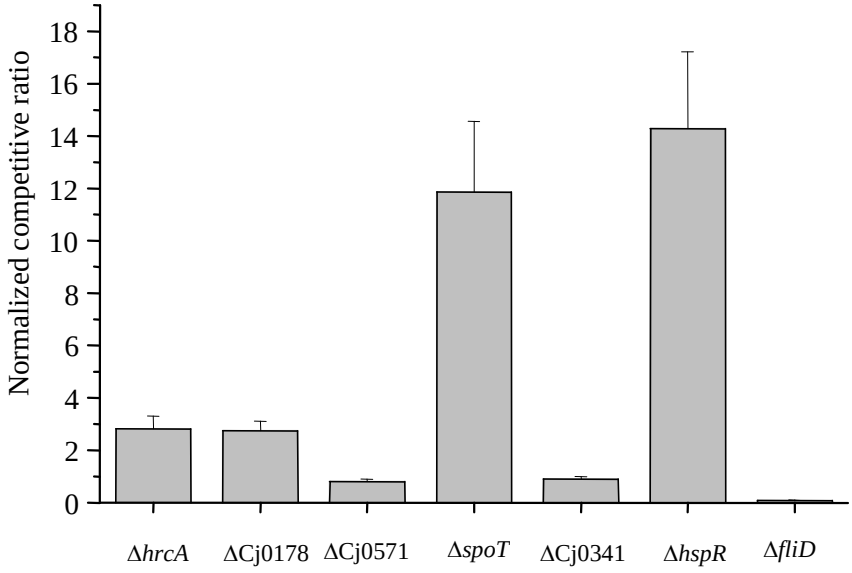
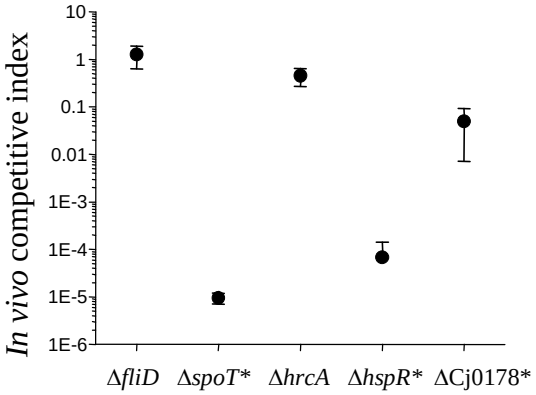

Normalized competitive ratio
0
2
4
6
8
10
12
14
16
18
ΔhrcA
ΔCj0178
ΔCj0571
ΔspoT
ΔCj0341
ΔhspR
ΔfliD

**Figure 6**

In vivo (panel A) and in vitro (panel B) competition assays. The in vivo competitive index is the ratio of the mutant to the wild-type strain recovered in the ileal loop 48 hours post-infection. Four loops were infected with a mixture of each mutant and the wild-type strain at a ratio of 1 to 1. The in vitro competitive index is the ratio of the mutant to the wild-type strain in MH broth at late log phase. The in vitro competition assay was performed in triplicate. The error bars indicate the standard deviations. The symbol * indicates a statistical significance (*P* value <0.001).

A)


*In vivo* competitive index
10
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
Δ*fliD*
Δ*spoT**
Δ*hrcA*
Δ*hspR**
ΔCj0178*

B)

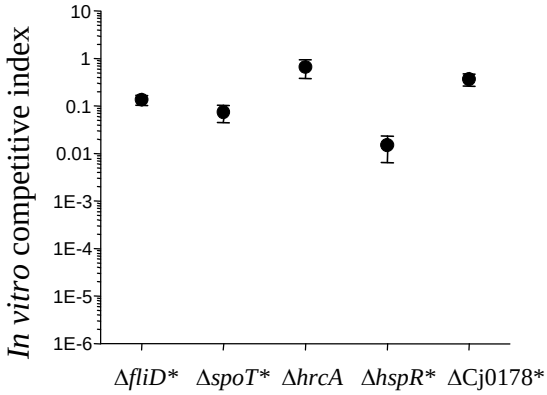
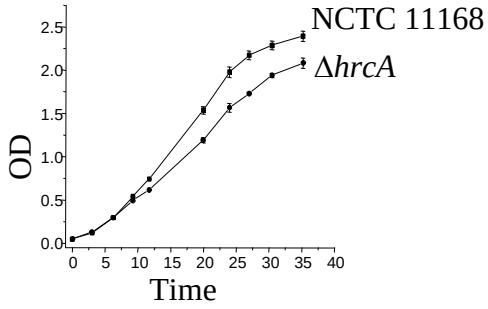

*In vitro* competitive index
10
1
0.1
0.01
1E-3
1E-4
1E-5
1E-6
Δ*fliD**
Δ*spoT**
Δ*hrcA*
Δ*hspR**
ΔCj0178*

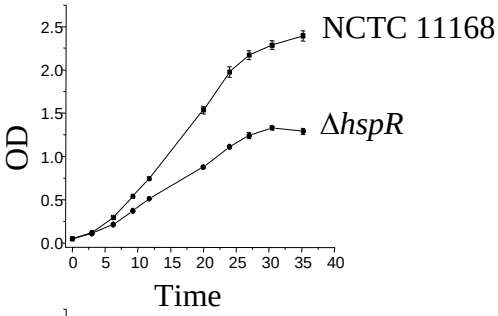
**Figure 7**

Growth kinetics of *C. jejuni* NCTC 11168 and 5 mutants, Δ*hrcA* (panel A), Δ*hspR* (panel B), Δ*spoT* (panel C), ΔCj0178 (panel D), and Δ*fliD* (panel E). Biphasic MH cultures were incubated at 37°C under microaerophilic conditions. The growth kinetics were performed in triplicate and the error bars represent the standard deviations.

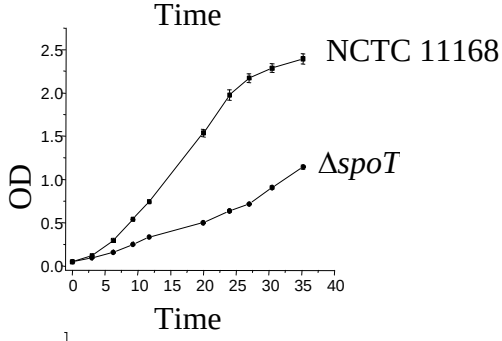
A)


NCTC 11168
Δ*hrcA*
OD
Time

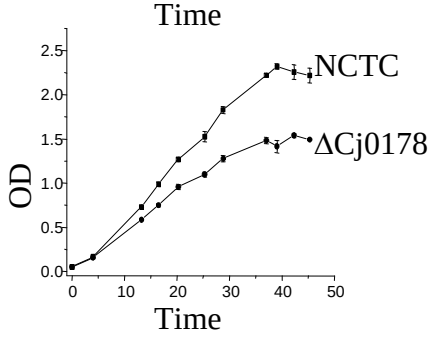
B)


NCTC 11168
Δ*hspR*
OD
Time

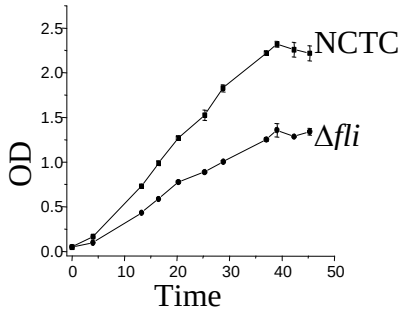
C)


NCTC 11168
Δ*spoT*
OD
Time

D)


NCTC
ΔCj0178
OD
Time

E)


NCTC
Δ*fli*
OD
Time

VITA

Kiran S Palyada

Candidate for the Degree of

Doctor of Philosophy

THESIS: RESPONSE OF *CAMPYLOBCTER JEJUNI* TO IRON AND HYDROGEN PEROXIDE.

Major Field: Veterinary Biomedical Sciences

Biographical:

**Education**

| Degree | Institution | Year |
|---|---|---|
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| Bachelor of Veterinary Science-B.V.Sc | University of Agricultural Sciences, Bangalore, India | 1998 |
| Pre-University Course | DRM Science college, Davangere, India | 1992 |

**Academic honors and awards**:

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- Graduate teaching assistantship during the year 2000-2001 at OSU
- Phi-Zeta award for the excellence in basic science research seminar at OSU.
- Pfizer Veterinary Award for securing first rank in BVSc
- Dr.S.Mohiyuddin Memorial Gold Medal for the best student in Veterinary Pathology
- The Lions International District 304-S2 Gold Medal for the Best BVSc student
- RG & FMK Gold Medal for the best student from among BVSc graduates
- NRSS Gold Medal for the best BVSc student in Veterinary Medicine
- Recipient of Merit Scholarship for four consecutive years during BVSc

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Member of American society of Microbiology

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Institution: Oklahoma State University Location: Stillwater, Oklahoma

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**Scope and Method of study:** Iron affects the physiology of bacteria in two different ways: as a micronutrient for bacterial growth and as a catalyst for the formation of hydroxyl radicals. The present study reports the response of *Campylobacter jejuni* to iron and oxidative stresses using DNA microarrays. *C.jejuni* response to hydrogen peroxide (HP), cumene hydroperoxide (CHP) and menadione was investigated. The response to hydrogen peroxide was investigated both in the presence and the absence of iron. Characterization of the ferric uptake regulator (Fur) and peroxidase regulator (PerR) mutants by transcriptome profiling allowed the identification of Fur and PerR regulated genes, respectively. Chicken colonization model was used to test different *C.jejuni* mutants for their ability to colonize chicken ceca.

**Findings and Conclusions**: *C. jejuni* responded to iron availability by differential expression of genes belonging to several functional groups, ranging from energy metabolism, to cell surface structures, to iron acquisition, and to oxidative stress defense. In addition to identifying several novel iron uptake genes, this study confirmed the function of many genes previously known to be involved in iron acquisition. Directed mutagenesis of several genes identified by microarray experiments allowed the characterization of CfrA as the enterobactin receptor and ChuA as the heme receptor. Both catalase (*katA*) and alkyl hydroxyperoxidase (*ahpC*) were induced upon oxidative stress in cells grown in presence of iron while only the catalase was induced in cells grown in absence of iron suggesting an additive effect of iron upon oxidative stress. In contrast to HP and CHP, menadione induced a high number of genes belonging to the DNA repair category suggesting that it is more damaging to the cell than the two other oxidative agents. Mutants in both transcriptional regulators-Fur and PerR were affected in their ability to colonize chicken ceca indicating their important role in the in vivo growth and survival. Further investigation on the numerous gene targets identified in this study would pave way to better understand the patho-physiological mechanism of *C. jejuni* and might help to develop a drug or vaccine target against this pathogen.

ADVISOR'S APPROVAL: _____Dr.Alain Stintzi_____