Acid Adaptive Mechanisms of *Campylobacter jejuni* in the Gastrointestinal Tract

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ABSTRACT

Campylobacter jejuni is a prevalent cause of bacterial gastroenteritis in humans worldwide. The mechanism by which *C. jejuni* survives stomach acidity remains unknown. In this study, we have demonstrated that the ferric uptake regulator Fur plays an important role in *Campylobacter* acid survival. *C. jejuni* with a *fur* deletion was more sensitive to acid than the wild-type. Profiling the acid stimulon of the *C. jejuni* Δ*fur* mutant allowed us to uncover Fur-regulated genes under acidic conditions. The up-regulation of heat shock genes and the down-regulation of genes involved in flagellar and cell envelope biogenesis in the *fur* mutant highlight the importance of Fur in *Campylobacter* acid survival. Furthermore, prior exposure of *C. jejuni* to acid increased its capacity to survive other stresses, such as oxidative stress. This enhanced survival in the presence of oxidative stress was shown to be Fur-dependent through the regulation of catalase *katA* expression. Interestingly, Fur-mediated repression of *katA* was alleviated under low-pH conditions, allowing for higher catalase expression and defense against oxidative stress. Additionally, the transcriptome of *C. jejuni* under acidic conditions revealed that many genes involved in *Campylobacter* pathogenesis were differentially expressed. Prior exposure of *C. jejuni* to acid significantly increased its adherence to and invasion of human epithelial cells. Furthermore, *in vivo* experiments using *Galleria mellonella* larvae showed that acid exposure markedly enhanced *Campylobacter* virulence potential. In conclusion, this study demonstrates that the ferric uptake regulator Fur is a potential regulator of *Campylobacter* acid survival and cross-protection against other stresses. Moreover, our results suggest that the obligate passage of *C. jejuni* through the stomach acid barrier modulates the expression of its virulence factors and predisposes the bacterium for efficient gut colonization.

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LIST OF ABBREVIATIONS

ATR = Acid Tolerance Response

ANOVA = Analysis Of Variance

ASPs = Acid Shock Proteins

BSA = Bovine Serum Albumin

CFU = Colony-Forming Unit

CDT = Cytolethal Distending Toxin

EDTA = Ethylenediaminetetraacetic Acid

ETC = Electron Transport Chain

FBS = Fetal Bovine Serum

GBS = Guillain-Barré Syndrome

GIT = Gastrointestinal Tract

HBSS = Hanks Buffered Saline Solution

LOS = Lipooligosaccharide

LD = Lethal Dose

MEMα = Minimal Essential Medium alpha

MH Medium = Mueller-Hinton Medium

MOI = Multiplicity Of Infection

OD = Optical Density

ROSs = Reactive Oxygen Species

SDS = Sodium Dodecyl Sulfate

TCRSs = Two-Component Regulatory Systems

VBNC = Viable But Nonculturable

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Chapter 1 : Introduction

1.1. *Campylobacter* infection

1.1.1. *Campylobacter jejuni*

Campylobacter is a Gram-negative, microaerophilic, curved-rod, motile bacterium (216). This organism was first recorded in 1886 by Escherich, who isolated organisms resembling *Campylobacter* "vibrionen" from children suffering from diarrheal disease (82). Thereafter, *Campylobacter* was characterized as a nonculturable, spiral bacterium that was frequently isolated from dysenteric disease; *Campylobacter* was first successfully cultured in 1913 by McFadyean and Stockman (82, 224). Later, *Campylobacters* were isolated from patients during a large milk-borne outbreak of diarrheal disease in the United States in 1938 and from pregnant women in France nine years later in 1947 (260, 461). Due to their morphological similarity with *Vibrio cholerae*, *Campylobacters* were initially labeled as "related vibrios" (222, 223).

Campylobacter is a member of the order *Campylobacterales*, which belongs to the epsilon class of Proteobacteria that consists of two other genera: *Helicobacter* and *Wolinella* (3). *C. jejuni* and *C. coli* are considered the most important human pathogens as compared to other species included within *Campylobacterales* (3). The genome sequence of *C. jejuni* NCTC11168 was completed in 2000 (345). *C. jejuni* has a circular chromosome (30.6% G+C) of 1,641,481 base pairs (bp) in length, with approximately, 20% of the *Campylobacter* coding sequences represent pseudogenes (345). Strikingly, the genome of *C. jejuni* does not show insertion sequences or phage-associated sequences (345). The genome of *C. jejuni* was characterized by the presence of hypervariable sequences, which are thought to play a role in

the survival strategy of this bacterium (345). By comparison with other bacteria, 55.4% of *C. jejuni* genes have orthologues in the closely related *H. pylori*, with strong similarities mainly in housekeeping functions (345). Moreover, 28.0%, 27.0%, 4.6% and 2.1% of *C. jejuni* genes show similarity to genes from *E. coli*, *B. subtilis*, *A. fulgidus* and *S. cerevisiae*, respectively (345).

Campylobacter spp. can grow over a wide range of temperatures (30 to 47°C), with most preferring an optimal temperature of 42°C (317, 425). *C. jejuni* is oxygen-sensitive and grows optimally in the presence of low oxygen concentrations of 3-15% (216). In unfavorable environments, such as higher oxygen levels or nutrient deficiency, *Campylobacters* can change into a coccoid shape, which is a viable but nonculturable (VBNC) form (384). *Campylobacters* are generally considered to be asaccharolytic organisms that are unable to metabolize carbohydrates and thus require complex nutritional environments for growth (276, 345). An exception to this rule are *C. jejuni* strains possessing the FucP genomic island (*cj0480c–cj0490*) which are able to utilize L-fucose as a substrate for growth (304, 421).

1.1.2. Campylobacteriosis

C. jejuni is one of the most prevalent causes of human gastroenteritis worldwide and results in approximately 400-500 million infections annually (324, 388). Humans acquire a *Campylobacter* infection by consumption of contaminated meat and/or other food products (73, 131). Meat contamination occurs primarily during slaughtering or meat processing (73, 131). In addition to contaminated meat, milk and water have been reported as sources of *Campylobacter* infection in humans (6, 131, 454). *C. jejuni* infection varies from a mild, noninflammatory, self-limiting diarrhea to severe, inflammatory diarrhea that lasts for

several weeks (488). The inflammatory diarrhea caused by *C. jejuni* is usually accompanied by a fever and bloody stools containing polymorphonuclear leukocytes (44, 488). Moreover, tissue invasion by *C. jejuni* has been reported in many studies using animal models (391). Although *C. jejuni* infection is considered self-limiting, it is associated with higher mortality in both developing and developed countries (284, 365).

Links between *C. jejuni* and post-infection complications, such as Miller-Fisher syndrome and Guillain-Barré syndrome (GBS), have been reported in some individuals (4, 488). GBS is a peripheral neuropathy that results from a structural similarity between *Campylobacter*'s outer membrane lipooligosaccharides (LOS) and human gangliosides on peripheral nerve axons; this mimicry triggers an autoimmune reaction against the peripheral nervous system (159).

C. jejuni infection is responsible for an estimated 5% of food-related deaths and 17% of foodborne-related hospitalizations (284). The clinical features and epidemiology of *Campylobacter* infection are distinct between developed and developing countries (41). Interestingly, the number of patients that have diarrheal illness in developed countries that are infected with *C. jejuni* exceeds those infected with *Salmonella*, *Shigella* and *E. coli* O157:H7 combined (45, 273). Considering that most *C. jejuni* infections are substantially underreported due to difficulties in bacterial culturing, the recognized number of *C. jejuni* infections is believed to be even higher than reported (4). The Centers for Disease Control and Prevention (CDC) estimates that there are 2.5 million *C. jejuni* infections per year in the United States (4, 6, 284). *Campylobacter* illnesses are believed to cost up to $8 billion each year in the United States alone (59). The incidence estimates of *Campylobacter* infection in the European Union and New Zealand are 51/100,000 and 396/100,000, respectively (20,

195). In Canada, the annual number of *Campylobacter* infection cases is estimated to range from 9.1 to 19.3 per 1,000 Canadians (447). By comparison, the annual number of cases due to *Salmonella* infection is approximately 2.5 to 6.9 cases per 1,000 Canadians (447). The actual number of cases of illness due to *Campylobacter* infection is likely higher than these estimates due to factors such as under-diagnosing and under-reporting (447).

C. jejuni infection is much more common in developing countries as compared to developed countries (3). In developing countries, Campylobacterosis predominantly affects young children and the incidence of infection declines with age (442). Previous studies have reported that *C. jejuni* is the main cause of diarrhea in children under the age of five in developing countries (73). Exposure to *C. jejuni* infection early in life results in the development of protective immunity that may account for the lower severity of *Campylobacter* infection among adults in developing countries (61).

1.1.3. Infection management and treatment

As *Campylobacter* infection is self-limiting, treatment with antibiotics should be considered only in the cases of immunodeficient children or when the infection lasts for more than one week (41). The administration of the antibiotics erythromycin (the drug of choice) or azithromycin early in the infection course provides good results and shortens the duration of the illness (210, 414). However, the risk of death from *Campylobacter*-associated bacteremia is higher in patients who had inadequate antibiotic treatment or remained untreated as compared to those patients who were treated appropriately with antibiotics (332). Moreover, emerging bacterial resistance to antibiotics is considered a major concern in the treatment of *Campylobacter* infection (104). The extensive use of fluoroquinolone antibiotics on poultry farms has dramatically increased *Campylobacter* resistance to these

drugs (102-104). For example, the increasing isolation of *C. jejuni* isolates highly resistant to quinolones in the period between 1996 and 1998 in the United States was associated with the large-scale use of antibiotics in agriculture during this period (414). Mutations in DNA gyrase A (GyrA) are the primary cause of *Campylobacter* resistance to fluoroquinolone antibiotics (348). For other antibiotics, such as tetracycline, *Campylobacter* resistance occurs mainly through the acquisition of a pTet plasmid that carries a *tetO* gene (14). *Campylobacter* resistance to macrolides (e.g., erythromycin) is chromosomally mediated and is conferred by target modification such as mutations in the 23S rRNA gene (157, 441, 483, 484). Unfortunately, the development of anti-*Campylobacter* vaccines has been hampered by our poor understanding of the virulence factors of this pathogen and the mechanisms surrounding *Campylobacter* pathogenesis (398).

Finally, limiting infection transmission from poultry to humans has been shown to be a critical step in the control of *Campylobacter* infection (82). This limited transmission could be achieved through different techniques, including the use of disinfectants and biosecurity measures in poultry houses (82).

1.1.4. *Campylobacter* virulence factors

1.1.4.1. Motility and chemotaxis

The importance of flagella in *Campylobacter* host colonization and pathogenesis has been demonstrated in previous studies (66, 313). Flagella have been shown to enhance *Campylobacter* movement into the mucus layer, allowing *Campylobacter* to efficiently colonize the intestine (253, 319, 434). Moreover, the contribution of the flagella to *Campylobacter* adhesion to and invasion of epithelial cells is well established (147, 464,

486). Interestingly, both nonflagellated and nonmotile mutants of *C. jejuni* were unable to colonize suckling mice and were easily cleared from the gastrointestinal tract (GIT), indicating the importance of flagella in *Campylobacter* host colonization (300, 318). The *Campylobacter* flagellum is composed mainly of a basal body, hook and filament. The filament consists mainly of two flagellin proteins: FlaA and FlaB (166, 323). Flagellar genes are regulated by FliA (σ^{28}) and RpoN (σ^{54}) in addition to the two-component signal transduction system FlgS/FlgR (193, 481). Components of the flagella have been successfully used as a component in vaccine preparation against *Campylobacter* infection in mice (257). However, the efficacy of flagellar components in a human vaccine has not been evaluated and remains to be tested.

In addition to bacterial motility, chemotaxis, which is the bacterial capacity to respond to specific chemicals or nutrients and alter their motility accordingly, is an important determinant for *Campylobacter* pathogenesis (454). *Campylobacters* are attracted to amino acids and mucus components that are present at higher concentrations in the GITs of chickens whereas they are repelled by bile acids (185). A genomic analysis has demonstrated that *C. jejuni* harbors many chemotactic genes that play a role in *Campylobacter* survival and host pathogenesis (277, 345). In addition to the chemotaxis regulatory protein, CheY (171, 487), *C. jejuni* contains two methyltransferases, CheB and CheR, which are important for methylation-dependent chemotaxis pathways (204). *C. jejuni* Δ*cheY* and Δ*cheBR* mutants were defective for chick colonization as compared to the wild-type strain (167, 204). Moreover, the genome of *C. jejuni* encodes at least ten methyl-accepting chemotaxis proteins (345, 488). Mutants lacking any of the methyl-accepting chemotaxis receptors were defective in animal colonization and pathogenesis (167, 436, 487).

1.1.4.2. Surface polysaccharide structures and protein glycosylation

C. jejuni displays several surface carbohydrate structures, such as the capsule, LOS and *O*- and *N*-linked glycans, that contribute to bacterial pathogenesis (171). Mutating the capsular polysaccharide transporter genes *kpsM* or *kpsE* leads to a defect in *Campylobacter*'s capacity to colonize chicks (13, 15, 200). Additionally, the *C. jejuni* LOS is important for bacterial evasion of the human immune system, along with host cell adhesion and invasion (208). Sialylation of the LOS outer core significantly reduces immunogenicity and contributes to successful *Campylobacter* host colonization (150, 381). In addition, the *C. jejuni* LOS is believed to be responsible for the development of GBS because of its mimicry of gangliosides (150, 312, 381). The formation of autoantibodies that are responsible for axon demyelination is thought to be the leading cause of GBS (33, 101, 240).

In *C. jejuni*, glycoproteins play a role in bacterial adhesion (11, 234), host colonization (433), protective immunity (137, 358) and antigenic variation (99, 132). Importantly, *C. jejuni* possesses systems for two different protein glycosylation pathways: *O*-linked glycosylation and *N*-linked glycosylation (435, 488). *O*-linked glycosylation of flagellin plays a role in the proper assembly of the flagellar filament in *C. jejuni*, and any defect in this process results in the loss of bacterial motility and decreased virulence in animal models (146, 151). The other type of protein glycosylation, *N*-linked glycosylation plays a role in the post-translational modification of multiple periplasmic and membrane-bound proteins in *C. jejuni* (2, 207, 489). Protein *N*-glycosylation plays a role in the *C. jejuni* virulence and host pathogenesis (2). A *C. jejuni pgl* mutant, in which general protein glycosylation is impaired, exhibited reduced cell adherence and invasion and was defective in animal colonization (167, 200,

206, 433). Altogether, it is clear that protein glycolysation is very important for *C. jejuni* host colonization and pathogenesis (246, 433).

1.1.4.3. Adhesion and invasion

Campylobacter adhesion to and invasion of the GIT epithelial cells are important for bacterial host colonization and disease development (171, 391). Moreover, the severity of *Campylobacter* infection and the degree of inflammation are directly correlated to *C. jejuni*'s ability to adhere to epithelial cells (112). The isolation of *C. jejuni* from patient tissues and its ability to invade intestinal epithelial cells indicate the importance of the *Campylobacter* invasive capacity for host pathogenesis (147, 226, 350, 379, 464). Much of the gastric inflammation and mucosal damage observed in *Campylobacter* infection may result from bacterial invasion of intestinal epithelial cells (454). For example, several inflammatory markers, such as the proinflammatory cytokine IL-8, are produced as a consequence of infection and epithelium invasion by *C. jejuni* (108).

While *C. jejuni* lacks most of the adherence proteins identified in other pathogens, it harbors CadF (*Campylobacter* adhesion to fibronectin), which binds specifically to fibronectin present on epithelial cells (227, 298, 299). CadF induces bacterial internalization by triggering signaling processes and the activation of the small GTPases Rac1 and Cdc42 (236). It is thought that *Campylobacter* invasion and internalization into intestinal cells is mediated via microtubule-dependent and actin filament-independent mechanisms (39, 299, 326, 401). In addition to CadF, *C. jejuni* also expresses PEB1, which acts as a bacterial adhesin (350). Interestingly, a *C. jejuni peb1* mutant strain was defective in cell adhesion, invasion and animal model colonization (350). Another known *Campylobacter* adhesin is the surface-exposed lipoprotein JlpA, which is required for cell binding and proinflammtory

cytokine production (196, 197). The contribution of other proteins, such as the *Campylobacter* invasion antigens (Cia) and the secreted protein FlaC, to *C. jejuni* cell invasion remains unclear and requires further research (379, 419).

1.1.4.4. Toxins

The characterization of *C. jejuni* toxins is important to fully understand bacterial pathogenesis and cytopathic effects in the host (454, 478). *C. jejuni* produces cytolethal distending toxin (CDT) which is produced by many pathogens including *E. coli* and *Haemophilus ducreyi* (488). CDT causes arrest at the G2 phase of the cell cycle by blocking the CDC2 kinase (160, 244, 474). Active CDT is a complex of three proteins: CdtA, CdtB and CdtC; CdtB and CdtC together without CdtA display cytotoxic activity (245, 259). The CdtB component, which is responsible for the CDT toxic activity, has DNase activity and causes DNA damage, while both CdtA and CdtC are thought to be involved in binding to host cells (160, 244, 245). *In vitro* studies showed that CDT interferes with cell viability by causing cell death through the induction of cell distension and swelling (474). CDT also interferes with intestinal absorption by disrupting crypt cells and inhibiting their maturation into functional villi, thus, resulting in the diarrhea that is associated with *Campylobacter* infection (474). Furthermore, CDT has a role in modulating the human immune response through the production of interleukin IL-8, which induces intestinal inflammation (173, 360). Interestingly, a *C. jejuni cdtB* mutant was able to colonize the GIT of immunodeficient mice but was defective in invading different body organs relative to the wild-type strain, highlighting the importance of CDT in *C. jejuni* virulence (360). Finally, the immune response to CDT is host-specific and depends on the host's capacity to recognize *Campylobacter* CDT antigens (1, 38, 488). In contrast to *Campylobacter* colonization in

chickens, in which CDT does not promote inflammation, CDT induces the production of neutralizing antibodies in humans (1, 488). Characterizing the role played by CDT in *Campylobacter* pathogenesis would enable us to understand the virulence mechanisms of *C. jejuni* and to develop strategies to control *Campylobacter* infection in the host.

1.1.4.5. Two-component regulatory systems

Two-component regulatory systems (TCRSs) are widely spread in bacteria, including *C. jejuni* (306, 454). TCRSs play essential roles in signal transduction by controlling gene expression to enhance bacterial survival in various environments (306, 454). TCRSs consist of two proteins: a sensor protein with histidine kinase activity and a response regulator (427, 488). The sensor protein is autophosphorylated upon detecting its environmental stimuli. Subsequently, the sensor protein transfers a phosphate group to the corresponding response regulator, which affects the expression of target genes (293, 346, 488).

Several TCRSs have been identified in *C. jejuni* that contribute to its survival in various environmental conditions, host colonization and pathogenesis (171). For example, the TCRS RacRS was demonstrated to be involved in thermoregulation and *Campylobacter* host colonization (50, 477). A *C. jejuni* Δ*racR* mutant was defective for growth at 42ºC and chick colonization as compared to the wild-type strain (50). *C. jejuni* also contains the *Campylobacter* planktonic growth regulation (CprRS) (431), and the phosphate-sensitive (PhosS/PhosR) TCRSs (480). CprRS regulates *Campylobacter* adaptation to various environmental stressors (e.g., osmotic and oxidative stress) and is important for *C. jejuni* pathogenesis (431). The PhosS/PhosR TCRS is stimulated by phosphate limitation and regulates the expression of many *Campylobacter* phosphate-acquisition genes (480). Many other TCRSs such as the flagellar biogenesis and motility regulator (FlgRS) (481), the

diminished capacity to colonize (DccRS) (274), and the response regulator (CbrR) (423) were found to be important for *C. jejuni* host colonization and response to various environmental stimuli (50, 368, 454). Since TCRSs play a role in *Campylobacter* adaptation to environmental changes, they could represent potential targets for the treatment and management of *C. jejuni* infections.

1.2. Enteric pathogens and acid stress survival

Enteric bacteria possess an extraordinary set of stress response mechanisms for surviving harsh conditions, such as fluctuations in environmental pH (387). Enteropathogens, such as *S. typhimurium*, *E. coli* and *S. flexneri* are neutralophiles that require a neutral pH for survival (26, 492). Being neutralophiles does not guarantee that enteric bacteria will not experience an unfavorable pH during their life (492). During host infection, enteric pathogens must transit through the stomach acid barrier, where the pH is low enough to kill them (297). In addition to stomach acidity, enteric pathogens are exposed to volatile fatty acids such as acetate, propionate, and butyrate that are present in the intestine (241). Volatile fatty acids can diffuse across the bacterial cell membrane, dissociate intracellularly and lower the internal pH (241). Moreover, facultative intracellular pathogens, such as *Salmonella* sp., must survive a highly acidic pH (pH 4.5-5) once they are engulfed by phagolysosomes (328, 370).

Enteropathogens also experience pH fluctuations outside of the host GIT. For example, enteric bacteria encounter low-pH conditions in industrial waste or decaying organic matter (26). Interestingly, the infective dose of enteric bacteria appears to be directly proportional to their capacity to cope with such acidic conditions. *S. flexneri*, non-typhi *Salmonella* and *V. cholerae* have oral infectious doses of 10^2, 10^5 and 10^9 colony-forming units (CFUs),

respectively, that correlate with the acid survival capacity exhibited by each organism; *S. flexneri* is the most acid-resistant, and *V. cholerae* is the most acid-sensitive (43). This examplifies the fact that the ability of bacteria to cope with acidic conditions not only enhances their survival of acid stress but also enhances their pathogenesis and host colonization capabilities (43).

1.2.1. Acid stress: definition and impact on bacteria

The term ‘acid stress’ is defined as the biological effects of low pH and weak acids encountered by bacteria (26). Acids have been extensively used in food industry to preserve food against microbial activity (144, 363). Nevertheless, acid-treated foods that were believed to be microbiologically safe were later found to be responsible for many bacterial diseases and revealed that acid-preserved food could act as a vehicle for various microbial infections (34, 278). Moreover, it has been suggested that the use of acid to decontaminate food could help bacteria develop adaptive mechanisms to various environmental stresses (52, 468).

The viability of bacterial cells exposed to a severe acid shock differs depending on whether they had been exposed to an earlier mild acid treatment (125). The capacity of bacteria to survive severe acidic conditions after they have adapted to a mild acid for one generation is described as acid tolerance (AT) (126). The exposure of bacteria to a low pH disrupts the outer membrane, interferes with the biosynthesis of cellular components and eventually leads to cell death (387). In addition, bacterial death could result from the perturbation of cytoplasmic pH homeostasis and subsequent damage to DNA and cellular enzymes (364).

Distinguishing between the stresses caused by organic and inorganic acids is very important. Bacteria employ different repair systems in response to each type of acid stress and this

indicates differences between acid stress caused by either organic or inorganic acids (25, 241). For example, the TCRS PhoP/PhoQ plays a role in *S. typhimurium* tolerance to inorganic acid stress but has a minor effect against organic acid stress (25). The exposure of bacteria to weak organic acids (e.g., benzoic acid) not only results in an increased intracellular concentration of protons that acidify the intracellular pH but could also result in anion accumulation within the cells (25, 26, 242). The accumulation of anions inside the cell interferes with the intracellular glutamate pool and consequently perturbs the anion balance during bacterial growth (383). As such, whether proteins involved in the *E. coli* response to benzoic acid are due to the accumulation of benzoate anion or a reduction in intracellular pH is unknown (242). An understanding of the bacterial response to acidic conditions is important for limiting foodborne infections in humans.

1.2.2. Different bacterial response strategies for acid stress

Enteric bacteria have developed numerous constitutive and/or inducible mechanisms to sense and adapt to acidic environments (26). These mechanisms include amino acid decarboxylases, the regulatory sigma factor σ^s (RpoS), the ferric uptake regulator Fur, PhoP and OmpR (26), as shown in Figure 1.1.

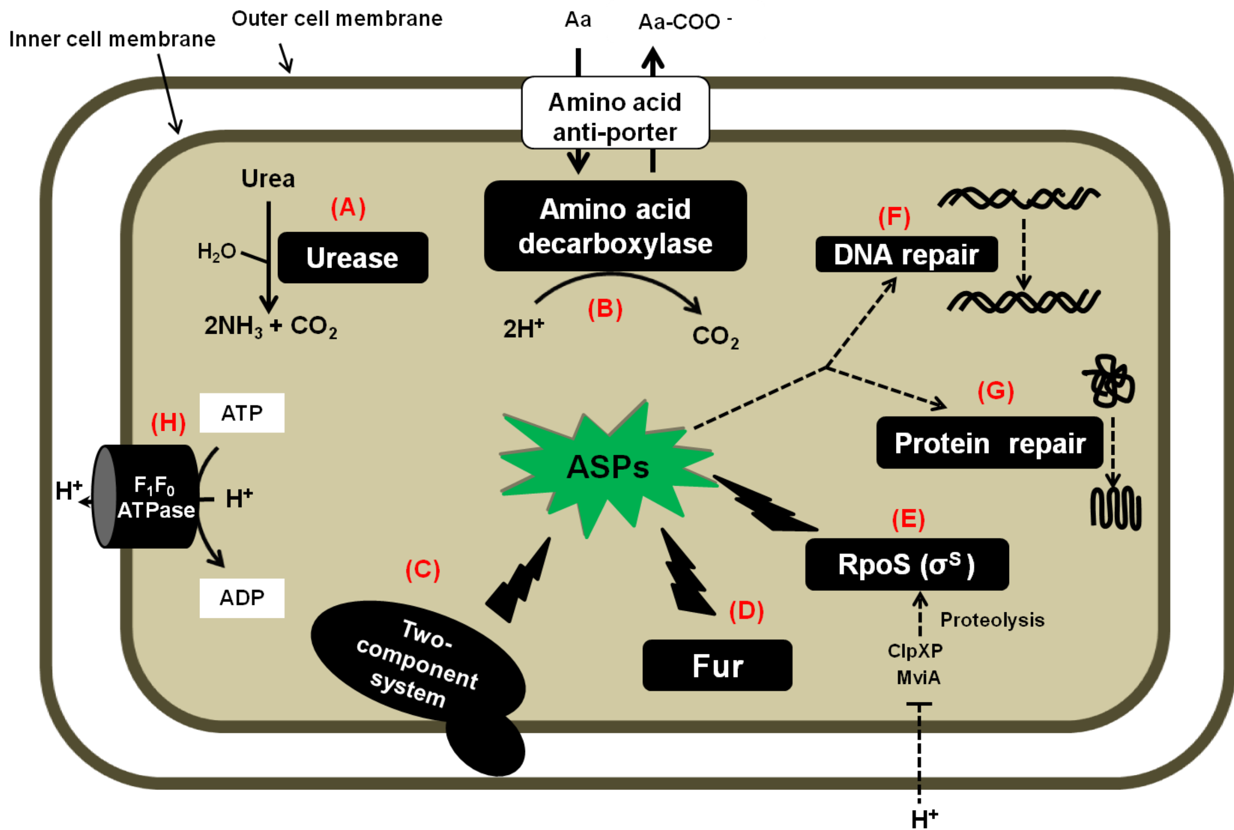


Figure 1.1: Different strategies for acid stress survival in enteric bacteria. The figure represents a composite cell, showing various mechanisms of acid response in enteric pathogens. Some bacteria [e.g., *H. pylori* (424)] encode a urease enzyme (A) that catalyzes ammonia production from urea and thereby alkalinizes the acidic cytoplasm. The exposure of bacteria to a low external pH also results in the activation of amino acid decarboxylases (B) that consume hydrogen protons during amino acid decarboxylation and exchange the end products for new substrates via membrane-bound antiporters [e.g., *E. coli* (172) and *Shigella* sp. (466)]. Acid exposure also activates the expression of many regulators, such as two-component systems (C) as in *Salmonella* sp. (25), the ferric uptake regulator Fur (D) as in *Shigella* sp. (327) and *Salmonella* sp. (127) and sigma factor σ^S (RpoS) (E) as in *Salmonella* sp. (254). Acid exposure increases RpoS concentration through prevention its degradation and proteolysis by MviA chaperone and ClpXP protease (27). The two-component systems, Fur and RpoS activate the expression of acid shock proteins (ASPs) that function to repair the damage that occurs to cellular components, such as DNA (F) and proteins (G), upon exposure to acid. Finally, the F_1F_0 ATPase (H) pumps protons outside the cellular cytoplasm, resulting in a reversal of intracellular acidity (76, 282, 430).

1.2.2.1. Urease

Ureases (urea amidohydrolase) are multi-subunit, nickel-containing enzymes that are produced by many bacteria (74, 295, 296) and catalyze urea hydrolysis to carbon dioxide and ammonia (54). Ureases are multi-subunit and require additional urease specific gene products for the biogenesis of active holoenzyme (54). The urease gene cluster contains seven genes; *ureA*, *ureB* and *ureC* encode the structural subunits of urease, and *ureE*, *ureF*, *ureG*, and *ureH* code for urease assembly proteins (54, 258, 295). Urease activity has been demonstrated to be important for many bacteria such as *H. pylori* (97, 424) and enterohemorrhagic *E. coli* (165). The enteric pathogen, *H. pylori* has to pass through the highly acidic environment of the stomach before it can reach the protective mucosa (424). Under such conditions, *H. pylori* is capable of maintaining its cytoplasmic pH at a value close to neutral (424). Urease elevates the local pH through the production of ammonia from urea hydrolysis, allowing the bacterium to survive in acidic environments (145, 424). In addition to its role in the acid tolerance of *H. pylori*, urease is important for both host colonization and pathogenesis (97, 205, 451). For example, the urease-negative *H. pylori* mutant is defective for animal colonization as compared to the wild-type strain (205, 451).

1.2.2.2. Amino acid decarboxylases

The importance of amino acid decarboxylases in the acid survival of enteric pathogens has been demonstrated extensively (68, 122, 129, 266). For example, three amino acid decarboxylases have been characterized in *Salmonella* sp. which are the lysine, ornithine and arginine decarboxylases (26, 344). Amino acid decarboxylases elevate the cellular pH through the consumption of protons during amino acid decarboxylation and exchange the end products for new substrates via a membrane-bound antiporter (344, 376).

For example, *Salmonella* lysine decarboxylase (CadA) consumes protons during lysine decarboxylation to cadaverine, which is exchanged for fresh lysine via a cadaverine antiporter (CadB) (344). In contrast to *Salmonella* sp., the activities of amino acid decarboxylases in *E. coli* and *Shigella* sp. depend primarily on whether these bacteria undergo oxidative or fermentative metabolism (26, 264). Under fermentation conditions, two systems are activated: the arginine (191) and glutamate decarboxylases (68). Both systems act similarly to lysine decarboxylase in *Salmonella* through the consumption of protons during amino acid decarboxylation and exchange the decarboxylation end product for fresh amino acids from the medium (26, 264). In contrast to the fermentation systems identified in *E. coli* and *Shigella* sp., an oxidative system that is repressed by glucose and depends on sigma factor (σ^S) for expression was observed in these bacteria when grown to the stationary phase (264). Once the oxidative system is induced, bacterial cells do not need the presence of amino acids in the medium upon further exposure to an acid challenge (26, 264, 466). Moreover, the cyclic AMP (cAMP) and cAMP receptor protein (CRP) are required for the oxidative glucose-repressed system (68, 376).

1.2.2.3. The ferric uptake regulator (Fur)

The ferric uptake regulator Fur is an iron-binding transcriptional regulator (156). Under iron-replete conditions, Fur binds to iron, and subsequently, the Fur-iron complex binds to the Fur box in the target genes promoters, thereby repressing their expression (16, 17, 107). More than 200 gene loci are directly bound by the Fur protein in *H. pylori*, reflecting the importance of Fur as a global regulator (85). In addition, Fur is involved in regulating the expression of several genes involved in oxidative stress defense such as *sodB* and *katA* in many enteric pathogens such as *H. pylori*, *E. coli*, and *C. jejuni* (84, 105, 106,

180, 280, 320, 337, 338, 440). Despite being a classical repressor for gene expression, Fur acts as a positive regulator for a subset of genes that encode proteins that are important for chemotaxis, motility, host interactions and redox equilibrium in *H. pylori* (85, 86, 105). Small regulatory RNAs (sRNAs) also play a role in indirect, positive gene regulation by Fur in many bacteria (327). The sRNAs act post-transcriptionally on the decay and translation of target mRNAs (280).

Importantly, links between Fur regulation and acid stress survival have been demonstrated in enteric pathogens. A *fur* mutant in avian septicemic *E. coli* is unable to survive under acidic conditions as compared to the wild-type strain (493). Furthermore, Fur positively regulates the expression of urease in both enterohemorrhagic *E. coli* and *H. pylori* (165, 455). While Fur is involved in acid survival in both *S. typhimurium* and *S. flexneri* (124, 154, 327), the pathways regulating the expression of acid-responsive genes are different in both organisms (264). In *S. typhimurium*, the ASPs are activated by Fur in an iron-independent manner (124, 154), which contrasts with what has been observed in *S. flexneri*, where both Fur and iron are essential for acid survival (327).

1.2.2.4. The sigma factor RpoS

Bacterial sigma factors play a role in activating transcription in response to various environmental signals (406). Importantly, seven sigma factors have been identified in *E. coli* (110). These sigma factors, σ^{70} (RpoD), σ^{54} (RpoN), σ^{38} (RpoS), σ^{32} (RpoH), σ^{28} (RpoF), σ^{24} (RpoE) and σ^{19} (FecI), are classified into two groups: the σ^{70} family and the σ^{54} group (479). At least four of these sigma factors are involved in the bacterial response to stress: RpoE and RpoH are involved in the heat shock response, RpoN is involved in nitrogen regulation and RpoS is important for bacterial survival in the stationary phase (110).

Importantly, RpoS is a global transcriptional regulator that plays an important role in the stationary phase-associated resistance to various stresses, such as heat, osmolarity, acid and oxidative stress (270). Upon bacterial entry into the stationary phase, the concentration of RpoS increases significantly (243). RpoS regulates the expression of many genes, known as the σ^S regulon, that are involved in bacterial survival in unfavorable conditions (169, 243). RpoS is important for the acid survival of many enteric pathogens, such as *E. coli*, *S. typhimurium* and *S. flexneri* (266, 411). For example, the *E. coli* and *S. flexneri* Δ*rpoS* mutants are defective in maintaining a higher internal pH in the face of external acidity as compared to the wild-type strains (411). The expression levels of at least ten ASPs involved in *Salmonella* acid survival are regulated by RpoS (12). The exposure of bacteria to low pH protects RpoS from degradation and proteolysis by the ClpXP protease (301, 397). The ClpXP protease is regulated by MviA (encoded by *m*ouse *vi*rulence gene; *mviA*), which is inactivated by acids (12) as shown in Figure 1.1. Additionally, mutating *mviA* results in the stabilization of RpoS and consequently increased bacterial resistance to acid due to the elevated expression of RpoS-dependent genes (27).

1.2.2.5. The two-component regulatory system PhoP-PhoQ

PhoP-PhoQ is a two-component regulatory system that has been identified in many bacteria (149). PhoP-PhoQ consists of a cytoplasmic regulator, PhoP and an inner membrane sensor, PhoQ (149). Transcription of PhoP-activated genes is enhanced in response to low concentrations of Mg^{2+} (139, 149). Under low concentrations of Mg^{2+}, PhoP is transphosphorylated by PhoQ and induces the expression of target genes (12). Moreover, the PhoP-PhoQ system is involved in the regulation of many genes involved in cellular activities and bacterial pathogenesis (149). In *Salmonella* sp., the expression of PhoP-regulated genes

is enhanced under low pH conditions, suggesting that the PhoQ protein is involved in acid detection (5). Moreover, PhoP itself is an ASP that is required for the expression of other ASPs (25). The acid shock induction of PhoP appears to occur at the transcriptional level and requires PhoQ (25). The mechanism of acid detection by PhoP-PhoQ remains unknown, and whether PhoQ senses the pH independently of Mg^{2+} or whether the conformation of the Mg^{2+} binding site is affected by protons is unclear (25). However, the hypothesis that PhoQ could be a pH sensor remains controversial. Bacterial exposure to mild acidic conditions induces transcription only in a subset of PhoP-activated genes, and this activation also occurs in a *phoQ* mutant (25, 139, 418).

1.2.2.6. The two-component signal transduction system EnvZ-OmpR

OmpR is involved in the acid response of many enteric pathogens, such as *S. typhimurium* (22, 23). OmpR which functions as an activator, is part of a two-component signal transduction system in which EnvZ is the inner membrane sensor (294). Bang *et al*. demonstrated that the level of OmpR increased primarily in acid-exposed stationary phase cells and to a lesser extent in log phase cells (22). OmpR itself is considered an ASP that is induced at the transcriptional level upon exposure to acid stress (22). Furthermore, OmpR can trigger the expression of numerous ASPs that are involved in the stationary phase AT response (23). Upon detecting a signal, EnvZ is autophosphorylated and transfers a phosphate to OmpR (22, 93, 186), which activates the expression of target genes (93, 186, 380).

1.2.2.7. DNA repair

Exposure of bacteria to acid stress results in DNA damage through depurination and depyrimidination (24, 267, 268, 272). Therefore, many bacteria have developed efficient mechanisms to correct the damage in DNA that occurs upon exposure to acid (129). The involvement of certain proteins in repairing damaged DNA within bacterial cells provides a valuable means to survive highly acidic conditions (12, 123, 129). Mutations in genes involved in the repair of acid-induced DNA damage, such as *polA* (DNA polymerase I) and *ada* (DNA methyl transferase) lead to increased bacterial sensitivity to acid (12, 129, 339, 364). Raja *et al*. demonstrated that mutant strains of *E. coli* defective in repairing damaged DNA were highly acid-sensitive (364). Moreover, an ***H.** pylori* Δ*recN* mutant was unable to survive a decrease in pH (463). RecN is involved in homologous recombination, one of the key mechanisms involved in repairing DNA double-strand breaks (463).

1.2.2.8. F_1F_0-ATPase proton pump

Many bacteria use proton pump systems to extrude protons out of the cytoplasm and consequently prevent its acidification (407). For example, the exposure of bacteria to acid stress results in the up-regulation of F_1F_0-ATPase genes that play an important role in the bacterial acid response (18, 36, 77, 128). F_1F_0-ATPase is a multi-subunit system that links ATP production and the transmembrane proton motive force (PMF), which facilitates the extrusion of protons from the cell cytoplasm (78). F_1F_0-ATPase is composed of two systems: a membrane-embedded F_0 complex that has proton-translocating activity and a peripherally bound F_1 complex that has ATPase activity (116). The importance of the F_1F_0-ATPase in proton extrusion and reversing the cytoplasm acidification in many food pathogens, such as *L. monocytogenes* and *E. coli*, has been demonstrated (87, 116). *L. monocytogenes* cells

treated with the ATPase inhibitor *N*, *N*'-dicyclohexylcarbodiimide (DCCD) were more sensitive to acid relative to untreated cells, suggesting that the F_1F_0-ATPase is essential for *L. monocytogenes* acid survival (87). Moreover, the F_1F_0-ATPase is important for the induction of an acid tolerance response (ATR) in *L. monocytogenes* and enables bacteria to survive severe acidic conditions following exposure to a mild acid (76).

1.3. *Campylobacter jejuni* stress response

In comparison with other enteric pathogens, such as *E. coli*, little is known about the mechanisms of *Campylobacter* stress responses (306, 342). Moreover, how *C. jejuni* regulates gene expression in response to different stress conditions remains ambiguous and not fully understood (342). However, the low infectious dose of *C. jejuni* in humans suggests that it has developed certain mechanisms to sense and cope with various stresses encountered either within or outside of the host (40, 382). For example, *C. jejuni* can survive for long periods in unfavorable environments, such as low temperature (56, 164, 384). In the following section, the mechanisms developed by *C. jejuni* to survive various stresses will be highlighted.

1.3.1. Mechanisms of *C. jejuni* stress survival

1.3.1.1. Viable but nonculturable (VBNC) state

The VBNC state is a survival mechanism that has been observed in many organisms, including *C. jejuni*, in response to stress conditions (75, 213, 330, 384). Simply, the VBNC state means that an organism cannot be cultured under unfavorable conditions but remains viable and metabolically active until the surrounding environment becomes more suitable for

growth and cell division (236). Morphologically, *C. jejuni* transforms from its characteristic spiral shape to a coccoid form during the VBNC state (384).

The contribution of the VBNC state of *Campylobacter* to bacterial survival in the presence of environmental stresses has been characterized (163). The coccoid form enables *C. jejuni* to be dormant under unfavorable conditions until the surrounding environment becomes supportive of its growth (384). For example, *C. jejuni* can escape severe acidic conditions by transforming into the VBNC form (71). *Campylobacters* could not be cultured even using enrichment culture media; however, viable cells were detected using a double-staining technique (71). The capacity of *C. jejuni* to transform into the VBNC state under stress was further confirmed by the detection of *C. jejuni* in the viable state following long-term exposure to low temperatures (248). This result was detected using indicators of cell viability, such as respiratory activity and cellular integrity (248).

The importance of the VBNC state of *Campylobacter* for bacterial pathogenesis and animal colonization remains controversial and likely depends on both the bacterial strain and the animal species (198, 285). The VBNC state could be a *Campylobacter* risk factor if the organism is capable of infecting a host during this state (306). A previous study indicated that nonculturable *Campylobacters* were able to colonize chicks following their consumption of water that was contaminated with VBNC *Campylobacter* (349). In contrast, other studies have shown that *Campylobacters* could not be isolated from the stool of chickens that were previously infected with nonculturable forms (285, 494). Moreover, no specific antibodies against *Campylobacter* were detected in animal models (mice and rabbits) following the administration of coccoid forms (35, 163). Clearly, more work is required to fully

characterize the contribution of VBNC state to *Campylobacter* pathogenesis and colonization.

1.3.1.2. Stress regulators

While *C. jejuni* harbors some regulators involved in stress responses, such as the heat shock regulators HspR and HrcA (306), it lacks most of the common regulators identified in many enteric pathogens (341). These regulators include SoxRS and OxyR, which protect bacteria against oxidative stress, the major cold shock protein CspA and the alternative sigma factor RpoH, which regulates the heat shock response (306). Moreover, *C. jejuni* lacks genes encoding the stationary phase-associated RpoS, which acts as a general stress regulator (345). The genome of *C. jejuni* contains only three sigma factors, *fliA*, *rpoD* and *rpoN* (345). The absence of RpoS in *C. jejuni* could account for the observation that *C. jejuni* is more sensitive to stress when growing in the stationary phase than in the mid-exponential phase (214). *C. jejuni* entry into the stationary phase is not associated with the physiological changes observed in other bacteria that are involved in the protection of these bacteria against various stresses (170, 279). For example, *C. jejuni* did not exhibit any increase in bacterial resistance to heat or acid stress upon entry into the stationary phase (279). In fact, modulations in the membrane fatty acid composition and increased cell membrane integrity were the only changes in *Campylobacters* upon entry into the stationary phase (279).

1.3.1.3. Two-component regulators

The two-component regulatory systems (TCRSs) are important for bacterial signal transduction and response to environmental stresses (306, 427). The TCRS is composed of a sensory histidine kinase (HK) that can transphosphorylate the corresponding response

regulator (RR) (427). The RR stimulates the differential expression of target genes, allowing bacteria to immediately respond to changes in environmental conditions (306, 427). The *C. jejuni* genome contains 7 HKs and 12 RRs (345), and among these are five TCRSs with an adjacent HK and RR such as CprRS, DccRS and RacRS (306, 431). The role of various TCRSs in *C. jejuni* survival during stress conditions and pathogenesis has been described in this chapter under *Campylobacter* virulence factors.

1.3.2. Survival of *Campylobacter jejuni* in the presence of major stresses

While *Campylobacter* lacks many proteins involved in the stress response in other organisms, *C. jejuni* has developed certain mechanisms to survive unfavorable conditions. This section highlights some of the characterized mechanisms *C. jejuni* employs to survive major stresses, such as heat, oxidative and osmotic stresses.

1.3.2.1. *Campylobacter* response to temperature stress

C. jejuni can grow over a wide temperature range (30 - 47°C) with an optimum growth temperature of 42°C (425). *C. jejuni* transforms into the VBNC form upon exposure to lower temperatures (248) and *C. jejuni* can survive at 4°C for extended periods (42, 248, 384). However, *C. jejuni* is sensitive to higher temperatures and its growth declines at temperatures above 42°C (306). The transcriptional profile of *C. jejuni* transitioned from an incubation temperature of 37°C to 42°C revealed that 20% of the *Campylobacter* genes were differentially expressed in response to change in temperature (425). Most of the alterations in gene expression occurred rapidly after the temperature change, indicating that *C. jejuni* can modulate gene expression rapidly in response to a new temperature (192). In a similar study,

exposure of *C. jejuni* to heat shock resulted in the up-regulation of at least 24 proteins; one of these proteins was DnaJ, a well-known heat shock chaperone (229).

With regard to the response to temperature stress, the genome of *C. jejuni* encodes a signal transduction system designated RacR-RacS, which is the reduced ability to colonize system that plays a role in a temperature-dependent signaling pathway (50). Interestingly, a *C. jejuni* Δ*racR* mutant had impaired growth at 42ºC as compared to the wild-type strain (50). In addition to this two-component regulator, *C. jejuni* harbors several heat shock proteins that enable the bacteria to respond to variations in temperature (229). Heat shock proteins act as chaperones that repair damaged proteins and degrade misfolded proteins upon exposure to heat stress (229). Many heat shock proteins and chaperone homologs, including the molecular chaperones GroELS, DnaK and DnaJ have been identified in *C. jejuni* (192, 306, 425). The importance of heat shock proteins for *C. jejuni* was further confirmed by the finding that a *C. jejuni* Δ*dnaJ* mutant had impaired growth at a higher temperature as compared to wild-type strain (229).

The contribution of extracellular proteins produced by *C. jejuni* to thermotolerance has also been previously characterized (192, 307). Strikingly, the survival of *C. jejuni* at 55°C increased 100-fold when the bacteria were grown in used medium that previously contained *Campylobacters* as compared to *Campylobacter* grown in fresh medium (307). Moreover, *C. jejuni* survival in used medium was very similar to its survival in fresh medium when the cell-free used medium was treated with proteinase (307). This finding suggests that protective extracellular protein(s) produced by *Campylobacters* enhance the survival capacity of other cells (192, 307). In contrast to other enteric pathogens, *C. jejuni* resistance to heat decreases upon entry into the stationary phase (216). As mentioned previously, *C.*

jejuni entry into the stationary phase is not accompanied by the physiological changes observed in other enteric bacteria that enhance their survival in the presence of various stresses, and this is most likely due to the absence of RpoS (170, 243, 345).

1.3.2.2. *Campylobacter* response to osmotic stress

Osmotic stress is considered one of the biggest challenges encountered by enteric pathogens during host infection (192). An optimal environmental osmolarity is crucial for proper bacterial cell growth and division (192). Therefore, enteric bacteria have developed different mechanisms in response to variations in environmental osmolarity (192). For example, bacteria modulate the expression of genes encoding various transporters and/or enzymes in response to the solute concentration in the growth environment (473). The minimum cytoplasmic solute concentration required for bacterial growth is 300 mOsm (473). The growth of bacteria under unfavorable osmotic conditions can inhibit physiological processes and can induce a VBNC state (192, 473). However, bacteria can maintain osmotic homeostasis in both low- and high-osmolarity environments (473). For example, bacteria produce oligosaccharides to avoid hypo-osmotic shock, while under higher osmotic conditions, bacteria activate the uptake of compatible solutes, such as K^+, to maintain a higher osmolarity within the cell (473).

How *C. jejuni* regulates different transport systems to adapt to variations in osmolarity has not been fully characterized (140, 192). *C. jejuni* lacks most osmoprotectants that have been identified in other bacteria, such as *E. coli* (140). Only one K^+ transport system has been identified in *C. jejuni*; however, the influence of osmotic stress on its induction has not yet been characterized (192, 340). Interestingly, entry into the VBNC state is one possible mechanism by which *Campylobacters* respond to low osmolarities (372). Additionally,

Campylobacter culturability significantly decreases in the presence of the higher concentrations of NaCl that are commonly used to inhibit microbial growth in the food industry (94). Moreover, *Campylobacter* survival in presence of salt is significantly influenced by both environmental temperature and pH (94, 212). For example, the survival of *Campylobacter* in a high concentration of NaCl [4.5% NaCl (w/v)] was enhanced by reducing the temperature (< 42°C) (94) and was inhibited when the pH was outside the range of 6.5-8.0 (212).

Recent studies have suggested additional mechanisms involved in *C. jejuni* response to osmotic stress (62, 202, 321). Nothaft *et al*. found that the levels of free oligosaccharides (fOS) derived from the *N*-linked protein glycosylation pathway in *C. jejuni* are dependent on the presence of salts and sucrose in the environment (321). These findings suggest a role of fOS in the survival of *C. jejuni* under osmotic stress (96, 321). Moreover, it has been demonstrated that *cj0263* encodes a putative mechanosensitive channel that plays a role in protection of *C. jejuni* against hypoosmotic stress (202). Cameron *et al*. have also characterized the transcriptional profiling of *C. jejuni* in response to hyperosmotic stress (62). The microarray analysis revealed the induced expression of the heat shock genes and genes that are important for osmoadaptation (e.g., *gltD* and *glnA*) as well as the capsule export gene *kpsM* (62). The Δ*kpsM* mutant demonstrated higher sensitivity to hyperosmotic stress indicating the importance of the capsule export apparatus for *C. jejuni* hyperosmotic stress survival (62). However, future work is needed in order to further elucidate the *Campylobacter* response to osmotic stress.

1.3.2.3. *Campylobacter* response to nutrient stress

In comparison with other enteric pathogens, *C. jejuni* is nutritionally fastidious and more susceptible to environmental stresses (292). Most *Campylobacter* spp. are asaccharolytic organisms that cannot metabolize sugars and depend on amino acids as both carbon and energy sources (216, 458). Recently, Muraoka *et al.* and Stahl *et al.* have shown that certain *C. jejuni* strains can harbor a functional L-fucose metabolic pathway as an exception to the asaccharolytic nature of this organism (304, 421). In addition to amino acids, *C. jejuni* can use pyruvate, small organic acids and metabolic intermediates that result from anaerobic fermentation as both energy and carbon sources (216, 251, 458, 470, 482). *Campylobacter* viability decreases significantly under nutrient-depleted conditions (292). Moreover, long-term nutrient insufficiency influences *Campylobacter* survival and virulence properties (225). Compared to other stresses, starvation is the most powerful stress that significantly affects *Campylobacter* viability and virulence in epithelial cells (63, 292). For example, while starved *C. jejuni* could survive in Caco-2 cells for up to 4 days and caused disease in an animal model, the bacterial load in infected organs was markedly low, and the infected animals recovered from campylobacteriosis rapidly (225). Previous studies have also described other *C. jejuni* strategies for surviving stresses such as nutrient insufficiency and/or starvation (141, 384, 446). For example, *C. jejuni* can transform into a VBNC state, which could help bacteria survive under low nutrient availability (384). Moreover, *C. jejuni* mounts a stringent response which is regulated by *spoT* and controls bacterial stress survival (141). The stringent response is a global stress response that helps bacteria survive under unfavorable conditions by the alteration of gene expression pathways (67, 141). SpoT catalyses the synthesis of guanosine penta-phosphate (pppGpp) which is subsequently

converted to guanosine tetra-phosphate (ppGpp) (67, 70). Guanosine tetra-phosphate (ppGpp) binds to RNA polymerase and redirects transcription from growth-related genes to stress-survival genes (70, 275). As such, *C. jejuni* survival under nutrient deprivation and carbon-limiting conditions was greatly enhanced by the *spoT*-dependent stringent response (141, 192).

1.3.2.4. *Campylobacter* response to oxidative stress

As a microaerophilic organism, *C. jejuni* needs to survive both oxidative stress and toxic compounds that result from oxygen metabolism (306). While high concentrations of oxygen are deleterious for *C. jejuni*, *Campylobacters* need to grow at reduced oxygen concentrations that are required for oxygen-dependent ribonucleotide reductase activity (120, 402). In addition, *C. jejuni* utilizes oxygen as a terminal electron acceptor in its respiratory chain (120, 415). *C. jejuni* is frequently exposed to different types of reactive oxygen species (ROS), such as superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\bullet}$), either inside or outside the host (306). These ROS could result from *Campylobacter*'s normal metabolic processes, the intestinal microbiota or the host immune system as a means of defense against microbial infection (8, 400). ROS are very harmful for bacteria, as they can damage both DNA and proteins in addition to their role in lipid peroxidation (111). Therefore, not surprisingly, the exposure of *C. jejuni* to oxidizing agents induces the expression of proteins involved in repairing damaged proteins and DNA (337).

Many studies characterized the mechanisms of *C. jejuni* adaptation to aerobic metabolism and oxidative stress responses (115, 119, 120, 199, 457). *C. jejuni* lacks both the superoxide- and peroxide-sensing regulators SoxRS and OxyR, respectively, that are involved in the regulation of oxidative defense mechanisms in other enteric pathogens (188, 453). Instead,

C. jejuni possesses the peroxide-sensing regulator PerR that regulates its response to oxidative stress (453). Many proteins involved in the defense against oxidative stress have been characterized in *C. jejuni* (306). For example, *C. jejuni* harbors a single catalase encoded by *katA* that degrades H_2O_2 to water and oxygen (148). In addition, the genome of *Campylobacter* encodes an alkyl hydroperoxide reductase (AhpC) that is involved in the detoxification of alkyl hydroperoxides (19). Mutations in either alkyl hydroperoxidase (AhpC) or catalase (KatA) result in significant defects in both *Campylobacter* oxidative stress survival and chick colonization (19, 337). The expression of both *katA* and *ahpC* is regulated by the peroxide-sensing regulator PerR (453). Upon H_2O_2 detection, two histidines in the PerR regulatory metal binding site undergo a metal-catalyzed oxidation reaction, resulting in protein inactivation and the subsequent derepression of PerR target genes (256). Interestingly, the *C. jejuni* catalase *katA* is also regulated by the transcriptional regulator Fur (338, 453). In addition to KatA and AhpC, the genome of *C. jejuni* encodes a superoxide dismutase SodB (176, 362). This superoxide dismutase catalyzes the dismutation of superoxide radicals into hydrogen peroxide and oxygen and protects *Campylobacter* from oxygen toxicity (361, 362). Furthermore, *Campylobacter* aerotolerance is thought to be directly correlated to superoxide dismutase and markedly increases with elevated SodB activity (220). Therefore, not surprisingly, a *C. jejuni* Δ*sodB* mutant was defective in both bacterial survival and host colonization relative to the wild-type strain (337, 361).

1.3.2.5. The importance of studying the acid stress response in *C. jejuni*

Due to the increasing incidence of *C. jejuni* as one of the major causes of bacterial gastroenteritis in humans (3, 324), and because stress survival significantly contributes to the virulence capacity of enteric pathogens (26, 412), it is necessary to characterize the acid

tolerance response of *C. jejuni*. *C. jejuni* encounters a wide range of acidic conditions while travelling through the GIT (250, 373). However, *C. jejuni* lacks most of the stress response regulators identified in other enteric pathogens which enhance their survival in adverse environments (308, 341, 342, 345). These regulators include the global stationary phase stress response factor RpoS, the osmotic shock protectant BetAB, and the oxidative stress response factor SoxRS (342, 345). Moreover, *C. jejuni* lacks many proteins involved in survival of enteric bacteria (e.g., *H. pylori* and *E. coli*) to acid stress such as urease and amino acid decarboxylases, respectively (172, 424).

Several studies have been recently conducted to determine the mechanisms of *C. jejuni* response to acid stress (37, 250, 308, 373, 374, 412). These studies employed various techniques including transcriptional profiling (250, 373, 374) and proteomic analysis (37) to characterize *Campylobacter* acid response. For example, Reid *et al*. determined the transcriptional profile of *C. jejuni* upon exposure to *in vitro* acid stress (373). *C. jejuni* gene expression was monitored over a 20-min period following exposure to acid stress in Muller-Hinton (MH) broth buffered to pH 4.5 (373). Moreover, the transcriptional profile of *C. jejuni* in response to *in vitro* acid stress was correlated with the gene expression of *C. jejuni* after *in vivo* oral inoculation into a neonatal piglet (373). In contrast to *in vitro* acid shock, many factors such as exposure to oxidative stress and nutrient availability could affect *Campylobacter* transcriptional profile in response to *in vivo* gastric conditions (373).

Importantly, the transcriptome of *C. jejuni* revealed some variations under both *in vivo* and *in vitro* acidic conditions (373). While the expression of formate dehydrogenase (*fdhBCD*) and NADH dehydrogenase (*nuoMN*) genes was repressed in the pig stomach only, expression of succinate dehydrogenases (*sdhAB*) genes was induced in the pig stomach and repressed

under *in vitro* acid stress (373). Moreover some genes encoding for the biogenesis of cell surface polysaccharides (e. g., *cj1413c*, *cj1423c* and *kpsM*) were down-regulated in the pig stomach and not affected under *in vitro* acid shock (373). These findings indicate that *C. jejuni* is capable of modulating the expression of its genes upon entry into the host (373). In addition to acid stress, *C. jejuni* faces unique conditions such as nutrient insufficiency and other stresses (e.g., oxidative stress) within the host which are not encountered under an *in vitro* environment (373).

Moreover, exposure of *C. jejuni* to acid shock resulted in the up-regulation of a number of genes involved in response to heat shock such as *hrcA*, *dnaK*, *groES*, *groEL*, *grpE* and *clpB* (373). The up-regulation of heat shock proteins in *C. jejuni* under low pH could help the cells deal with the aggregated proteins accumulated in the cytoplasm upon its acidification (373). Similarly, acid exposure of *C. jejuni* induced the expression of many genes involved in the defense against oxidative stress such as catalase (*katA*) and ferritin (*cft*) (373). These results suggest an overlap between *Campylobacter* acid response and its responses to other stresses such as the oxidative stress (373).

A recent study by Le *et al*. characterized the effect of acid stress on *C. jejuni* viability, gene expression as well as bacterial virulence in epithelial cells (250). There was no loss in *C. jejuni* viability upon exposure to acidic conditions at pH 3.5 and pH 5 for 10 min (250). The study of *C. jejuni* transcriptional profiles under such acidic conditions revealed that many genes were differentially expressed (250). Importantly and similar to the study by Reid *et al*. (373), the down-regulation of genes encoding ribosomal proteins further confirms that *C. jejuni* switches from the exponential growth to the stress survival mode upon exposure to stressful conditions (250). Moreover, the up-regulation of antioxidative stress genes (e.g.,

katA) and genes involved in heat shock response (e.g., *clpB*, *dnaK*, *hrcA* and *htrA*) indicates that specific stress response proteins may also have roles in general stress responses in *C. jejuni* (250). In addition, exposure of *C. jejuni* to acid enhanced the expression of many genes involved in bacterial pathogenesis such as flagellar genes, *ciaB* and *peb1A* (250). An important finding by Le *et al*. is that acid exposure increases *Campylobacter* invasion into epithelial cells mainly through the basolateral route (250). *C. jejuni* cell-invasion was significantly higher when the intestinal cells were grown in a transwell model than on flat-bottomed wells (250).

At the proteomic level, Birk *et al*. characterized the proteome of three *C. jejuni* strains (NCTC11168 and two strains that were originally isolated from turkeys) using radioactive methionine labelling and 2D-gel-electrophoresis after exposure to relatively mild acid conditions (HCl, pH 5.2 and acetic acid, pH 5.7) for 20 min (37). Up to 7 proteins including those involved in iron metabolism (e.g., P19) and oxidative stress defense (e.g., SodB, AhpC and Dps) were increased in *C. jejuni* under acidic conditions (37). Birk *et al*. (37) suggested that the increased expression of oxidative stress defense proteins was due to the need to defend against the iron-mediated lipid peroxidation at low pH (36, 394). Iron solubility is enhanced under acidic conditions which could affect bacterial growth (36). The elevated iron concentration promotes the generation of damaging hydroxyl radicals through the Fenton reaction (36, 337). Therefore, *C. jejuni* induces the expression of antioxidative stress proteins to overcome the iron-mediated oxidative stress generated at low pH (36, 37, 394). In contrast to other studies (250, 373), the expression of heat shock proteins, including chaperones and proteases, was not observed in *C. jejuni* under acidic conditions (37). This could be due to the molecular size and isoelectric point (pI) of these proteins falling outside the detection

limit of the proteomic experiment (37). For example GroES and ClpB have molecular masses close to the minimum and maximum detection sizes, respectively, and the pI of HtrA is outside the pI range of the system used in this study (37).

Importantly, Murphy *et al*. demonstrated that the extracellular proteins produced by *C. jejuni* during growth could contribute to the induction of bacterial acid tolerance (307). The acid survival capacity of *C. jejuni* was significantly enhanced when cells were grown in a cell-free spent medium as compared to a freshly prepared medium (307). The extracellular protein(s) accumulated by *C. jejuni* during growth may play a role in the induction of stress tolerance response through signaling mechanisms (307). However, these proteins or the mechanisms of either their secretion or recognition by *C. jejuni* were not identified which await further investigations. Despite previous studies that have provided us with a wealth of information about *C. jejuni* gene expression in response to *in vitro* and *in vivo* acidic conditions, detailed mechanisms of *Campylobacter* acid stress response are still lacking which warrants more research.

1.4. Hypotheses and objectives of the study

Campylobacter jejuni is considered one of the most common causes of bacterial gastroenteritis in humans worldwide (3, 324, 388). During its life, *C. jejuni* encounters many challenges either inside or outside of the host including fluctuations in pH (250, 373, 374). In contrast to other enteric pathogens, such as *E. coli*, *Salmonella* sp., and *Shigella* sp. the mechanism of *Campylobacter* acid survival remains unknown. Furthermore, the impact of prior acid exposure on *Campylobacter* pathogenesis and survival to other stresses has not been characterized. While *C. jejuni* lacks many of the classical regulators and proteins involved in stress defense that have been identified in other enteric pathogens (341, 345), the

low infectious dose of *C. jejuni* indicates that it has evolved different strategies to survive hostile conditions (307, 373).

Importantly, the genome of *C. jejuni* encodes the transcriptional regulator Fur which regulates iron metabolism genes (57, 58, 180, 338, 345, 456). The relative paucity of the transcriptional regulators in *C. jejuni* (342, 345) and the involvement of Fur in the acid survival of other bacteria [e.g., *E. coli* (493) and *Salmonella* sp. (127)], let us hypothesize that Fur is involved in *Campylobacter* acid survival. In contrast to other enteric pathogens such as *Salmonella* sp. (127), *S. flexneri* (327), and *H. pylori* (36), the role of Fur in *C. jejuni* acid survival has not been characterized. This is the first attempt to investigate the involvement of Fur in *C. jejuni* acid survival. In addition, the contribution of acid exposure and Fur to *Campylobacter* protection against other stresses will be assessed. Previous works demonstrated that prior acid exposure cross-protects enteric pathogens (e.g., *Salmonella* sp.) against other stresses including heat and salt (26, 261). However, the precise mechanisms of acid-mediated cross-protection and the proteins involved in this process are still unknown. Moreover, it has been previously shown that many oxidative stress defense genes (e.g., *katA*) were up-regulated in *C. jejuni* upon acid exposure (250, 373), yet the regulator(s) that are responsible for antioxidative stress gene induction under low pH are unknown. The enhanced expression of *katA* in *C. jejuni* upon acid exposure (250, 373) suggests that there is a link between *Campylobacter* acid survival and its capacity to survive oxidative stress. In the present study we investigated whether Fur is involved in protection of *C. jejuni* against oxidative stress under low pH, thereby providing, for the first time, a possible mechanism of the acid-mediated cross-protection of *C. jejuni* against other stresses. In addition, although previous studies demonstrated that prior acid exposure enhances *C. jejuni* virulence in

eukaryotic cells (239, 250), these studies did not characterize the effect of acid stress on *Campylobacter* pathogenesis in infection models. Therefore, we hypothesized that the obligate passage of *C. jejuni* through the stomach acidity modulates the expression of its colonization factors and predisposes the bacterium for efficient gut colonization. In the present work we characterized the impact of acid stress on *C. jejuni* pathogenesis using *G. mellonella* as an infection model. The results of the *in vivo* experiments using *G. mellonella* would significantly help us determine how acid exposure modulates the pathogenesis of *C. jejuni* in the host.

Chapter 2 : *C. jejuni* acid stress response and the influence of acid exposure on *Campylobacter* pathogenesis

2.1. Introduction

The Gram-negative *C. jejuni* is one of the most common causes of foodborne bacterial gastroenteritis in humans worldwide (3). To successfully infect the host, enteric pathogens, including *C. jejuni*, must survive various stress conditions (47). One drastic condition encountered by enteropathogens during host infection is the low gastric pH (26). Therefore, enteric pathogens have evolved different strategies to combat acid stress (these strategies have been described in detail in Chapter 1).

While the low oral infection dose for *C. jejuni* (500-800 organisms) indicates that *C. jejuni* has developed specific strategies to respond to acid stress (40, 382), the mechanisms of how *C. jejuni* responds to acid stress remain unknown (308). Only a few studies have demonstrated the capacity of some strains of *C. jejuni* to develop ATR to acidic pH conditions (305, 308). *C. jejuni* bacterial cells that were adapted to mild acid were able to survive severe acid stress better than unadapted cells (308). *C. jejuni* lacks regulatory proteins (e.g., RpoS, SoxRS and OxyR) common in other enteric pathogens that govern their adaptive responses to various stresses such as acid stress (37, 188, 254, 345, 453). This difference highlights our limited understanding of *C. jejuni* stress responses and the need for further research on its survival mechanisms (308).

In addition to characterizing the bacterial acid stress response, previous studies demonstrated that the exposure of enteric bacteria to acid stress appears to be interconnected with their response to other stresses (26, 261, 422). The expression of proteins involved in bacterial defenses against oxidative stress, such as AhpC, Dps and SodB was induced under acidic

conditions in several bacteria suggesting a mechanism of cross-protection (19, 190, 353, 361, 373). For example, acid adaptation increases the tolerance of *S. typhimurium* towards other environmental stresses, such as oxidative, bile and osmotic stress (261). However, the cross-protection does not work both ways. Exposure of enteric pathogens to other stresses, such as thermal shock and osmotic stress could lead to more specific responses and does not induce acid tolerance (26). Changes in the outer membrane composition and cell surface hydrophobicity of acid-adapted cells could account for their increased tolerance to other stresses, such as oxidative stress and bile salts (261).

The finding that acid exposure cross-protects enteropathogens such as *Salmonella* spp. (261) against other stresses suggests that the obligate passage of the acidic environment of the stomach might prepare enteric bacteria to better survive subsequent stresses encountered in the intestine (26). Moreover, a strong correlation exists between a bacterium's capacity to survive acidic conditions and its capacity to survive within the host and cause disease (117, 182). For example, mutations that increase acid sensitivity attenuate the colonization potential of bacteria and negatively influence their pathogenesis (377). Not surprisingly, the low infectious dose of enterohemorrhagic *E. coli* is directly correlated with its capacity to survive acidic conditions (10, 28, 262). As another example, the transcription of virulence genes of intracellular pathogens, such as *S. typhimurium*, is greatly enhanced in the low pH of phagosomes following the bacterial invasion of epithelial cells and macrophages (5, 138, 370).

The current study was performed to understand how *C. jejuni* modulates the expression of its genes upon exposure to acidic conditions. Although previous studies have characterized the transcriptome of *C. jejuni* upon acid exposure (250, 373, 374); these studies did not

characterize the effect of acid stress on *C. jejuni*'s capacity to survive in the presence of other environmental stresses (e.g., oxidative stress) neither did they determine the contribution of acid stress to *C. jejuni* host pathogenesis. While Le *et al*. (250) demonstrated that prior exposure to acid enhances the invasion capacity of *C. jejuni* into cultured intestinal cells, this study did not characterize the effect of acid stress on *C. jejuni* virulence in animal models. The main objective of our study was to characterize the transcriptional profile of *C. jejuni* in response to acid shock using microarray profiling. Microarrays have been widely used to study bacterial transcriptional responses to stressful conditions and to characterize microbial gene expression under *in vitro* conditions mimicking those encountered by pathogens *in vivo* within the host (7, 72, 250, 252, 327, 373, 374, 452). We also characterized the contribution of prior acid exposure to *C. jejuni* survival to other stresses, such as oxidative, bile and osmotic stress. In addition, the effect of acid stress on *C. jejuni* virulence capacity was determined. We investigated the effect of acid stress on *C. jejuni* adherence, invasion and intracellular survival within human epithelial cells. Finally, the effect of acid stress on *C. jejuni* pathogenesis was characterized using the *Galleria mellonella* virulence model which significantly improved our knowledge about how acid stress could modulate *C. jejuni* host pathogenesis.

2.2. Materials and Methods

2.2.1. Bacterial strains and growth conditions

The highly invasive *C. jejuni* 81-176 strain isolated from a raw milk outbreak of *Campylobacter* (178) was used in the present study. *C. jejuni* was routinely grown on

Mueller-Hinton (MH) agar plates and MH biphasic flasks (MH; Oxoid) under microaerophilic conditions (83% N_2, 4% H_2, 8% O_2 and 5% CO_2). The bacterial culture was maintained in a MACS-VA500 microaerophilic workstation (Don Whitley, West Yorkshire, England) at 37°C. The bacterial strains used in this study are listed in Appendix I.

2.2.2. Acid survival assay

C. jejuni 81-176 was grown to the mid-exponential phase in MH medium (385). The acid survival assays were performed as previously described by Reid *et al*. (373) and Le *et al*. (250). A 5 mL aliquot of *C. jejuni* culture was added to 10 mL of MH broth previously adjusted to pH 3 using HCl. The final pH of the solution was measured using a pH meter and found to be equal to pH 4. Samples were withdrawn immediately and 2, 4, 6 and 8 min after exposure to acid, serially diluted into PBS (pH 7.4) and plated on MH agar plates. Plates were incubated at 37°C under microaerophilic conditions for 48 h and colonies were counted.

The role of D-gluconate in *C. jejuni* acid survival was determined by examining the capacity of exponentially growing cells to survive acid stress (pH 3) in the presence and/or absence of 20 mM D-gluconate. *C. jejuni* 81-176 was grown to logarithmic phase in biphasic MH medium and an aliquot of 2.5 mL of *C. jejuni* culture was added to 10 mL of MH broth adjusted to pH 2.6 using concentrated HCl with and without 20 mM D-gluconate (resulting in a final pH of 3). Samples were withdrawn immediately and 2, 8 and 15 min after exposure to acid for viable counting as described above. The percentage of bacterial survival was determined as a function of the duration of acid exposure. The results are expressed as the means of three independent experiments ± standard errors of the mean. The data were

statistically analyzed using a Student unpaired *t*-test (GraphPad Prism version 5.03 for Windows, www.graphpad.com). *P* value below 0.05 was considered significant.

2.2.3. RNA extraction

C. jejuni 81-176 was grown to logarithmic phase in biphasic MH medium (pH 7.4) under microaerophilic conditions. Bacterial cells were exposed to acid stress at pH 4 for 8 min in HCl-adjusted MH broth as described above for the acid survival assay (the final pH was confirmed to be equal to 4 using a pH meter). RNA turnover was prevented by adding a 1/10 volume of cold RNA degradation stop solution (10% [vol/vol] buffer-saturated phenol pH 4.3 in absolute ethanol) (31). Bacterial cells were collected by centrifugation (8,000 X *g*, 10 min), and the cell pellet was resuspended in TE buffer (50 mM Tris-HCl pH 8.0, 1 mM EDTA). Total RNA was extracted using a hot phenol-chloroform method (438) and precipitated with absolute ethanol by overnight incubation at 80°C. The RNA was washed three times with 80% cold ethanol to remove any impurities and was resuspended in RNase-free ddH_2O. The contaminating genomic DNA was removed from the RNA preparation with a RNase-free DNase I treatment. Briefly, 0.5 µl of RNase inhibitor (RNaseout® invitrogen) was added to 60 µg of RNA. Next, 10 µl 10X buffer (50 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, 5 mM EDTA, 5 mM DTT) and 4 µl of RNase-free DNase (Epicenter Biotechnologies, Madison, WI) were added to RNA followed by incubation at 37ºC for 30 min. Another 4 µl DNase was added to RNA and incubated for another 30 min. RNA was purified using a Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) and DNase treatment was repeated once more. Finally, the RNA was purified as mentioned above and PCR amplification was used to confirm that the preparation was free of genomic DNA. The RNA quality and quantity were ascertained using the BioRad's Experion RNA StdSens Analysis Kit following the

manufacturer's protocols. Moreover, RNA integrity was assessed by agarose gel electrophoresis to ensure high quality RNA that had a 23S band with double the intensity of the 16S band and no smears on the gel. RNA samples were stored at -80°C until further use.

2.2.4. Microarray probe labeling and slide hybridization

The protocol described by Palyada *et al*. (338) for probe labeling and slide hybridization was followed herein. Briefly, 10 µg of total RNA samples from each control (unstressed *C. jejuni*) and test sample (acid-stressed *C. jejuni*) were converted to cDNA using Superscript II (Invitrogen); 10 µg of random hexamers (Amersham Biosciences); a dNTP mixture of 0.5 mM dGTP, dATP and dCTP each; 0.16 mM dTTP; and 0.34 mM aminoallyl-dUTP. Aminoallyl-dUTP was incorporated in the reaction to allow cDNA labeling with the monoreactive fluors indocarbocyanine (Cy3) and indodicarbocyanine (Cy5) (GE Healthcare) (338). The aminoallyl-labeled cDNA was purified from free amines and unincorporated aminoallyl-dUTP by adding 350 µL of H_2O and spinning through a Microcon YM-30 filter (Millipore) at 10000 rpm, followed by washing with sterile ddH_2O. Following concentration and resuspension into $NaHCO_3$ pH 9.0, the aminoallyl-labeled cDNA was coupled to either Cy3 (control samples) or Cy5 dye (test samples) by adding 10 µL of Cy3 or Cy5 (GE Healthcare) in dimethyl sulfoxide followed by incubation in the dark for 1 h at room temperature. Next, the fluorescently labeled cDNA was purified using QIAquick PCR purification kit according to the manufacturer's instructions (Qiagen). Fluorescent Cy3- and Cy5-labeled cDNAs were combined and the fluor-labeled cDNA mix was dried under vacuum and resuspended in 15.1 µL of H_2O, to which the following was added: 9 µL of 20X SSC, 2.5 µL of salmon sperm DNA (10 mg/mL), 9 µL of formamide, and 0.5 µL of 10% sodium dodecyl sulfate (SDS). The microarray slides used in this study were constructed

using PCR-amplified fragments that represent the open reading frames (ORFs) identified in the *C. jejuni* NCTC11168 genome, as described previously (425), and summarized in Figure 2.1. Details of the microarray construction and a complete list of the genes represented on the microarrays are available online (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE9920). Prior to hybridization the arrays were prehybridized at 42°C for 45 min in prehybridization buffer [5X SSC buffer (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7), 25% formamide, 0.1% SDS, and 1% bovine serum albumin (BSA)], rinsed with water, and dried by spinning. The combined probes were denatured for 2 min at 99°C, and applied to the microarray slide underneath a coverslip. The slides were placed in a humidified chamber (Arrayit, Sunnyville, CA), and incubated in the dark overnight at 42°C. Next, the slides were washed once for 5 min at 42°C in 100 mL 2X SSC, 0.1% SDS, once for 10 min in 100 mL 0.1X SSC, 0.1% SDS and four times for 1 min each at room temperature in 400 mL 0.1X SSC. Finally, slides were rinsed with distilled H_2O, dried and scanned using a laser-activated scanner (Scan-Array Gx, PerkinElmer) at 10-µm resolution.

2.2.5. Data collection and analysis

The microarray data collection and analysis were performed as previously described (337, 338, 426) and summarized in Figure 2.1. The signal intensities of each spot were collected using ScanArray software (PerkinElmer). Spots exhibiting hybridization anomalies and spots with background-subtracted intensities less than three times the standard deviation of the background in both channels were excluded from the analysis. The spot intensities were normalized via locally weighted linear regression (LOWESS) using MIDAS (http://www.tigr.org/software/) as previously described (337, 338, 426). Microarray data

were collected from three independent biological replicates for both the test and control samples. The ratio of channel 2 (Cy5) to channel 1 (Cy3) was converted to $\log_2$, and the data were analyzed using the Bayesian statistics (21). The differentially expressed genes (> 1.5-fold differential expression; $P < 10^{-4}$) were subjected to hierarchical clustering using Genesis (http://genome.tugraz.at).

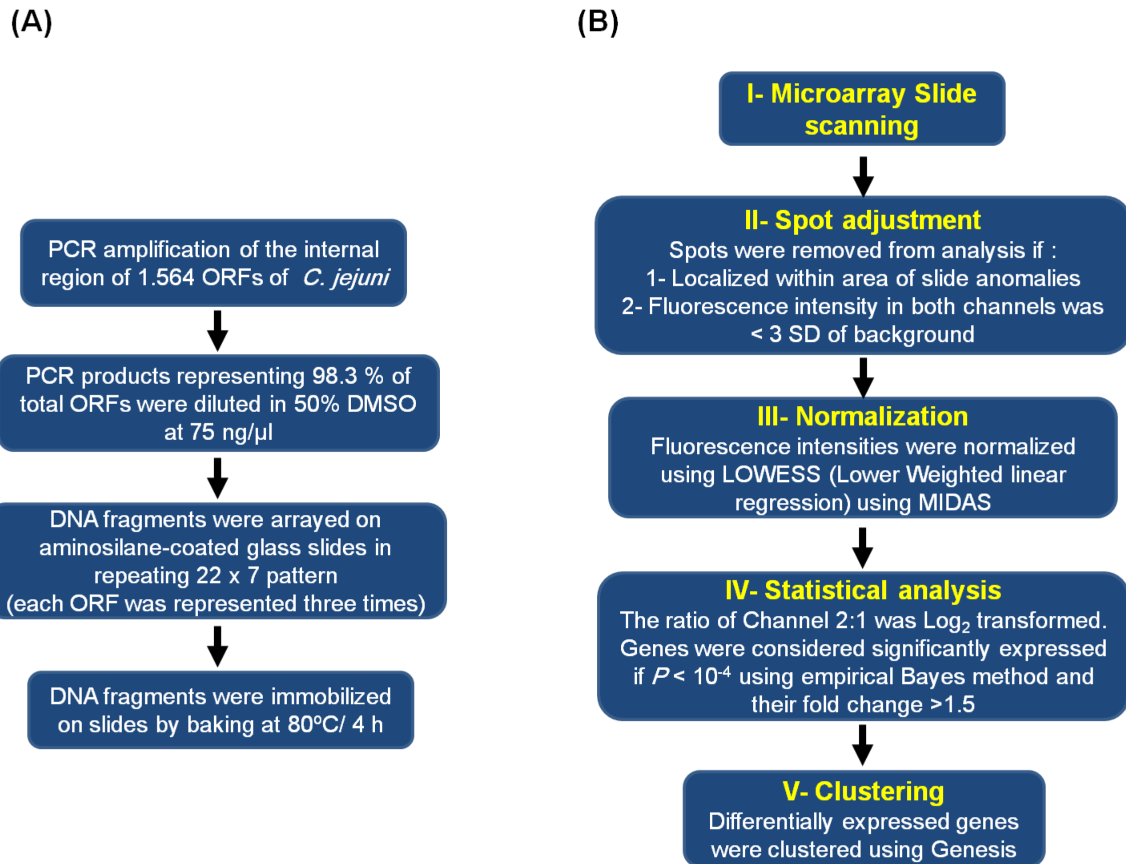


Figure 2.1: Flowchart of the microarray slide construction as well as data collection and analysis. (A) The microarray slides previously constructed (425) were used in the present study. The flowchart summarizes the steps employed by Stintzi, (2003) (425) to construct the microarray slides. (B) The microarray data collection and analysis were performed as previously described (337, 338, 426).

2.2.6. Validation of microarray results by qRT-PCR

The relative expression levels of eight differentially expressed genes (*cft*, *cj0264c*, *cj0265c*, *cj0414*, *cj0415*, *cj0448c*, *dapB* and *rpoA*) and another six genes that did not change significantly in *C. jejuni* 81-176 after acid treatment (*ahpC*, *cj1583c*, *slyD*, *uvrB*, *uvrA* and *zupT*) were further analyzed using qRT-PCR. The relative expression level of each gene was normalized to *rpsL* as an endogenous control. The analysis was conducted as described previously (425, 426) and followed the protocol described in the QuantiTect SYBR green RT-PCR kit (Qiagen) using a 7300 real-time PCR system (Applied Biosystems). The primers for qRT-PCR were designed using Primer3 software and are listed in Appendix II. PCR amplification was confirmed by both agarose gel electrophoresis and a melting curve analysis of the products. The relative gene expression was calculated using the comparative threshold cycle ($\Delta\Delta C_T$) method (425, 426). The $\log_2$ ratio values of gene expression obtained by the microarray analysis were plotted against to the $\log_2$ ratio of relative quantity values from qRT-PCR. Finally, the coefficient of determination (R^2) was determined as a measure of the degree of correlation between the microarray data and real-time qRT-PCR data.

2.2.7. Oxidative stress experiments

2.2.7.1. Disk inhibition assay

The capacities of both acid-stressed and unstressed *C. jejuni* 81-176 to survive H_2O_2 were assessed using disk inhibition assays. *C. jejuni* bacteria were acid-stressed by exposing an overnight culture of *C. jejuni* to acidic conditions (pH 4 for 8 min) in HCl-adjusted MH broth as described above. After centrifugation at 8,000 X *g* for 5 min at room temperature, the bacterial pellets were washed twice in MH broth (pH 7.4) and resuspended in MH broth

to an optical density of 1.0 at 600 nm (OD_{600}). For the control (unstressed *C. jejuni*), the same steps were performed but without the exposure to acid. Next, 1 mL of bacterial suspension in MH broth (acid-stressed or unstressed bacteria) was added to 24 mL of molten MH agar, poured into Petri dishes and allowed to solidify. Ten microliters of different molar concentrations of H_2O_2 (250-1000 mM) were pipetted on top of a 6 mm diameter paper disk placed on the surface of each MH agar plate, and the plates were then incubated for 24 h at 37°C under microaerophilic conditions (337). The H_2O_2 sensitivities of both acid-stressed and unstressed *C. jejuni* were determined by measuring the diameters of the growth inhibition zones around the paper disks. The results are expressed as the means of three independent experiments ± standard errors of the mean. The data were statistically analyzed using a Student unpaired *t*-test. A P value < 0.05 was considered significant.

2.2.7.2. Kill curve of *C. jejuni* to H_2O_2

In addition to the disk inhibition assay, the capacity of both acid-stressed and unstressed *C. jejuni* 81-176 to survive oxidative stress was assessed by performing a kill curve of bacteria against H_2O_2. Briefly, acid-stressed and unstressed *C. jejuni* 81-176 (prepared as described for the disk inhibition assay) were exposed to 10 mM H_2O_2 in MH broth. The percentage of bacterial survival for both acid-stressed and unstressed *C. jejuni* was determined immediately and 4, 8, 15 and 30 min after exposure to H_2O_2. The percentage of bacterial survival was determined by counting viable cells after serially diluting into PBS (pH 7.4), plating on MH agar plates and incubating for 48 h at 37°C under microaerophilic conditions. The bacterial survival to H_2O_2 was expressed as the percentage of survival as a function of the duration of exposure to H_2O_2. The results are expressed as the means of three independent biological experiments ± standard errors of the mean. The difference between

the capacities of both acid-stressed and unstressed *C. jejuni* to survive H_2O_2 was considered significant at a P value < 0.05 using a Student unpaired t-test.

2.2.8. Growth of *C. jejuni* in the presence of osmotic stress

The influence of acid exposure on *C. jejuni* growth in the presence of osmotic stress was assessed by monitoring the growth curves of both acid-stressed and unstressed *C. jejuni* in the presence of NaCl. Acid-stressed and unstressed *C. jejuni* cells were prepared as described above for oxidative stress experiments. The bacterial pellets were washed in MH broth (pH 7.4) and resuspended in fresh MH medium to an OD_{600} of 0.2. The bacterial suspension was further diluted 1/10 in MH broth, and 150 µL aliquots of these suspensions were dispensed into 100-well plates containing 150 µL of MH broth supplemented with NaCl. The final molar concentration of NaCl in the wells was 0.03 M or 0.06 M. The growth of both acid-stressed and unstressed *C. jejuni* was monitored by measuring the optical density (OD) every 10 min over 36 h using a Bioscreen C plate reader with shaking at 37°C. The results were displayed as the maximum OD obtained during 36 h of growth for both acid-stressed and unstressed *C. jejuni*. In addition, the growth rates of both acid-stressed and unstressed *C. jejuni* in the presence of NaCl were calculated for the exponential phases as previously described (393, 471) using the following equation: $\mu = 2.303\ [(\log_{10} OD_2 - \log_{10} OD_1)/(t_2 - t_1)]$, where μ is the growth rate, OD_1 is the optical density at time point 1 (t_1) and OD_2 is the optical density at time point 2 (t_2). The results are expressed as the means of three independent biological experiments ± standard errors of the mean. The difference between the capacities of both acid-stressed and unstressed *C. jejuni* to survive and grow in presence of osmotic stress was considered significant at a P value < 0.05 using a Student unpaired t-test.

2.2.9. Sensitivity of *C. jejuni* to bile salts

The capacity of both acid-stressed and unstressed *C. jejuni* 81-176 to survive bile stress was determined as follow. The minimum inhibitory concentrations (MICs) of both bovine bile and sodium deoxycholate were determined for *C. jejuni* 81-176 before and after exposure to acid using the microdilution broth method. Acid-stressed (test) and unstressed (control) *C. jejuni* were prepared in the same manner as described for the oxidative stress experiments. The bacterial pellets were washed and resuspended in MH broth (pH 7.4) to an OD_{600} of 0.2. The bacterial suspension was then diluted 1/1000 using MH broth to achieve a bacterial count of 10^6 CFU/mL. Next, 100 µL of either the control or the test sample was added to 100 µL of a two-fold serially diluted solution of bile salt (sodium deoxycholate or bovine bile) in a 96-well plate. The final concentration of sodium deoxycholate and bovine bile in the wells ranged from 0 to 100 mg/mL and 0 to 10 mg/mL, respectively. The plates were incubated in a MACS-VA500 workstation under microaerophilic conditions with shaking at 37°C for 36 h. The lowest concentration of bile salt (sodium deoxycholate or bovine bile) that inhibited bacterial growth (acid-stressed and unstressed *C. jejuni*) was determined. The results are expressed as the means of three independent biological experiments ± standard errors of the mean. The difference between the capacities of both acid-stressed and unstressed *C. jejuni* to grow in the presence of bile salt stress was considered significant at a P value < 0.05 using a Student unpaired t-test.

2.2.10. Bacterial interaction with Caco-2 cells

2.2.10.1. Epithelial cells

Caco-2 cells were grown in minimum essential medium alpha (MEMα; Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% non-essential amino acids. The cells were grown and maintained without antibiotics at 37°C in a 5% CO_2 humidified atmosphere. For adherence and invasion assays, confluent Caco-2 cells were harvested by trypsinization in 0.01% EDTA. Caco-2 cells were adjusted to 10^6 cells/mL by counting in a hemocytometer, seeded in a 24-well tissue culture plate and incubated at 37°C in a 5% CO_2 humidified atmosphere until a confluent monolayer formed. Prior to adhesion and invasion assays, the cell monolayers were washed twice with Hank's balanced salt solution (HBSS) composed of KCl (400 mg/L), $CaCl_2.2H_2O$ (186 mg/L), $MgSO_4.7H_2O$ (200 mg/L), KH_2PO_4 (60 mg/L), $NaH_2PO_4.7H_2O$ (90 mg/L), NaCl (8000 mg/L), $NaHCO_3$ (350 mg/L) and glucose (1000 mg/L) (155) at pH 7.4.

2.2.10.2. Adherence and invasion assays

The adhesion and invasion assays were performed as described by Poly *et al*. (356). *C*. *jejuni* 81-176 was cultured overnight in MH broth at 37°C under microaerophilic conditions. Acid-stressed and unstressed bacteria were prepared as described above for the oxidative stress experiments. The bacterial pellets were washed in MH broth (pH 7.4) and resuspended in 10% FBS-MEMα. Both acid-stressed and unstressed *C*. *jejuni* suspensions were inoculated separately into wells containing confluent monolayers of Caco-2 cells at a targeted multiplicity of infection (MOI) of 100:1. The actual numbers of bacteria in the inocula added to the monolayers were confirmed retrospectively by serial dilution and plate

counting. Bacteria-infected Caco-2 cells were incubated for 3 h at 37°C and 5% CO_2 to allow for bacterial adherence and internalization. To determine the number of bacteria adhering to and internalized in the eukaryotic cells, the epithelial cells were washed twice with HBSS and lysed with 0.1% Triton X-100. The total number of bacteria associated with the eukaryotic cells (extracellular and intracellular bacteria) was determined by serial dilutions of lysates in PBS (pH 7.4), plating on MH agar plates and counting the resultant colonies after incubation for 48 h under microaerophilic conditions.

To measure bacterial invasion, a gentamicin protection protocol was employed (228, 356). *C. jejuni*-infected eukaryotic cells were washed twice with HBSS and incubated in fresh 10% FBS-MEMα containing 250 µg/mL gentamicin sulfate for 1 h to kill the remaining viable extracellular bacteria. Intracellular bacteria were determined by washing the infected Caco-2 cells twice with HBSS, and the cells were lysed with 0.1% Triton X-100. Following serial dilution in PBS, the released intracellular bacteria were counted as described above by plate counting. The number of adhering bacteria to Caco-2 cells was determined by subtracting the number of intracellular bacteria from the total number of bacteria recovered from eukaryotic cells not treated with gentamicin. The adhesion and invasion results are expressed as the percentages of adhering and invading bacteria relative to the infection dose. The results are expressed as the means of three independent experiments ± standard errors of the mean. The difference between acid-stressed and unstressed *C. jejuni* in adhesion and invasion into Caco-2 cells was considered significant at a P value < 0.05 using a Student unpaired t-test.

2.2.10.3. Intraepithelial cell survival assays

The intracellular survival of both acid-stressed and unstressed *C. jejuni* within Caco-2 cells was determined as previously described (314). Acid-stressed (test) and unstressed

(control) *C. jejuni* 81-176 were prepared as described above. Each bacterial suspension was overlaid onto wells containing confluent monolayers of Caco-2 cells at a targeted MOI of 100:1. Infected Caco-2 cells were incubated for 3 h at 37°C in a 5% CO_2 humidified atmosphere. Next, the infected monolayers were washed twice with HBSS, cultured in 10% FBS-MEMα containing gentamicin sulfate (250 µg/mL) and incubated for an additional 1 h to kill the extracellular bacteria. Then, the monolayers were washed twice with HBSS and either lysed with 0.1% Triton X-100 to recover the intracellular bacteria (4 h time point) or further incubated for 24, 48 and 72 h in 10% FBS-MEMα with no antibiotics. Following incubation, the monolayers were washed twice with HBSS and lysed with 0.1% Triton X-100 to determine the numbers of intracellular bacteria at different time points. The numbers of viable intracellular bacteria were determined as described for the adhesion and invasion assays by serial dilution in PBS and plating on MH agar plates. The results are expressed as the means of three independent experiments ± standard errors of the mean. The difference between both acid-stressed and unstressed *C. jejuni* for intracellular survival within Caco-2 cells was considered significant at a P value < 0.05 using a Student unpaired *t*-test.

2.2.11. *Galleria mellonella* larvae infection

The effect of acid stress on *C. jejuni* pathogenesis was determined using *in vivo Galleria mellonella* killing assays, as described previously (69, 331). Briefly, larval survival and 50% lethal dose (LD_{50}) assays were determined for both acid-stressed (test) and unstressed (control) *C. jejuni* 81-176 by injection in *G. mellonella*. To prepare bacterial stocks, *C. jejuni* was grown in MH broth at 37°C under microaerophilic conditions. Test and control *C. jejuni* bacteria were prepared as described above. Bacterial pellets were washed in PBS (pH 7.4) and resuspended in the same buffer to the desired bacterial density. The

infectious dose of both the test and control (CFU/mL) were determined by serial dilution and colony counting on MH agar plates. *G. mellonella* larvae were obtained from Gecko Gurl, (Ottawa, ON, http://www.geckogurl.com/). The larvae were allowed to acclimate in the lab after delivery for at least 24 h by storage at 10-15°C. For *G. mellonella* larvae infection, cohorts of ten larvae were injected with 10 µL of ten-fold serial dilutions of *C. jejuni* (10^7-10^5 CFU in PBS) into the hemocoel using a Hamilton 10 µL 901RN syringe (Hamilton® Microliter™). The infected larvae were maintained in vented Petri dishes and were incubated at room temperature under aerobic conditions. Mortality, survival and the appearance of *G. mellonella* larvae were monitored every 24 h for 6 days following inoculation. As negative controls, a cohort of ten larvae was injected with 10 µL of sterile PBS, and another 10 larvae were not injected. For microscopic examination of *C. jejuni*-infected larvae, the rear 2 mm of each larva was removed and the hemocoel was collected into a microcentrifuge tube. The hemocoel was subjected to Gram staining, and the slides were examined using a BX5 microscope (Olympus Inc., Center Valley, PA). Representative images were photographed with an Olympus DP70 camera. For both acid-stressed and unstressed *C. jejuni*, the experiment was performed in at least three independent replicates. Survival curves of both the control and bacteria-infected larvae were plotted using the Kaplan-Meier method. The difference in larvae survival was determined using the log-rank test (GraphPad Prism, www.graphpad.com). The LD_{50} was calculated using the Probit method (XLstat 2010, Addinsoft, New York, NY, USA) as previously described (331), and differences in bacterial virulence were compared using the Mann-Whitney test. A value of $P < 0.05$ was considered statistically significant.

2.3. Results

2.3.1. Acid survival of *C. jejuni*

As mentioned before, the aim of this study was to characterize the transcriptome of *C. jejuni* in response to acid stress. Initially, it was important to determine the capacity of *C. jejuni* to survive under our experimental conditions. The acid survival assay was performed in order to ascertain that the exposure of *C. jejuni* to acid did not affect bacterial cell viability. The ability of *C. jejuni* 81-176 to survive acid stress at pH 4 was determined using the dilution plate count technique as described in Materials and Methods. As shown in Figure 2.2 no significant loss in *C. jejuni* viability was detected following acid exposure at pH 4 for 8 min. These results indicate that *C. jejuni* is able to survive the experimental conditions and that changes in *C. jejuni* gene expression would be only due to a response to low pH and not bacterial death under the applied conditions.

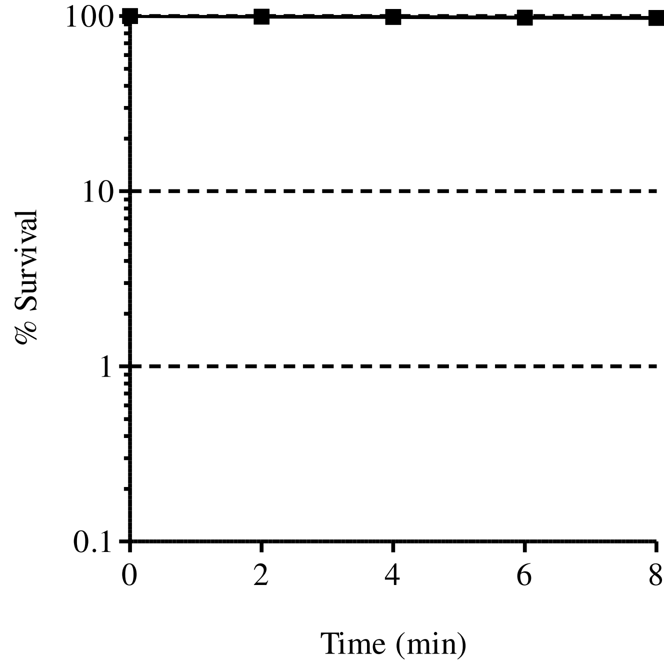


Figure 2.2: *C. jejuni* 81-176 remains viable during an 8-min exposure to acidic condition at pH 4. *C. jejuni* was grown to the logarithmic phase and was then exposed to acid at pH 4. Samples were withdrawn immediately and 2, 4, 6 and 8 min after acid exposure to determine the percentage of bacterial survival. The data shown are the means of three independent experiments ± standard error of the mean. Standard errors are present but are too small to be seen.

2.3.2. Transcriptional profiling of *C. jejuni* at low pH

In this study, we aimed to characterize the transcriptional profile of *C. jejuni* 81-176 in response to *in vitro* acid shock using microarray profiling. The acid stimulon of *C. jejuni* enabled us to determine the genes that are differentially expressed in *C. jejuni* under acidic conditions. These genes could play a role in *C. jejuni* acid stress response as well as host pathogenesis. A total of 267 genes were differentially expressed in *C. jejuni* 81-176 in response to acid exposure. These genes were divided into up-regulated genes (group A; 117 genes) and down-regulated genes (group B; 150 genes) (Appendix III), which could be further categorized into 20 sub-groups according to their clusters of orthologous genes (COG) functions (Figure 2.3).

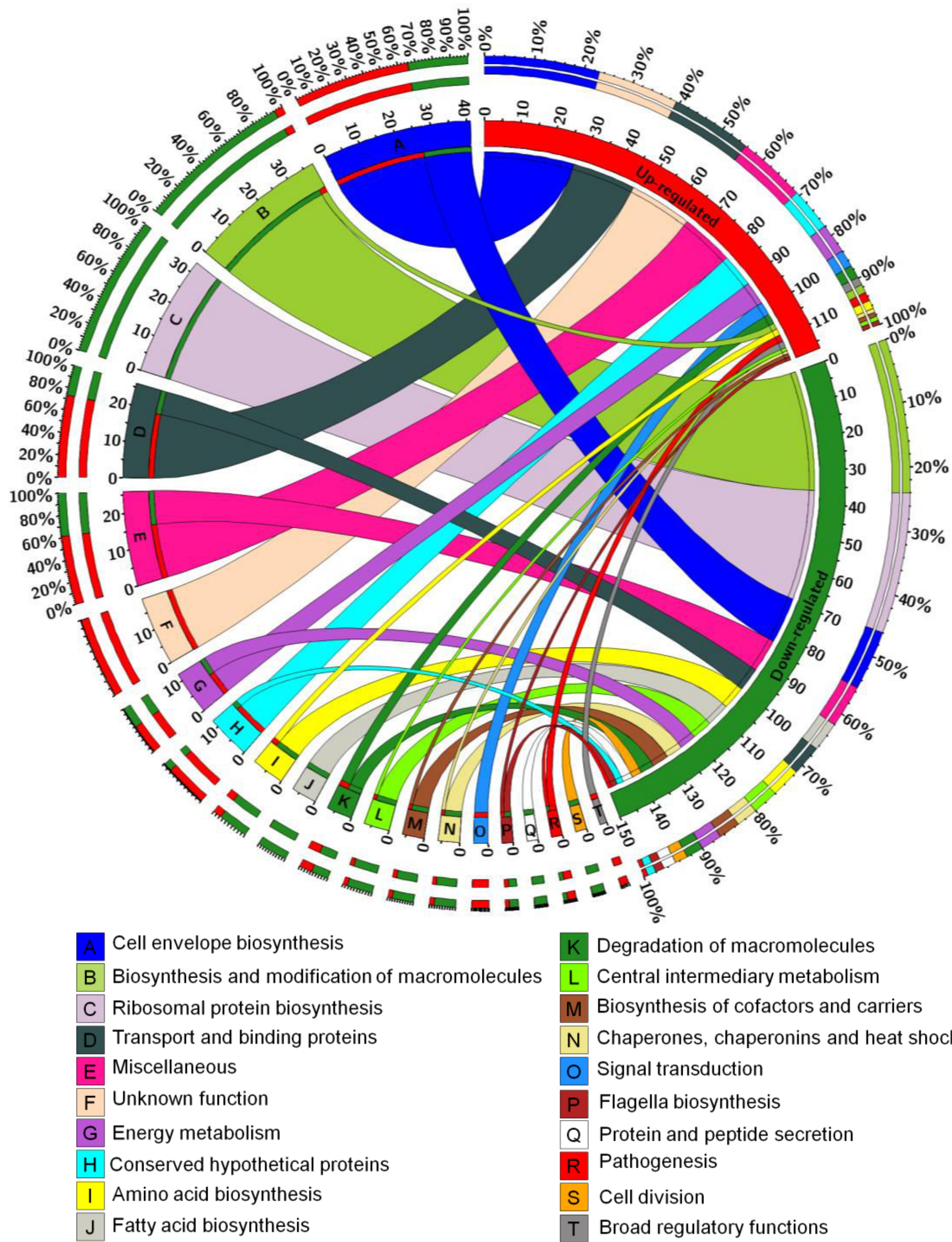


Figure 2.3: Functional categorization of differentially expressed genes in *C. jejuni* 81-176 in response to acid stress (pH 4 for 8 min). The total numbers of up- and down-regulated genes are represented in red and green segments, respectively. Each functional category is represented by a colored segment (A-T), and the ribbon size indicates the number of genes that are either up- or down-regulated within each category. The outermost colored segments represent the relative contribution of each functional category to the total number

of up- and down-regulated genes. The figure was constructed using Circos Table Viewer version 0.54 (237).

Group A contains genes that were up-regulated in *C. jejuni* under acidic conditions (pH 4 for 8 min). The 117 genes in this cluster were up-regulated in *C. jejuni* 81-176 in response to acidic conditions. This cluster includes a number of genes associated with energy generation and general metabolism, such as acetate kinase (*ackA*), aconitate hydratase (*acnB*), L-lactate dehydrogenase complex (*cj0073c*, *cj0074c* and *cj0075c*), amino acid metabolism (L-asparaginase; *ansA*), genes involved in trimethylamine *N*-oxide/dimethyl sulfoxide (TMAO/DMSO) respiration (*cj0264c* and *cj0265c*), the putative cytochrome *c* (*cj0874c*), oxidoreductase (*cj0833c*) and a malate:quinone oxidoreductase (*mqo*; *cj0393c*). In addition, this cluster contains genes encoding for products required for cofactor biosynthesis (*hemD*), a molecular chaperone DnaJ-like protein (*cj1034c*), hemerythrin (*herB*; *cj1224*), iron storage (ferritin; *cft*), transport and binding proteins (e.g., *cjaB*, *cj0919*, *cj0850c* and *cj0934c*), potassium transport ATPase (*kdpA*); and genes encoding proteins involved in regulatory functions, such as a putative transcriptional regulator (*cj0883c*). A large number of up-regulated genes included in this group were those genes encoding products involved in cell envelope biogenesis, such as genes involved in pseudaminic acid biogenesis (*pseB*), the putative periplasmic proteins (*cj0420*, *cj0735*, *cj0776c*, *cj0834c*, *cj0864*, *cj0876c* and *cj0909*), putative integral membrane proteins (*cj0343c*, *cj0553*, *cj0721c*, *cj0830*, *cj0852c*, *cj0986c* and *cj1022c*) and putative lipoproteins (*cj0591c*, *cj0770c*, *cj0771c* and *cj0772c*).

Other up-regulated genes in this group included *cj0414* and *cj0415* which encode orthologous to gluconate dehydrogenase (GADH) from *P. cypripedii* (336), and those involved in *Campylobacter* phosphate uptake (*cj1194*) and storage (*cj0604*). Interestingly, acid exposure resulted in the up-regulation of genes known to contribute to *Campylobacter*

pathogenesis, such as the *Campylobacter* invasion antigen (*ciaB*), a putative CinA-like protein (*cj1062*) and an aspartate aminotransferase (*aspB*). Furthermore, some genes encoding for proteins involved in signal transduction, such as the putative methyl-accepting chemotaxis protein-type (MCP) signal transduction proteins (*cj0448c*, *cj0951c* and *cj1110c*) and the two-component sensor histidine kinase (*racS*) were up-regulated in *C. jejuni* upon acid exposure. A large number of genes in *C. jejuni* have no ascribed function; group A includes 29 genes encoding for proteins with conserved hypothetical or unknown functions, such as *cj0449c*, *cj0681*, *cj0797c*, *cj0849c*, *cj0900c*, *cj0916c*, *cj0939c*, *cj1057c* and *cj1100*.

Group B contains genes that were down-regulated in *C. jejuni* under acidic conditions (pH 4 for 8 min). Acid exposure repressed the expression of 150 *C. jejuni* genes assembled into group B. Among these genes are genes encoding products for amino acid biogenesis, such as dihydropicolinate reductase (*dapB*), branched-chain amino acid aminotransferase (*ilvE*) and cofactor biosynthesis (*folCDP* and *hemA*). In addition, this cluster includes genes encoding products involved in cell division, such as putative cell division proteins (*ftsKZ*) and a homolog of *E. coli* rod shape-determining protein (*mreB*), and genes encoding products involved in energy generation and central intermediary metabolism, such as carbonic anhydrase (*cynT*), S-adenosylmethionine synthetase (*metK*), carbamoyl transferase (*hypF*), fructose-bisphosphate aldolase (*fba*) and cytochrome *bd* oxidase subunits I and II (*cydA* and *B*). A large number of genes in group B encode for ribosomal proteins (*rplABCDEFKMNOPRTVWX, rpmCG* and *rpsBCDEFGHIJKMNQT*), transcription (*rpoAB* and *nusAG*), translation (*efp, def, fusA*, *infAC*, *prfA*, *tsf*, *tuf* and *cj1453c*), DNA replication (*dnaAGQ*, *gyrA*, *ligA* and *ssb*), uptake (*exbB3*), transport (*cj1648* and *livGHFJM*) and fatty acid biogenesis (*aas*, *fabDHH2F* and *plsX*). This group includes genes involved in cell

envelope biogenesis (*cj1412c*, *cj1500*, *cj1668c*, *cj1626c*, *cj1637*, *cj1666c*, *ddlA*, *flhA*, *fliI*, *kdtA*, *lpxABK*, *murC*, *peb3* and *pbpC*), the heat shock response (*clpA*, *dnaKJ*, *groEL* and *htpG*), purine and nucleoside biogenesis (*purBH*, *guaA* and *ndk*) and protein secretion (*ffh*, *lepP* and *mttB*). Finally, group B includes genes encoding products involved in the degradation of macromolecules, such as the ATP-dependent proteases (*clpXP*) and a putative serine protease (*cj1365c*).

2.3.3. Validation of microarray data by qRT-PCR

The microarray results were validated using qRT-PCR on a subset of differentially expressed genes. This analysis was accomplished by plotting the $\log_2$ ratio values of gene expression from the microarray experiment against the $\log_2$ ratio of the relative quantity values obtained from qRT-PCR. A strong correlation ($R^2 = 0.78$) was identified between the two data sets validating the microarray experiment (Figure 2.4).

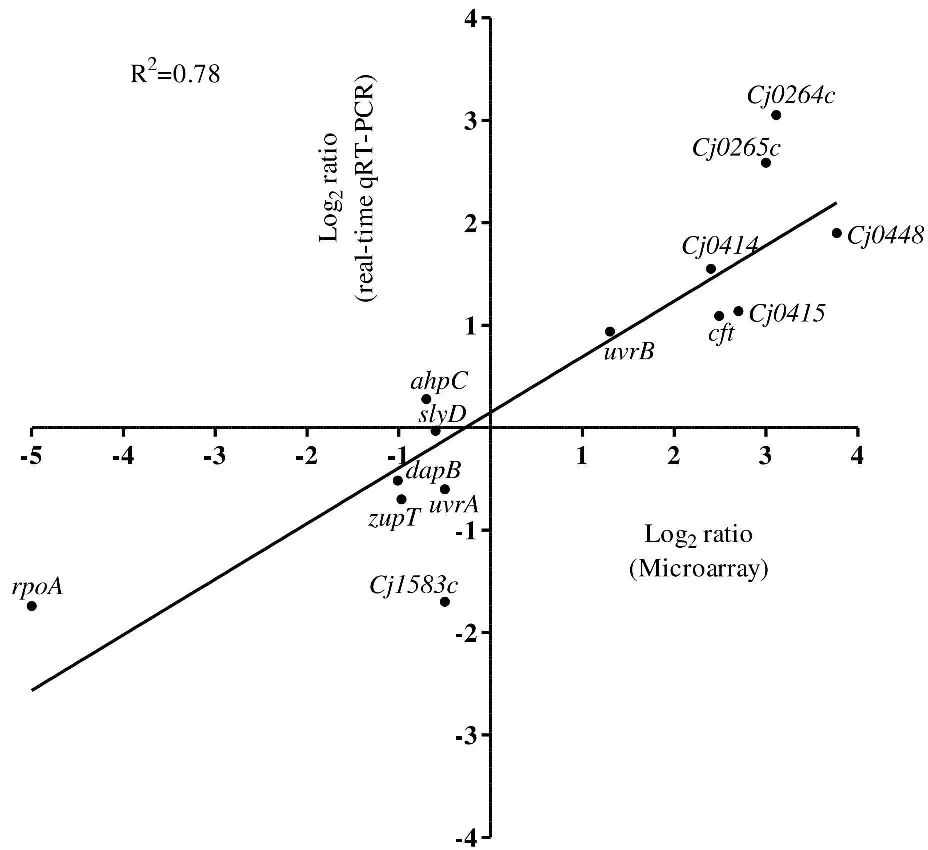


Figure 2.4: Validation of microarray results by qRT-PCR. The $\log_2$ ratio values of gene expression levels measured by microarray were plotted against the $\log_2$ relative quantity values obtained from qRT-PCR. A strong correlation ($R^2 = 0.78$) was identified between the data obtained from the microarray experiment and the qRT-PCR data.

2.3.4. *Campylobacter* acid survival is enhanced in the presence of D-gluconate

As shown in Appendix III the transcriptome of *C. jejuni* in response to acid stress revealed the up-regulation of two genes, *cj0414* and *cj0415*, which encode for gluconate dehydrogenase (336). Therefore, we tested the contribution of Cj0414 and Cj0415 to *C. jejuni* acid survival by assessing the capacity of *C. jejuni* to grow at low pH in the presence or absence of 20 mM D-gluconate. As shown in Figure 2.5, the capacity of *C. jejuni* to survive an acid shock at pH 3 was significantly enhanced in the presence of gluconate suggesting a possible role of Cj0414 and Cj0415 in *C. jejuni* acid survival.

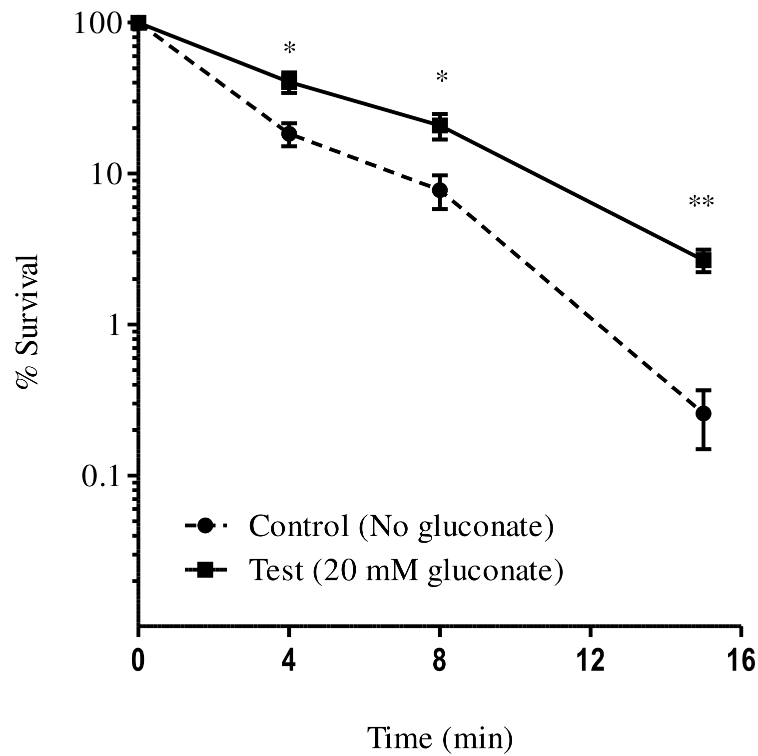


Figure 2.5: D-gluconate enhances *C. jejuni* survival in the presence of acid stress. *C. jejuni* grown to logarithmic phase in biphasic MH culture was exposed to acidic condition at pH 3 either in the presence or in absence of 20 mM D-gluconate. Samples were withdrawn immediately and 4, 8 and 15 min after acid exposure to determine the percentage of bacterial survival. The data shown are the means of three independent experiments ± standard error of the mean. A $P < 0.05$ was considered significant using a Student unpaired *t*-test.

2.3.5. Prior exposure of *C. jejuni* to acid enhanced its capacity to survive H_2O_2

The transcriptomic profile of *C. jejuni* under acidic conditions revealed the up-regulation of two genes involved in the oxidative stress defense [ferritin; *cft* (462) and hemerythrin; *herB* (218)]. This finding suggests an overlap in the response of *C. jejuni* to acid and oxidative stresses. To determine a possible effect of acid exposure on the cross-protection of *C. jejuni* against oxidative stress, we compared both acid-stressed and unstressed *C. jejuni* for their capacity to survive H_2O_2 using disk inhibition assays. Intriguingly, acid-stressed *C. jejuni* were significantly more resistant to H_2O_2 than unstressed bacteria (Table 2.1).

The results of the disk inhibition assay were further confirmed by examining the kill curves of both acid-stressed and unstressed *C. jejuni* in the presence of H_2O_2. The results were obtained by counting viable bacterial cells immediately and 4, 8, 15 and 30 min after exposure to 10 mM H_2O_2. Consistent with the disk inhibition assay, acid-stressed *C. jejuni* were significantly more resistant to H_2O_2 than unstressed bacteria ($P < 0.05$, Student unpaired t-test) (Figure 2.6).

Table 2.1: Sensitivity of *C. jejuni* 81-176 to H_2O_2 before and after exposure to acid

Strain	Diameter[a] (mm) of inhibition zone after exposure to different molar concentrations of H_2O_2		
	250 mM	500 mM	1000 mM
***C. jejuni* 81-176**			
Acid-stressed	13.2 ± 0.22**	16.8 ± 0.22***	22.0 ± 0.33**
Unstressed	15.2 ± 0.44	20.7 ± 0.29	24.3 ± 0.19

[a] The diameter of the inhibition zone is represented as the mean clear zone (in mm) ± standard error of three independent experiments for *C. jejuni* (acid-stressed and unstressed) after exposure to different molar concentrations (250-1000 mM) of H_2O_2. An asterisk (*) indicates $P < 0.05$ using a Student unpaired t-test.

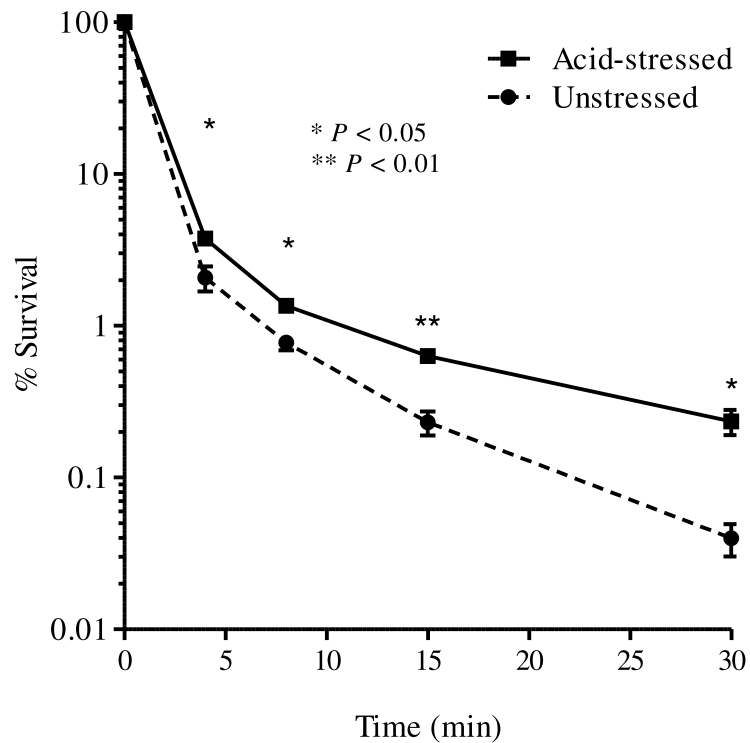


Figure 2.6: Survival of both acid-stressed and unstressed *C. jejuni* 81-176 in the presence of H_2O_2. Prior exposure of *C. jejuni* to acid enhanced its capacity to survive H_2O_2. Both acid-stressed and unstressed *C. jejuni* were exposed to 10 mM H_2O_2. The percentage of bacterial survival was determined immediately and 4, 8, 15 and 30 min following exposure to H_2O_2. The data shown are the means of three independent experiments ± standard error of the mean. An asterisk (*) indicates $P < 0.05$ using a Student unpaired *t*-test.

2.3.6. Acid stress does not improve *C. jejuni* growth or survival in the presence of either osmotic or bile stress

In addition to stomach acidity, *C. jejuni* encounters other stresses in the GIT during host colonization such as oxidative, osmotic and bile stress (57, 119-121, 373, 426). Our finding showing that *C. jejuni* H_2O_2 survival was enhanced following acid exposure suggests that acid stress could be perceived by *C. jejuni* as a general stress indicator. Acid exposure of *C. jejuni* could trigger the expression of proteins that contribute to bacterial tolerance to other environmental stressors in addition to acid stress. We hypothesized that acid-stressed *C. jejuni* would show enhanced survival to other stresses such as osmotic and/or bile stress as compared to unstressed bacteria. Therefore, it was important to characterize the effect of prior acid exposure on the capacity of *C. jejuni* to survive osmotic and bile stress. This would significantly help us understand the mechanisms of *C. jejuni* stress response and host pathogenesis.

The capacity of both acid-stressed and unstressed *C. jejuni* to grow at different molar concentrations of NaCl was monitored by measuring the optical density of growing bacteria over 36 h. As shown in Figure 2.7, no difference was noted between acid-stressed and unstressed *C. jejuni* for their capacity to grow under osmotic stress. Both acid-stressed and unstressed *C. jejuni* were able to grow to similar maximum optical densities in the presence of different concentrations of NaCl. Moreover, acid-stressed *C. jejuni* exhibited growth rates (μ) of 0.27 ± 0.06 h^{-1} and 0.25 ± 0.04 h^{-1} which were not significantly different from those of unstressed bacteria (0.27 ± 0.04 h^{-1} and 0.23 ± 0.06 h^{-1}) when bacteria were grown in the presence of 0.03 M and 0.06 M NaCl, respectively ($P > 0.05$; Student unpaired t-test).

Similar to *C. jejuni*'s response to osmotic stress, prior acid exposure did not enhance the capacity of *C. jejuni* to survive bile stress. *C. jejuni* growth in the presence of bile salts was investigated by determining the MICs of different bile salts for both acid-stressed and unstressed bacteria. Acid-stressed *C. jejuni* MICs for both bovine bile and sodium deoxycholate were not significantly different from the MICs of unstressed bacteria (Table 2.2). Our results indicate that the prior acid exposure of *C. jejuni* does not enhance its capacity to grow and survive in the presence of either osmotic or bile stress.

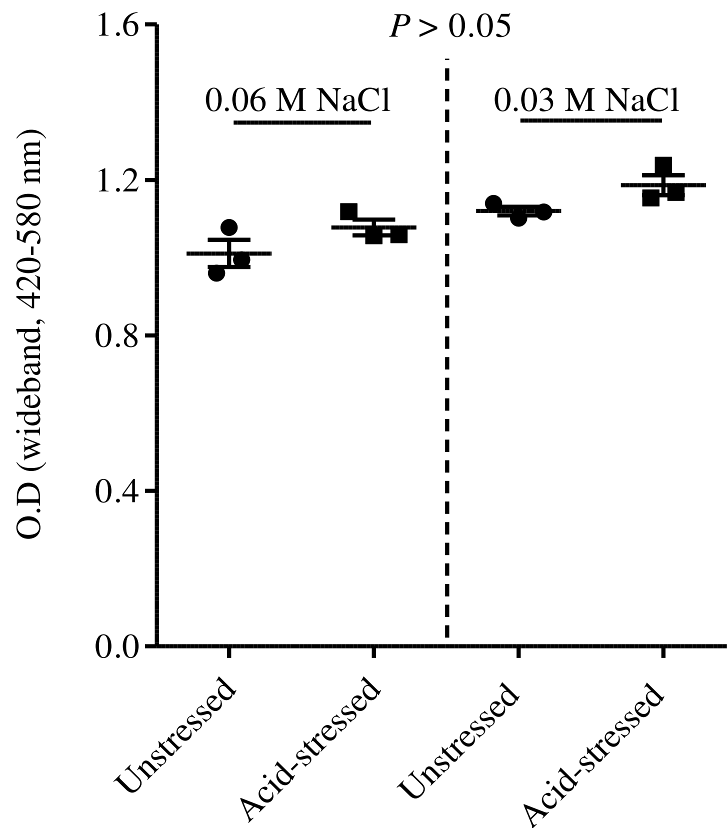


Figure 2.7: Growth of *C. jejuni* 81-176 in the presence of osmotic stress was not enhanced upon acid exposure. Bacterial growth was determined by measuring the maximum OD attained over 36 h in MH broth supplemented with 0.03 M or 0.06 M NaCl. The data points represent the results of three independent experiments with ten technical replicates each for both acid-stressed (black squares) and unstressed (black circles) *C. jejuni*. The solid line represents the mean ± standard error. No significant difference was found between acid-stressed and unstressed bacteria in growth in the presence of osmotic stress using a Student unpaired *t*-test.

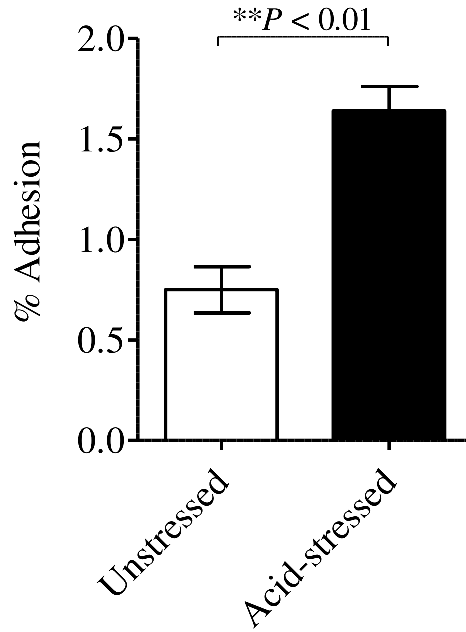
Table 2.2: Sensitivity of acid-stressed and unstressed *C. jejuni* 81-176 to bile salts

Strain	MIC (mg/mL)[a]	
	Bovine bile	**Sodium deoxycholate**
***C. jejuni* 81-176**		
Acid-stressed	1.3 ± 0.26	13.9 ± 3.7
Unstressed	1.2 ± 0.35	11.8 ± 2.5

[a] MIC indicates the minimum inhibitory concentration of bile salts (mg/mL) ± standard error that inhibits bacterial growth. Each experiment was repeated with at least three biological replicates with three technical replicates each. No significant difference in survival of bile salts was found between acid-stressed and unstressed *C. jejuni* using a Student unpaired *t*-test.

2.3.8. *C. jejuni* adhesion to and invasion of Caco-2 cells

Interestingly, acid exposure resulted in the up-regulation of many genes involved in *C. jejuni* pathogenesis such as *cj0914c* (*ciaB*), *cj1062* (putative CinA-like protein) and *cj0762c* (*aspB*; aspartate aminotransferase). All these genes encode proteins that are required for *C. jejuni* invasion or survival within eukaryotic cells (143, 230, 322). These findings suggest that prior acid exposure could play a role in *C. jejuni* virulence in human epithelial cells. To determine the influence of acid stress on *C. jejuni* virulence phenotypes, the capacities of both acid-stressed and unstressed *C. jejuni* to adhere to and invade Caco-2 cells were characterized. Interestingly, the capacities of *C. jejuni* to adhere to and invade Caco-2 cells were greatly enhanced following acid exposure. As shown in Figure 2.8, the prior exposure of *C. jejuni* to acid increased its capacities for both the adhesion to and invasion of Caco-2 cells by at least 2-fold. Unstressed *C. jejuni* had significantly ($P < 0.05$, Student unpaired *t*-test) reduced capacities to adhere to and invade Caco-2 cells (0.74% ± 0.11 and 0.08% ± 0.005, respectively) relative to acid-stressed bacteria (1.6% ± 0.12 and 0.15% ± 0.011, respectively).

A 

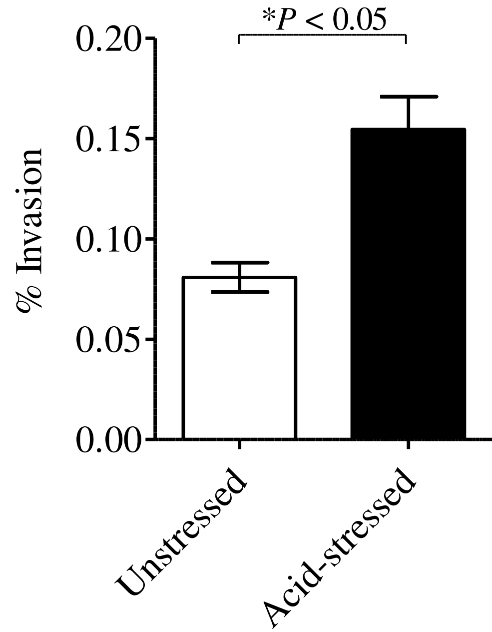
B 

Figure 2.8: Role of acid stress in *C. jejuni* 81-176 adhesion to and invasion of Caco-2 cells. Acid-stressed *C. jejuni* showed higher capacities to (A) adhere to and (B) invade Caco-2 cells relative to unstressed bacteria. *C. jejuni* was exposed to acid (pH 4 for 8 min), and Caco-2 cells were infected with acid-stressed and unstressed *C. jejuni* at an MOI of 100:1. The percentages of adhering and invading bacterial cells into Caco-2 cells were determined after 3 hours of coculture by lysing epithelial cells with 0.1% Triton X-100, serially diluting in PBS and plating on MH agar. The data shown are the means of three independent experiments ± standard error of the mean. An asterisk (*) indicates $P < 0.05$ using a Student unpaired *t*-test.

2.3.9. Intracellular survival of *C. jejuni* within Caco-2 cells

In addition to characterizing the adhesion and invasion capacities of *C. jejuni* into eukaryotic cells, the contribution of acid stress to *C. jejuni* intracellular survival within human epithelial cells was further studied. The capacity of *C. jejuni* to invade and survive intracellularly within epithelial cells is a critical step for disease development (324). Intracellular survival could enable *C. jejuni* to evade host immune responses and establish long-term persistent infections (88, 177, 432). Importantly, *C. jejuni* is capable of surviving intracellularly and inducing cytotoxic responses such as tissue damage in infected humans (46, 324). The improved adherence and invasion capacities of acid-stressed *C. jejuni* as compared to unstressed bacteria let us hypothesize that *C. jejuni* intracellular survival within human cells would be enhanced following acid exposure.

Caco-2 cells were infected with either acid-stressed or unstressed *C. jejuni*, and bacterial counts within infected cells were analyzed at 4, 24, 48 and 72 h post-infection. Interestingly, the acid-stressed *C. jejuni* was detected in significantly higher numbers within Caco-2 cells as compared to unstressed bacteria at different time points following coculture. At 4 h post-infection, the intracellular percentages of the acid-stressed and unstressed *C. jejuni* were 0.15% ± 0.01 and 0.06% ± 0.01, respectively. However, there was a decrease in *C. jejuni* numbers for both acid-stressed and unstressed *C. jejuni* at 24 h post-infection, as the percentages of intracellular bacteria decreased from 0.15% ± 0.01 to 0.02% ± 0.001 and 0.06% ± 0.01 to 0.005% ± 0.001, respectively. Forty-eight hours post-infection, both acid-stressed and unstressed bacteria were able to multiply intracellularly, and their numbers increased to 0.16% ± 0.02 and 0.08% ± 0.01, respectively. At 72 h post-infection, the intracellular percentages for both acid-stressed and unstressed *C. jejuni* were 0.05% ± 0.01

and 0.02% ± 0.001, respectively (Figure 2.9). Our results show that both acid-stressed and unstressed *C. jejuni* follow a similar pattern of bacterial survival intracellularly. The cell number ratio between acid-stressed and unstressed *C. jejuni* was ~ 3:1 which remains almost constant at different time points following coculture with Caco-2 cells. If there was an improvement in *C. jejuni* intracellular survival by acid exposure, the cell number ratio between acid-stressed and unstressed *C. jejuni* should have increased following infection of eukaryotic cells. However, the observed higher intracellular numbers of acid-stressed *C. jejuni* relative to unstressed bacteria at different time points could be related to the increased invasive capacity of *C. jejuni* following acid exposure and not due to an enhanced capacity of *C. jejuni* intracellular survival.

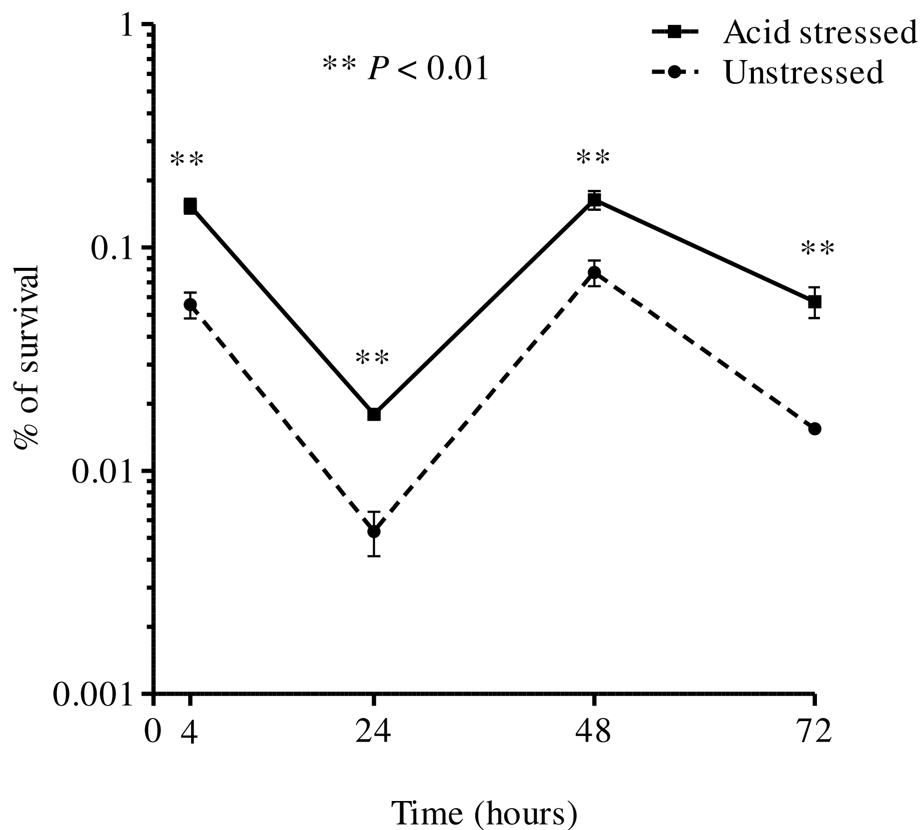


Figure 2.9: Intracellular survival of *C. jejuni* 81-176 within Caco-2 cells upon acid exposure. *C. jejuni* was exposed to acid (pH 4 for 8 min), and Caco-2 cells were infected with acid-stressed and unstressed *C. jejuni* at an MOI of 100:1. The percentages of surviving *C. jejuni* within epithelial cells were determined over a 72-h period as described for the invasion assay. The results are shown as the means of three independent experiments ± standard error of the mean. An asterisk (*) indicates $P < 0.05$ using a Student unpaired *t*-test.

2.3.10. Exposure to acid increases *C. jejuni* pathogenesis in *G. mellonella*

As mentioned above, many genes involved in *C. jejuni* pathogenesis were up-regulated upon the exposure to acidic conditions (pH 4 for 8 min). Moreover, the enhanced capacity of the acid-stressed *C. jejuni* to adhere to and invade epithelial cells suggests that the virulence of *C. jejuni* in the host would be enhanced following acid exposure. The influence of acid stress on *C. jejuni* pathogenesis was characterized using the *G. mellonella* (waxworm) infection model. In contrast to uninoculated and PBS-inoculated larvae, all larvae infected with *C. jejuni* (acid-stressed or unstressed) showed signs of invasive infection (e.g., melanization) and higher mortality rates (Figure 2.10). Both acid-stressed and unstressed *C. jejuni* bacteria caused dose-dependent killing in waxworm larvae (Figure 2.10 A and B). At the highest and lowest bacterial doses (10^7/10^5 CFU), there was no difference in the *C. jejuni*-induced mortality rate between the acid-stressed or unstressed bacteria (Figure 2.10 C). In contrast, at the 10^6 CFU infection dose, the acid-stressed *C. jejuni* killed significantly more waxworms than the unstressed bacteria (Figure 2.10 C). In addition, the LD_{50}s of both acid-stressed and unstressed *C. jejuni* in waxworms were determined. In comparison with acid-stressed bacteria, which had an LD_{50} of 2.9 x 10^4 CFU, the LD_{50} of unstressed *C. jejuni* was significantly higher (1.39 x 10^5 CFU); thus, the unstressed bacteria were significantly less virulent in *G. mellonella* (Figure 2.10 D). A histopathological examination of *C. jejuni*-infected larvae revealed bacterial cells surrounded by larval hemoctyes, hemolymph and melanin pigments (Figure 2.10 E). Melanization is an insect immune response that results from the cleavage of prophenoloxidase to active phenoloxidase to limit pathogen growth within the hemocoel (211). Our results indicate that the virulence of *C. jejuni* in *G. mellonella* larvae was significantly enhanced by acid exposure.

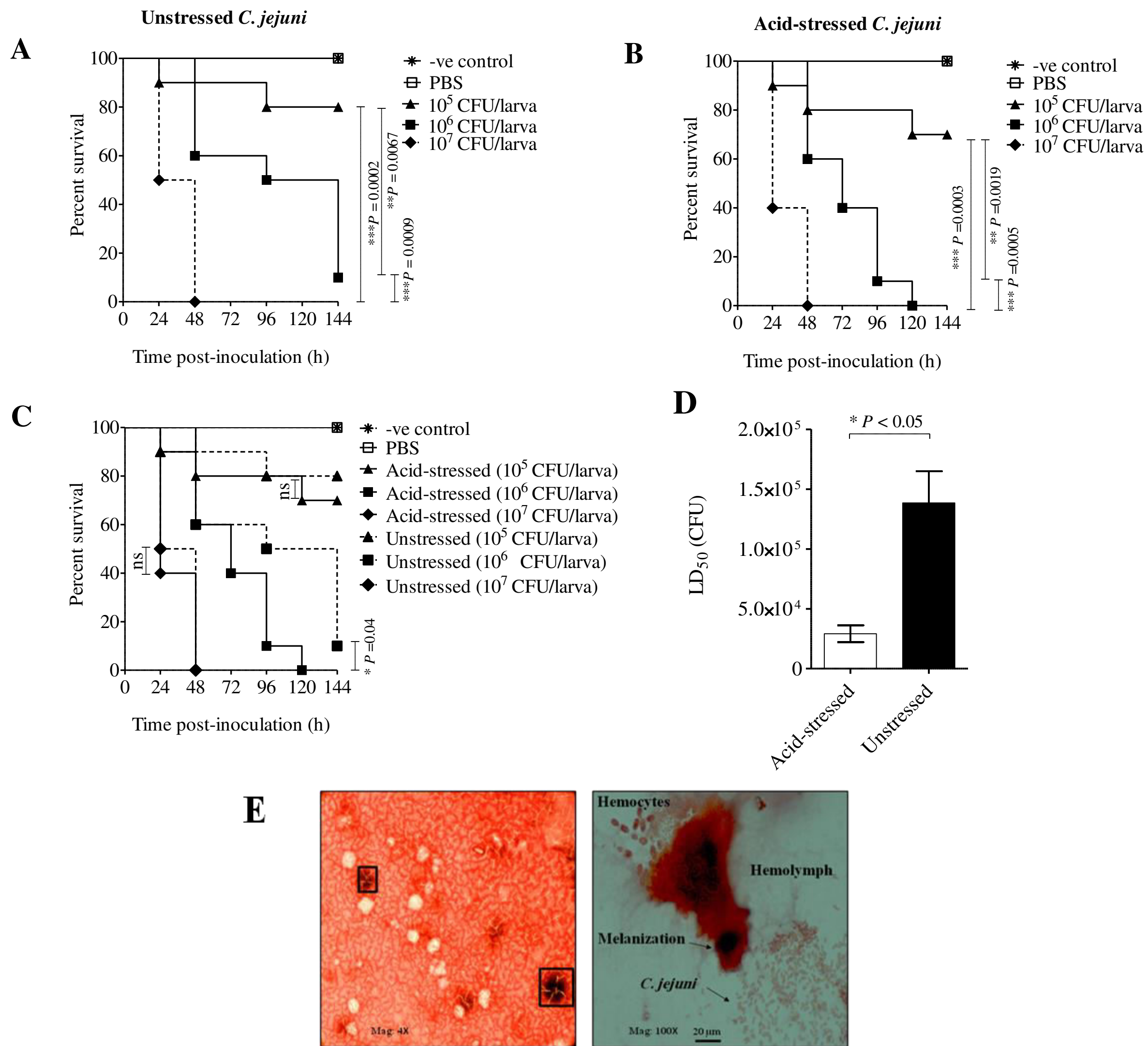


Figure 2.10: Acid stress enhances *C. jejuni* pathogenesis in *G. mellonella* larvae. Larvae (n = 10 larvae/group) were inoculated with 10 µL of serial dilutions (10^7, 10^6 and 10^5 CFU) of acid-stressed or unstressed *C. jejuni*. Larval survival was monitored every 24 h for 6 days and plotted using Kaplan-Meier survival curves. No deaths occurred in uninfected larvae or larvae injected with sterile saline (A-C). Both acid-stressed and unstressed *C. jejuni* exhibited dose-dependent killing of larvae (A and B, respectively). Survival of *G. mellonella* larvae injected with acid-stressed and unstressed *C. jejuni* was compared at different bacterial doses (C). At the highest and lowest doses (10^7 and 10^5 CFU, respectively), no significant variations in larval killing by acid-stressed or unstressed *C. jejuni* were identified. However, at the 10^6 CFU dose, acid-stressed *C. jejuni* killed more larvae than unstressed bacteria (P = 0.04, log-rank test). (D) The LD_{50} was determined using a Probit analysis (331). A P value < 0.05 was considered significant using a Mann-Whitney test. (E) Microscopic examination of *C. jejuni*-infected larvae revealed bacterial cells surrounded by larval hemoctyes, hemolymph and melanin pigment (boxed regions) (Gram staining, 4X (left panel) and 100X (right panel) original magnification). Size bars, 20 µm. Magnification, mag.

2.4. Discussion

C. jejuni is a major cause of foodborne gastroenteritis in humans worldwide (3, 41, 324). The capacity of *C. jejuni* to sense and adapt to various stresses in the GIT is crucial for its pathogenesis (373, 374). One of the inevitable stressors that are encountered by *C. jejuni* shortly after being ingested with contaminated food or water is the fluctuations in pH (250, 373, 374). For successful colonization of the intestine, *C. jejuni* must first survive the low pH of the stomach. However, the mechanism by which *C. jejuni* copes with stomach acidity remains unknown (250, 373). This work aimed to characterize the transcriptional profile of *C. jejuni* in response to low pH, the effect of acid exposure on *C. jejuni* survival in the presence of other stresses as well as on *C. jejuni* host pathogenesis.

Acid stress induces the expression of genes involved in energy generation and bacterial metabolism. Interestingly, the exposure of *C. jejuni* to acid resulted in the up-regulation of genes encoding proteins involved in energy production and various electron transport pathways. For example, acid shock induced the expression of the aconitase gene *acnB* that could help *C. jejuni* survive acidic conditions by increasing acid consumption (e. g., acetate) in the tricarboxylic acid (TCA) cycle (373). Moreover, our data revealed the up-regulation of genes involved in amino acid metabolism such as L-asparaginase (*ansA*). L-asparaginase catalyzes the hydrolysis of asparagine to L-aspartic acid and ammonia, which can buffer the cellular environment and/or reverse cytoplasm acidification (26). Importantly, our data revealed the up-regulation of genes involved in the electron transport pathways, such as malate:quinone oxidoreductase (*mqo*; *cj0393c*), and genes involved in TMAO/DMSO respiration (*cj0264c* and *cj0265c*). Electron transfer within electron transport pathways is accompanied with proton translocation across the cytoplasmic membrane, which could

contribute to *C. jejuni* acid survival (203, 374). In addition, the acid exposure of *C. jejuni* induced the expression of *Campylobacter* gluconate dehydrogenases (Cj0414 and Cj0415), which convert D-gluconate to 2-keto-D-gluconate (336, 490). Interestingly, our data revealed that the acid survival of *C. jejuni* was markedly enhanced in the presence of D-gluconate. These results suggest that gluconate dehydrogenases (Cj0414 and Cj0415) could contribute to *C. jejuni*'s survival at low pH. This hypothesis is supported by many studies that have demonstrated that *cj0414* and *cj0415* were up-regulated in acid-exposed *C. jejuni* (250, 373, 374). Moreover, a Δ*cj0415* mutant was acid-sensitive, indicating that Cj0415 is important for *C. jejuni*'s response to acid stress (374). Indeed, this is the first study demonstrating that *C. jejuni* acid survival is enhanced in the presence of gluconate. Gluconate can serve as an electron donor in highly branched electron transport chains which in the presence of dehydrogenases (Cj0414 and Cj0415) can initiate electron transport and energy conservation (215, 310, 336, 354, 472). As mentioned above, electron transport is important for bacterial acid survival. Therefore the capacity of *C. jejuni* to use gluconate as an electron donor via GADH activity might contribute to *C. jejuni* acid survival.

Importantly, the transcriptome of *C. jejuni* under low pH revealed the up-regulation of three genes (*cj0073c*, *cj0074c* and *cj0075c*). These genes encode for non-flavin iron-sulfur containing oxidoreductase complex that display L-lactate dehydrogenase activity converting L-lactate to pyruvate (448). Previous studies showed that *C. jejuni* has the capacity to use L-lactate as both carbon source and electron donor (448). Once inside the cell, L-lactate can be catabolised into pyruvate by the activity of lactate dehydrogenases (420, 448). The importance of pyruvate for *C. jejuni* metabolism and growth in the environment is well established (459, 460). Pyruvate is fed directly into the TCA cycle, which is an important

energy source for *C. jejuni* (420). Moreover, L-lactate can act as an electron donor within the electron transport chains (448). The released electrons during the intracellular L-lactate oxidation to pyruvate are used for oxygen reduction with the consumption of protons (448). The role of L-lactate dehydrogenase activity in energy metabolism and its involvement in the electron transport chain could explain its importance for *C. jejuni* acid survival.

Finally, in contrast to the study by Reid *et al*. (374), the putative Na^+/ H^+ antiporter (*cj0832c*) was up-regulated in the present study. Interestingly, Na^+/H^+ antiporters are thought to play an essential role in pH homeostasis in bacterial cells because these antiporters are involved in Na^+ and H^+ circulation within bacteria (333). The importance of Na^+/ H^+ antiporters for intracellular pH homeostasis in *E. coli* (334, 335) and *B. subtilis* has been demonstrated (235). The differences between our results and previous finding (374) could be attributed to differences in experimental design, growth conditions and natural variations between bacterial strains. Reid *et al*. studied the transcriptome of *C. jejuni* NCTC11168 at pH 5.5, while our study characterized gene expression in *C. jejuni* 81-176 at more acidic conditions (pH 4).

A possible role of the heat shock proteins in *C. jejuni* acid survival. Our data revealed that a putative DnaJ-like protein (Cj1034c), which acts as a molecular chaperone (118), was up-regulated in *C. jejuni* upon acid exposure. Molecular chaperones, including the heat shock proteins play a role in protein quality control in *C. jejuni* in response to increased temperatures (179, 425). The induced expression of Cj1034c might repair the damage that occurs upon acidification of the bacterial cytoplasm and reduce the accumulation of misfolded proteins. In agreement with our finding, the up-regulation of heat shock proteins was previously observed in *C. jejuni* NCTC11168 following acid exposure (250, 373).

Importantly, our observation suggests that there is a similarity in *C. jejuni*'s responses to various stresses. Although *C. jejuni* did not encounter a heat stress under our experimental conditions, the heat shock proteins could play a role in the protection of cells against acid stress. Acid exposure of *C. jejuni* is accompanied by an increase in intracellular damaged and misfolded proteins (1, 26, 37, 125, 271). Therefore, not surprisingly, heat shock proteins are induced in *C. jejuni* upon acid exposure to reduce the intracellular level of unfolded and aggregated proteins.

Acid shock repressed the expression of genes encoding ribosomal proteins in *C. jejuni*. The transcriptional profile of *C. jejuni* under acidic conditions revealed that 32 genes encoding for ribosomal protein biogenesis (*rplABCDEFKMNOPRTVWX, rpmCG* and *rpsBCDEFGHIJKMNQT*) were down-regulated. This finding agrees with a previous study showing that exposure of *C. jejuni* to acid resulted in the repression of 12 ribosomal genes (374). Similarly, *in vitro* exposure of *H. pylori* (469), *S. aureus* (48) and *S. oneidensis* (252) to acid resulted in the down-regulation of ribosomal genes. The down-regulation of protein biogenesis genes in *C. jejuni* NCTC11168 upon exposure to acid has been demonstrated previously (250). It is therefore tempting to propose that *C. jejuni* responds to acid shock by changing from a state of protein synthesis required for bacterial growth to a survival mode to withstand this stress. This hypothesis is further supported by the finding that genes involved in cell division and replication, such as the putative cell division proteins (FtsKZ), were down-regulated in acid-stressed *C. jejuni,* as noted in both the present study and a previous study (250).

Acid exposure affects the expression of genes involved in cell surface composition in *C. jejuni*. Our data revealed that the exposure of *C. jejuni* to acid resulted in the differential

expression of many genes encoding proteins involved in cell envelope biogenesis. In agreement with a previous study (373), both the *lpxB* and *kdtA* genes, which encode for lipid A biogenesis, were down-regulated in *C. jejuni* upon exposure to acid. In addition, the exposure of *C. jejuni* to acid stress led to the down-regulation of the *aas*, *fabDHH2F* and *plsX* genes which are involved in fatty acid biogenesis as well as genes that are involved in cell envelope and LOS biogenesis (*cj1412c*, *cj1500*, *cj1668c*, *cj1626c*, *cj1637*, *cj1666c*, *ddlA*, *lpxAK*, *murC*, *peb3* and *pbpC*). The cell surface components play an essential role in bacterial survival to various stresses (52, 389). Bacteria generally modify their cell membrane composition to maintain both membrane integrity and functionality against various stresses, especially acid stress (52, 389). Therefore, bacteria either repress or activate genes encoding proteins involved in membrane composition to survive such unfavorable conditions (286, 287). For example, *H. pylori* represses the expression of membrane proteins to change cell permeability as a protective means against acid stress (287).

Acid stress activates the expression of virulence genes in *C. jejuni*. Interestingly, the exposure of *C. jejuni* to acidic conditions (pH 4 for 8 min) led to the up-regulation of several pathogenic determinants such as Cj0914c (CiaB protein), Cj1062 (putative CinA-like protein) and Cj0762c (AspB; aspartate aminotransferase). This finding indicates that acid exposure could modulate the virulence capacity of *C. jejuni*. A previous study by Konkel *et al*. (230) indicated that the *C. jejuni* CiaB protein is required for bacterial internalization into cultured mammalian cells. Moreover, the *C. jejuni* Δ*ciaB* mutant was defective for the capacity to colonize the cecum in a chick colonization model (495). Similarly, both Cj1062 (putative CinA-like protein) and Cj0762c (AspB; aspartate aminotransferase) have been shown to be important for *C. jejuni* host colonization and pathogenesis (143, 322). The *C.*

jejuni Δ*cj1062* mutant was unable to invade the intestinal epithelial cells suggesting a potential role of Cj1062 in bacterial virulence (143). The importance of AspB (aspartate aminotransferase) for *C. jejuni* pathogenesis has been demonstrated recently (322). AspB is involved in fumarate production, which constitutes both a carbon source and an alternative electron acceptor during *Campylobacter* anaerobic respiration (402, 416). Importantly, mutations in *aspB* led to a significant reduction in the virulence of *C. jejuni* in eukaryotic cells, along with an impaired host colonization (322). Our data revealing that many virulence genes were up-regulated in *C. jejuni* grown in acidic environment clearly suggest that acid stress could enhance *C. jejuni* host pathogenesis.

Finally, our data revealed the induced expression of Cj0448c in *C. jejuni* upon acid exposure. Cj0448c is a chemotaxis signal transduction protein, which is thought to sense a cytoplasmic acidification or a consequence of this acidification (373). Cj0448c was also up-regulated in *C. jejuni* in the chick cecum suggesting that Cj0448c could be important for *C. jejuni* host colonization (477). Importantly, mutation in genes encoding the chemotaxis signal transduction proteins in *C. jejuni* led to an acid-sensitive phenotype (374). The up-regulation of Cj0448c in *C. jejuni* under acidic conditions could help bacteria survive in acidic environment and improve its capacity to colonize the host.

Acid stress increases the expression of phosphate acquisition genes in *C. jejuni*. Our data indicate that genes involved in both phosphate uptake (*cj1194*) and storage (*cj0604*) were up-regulated in *C. jejuni* following acid exposure. Most bacterial pathogens such as *E. coli*, *C. jejuni* and *S. flexneri* are capable of synthesizing polyphosphate (Poly-P) from phosphate residues (221, 233, 366). Poly-P is a polymer of many phosphate residues which are linked by high-energy bonds (366). Our findings might indicate a role for poly-P accumulation in *C.*

jejuni survival under acidic conditions. Poly-P acts as both energy reservoir and ATP substitute in many bacteria (e.g., *E. coli* and *Salmonella* spp.), thereby enhances their capacity to survive under stress conditions (53, 221, 232, 238, 367, 405, 475). A mutant of *E. coli*, which is incapable of synthesizing poly-P, is also impaired for the capacity to survive various stressors such as osmotic, oxidative and heat stress (367). Similarly, poly-P is involved in *Campylobacter* stress survival and adaptation (95, 135). A *C. jejuni* mutant, which is deficient in poly-P accumulation, is markedly defective for the capacity to survive various stresses such as osmotic and acid stress (135). The up-regulation of phosphate acquisition genes could help *C. jejuni* survive acid stress. Poly-P could be an energy source for *C. jejuni* providing it with energy required for acid tolerance. Moreover, poly-P could be required for the formation of biofilm and VBNC cells in *C. jejuni* (95, 135), and consequently it could play a role in *C. jejuni* acid stress survival. In agreement with our results, the genes involved in phosphate uptake were up-regulated in *C. jejuni* (374), *S. oneidensis* (252) and *B. cepacia* (303) under acidic conditions.

Acid stress cross-protects *C. jejuni* against oxidative stress. The transcriptome of *C. jejuni* under acidic condition revealed the up-regulation of two genes involved in oxidative stress defense [ferritin; *cft* (462) and hemerythrin; *herB* (218)]. Based on this observation, we hypothesized that prior acid exposure could enhance *C. jejuni* capacity to survive oxidative stress. To test this hypothesis we assessed the capacities of acid-stressed and unstressed *C. jejuni* to survive H_2O_2. As anticipated, acid-stressed *C. jejuni* was significantly more resistant to H_2O_2 than unstressed bacteria. The roles of Cft and HerB in *C. jejuni* survival to oxidative stress have been previously demonstrated (218, 462). Wai *et al.* showed that a ferritin-deficient mutant of *C. jejuni* is more sensitive to killing by H_2O_2 as compared to the wild-

type strain (462). Ferritin (Cft) plays a significant role in storing cytosolic iron and avoiding cellular toxicity under iron- replete conditions (462). The intracellular iron concentration increases in the absence of ferritin, which in the presence of O_2 could generate oxidative stress via the Fenton reaction causing cell damage and death (57, 188, 462). Similar to ferritin, hemerythrins such as HerB are important for *C. jejuni* survival under high oxygen conditions (218). Hemerythrins help protect key iron-sulphur cluster enzymes such as pyruvate and 2-oxoglutarate:acceptor oxidoreductases (Por and Oor, respectively) from oxidative damage (218). Therefore, not surprisingly, the acid-stressed *C. jejuni*, in which both Cft and HerB were up-regulated, exhibited a higher H_2O_2 resistance as compared to unstressed bacteria. Of note, the catalase gene *katA*, which encodes the main enzyme responsible for H_2O_2 resistance in *C. jejuni*, was not identified as up-regulated by our microarray analysis. It is possible that *C. jejuni* exposure to our experimental conditions (pH 4 for 8 min) was not drastic enough to induce KatA expression. It is more likely that an induced KatA expression would have been seen in *C. jejuni* under higher acidic conditions or upon acid exposure for longer time as that has been shown in other studies (250, 373).

The improved capacity of acid-stressed *C. jejuni* to survive H_2O_2 might indicate that acid exposure could prepare the cells to tolerate additional stresses encountered later during their life. The contribution of acid exposure to the cross-protection against other stressors (e.g., heat and osmotic stress) has been observed in many bacteria such as *Salmonella* spp. (26, 261, 409, 485) and *E. coli* (347, 409). The enteric pathogen *C. jejuni* is exposed to various stress conditions including osmotic and bile stress either inside or outside the host (62, 121, 265, 426). Survival of *C. jejuni* to both osmotic and bile stress is critical for host pathogenesis (62, 265). Exposure to osmotic stress could cause physiological changes in

bacterial cells such as dehydration of the cytoplasm and consequently cell death (62, 217, 283, 410, 476). Similarly, bile salts can disaggregate the lipid bilayer of bacterial cell membrane and thereby serve as effective antimicrobial agents (153). Therefore, we tested the hypothesis that acid exposure could play a role in cross-protection of *C. jejuni* against other environmental stresses such as osmotic and bile stress. However, in contrast to oxidative stress, there was no difference between acid-stressed and unstressed *C. jejuni* in survival either osmotic or bile stress. These results are in agreement with our microarray data showing that the expression of genes involved in *C. jejuni* response to osmotic or bile stress was not induced by acid exposure. Indeed, the transcriptome of *C. jejuni* revealed that none of the genes involved in *C. jejuni* osmotic stress survival (e.g., *gltD*, *glnA* and *kpsM*) or bile survival (e.g., *cmeABC* and *cbrR*) were up-regulated upon acid exposure.

The role of acid stress in *C. jejuni* virulence in Caco-2 cells. The transcriptional profile of *C. jejuni* revealed that many virulence genes were up-regulated upon acid exposure. These genes include *cj0914c* (*ciaB*), *cj1062* (putative CinA-like protein) and *aspB* (aspartate aminotransferase). All these genes have been shown to be involved in *C. jejuni* host colonization and pathogenesis (143, 230, 322, 373, 374, 467, 495). Based on these findings, we hypothesized that prior acid exposure would enhance *C. jejuni* virulence phenotypes. Therefore, we characterized the contribution of acid stress to *C. jejuni* virulence in eukaryotic cells using the Caco-2 cell line. Caco-2 cells are derived from a human colon carcinoma that have the capacity to differentiate into microvillated monolayers, as in the normal intestinal epithelium (109, 386) and have been extensively used for the study of *Campylobacter* adherence and invasion (289, 390, 434). Interestingly, prior acid exposure significantly increased *C. jejuni*'s capacity to adhere to and invade Caco-2 cells. The

observed up-regulation of many virulence genes in acid-stressed *C. jejuni* could account for this phenotype.

A concurrent and recent study by Le *et al*. (250) demonstrated that prior acid exposure enhanced the invasion capacity of *C. jejuni* in intestinal epithelial cells grown in transwell model. However and in contrast to our finding, Le *et al*. showed that there was no significant effect of acid stress on the invasive capacity of *C. jejuni* when the epithelial cells were grown on flat-bottomed wells (250). Le *et al*. suggested that *C. jejuni* invades epithelial cells at the basolateral side, possibly after paracellular passage (250). The difference between our finding and that by Le *et al*. could be attributed to variations in bacterial strains and experimental design employed in each experiment. Le *at al*. (250) used *C. jejuni* NCTC11168 which is characterized by low invasion capacity (356), while in the present study we used *C. jejuni* 81-176 which was shown to be highly invasive (355). Moreover, Le *et al*. exposed *C. jejuni* NCTC11168 cells to acid stress at pH 5 (250) while in the present study, *C. jejuni* 81-176 was exposed to more acidic conditions (pH 4). Exposure of *C. jejuni* to acid stress at pH 4 is undoubtedly more stressful for the cells and could induce the expression of more virulence genes than acid exposure at pH 5. Finally, Le *et al*. (250) incubated *C. jejuni* in coculture with the intestinal cells for only 2 h, while in the present study, the incubation time was 3 h prior to the gentamicin protection assay. Longer incubation time provides a higher chance for *C. jejuni* to adhere to and invade the epithelial cells potentially explaining the enhanced capacity of *C. jejuni* to invade epithelial cells grown on flat-bottomed wells. Despite these differences, both studies clearly demonstrate that acid stress enhances *C. jejuni* virulence in human epithelial cells.

The capacity of *C. jejuni* to adhere to and invade human cells was shown to be important for host colonization and pathogenesis (408). Binding to epithelial cells prevents *C. jejuni* clearance from the intestine by peristalsis and fluid flow (231). Furthermore, cell invasion could enable *C. jejuni* to persist within the host, evade the immune response and damage host cells (88, 91, 391). It has been shown that acid stress triggers *C. jejuni* to become more motile and consequently increases its capacity to invade human cells (250). Altogether, our results suggest that the obligate transit of *C. jejuni* through the stomach acidity could prepare the bacteria for efficient colonization of the intestine potentially leading to pathogenesis.

Acid stress enhances *C. jejuni* pathogenesis in a *G. mellonella* infection model. As mentioned above, the transcriptional profile of *C. jejuni* under acidic conditions revealed the up-regulation of many virulence genes. Moreover, the adherence and invasion capacities of *C. jejuni* into human epithelial cells were significantly improved by acid exposure suggesting enhanced virulence. Therefore it was important to characterize the effect of acid stress on *C. jejuni* pathogenesis using a disease model. The aim of this study was to demonstrate how prior acid exposure could enhance the pathogenesis of *C. jejuni*. Avian colonization models are not suitable to identify *C. jejuni* virulence mechanisms involved in human infection as *C. jejuni* is commensal for avian hosts (195, 317). Moreover, the transit of *C. jejuni* through the stomachs of animal models that are routinely used for *C. jejuni in vivo* experiments such as the colostrum-deprived piglet would be highly challenging and inappropriate to test our hypothesis. *C. jejuni* would be obligated to face the gastric acidity that would not allow us to compare between acid-stressed and unstressed *C. jejuni* in terms of host pathogenesis. Therefore, these animal models would not be helpful in understanding the influence of acid stress on *C. jejuni* infectivity.

Several studies have reported that *G. mellonella* is susceptible to *C. jejuni* infection and provides a valuable model to elucidate *C. jejuni* virulence factors (69, 403). In contrast to traditional animal models for *C. jejuni* (e.g., chick colonization model), *G. mellonella* lacks a typical GIT, providing the advantage of escaping the obligate passage through the stomach acidity. Therefore, this insect model was more suitable for investigating the impact of acid exposure on *Campylobacter* pathogenesis than traditional animal models. Importantly, *G. mellonella* harbors phagocytic cells known as hemocytes (211, 247). Hemocytes phagocytose and kill pathogens using antimicrobial peptides and ROS generated during a respiratory burst (30, 247). Therefore, *G. mellonella* hemocytes have many characteristics in common with mammalian phagocytes (30, 247). *G. mellonella* has been employed as a model to evaluate the infectivity of many pathogens, such as *Aspergillus* spp. (375), *A. baumannii* (351), *B. cereus* (113), *C. albicans* (51), *C. neoformans* (311), *P. aeruginosa* (194), *Listeria* spp. (302), *S. pyogenes* (331), *B. mallei* (396), *B. cepacia* (399), *S. aureus* (352) and *F. tularensis* (9).

Interestingly, the acid-stressed *C. jejuni* was more virulent in *G. mellonella* larvae as compared to unstressed bacteria. Prior exposure of *C. jejuni* to acid greatly enhanced its capacity to kill *G. mellonella* larvae. Moreover, the LD_{50} of acid-stressed *C. jejuni* was markedly lower than that of unstressed bacteria. This phenotype could be explained by the up-regulation of *C. jejuni* virulence genes in response to acid exposure increasing the bacterial capacity to destroy larval tissues. In addition to the induced expression of virulence genes, the acid stimulon of *C. jejuni* also revealed the up-regulation of two genes involved in oxidative stress defense (*cft* and *herB*) (218, 462). The increased expression of both Cft and HerB in acid-stressed *C. jejuni* might help bacteria survive and resist killing by the ROS

generated in the larval hemocytes. In support of our results, a correlation has been observed between acid exposure and the increase in bacterial virulence in the host (26, 184, 370, 377). For example, the exposure of enterohemorrhagic *E. coli* to acid enhanced its adhesion to epithelial cells and the bacterial induction of host-cell apoptosis (184). Similarly, systems associated with the pathogenesis and expression of virulence genes in *Salmonella* sp. are highly induced in acidic conditions (5, 370, 377). Merrell *et al*. demonstrated a link between *V. cholerae*'s capacity to survive acid stress and intestinal colonization (288). Moreover, acid exposure modulates the expression of several virulence factors in *H. pylori*, suggesting a link between the acid response and pathogenesis in this gastric pathogen (55).

Altogether, our data indicate that the exposure of *C. jejuni* to acid results in the differential expression of genes involved in acid stress survival such as genes involved in cell surface composition, energy metabolism and electron transport. In addition, the repression of ribosomal genes in *C. jejuni* under acidic conditions could be a bacterial strategy to cope with stressful conditions. The down-regulation of ribosomal genes would allow bacteria to reallocate their energy and resources toward the production of proteins that are required for *C. jejuni* stress survival such as heat shock and oxidative stress defense proteins. Importantly, the up-regulation of some heat shock proteins and the improved survival of acid-stressed *C. jejuni* to oxidative stress as compared to unstressed cells suggest a link between *C. jejuni* acid stress response and its responses to other stresses. Interestingly, the induced expression of virulence genes in *C. jejuni* upon acid exposure indicates that acid stress plays a role in *C. jejuni* host pathogenesis. The acid exposure significantly enhanced the virulence potential of *C. jejuni* in both human epithelial cells and *G. melonella* larvae. It

is worth mentioning that the present study is the first attempt to characterize the influence of acid stress on *C. jejuni* pathogenesis using *G. mellonella* as an infection model.

In summary, our results indicate that acid stress plays a critical role in *C. jejuni* host pathogenesis. The inevitable passage of *C. jejuni* through the stomach acidity after being ingested with contaminated food or water not only improves its capacity to survive other stresses encountered later in the intestine, but also enhances its pathogenesis. This finding and in agreement with other studies (32, 262, 378, 392), indicates that acid decontamination of food might not be an appropriate method for food preservation. Based on our data, food pathogens including *C. jejuni* could benefit from acid treatment of food by improving their stress survival and pathogenesis in the hosts.

Chapter 3 : The ferric uptake regulator Fur plays an important role in *C. jejuni* acid survival and host pathogenesis

3.1. Introduction

Enteric pathogens have developed various strategies to sense and combat gastric acidity (26) (as described in detail in Chapter 1). Several studies have correlated the ferric uptake regulator Fur and enteric pathogens acid survival (124, 127, 154, 165, 327, 493). The ferric uptake regulator Fur (17 kDa) is an iron-binding transcriptional repressor (156). Under iron-enriched environments, Fur binds to Fe^{2+} and the Fur-Fe^{2+} complex binds to a DNA sequence known as the Fur-box in the promoters of target genes (16, 17, 90, 107). Fur binding to the target genes prevents the recruitment of RNA polymerase and thereby represses gene expression (16, 89).

Interestingly, Fur has been recently shown to be the first transcriptional regulator in *H. pylori* that is required for growth under acidic conditions (36). Likewise, a *fur* mutant of *E. coli* was impaired for acid survival and was unable to trigger an acid tolerance response (ATR) as observed in the wild-type strain (493). In addition, Fur was found to regulate the urease activity that is involved in the acid response of some strains of enterohemorrhagic *E. coli* (165). Similarly, Fur plays a role in the acid survival of both *S. flexneri* and *S. typhimurium* (124, 127, 154, 165, 327, 493). Fur represses the expression of the small regulatory RNA *ryhB*, that negatively regulates *evgA* and *ydeP*, which are involved in *Shigella* acid survival (327). The *fur* mutant of *S. typhimurium* was defective for acid survival and lacks the inducible pH homeostasis system that is associated with the ATR in wild-type bacteria (127).

In addition to its role in acid stress survival, Fur plays an important role in the survival of many bacteria, such as *E. coli* (175), *H. pylori* (29, 65, 329), *Y. pestis* (136), *S. aureus* (183)

and *P. aeruginosa* (161) in the presence of various environmental stresses (e.g., oxidative stress). Importantly, Fur regulates the expression of many oxidative stress defense genes (e.g., *sodB* and *katA*) in enteric pathogens such as *E. coli* and *C. jejuni* (58, 65, 337, 453). In addition to oxidative stress genes, Fur also regulates *hmp* which encodes a flavohemoglobin that protects *S. typhimurium* from nitric oxide stress (81). Therefore, it is suggested that Fur could help bacteria survive harsh conditions and thereby enhances bacterial pathogenesis in the host (65).

Interestingly, Fur plays an important role in the virulence of many pathogenic bacteria such as *V. cholerae*, *E. coli* and *H. pylori* (65). For example, Fur regulates hemolysin production in both *V. cholerae* and *E. coli* (130, 428); Fur regulates adhesion and shiga toxin production in enterohemorrhagic and uropathogenic strains of *E. coli* (60, 369); and Fur regulates genes involved in quorum sensing and toxin production in *P. aeruginosa* (325, 359). Therefore, not surprisingly, *fur* mutations result in severe defects in virulence for many enteric pathogens in animal models (65). For example, a Δ*fur* mutant of *H. pylori* was significantly less virulent in both Mongolian gerbil and murine models of infection (55, 134). Similarly, the *fur* mutants of both *S. aureus* and *L. monocytogenes* were attenuated in a murine infection model as compared to wild-type strains (183, 371).

As an enteric pathogen, the capacity to survive gastric acidity is a fundamental requirement for *C. jejuni* to colonize the host and cause disease (373). In *C. jejuni*, Fur regulates the expression of genes belonging to several functional groups including energy metabolism, iron acquisition, cell membrane biogenesis and oxidative stress defense (58, 180, 338). Importantly, the crystal structure of *C. jejuni* Fur (CjFur) has recently been characterized providing a greater understanding of its regulatory function (58). Four modes of Fur-

regulation of target genes have been identified in *C. jejuni*; apo- and holo-CjFur gene activation and repression (58). Moreover, characterizing the CjFur metal binding sites demonstrated that CjFur contains two occupied Zn^{2+}-binding sites (S1 and S3) in addition to the regulatory iron-binding S2 site per protomer (58).

In the present study, we aimed to characterize the role of Fur in *Campylobacter* acid survival. We studied the transcriptional profile of a *C. jejuni* Δ*fur* mutant under acidic conditions using a microarray based approach. We identified *C. jejuni* genes that were regulated by Fur and acid stress. The contribution of the iron-binding S2 site to *Campylobacter* acid survival was also determined. Moreover, we characterized the involvement of Fur in the acid-induced protection of *C. jejuni* against other stresses. Finally, the importance of Fur for *C. jejuni* virulence in human epithelial cells and host pathogenesis was investigated.

3.2. Materials and Methods

3.2.1. Bacterial strains and growth conditions

C. jejuni NCTC11168 was acquired from the National Collection of Type Cultures. The bacterial strains used in this study are listed in Appendix I. The growth of *C. jejuni* strains was conducted on MH agar plates and MH biphasic cultures. The cultures were incubated at 37°C under microaerophilic conditions (8% O_2, 4% H_2, 5% CO_2 and 83% N_2) in a MACS-VA500 workstation.

3.2.2. CjFurΔS2 site-directed mutagenesis

CjFurΔS2 site-directed mutagenesis was conducted by Sabina Sarvan (University of Ottawa) using the Quick Change Site-Directed Mutagenesis Kit (Stratagene). The *fur*ΔS2

mutant was constructed by mutating two histidine residues to alanines (His43Ala-His102Ala) using *pStrepSumofur* (58) as a template and primers H43A-For, H43A-Rev, H102A-For and H102A-Rev listed in Appendix II. The resulting mutants were confirmed by DNA sequencing (Centre de Recherche du CHUL [CHUQ], Québec, Canada).

3.2.3. Complementation of a *C. jejuni* Δ*fur* mutant with Cj*fur*ΔS2

To study the role of the iron-binding S2 site in acid sensing, we introduced the Cj*fur*ΔS2 gene construct into a previously constructed *C. jejuni* Δ*fur* mutant (338) using the pRR-Km plasmid (373) and following the methodology described previously (209). The Cj*fur*ΔS2 nucleotide sequence was PCR-amplified from a pCDF dual plasmid (Novagen®) containing the Cj*fur*ΔS2 mutated insert using the Phusion® Hot Start II High-Fidelity DNA polymerase (Thermo Scientific) and the primers JBCL-Fur4 and JBCL-Fur2R listed in Appendix II. The *fur*ΔS2 PCR product was directionally cloned into the pRR-Km plasmid using the In-Fusion PCR cloning kit (Clontech). The resulting construct was sequenced to confirm the absence of PCR-induced errors in the insert. This final construct was used to transform *C. jejuni* Δ*fur* mutant as previously described (314, 338, 373), and transformants were selected on MH agar plates containing both chloramphenicol (20 µg/mL) and kanamycin (10 µg/mL). Finally, the insertion of the Cj*fur*ΔS2 gene construct into the rRNA locus of *C. jejuni* Δ*fur* was confirmed by PCR analysis using the primers AR55 and JBCL-Fur2R listed in Appendix II and as described previously (373).

3.2.4. Acid survival assays

C. jejuni cells (wild-type and mutants) were grown to the logarithmic phase in biphasic MH medium. The acid survival assays were performed as described in Chapter 2.

Briefly, bacterial strains were exposed to pH 3 or pH 4 in MH-HCl medium. Samples were withdrawn at different time points following acid exposure. The bacterial survival was determined by counting viable cells after serial dilution in PBS pH 7.4 and plating on MH agar plates. The results of the acid survival are expressed as the percentage of survival as a function of the duration of acid exposure. The results are expressed as the means of three independent experiments ± standard error of the mean. An asterisk (*) indicates $P < 0.05$ using a two-way ANOVA followed by a Bonferroni multiple comparison test.

3.2.5. Total RNA extraction for real-time qRT-PCR and microarray

Wild-type and a Δ*fur* mutant of *C. jejuni* NCTC11168 were grown to the logarithmic phase in MH broth (pH 7.4) under microaerophilic conditions. Exposure of bacteria to acid stress was performed as described in Chapter 2. Bacteria were exposed to neutral (pH 7) or acidic conditions (pH 3 or 4 for 8 min) in HCl-adjusted MH broth. RNA integrity was preserved by adding a 1/10 volume of cold RNA degradation stop solution (31). Bacterial cells were collected by centrifugation (8,000 X *g*, 10 min, 4°C), and the cell pellet was resuspended in TE buffer. Total RNA was extracted from bacteria grown under neutral conditions (pH 7) as well as from the acid-stressed bacteria using a hot phenol-chloroform extraction method (438) as described previously in Chapter 2. RNA was treated twice with DNase I (Epicenter), and the absence of genomic DNA was confirmed by PCR. Final RNA quality and quantity were ascertained using BioRad's Experion RNA StdSens Analysis Kit.

3.2.6. Probe labeling, slide hybridization and microarray data analysis

The microarray hybridization and analysis were conducted as previously described (337, 338, 373) and in Chapter 2. Briefly, RNA samples from each control (*C. jejuni*

NCTC11168 wild-type) and test sample (Δ*fur* mutant) were converted to cDNA using Superscript II. cDNA labeling was accomplished by incorporating aminoallyl-dUTP in the reaction and the aminoallyl-labeled cDNA was coupled to either Cy3 or Cy5 dye. Differentially labeled probes from both the test and control samples were cohybridized to a *C. jejuni* microarray. Finally, the microarray slides were scanned using a laser-activated scanner (Scan-Array Gx, PerkinElmer). Microarray data were collected from three independent biological replicates with three technical replicates each for both the test and control samples. Finally, the ratio of channel 2 (Cy5) to channel 1 (Cy3) was converted to $\log_2$, and the data were analyzed using the Bayesian statistics (21).

3.2.7. Motility assay

The motility of *C. jejuni* wild-type and mutants (Δ*flgD*, Δ*flgE*, Δ*flgH*, Δ*flgK*, Δ*flgP*, Δ*flhB* and Δ*motAB*) was assayed on 0.4% MH agar plates as previously described (119, 143). Bacterial strains were cultured overnight in biphasic MH medium under microaerophilic conditions. Bacterial cultures were then diluted to an optical density of 0.02 at 600 nm. Ten µL of bacterial suspension was stabbed into a 0.4% MH agar plate and incubated at 37°C under microaerophilic conditions for 24 h. The motility of *C. jejuni* strains was characterized by an area of growth around the site of bacterial inoculation in the soft agar. Results are represented as the mean ± standard error of diameter (in mm) of bacterial migration from the site of inoculation for three biological experiments with three technical replicates each. Statistical analysis was performed using one-way ANOVA followed by a Bonferroni multiple comparison test and a P value < 0.05 was considered significant.

3.2.8. Validation of microarray results by qRT-PCR

The relative expression levels of four genes (*cj0448c*, *clpB*, *grpE* and *katA*) that were up-regulated in the Δ*fur* mutant relative to wild-type strain under acidic conditions and four genes (*cj0414*, *cj0415*, *flaB* and *metB*) that were down-regulated relative to the wild-type were analyzed by qRT-PCR. The relative expression level of each gene was normalized to *rpsL*, for which the expression levels remained unchanged in the microarray analysis between the Δ*fur* mutant and wild-type strain. Primers were designed using Primer3 software and are listed in Appendix II. Validation of the microarray data was conducted as described in Chapter 2, and the coefficient of determination (R^2) was determined as a measure of the degree of correlation between the microarray data and real-time qRT-PCR data.

3.2.9. Oxidative stress survival

The impact of acid stress on the oxidative stress survival of *C. jejuni* wild-type, Δ*fur* and Δ*katA* mutants was determined using disk inhibition analysis, as described in Chapter 2. Briefly, the disk inhibition assay was performed by measuring the diameters of the growth inhibition zones of both acid-stressed and unstressed *C. jejuni* strains around paper disks saturated with different molar concentrations of H_2O_2 solution. In addition, the capacity of both acid-stressed and unstressed *C. jejuni* wild-type to survive oxidative stress was assessed by performing a kill curve of bacteria against H_2O_2. The percentages of bacterial survival for both acid-stressed and unstressed *C. jejuni* were determined at different time intervals following the exposure to 10 mM H_2O_2 by serial dilution and plate counting. The results are shown as the means of three independent experiments ± standard error of the mean. The differences between the capacities of both acid-stressed and unstressed *C. jejuni* strains to survive H_2O_2 were considered significant at a P value < 0.05 using a Student unpaired *t*-test.

3.2.10. Quantitative RT-PCR

The relative expression levels of *katA* transcripts in both acid-stressed and unstressed *C. jejuni* NCTC11168 were determined as described previously (338), using the QuantiTect SYBR green RT-PCR kit (Qiagen) and a 7300 real-time PCR system (Applied Biosystems). The relative *katA* expression levels were normalized to *metC* (putative cystathionine beta-lyase), for which the expression levels remained unchanged in acid-stressed and unstressed *C. jejuni*. The extent of induction of *katA* expression was measured using the comparative threshold cycle ($\Delta\Delta C_T$) method by calculating the relative fold change of the *katA* transcript. Experiments were conducted in at least biological triplicate with the specificity of the PCR amplification confirmed by agarose gel electrophoresis as well as melt curve analysis of the PCR products according to the manufacturer's recommendations.

3.2.11. Western blot analysis

Five micrograms of protein lysates from acid-stressed and unstressed *C. jejuni* were separated by SDS-PAGE on a 12% denaturing gel. Proteins were immediately transferred from the gel to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were blocked by overnight incubation in 5% (wt/vol) skim milk and 0.1% Tween-20 in PBS at 4°C. Next, the membranes were incubated with 0.1 µg/mL anti-KatA antiserum (120) for 1 h followed by three washes with 0.1% Tween-20. Finally, the membranes were incubated for 1 h with a 1:3,000 dilution of anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (Invitrogen) followed by three washes with 0.1% Tween-20. The immunoblot membrane was developed with a 1:1 mixture of luminol-peroxide solution (Thermo Scientific) for 1 min, and chemiluminescence was detected by X-ray film (Thermo

Scientific). Densitometric analysis of the immunoblot results was performed using ImageJ 1.45s (http://imagej.nih.gov/ij/).

3.2.12. Electrophoretic mobility shift assays and calculation of dissociation constants (K_ds)

The binding of CjFur to the Cy5-labeled *katA* promoter region under different pH conditions (pH 7, 6.5 and 6) was characterized using an electrophoretic mobility shift assay (EMSA). The gel-shift assays were performed as previously described (58) using the JFC1584 and JFC1585 primers listed in Appendix II and purified recombinant CjFur provided by Sabina Sarvan (University of Ottawa). Forward and reverse Cy5-labeled primers corresponding to 40-bp DNA fragment of the *katA* promoter region were purchased from Eurofins MWG Operon. Oligonucleotides (10 µM each) were annealed by incubation at 95°C for 10 minutes and slowly cooled down to room temperature in 50 µL of annealing buffer (10 mM Tris pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT). For the gel shift assay, 700 nM purified recombinant CjFur was incubated in a final assay volume of 20 µL binding buffer (20 mM Bis-Tris borate, 50 mM KCl, 50 µM $MnCl_2$, 0.1% Triton X-100, 5% glycerol and 3 mM $MgCl_2$) for 30 min on ice. The binding buffer was adjusted to the desired pH using a saturated solution of boric acid. Next, the Cy5-*katA* fragment (1 nM) was added to CjFur and incubated for 30 min on ice in the presence of 1 µg poly dI-dC. The samples were run on a 6% wt/vol nondenaturing polyacrylamide gel (19:1) for 50 min at 100 V and 4°C. The gels were freshly prepared with 100 mM Bis-Tris borate and 100 µM $MnCl_2$, and were pre-electrophoresed at 150 V for 30 min at 4°C. As a control, the binding affinity between CjPerR and the *katA* promoter region under the same conditions was determined (purified recombinant CjPerR was also provided by Sabina Sarvan). The dissociation

constant (K_d) of Fur-*katA* binding under neutral or acidic conditions was determined as described previously (64, 269). The concentration of CjFur (in nM) required to reach half-maximal binding to the *katA* promoter was calculated using SigmaPlot software (www.sigmaplot.com/). The difference between dissociation constants of CjFur-*katA* binding under neutral or acidic conditions was considered statistically significant at $P < 0.05$ using a Student unpaired *t*-test. All gel shift assays were scanned using a Typhoon scanner (GE Healthcare; Typhoon Trio) and analyzed using ImageQuant TL (www.gelifesciences.com/iqtl).

3.2.13. Adhesion, invasion and intracellular survival of *C. jejuni* strains within HCT116 cells

Human colonic epithelial HCT116 cells were obtained from the American Type Culture Collection and were routinely maintained in MEMα (Invitrogen) supplemented with 10% FBS. Cells were grown and maintained without antibiotics at 37°C in a 5% CO_2 humidified atmosphere. The adherence, invasion and intracellular survival of wild-type, *fur* mutant and *fur*-complemented *C. jejuni* NCTC11168 strains within HCT116 cells were performed as described in Chapter 2. Briefly, suspensions of *C. jejuni* strains were inoculated separately into wells containing confluent monolayers of HCT116 cells at a targeted multiplicity of infection (MOI) of 1000:1. After incubation for 3 h, the bacteria-infected HCT116 cells were lysed with 0.1% Triton X-100. The total number of bacteria associated with the eukaryotic cells (intracellular and extracellular bacteria) was determined by serial dilutions of lysates in PBS and plating on MH agar plates. The bacterial invasion was determined by following a gentamicin protection protocol as previously described (228, 356). The number of adhering bacteria to HCT116 cells was determined by subtracting the

intracellular bacteria number from the total number of bacteria recovered from cells not treated with gentamicin. For intracellular survival, each bacterial suspension of *C. jejuni* strains was overlaid onto wells containing confluent monolayers of HCT116 cells at a targeted MOI of 1000:1. The numbers of intracellular bacteria at different time intervals were determined as described for the invasion assays and following the gentamicin protection protocol. For adhesion, invasion and intracellular survival assays, the results are expressed as the means of three independent biological experiments with at least three technical replicates each ± standard error of the mean. The difference between *C. jejuni* strains was considered significant at a P value < 0.05 using a two-ways ANOVA followed by a Bonferroni multiple comparison test.

3.2.14. *Galleria mellonella* larvae infection

The pathogenesis of *C. jejuni* strains (NCTC11168 wild-type, Δ*fur* and Δ*fur* + *fur*) was characterized using *in vivo G. mellonella* killing assays as described in Chapter 2. Briefly, larval survival and LD_{50} assays were determined for *C. jejuni* strains by injection in *G. mellonella*. Mortality and survival of *G. mellonella* larvae were monitored over 6 days following inoculation. The experiment was performed in at least three independent replicates. Survival curves of both the control and bacteria-infected larvae were plotted using the Kaplan-Meier method. The difference in larvae survival was determined using the log-rank test. The LD_{50} was calculated using the Probit method and differences in bacterial virulence were compared using the Mann-Whitney test. A value of $P < 0.05$ was considered statistically significant.

3.3. Results

3.3.1. *C. jejuni* Δ*fur* is more sensitive to acid than the wild-type strain

The ferric uptake regulator Fur has been shown to be involved in the acid survival of few enteric pathogens such as *S. flexneri* (327) and *Salmonella* spp. (127). However, the contribution of Fur to the acid survival of other enteric bacteria such as *Listeria* spp., *V. cholerae* as well as *C. jejuni* has not been previously characterized. *C. jejuni* lacks many stress regulators (e.g., RpoS) identified in other enteropathogens (37, 342, 345). Fur is involved, directly or indirectly, in the regulation of genes encoding proteins involved in the stress response of many bacteria such as *H. pylori* (36, 65). Therefore, and based on the absence of known stress regulators in *C. jejuni*, we hypothesize that Fur might play a role in the regulation of the acid response genes in this bacterium. To characterize the role of Fur in *C. jejuni* acid survival, we compared the capacities of the *C. jejuni* Δ*fur* mutant and the wild-type to survive acidic conditions.

As shown in Figure 3.1, the *C. jejuni* Δ*fur* mutant was significantly more sensitive to severe acidic conditions (pH 3) than the wild-type strain. However, no difference in acid sensitivity was observed between the Δ*fur* mutant and *C. jejuni* wild-type at pH 4 (Figure 3.2). Complementation with the *fur* gene restored the acid sensitivity of the Δ*fur* mutant (Figure 3.3), confirming that the acid sensitivity phenotype results mainly from the disruption of the *fur* gene. Next, to test the role of the S2 iron-binding site in acid sensitivity, we complemented the Δ*fur* mutant with the *fur*ΔS2 gene and tested the acid survival capacity of *C. jejuni fur*ΔS2 relative to the wild-type strain. As shown in Figure 3.3, the complementation with the *fur*ΔS2 gene restored the acid sensitivity of the Δ*fur* mutant indicating that the role of Fur in *C. jejuni* acid stress response is iron independent.

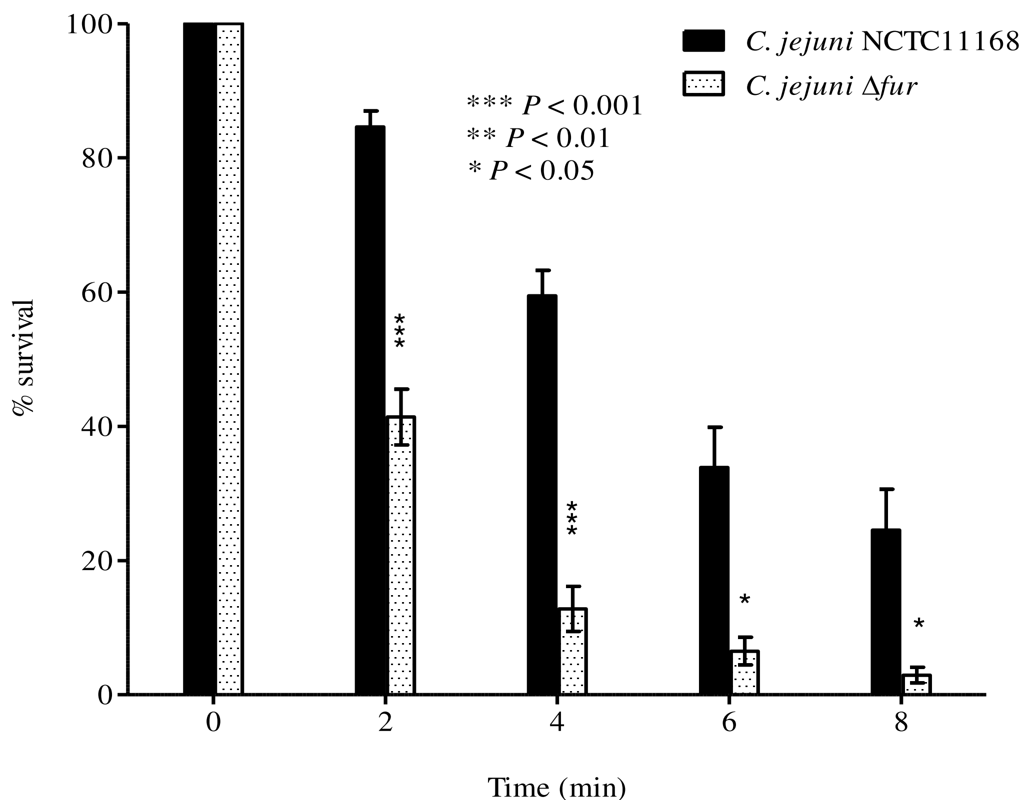


Figure 3.1: The *C. jejuni* Δ*fur* mutant is more acid sensitive relative to NCTC11168 wild-type. Bacteria grown to logarithmic phase in biphasic MH culture was exposed to acidic condition at pH 3. Samples were withdrawn immediately and 2, 4, 6 and 8 min after acid exposure to determine cell viability. Data from a minimum of three independent experiments are shown as the percentage of survival ± standard error of the mean. An asterisk (*) indicates $P < 0.05$ using a Student unpaired *t*-test.

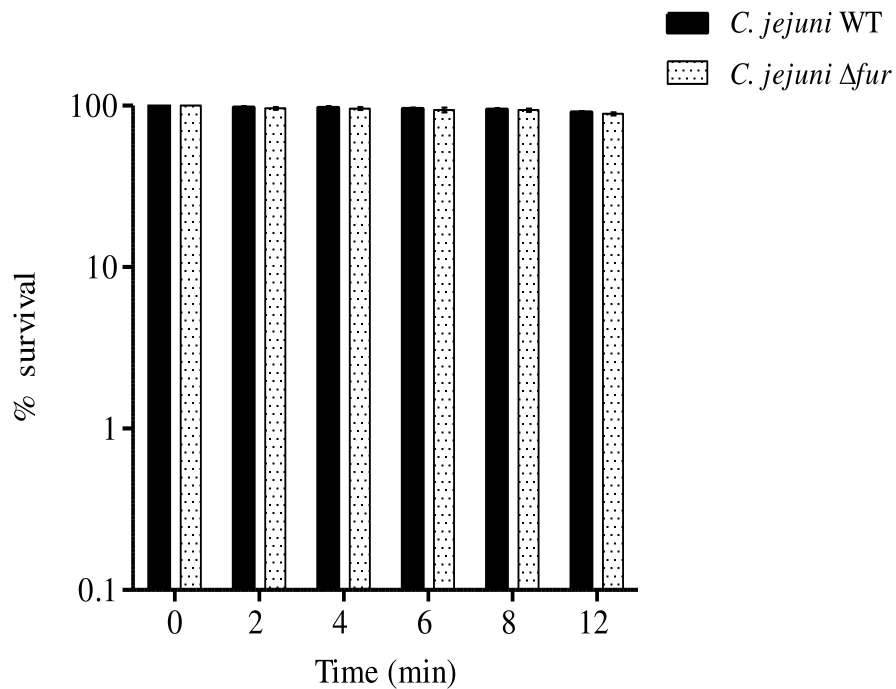


Figure 3.2: *C. jejuni* NCTC11168 wild-type and the Δ*fur* mutant survive acid stress at pH 4 for 12 min. Strains were grown to logarithmic phase in biphasic MH culture and were then exposed to acidic condition at pH 4. Samples were withdrawn immediately and 2, 4, 6, 8 and 12 min after acid exposure to determine cell viability. Data from a minimum of three independent experiments are shown as the percentage of survival ± standard error of the mean. Standard errors are present but are too small to be seen.

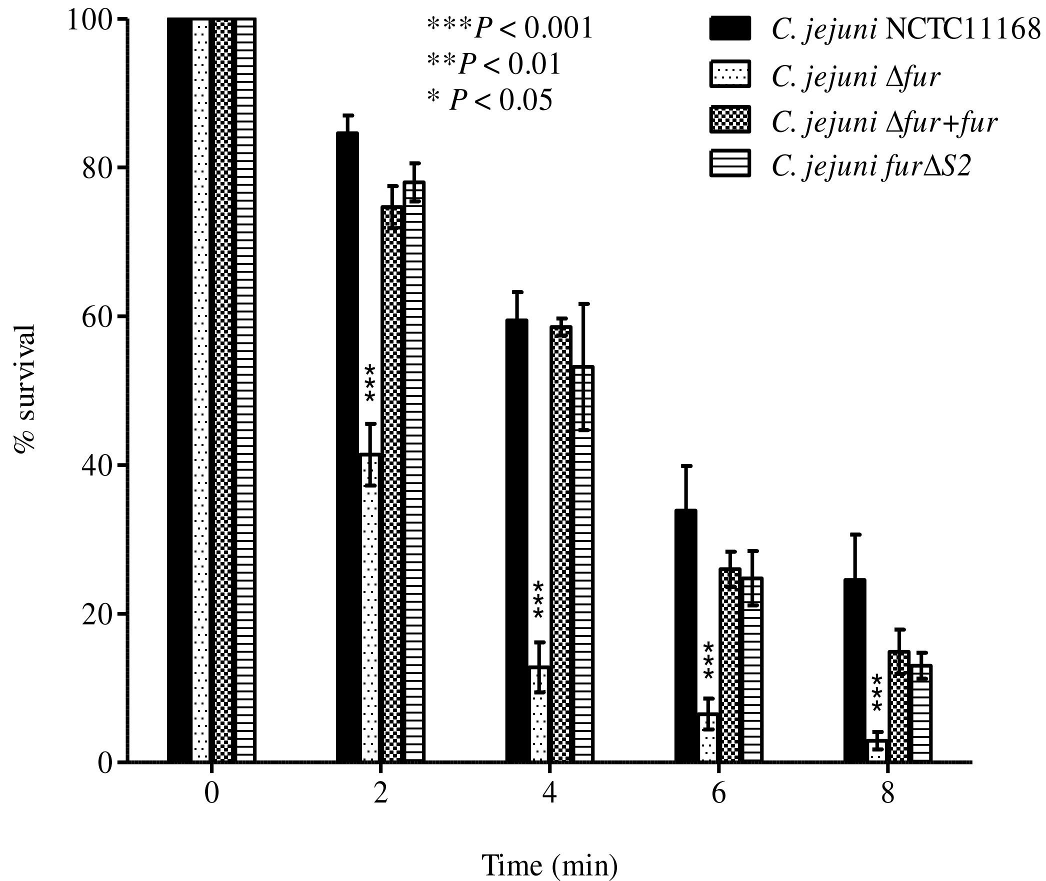


Figure 3.3: Complementation with the wild-type *fur* or the *fur*ΔS2 gene restores the acid sensitivity of the *C. jejuni* Δ*fur* mutant. Strains were grown to logarithmic phase in biphasic MH culture and were then exposed to acidic condition at pH 3. Samples were withdrawn immediately and 2, 4, 6 and 8 min after acid exposure to determine cell viability. Data from a minimum of three independent experiments are shown as the percentage of survival ± standard error of the mean. An asterisk (*) indicates $P < 0.05$ using a two-way ANOVA followed by a Bonferroni multiple comparison test.

3.3.2. Transcriptional profile of the *C. jejuni* Δ*fur* mutant at low pH

The acid survival assays showed that the *C. jejuni* Δ*fur* mutant was affected in the acid survival capacity as compared to the wild-type strain. Based on the fact that Fur functions as a transcriptional regulator, it was important to investigate the transcriptional profiles of both *C. jejuni* wild-type and the Δ*fur* mutant under low-pH conditions. This experiment revealed the *C. jejuni* genes that are regulated by Fur and that are differentially expressed under acidic conditions. The transcriptional profiles of the *C. jejuni* Δ*fur* mutant and wild-type were analyzed by selecting genes that were Fur-regulated and differentially expressed (≥ 1.5-fold change; $P < 10^{-4}$) in at least one of the two conditions (pH 3 and pH 4). A total of 141 genes were differentially expressed which were subjected to hierarchical clustering analysis (Figure 3.4) and were grouped into three major clusters (A, B and C; Appendix IV). Also, up- and down-regulated genes were grouped by functional category (Figure 3.5).

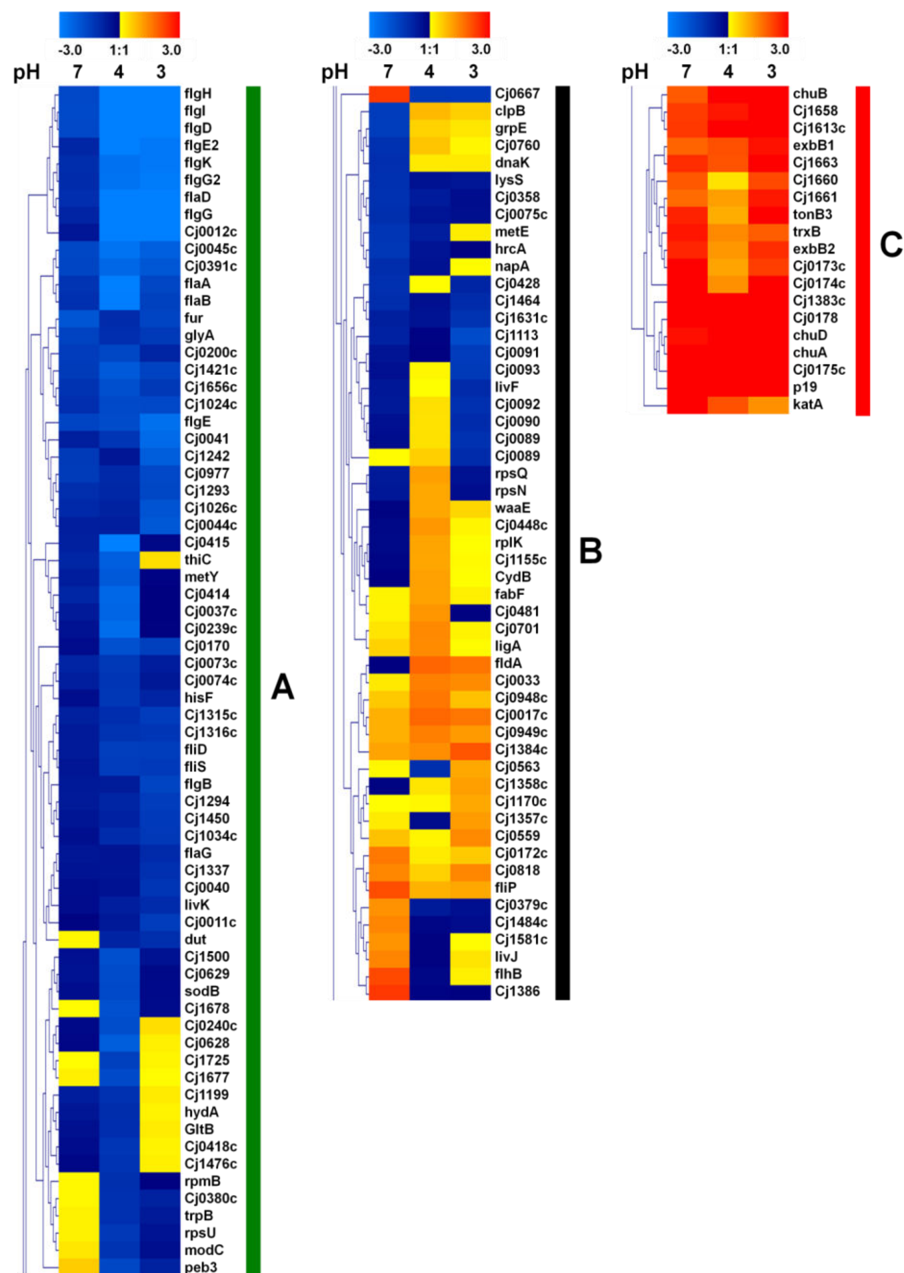


Figure 3.4: Hierarchical clustering of genes differentially expressed in the *C. jejuni* ∆*fur* mutant as compared to wild-type in response to neutral and acidic conditions. Bacterial cells were exposed to neutral pH 7 and acid stress (pH 4 or 3 for 8 min). Differentially expressed genes were subjected to hierarchical clustering using Genesis and were grouped into three major clusters A, B and C. The first column represents gene expression at pH 7. The second and third columns represent gene expression at pH 4 and 3, respectively. The red boxes represent the genes up-regulated in the *fur* mutant; the blue boxes represent the genes down-regulated in the *fur* mutant.

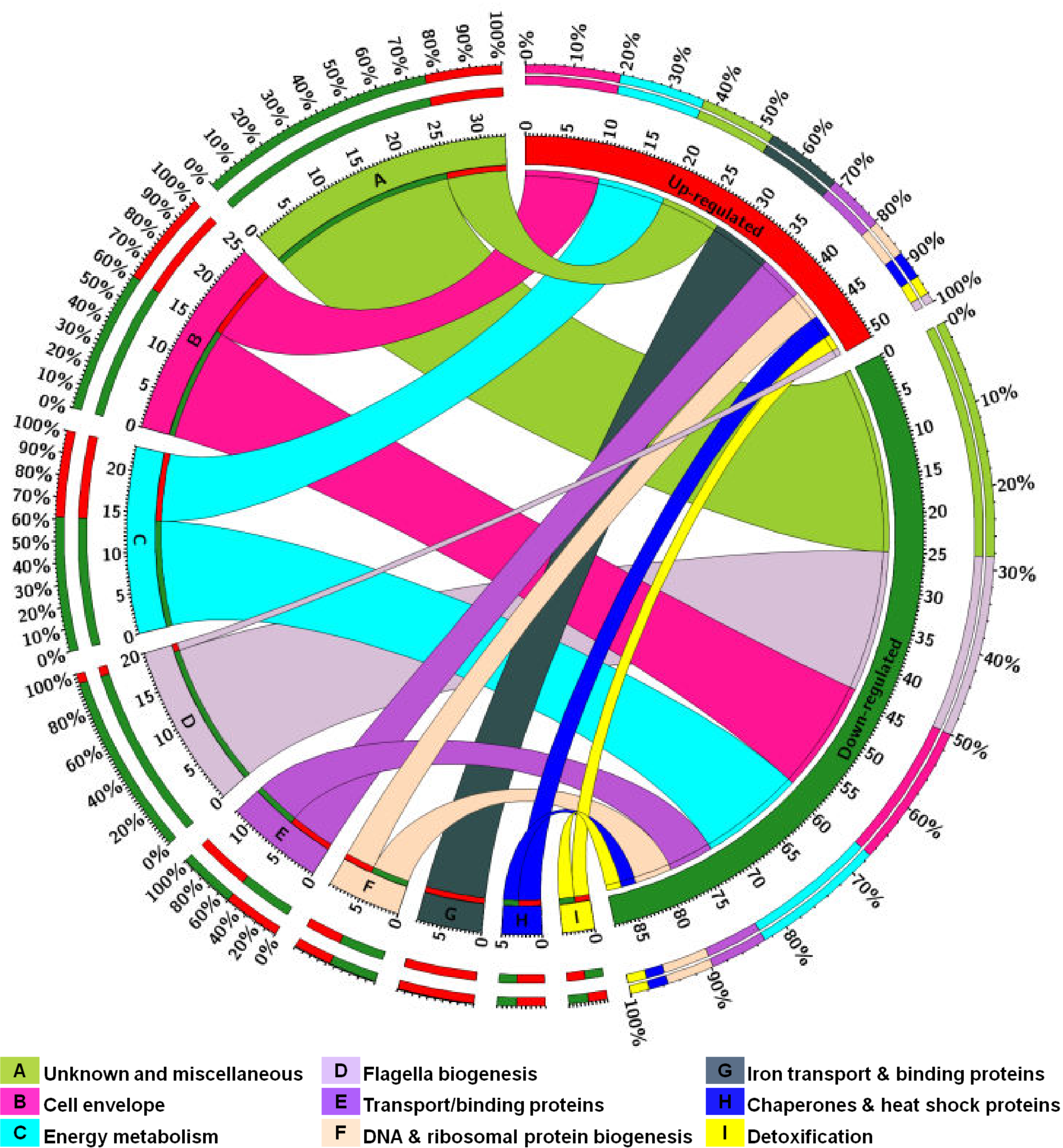


Figure 3.5: Functional categorization of Fur- and acid-responsive genes. The total numbers of up- and down-regulated genes are represented in red and green segments respectively. A colored segment (A-I) represents each functional category, and the ribbon size indicates the number of genes that are either up- or down-regulated within each category. The outermost colored segments represent the relative contribution of each functional category to the total number of up- and down-regulated genes. The figure was constructed using Circos Table Viewer version 0.54 (237).

Cluster A contains the genes that were down-regulated at pH 4 and mostly at pH 3 and/or pH 7. This cluster includes genes associated with bacterial motility and flagellar biogenesis (*flaABDG*, *flgBDEGHIKR*, *flgE2*, *flgPG2* and *fliKDS*), genes involved in cell membrane biogenesis (*cj0200c*, *cj0628*, *cj0629*, *cj1421c*, *cj1500*, *cj1677*, *cj1678*, *cj1725*, *pseABC*, and *peb3*), genes associated with the heat shock response (adenylosuccinate lyase; *cj1034c*), genes involved in amino acid metabolism, such as serine hydroxymethyltransferase (*glyA*), tryptophan synthase beta chain (*trpB*), and a putative O-acetylhomoserine (thiol) lyase (*metB*), as well as genes encoding for ribosomal proteins biosynthesis (*rpmB* and *rpsU*). Also, this cluster contains genes involved in energy metabolism, such as a putative cytochrome *c* (*cj0037c*), pyruvate-flavodoxin oxidoreductase (*cj1476c*), lactate dehydrogenase (*cj0073c* and *cj0074c*), genes involved in cell metabolism (*dut*, *hydA*, *gltB* and *thiC*) and genes encoding the gluconate dehydrogenase (*cj0414* and *cj0415*). This cluster also includes some genes involved in detoxification, such as superoxide dismutase (*sodB*), non-heme iron protein (*rrc*), genes involved in DNA repair and modification (*cj0011c*) and genes encoding transport and binding proteins (*cj0045c*, *livK* and *modC*). In addition to previously mentioned genes, this cluster includes some conserved hypothetical proteins (Cj0044c, Cj0239c and Cj0977), genes with unknown function (*cj0040*, *cj0170*, *cj0380c*, *cj0391c*, *cj0418c*, *cj1242*, *cj1450* and *cj1656c*) and genes with miscellaneous functions (*cj1199*, *cj1337*, *hisHF* and *iscS*).

Cluster B comprises genes for most of which expression was slightly up-regulated at acidic conditions (pH 3 and pH 4). Included in this cluster are genes involved in energy metabolism such as a flavodoxin (*fldA*), cytochrome oxidase (*cydB*), nitrate reductase (*napA*), lactate dehydrogenase (*cj0075c*), and putative periplasmic cytochrome *c* (*nrfAH*).

Also, this cluster contains genes involved in cell membrane biogenesis (*cj0033*, *cj0089*, *cj0090*, *cj0091*, *cj0092*, *cj0093*, *cj0818*, *cj1170c* and *waaE*), flagella biogenesis (*flhB* and *fliP*), signal transduction (*cj0448c*), genes encoding for transport and binding proteins (*cj1155c* and *cj1581c*), branched-chain amino acid ABC transport system (*livFJ*) as well as genes involved in ribosomal protein biogenesis (*rplK* and *rpsNQ*). This cluster includes some genes involved in detoxification, such as the putative cytochrome C551 peroxidase (*cj0358*), an ankyrin-containing protein (*cj1386*) that was recently shown to be involved in defense against oxidative stress in *C. jejuni* (120), and genes with either miscellaneous or unknown functions (*dapA*, *cj0172c*, *cj0379c*, *cj0428*, *cj0563*, *cj0559*, *cj0667*, *cj0949c*, *cj1384c*, *cj1464* and *cj1631c*). This cluster also contains genes encoding proteins involved in the heat shock response, namely a heat shock protein (*grpE*), a molecular chaperone (*dnaK*), the heat-inducible transcription repressor (*hrcA*) and an ATP-dependent Clp protease (*clpB*). In addition to previously mentioned genes, this cluster includes genes involved in the biosynthesis of macromolecules (*cj0701*, *dsbI*, *ligA* and *lysS*), amino acid biogenesis (*metE*), fatty acid biogenesis (*fabF*), a cation efflux protein (*cj0948c*) and genes encoding for conserved hypothetical proteins (*cj0760*, *cj1113* and *cj1484c*).

Cluster C contains genes that were up-regulated at both neutral (pH 7) and acidic conditions (pH 3 and 4). This cluster includes genes involved in iron metabolism and transport, such as the hemin uptake system (*chuABDZ*), a putative iron permease (*cj1658*), periplasmic protein (*p19*) and a putative iron-uptake ABC transport system (*cfbpABC*). In addition, this cluster contains genes involved in cell membrane biogenesis (*cj1660*), transport and binding proteins [*exbB1/B2*, *cj1661*, *cj1663*, *cj0178* (*ctuA*) and *tonB3*], and a gene with unknown function (*cj1383c*). Finally, this cluster contains some genes that are involved in

Campylobacter response to oxidative stress, such as catalase (*katA*) and thioredoxin reductase (*trxB*).

Comparison of the acid stimulon of *C. jejuni* Δ*fur* mutant with the CjFur regulons identified in previous studies. In addition to characterizing the acid stimulon of the *C. jejuni* Δ*fur* mutant, it was important to compare the Fur-regulated genes under acidic conditions with the previously characterized CjFur regulons. This should reveal the impact of acid exposure on the expression of the Fur-regulated genes in *C. jejuni*. The acid stimulon of the *C. jejuni* Δ*fur* mutant was compared to the CjFur regulons previously characterized by Palayda *et al*. (338), Holmes *et al*. (180) and to the CjFur-enriched genes that were identified by Butcher *et al*. using ChIP-chip analysis (58) (Figure 3.6 and Appendix V). Our data revealed that 141 genes were differentially expressed in the *fur* mutant in response to acid stress. However, 53 and 117 genes were differentially expressed in the *fur* mutant in response to iron as shown by Palayda *et al*. (338) and Holmes *et al*. (180), respectively, while 95 genomic loci bound by CjFur were identified by Butcher *et al*. using the ChIP-chip analysis (58). Only 25 and 37 genes out of the 141 genes of the *fur* mutant acid stimulon were members of the CjFur regulons in response to iron identified by Palayda *et al*. (338) and Holmes *et al*. (180), respectively. In addition, our data revealed that 18 acid-responsive genes identified in the *fur* mutant were members of CjFur targets by ChIP-chip analysis (58). Notably, 88 genes differentially expressed in the *fur* mutant under acid were not members of either the Fur regulons in response to iron or the CjFur targets (Figure 3.6 and Appendix V). Interestingly, many genes that were found to be only acid-responsive encode for proteins important for *C. jejuni* stress responses. For example, many of these genes encode for proteins involved in cell envelope and flagella biogenesis (e.g., *cj1170c* and *flhB*), heat shock

response (*cj1034c*, *grpE*, *clpB* and *hrcA*), signal transduction (*cj0448c* and *flgR*), detoxification (*cj0358* and *sodB*), as well as genes encoding for L-lactate dehydrogenase complex (*cj0075c-cj0073c*) which were found to be involved in *C. jejuni* acid response herein and elsewhere (250, 374). Importantly, only 5 genes were found to be common in all studies; our study and those by Palayda *et al*. (338), Holmes *et al*. (180) and Butcher *et al*. (58). Three of these genes are involved in iron metabolism [*chuA*, *chuB* and *cj0175c* (*cfbpA*)] and two genes are involved in oxidative stress response [*cj0012c* (*rrc*) and *trxB*]. Indeed, this comparison of the acid stimulon of the *C. jejuni* Δ*fur* mutant to the previously characterized CjFur regulons and CjFur targets revealed many genes which were only differentially expressed at low pH and therefore these genes may play a role in *C. jejuni* acid response.

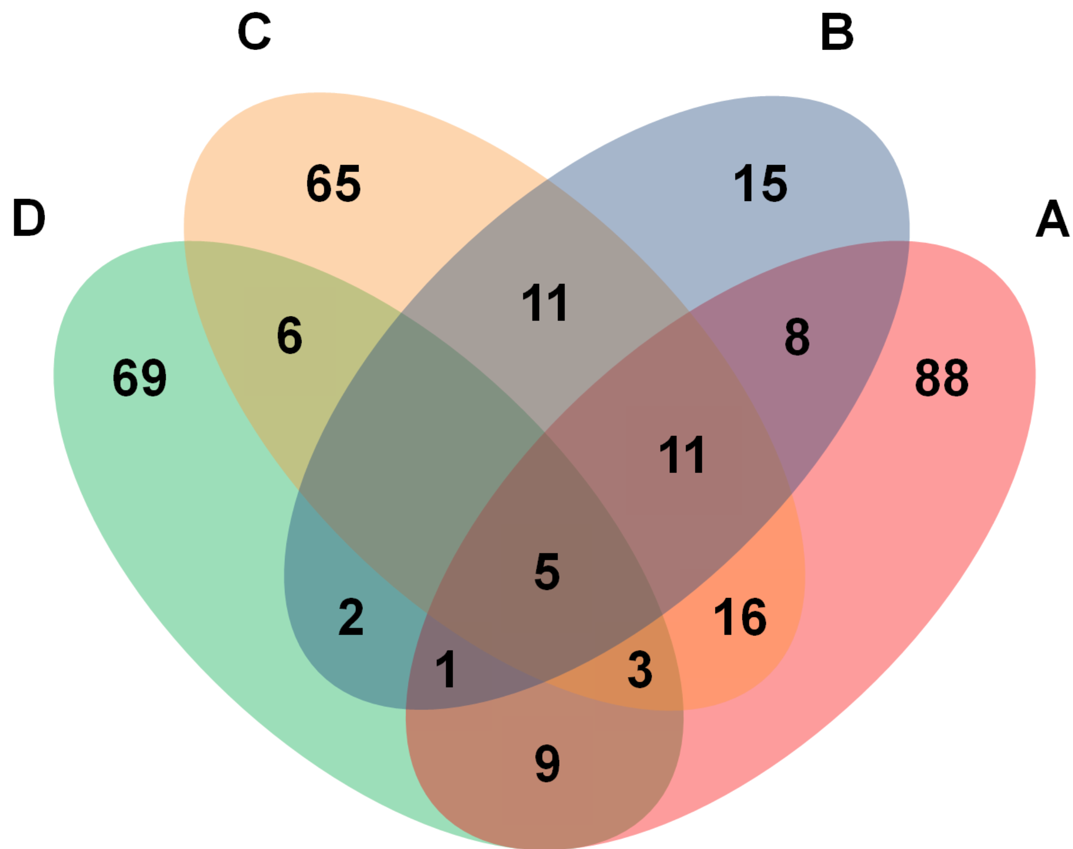


Figure 3.6: Venn diagram showing overlap among *C. jejuni* genes from the Fur regulons identified in the current study and previous studies. The numbers in the Venn diagram represent the number of genes. The acid stimulon of the *C. jejuni* Δ*fur* mutant (A) was compared to the CjFur regulons previously characterized by (B) Palayda *et al*. (338), (C) Holmes *et al*. (180) in response to iron as well as to (D) the CjFur target genes that were identified by Butcher *et al*. (58) using ChIP-chip analysis.

3.3.3. The role of differentially expressed genes in *Campylobacter* acid survival

To further characterize the contribution of differentially expressed genes that were identified by our microarray analysis to *C. jejuni* acid survival, we tested the impact of deletion mutations of some of those genes on the acid survival capacity of *C. jejuni*. The tested mutants included mutants into genes encoding for proteins involved in flagellar biogenesis and motility (Δ*flgD*, Δ*flgE,* Δ*flgH*, Δ*flgK*, Δ*flhB* and Δ*flgP*), cell membrane structure (Δ*cj0818*), oxidative stress response (Δ*rrc*) and signal transduction (Δ*flgR*). Moreover, the acid sensitivity of *C. jejuni* Δ*motAB* was compared to that of the wild-type strain to further determine the importance of motility for *C. jejuni* acid survival. Both *motA* and *motB* genes, which encode for the flagellar motor apparatus, are required for *C. jejuni* motility but not for flagellar biogenesis (289).

In contrast to Δ*flgE* mutant which was not sensitive to acid, deletion mutations of other genes (*rrc*, *cj0818*, *flgD*, *flgH*, *flgK*, *flgP*, *flgR*, *flhB* and *motAB*) significantly increased the acid sensitivity of *C. jejuni* (Figure 3.7). In addition, we characterized the influence of deletion mutation of flagellar genes on *C. jejuni* motility on soft agar. Interestingly, *C. jejuni* Δ*flgD,* Δ*flgH*, Δ*flgK*, Δ*flgP*, Δ*flgR*, Δ*flhB* and Δ*motAB* mutants, which were acid sensitive, were defective for bacterial motility as compared to the wild-type strain (Figure 3.8). Our data suggest that there is a link between *C. jejuni* motility and the bacterial capacity to survive acid stress.

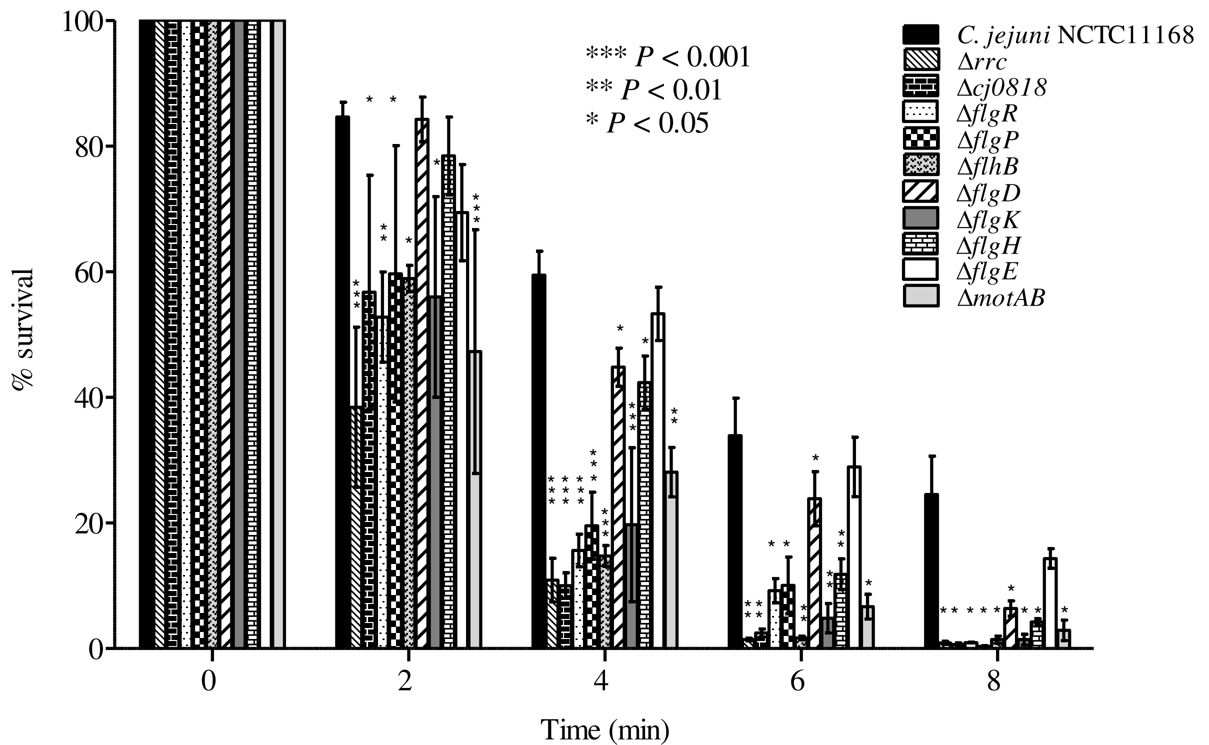


Figure 3.7: Acid survival of *C. jejuni* mutants relative to the wild-type strain. *C. jejuni* mutants (Δ*rrc*, Δ*cj0818*, Δ*flgR*, Δ*flgP*, Δ*flhB*, Δ*flgD*, Δ*flgK*, Δ*flgH* and Δ*motAB*) but not Δ*flgE* were more sensitive to acid as compared to the wild-type strain. *C. jejuni* NCTC11168 and mutants were grown to logarithmic phase in biphasic MH culture and were then exposed to acidic condition at pH 3.0. Samples were withdrawn immediately and 2, 4, 6 and 8 min after acid exposure to determine cell viability. Data from a minimum of three independent experiments are shown as the percentage of survival ± standard error of the mean. An asterisk (*) indicates $P < 0.05$ using a two-way ANOVA followed by a Bonferroni multiple comparison test.

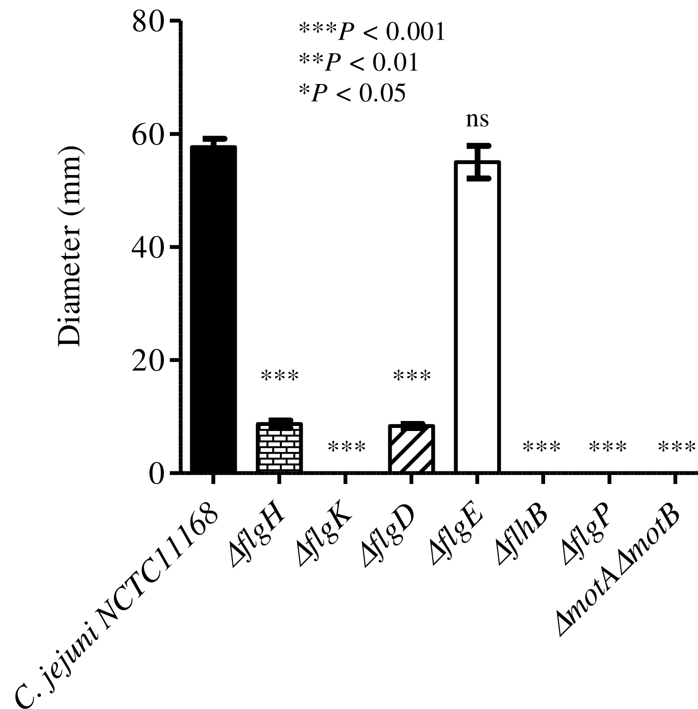


Figure 3.8: Motility assay of *C. jejuni* NCTC11168 and flagella mutants on soft MH agar. *C. jejuni* strains grown overnight in biphasic MH medium were diluted to an optical density of 0.02 at 600 nm. Ten µL of bacterial suspension was stabbed into a 0.4% MH agar plate and incubated at 37°C under microaerophilic conditions for 24 h. Results are represented as the means ± standard errors of diameter (in mm) of bacterial migration from the site of inoculation for three biological experiments with three technical replicates each. An asterisk (*) indicates $P < 0.05$ using one-way ANOVA followed by a Bonferroni multiple comparison test.

3.3.4. Validation of microarray results by qRT-PCR

The microarray data were validated using qRT-PCR for a subset of genes that was either up- or down-regulated by transcriptome profiling. This analysis was performed by plotting the log_2 ratio values of gene expression from the microarray experiment against the log_2 ratio of the relative quantity values obtained by qRT-PCR. A strong correlation (R^2 = 0.85) was identified between the data obtained from the microarray experiment and qRT-PCR (Figure 3.9).

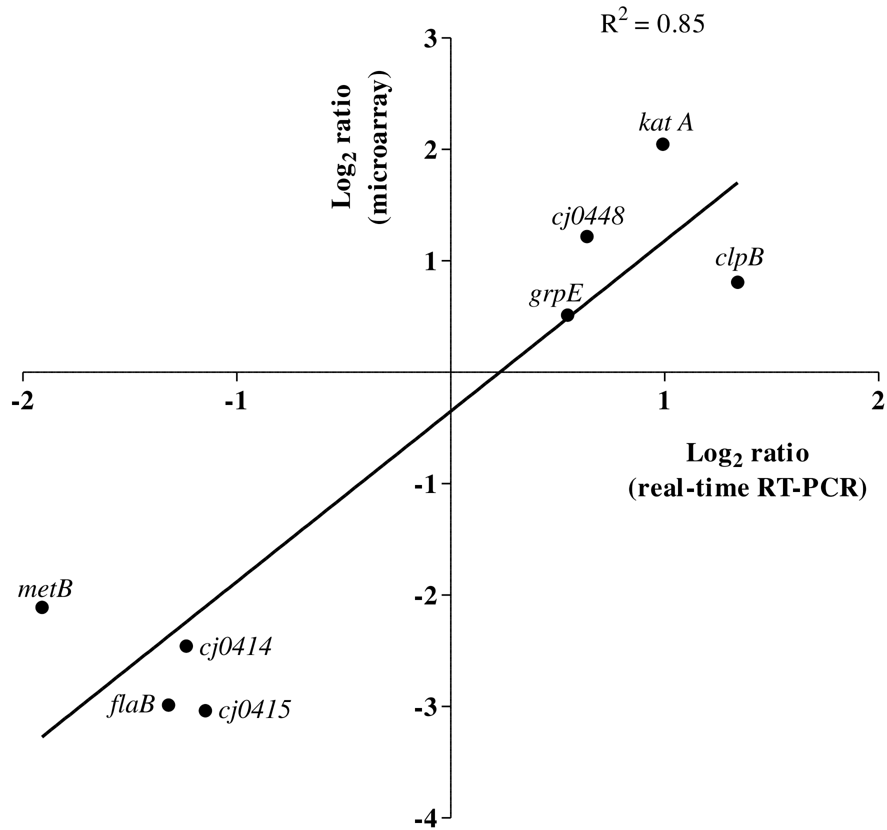


Figure 3.9: Validation of microarray results by qRT-PCR. The log_2 ratio values of gene expression levels measured by microarray analysis were compared to the log_2 relative quantity values obtained from qRT-PCR. A correlation coefficient (R^2) of 0.85 indicates a strong correlation between the data obtained from the microarray experiment and qRT-PCR.

3.3.5. Fur protects *C. jejuni* against oxidative stress upon acid exposure

The transcriptome analysis of the *C. jejuni* Δ*fur* mutant in acidic conditions revealed that the catalase gene *katA* was up-regulated in the Δ*fur* mutant under neutral conditions and to a lesser extent under acidic conditions (pH 4 and 3) (Appendix IV and Figure 3.4). This suggests that *katA* expression was induced in *C. jejuni* wild-type at low pH. This agrees with previous studies showing that *katA* is up-regulated in *C. jejuni* upon acid exposure (250, 373). Therefore, we compared between acid-stressed and unstressed *C. jejuni* NCTC11168 for their capacity to survive H_2O_2 stress. Interestingly, acid-stressed *C. jejuni* was more resistant to oxidative stress than unstressed bacteria, as indicated by a disk inhibition assay (Table 3.1). The results of the disk inhibition assay were further confirmed by performing kill curves for both acid-stressed and unstressed *C. jejuni* in response to H_2O_2 exposure. Consistent with the disk inhibition assay, acid-stressed *C. jejuni* survived oxidative stress better than unstressed bacteria (Figure 3.10).

It is well known that the catalase KatA is the main protein responsible for the detoxification of H_2O_2 in *C. jejuni* (88, 120). Given that the ferric uptake regulator Fur is involved in the regulation of *katA* expression (58, 88, 120, 337, 453, 456), we aimed to confirm that the enhanced survival of *C. jejuni* wild-type to H_2O_2 in acidic environments is mediated by Fur and KatA. Toward this end, we compared acid-stressed and unstressed *C. jejuni* mutants of *fur* and *katA* for their capacity to survive oxidative stress by H_2O_2. In contrast to the wild-type, no differences were identified between the acid-stressed or unstressed *C. jejuni* Δ*fur* or Δ*katA* mutants in their capacity to survive oxidative stress (Table 3.1). Our results clearly indicate that both Fur and KatA are essential for *C. jejuni* H_2O_2 survival under acidic conditions.

Table 3.1: Sensitivity of *C. jejuni* NCTC11168, a Δ*fur* mutant and a Δ*katA* mutant to H_2O_2 before and after exposure to acid stress

Strain	Diameter[a] (mm) of inhibition zone after exposure to different molar concentrations of H_2O_2		
	250 mM	500 mM	1000 mM
***C. jejuni* NCTC11168**			
Acid-stressed	12.11 ± 0.44**	18.22 ± 0.44*	22.44 ± 0.44*
Unstressed	15.00 ± 0.19	20.22 ± 0.11	24.00 ± 0.19
***C. jejuni* Δ*fur* mutant**			
Acid-stressed	12.22 ± 0.22	14.88 ± 0.11	17.99 ± 0.19
Unstressed	12.10 ± 0.29	14.55 ± 0.29	17.88 ± 0.22
***C. jejuni* Δ*katA* mutant**			
Acid-stressed	20.77 ± 0.22	26.44 ± 0.29	32.22 ± 0.11
Unstressed	20.66 ± 0.33	26.44 ± 0.22	32.00 ± 0.19

[a] The diameter of the inhibition zone is represented as the mean clear zone (in mm) ± standard error of three independent experiments for *C. jejuni* strains after exposure to different molar concentrations (250-1000 mM) of H_2O_2. *P* values < 0.05 (Student unpaired *t*-test) were considered significant (*).

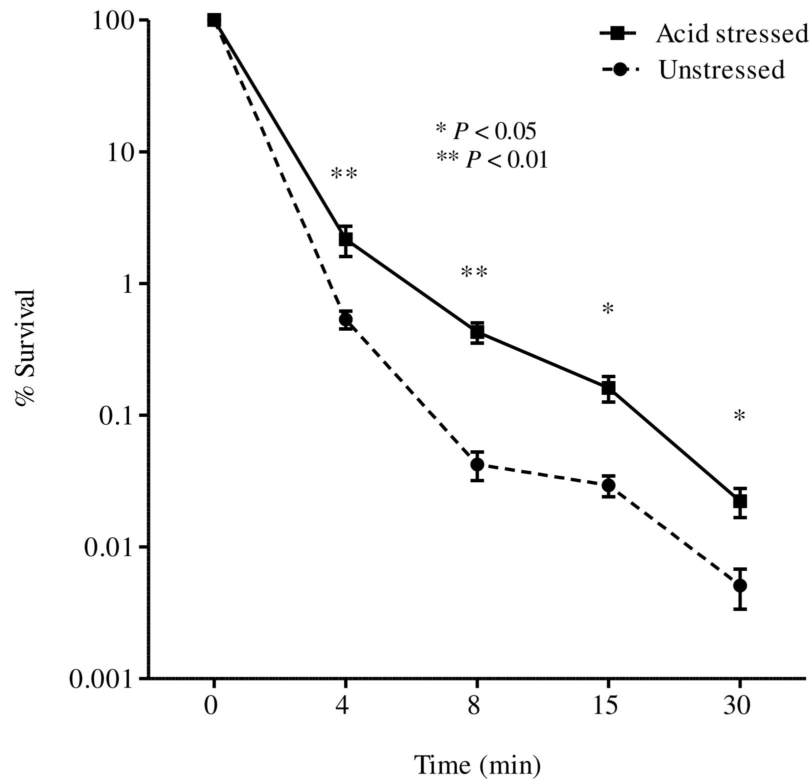


Figure 3.10: Survival of both acid-stressed and unstressed *C. jejuni* NCTC11168 in the presence of H_2O_2. Both acid-stressed and unstressed *C. jejuni* were exposed to 10 mM H_2O_2, and the percentages of bacterial survival were determined immediately and 4, 8, 15 and 30 min after H_2O_2 exposure by counting viable cells. The data shown are the means ± standard errors of three biological experiments with three technical replicates each. *P* values < 0.05 (Student unpaired *t*-test) were considered significant (*).

3.3.6. Western blot analysis

The survival of *C. jejuni* to oxidative stress was greatly enhanced upon acid exposure suggesting that the level of KatA protein was increased in the acid-stressed *C. jejuni* as compared to unstressed bacteria. Therefore, the KatA levels were quantified using Western blot analysis in both acid-stressed and unstressed *C. jejuni*. An anti-CmeB antibody was used to ensure equal loading of each protein lysate sample (Figure 3.11 B). As shown in panels A and C of Figure 3.11, the KatA levels were significantly higher in acid-stressed *C. jejuni* relative to unstressed bacteria (2.81 ± 0.63 fold increase, $P < 0.05$).

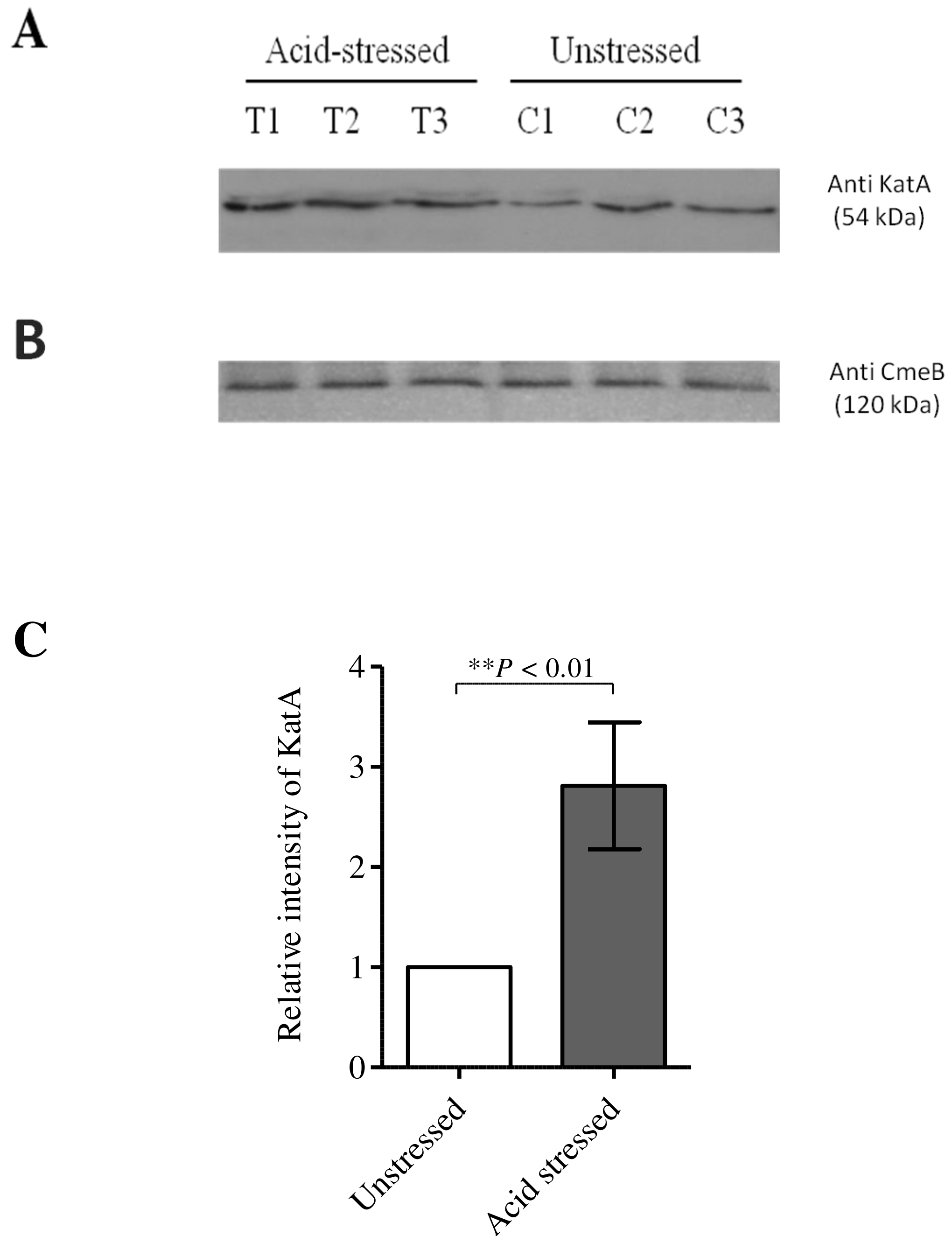


Figure 3.11: Quantification of KatA expression in both acid-stressed and unstressed *C. jejuni* NCTC11168 by Western blot analysis. KatA levels from three biological replicates of acid-stressed (T1, T2 and T3) and unstressed *C. jejuni* (C1, C2 and C3) were quantified by immunoblotting using anti-KatA antiserum (A) and anti-CmeB antibodies were used as a loading control for total protein content (B). (C) There was a significant increase of 2.81 ± 0.63 fold in the KatA levels in acid-stressed *C. jejuni* relative to unstressed bacteria as determined by the densitometry quantification of KatA levels. The error bars represent the standard errors of three biological replicates with three technical replicates each. An asterisk (*) indicates $P < 0.05$ using a Student unpaired t-test.

3.3.7. Quantitative RT-PCR

The microarray data suggest that *katA* expression is highly induced in *C. jejuni* in acidic environments. Moreover, the Western blot analysis indicates that KatA levels were significantly higher in acid-stressed *C. jejuni* relative to unstressed bacteria. We also characterized the relative expression levels of *katA* transcripts in both acid-stressed and unstressed *C. jejuni* using qRT-PCR. As expected and shown in Figure 3.12, there was a significant increase of 6.7 ± 0.4 fold in *katA* transcript abundance in acid-stressed *C. jejuni* as compared to unstressed *C. jejuni* (Student unpaired *t*-test, $P < 0.001$). The qRT-PCR findings further confirm the results of our Western blot analysis and demonstrate that prior acid exposure enhances *katA* expression in *C. jejuni*.

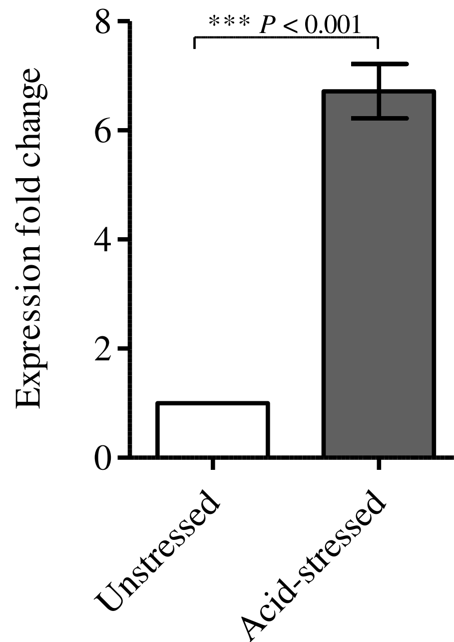


Figure 3.12: Quantification of *katA* expression in acid-stressed and unstressed *C. jejuni* NCTC11168 by qRT-PCR. qRT-PCR revealed that *KatA* expression increased 6.7-fold in acid-stressed *C. jejuni* compared with unstressed bacteria. The error bars represent the standard errors of three biological replicates. A value of $P < 0.001$ was considered significant using a Student unpaired *t*-test.

3.3.8. Alleviation Fur-repression of *katA* under acidic conditions

Our results demonstrating that *katA* expression was highly induced in acid-stressed *C. jejuni* suggest that acid exposure modulates the expression of *katA* in this bacterium. Therefore, it was important to characterize the effect of low pH on the regulation of *katA* expression by Fur. The influence of acid on CjFur binding to the promoter region of *katA* was determined using EMSAs. As shown in Figure 3.13 A, the binding affinity of CjFur to the *katA* promoter was higher at neutral pH 7 than under acidic conditions (pH 6.5 and pH 6). As a control, no difference in binding affinity between CjPerR and the *katA* promoter was detected under the same conditions (Figure 3.13 B). Moreover, the dissociation constant (K_d) of CjFur binding to the *katA* promoter was determined at both pH 7 and pH 6.5. EMSAs were performed in the presence of fixed amounts of Cy5-*katA* and increasing amounts of CjFur protein under both neutral and acidic conditions (Figure 3.13 C and D, respectively). Interestingly, in neutral conditions, CjFur binds to the *katA* promoter with a K_d of 293.73 ± 38.66 nM, which is significantly lower ($P < 0.01$, *t*-test) than the K_d value under acidic conditions (584.03 ± 33.82 nM). Our results clearly indicate that the binding between CjFur and the *katA* promoter is reduced under acidic conditions leading to release of *katA* repression.

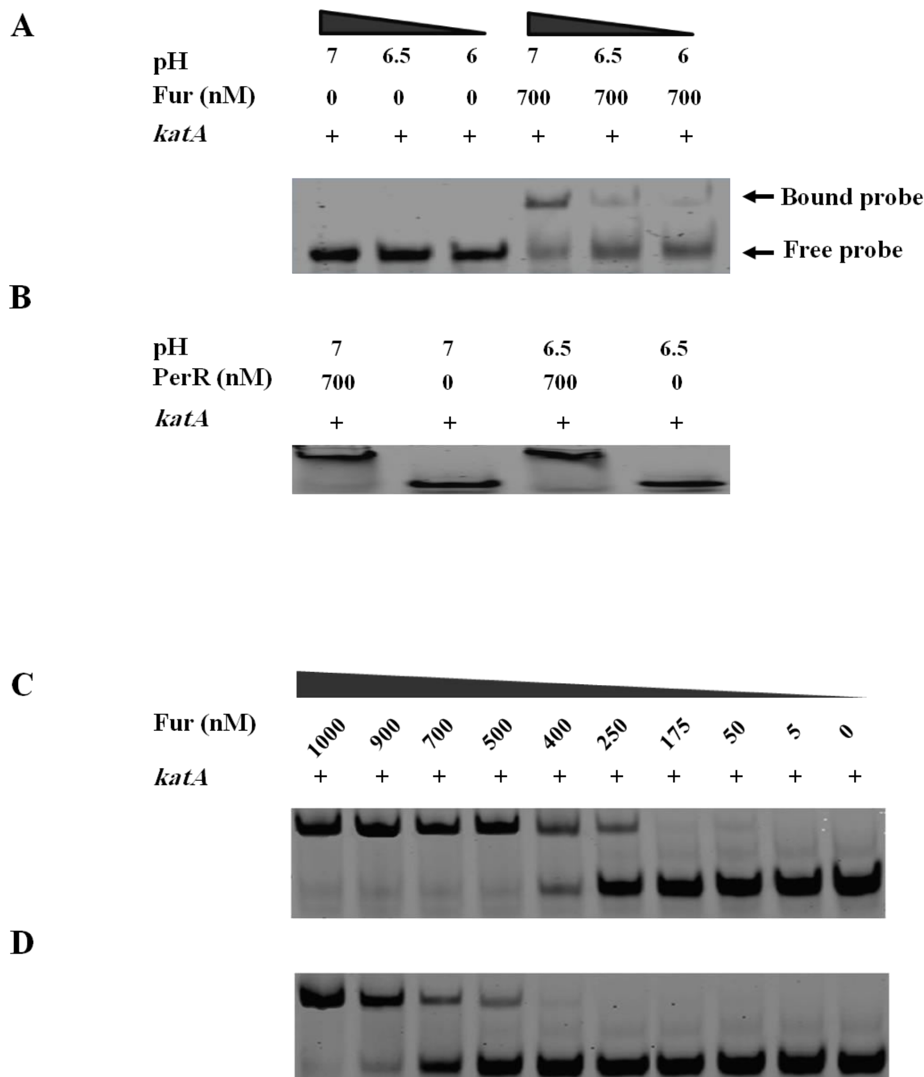
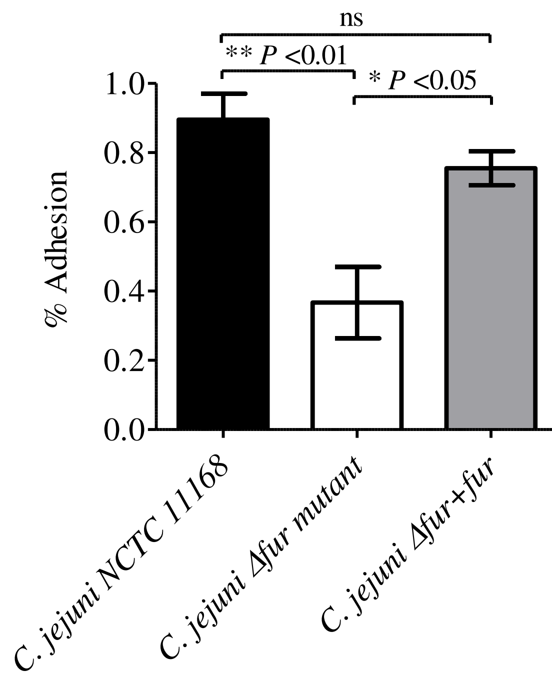


Figure 3.13: Electrophoretic mobility shift assays (EMSAs) of CjFur binding to Cy5-*katA* at different pH conditions. (A) The binding of CjFur (700 nM) to the promoter region of *katA* (1 nM) was determined under both neutral and acidic conditions. The capacity of CjFur to retard the electrophoretic mobility of Cy5-*katA* in 6% nondenaturing polyacrylamide gel was caharcterized at different pH values (pH 7, 6.5 and 6). CjFur binding to the *katA* promoter was higher at neutral pH 7, as indicated by enhanced retardation of *katA*, than under acidic conditions (pH 6.5 and pH 6). No shift of *katA* was observed in the lanes devoid of CjFur either under neutral or acidic conditions. (B) As a control, the binding between CjPerR (700 nM) and the *katA* promoter (1 nM) was examined under the same conditions employed above for CjFur-*katA* binding. The dissociation constant (K_d) of CjFur-*katA* binding was determined by performing EMSA reactions in the presence of fixed amounts of Cy5-*katA* and increasing the amounts of CjFur protein at both neutral (C) and acidic conditions (D). A significant difference in CjFur-*katA* binding was observed at neutral pH 7 (K_d = 293.73 ± 38.66 nM) and acidic pH 6.5 (K_d = 584.03 ± 33.82 nM) ($P < 0.01$, Student unpaired *t*-test).

3.3.9. Fur plays a role in *Campylobacter* adhesion to and invasion of HCT116 cells

The microarray data revealed that many genes involved in pathogenesis were differentially expressed in the *C. jejuni* Δ*fur* mutant upon acid exposure. These genes include genes involved in flagella biogenesis, energy metabolism, in addition to the iron acquisition genes. Therefore we hypothesized that acid exposure of *C. jejuni* could enhance its pathogenesis and Fur plays a significant role in this process by regulating the expression of many virulence genes. To characterize the contribution of Fur to *Campylobacter* pathogenesis, the capacity of a *fur* mutant to adhere to and invade eukaryotic cells was examined and compared to the wild-type strain. As shown in Figure 3.14, the *C. jejuni* Δ*fur* mutant exhibited a significantly reduced capacity to adhere to and invade HCT116 cells (0.37% ± 0.1 and 0.02% ± 0.002, respectively) relative to wild-type strain (0.89% ± 0.07 and 0.07% ± 0.01, respectively). Complementation of the Δ*fur* mutant with the *fur* gene (strain Δ*fur* + *fur*) restored the wild-type adhesion and invasion phenotypes. Our results indicate that Fur is important for *C. jejuni* adhesion to and invasion of human epithelial cells.

A



B

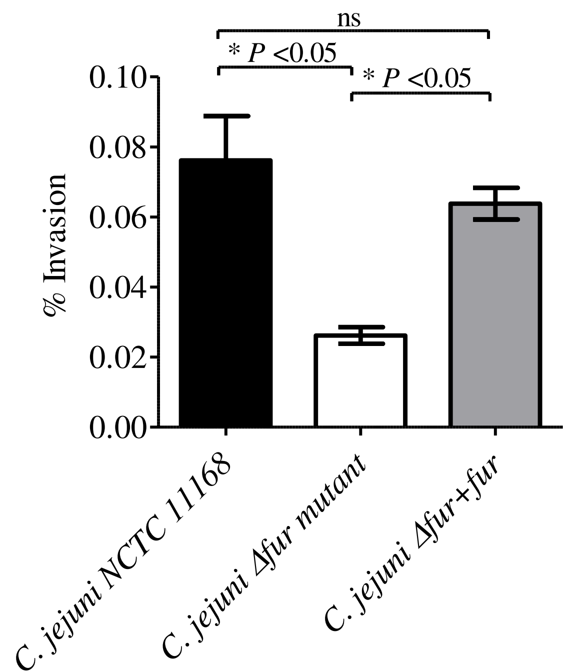


Figure 3.14: Role of Fur in *C. jejuni* adhesion to and invasion of HCT116 cells. The *C. jejuni* Δ*fur* mutant is affected in its capacity to (A) adhere to and (B) invade HCT116 cells relative to the wild-type strain. A monolayer of HCT116 cells was infected with wild-type, a Δ*fur* mutant or a Δ*fur* + *fur C. jejuni* strain at an MOI of 1000:1. The percentages of bacterial adherence to (A) and invasion of HCT116 cells (B) were determined by lysing the epithelial cells with 0.1% Triton X-100, serially diluting in PBS and plating on MH agar. The data shown are the means of three independent experiments ± standard error of the mean. A *P* value < 0.05 was considered significant using a one-way ANOVA followed by a Bonferroni multiple comparison test.

3.3.10. Fur is required for the intracellular survival of *C. jejuni*

As mentioned above, many genes important for bacterial pathogenesis were differentially expressed in the *C. jejuni* Δ*fur* mutant upon acid exposure. In addition, our results revealed that Fur plays a role in *C. jejuni* adhesion and invasion of epithelial cells. These findings suggest that Fur could be involved in *Campylobacter* intracellular survival especially following bacterial acid exposure. To understand the role of Fur in *Campylobacter* survival within eukaryotic cells, the *in vitro* intraepithelial cell survival was determined for *C. jejuni* wild-type and the *fur* mutant. Interestingly, wild-type *C. jejuni* exhibited a significant increase in its capacity to persist within HCT116 cells as compared to the *fur* mutant (Figure 3.15). Both *C. jejuni* wild-type and the *fur* mutant were characterized by an initial death phase (24 h post-infection), as the percentages of intracellular bacteria decreased from 5 x 10^{-2} % ± 9 x 10^{-3} to 2 x 10^{-2} % ± 4 X 10^{-3} and 2 x 10^{-2} % ± 2 x 10^{-3} to 0.1 x 10^{-2} % ± 0.2 x 10^{-3}, respectively. Forty-eight hours post-infection, both the wild-type strain and the *fur* mutant were able to multiply intracellularly, and their numbers increased to 5 x 10^{-2} % ± 0.01 and 0.1 x 10^{-2} % ± 0.2 x 10^{-3}, respectively. At 72 h post-infection, *C. jejuni* was not detected at higher numbers in infected epithelial HCT116 cells, and the percentages of intracellular bacteria for both the wild-type strain and the *fur* mutant were 4.5 x 10^{-2} % ± 1 x 10^{-2} and 0.1 x 10^{-2} % ± 0.2 x 10^{-3}, respectively. As expected, the defect in the capacity of the *fur* mutant to survive intracellularly was restored by complementation with the *fur* gene. Our results indicate that Fur is important for *C. jejuni* intracellular survival within human epithelial cells.

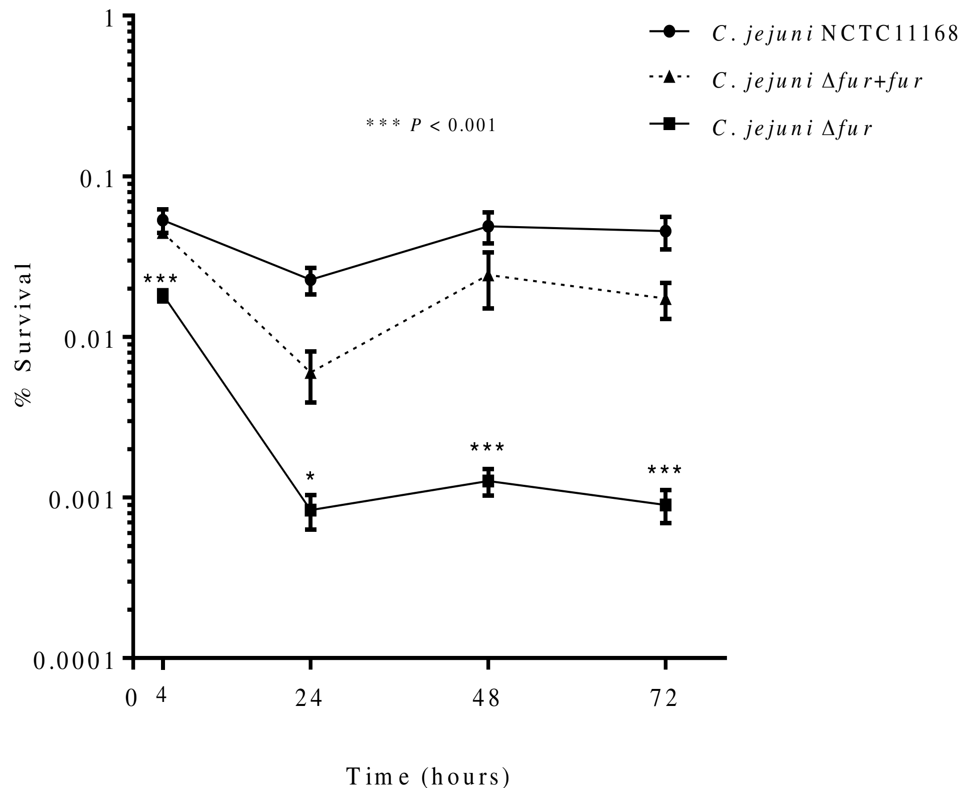


Figure 3.15: Role of Fur in the intracellular survival of *C. jejuni* within HCT116 cells. The *C. jejuni* Δ*fur* mutant is affected in its capacity to survive intracellularly in HCT116 cells relative to the wild-type strain. A monolayer of HCT116 cells was infected with *C. jejuni* wild-type, the Δ*fur* mutant or the Δ*fur* + *fur* strain at an MOI of 1000:1. The percentage of intracellular bacterial survival was determined over a 72-h period, as described for the invasion assay. The results are the means of three biological experiments ± standard errors. A P value < 0.05 was considered significant using a two-way ANOVA test followed by a Bonferroni multiple comparison test.

3.3.11. Fur is required for *C. jejuni* pathogenesis in *Galleria mellonella*

Importantly, our data revealed that Fur is important for *C. jejuni* virulence in eukaryotic cells. Therefore it was important to characterize the contribution of Fur for *C. jejuni* pathogenesis in a disease model. The contribution of Fur to *Campylobacter in vivo* pathogenesis was examined using *G. mellonella* as an infection model. In contrast to larvae infected with *C. jejuni* strains (wild-type, *fur* mutant or *fur*-complemented) that had distinct signs of invasive infection, no death was recorded for the control groups, either uninoculated or PBS-inoculated larvae. All *C. jejuni* strains caused dose-dependent killing in *Galleria* larvae (Figure 3.16 A-C). At the 10^6 CFU infection dose, wild-type *C. jejuni* killed significantly more waxworms than the *fur* mutant ($P = 0.01$) (Figure 3.16 D). The defect in the capacity of the *C. jejuni* Δ*fur* mutant to kill larvae was restored by complementation with the *fur* gene (Figure 3.16 D). Moreover, as shown in Figure 3.16 E, both *C. jejuni* wild-type and the Δ*fur* + *fur* strains had significantly lower LD_{50} values (3.1×10^5 and 4.6×10^5 CFU, respectively) relative to the Δ*fur* mutant (2.6×10^6 CFU). Finally, histopathological examination of *C. jejuni*-infected larvae revealed bacterial cells surrounded by larval hemocytes, coagulated hemolymph and melanin pigments (Figure 3.16 F). Our data demonstrate that Fur plays a significant role in *C. jejuni* pathogenesis.

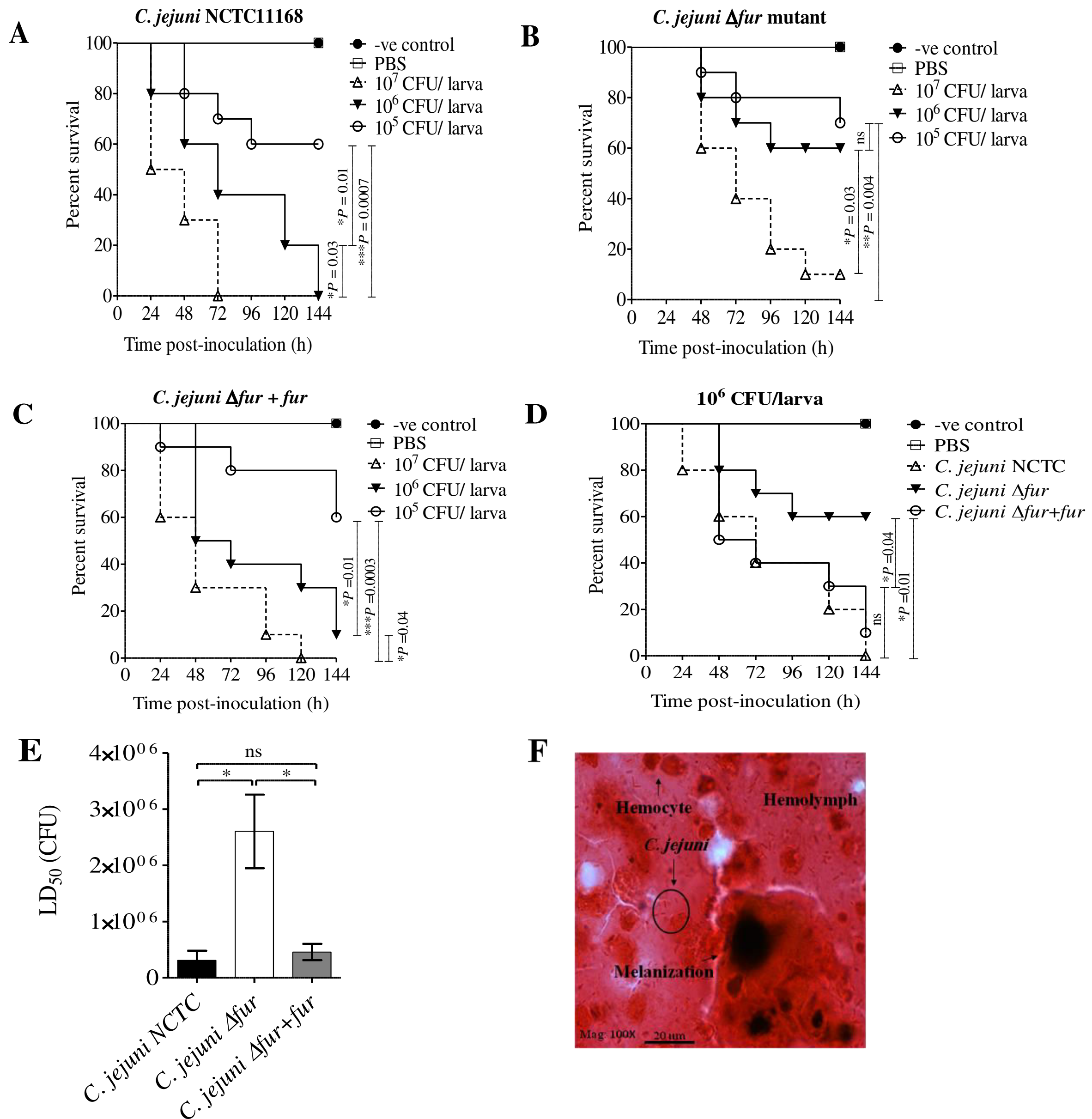


Figure 3.16: The ferric uptake regulator Fur is important for *C. jejuni* pathogenesis in *G. mellonella* larvae. *G. mellonella* larvae (n = 10 larvae/group) were inoculated with 10 µL of serially diluted (10^7, 10^6 and 10^5 CFU) wild-type, Δ*fur* mutant, or Δ*fur* + *fur* *C. jejuni* NCTC11168. Larval survival was monitored every 24 h for 6 days and was plotted using Kaplan-Meier survival curves. (A-C) Survival curves of *G. mellonella* larvae inoculated with different *C. jejuni* strains: (A) wild-type, (B) the Δ*fur* mutant or (C) the Δ*fur* + *fur* strain. (D) The *C. jejuni* wild-type and *fur*-complemented strains killed more larvae than the Δ*fur* mutant at the 10^6 CFU dose, (P = 0.007 and 0.04, respectively, log-rank test). (E) The LD_{50} was determined by Probit analysis. The *C. jejuni* Δ*fur* mutant has a significantly higher LD_{50} than either wild-type or *fur*-complemented strains; thus, this mutant is less virulent. A P value < 0.05 was considered significant using a Mann-Whitney rank-sum test. (F) Microscopic examination of *C. jejuni*-infected larvae revealed *C. jejuni* cells surrounded by larval hemocytes, hemolymph and melanin pigment (Gram staining, 100 X original magnification). Size bar, 20 µm. Magnification, mag.

3.4. Discussion

In addition to the classical role of Fur in the regulation of iron metabolism, Fur has been linked to the oxidative, nitrosative, and acid stress responses in several bacteria (84, 105, 106, 165, 180, 280, 327, 337, 338, 455, 493). Fur has been shown to regulate the expression of genes involved in acid survival in *S. typhimurium* in an iron-independent manner (127, 154). More recently, transcriptome analyses have revealed the importance of Fur in the regulation of pH homeostasis in *H. pylori* (36, 55, 134). Indeed, Fur was found to be required for *H. pylori* growth at low pH and up to 93 genes were found to exhibit altered expression in a Fur-deficient strain in response to acid shock (134). As mentioned before, many regulators (e.g. RpoS) which help enteric pathogens survive in the presence of various stresses are absent in *C. jejuni* (37, 342, 345). However, *C. jejuni* harbors a ferric uptake regulator Fur that has been shown to be involved in the regulation of iron metabolism and oxidative stress response genes (57, 58, 180, 337, 338, 453, 456). The lack of many stress response regulators in *C. jejuni* in addition to the importance of Fur in the acid survival of other bacteria let us hypothesize that Fur could be involved in *Campylobacter* acid survival. While previous studies focused on the role of Fur in iron metabolism (180, 337, 338, 456), its potential role in *C. jejuni*'s acid response has not been yet characterized. This study was conducted to define the role of Fur in the control of the acid stress response in *C. jejuni*. Moreover, we aimed to determine the importance of Fur in *C. jejuni* virulence and host pathogenesis.

Role of Fur in *C. jejuni* acid survival. A *C. jejuni* Δ*fur* mutant was found to be significantly affected in its ability to survive acid shock exposure suggesting a role for Fur as a regulator of the *C. jejuni* acid stress response. This observation was further confirmed by the

restoration of the acid sensitivity of the Δ*fur* mutant by complementation with the *fur* gene. Complementation of the Δ*fur* mutant with the *fur*ΔS2 gene restored the wild-type phenotype, suggesting that the iron-binding S2 site within CjFur has no role in *C. jejuni* acid survival. Similarly, Fur contributes to *S. typhimurium* acid survival in an iron-independent mechanism (124, 154). However, this mechanism contrasts with what has been observed in *S. flexneri*, where the iron-independent Fur regulation of acid-response genes was not evident (327).

Transcriptional profile of a Δ*fur* mutant and wild-type *C. jejuni* under acidic conditions. In other enteric bacteria, Fur is a known regulator of acid shock proteins (ASPs) which protect and repair the damages that occur in cellular components following acid exposure (124, 154, 404, 493). Therefore, it is tempting to speculate that the acid survival defect of the *C. jejuni* Δ*fur* mutant results from the absence of ASPs expression. To test this hypothesis and identify these ASPs, we compared the transcriptomes of the Δ*fur* mutant to the wild-type *C. jejuni* under acidic conditions using genome-wide microarrays. Notably, the changes in gene expression in both the Δ*fur* mutant and wild-type *C. jejuni* were not a result of cells dying at low pH; rather, they reflect the bacterial response to acid stress. Indeed, both the *C. jejuni* Δ*fur* mutant and the wild-type remained viable at pH 4 for 8 min. In contrast a significant decrease in cell viability occurred in both the Δ*fur* mutant and the wild-type strain at pH 3 which could result from bacteria entering into a VBNC state upon exposure to strong acidic conditions (35, 49, 198, 437). Nevertheless, the up-regulation of many genes at pH 3 and the similarities in gene expression between pH 3 and 4 indicate that *C. jejuni* remains transcriptionally active at pH 3 despite a decrease in cell viability. Our transcriptomic results indicate that flagellar biogenesis genes (*flaABDG, flgBDEGHIKMG2K2E2* and *fliKDS*) were repressed in the *C. jejuni* Δ*fur* mutant at low pH as compared to the wild-type strain. Flagella

can confer protection for bacteria against acid through the rapid movement from an acidic environment to a more suitable pH (287, 429). A previous study by Reid *et al*. (373) demonstrated that a decrease in pH signals the entry into the host, causing *C. jejuni* to highly express flagellar genes that are required to escape the stomach acidity and rapidly localize the bacterium to the protective mucus layer. Similarly, many flagellar genes were up-regulated in the closely related bacterium *H. pylori* upon exposure to acid (287, 469).

The role of the flagellar genes in *C. jejuni* acid resistance was clearly demonstrated by the phenotype of the mutants into the flagellar genes *flgD*, *flgH*, *flgK*, *flgR* or *flhB* which were significantly impaired in their capacity to survive acidic conditions. Importantly, the acid-sensitive flagellar mutants Δ*flgD*, Δ*flgH*, Δ*flgK* and Δ*flhB* were also defective for bacterial motility as compared to *C. jejuni* wild-type. In addition, *C. jejuni* Δ*flgR* mutant (*flgR* encodes the sigma-54 transcriptional activator FlgR) was found to be defective for motility as compared to the wild-type strain (119, 201). However, the Δ*flgE* mutant that was still motile was not sensitive to acid. These findings indicate that it is the bacterial motility and not the flagellum itself that plays a role in the acid survival of *C. jejuni*. To further investigate this finding, we characterized the capacity of Δ*flgP* and Δ*motAB* mutants to survive acidic conditions. FlgP and MotAB have been shown to be required for *C. jejuni* motility but not for flagellum biogenesis (289, 417). The *flgP* gene encodes an outer membrane lipoprotein, which is important for *Campylobacter* motility (289, 417). Both *motA* and *motB* genes encode for the flagellar motor apparatus which utilizes the transmembrane proton motive force (PMF) to drive flagellar rotation (289, 444). Testing the acid sensitivity of the Δ*flgP* and Δ*motAB* mutants would help us characterize the link between *Campylobacter* motility

and acid sensitivity. Interestingly, both Δ*flgP* and Δ*motAB* displayed significantly increased acid sensitivity as compared to *C. jejuni* wild-type.

The flagellar motor functions as a torque-generating unit and depends mainly on the PMF generated across the cell membrane to provide energy required for flagellar rotation (444). Flagellar mutation could disrupt the proton potential of the inner cell membrane and thereby interferes with the electron transport chain (ETC) (119). As mentioned before in Chapter 2, the electron transport chain plays an important role in *Campylobacter* acid survival. Proton translocation across the cytoplasmic membrane within electron transport pathways (98, 215, 354) could decrease the intracellular proton concentration and thereby reverses the cytoplasmic acidity (203, 374). In addition, it has been recently shown that flagellar mutation increases *C. jejuni* sensitivity to oxidative stress (119). *C. jejuni* flagellar mutants that were defective for motility, were more sensitive to oxidative stress as compared to the wild-type strain (119). Flint *et al*. demonstrated that disturbance of the ETC, which is the main source of endogenous ROS production (187), may increase the production of ROS through electron leakage (119, 309). Therefore, it is tempting to speculate that in addition to the acid-mediated cell damage, the accumulation of intracellular ROS within flagellar mutants could increase bacterial death in acidic environments.

Notably, some genes involved in cell membrane biogenesis (e. g., *cj0818*) were up-regulated in the *C. jejuni* Δ*fur* mutant under low pH. Cj0818 is a putative lipoprotein which is involved in *Campylobacter* cell membrane biogenesis (338, 345). To test the importance of cell membrane composition for *C. jejuni* acid survival, we compared the acid survival capacity of Δ*cj0818* mutant relative to the wild-type strain. Interestingly, the Δ*cj0818* mutant was more acid sensitive as compared to *C. jejuni* wild-type asserting the role of Cj0818 in acid survival

and consequently the importance of Fur in *C. jejuni* acid survival. The up-regulation of cell membrane biogenesis genes such as Cj0818 in the Δ*fur* mutant could decrease bacterial sensitivity to acid. The role of the membrane composition in bacterial resistance to various stresses has been well-characterized (373). Cell membrane proteins play a role in membrane integrity and rigidity and therefore enhance bacterial resistance to acid stress (52, 389).

Moreover, our results revealed the differential expression of five heat shock genes in the *C. jejuni* Δ*fur* mutant under acidic conditions. Two genes, the heat-inducible transcriptional repressor *hrcA* and the adenylosuccinate lysate (Cj1034c) were down-regulated while the expression levels of three genes, namely *grpE*, *clpB* and *dnaK* were up-regulated. Interestingly, Butcher *et al*. (58) demonstrated that *dnaK* is under direct regulation by Fur highlighting the importance of Fur in *Campylobacter* acid survival. The role of heat shock proteins in the protection of bacteria against acid stress has been previously described in Chapter 2 and by Reid *et al*. (373). It is most likely that the increased acid sensitivity of the *C. jejuni* Δ*fur* mutant triggers the activation of heat shock proteins to protect bacterial cells against acid stress. Heat shock proteins could repair damaged and misfolded intracellular proteins as a result of acid exposure and acidification of the cytoplasm (373, 439). Importantly, the repression of HrcA in the *fur* mutant at low pH could account for the induced expression of some heat shock genes observed herein. Previous studies demonstrated that HrcA is a known repressor of heat shock genes (179, 315). HrcA binds to the CIRCE (controlling inverted repeat of chaperone expression) element upstream of the regulated genes preventing their transcription (179, 315, 425). An inverted repeat sequence similar to CIRCE has been identified preceding the *dnaK* gene, suggesting that *dnaK* may be regulated by HrcA (179, 445). Stress conditions increase HrcA instability leading to the

dissociation of HrcA from the CIRCE element and an increased expression of the heat shock genes (174, 465). Thus, exposure of the *C. jejuni* Δ*fur* mutant to acid stress may repress HrcA and result in de-repression of heat shock genes to protect bacteria against acid-induced cell damage. In agreement with our findings, a number of heat shock proteins were up-regulated in *C. jejuni* following acid exposure (250, 373). Similarly, the heat shock protein ClpB in *S. aureus* and the periplasmic chaperones *hdeA* and *hdeB* in *E. coli* were up-regulated in response to acid (48, 133, 162, 181, 219, 281, 452).

Interestingly, our data indicate that KatA expression was significantly higher in the acid-stressed *C. jejuni* relative to unstressed bacteria as revealed by the Western blot and qRT-PCR analyses. The up-regulation of KatA in the acid-stressed *C. jejuni* suggests that acid exposure induces catalase expression which agrees with previous studies (250, 337). Moreover, the enhanced H_2O_2 survival by *C. jejuni* wild-type following acid exposure, a finding that was not seen in either the Δ*fur* or Δ*katA* mutant, suggests that both Fur and KatA are essential for the acid-improved *C. jejuni* H_2O_2 resistance. These results indicate that Fur plays an important role in the acid-induced cross-protection of *C. jejuni* against oxidative stress. The expression of the catalase *katA* is primarily regulated by the peroxide response regulator PerR (337, 453). PerR senses H_2O_2 by iron-catalyzed oxidation of histidine residues which coordinate Fe^{+2} in the regulatory binding site leading to Fe^{2+} release and the subsequent de-repression of *katA* (256, 337). In addition to PerR, the ferric uptake regulator Fur has been shown to be involved in the direct regulation of *katA* (58, 337, 453). Iron solubility increases under acidic conditions to a limit that could negatively affect bacterial growth by promoting the generation of damaging hydroxyl radicals through the Fenton's reaction (36, 337). Therefore, the acid induction of KatA expression might enable *C. jejuni*

to cope with iron-mediated oxidative stress at low pH. While a previous study revealed that *perR* was highly up-regulated in *C. jejuni* under acid (pH 5.5), the expression of two PerR-regulated genes, *katA* and *ahpC*, remained unchanged (374). It was suggested that PerR might be post-transcriptionally controlled under low pH and unable to regulate gene expression (374). Therefore, we hypothesized that, under acidic conditions, the acid-induced expression of *katA* in *C. jejuni* is mediated mainly by Fur. In addition, we proposed that the Fur regulation of *katA* can be modulated at low pH. In order to test this hypothesis, we compared the binding affinities of CjFur to the promoter region of *katA* under both neutral and acidic conditions using EMSAs. Interestingly, the binding affinity of CjFur to the *katA* promoter was significantly reduced at acidic pH, with a higher dissociation constant (K_d) as compared to neutral conditions. These results indicate that the Fur-mediated repression of *katA* could be released in acidic environment. Acid exposure of *C. jejuni* can alleviate Fur-repression of *katA* enabling over expression of catalase to defend against oxidative stress under acidic conditions.

It has been previously demonstrated that the Fur protein exists mainly as a dimer in solution at neutral conditions (291, 316). D'Autreaux *et al*. characterized the different oligomeric forms of the Fur protein as a function of pH by size exclusion chromatography and mass spectrometry analysis (83). In contrast to neutral conditions where Fur is found as a dimer, oligomerization of Fur was enhanced when the pH of solution was decreased below 7.0 (83). These results are in agreement with other studies showing that Fur protein is able to oligomerize in a pH-dependent manner (130, 249). The increased tendency of Fur to form higher-order oligomers under acidic condition could be attributed to the protonation effect (83). The increased protonation level in acidic environment may neutralize the repulsive

charges on Fur proteins or lead to additional attractive forces thus enhancing Fur oligomerization (83). Fur is rich in histidine residues (79, 80, 343, 395, 413) which are thought to be involved in the oligomerization process as they have a p*K*a of around 6.5 (83).

The reduced binding affinity of CjFur to the *katA* promoter at acidic pH as compared to neutral conditions could be related to interference with the characteristic dimeric state of CjFur at low pH. It is more likely that CjFur oligomerization is enhanced under low pH to form higher-order oligmers (e.g., tetramer and hexamer). These oligomers were found to be defective for DNA binding capacity as compared to the dimer form of Fur protein (83). Therefore, the binding between CjFur and the *katA* promoter would be decreased under acidic conditions. As shown in figure 3.17, the pH-induced oligomerization may explain how Fur plays a role in the acid-mediated cross-protection of *C. jejuni* against oxidative stress. Oligmerization of CjFur under low pH could enable *C. jejuni* to tune the Fur-regulation of *katA* upon acid exposure. Dissociation of CjFur from the *katA* promoter would enhance *katA* transcription by the RNA polymerase and consequently elevates *katA* expression to defend against oxidative stress.

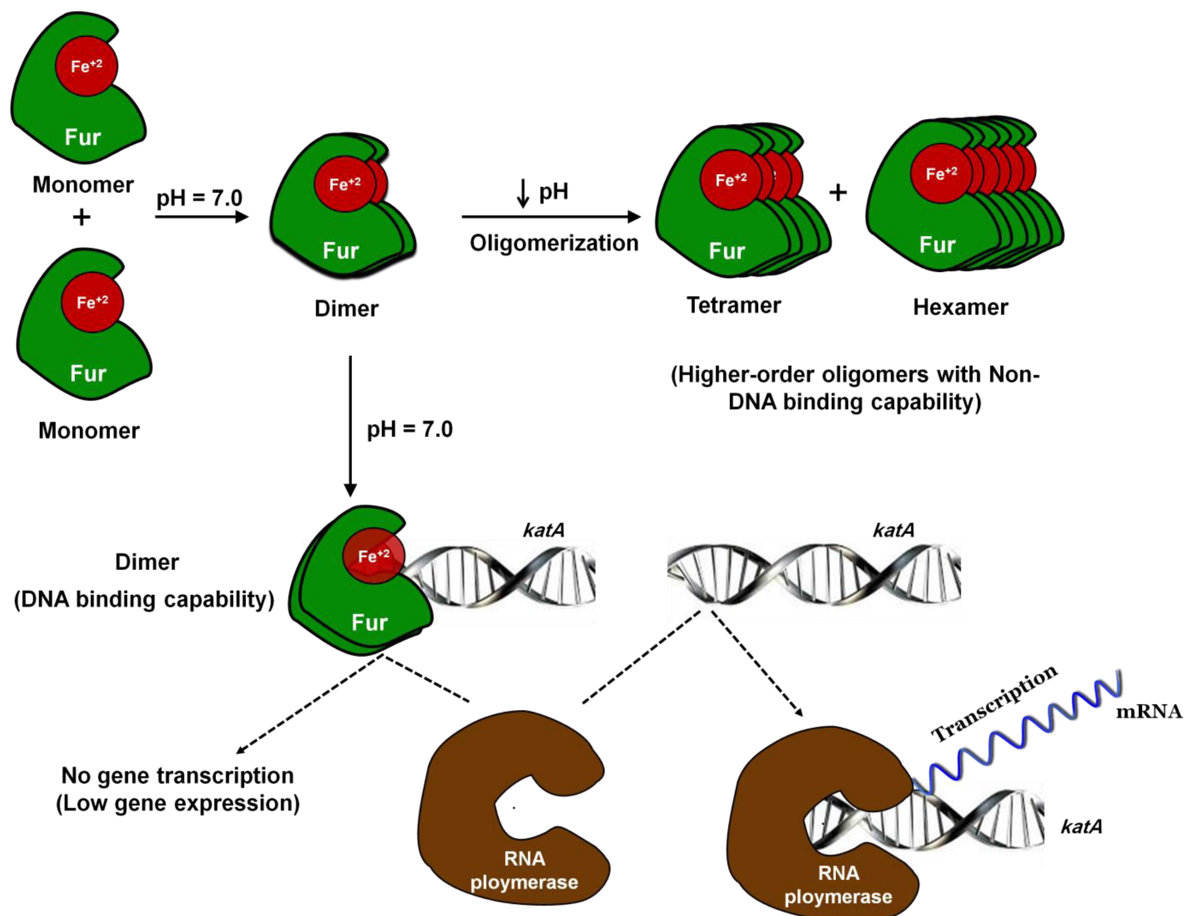


Figure 3.17: Proposed model for the acid-induced alleviation of Fur-repression of *katA*. Fur exists mainly at neutral pH as a dimer composed of two Fur monomers. Oligomerization of Fur is enhanced under acidic conditions ($pH < 7.0$) to form higher-order oligomers such as tetramer and hexamer. These oligomers are unable to bind to the *katA* promoter which allows RNA polymerase to bind to DNA and initiate gene transcription. On the other hand, Fur dimer will bind to the *katA* promoter and consequently prevents gene transcription by RNA polymerase resulting in a decrease in *katA* gene expression.

The ferric uptake regulator Fur is important for *C. jejuni* host pathogenesis. The transcriptional profile of the Δ*fur* mutant under acidic conditions revealed the differential expression of many genes involved in *C. jejuni* virulence. In addition to the iron acquisition genes, many genes involved in flagella biogenesis and energy metabolism were differentially expressed. Our data indicate that Fur could play a role in the pathogenesis of *C. jejuni* especially upon exposure to acidic conditions as those encountered in the host GIT. The contribution of Fur to *C. jejuni* virulence in either human epithelial cells or infection models has not been previously characterized. Our results show that Fur plays a critical role in *Campylobacter* virulence in eukaryotic cells. A deletion mutation of the *fur* gene significantly reduced *C. jejuni*'s capacity to adhere to, invade and survive intracellularly within HCT116 cells. Moreover, a significant difference was noted in waxworm larvae lethality caused by the *C. jejuni* wild-type as compared to the *fur* mutant with the LD_{50} of wild-type *C. jejuni* found to be markedly lower than that of the *fur* mutant.

The importance of Fur for *C. jejuni* virulence is most likely multifactorial and could be related to its role in regulating the expression of genes belonging to several biological functions, such as iron acquisition, energy metabolism and flagella biogenesis (58, 180, 338). Most of these genes encode proteins that contribute significantly to *C. jejuni* pathogenesis (338). For example, Fur is involved in the regulation of iron transport proteins, such as the ferric-enterobactin transporter system (CfrA-CeuBCDE), the hemin transporter system (ChuABCD), and a putative iron acquisition system (Cj1658-P19) (180, 338, 456). Under iron replete conditions, Fur binds Fe^{2+}, consequently the Fur-Fe^{2+} complex recognizes a DNA sequence (Fur box) in the promoters of target genes and represses their transcription (255). However, the expression of iron-regulated genes will be highly induced in the *C.*

jejuni Δ*fur* mutant as compared to the wild-type strain (180, 338, 456). The constitutive activation of Fur-regulated genes in the *C. jejuni* Δ*fur* mutant may not be entirely beneficial and could have an adverse impact on bacterial pathogenesis. The over expression of iron acquisition proteins in the Δ*fur* mutant could provide attractive targets for the host antibodies which would facilitate bacterial clearance by the immune system. For example, the *Campylobacter* ferric enterobactin receptor CfrA has been shown to be highly immunogenic and therefore could be a potential candidate for vaccine development against *C. jejuni* in poultry (491). Importantly, the reduced levels of *C. jejuni* colonization in poultry was found to be correlated with the elevated levels of *Campylobacter*-specific antibodies (263).

As mentioned above, Fur regulates the expression of iron acquisition proteins and thereby helps bacteria tightly control the cytoplasmic iron levels (57, 255). Despite its critical role for biological systems, iron can be toxic under certain conditions (58, 338). Iron plays a critical role in the formation of reactive oxygen species (ROS) such as hydroxyl radical ($^{\cdot}OH$) through the Fenton or Haber-Weiss chemistry (57). These ROS are biotoxic and are capable of oxidizing macromolecules and lipids causing cell damage and death (189, 357). When iron concentrations exceed those required for biological functions, Fur represses iron uptake and thereby protects the cells against the ROS-mediated cell damage (57, 255, 449). The *fur* mutation may increase oxidative stress due to the iron overload in the cells, which could be another reason for the virulence defect phenotype of the *C. jejuni* Δ*fur* mutant as compared to the wild-type strain.

Finally, our data revealed that many flagellar genes such as *flaA* and *flaB* were down-regulated in the *fur* mutant as compared to the wild-type strain. The involvement of Fur in the regulation of flagellar genes has been previously demonstrated (58, 180, 338).

Importantly, some of the flagellar biogenesis genes including the major *C. jejuni* flagellins (*flaAB*) were identified as CjFur targets (58). The *Campylobacter* flagellum is an important virulence determinant that enhances bacterial motility and acts as a type III secretion system (TTSS) (114). Mutation in flagellar genes significantly impaired *C. jejuni* invasion into human epithelial cells (419, 486). The importance of flagella for *C. jejuni* pathogenesis (114, 147, 419, 464, 486) could account for the virulence defect of the Δ*fur* mutant in both the eukaryotic cells and *G. mellonella* larvae relative to the wild-type strain. From all mentioned above, it is clear that the ferric uptake regulator Fur plays a critical role in *C. jejuni* host pathogenesis. Similarly, Fur is involved in the regulation of several virulence-associated genes in other microbes (65, 255). For example, deletion of the *fur* gene in a number of pathogenic organisms, such as *L. monocytogenes* (371), *V. cholerae* (290), *H. pylori* (55), *Actinobacillus* sp. (158), *N. meningitidis* (92), *S. typhimurium* (100, 443, 450) and *S. aureus* (183) leads to reduced virulence.

In summary, our data indicate that in addition to its role in iron metabolism, Fur is an important regulator of *Campylobacter* acid stress response. Moreover, we showed that Fur plays a role in the acid-induced cross-protection of *C. jejuni* against oxidative stress. We demonstrated that under low pH, the binding affinity between CjFur and the *katA* promoter is reduced allowing the higher expression of catalase and thereby defense against oxidative stress. Interestingly, we demonstrated that Fur significantly contributes to *C. jejuni* host pathogenesis. Enhanced immune clearance by the host and the defect in expression of virulence genes could account for the virulence attenuation of the Δ*fur* mutant as compared to *C. jejuni* wild-type.

Chapter 4 : General discussion

4.1. Conclusions

The current work was conducted to understand how the enteric pathogen *C. jejuni* survives acidic conditions, such as those encountered in the GIT during host infection. We also aimed to characterize the role of the ferric uptake regulator Fur in *Campylobacter* acid survival. In addition, the contribution of acid exposure and Fur to the protection of *C. jejuni* against other stresses and host pathogenesis was investigated herein.

To understand *C. jejuni*'s acid response, we characterized the transcriptome of *C. jejuni* under acidic conditions. The transcriptional profile of *C. jejuni* at low pH revealed that many genes involved in bacterial acid response were up-regulated. For example, acid exposure of *C. jejuni* resulted in up-regulation of gluconate dehydrogenases (Cj0414 and Cj0415). Importantly, we showed for the first time that *C. jejuni*'s capacity to survive acid stress is significantly enhanced in the presence of D-gluconate. Previous studies (250, 373, 374) showed that gluconate dehydrogenases were highly expressed in acid-stressed *C. jejuni*. Moreover, Reid *et al*. showed that a Δ*cj0415* mutant was acid sensitive as compared to *C. jejuni* wild-type (374). However, these studies did not demonstrate the role of gluconate dehydrogenase in *C. jejuni* acid survival. *C. jejuni* can utilize gluconate as an electron donor via the gluconate dehydrogenase activity of Cj0414 and Cj0415 (336). It is likely that gluconate's use as an electron donor in electron transport chains (472) plays a role in *C. jejuni* acid survival. The electron transport is coupled with proton translocation across the membrane (98), which could decrease the cytoplasmic proton concentration and thereby increase the intracellular pH. In addition, our results revealed the up-regulation of genes

encoding for an L-lactate dehydrogenase complex (Cj0075c-Cj0073c) which are thought to play a similar role as gluconate dehydrogenase in *C. jejuni* acid survival.

Importantly, we showed that acid exposure significantly enhances the capacity of *C. jejuni* to survive oxidative stress. The up-regulation of the oxidative stress genes such as ferritin (*cft*) and hemerythrin (*herB*) could account for the enhanced H_2O_2 survival by acid-stressed *C. jejuni* as compared to unstressed bacteria. The induced expression of oxidative stress defense genes in the acid-stressed *C. jejuni* indicates that there is a similarity in bacterial responses to various stresses.

Our findings suggest that *C. jejuni* can benefit from the obligate transit through the acidic environment of the stomach, as this passage will prepare *C. jejuni* for its exposure to other stresses such as oxidative stress encountered later in the intestine. Moreover, the transcriptome of *C. jejuni* at low pH revealed that many genes involved in bacterial virulence were highly expressed. These genes include *ciaB* (*Campylobacter* invasion antigen), *aspB* (aspartate aminotransferase) and *cj1062* (putative CinA-like protein) as well as signal transduction genes (e.g. *cj0448c*) which are important pathogenic determinants for *C. jejuni*. Therefore, we investigated the effect of acid stress on *Campylobacter* pathogenesis using *G. mellonella* larvae as an infection model. Interestingly, the acid-stressed *C. jejuni* was more virulent in larvae as compared to unstressed bacteria. Prior exposure to acid enhanced the capacity of *C. jejuni* to kill larvae, possibly by modulating the expression of its colonization and virulence factors. Based on our data, we can conclude that prior acid exposure predisposes *C. jejuni* for efficient gut colonization and host pathogenesis. To our knowledge, this is the first study that characterizes the influence of prior acid exposure on *C. jejuni* pathogenesis *in vivo* using an infection model. Previous studies have either investigated gene

expression in *C. jejuni* under acidic conditions (37, 250, 373, 374) or tested the impact of acid stress on *C. jejuni*'s interaction with human epithelial cells *in vitro* (250).

Next, we aimed to characterize the contribution of Fur to *C. jejuni* acid survival and host pathogenesis. Previous studies by Reid *et al*. (373, 374) studied the transcriptome of *C. jejuni* under acidic conditions and revealed that many iron acquisition genes were differentially expressed under such conditions. Given that iron acquisition genes are Fur-regulated, we hypothesized that Fur plays a role in *C. jejuni* acid survival. Our data revealed that the *C. jejuni* Δ*fur* mutant was significantly defective for acid survival as compared to the wild-type strain. Because Fur functions as a transcriptional regulator, we employed a microarray-based approach to characterize the transcriptome of both a Δ*fur* mutant and wild-type *C. jejuni* at low pH. The transcriptome of the *C. jejuni* Δ*fur* mutant at low pH revealed that many genes were differentially expressed. Many of these genes are involved in bacterial acid survival, including genes involved in heat shock response and flagella biogenesis. Importantly, our study showed that there is a link between *C. jejuni* motility and the bacterial capacity to survive acid stress. On contrast to the Δ*flgE* mutant that was motile and acid-resistant, the acid-sensitive flagellar mutants Δ*flgD*, Δ*flgH*, Δ*flgK*, Δ*flgP*, Δ*flgR*, Δ*motAB* and Δ*flhB* were defective for bacterial motility as compared to *C. jejuni* wild-type. Disruption of the ETC and increased sensitivity to oxidative stress (119) could account for acid sensitivity of the flagellar mutants *C. jejuni* relative to wild-type strain. Interestingly, the flagellar biogenesis genes in *C. jejuni* have been shown to be under both spatial and numerical regulation by a regulatory cascade (142). As shown in Figure 4.1, two different alternative σ factors, *fliA* (σ^{28}) and *rpoN* (σ^{54}) regulate the expression of flagellar genes in *C. jejuni* (152, 168).

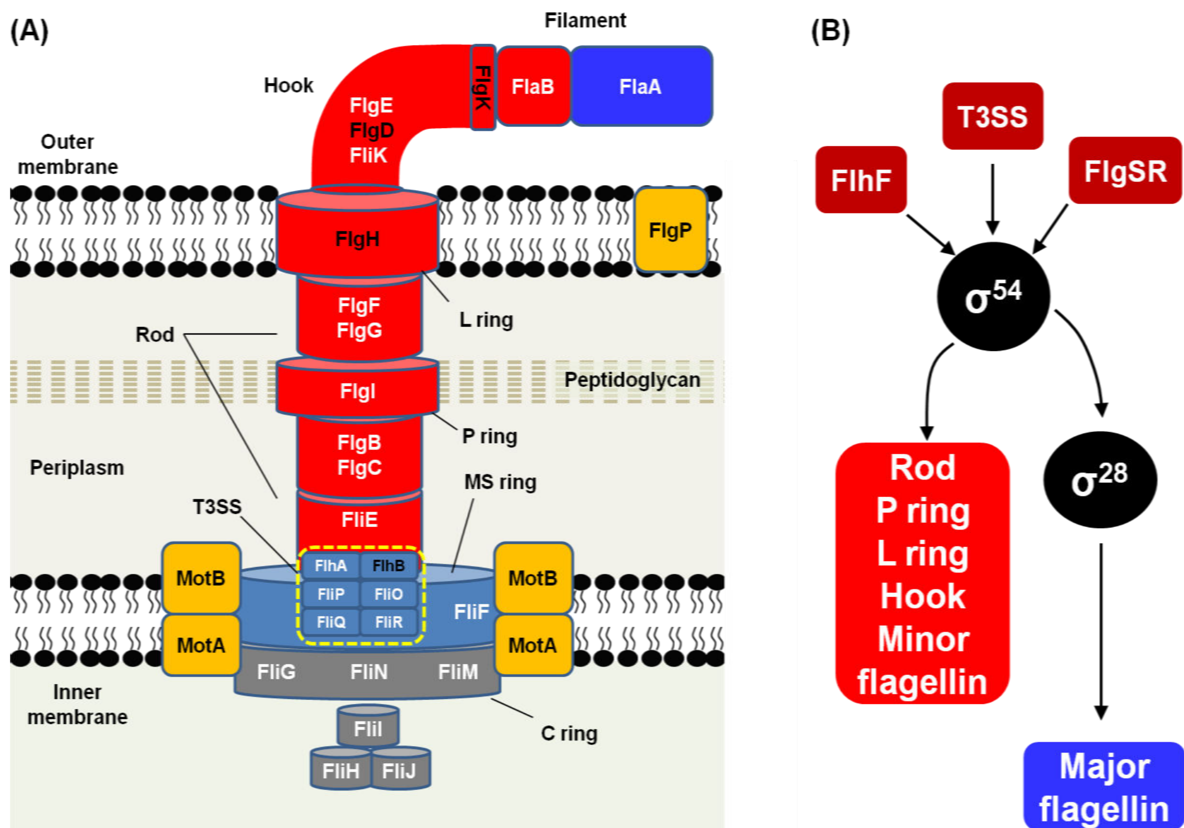


Figure 4.1: Main components of flagellar apparatus and transcriptional regulatory cascades of flagella biogenesis genes in *C. jejuni*. (A) Many genes involved in flagellar biogenesis and motility were differentially expressed in *C. jejuni* Δ*fur* mutant relative to the wild-type strain in response to acid. Flagellar genes written in black were found to be essential for both *Campylobacter* motility and acid survival in the present study. Similarly, the outer-membrane lipoprotein (FlgP) (417) is important for *C. jejuni* motility and acid survival. However, deletion mutation of FlgE (flagellar hook) did not affect either *Campylobacter* motility or acid survival. (B) The expression of flagella biogenesis genes is under regulation by two alternative σ factors, *fliA* (σ^{28}) and *rpoN* (σ^{54}) in *C. jejuni*. σ^{28} regulates mainly the expression of major falgellin (FlaA), while σ^{54} regulates the expression of genes involved in flagellar rod, P ring, L ring, hook and minor flagellin (FlaB) biogenesis. The regulation of σ^{54}-regulon expression is under tight control by T3SS, FlgRS and FlhB. T3SS; Type III secretion system. Figure is adapted and modified from Gilbreath *et al*. (142).

While σ^{28} is required for the transcription of a small number of flagellar genes, (e.g., *flaA*) (66, 166, 193), σ^{54} plays a significant role in the expression of flagellar genes in *C. jejuni* (66). The σ^{54} activity is regulated by the flagellar type III secretion system (T3SS) and the two-component regulatory system (FlgSR) along with FlhF (142, 168). This tight regulation of σ^{54} ensures that production of the rod and hook proteins in *C. jejuni* does not occur except in bacteria that are capable of secreting flagellar substrates (142, 168).

Importantly, we performed a comparative analysis of the acid stimulon of *C. jejuni* Δ*fur* mutant with the previously characterized Fur regulons in response to iron (180, 338) and Fur-targets (58). There was a significant variation between the acid stimulon of *C. jejuni* Δ*fur* mutant identified in the present study and the previously characterized Fur regulons and Fur targets. Many genes were differentially expressed in the *fur* mutant in response to acid that were not among the previously characterized Fur regulons (180, 338) or the Fur direct-binding targets (58). Only 5 genes; three genes involved in iron metabolism [*chuA*, *chuB* and *cj0175c* (*cfbpA*)] and two genes involved in oxidative stress response (*rrc* and *trxB*) were common in the present study and the studies by Palyada *et al*. (338), Holmes *et al*. (180) and Butcher *et al*. (58). This lack of overlap between the acid stimulon of the *C. jejuni* Δ*fur* mutant and the Fur regulons in response to iron (180, 338) could be explained by the difference in the experimental growth conditions. Fur is a global transcriptional regulator that is involved in regulation of genes belonging to diverse biological functions (58). It is likely that the environmental conditions under which *C. jejuni* grows modulate the Fur-regulation of targeted genes to express only genes required for growth under these conditions. Therefore, it is not surprising to note the difference between the Fur-regulated genes in response to acid and those genes expressed in response to iron. Under acidic conditions, *C.*

jejuni will normally tend to induce the expression of stress response proteins such as the heat shock proteins, which protect the cells against the acid-mediated cell damage. However, under iron-replete or deficient conditions, Fur will repress or induce the expression of iron acquisition proteins, respectively in order to tightly control the intracellular iron levels in *C. jejuni*. On the other hand, while our results indicate that the acid stimulon of the *C. jejuni* Δ*fur* mutant comprised 141 genes, Butcher *et al*. showed that only 95 transcriptional units were bound by Fur using ChIP-chip analysis (58). The difference between our finding and that by Butcher *et al*. (58) could be attributed to variations in techniques employed in each study. Our study used the transcriptional profiling approaches, which identify the differentially expressed genes in *C. jejuni* Δ*fur* mutant relative to the wild-type strain under tested conditions regardless that they are directly or indirectly regulated by Fur. However, Butcher *et al*. (58) used the ChIP-chip analysis which determines only the genes that are directly bound by Fur under specific conditions.

Next, we aimed to characterize the contribution of Fur to the acid-induced cross-protection of *C. jejuni* against oxidative stress. Our microarray data found that catalase (*katA*) was among the genes that were differentially expressed in the *C. jejuni* Δ*fur* mutant under acid. The finding that *katA* was up-regulated in the Δ*fur* mutant was not surprising because previous studies indicated that Fur is a transcriptional repressor of *katA* (337, 453). However, *katA* was up-regulated at low pH in the Δ*fur* mutant relative to *C. jejuni* wild-type to a lesser extent than at neutral pH. This finding suggests that *katA* expression was highly induced in *C. jejuni* wild-type at low pH. In support of our hypothesis, we showed that the expression levels of KatA were higher in acid-stressed *C. jejuni* as compared to unstressed bacteria by Western blot analysis and qRT-PCR. This finding led us to hypothesize that Fur-repression

of *katA* in *C. jejuni* can be modulated under acidic conditions. At low pH, the binding of Fur to the *katA* promoter is decreased as revealed by EMSA. Previous studies demonstrated that Fur protein tends to oligomerize at low pH forming higher-order oligomers (83, 130, 249), which, in contrast to the dimer state of Fur, are unable to bind to DNA (83). Therefore, it is tempting to speculate that *C. jejuni* Fur oligomerizes under acid leading to a decrease in the binding affinity of Fur to the *katA* promoter. Dissociation of Fur from the *katA* promoter would enhance *katA* transcription and consequently elevate *katA* expression. This observation itself was novel because how prior acid exposure cross-protects bacteria against subsequent stresses has never been illustrated before. This is the first study that provides a mechanism of the acid-mediated cross-protection of bacteria against oxidative stress. Moreover, our data clearly indicate that *C. jejuni* is able to fine-tune the Fur-regulation of *katA* according to the surrounding conditions. Finally, our data revealed that the *fur* mutant was impaired for virulence in human epithelial cells and *G. mellonella* larvae as compared to the wild-type strain. The enhanced immune clearance by the host (263, 338, 491) in addition to the role of Fur in regulation of virulence genes in *C. jejuni* such as flagellar genes could account for the virulence attenuation of the Δ*fur* mutant as compared to *C. jejuni* wild-type.

Taken together, we have shown that exposure of *C. jejuni* to acid induces the expression of genes involved in defense against acid stress. Moreover, our results indicate that the obligate passage of *C. jejuni* through the stomach acidity could prepare the bacterium to survive subsequent stresses later in the small intestine. Our data clearly demonstrate that in addition to its role in regulating iron metabolism, Fur is a major contributor to *C. jejuni* acid survival and plays a significant role in *C. jejuni* host pathogenesis. The mechanisms of *C. jejuni* acid survival identified in the present study and the contribution of Fur to *C. jejuni* acid survival and host pathogenesis are summarized in Figure 4.2.

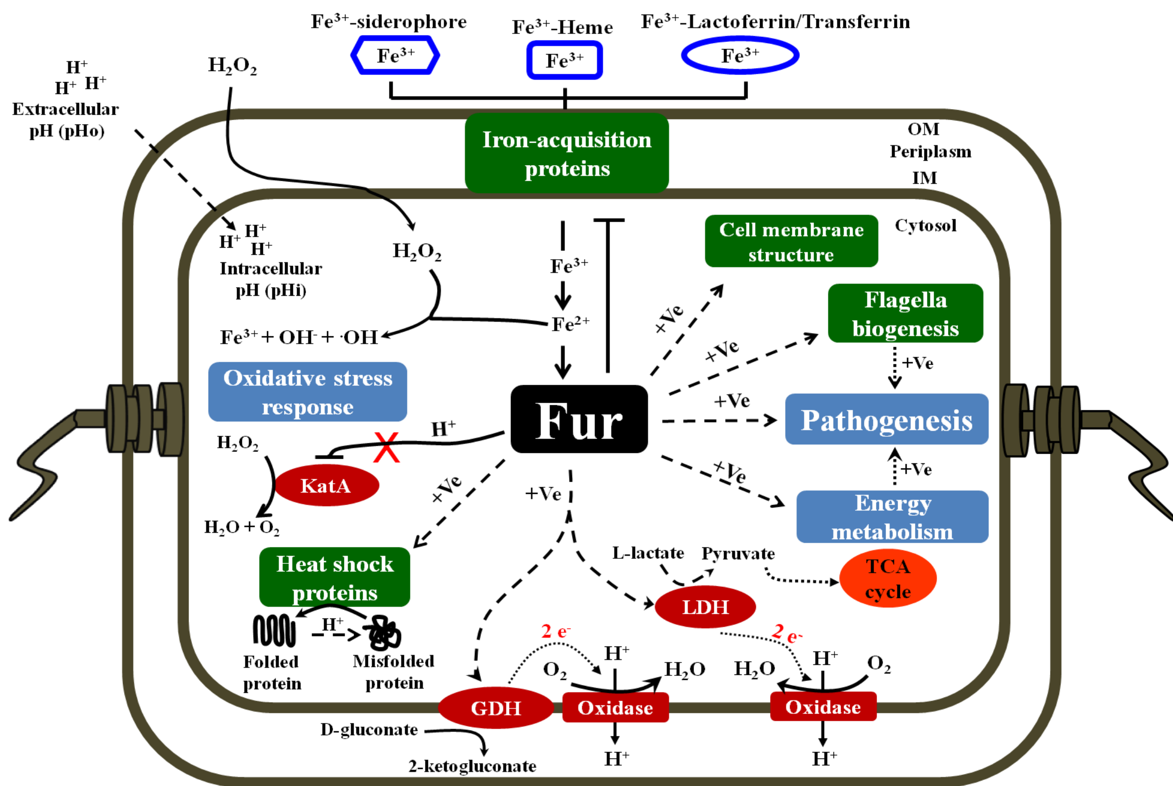


Figure 4.2: The mechanisms of *C. jejuni* acid survival and the contribution of Fur to *C. jejuni* acid survival and host pathogenesis. Acid exposure of *C. jejuni* results in up-regulation of genes important for *C. jejuni* acid survival. These genes encode for proteins involved in cell membrane biogenesis, heat shock response and flagella biogenesis. In addition, acid stress induces the expression of D-gluconate (Cj0414 and Cj0415) and L-lactate (Cj0075c-Cj0073c) dehydrogenases in *C. jejuni*. Gluconate dehydrogense catalyzes gluconate oxidation to 2-keto-D-gluconate (336) with the release of electrons which are accepted by oxygen as an electron acceptor via oxidase. The electron transport is coupled with proton transfer over the membrane (98, 215, 354) which decreases the intracellular acidity. Similarly, L-lactate dehydrogenase catalyzes the oxidation of L-lactate to pyruvate with the realase of electrons (448). The realsed electons enter the electron transport chain as mentioned above and thereby enhance *C. jejuni* acid survival. Pyruvate is directly fed into the TCA cycle which is an important energy source for *C. jejuni* improving its stress survival and pathogenesis (420). Importantly, Fur represses the iron acquisition proteins to limit the cellular iron overload at low pH and consequently protects the cell against the iron-mediated oxidative stress. In addition, the Fur-repression of *katA* is alleviated at low pH which allows for higher catalase expression. Catalase KatA catalyzes hydrolysis of H_2O_2 into H_2O and O_2 and thereby protects the cell against oxidative stress. Finally, acid stress induces the expression of many virulence genes in *C. jejuni*. Our study shows that Fur is involved in regulation of many genes important for *C. jejuni* pathogenesis such as the flagella biogenesis genes. Fur; Ferric uptake regulator, GDH; Gluconate dehydrogenase, IM; Inner membrane, KatA; Catalase, LDA; L-lactate dehydrogenase, OM; Outer membrane, ROS; reactive oxygen species, TCA cycle; Tricarboxylic Acid cycle.

4.2. Future directions

In this study, we demonstrated that the role of Fur extends beyond iron regulation and is involved in *C. jejuni* acid survival. Despite identifying the genes that are regulated by Fur in an acidic environment, the exact contribution of most of these genes to *C. jejuni* acid survival remains unknown. Moreover, many of the differentially expressed genes in the *fur* mutant encode proteins with hypothetical or unknown functions. Future work should focus on the characterization of these genes and their products to better understand their importance for *C. jejuni* acid survival and host pathogenesis. Moreover, while microarray profiling was helpful in characterizing the Fur regulon under acidic conditions, it was unable to determine the specifics of Fur regulation. Therefore, the mode of Fur regulation, either directly or indirectly, of differentially expressed genes identified by the microarray should be further illustrated. This question could be addressed using EMSA and/or DNase I footprinting of the identified Fur targets. Strikingly, our study characterized how prior acid exposure cross-protects *C. jejuni* against oxidative stress. However, the link between acid exposure and *C. jejuni*'s response to other stresses, such as osmotic and bile stresses should be further studied. Finally, more research should be conducted to identify any other proteins that could participate in *C. jejuni* acid survival. Determining the mechanisms of *C. jejuni* survival in the presence of various stresses, especially acid stress, will enrich our understanding of the pathogenesis of this bacterium.

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Contributions of collaborators

Sabina Sarvan (Couture lab, Department of Biochemistry, Microbiology and Immunology, University of Ottawa), performed the site directed mutagenesis in CjFur to generate *fur*ΔS2 mutant. In addition, Sabina prepared CjFur and Cy_5-labelled *katA* that were used in electrophoretic shift assays (EMSA). James Butcher (Stintzi lab, Department of Biochemistry, Microbiology and Immunology, University of Ottawa), designed the primers for inserting the *fur*ΔS2 mutant gene into the pRRK complementation vector.

Dr. Alain Stintzi: Dr. Alain Stintzi (Department of Biochemistry, Microbiology and Immunology, University of Ottawa) contributed as a supervisor to the project. Also, Dr. Stintzi contributed to the analysis of microarray data, maintenance, euthanasia, and necropsies of chicks for *in vivo* chick colonization experiments.

Appendix I: Strains and plasmids used in this study

Strain or plasmid	Genotype[a]	Source or reference
Strains		
***E. coli* DH5α**	endA1 hsdR17(r_k^- m_k^-) supE44 thi-1 recA1 gyrA relA1 Δ(lacZYA argF U169 deoR [ϕ80dlacΔ(lacZΔM15)]	Invitrogen
C. jejuni		
AS144	*C. jejuni* NCTC11168	National Collection of Type Cultures
AS1297	*C. jejuni* 81-176	National Collection of Type Cultures
AS230	AS144 Δ*fur* ::Cam^r	Palyada *et al*. (338)
AS960	AS230 + *fur* ::Cam^r Kan^r	Palyada *et al*. (337)
AS433	AS144 Δ*katA*::Cam^r	Palyada *et al*. (338)
AS1350.	AS230 + *fur*ΔS2 ::Cam^r Kan^r	This study
AS1071	AS144 Δ*cj0012c* ::Cam^r	Flint *et al*. (119)
AS742	AS144 Δ*cj1026c* (*flgP*) ::Cam^r	Flint *et al*. (119)
AS748	AS144 Δ*cj0818* ::Cam^r	Flint *et al*. (119)
AS751	AS144 Δ*cj1024c* ::Cam^r	Flint *et al*. (119)
AS757	AS144 Δ*flgG* ::Cam^r	Flint *et al*. (119)
AS761	AS144 Δ*flhB* ::Cam^r	Flint *et al*. (119)
AS764	AS144 Δ*flgD* ::Cam^r	Flint *et al*. (119)
AS765	AS144 Δ*flgK* ::Cam^r	Flint *et al*. (119)
AS768	AS144 Δ*flgE* ::Cam^r	Flint *et al*. (119)
AS1336	AS144Δ*motA*Δ*motB*::Kan^r	Flint *et al*. (119)
Plasmids		

pRR-Km	Cloning vector used for complementation of mutants, Kan^r	Reid et al. (373)
pStrepSumofur	Vector used for Cj*fur*ΔS2 site-directed mutagenesis	Butcher et al. (58)
pCDF	Vector used for Cj*fur*ΔS2 PCR-amplification	Provided by Sabina Sarvan (University of Ottawa)

[a] Cam^r, chloramphenicol resistance gene; Kan^r, kanamycin resistance gene.

Appendix II: Primer List

Primer Name	Primer sequence (5'-3')
CHAPTER 2	
Quantitative RT PCR	
ahpCF (+)	CTGCAGGACAAACTTCACCA
ahpCR (-)	TTCTTGCTTGATGCTGATGG
cftF (+)	GAGTTCTTGGTGCTATGAAAACAG
cftR (-)	TCTTGAAGCTCTACATGAGAATCTG
Cj0264cF (+)	TCTTAAATGCTCTGGGTACAG
Cj0264cR (-)	GTAAGTAAGTGGAAAGGTGCTTC
Cj0265cF (+)	GCCTATACCGATGAAGTTGTATC
Cj0265cR (-)	CACAGAAGGTGCTTTAGGATTTAC
Cj0414F (+)	CTGTTTTAAAGGCAGCAGAACTTAC
Cj0414R (-)	CTTCTCCTTGCTCATCTTTAGG
Cj0415F (+)	GCTTTAGGTTCTATGGTGGCTTT
Cj0415R (-)	AAAAGTGGATCACCCCAAGA
Cj0448cF (+)	GGAACATTGCATAGAAGTGTAGATG
Cj0448cR (-)	CTAGTTTTCTTACTTCATCGGCAAC
Cj1583c F (+)	CCTGTTTTGGTGCTCGTTTT
Cj1583c R (-)	CAAGCCCCTTGTAAGCAAAG
dapBF (+)	TAAGTGGAAGAGATGGCATTATAGG
dapBR (-)	CCTTGAAGTAGCGGTATGATTAAG
rpoAF (+)	CGAGCTTGCTTTGATGAGTG
rpoAR (-)	AGTTCCCACAGGAAAACCTA

rpsLF (+)	CAAAGAAGGGGAGTTTGCAC
rpsLR (-)	TATCAAGAGCACCACGAACG
slyDF (+)	TACGATGAAAATGCCGTTCA
slyDR (-)	TTCGCCAAAAAGCTCCATAC
uvrAF (+)	CACACCACGTTCAAATCCTG
uvrAR (-)	CCCCGCAAGTATCACAAACT
uvrBF (+)	CATTGCCATTATGGATGCAG
uvrBR (-)	GCGGCGTTCATTGGTAGTAT
zupTF (+)	CTCTTTCGGGTTTTGCAGAG
zupTR (-)	ATTGCCATTCCTGCGATAAG
CHAPTER 3	
Quantitative RT-PCR	
Cj0414F (+)	CTGTTTTAAAGGCAGCAGAACTTAC
Cj0414R (-)	CTTCTCCTTGCTCATCTTTAGG
Cj0415F (+)	GCTTTAGGTTCTATGGTGGCTTT
Cj0415R (-)	AAAAGTGGATCACCCCAAGA
Cj0448cF (+)	GGAACATTGCATAGAAGTGTAGATG
Cj0448cR (-)	CTAGTTTTCTTACTTCATCGGCAAC
clpBF (+)	AAGCCGTACGAAGAAAACCTTATAG
clpBR (-)	AATCCACTGTTACACCTTTGCTATC
flaBF (-)	CGAACCAATGTCTGCTCTGA
flaBF (+)	GCAGGCTCAGGTTTTTCAG
grpEF (+)	GCTTTAGAAGCAGCTGTTAATG
grpER (-)	CATCTTTGATAAGAGCCACTCC
katAF (+)	CTTTAGTCCAAGCAATATCGTTCC

katAR (-)	CAGCGACATTGTAAGTATTCACTTC
metBF (+)	AAACTTTAGCATTACACGGAGCTT
metCF (+)	CTAAACTTATTCATTGTGGCAGAGG
metCR (-)	CTCTGTATTTTTCCAAGTTGCGTG
metBR (-)	CCCTCCTTCGACATTAGCAA
rpsLF (+)	CAAAGAAGGGGAGTTTGCAC
rpsLR (-)	TATCAAGAGCACCACGAACG
Directed mutagenesis (generation of Cj*fur*ΔS2 mutant)	
*fur*ΔS2-H43A(F)	AAAACTCTTTATCACAGTGATACTGCCTACACACCCGAAAGT
*fur*ΔS2-H43A(R)	CATATATAAACTTTCGGGTGTGTAGGCAGTATCACTGTGATAAAGAGTTTT
*fur*ΔS2-H102A(F)	CTTGCCAATAAACCTCACCATGATGCCATGATATGTAAAAATTGCGGAAAA
*fur*ΔS2-H102A(R)	TTTTCCGCAATTTTTACATATCATGGCATCATGGTGAGGTTTATTGGCAAG
Transformation of Cj*fur*ΔS2 into *C. jejuni* Δ*fur*	
JBCL-Fur4	GATTTAGATGTCTAGACTCAAAAAGGGAGTGATATGCTGATAGAAAATGTGGA
JBCL-Fur2R	GGGGAAGCTTTCTAGTTATATTTTTACCTTTGCTT
AR55	ATGACATTGCCTTCTGCGT
Electrophoretic mobility shift assays (EMSAs)	
JFC1584	5'Cy5-TGCATTTTATTGATAATAAATTTCAAAATAAATTTAGTTT
JFC1585	5'Cy5-AAACTAAATTTATTTTGAAATTTATTATCAATAAAATGCA

Appendix III: *C. jejuni* transcriptome in acidic medium (pH 4/8 min)

Name	Gene	Fold Change (log_2)	Bayes.p	Annotation
Up-regulated genes				
Cj0835c	acnB	5.02	1.11E-04	Aconitate hydratase
Cj0203	Cj0203	4.76	5.85E-06	Putative citrate transporter
Cj0075c	Cj0075c	4.56	1.17E-05	L-lactate dehydrogenase
Cj0021c	Cj0021c	4.44	3.00E-03	Putative fumarylacetoacetate (FAA) hydrolase family protein
Cj0553	Cj0553	4.44	9.42E-04	Putative integral membrane protein
Cj0735	Cj0735	4.34	1.11E-04	Putative periplasmic protein
Cj0920c	Cj0920c	4.15	2.69E-04	Putative ABC-type amino acid transporter permease protein
Cj0735	Cj0735	4.09	1.23E-04	Putative periplasmic protein
Cj0552	Cj0552	4.07	2.69E-04	Putative membrane protein
Cj0554	Cj0554	4.01	8.46E-03	Hypothetical protein Cj0554
Cj0074c	Cj0074c	3.94	5.27E-05	L-lactate dehydrogenase
Cj1262	racS	3.93	6.14E-04	Two-component sensor (histidine kinase)
Cj0448c	Cj0448c	3.77	5.85E-06	Putative MCP-type signal transduction protein
Cj0762c	aspB	3.74	1.81E-04	Aspartate aminotransferase
Cj0073c	Cj0073c	3.63	7.55E-04	L-lactate dehydrogenase
Cj0903c	Cj0903c	3.50	2.93E-04	Putative amino acid transport protein

Cj0369c	Cj0369c	3.26	1.76E-05	Ferredoxin domain-containing integral membrane protein
Cj0689	ackA	3.23	9.42E-04	Acetate kinase
Cj0264c	Cj0264c	3.11	1.17E-05	Molybdopterin-containing oxidoreductase
Cj0866	Cj0866	3.03	1.23E-04	Pseudogene (arylsulfatase)
Cj0449c	Cj0449c	3.02	5.85E-06	Hypothetical protein Cj0449c
Cj0265c	Cj0265c	3.01	1.17E-05	Putative cytochrome C-type haem-binding periplasmic protein
Cj0951c	Cj0951c	2.94	7.26E-04	Putative MCP-domain signal transduction protein
Cj0393c	Cj0393c (*mqo*)	2.72	1.99E-04	Putative malate:quinone oxidoreductase
Cj0919c	Cj0919c	2.72	5.85E-06	Putative ABC-type amino acid transporter permease protein
Cj0415	Cj0415	2.71	6.96E-04	Putative oxidoreductase subunit
Cj0833c	Cj0833c	2.68	5.85E-06	Oxidoreductase
Cj0883c	Cj0883c	2.68	5.85E-06	Putative transcriptional regulator
Cj0874c	Cj0874c	2.57	1.11E-04	Cytochrome C
Cj0612c	cft	2.49	2.34E-03	Ferritin
Cj0029	ansA	2.40	3.41E-03	Cytoplasmic L-asparaginase
Cj0414	Cj0414	2.40	1.04E-03	Putative oxidoreductase subunit
Cj0069	Cj0069	2.34	1.72E-03	Hypothetical protein Cj0069
Cj0876c	Cj0876c	2.32	5.85E-06	Putative periplasmic protein

Cj0834c	Cj0834c	2.32	1.17E-05	Ankyrin repeat-containing possible periplasmic protein
Cj0952c	Cj0952c	2.29	1.17E-05	Putative HAMP containing membrane protein
Cj1110c	Cj1110c	2.28	1.23E-04	Putative MCP-type signal transduction protein
Cj1194	Cj1194	2.24	5.27E-05	Possible phosphate permease
Cj0555	Cj0555	2.21	1.17E-05	Putative dicarboxylate carrier protein MatC
Cj0981c	Cj0981c (*cjaB*)	2.12	2.69E-04	Putative MFS (Major Facilitator Superfamily) transport protein
Cj0559	Cj0559	2.07	2.93E-05	Putative pyridine nucleotide-disulphide oxidoreductase
Cj0005c	Cj0005c	2.05	1.86E-03	Putative molybdenum containing oxidoreductase
Cj0986c	Cj0986c	2.02	3.45E-04	Putative integral membrane protein
Cj0987c	Cj0987c	2.02	2.81E-04	Putative MFS (Major Facilitator Superfamily) transport protein
Cj0809c	Cj0809c	1.84	1.29E-04	Putative hydrolase
Cj0676	Cj0676 (*kdpA*)	1.82	7.26E-04	potassium-transporting ATPase A chain
Cj0989	Cj0989	1.81	1.18E-03	Putative membrane protein
Cj0854c	Cj0854c	1.81	5.38E-04	Putative periplasmic protein
Cj1293	Cj1293 (PseB)	1.79	8.46E-03	UDP-GlcNAc-specific C4,6 dehydratase
Cj0539	Cj0539	1.78	9.03E-03	Hypothetical protein Cj0539
Cj0739	Cj0739	1.71	3.84E-03	Hypothetical protein Cj0739

Cj0794	Cj0794	1.69	2.93E-04	Hypothetical protein Cj0794
Cj0604	Cj0604	1.69	1.58E-04	Putative polyphosphate kinase
Cj0980	Cj0980	1.69	2.93E-04	Putative peptidase
Cj0864	Cj0864	1.68	4.68E-05	Putative periplasmic protein
Cj1316c	Cj1316c (*PseA*)	1.66	6.15E-03	Pseudaminic acid biosynthesis PseA protein
Cj0909	Cj0909	1.64	2.43E-03	Putative periplasmic protein
Cj1163c	Cj1163c	1.57	5.66E-03	Putative cation transport protein
Cj1224	Cj1224	1.55	8.89E-04	Putative iron-binding protein
Cj0997	Cj0997	1.50	8.99E-03	Putative methyltransferase (GidB homolog)
Cj0935c	Cj0935c	1.47	3.57E-03	Putative sodium:amino acid symporter family protein
Cj0420	Cj0420	1.38	3.53E-03	Putative periplasmic protein
Cj0982c	Cj0982c (*cjaA*)	1.34	9.19E-04	Putative amino acid transporter periplasmic solute-binding protein
Cj0877c	Cj0877c	1.33	9.07E-04	Hypothetical protein Cj0877c
Cj0350	Cj0350	1.33	5.10E-03	Hypothetical protein Cj0350
Cj0859c	Cj0859c	1.31	1.39E-03	Hypothetical protein Cj0859c
Cj0344	Cj0344	1.30	5.93E-03	Hypothetical protein Cj0344
Cj0740	Cj0740	1.30	1.57E-03	Hypothetical protein Cj0740

Cj0934c	Cj0934c	1.28	9.04E-03	Putative sodium:amino acid symporter family protein
Cj1005c	Cj1005c	1.27	1.00E-03	Putative membrane bound ATPase
Cj0343c	Cj0343c	1.27	3.42E-03	Putative integral membrane protein
Cj0040	Cj0040	1.27	8.87E-03	Hypothetical protein Cj0040
Cj1161c	Cj1161c	1.25	4.34E-03	Putative cation-transporting ATPase
Cj1080c	Cj1080c (*hemD*)	1.25	1.16E-03	Putative uroporphyrinogen-III synthase
Cj0988c	Cj0988c	1.24	5.59E-03	Hypothetical protein Cj0988c
Cj0776c	Cj0776c	1.24	2.66E-03	Putative periplasmic protein
Cj0270	Cj0270	1.21	4.38E-03	Putative tautomerase family protein
Cj0900c	Cj0900c	1.20	2.15E-03	Small hydrophobic protein
Cj1112c	Cj1112c	1.20	6.11E-03	Putative SelR domain containing protein
Cj0832c	Cj0832c	1.19	3.80E-04	Putative Na^+/H^+ antiporter family protein
Cj0939c	Cj0939c	1.19	8.68E-03	Hypothetical protein Cj0939c
Cj0617	Cj0617	1.18	4.78E-03	Hypothetical protein Cj0617
Cj1154c	Cj1154c	1.18	7.27E-03	Putative cytochrome oxidase maturation protein cbb3-type
Cj0748	Cj0748	1.17	1.33E-03	Hypothetical protein Cj0748
Cj0772c	Cj0772c	1.15	3.14E-03	Putative NLPA family lipoprotein

Cj1022c	Cj1022c	1.14	4.49E-03	Putative integral membrane protein
Cj0916c	Cj0916c	1.13	1.46E-03	Hypothetical protein Cj0916c
Cj0426	Cj0426	1.11	2.69E-04	ABC transporter ATP-binding protein
Cj0591c	Cj0591c	1.10	4.72E-03	Putative lipoprotein
Cj0427	Cj0427	1.10	8.60E-03	Hypothetical protein Cj0427
Cj0685c	Cj0685c (*cipA*)	1.10	1.80E-03	Possible sugar transferase
Cj1034c	Cj1034c	1.05	2.48E-03	Possible DnaJ-like protein
Cj0914c	Cj0914c (*ciaB*)	1.05	3.32E-03	CiaB protein
Cj0852c	Cj0852c	1.04	2.98E-03	Putative integral membrane protein
Cj0965c	Cj0965c	1.02	6.79E-03	Putative acyl-CoA thioester hydrolase
Cj0771c	Cj0771c	1.01	3.29E-03	Putative NLPA family lipoprotein
Cj0797c	Cj0797c	1.00	4.94E-03	Hypothetical protein Cj0797c
Cj1152c	Cj1152c (*gmhB*)	1.00	5.36E-03	D,D-heptose 1,7-bisphosphate phosphatase
Cj0653c	Cj0653c	0.99	3.08E-03	Putative aminopeptidase
Cj0878	Cj0878	0.98	2.65E-03	Hypothetical protein Cj0878
Cj1036c	Cj1036c	0.98	2.12E-03	Hypothetical protein Cj1036c
Cj0721c	Cj0721c	0.98	1.42E-03	Putative integral membrane protein

Cj0618	Cj0618	0.96	8.26E-03	Hypothetical protein Cj0618
Cj1060c	Cj1060c	0.95	4.32E-03	Putative membrane protein
Cj1159c	Cj1159c	0.89	7.78E-03	Small hydrophobic protein
Cj0973	Cj0973	0.88	6.60E-03	Hypothetical protein Cj0973
Cj0850c	Cj0850c	0.86	3.45E-03	Putative MFS (Major Facilitator Superfamily) transport protein
Cj0770c	Cj0770c	0.84	8.28E-03	Putative NLPA family lipoprotein
Cj0681	Cj0681	0.79	6.10E-03	Hypothetical protein Cj0681
Cj1057c	Cj1057c	0.76	6.97E-03	Putative coiled-coil protein
Cj1062	Cj1062	0.72	5.10E-03	Putative CinA-like protein
Cj1100	Cj1100	0.68	4.17E-03	Hypothteical protein Cj1100
Cj0830	Cj0830	0.65	7.14E-03	Putative integral membrane protein
Cj1035c	Cj1035c (ate)	0.44	5.67E-03	Putative arginyl-tRNA-protein transferase
Cj0769c	Cj0769c (*flgA*)	0.23	1.00E-03	Putative flagellar protein FlgA
Cj0001	dnaA	0.03	4.37E-03	Chromosomal replication initiator protein
Cj0237	cynT	0.01	8.99E-03	Carbonic anyhydrase

Down-regulated genes

Cj0938c	aas	-0.26	1.41E-03	Acyl-acyl carrier protein synthetase
Cj0257	dgkA	-0.32	5.85E-06	Diacylglycerol kinase

Cj0840c	fbp	-0.37	5.97E-03	Putative fructose-1,6-bisphosphatase
Cj0849c	Cj0849c	-0.44	7.74E-03	Hypothetical protein Cj0849c
Cj0822	dfp	-0.44	6.42E-03	Phosphopantothenoylcysteine decarboxylase
Cj0798c	ddlA	-0.45	6.79E-03	Putative D-alanine-D-alanine ligase
Cj0191c	def	-0.51	3.69E-03	polypeptide deformylase
Cj0481	dapA	-0.57	2.94E-03	Putative dihydrodipicolinate synthase
Cj1054c	murC	-0.58	8.41E-03	UDP-N-acetylmuramate--alanine ligase
Cj0325	xseA	-0.66	1.38E-03	Exodeoxyribonuclease VII large subunit
Cj1605c	dapD	-0.66	3.10E-03	Tetrahydrodipicolinate N-succinyltransferase
Cj1668c	Cj1668c	-0.70	7.22E-03	Putative periplasmic protein
Cj1637c	Cj1637c	-0.71	3.55E-03	Putative periplasmic protein
Cj0192c	clpP	-0.72	7.36E-03	ATP-dependent clp protease proteolytic subunit
Cj0759	dnaK	-0.74	2.28E-03	Heat shock protein dnaK
Cj0321	dxs	-0.74	2.16E-03	1-deoxyxylulose-5-phosphate synthase
Cj1508c	fdhD	-0.75	6.69E-03	FdhD protein
Cj1604	hisI	-0.80	6.68E-03	Phosphoribosyl-AMP cyclohydrolase
Cj1027c	gyrA	-0.82	5.90E-03	DNA gyrase subunit A
Cj1048c	dapE	-0.83	2.00E-03	Succinyl-diaminopimelate desuccinylase

Cj0585	folP	-0.85	6.44E-03	Putative dihydropteroate synthase
Cj0542	hemA	-0.85	2.12E-03	Glutamyl-tRNA reductase
Cj0195	fliI	-0.87	4.19E-03	Flagellum-specific ATP synthase
Cj0166	miaA	-0.89	6.67E-03	tRNA delta (2)-isopentenylpyrophosphate transferase
Cj0452	dnaQ	-0.91	1.11E-04	Exonuclease, possibly DNA polymerase III epsilon subunit
Cj1666c	Cj1666c	-0.94	4.32E-03	Putative periplasmic protein
Cj1648	Cj1648	-1.01	7.27E-03	Possible ABC transport system periplasmic substrate-binding protein
Cj0197c	dapB	-1.01	8.02E-04	Dihydrodipicolinate reductase
Cj1626c	Cj1626c	-1.02	2.08E-03	Putative periplasmic protein
Cj1067	pgsA	-1.03	9.74E-03	CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidyltransferase
Cj0578c	mttB	-1.05	7.03E-03	Sec-independant protein translocase
Cj1303	fabH2	-1.05	3.22E-03	Putative 3-oxoacyl-[acyl-carrier-protein] synthase
Cj0622	hypF	-1.07	8.50E-03	Carbamoyltransferase
Cj1599	hisB	-1.07	3.20E-03	Imidazoleglycerol-phosphate dehydratase
Cj1221	groEL	-1.09	2.86E-03	60 kD chaperonin (cpn60)
Cj1482c	Cj1482c	-1.13	8.03E-03	Hypothetical protein Cj1482c
Cj1248	guaA	-1.13	1.22E-03	GMP synthase (glutamine-hydrolyzing)
Cj1365c	Cj1365c	-1.16	8.24E-03	Putative secreted serine protease

Cj0551	efp	-1.16	1.81E-04	Elongation factor P
Cj1346c	dxr	-1.18	5.62E-04	Putative 1-deoxy-D-xylulose 5-phosphate reductoisomerase
Cj1366c	glmS	-1.18	1.33E-03	Glucosamine-fructose-6-phosphate aminotransferase
Cj1597	hisG	-1.20	1.90E-03	ATP phosphoribosyltransferase
Cj1213c	glcD	-1.21	1.15E-03	Putative glycolate oxidase subunit D
Cj0811	lpxK	-1.23	6.63E-03	Putative tetra- acyldisaccharide 4'-kinase
Cj1260c	dnaJ	-1.26	1.11E-04	Chaperone DnaJ
Cj1315c	hisH	-1.27	1.75E-03	Imidazole glycerol phosphate synthase subunit
Cj1019c	livJ	-1.28	8.27E-03	Branched-chain amino acid ABC transport system periplasmic binding protein
Cj0597	fba	-1.29	1.64E-03	Fructose-bisphosphate aldolase
Cj1419c	Cj1419c	-1.30	9.30E-03	Possible methyltransferase
Cj0518	htpG	-1.33	7.67E-04	Hsp90 family heat shock protein
Cj1638	dnaG	-1.35	1.99E-04	DNA primase
Cj0116	fabD	-1.36	1.39E-03	Malonyl CoA-acyl carrier protein transacylase
Cj0882c	flhA	-1.36	3.63E-04	Flagellar biosynthesis protein
Cj0856	lepP	-1.39	6.09E-03	Signal peptidase I
Cj0704	glyQ	-1.39	7.92E-03	Glycyl-tRNA synthetase alpha chain

Cj1453c	Cj1453c	-1.40	4.00E-03	Putative tRNA(Ile)-lysidine synthase
Cj0081	cydA	-1.43	6.44E-05	Cytochrome bd oxidase subunit I
Cj0442	fabF	-1.44	4.56E-04	3-oxoacyl-[acyl-carrier-protein] synthase
Cj1088c	folC	-1.45	7.49E-04	Dihydrofolate synthase
Cj1454c	Cj1454c	-1.47	9.05E-03	Putative radical SAM domain family protein
Cj0098	fmt	-1.49	1.74E-03	Methionyl-tRNA formyltransferase
Cj0460	nusA	-1.49	6.76E-03	Transcription termination factor
Cj1030c	lepA	-1.49	7.47E-03	LepA GTP-binding protein homolog
Cj1418c	Cj1418c	-1.50	6.18E-03	Putative transferase
Cj0273	fabZ	-1.52	2.22E-04	(3R)-hydroxymyristoyl-[acyl carrier protein] dehydratase
Cj1633	Cj1633	-1.52	4.56E-04	Putative ATP-binding protein
nrdB	nrdB	-1.54	4.09E-03	Ribonucleoside-diphosphate reductase alpha chain
Cj0024	nrdA	-1.54	4.09E-03	Ribonucleoside-diphosphate reductase alpha chain
Cj1096c	metK	-1.57	7.26E-04	S-adenosylmethionine synthetase
Cj1108	clpA	-1.58	4.68E-05	ATP-dependent CLP protease ATP-binding subunit
Cj0207	infC	-1.59	2.79E-03	Translation initiation factor IF-3
Cj1412c	Cj1412c	-1.60	2.01E-03	Putative integral membrane protein
Cj1590	infA	-1.62	7.91E-03	Translation initiation factor IF-1

Cj0269c	ilvE	-1.63	3.07E-03	Branched-chain amino acid aminotransferase
Cj0897c	pheS	-1.65	9.99E-03	Phenylalanyl-tRNA synthetase alpha chain
Cj0855	folD	-1.67	1.54E-03	Methylenetetrahydrofolate dehydrogenase
Cj0652	pbpC	-1.72	9.99E-01	Penicillin-binding protein
Cj0288c	lpxB	-1.78	1.35E-03	Lipid-A-disaccharide synthase
Cj0894c	lytB	-1.84	8.12E-03	LytB homolog, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase
Cj1371	Cj1371	-1.85	1.45E-03	Putative periplasmic protein (vacJ homolog)
Cj1500	Cj1500	-1.85	7.26E-04	Putative integral membrane protein
Cj1188c	gidA	-1.89	4.10E-05	tRNA uridine 5-carboxymethylaminomethyl modification enzyme
Cj1611	rpsT	-1.94	9.11E-03	30S ribosomal protein S20
Cj1091c	leuS	-1.99	1.47E-03	Leucyl-tRNA synthetase,
Cj0095	rpmA	-2.02	9.41E-03	50S ribosomal protein L27
Cj1015c	livG	-2.05	2.88E-03	Branched-chain amino acid ABC transport system ATP-binding protein
Cj1709c	Cj1709c	-2.06	7.61E-05	Putative ribosomal pseudouridine synthase
Cj0332c	ndk	-2.14	6.44E-05	Nucleoside diphosphate kinase
Cj0023	purB	-2.19	5.10E-03	Adenylosuccinate lyase
Cj0275	clpX	-2.27	5.85E-06	ATP-dependent clp protease ATP-binding subunit clpX

Cj1696c	rplX	-2.27	8.99E-03	50S ribosomal protein L24
Cj1017c	livH	-2.35	1.95E-03	Branched-chain amino acid ABC transport system permease protein
Cj0586	ligA	-2.35	2.22E-04	DNA ligase
Cj0289c	peb3	-2.35	1.05E-03	Major antigenic peptide PEB3
Cj0293	surE	-2.35	5.88E-03	SurE protein homolog
Cj1070	rpsF	-2.42	6.04E-03	30S ribosomal protein S6
Cj0117	pfs	-2.43	2.07E-03	S-adenosylhomocysteine nucleosidase
Cj0245	rplT	-2.44	8.03E-03	50S ribosomal protein L20
Cj0082	cydB	-2.45	1.11E-04	Cytochrome bd oxidase subunit II
Cj0206	thrS	-2.45	3.76E-03	Threonyl-tRNA synthetase
Cj1071	ssb	-2.46	8.82E-03	Single-strand DNA binding protein
Cj1699c	rpmC	-2.46	8.34E-03	50S ribosomal protein L29
Cj0478	rpoB	-2.48	3.57E-03	DNA-directed RNA polymerase beta chain
Cj0471	rpmG	-2.52	3.92E-03	50S ribosomal protein L33
Cj0276	mreB	-2.54	5.85E-06	Homolog of *E. coli* rod shape-determining protein
Cj0109	exbB3	-2.57	8.46E-03	Putative MotA/TolQ/ExbB proton channel family protein
Cj1014c	livF	-2.59	2.17E-04	Branched-chain amino acid ABC transport system ATP-binding protein
Cj0709	ffh	-2.61	1.11E-04	Signal recognition particle protein

Cj0328c	fabH	-2.68	1.17E-05	3-oxoacyl-[acyl-carrier-protein] synthase
Cj1592	rpsM	-2.70	6.11E-03	30S ribosomal protein S13
Cj1691c	rplR	-2.73	1.61E-03	50S ribosomal protein L18
Cj0696	ftsZ	-2.75	6.85E-04	Cell division protein
Cj0886c	ftsK	-2.75	6.85E-04	Putative cell division protein
Cj0953c	purH	-2.75	4.33E-04	Phosphoribosylaminoimidazolecarbox amide formyltransferase/IMP cyclohydrolase
Cj1690c	rpsE	-2.84	1.86E-03	30S ribosomal protein S5
Cj1479c	rpsI	-2.85	1.20E-03	30S ribosomal protein S9
Cj1612	prfA	-2.88	5.44E-04	Peptide chain release factor 1
Cj1016c	livM	-2.94	4.68E-05	Putative branched-chain amino acid ABC transport system permease protein
Cj1705c	rplW	-2.96	2.03E-03	50S ribosomal protein L23
Cj0329c	plsX	-2.97	6.67E-04	Putative fatty acid\phospholipid synthesis protein
Cj1710c	Cj1710c	-3.05	5.85E-06	Putative metallo-beta-lactamase family protein
Cj0492	rpsG	-3.08	1.22E-03	30S ribosomal protein S7
Cj1702c	rplV	-3.11	1.56E-03	50S ribosomal protein L22
Cj1697c	rplN	-3.17	1.61E-03	50S ribosomal protein L14
Cj0707	kdtA	-3.20	1.17E-05	3-deoxy-D-manno-octulosonic-acid transferase

Cj0274	lpxA	-3.22	1.17E-05	UDP-N-acetylglucosamine acyltransferase
Cj1689c	rplO	-3.31	8.60E-04	50S ribosomal protein L15
Cj1692c	rplF	-3.48	3.51E-04	50S ribosomal protein L6
Cj1694c	rpsN	-3.74	2.33E-03	30S ribosomal protein S14
Cj1711c	ksgA	-3.77	5.79E-04	Putative dimethyladenosine transferase (16S rRNA dimethylase)
Cj1693c	rpsH	-3.94	7.61E-05	30S ribosomal protein S8
Cj1593	rpsK	-4.02	1.64E-04	30S ribosomal protein S11
Cj1480c	rplM	-4.07	1.40E-04	50S ribosomal protein L13
Cj1182c	rpsB	-4.14	2.81E-04	30S ribosomal protein S2
Cj0493	fusA	-4.20	6.67E-04	Elongation factor G
Cj1181c	tsf	-4.27	4.04E-04	Elongation factor TS
Cj0474	rplK	-4.29	1.11E-04	50S ribosomal protein L11
Cj0473	nusG	-4.49	4.68E-05	Putative transcription anti-termination protein
Cj0470	tuf	-4.73	4.68E-05	Elongation factor TU
Cj1707c	rplC	-4.84	4.10E-05	50S ribosomal protein L3
Cj1708c	rpsJ	-4.87	5.27E-05	30S ribosomal protein S10
Cj1594	rpsD	-4.96	1.17E-05	30S ribosomal protein S4
Cj1706c	rplD	-5.05	4.10E-05	50S ribosomal protein L4
Cj1595	rpoA	-5.05	6.44E-05	DNA-directed RNA polymerase alpha chain

Cj1700c	rplP	-5.12	4.10E-05	50S ribosomal protein L16
Cj1698c	rpsQ	-5.23	1.17E-05	30S ribosomal protein S17
Cj1695c	rplE	-5.34	5.85E-06	50S ribosomal protein L5
Cj0475	rplA	-5.51	6.67E-04	50S ribosomal protein L1
Cj1701c	rpsC	-6.08	5.85E-06	30S ribosomal protein S3
Cj1704c	rplB	-6.25	1.11E-04	50S ribosomal protein L2

Appendix IV: Transcriptomic data of the *C. jejuni* Δ*fur* mutant under different pH conditions

Name	Gene	Genome annotation	Gene function & Class (According to the Sanger Center annotation)	Fold change (log_2) at pH: 7	3	4
Cluster A						
Cj0402	glyA	Serine hydroxymethyltransferase	Amino acid biosynthesis	-1.5	-1.3	-1.0
Cj1727c	metB	Putative O-acetylhomoserine (thiol)-lyase	Amino acid biosynthesis	-0.7	-0.1	-2.1
Cj0400	fur	Ferric uptake regulator	Broad regulatory functions	-2.0	-1.6	-1.0
Cj1462	flgI	Flagellar basal body P-ring protein	Cell envelope	-1.7	-3.2	-4.0
Cj0687c	flgH	Flagellar basal body L-ring protein	Cell envelope	-1.7	-3.4	-4.1
Cj0042	flgD	Flagellar basal body rod modification protein	Cell envelope	-1.7	-3.2	-3.9
Cj0043	flgE	Flagellar hook protein	Cell envelope	-1.6	-2.7	-1.8
Cj0200c	Cj0200c	Putative periplasmic protein	Cell envelope	-1.4	-0.9	-1.7
Cj1421c	Cj1421c	Putative sugar transferase	Cell envelope	-1.4	-1.6	-2.1
Cj1339c	flaA	Flagellin	Cell envelope	-1.2	-1.7	-3.1
Cj1338c	flaB	Flagellin	Cell envelope	-1.1	-1.5	-3.0
Cj0887c	flaD	Putative flagellin	Cell envelope	-1.1	-3.6	-3.1
Cj1293	pseB	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	Cell envelope	-1.1	-1.7	-0.9
Cj1466	flgK	Flagellar hook-associated protein FlgK	Cell envelope	-1.1	-2.8	-2.7

Cj0697	flgG2	Flagellar basal-body rod protein	Cell envelope	-1.1	-2.9	-2.7
Cj1026c	flgP	Putative lipoprotein	Cell envelope	-1.0	-2.0	-0.8
Cj1729c	flgE2	Flagellar hook protein FlgE	Cell envelope	-0.9	-2.8	-4.3
Cj0697	flgG	Flagellar basal-body rod protein	Cell envelope	-1.1	-2.9	-2.7
Cj0041	fliK	Flagellar hook-length protein	Cell envelope	-0.8	-2.5	-1.3
Cj1316c	pseA	Pseudaminic acid biosynthesis PseA protein	Cell envelope	-0.6	-1.3	-1.2
Cj1294	pseC	C4 aminotransferase specific for PseB product	Cell envelope	-0.6	-1.4	-0.9
Cj0548	fliD	Flagellar capping protein	Cell envelope	-0.6	-1.5	-1.5
Cj0528c	flgB	Flagellar basal body rod protein	Cell envelope	-0.6	-1.6	-0.7
Cj0549	fliS	Flagellar protein FliS	Cell envelope	-0.5	-1.4	-1.4
Cj0547	flaG	Flagellar protein FlaG	Cell envelope	-0.5	-1.0	-0.5
Cj0628	Cj0629	Putative lipoprotein	Cell envelope	-0.4	-0.2	-1.8
Cj1500	Cj1500	Putative inner membrane protein	Cell envelope	-0.4	-0.4	-1.9
Cj1034c	Cj1034c	Adenylosuccinate lyase	Chaperones, chaperonins & heat shock	-0.4	-1.3	-1.0
Cj0977	Cj0977	Hypothetical protein Cj0977	Hypothetical proteins	-1.4	-1.7	-1.0
Cj0073c	Cj0073c	L-lactate dehydrogenase	Energy metabolism	-0.9	-0.7	-1.3
Cj0044c	Cj0044c	Hypothetical protein Cj0044c	Hypothetical proteins	-0.7	-2.1	-0.7
Cj0239c	Cj0239c	NifU protein homolog	Hypothetical proteins	-0.4	-0.05	-2.6
Cj0169	sodB	Superoxide dismutase (Fe)	Detoxification	-0.3	-0.2	-1.7

Cj0011c	Cj0011c	Putative non-specific DNA binding protein.	DNA replication, restriction/modification, recombination and repair	-0.1	-1.4	-0.6
Cj0074c	Cj0074c	L-lactate dehydrogenase	Energy metabolism	-0.7	-0.6	-1.3
Cj0037c	Cj0037c	Putative cytochrome C	Energy metabolism	-0.6	-0.02	-2.4
Cj0414	Cj0414	Putative oxidoreductase subunit	Miscellaneous	-0.9	-0.03	-2.5
Cj0415	Cj0415	Putative oxidoreductase subunit	Miscellaneous	-0.8	-0.2	-3.0
Cj1315c	hisH	Imidazole glycerol phosphate synthase subunit HisH	Miscellaneous	-0.8	-1.4	-1.1
Cj1337	pseE	PseE protein	Miscellaneous	-0.5	-1.1	-0.5
Cj1314c	hisF	Imidazole glycerol phosphate synthase subunit	Miscellaneous	-0.3	-0.9	-1.3
Cj1024c	flgR	Sigma-54 transcriptional activator	Signal transduction	-1.1	-1.7	-1.7
Cj0045c	Cj0045c	Putative iron-binding protein	Transport/binding proteins	-1.7	-2.3	-2.7
Cj0012c	rrc	Non-haem iron protein	Transport/binding proteins	-0.5	-4.1	-4.1
Cj1018c	livK	Branched-chain amino acid ABC transport	Transport/binding proteins	-0.3	-1.0	-0.7
Cj0391c	Cj0391c	Hypothetical protein Cj0391c	Unknown	-1.6	-2.1	-2.4
Cj1242	Cj1242	Hypothetical protein Cj1242	Unknown	-1.4	-2.2	-0.5
Cj1656c	Cj1656c	Hypothetical protein Cj1656c	Unknown	-1.2	-1.4	-1.9
Cj1450	Cj1450	Putative ATP/GTP-binding protein	Unknown	-0.5	-1.4	-0.8
Cj0040	Cj0040	Hypothetical protein Cj0040	Unknown	-0.3	-1.3	-0.4

Cj0170	Cj0170	Hypothetical protein Cj0170	Unknown	-0.3	-1.5	-1.9
Cj0453	thiC	Thiamine biosynthesis protein ThiC	Biosynthesis of cofactors, prosthetic groups and carriers	-0.9	0.4	-2.2
Cj1725	Cj1725	Putative periplasmic protein	Cell envelope	0.02	0.1	-1.5
Cj1677	Cj1677	Putative lipoprotein	Cell envelope	0.2	0.05	-1.8
Cj0007	gltB	Glutamate synthase (NADPH) large subunit	Central intermediary metabolism	-0.4	0.2	-1.1
Cj1267c	hydA	Ni/Fe-hydrogenase small chain	Energy metabolism	-0.5	0.1	-1.1
Cj1476c	Cj1476c	Pyruvate-flavodoxin oxidoreductase	Energy metabolism	-0.2	0.2	-1.2
Cj1199	Cj1199	Putative iron/ascorbate-dependent oxidoreductase	Miscellaneous	-0.7	0.2	-1.2
Cj0240c	iscC	Cysteine desulfurase (NifS protein homolog)	Miscellaneous	-0.2	0.4	-1.8
Cj0418c	Cj0418c	Hypothetical protein Cj0418c	Unknown	-0.3	0.1	-1.3
Cj0348	trpB	Tryptophan synthase subunit beta	Amino acid biosynthesis	0.1	-0.6	-1.2
Cj0289c	peb3	Major antigenic peptide PEB3	Cell envelope	0.6	-0.8	-1.7
Cj1451	dut	dUTPase	Purines, pyrimidines, nucleosides and nucleotides	0.1	-1.1	-0.8
Cj0450c	rpmB	50S ribosomal protein L28	Synthesis and modification of macromolecules	0.1	-0.1	-1.1
Cj0370	rpsU	30S ribosomal protein S21	Synthesis and modification of macromolecules	0.1	-0.5	-1.3
Cj0300c	modC	Putative molybdenum transport ATP-binding protein	Transport/binding proteins	0.3	-0.4	-1.2

Cj0380c	Cj0380c	Hypothetical protein Cj0380c	Unknown	0.1	-0.8	-1.2

Cluster B

Cj0091	Cj0091	Putative lipoprotein	Cell envelope	-0.5	-1.4	-0.1
Cj0757	hrcA	Heat-inducible transcription repressor	Chaperones, chaperonins & heat shock	-1.3	-0.1	-0.5
Cj1113	Cj1113	Conserved Hypothetical protein Cj1113	Conserved Hypothetical proteins	-0.8	-1.8	-0.1
Cj0358	Cj0358	Putative cytochrome C551 peroxidase	Detoxification	-1.2	-0.3	-0.7
Cj0075c	Cj0075c	L-lactate dehydrogenase	Energy metabolism	-1.1	-0.4	-0.5
Cj0401	lysS	Lysyl-tRNA synthetase	Synthesis and modification of macromolecules	-1.1	-0.5	-0.4
Cj1464	flgM	Hypothetical protein Cj1464	Unknown	-1.1	-1.0	-0.4
Cj1631c	Cj1631c	Conserved Hypothetical protein	Unknown	-0.7	-1.2	-0.5
Cj1201	metE	Homocysteine methyltransferase	Amino acid biosynthesis	-1.3	0.2	-0.7
Cj0092	Cj0092	Putative periplasmic protein	Cell envelope	-0.6	-1.2	0.4
Cj1170c	Cj1170c	putative periplasmic protein	Cell envelope	0.02	1.0	0.1
Cj0090	Cj0090	Putative lipoprotein	Cell envelope	-0.4	-1.0	0.3
Cj0093	Cj0093	Putative periplasmic protein	Cell envelope	-0.4	-1.4	0.1
Cj0089	Cj0089	Putative lipoprotein	Cell envelope	0.04	-1.0	0.6
Cj0335	flhB	Flagellar biosynthesis protein	Cell envelope	2.1	0.2	-0.2

Cj0082	cydB	Cytochrome bd oxidase subunit II	Energy metabolism	-0.02	-0.01	1.1
Cj1357c	nrfA	Periplasmic cytochrome C	Energy metabolism	0.2	1.2	-0.3
Cj0780	napA	Nitrate reductase	Energy metabolism	-1.1	0.03	-0.4
Cj0481	dapA	Putative dihydrodipicolinate synthase	Miscellaneous	0.1	-0.1	1.2
Cj1698c	rpsQ	30S ribosomal protein S17	Synthesis and modification of macromolecules	-0.6	-0.4	1.1
Cj1694c	rpsN	30S ribosomal protein S14	Synthesis and modification of macromolecules	-0.6	-0.3	1.0
Cj1014c	livF	Branched-chain amino acid ABC transport system ATP-binding protein	Transport/binding proteins	-0.6	-1.0	0.01
Cj1581c	Cj1581c	Putative peptide ABC-transport system ATP-binding protein	Transport/binding proteins	1.3	0.04	-0.05
Cj1019c	livJ	Branched-chain amino acid ABC transport system periplasmic binding protein	Transport/binding proteins	1.4	0.3	-0.1
Cj0428	Cj0428	Hypothetical protein	Unknown	-1.1	-0.9	0.02
Cj0563	Cj0563	Hypothetical protein	Unknown	0.1	1.0	-1.1
Cj1484c	Cj1484c	Hypothetical protein	Cell envelope	1.4	-0.3	-0.2
Cj0379c	Cj0379c	Putative sulfite oxidase subunit	Miscellaneous	1.3	-0.4	-0.6
Cj0667	Cj0667	Putative S4 domain protein	Miscellaneous	2.3	-1.4	-1.4
Cj1386	Cj1386	Ankyrin-repeat containing protein	Miscellaneous	2.3	-0.05	-0.1
Cj1150c	hdlE (waaE)	D-beta-D-heptose 7-phosphate kinase	Cell envelope	-0.1	0.5	1.0

Cj0758	grpE	Heat shock protein GrpE	Chaperones, chaperonins & heat shock	-1.4	0.3	0.5
Cj0509c	clpB	ATP-dependent Clp protease ATP-binding subunit	Chaperones, chaperonins & heat shock	-1.4	0.6	0.8
Cj0759	dnaK	Molecular chaperone DnaK	Chaperones, chaperonins & heat shock	-1.1	0.2	0.2
Cj0760	Cj0760	Hypothetical protein	Conserved Hypothetical proteins	-1.2	0.1	0.7
Cj1358c	nrfH	Periplasmic cytochrome C	Energy metabolism	-0.1	1.1	0.3
Cj1382c	fldA	Flavodoxin FldA	Energy metabolism	-0.02	1.6	1.8
Cj0474	rplK	50S ribosomal protein L11	Synthesis and modification of macromolecules	-0.2	0.01	1.0
Cj1155c	Cj1155c	Putative cation-transporting ATPase	Transport/binding proteins	-0.1	0.1	1.0
Cj0448c	Cj0448c	Putative MCP-type signal transduction protein	Signal transduction	-0.2	0.1	1.2
Cj0033	Cj0033	Putative integral membrane protein	Cell envelope	0.3	1.4	1.5
Cj0818	Cj0818	Putative lipoprotein	Cell envelope	1.4	1.4	0.5
Cj0820c	fliP	Flagellar biosynthesis protein	Cell envelope	2.1	1.0	0.9
Cj0701	Cj0701	Putative protease	Degradation of macromolecules	0.3	0.1	1.4
Cj0948c	Cj0948c	Putative cation efflux family protein	Drug/analogue sensitivity	0.6	0.7	1.6
Cj0442	fabF	3-oxoacyl-(acyl carrier protein) synthase II	Fatty acid biosynthesis	0.1	0.2	1.1
Cj0559	Cj0559	Putative pyridine nucleotide-disulphide oxidoreductase	Miscellaneous	0.7	1.4	0.1

Cj0949c	Cj0949c	Putative peptidyl-arginine deiminase family protein	Miscellaneous	0.9	1.2	1.5
Cj0172c	Cj0172c	Putative saccharopine dehydrogenase	Miscellaneous	1.6	0.6	0.2
Cj0586	ligA	NAD-dependent DNA ligase LigA	Synthesis and modification of macromolecules	0.5	0.05	1.4
Cj0017c	dsbI	Disulphide bond formation protein	Synthesis and modification of macromolecules	0.9	1.6	1.8
Cj1384c	Cj1384c	Hypothetical protein Cj1384c	Unknown	1.1	2.0	1.3

Cluster C

Cj1613c	chuZ	Haem oxygenase	Haemin-uptake system	2.3	4.6	3.4
Cj0146c	trxB	Thioredoxin reductase	Biosynthesis of cofactors, prosthetic groups and carriers	2.8	1.9	1.4
Cj1660	Cj1660	Putative integral membrane protein	Cell envelope	2.0	2.1	0.3
Cj1659	p19	Periplasmic protein p19	Cell envelope	6.4	6.5	3.0
Cj1385	katA	Catalase	Detoxification	8.0	1.3	2.0
Cj1661	Cj1661	Possible ABC transport system permease	Transport/binding proteins	1.7	2.7	1.1
Cj0179	exbB1	Biopolymer transport protein	Transport/binding proteins	1.8	2.8	2.1
Cj1615	chuB	Putative haemin uptake system permease protein	Transport/binding proteins	1.9	3.7	3.3
Cj1658	Cj1658	Putative iron permease	Transport/binding proteins	2.3	4.2	2.7
Cj1663	Cj1663	Putative ABC transport system ATP-binding protein	Transport/binding proteins	2.5	3.0	2.0

Cj1628	exbB2	Putative exbB/tolQ family transport protein	Transport/binding proteins	2.6	2.5	1.2
Cj0753c	tonB3	TonB transport protein	Transport/binding proteins	2.6	3.1	0.9
Cj1617	chuD	Putative haemin uptake system periplasmic haemin-binding protein	Transport/binding proteins	2.8	6.3	4.7
Cj0173c	cfbpC	Putative iron-uptake ABC transport system ATP-binding protein	Transport/binding proteins	3.0	2.3	1.1
Cj0178	ctuA	Putative TonB-denpendent outer membrane receptor	Transport/binding proteins	3.2	4.6	4.4
Cj0174c	cfbpB	Putative iron-uptake ABC transport system permease protein	Transport/binding proteins	3.7	3.1	1.3
Cj1614	chuA	Haemin uptake system outer membrane receptor	Transport/binding proteins	3.9	6.0	5.0
Cj0175c	cfbpA	Putative iron-uptake ABC transport system,periplasmic iron-binding protein	Transport/binding proteins	4.6	5.7	3.5
Cj1383c	Cj1383c	Hypothetical protein Cj1383c	Unknown	4.3	7.6	6.3

Appendix V: Comparison of the acid stimulon of the *C. jejuni* ∆*fur* mutant identified in the present study with the previously characterized CjFur regulons and CjFur targets

Gene	Gene name	Gene function	*A	B	C	D	E
accA	acetyl-CoA carboxylase carboxyltransferase subunit α	Fatty acid biosynthesis			+		
bioA	adenosylmethionine-8-amino-7-oxononanoate transferase	Biosynthesis of cofactors, and prosthetic groups			+		
ceuB	enterochelin uptake permease	Cations transport/binding proteins			+	+	
chaN	putative iron transport protein	Cations transport/binding proteins		+	+		
chuA	haemin uptake system outer membrane receptor	Transport/binding proteins	+	+	+	+	+
chuB	putative haemin uptake system permease protein	Transport/binding proteins	+	+	+	+	+
ChuC	putative haemin uptake system ATP-binding protein	Transport/binding proteins				+	
chuD	putative haemin uptake system periplasmic haemin-binding protein	Transport/binding proteins	+			+	
Cj0011c	Putative non-specific DNA binding protein.	DNA modification, recombination and repair	+			+	
Cj0012c (rrc)	non-haem iron protein	Transport/binding proteins	+	+	+	+	+
Cj0017c (dsbI)	disulphide bond formation protein	Synthesis and modification of macromolecules	+				
Cj0030	Hypothetical protein	Unknown		+			
Cj0033	putative integral membrane protein	Cell envelope	+				
Cj0037c	putative cytochrome C	energy metabolism	+				
Cj0040	hypothetical protein Cj0040	Unknown	+	+			
Cj0041 (fliK)	putative flagellar hook-length control protein	Cell envelope	+				
Cj0044c	hypothetical protein Cj0044c	Conserved hypothetical proteins	+				
Cj0045c	putative iron-binding protein	Transport/binding proteins	+	+			

Cj0073c	L-lactate dehydrogenase	Energy metabolism	+				
Cj0074c	L-lactate dehydrogenase	Energy metabolism	+				
Cj0075c	L-lactate dehydrogenase	Energy metabolism	+				
Cj0088	Putative anaerobic C4-dicarboxylate transporter	Transport/binding proteins		+			
Cj0089	putative lipoprotein	Cell envelope	+				
Cj0089	putative lipoprotein	Cell envelope	+				
Cj0090	putative lipoprotein	Cell envelope	+				
Cj0091	putative lipoprotein	Cell envelope	+				
Cj0092	putative periplasmic protein	Cell envelope	+				
Cj0093	putative periplasmic protein	Cell envelope	+				
Cj0145	putative TAT pathway signal sequence domain protein	Hypothetical unknown proteins			+		
Cj0150c	aminotransferase	Miscellaneous			+		
Cj0159c	Hypothetical protein	Miscellaneous		+	+		
Cj0160c	putative radical SAM domain protein	Miscellaneous			+		
Cj0168c	Putative periplasmic protein	Cell envelope		+			
Cj0170	hypothetical protein Cj0170	Unknown	+				
Cj0172c	putative saccharopine dehydrogenase	Miscellaneous	+				
Cj0173c (cfbpC)	putative iron-uptake ABC transport system ATP-binding protein	Transport/binding proteins	+			+	
Cj0174c (cfbpB)	putative iron-uptake ABC transport system permease protein	Transport/binding proteins	+		+	+	
Cj0175c (cfbpA)	putative iron-uptake ABC transport system,periplasmic iron-binding protein	Transport/binding proteins	+	+	+	+	+
Cj0176c	putative lipoprotein	Cell envelope		+			
Cj0177	putative iron transport protein	Transport/binding proteins		+		+	
Cj0178 (ctuA)	putative TonB-denpendent outer membrane receptor	Transport/binding proteins	+	+			
Cj0179 (exbB1)	iron transport	Transport/binding proteins		+			

Cj0180 (exbD1)	iron transport	Transport/binding proteins		+		+
Cj0181 (tonB1)	iron transport	Transport/binding proteins		+		+
Cj0200c	putative periplasmic protein	Cell envelope	+			
Cj0239c	NifU protein homolog	Conserved hypothetical proteins	+			
Cj0240c (iscS)	cysteine desulfurase (NifS protein homolog)	Miscellaneous	+			
Cj0262c	putative methyl-accepting chemotaxis protein	Chemotaxis and mobility			+	
Cj0264c	molybdopterin containing oxidoreductase	Miscellaneous				+
Cj0265c	putative cytochrome C-type haem-binding periplasmic protein	energy metabolism		+		+
Cj0284c (cheA)	Chemotaxis histidine kinase	Chemotaxis and mobility		+		
Cj0294	moeB/thiF family protein	Miscellaneous			+	
Cj0333c (fdxA)	Ferredoxin	energy metabolism		+		+
Cj0334 (ahpC)	alkyl hydroperoxide reductase	Detoxification		+		
Cj0339	putative transmembrane transport protein	Other transport/binding proteins			+	
Cj0358	putative cytochrome C551 peroxidase	Detoxification	+			
Cj0378c	Putative integral membrane proteins	Cell envelope		+		
Cj0379c	putative sulfite oxidase subunit YedY	Miscellaneous	+	+		
Cj0380c	hypothetical protein Cj0380c	Unknown	+			
Cj0391c	hypothetical protein Cj0391c	Unknown	+			
Cj0401 (lysS)	Lysyl-tRNA synthetase	Macromolecule metabolism		+		
Cj0403	Hypothetical protein	Conserved hypothetical proteins		+		+
Cj0414	putative oxidoreductase subunit	Miscellaneous	+			+
Cj0415	putative GMC oxidoreductase subunit	Miscellaneous	+		+	
Cj0418c	hypothetical protein Cj0418c	Unknown	+		+	

Cj0421c	Putative integral membrane protein	Cell envelope		+		
Cj0423	Putative integral membrane proteins	Cell envelope		+		
Cj0424	Putative periplasmic protein	Cell envelope		+		
Cj0425	Putative periplasmic protein	Cell envelope		+		
Cj0428	hypothetical protein Cj0428	Unknown	+			
Cj0444	Pseudogene (probable tonB-dependent outer membrane receptor)	Transport/binding proteins		+		
Cj0448c	putative MCP-type signal transduction protein	Signal transduction	+			
Cj0454c	Putative membrane protein	Cell envelope		+		
Cj0481	Putative lyase	Miscellaneous		+		
Cj0481 (dapA)	putative dihydrodipicolinate synthase	Miscellaneous	+	+		
Cj0485	putative oxidoreductase	Miscellaneous			+	
Cj0499	putative histidine triad (HIT) family protein	Miscellaneous			+	
Cj0524	Hypothetical protein	Unknown		+		
Cj0528c (flgB)	Flagellar genes	Cell envelope		+		
Cj0547 (flaG)	Flagellar genes	Cell envelope		+		
Cj0550	Hypothetical protein	Unknown		+		
Cj0559	putative pyridine nucleotide-disulphide oxidoreductase	Miscellaneous	+	+		
Cj0560	Putative integral membrane proteins	Cell envelope		+		
Cj0563	hypothetical protein Cj0563	Unknown	+			
Cj0567	Hypothetical protein	Unknown		+		
Cj0569	hypothetical protein	Unknown				+
Cj0571	Putative transcriptional regulator	Broad regulatory functions		+		
Cj0614 (pstC)	Putative phosphate transport system permease protein	Transport/binding proteins		+		
Cj0628	putative lipoprotein	Cell envelope	+			

Cj0629	putative lipoprotein	Cell envelope	+			
Cj0634 (dprA)	DNA processing protein A	DNA replication, restriction/modification, recombination and repair				+
Cj0644	putative TatD-related deoxyribonuclease protein	Miscellaneous		+	+	
Cj0654c	pseudogene (transmembrane transport protein)	Transport/binding proteins		+		
Cj0667	putative S4 domain protein	Miscellaneous	+			
Cj0688 (pta)	Putative phosphate acetyltransferase	small molecule metabolism		+		
Cj0701	putative protease	Degradation of macromolecules	+			
Cj0720c (flaC)	Flagellar genes	Cell envelope		+		
Cj0723c	putative integral membrane zinc-metalloprotease	Degradation of macromolecules			+	
Cj0742	pseudogene (putative outer membrane protein)	Membranes, lipoproteins and porins			+	
Cj0748	Hypothetical protein	Unknown		+		
Cj0752	Pseudogenes (transmembrane transport protein)	Transport/binding proteins		+		
Cj0755 (cfrA)	Putative iron uptake protein	Transport/binding proteins		+		+
Cj0760	hypothetical protein Cj0760	Conserved hypothetical proteins	+			
Cj0770c	Putative periplasmic proteins	Cell envelope				+
Cj0818	putative lipoprotein	Cell envelope	+	+		+
Cj0819	Hypothetical protein	Unknown		+		
Cj0851c	Putative integral membrane	Cell envelope		+		
Cj0859c	Hypothetical protein	Unknown		+		
Cj0909	Putative periplasmic proteins	Cell envelope		+		
Cj0947c	putative carbon-nitrogen hydrolase	Miscellaneous				+
Cj0948c	putative cation efflux family protein	Drug/analogue sensitivity	+			
Cj0949c	putative peptidyl-arginine deiminase family protein	Miscellaneous	+	+		+

Cj0977	hypothetical protein Cj0977	Conserved hypothetical proteins	+		
Cj0980	putative peptidase	Degradation of macromolecules			+
Cj1005c	putative membrane bound ATPase	Miscellaneous			+
Cj1024c (flgR)	sigma-54 associated transcriptional activator	Signal transduction	+		
Cj1026c (flgP)	putative lipoprotein	Cell envelope	+		
Cj1034c	adenylosuccinate lyase	Chaperones, chaperonins, heat shock	+		
Cj1077	Putative periplasmic protein	Cell envelope		+	
Cj1110c	putative MCP-type signal transduction protein	Signal transduction			+
Cj1113	conserved hypothetical protein Cj1113	Conserved hypothetical proteins	+		
Cj1155c	putative cation-transporting ATPase	Transport/binding proteins	+		
Cj1170c	putative periplasmic protein	Cell envelope	+		
Cj1199	putative iron/ascorbate-dependent oxidoreductase	Miscellaneous	+		
Cj1200	Putative periplasmic proteins	Cell envelope		+	
Cj1201 (metE)	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	Amino acid biosynthesis		+	
Cj1224	Putative iron-binding protein	Transport/binding proteins		+	
Cj1225	Hypothetical protein	Conserved hypothetical proteins		+	
Cj1226c	Putative regulatory functions	Signal transduction		+	
Cj1230 (hspR)	Putative heat shock transcriptional regulator	Chaperones, chaperonins, heat shock		+	
Cj1237c	putative phosphatase	Miscellaneous			+
Cj1242	hypothetical protein Cj1242	Unknown	+		
Cj1287c	malate oxidoreductase	General intermediary metabolism			+
Cj1293 (pseB)	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase	Cell envelope	+		
Cj1294 (pseC)	C4 aminotransferase specific for PseB product	Cell envelope	+		

Cj1295	conserved hypothetical protein	Hypothetical unknown proteins			+	
Cj1306c	hypothetical protein	Hypothetical unknown proteins			+	
Cj1315c (hisH)	imidazole glycerol phosphate synthase subunit HisH	Miscellaneous	+			
Cj1316c (pseA)	pseudaminic acid biosynthesis PseA protein	Cell envelope	+		+	
Cj1324	Hypothetical protein	Cell envelope		+		
Cj1325	Hypothetical protein	Miscellaneous		+		
Cj1327 (neuB2)	N-acetylneuraminic acid synthetase	Cell envelope		+		
Cj1330	hypothetical protein	Hypothetical unknown proteins			+	
Cj1337 (pseE)	PseE protein	Miscellaneous	+			
Cj1340c	hypothetical protein	Conserved hypothetical proteins [122]				+
Cj1341c (maf6)	motility accessory factor	Miscellaneous				+
Cj1342c (maf7)	motility accessory factor	Miscellaneous				+
Cj1343c	putative periplasmic protein	Cell envelope				+
Cj1345c	putative periplasmic protein	Hypothetical unknown proteins			+	+
Cj1353 (ceuC)	Enterochelin uptake	Transport/binding proteins		+		+
Cj1354 (ceuD)	Enterochelin uptake	Transport/binding proteins		+		
Cj1355 (ceuE)	Enterochelin uptake	Transport/binding proteins		+		
Cj1356c	Putative integral membrane protein	Cell envelope		+		+
Cj1357c (nrfA)	putative periplasmic cytochrome C	energy metabolism	+			
Cj1358c (nrfH)	putative periplasmic cytochrome C	energy metabolism	+			
Cj1364 (fumC)	Fumarate hydratase	energy metabolism		+		
Cj1374c	Hypothetical protein	Conserved hypothetical proteins		+		
Cj1375	Putative efflux protein	Drug/analogue sensitivity		+		+

Cj1377C	putative ferredoxin	energy metabolism				+
Cj1383c	hypothetical protein Cj1383c	Unknown	+	+		+
Cj1384c	hypothetical protein Cj1384c	Unknown	+	+		+
Cj1386	ankyrin-repeat containing protein	Miscellaneous	+	+		
Cj1394	putative fumarate lyase	Miscellaneous		+	+	
Cj1407c	Putative phospho-sugar mutase	Central intermediary metabolism		+		
Cj1420c	putative methyltransferase	Hypothetical unknown proteins			+	
Cj1421c	putative sugar transferase	Cell envelope	+			
Cj1422c	possible sugar transferase	Surface polysaccharides, lipopolysaccharides/antigens			+	
Cj1427c	putative sugar-nucleotide epimerase/dehydratease	Surface polysaccharides, lipopolysaccharides/antigens			+	
Cj1450	putative ATP/GTP-binding protein	Unknown	+			
Cj1463	Hypothetical protein	Conserved hypothetical proteins		+		
Cj1464 (flgM)	hypothetical protein Cj1464	Unknown	+			
Cj1465	Hypothetical protein	onserved hypothetical proteins		+		
Cj1476c	pyruvate-flavodoxin oxidoreductase	energy metabolism	+			
Cj1479c (rpsI)	30S ribosomal protein	Ribosomal protein synthesis and modification		+		
Cj1484c	hypothetical protein Cj1484c	Cell envelope	+			
Cj1500	putative inner membrane protein	Cell envelope	+			
Cj1509c (fdhC)	Formate dehydrogenase	energy metabolism		+		
Cj1510c (fdhB)	Formate dehydrogenase	energy metabolism		+		
Cj1511c (fdhA)	Formate dehydrogenase	energy metabolism		+		

Cj1533c	Putative periplasmic protein	Broad regulatory functions		+		
Cj1537c (acs)	acetyl-coenzyme A synthetase	Fatty acid biosynthesis				+
Cj1548c	putative NADP-dependent alcohol dehydrogenase	Miscellaneous			+	
Cj1560	Putative membrane protein	Transport/binding proteins		+		
Cj1581c	putative peptide ABC-transport system ATP-binding protein	Transport/binding proteins	+			
Cj1583c	putative peptide ABC-transport system permease protein	Other transport/binding proteins			+	
Cj1587c	Putative ABC transporter	Transport/binding proteins		+		
Cj1588c	putative MFS transport protein	Other transport/binding proteins			+	
Cj1613c	putative pyridoxamine 5'-phosphate oxidase	Biosynthesis of cofactors, prosthetic groups and carriers	+	+		+
Cj1622 (ribD)	Putative riboflavin-specific deaminase	small molecule metabolism		+		
Cj1627c	hypothetical protein	Hypothetical unknown proteins			+	
Cj1628 (exbB2)	iron transport	Transport/binding proteins		+		+
Cj1629 (exbD2	iron transport	Transport/binding proteins		+		
Cj1631c	conserved hypothetical protein Cj1631c	Unknown	+			
Cj1634c (aroC)	chorismate synthase	Amino acid biosynthesis				+
Cj1637c	Putative periplasmic proteins	Cell envelope		+		
Cj1638 (dnaG)	DNA primase	DNA modification, recombination and repair		+		
Cj1644 (ispA)	Geranyltransferase	small molecule metabolism		+	+	
Cj1650	hypothetical proteins	Conserved hypothetical proteins				+
Cj1656c	hypothetical protein Cj1656c	Unknown	+			
Cj1658	putative iron permease	Transport/binding proteins	+	+		+

Cj1660	putative integral membrane protein	Cell envelope	+	+		
Cj1661	possible ABC transport system permease	Transport/binding proteins	+	+		+
Cj1662	Putative ABC transport system	Transport/binding proteins		+		
Cj1663	putative ABC transport system ATP-binding protein	Transport/binding proteins	+	+		
Cj1664	Thioredoxins	energy metabolism		+		
Cj1665	Thioredoxins	energy metabolism		+		
Cj1669c	Putative ATP-dependent DNA ligase	DNA modification and repair		+		
Cj1677	putative lipoprotein	Cell envelope	+			
Cj1678	putative lipoprotein	Cell envelope	+			
Cj1710c	putative metallo-beta-lactamase family protein	Miscellaneous			+	
Cj1713	putative radical SAM domain protein	Hypothetical unknown proteins		+	+	
Cj1721c	possible outer membrane protein	Membranes, lipoproteins and porins			+	
Cj1724c	putative GTP cyclohydrolase I	Biosynthesis of cofactors, and prosthetic groups			+	
Cj1725	putative periplasmic protein	Cell envelope	+		+	
clpB	ATP-dependent Clp protease ATP-binding subunit	Chaperones, chaperonins, heat shock	+			
ctsD	putative type II protein secretion system D protein	Protein and peptide secretion			+	
ctuA	putative tonB dependant outer membrane receptor	Cations transport/binding proteins			+	
CydB	cytochrome bd oxidase subunit II	energy metabolism	+			
dfp	DNA /pantothenate metabolism flavoprotein	DNA replication, restriction/modification, and repair			+	
dnaK	molecular chaperone DnaK	Chaperones, chaperonins, heat shock	+		+	
dsbB	putative disulfide oxidoreductase	Protein translation and modification			+	
dut	dUTPase	Purines, pyrimidines, nucleosides and nucleotides	+			

exbB1	biopolymer transport protein	Transport/binding proteins	+	+		+
exbB2	putative exbB/tolQ family transport protein	Transport/binding proteins	+	+		
fabF	3-oxoacyl-(acyl carrier protein) synthase II	Fatty acid biosynthesis	+			
fabG	3-oxoacyl-[acyl-carrier protein] reductase	Fatty acid biosynthesis			+	
fbp	putative fructose-1,6-bisphosphatase	General intermediary metabolism			+	
flaA	flagellin	Cell envelope	+		+	
flaB	flagellin	Cell envelope	+	+	+	
flaD (flgL)	putative flagellin	Cell envelope	+	+		+
flaG	flagellar protein FlaG	Cell envelope	+	+		
fldA	flavodoxin FldA	Energy metabolism	+		+	
flgB	flagellar basal body rod protein FlgB	Cell envelope	+			
FlgD	flagellar basal body rod modification protein	Cell envelope	+	+		
FlgE	flagellar hook protein	Cell envelope	+			
FlgE2	flagellar hook protein FlgE	Cell envelope	+			+
flgG	flagellar basal-body rod protein	Cell envelope	+			
flgG2	flagellar basal-body rod protein	Cell envelope	+			+
flgH	flagellar basal body L-ring protein	Cell envelope	+	+		+
flgI	flagellar basal body P-ring protein	Cell envelope	+			+
flgK	flagellar hook-associated protein FlgK	Cell envelope	+	+		
flhB	flagellar biosynthesis protein FlhB	Cell envelope	+			
fliD	flagellar capping protein	Cell envelope	+	+	+	
fliP	flagellar biosynthesis protein FliP	Cell envelope	+			
fliS	flagellar protein FliS	Cell envelope	+			
ftsH	membrane bound zinc metallopeptidase	Degradation of macromolecules			+	

fur	ferric uptake regulator	Broad regulatory functions	+		
glf	UDP-galactopyranose mutase	Surface polysaccharides, lipopolysaccharides/antigens			+
glnH	glutamine-binding periplasmic protein	Amino acids and amines transport/binding proteins			+
GltB	glutamate synthase (NADPH) large subunit	Central intermediary metabolism	+		
glyA	serine hydroxymethyltransferase	Amino acid biosynthesis	+	+	
gmhA2	putative phosphoheptose isomerase	Surface polysaccharides, lipopolysaccharides/antigens			+
grpE	heat shock protein GrpE	Chaperones, chaperonins, heat shock	+		
hddA	putative sugar kinase	Surface polysaccharides, lipopolysaccharides/antigens			+
hddC	putative sugar-phosphate nucleotidyltransferase	Surface polysaccharides, lipopolysaccharides/antigens			+
hipO	hippurate hydrolase	Miscellaneous			+
hisF	imidazole glycerol phosphate synthase subunit	Miscellaneous	+		
hisS	histidyl-tRNA synthetase	Aminoacyl tRNA synthetases and their modification			+
hrcA	heat-inducible transcription repressor	Chaperones, chaperonins, heat shock	+		
hydA	Ni/Fe-hydrogenase small chain	Energy metabolism	+		
katA	catalase	Detoxification	+	+	+
kdtA	3-deoxy-D-manno-octulosonic-acid transferase	Surface polysaccharides, lipopolysaccharides/antigens			+
kpsT	capsule polysaccharide export ATP-binding protein	Surface polysaccharides,			+

		lipopolysaccharides/antigens				
leuC	3-isopropylmalate dehydratase large subunit	Amino acid biosynthesis			+	
ligA	NAD-dependent DNA ligase LigA	Synthesis and modification of macromolecules	+			
livF	branched-chain amino-acid ABC transport system ATP-binding protein	Transport/binding proteins	+			
livH	amino-acid ABC transport system permease	Amino acids and amines transport/binding proteins			+	
livJ	branched-chain amino-acid ABC transport system periplasmic binding protein	Transport/binding proteins	+			
livK	branched-chain amino-acid ABC transport system,periplasmic binding protein	Transport/binding proteins	+			
lysS	lysyl-tRNA synthetase	Synthesis and modification of macromolecules	+	+		
maf6	motililty accessory factor	Surface structures			+	
map	methionine aminopeptidase	Protein translation and modification			+	
metA	putative homoserine O-succinyltransferase	Amino acid biosynthesis			+	
metE	homocysteine methyltransferase	Amino acid biosynthesis	+	+		
metY (metB)	putative O-acetylhomoserine (thiol)-lyase	Amino acid biosynthesis	+		+	
modB	putative molybdenum transport system permease protein	Anions transport/binding proteins			+	
modC	putative molybdenum transport ATP-binding protein	Transport/binding proteins	+			
murD	UDP-N-acetylmuramoylalanine-D-glutamate ligase	Murein sacculus and peptidoglycan			+	
murE	UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	Cell envelope				+

napA	nitrate reductase	Energy metabolism	+		+	
napG	putative ferredoxin	Energy metabolism			+	
neuA1	acylneuraminate cytidylyltransferase	Surface polysaccharides, lipopolysaccharides/antigens			+	
p19	periplasmic protein p19	Cell envelope	+	+		+
peb3	major antigenic peptide PEB3	Cell envelope	+			
proS	prolyl-tRNA synthetase	Aminoacyl tRNA synthetases and their modification			+	
pseF	acylneuraminate cytidylyltransferase	Surface polysaccharides, lipopolysaccharides/antigens			+	
ptmB	acylneuraminate cytidylyltransferase	Surface structures			+	
purU	formyltetrahydrofolate deformylase	Purines, pyrimidines, nucleosides and nucleotides			+	
pyrB	aspartate carbamoyltransferase	Purines, pyrimidines, nucleosides and nucleotides			+	
rplK	50S ribosomal protein L11	Synthesis and modification of macromolecules	+			
rpmB	50S ribosomal protein L28	Synthesis and modification of macromolecules	+			
rpsJ	30S ribosomal protein S10	Ribosomal protein synthesis and modification			+	
rpsN	30S ribosomal protein S14	Synthesis and modification of macromolecules	+			
rpsQ	30S ribosomal protein S17	Synthesis and modification of macromolecules	+			
rpsU	30S ribosomal protein S21	Synthesis and modification of macromolecules	+			
secA	preprotein translocase SECA subunit	Protein and peptide secretion			+	

serC	phosphoserine aminotransferase	Amino acid biosynthesis			+		
sodB	superoxide dismutase (Fe)	Detoxification	+				
surE	surE protein homolog	Drug/analogue sensitivity and antibiotic resistance			+		
thiC	thiamine biosynthesis protein ThiC	Biosynthesis of cofactors, prosthetic groups and carriers	+				
thyX	thymidylate synthase	Miscellaneous			+		
tonB3	TonB transport protein	Transport/binding proteins	+			+	
trpB	tryptophan synthase subunit beta	Amino acid biosynthesis	+				
trxB	thioredoxin reductase	Biosynthesis of cofactors, prosthetic groups and carriers	+	+	+	+	+
tupB	putative anion-uptake ABC-transport system permease	Anions transport/binding proteins			+		
uvrA	excinuclease ABC subunit A	DNA replication, restriction/modification, and repair			+		
uvrB	excinuclease ABC subunit B	DNA modification, and repair			+		
waaE (hldE)	D-beta-D-heptose 7-phosphate kinase/D-beta-D-heptose 1-phosphate adenylyltransferase	Cell envelope	+				
zupT	putative zinc transporter	Hypothetical unknown proteins			+		

[*]**A:** CjFur regulon identified in the present study

B: CjFur regulon identified by Holmes *et al*. (180)

C: CjFur-targets identified by Butcher *et al*. (58)

D: CjFur regulon identified by Palayda *et al*. (338)

E: Common genes in all studies.

Momen Askoura

Curriculum vitae

Personal information

Name: Momen Mahmoud Ez ElArab Abd ElAziz Askoura

Office address: Department of Biochemistry, Microbiology and Immunology. Microbiology & Immunology program. Faculty of Medicine.University of Ottawa, Canada.

Educational Background

1 **October 1996 to 2000:** Zagazig University, Egypt. Bachelor of Pharmaceutical Science; with general grade excellent with honor degree.
2 **October 2000 to December 2001:** Zagazig University, Egypt. passed the prerequisite graduate courses towards Master's of Science degree in Pharmaceutical Sciences.
3 **January 2001 to December 2005:** Zagazig University, Egypt. Master's degree in Pharmaceutical sciences (Microbiology)
4 **May 2009 to present:** Study my PhD in Microbiology and immunology program, department of Biochemistry, Microbiology and Immunology, university of Ottawa, Ottawa, ON, Canada

Work Experience

1- December 2001 to 2005: Teaching and Research assistant, Department of Microbiology, Zagazig University, Egypt.

Duties include - Demonstrating laboratory activities
- Marking laboratory reports
- Performing Molecular biology and Microbiology research under the direct supervision of principal investigator

2**- Dec. 2005 to May 2009:** Pre-doctoral research; Zagazig University, Egypt.

Duties include - Teaching different Microbiology courses to undregraduate students at the Faculty of Pharmacy.
- Demonstrating laboratory activities
- Preparing course materials.

- Demonstrating Microbiology laboratory activities.
- Preparing laboratory reagents for teaching purposes
- Marking laboratory reports.

3- **May 2009 to present:** Doctoral research; Faculty of Medicine, Department of Biochemistry and Microbiology, Ottawa University, Ottawa, Canada

Laboratory skills acquired:

1-Microbiological and biotechnological techniques

2-Production and assays of enzymes

3-Isolation of plasmid DNA

4- Screening of plasmid DNA by agarose gel electrophoresis

5-Protein analysis by polyacrylamide gel electrophoresis

6-Western blotting technique

7-Bacterial transformation

8- Microarray techniques

Language skills

Arabic and English

Awards and honors

Award of success from the Egyptian Pharmacists Syndicate, 2006.

1ST place in BMI poster day, University of Ottawa, Ottawa, Canada, 2011

Scholarships

Scholarship from the Egyptian Government to study Ph.D in Canada, 2009-2014

Personal training and academic career:

1-Intensive training course on applications of biotechnology, 2006 at the Department of Biotechnology, Institute Of Graduate Studies And Research Alexandria University, Egypt.

2- Course in computer science, 2002, Zagazig University, Zagazig, Egypt.

3- ICDL (International Computer Driving Licence) , 2008.

4- Ottawa Institute of Systems Biology (OISB) Symposium, Montabello, Quebec, Canada, May 8th and 9th, 2011.

Courses

Courses are Taken in Faculty and Leadrship Development Project (FLDP) Centre, Zagazig University, Egypt.

1-Effective connection skills course (20: 22/12/2005)

2-Thinking skills course (16:18/5/2006)

3-Techniques of scientific research course (28: 31/1/2006)

4-Job ethics and rules course (3:5/2/2007)

5-University lecturer preparation course (3: 18/3/2007).

6-Using technology in teaching course (1: 3 /7 /2007)

7-Effective presentation course (8: 10 /7 /2007).

Courses taken in Faculty of Medicine, Ottawa University, Ottawa, Canada.

8- Course in Bacteriology September: December, 2009 (Final grade A).

9- Course in HIV December: April 2010 (Final grade A).

10- Animal training course 2010.

11- Autoclaving, Lab. Safety and (WHMIS) Workplace Hazardous Materials Information System 2009:2010.

Selected Presentations

1- Departmental seminar in 2005 at the Department of Microbiology, University of Zagazig, Egypt about my Master research project "*In vitro* study on the pathogenicity of *Proteus spp* isolated from urinary tract infections"

2- Poster presentation in the Microbiology and immunology poster day of Faculty of Medicine, Ottawa University, Ottawa, Canada. Under the title of (Acid adaptive mechanisms *of Campylobacter jejuni* in the gastrointestinal tract), 2010.

3- Presentation in the Microbiology and immunology Symposium day of Faculty of Medicine, Ottawa University, Ottawa, Canada. Under the title of (Acid adaptive mechanisms *of Campylobacter jejuni* in the gastro intestinal tract), 2011.

2- Poster presentation in the Microbiology and immunology poster day of Faculty of Medicine, Ottawa University, Ottawa, Canada. Under the title of (Acid adaptive mechanisms *of Campylobacter jejuni* in the gastrointestinal tract), 2012.

3- Presentation in the Microbiology and immunology Symposium day of Faculty of Medicine, Ottawa University, Ottawa, Canada. Under the title of (Acid adaptive mechanisms *of Campylobacter jejuni* in the gastro intestinal tract), 2013.

<u>Conference Presentations</u>

- **Momen Askoura and Alain Stintzi.** '‘Acid adaptive mechanisms of *Campylobacter jejuni* in the gastrointestinal tract”.*Campylobacter*, *Helicobacter* and Related Organisms (CHRO) Conference, Aberdeen, Scotland, September, 2013, poster presentation
- **Momen Askoura and Alain Stintzi.** ‘Acid adaptive mechanisms of *Campylobacter jejuni* in the gastrointestinal tract”. Canadian Society of Microbiology (CSM), Carleton University, Ottawa, Canada, 2013, poster presentation.
- **Momen Askoura and Alain Stintzi.** ‘Acid adaptive mechanisms of *Campylobacter jejuni* in the gastrointestinal tract”. Ottawa Institute of Systems biology (OISB) Symposium, Mont-tremblant, QC, Canada, 2013, poster presentation.
- **Momen Askoura and Alain Stintzi.** ‘Acid adaptive mechanisms of *Campylobacter jejuni* in the gastrointestinal tract”. Canadian Campylobacter Workshop (CCW), ON, Canada, 2012, poster presentation.
- **Momen Askoura and Alain Stintzi.** ‘Acid adaptive mechanisms of *Campylobacter jejuni* in the gastrointestinal tract”. Ottawa Institute of Systems Biology (OISB), Montebello, QC, Canada, 2011, poster presentation

<u>Master’s degree thesis research project (Completed)</u>

In vitro study on the pathogenicity of *Proteus spp* isolated from urinary tract infections

<u>PhD thesis research project</u>

Acid adaptive mechanisms of *Campylobacter jejuni* in the gastrointestinal tract

<u>Publications</u>

F. M. E. Serry, M. K. Okasha, H. A. Abdel Salam and **<u>M. M. Ez ElArab Askoura.</u>** **(2006):** The role of adherence and urease in pathgenicity of *Proteus* isolates from urinary tract infections. **New Egyptian Journal of Microbiology. 14:**331-343.