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Campylobacter jejuni NCTC 11168 Response to Protamine Sulfate

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CAMPYLOBACTER JEJUNI NCTC 11168 RESPONSE TO PROTAMINE SULFATE

By

Nabil Hayek

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the M.Sc. degree in Microbiology and Immunology

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Abstract

Campylobacter jejuni is the principal cause of gastroenteritis worldwide. Contaminated chicken is the most important source of human infection by *Campylobacter.* In its hosts, *C. jejuni* colonizes the gastrointestinal tract despite the constitutive and induced secretion of cationic antimicrobial peptides (CAMPs). These peptides are a major component of the host's innate immune system and have been found to be effective against different bacterial species including *Campylobacter.* These observations suggest that *Campylobacter* has developed mechanisms to evade CAMPs. To test this hypothesis and identify genes presumably involved in adaptation and/or resistance to CAMPs, two approaches were pursued. In the first approach, the transcriptome profile of *C. jejuni* NCTC 11168 was analyzed upon exposure to increasing concentrations of protamine sulfate, which is a known antimicrobial peptide. In the second approach, a *Campylobacter* transposon mutant library was screened against two concentrations of protamine. The data obtained from both approaches revealed genes that could be important to overcome the effect of the studied antimicrobial agent and potentially other antimicrobial peptides. The transcriptome profile study unveiled the importance of two genes Cj1721c and Cj0355c. Cj1721c is an outer membrane protein with an unknown function, and Cj0355c, is an essential two-component system regulator. Phenotypic studies demonstrated that the Cj1721c knockout was more resistant to protamine, more motile, and had reduced biofilm-forming ability compared to the wild-type strain. In contrast, the strain that overexpresses Cj0355c was more sensitive to protamine and had greater biofilm-forming ability compared to the wild-type strain. Furthermore, it was attenuated in its ability to colonize the gastrointestinal tract of new born

piglets. Moreover, transcriptome profiling of the strain that overexpresses Cj0355c in MH and MEM medium revealed potential pathways that could be regulated by this regulator. This observation suggests that membrane integrity could be sensed by a still unknown sensor and then the signal transferred to Cj0355c, which affects the expression of key genes that are involved in resistance to protamine sulfate.

In summary, this study identifies previously uncharacterized genes (Cj1721c and Cj0355c) that are involved in the resistance to protamine sulfate and potentially to other antimicrobial peptides. In addition, the performed transposon mutants library screening indicates that *Campylobacter jejuni* NCTC 11168 can intrinsically evade the effect of protamine sulfate. On the other hand, we showed that *C. jejuni* does not use the multidrug efflux pump (MATE) to resist the effect of protamine.

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LIST OF ABBREVIATIONS

ABC	ATP-Binding Cassette
Amp	Ampicillin
AMP	Adenosine MonoPhosphate
Aps	Antimicrobial peptide sensor
Arg	Arginine
bp	Base Pairs
BPI	Bactericidal/Permeability-Inducing protein
CAMH	Cation Adjusted Mueller Hinton
CAMP	Cationic Antimicrobial Peptide
CAT	Chloramphenicol acetyltransferase (resistance cassette)
CDS	Coding Sequence
CDT	Cytolethal Distending Toxin
CFIA	Canadian Food Inspection Agency
CFU	Colony Forming Unit
CLSI	Clinical and Laboratory Standards Institute
Cm	Chloramphenicol
CRISPR	Clustered Regularly Interspaced Short Palindromic Repe
DccRS	Diminished Capacity to Colonize
DEPC	Diethylpyrocarbonate
EDTA	Ethylene Diamine Tetraacetic Acid
Fur	Ferric Uptake Regulator
GBS	Guillain-Barré Syndrome
GI	Gastrointestinal
hCAP18	human Cationic Peptide 18 KD
HD	Human Defensin
HBD	Human Beta Defensin
HK	Histidine Kinase
HNP	Human Neutrophil Peptides
IPTG	Isopropyl β-D-1-Thiogalactopyranoside
Km	Kanamycin
LB	Luria Bertani
LOS	Lipooligosaccharide

LOWESS	Locally Weighted Scatterplot Smoothing
LPS	Lipopolysaccharide
MATE	Multidrug And Toxic compound Extrusion
MEM	Minimum Essential Medium
MF	Major Facilitator
MH	Mueller Hinton
MIC	Minimum Inhibitory Concentration
Mtr	MultiTransferable Resistance
NCTC	National Collection of Type Cultures
NLPA	New LipoProtein A
O.D.	Optical Density
ORF	Open Reading Frame
PAMP	Pathogen-Associated Molecular Pattern
PA β N	Phenylalanine-Arginine β-Naphthylamide
PCR	Polymerase Chain Reaction
PES	Polyethersulfone
PRR	Pattern Recognition Receptor
qRT-PCR	Quantitative Reverse-Transcriptase PCR
RacRS	Reduced Ability to Colonize
RELM β	Resistin-Like Molecule β
Rpm	Revolutions Per Minute
RND	Resistance-Nodulation-Division
ROS	Radical Oxygen Species
RR	Response Regulator
SDS	Sodium Dodecyl Sulfate
SMR	Small Multidrug Resistance
spp	Species
T4SS	Type 4 Secretion System
TCS	Two-Component signal transduction System
TLR	Toll-Like Receptor
VBNC	Viable But Not Culturable
WT	Wild-type

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CHAPTER ONE: GENERAL INTRODUCTION

Campylobacter fetus was first identified in Britain in 1906 in reported cases of sheep and cattle abortion (McFadyean and Stockman, 1913). In 1947, the organism was isolated from the blood of pregnant women (Vincent et al., 1947). In 1963 the *Campylobacter* genus was proposed (Sebald and Véron, 1963); however, the culturing of this bacterium was not possible in the lab until the early 1970s (Butzler et al., 1973).

Bacteria of the family Campylobacteriaceae are small, spiral-formed, Gram-negative, microaerophilic bacteria that are able to grow at a temperature range between 30°C and 47°C (Boone et al., 2005). This family includes 18 species, six subspecies and two biovars (Humphrey et al., 2007). Although *Campylobacter* spp. can often exist in the gastrointestinal tract of animals without causing any disease, it is the leading cause of diarrhea in humans (Park, 2002). Moreover, *Campylobacter jejuni* is thought to be a causative agent of Guillain-Barré (Rhodes and Tattersfield, 1982) and Miller-Fisher syndromes (Clavelou et al., 1989). Of the *Campylobacter* spp., *C. jejuni* and *C. coli* are considered to be the most ubiquitous in the developed world, accounting for about 95% of *Campylobacter* infections in humans (Park, 2002). Only a few hundred bacteria are required for infection, and the incubation period is between 1 to 10 days, with the majority of patients showing onset of symptoms by day 4 (Humphrey et al., 2007). The gastrointestinal manifestations are common and include profuse and often bloody diarrhea, acute abdominal pain, and fever (Drake et al., 1981).

The severity and duration of the symptoms might vary among individuals. Recovery is generally rapid and occurs after few days of infection with bed rest and the maintenance of body fluid balance (Humphrey et al., 2007).

Campylobacter spp. can be present in a variety of environmental niches despite the fact that they are microaerophilic organisms. The most known and studied sources of infection for humans include undercooked poultry (Fields and Swerdlow, 1999), contaminated water (Blaser et al., 1980), and unpasteurized dairy products (Klein et al., 1986).

1.1. Epidemiology

In the recent 2006 American FoodNet report of enteropathogens infection, *Campylobacter* cases (5,712) were second after *Salmonella* (6,655), with *Shigella* in third place (2,736) (www.cdc.gov/factsandfigures). The estimation of the overall incidence in 10 U.S. States was 12.71 per 100,000 persons, dropping from 15 in 2004. However, these cases do not reflect the exact number of infected patients since they only account for patients who have sought medical attention. Most cases are sporadic, isolated and occur more frequently during the summer (Tee et al., 1986). Infection with *Campylobacter* is seen more frequently in infants and young adults than in other age groups (Hollander, 1981). In Canada, according to the report of the Public Health Agency 2006, *Campylobacter* cases are still the highest among all enteropathogens (Laboratory Surveillance Data For Enteric Pathogens in Canada, 2006). In Ontario, the average incidence rate is 42.3 cases per 100,000 people during 1997-2001 (Varela et al., 2007). Higher disease rates were reported in other developed countries such as England and Wales, in which the number of cases was estimated to be around 80 per 100,000 in 2005 (Department for Environment Food and Rural Affairs).

1.2. *Campylobacter* genome and virulence factors

The genome of *C. jejuni* NCTC 11168 was sequenced in 2000 (Parkhill et al., 2000), reanalyzed and re-annotated in 2007 (Gundogdu et al., 2007). According to the latter study *C. jejuni* genome is 1.6 Mb in length with 1643 potential coding sequences (CDS). *C. jejuni* is naturally competent, a feature that facilitates the uptake of foreign DNA and results in the increased genetic diversity of this microorganism. This phenomenon leads to a faster capacity of adaptation to various niches (Wang and Taylor, 1990). Moreover, an important encountered feature of the genome is the presence of homopolymeric tracts which play a role in phase variation of genes expression (Hendrixson, 2006). In addition, hypervariable regions were identified. These regions code for proteins that are required for the synthesis of components of the outer membrane structure such as lipooligosaccharides (LOS), flagellum, and the capsule (Young et al., 2007). These components are major virulence factors in *Campylobacter* (Table 1), and their structural variation allows the bacterium to resist and adapt to different encountered stresses in the environment and hosts by altering the recognized or affected bacterial patterns and molecules (Bacon et al., 2001; Fry et al., 2000; Park et al., 2000). These key virulence factors will be discussed further in this section.

First, the two post-translational proteins glycosylation systems (Szymanski et al., 2003); The *O*-linked system, which is involved in the flagellin glycosylation and the *N*-linked system, which is responsible for general protein glycosylation. The *N*-glycosylation is an essential feature of protein modifications in eukaryotes. However, it is observed in the bacterium *C. jejuni.* A total of 14 genes (Cj1119c-Cj1130c), named *pgl* for protein glycosylation, were related to this system in *C. jejuni* (Szymanski and Wren, 2005). The

glycosylation occurs at the protein Asn-X-Ser/Thr consensus sequence (X represent any amino acid except proline) (Szymanski and Wren, 2005). On the other hand the *O*-linked system consists of 50 genes (Cj1293-Cj1342) that were found to play a role in either motility or glycan biosynthesis. In this system the glycosylation occurs at the Ser/Thr residues (Doig et al., 1996) Together the *O-, N-* linked glycosylation systems play several important roles in bacteria-host interaction. For instance, they are crucial in modifying antigenic proteins in flagellum filament assembly (Logan et al., 2002), in adhesion and invasion (Szymanski et al., 2002), and in protection against proteolytic cleavage (Szymanski and Wren, 2005).

Another important virulence factor is the flagellum. In *Campylobacter,* the flagellum is not only crucial for motility and colonization but it is also important for protein secretion (Konkel et al., 2004). The flagellum is used as a secretory machine for certain flagellar and non-flagellar proteins such as *Campylobacter* invasion antigens (Cia) that are required for invasion of the epithelial cells (Konkel et al., 2004).

Lastly, the capsule genes *(kps)* also play a role in virulence (Zilbauer et al., 2005). However, the capsule's role in protecting *Campylobacter* against the innate immune system, especially antimicrobial peptides, has not been fully elucidated (Zilbauer et al., 2005; Zilbauer et al., 2008). The authors suggested that the capsule importance in resistance depends on the type of the secreted CAMPs (cationic antimicrobial peptides) during the colonization process since capsule-lacking *C. jejuni* exhibited different susceptibilities to different antimicrobial peptides.

Table 1. Identified virulence factors in *Campylobacter jejuni.*

The table indicates the major described virulence factors and their functions

Table 1. Identified virulence factors in *Campylobacter jejuni*

Virulence factor	Gene I.D	Annotation/Role	Reference
Adherence	*jlpA*	*jejuni* lipoprotein A	Jin et al., 2003
	cadF	Outer membrane fibronectin-binding protein	Konkel et al., 1997
	peb1	Asp/Glu-binding ABC transporter protein	Pei et al., 1998
	capA	*Campylobacter* adhesion protein A	Ashgar et al., 2007
Colonization/Invasion	*cia*	*Campylobacter* invasion antigen	Konkel et al., 2004
	dksA	*dnaK* suppressor	Yun et al., 2008
	ccpA-1 / ccpA-2	Cytochrome C peroxidase	Bingham-Ramos and Hendrixon, 2008
	ppk1	Polyphosphate kinase 1	Candon et al., 2007
Flagella	*flaC / Cj0977 / fliA / FspA*	• Colonization • Invasion of epithelial cells in vitro • Secretion of cia, FlaC, FspA • Regulation non flagellar genes cj0977/FspA	Song et al., 2004 / Yokoyama et al., 2008 / Fernando et al., 2007 / Goon et al., 2006
Cytolethal Distending Toxin	*cdtABC*	• Eukaryotic DNA breakage • Cells arrest in the G_2 phase • Induces apoptosis of macrophages • Induces IL-8 secretion	Johnson and Lior, 1988
Capsules	*Cj1413c-Cj1448c*	• Adherence in vitro • Serum resistance • Colonization of ferret model	Bacon et al., 2001
Lipooligosaccharide Flagellar glycosylation	*Cj1119c - Cj1130c* *Cj1293 - Cj1342*	• Hypervariable • Immune system avoidance • Serum resistance	Guerry et al., 2002
Iron acquisition	*cfrA*	*Campylobacter* ferric enterobactin uptake	Palyada et al., 2004
	Cj0178	Lactoferrin, transferrin-bound iron utilization	Miller et al., 2008
	feoB	Ferrous iron transport protein	Naikare et al., 2006
	ceuBCDE	*Campylobacter* enterochelin uptake system	Palyada et al., 2004

Virulence factor	Gene I.D	Annotation/Role	Reference
Oxidative stress	*csrA*	Carbon storage regulator	Fields and Thompson, 2008
DNA modification	*Cj1461*	DNA methyltransferase	Kim et al., 2008
Transporters	*cmeABC*	Bile and antimicrobials resistance	Lin et al., 2002
Two component system	*dccR*	Decrease capacity to colonize	Mackichan et al., 2004
	cprS	Campylobacter planktonic growth regulation	Svensson et al., 2009
	cbrR	Sodium deoxycholate resistance / colonization	Raphael et al., 2005

1.3. Characteristics of *C. jejuni* colonization

Interestingly, *C. jejuni* can colonize and invade human gastrointestinal epithelial cells causing an acute infection (Prasad et al., 1996) while it is merely commensal in chicken (Hendrixson and DiRita, 2004). To explain this variation, two recent studies have tried to study the chicken immune response at day-of-hatch and at two weeks post hatch after being exposed to *C. jejuni* (Smith et al., 2008; Van Deun et al., 2008). These two studies have not detected any increased heterophil influx, indicating that the epithelial cells are not damaged by *C. jejuni.* However, it was found that the invasion had occurred locally at the intestinal level and then systemically affected the spleen and liver. In their study, Smith and colleagues demonstrated that proinflammatory cytokines such as IL-6, CXCL 1-2, and IL-1, were induced in infected birds. The authors concluded that *Campylobacter* leads to an inflammatory response in poultry, but that it was not eliminated from the chicken and it did not cause disease. Interestingly, they implied that the difference in the innate immunity repertoire between humans and chickens might explain the difference between the pathology in chicken and in humans. On the other hand, Van Deun's group suggested an alternate mechanism for *Campylobacter* persistence in the chicken gut. They proposed that *Campylobacter* can temporally invade and evade epithelial cells response and during these cycles it can multiply in the intestinal mucus. In addition, the authors speculated that *Campylobacter* might be able to manipulate the chicken immune response like other commensal bacteria to escape host immune surveillance.

1.4. Bacterial cell surfaces

The bacterial cell membrane forms the part that is in contact with the cell's external and internal environment. Therefore, its design and components give bacteria not only their shape but more importantly, protect them against changes that might be deleterious for their survival. There are 2 types of bacterial cell walls, one for Gram positive bacteria and one for Gram negative bacteria. In Gram negative bacteria, the cell wall is composed of a cytoplasmic membrane, a thin peptidoglycan layer, and an outer membrane. In Gram positive bacteria, the cell wall is composed of a thick peptidoglycan layer and a cytoplasmic membrane. Conserved proteins embedded in the outer membrane are important factors in triggering the host-pathogen interaction (Akira et al., 2001). These special motifs, which are known as "pathogen-associated molecular pattern" (PAMPs), are recognized by the host "pattern recognition receptors" (PRRs) (Kaisho and Akira, 2000), such as Toll-Like receptors (Anderson, 2000). This interaction prompts the host innate immune response. Therefore, the bacterial membrane can play two opposite functions, it can trigger the host immune response as well as protect the bacteria from the host immune system.

1.4.1. Cytoplasmic membrane

The cytoplasmic membrane is composed of a phospholipid layer and acts as a selective barrier for certain ions and nutrients. In addition, it is the location for different metabolic processes such as respiration, lipid synthesis, and cell wall building block synthesis (Willey et al., 2007).

1.4.2. The outer membrane and peptidoglycan layer

Gram positive and Gram negative bacteria differ in their cell wall structural arrangement. The Gram positive bacteria have a thick (20-80 nm) homogenous peptidoglycan layer above the cytoplasmic membrane while the Gram negative bacteria have a thin layer of peptidoglycan (2-7 nm) that is embedded in the periplasm, which is the region between the outer and the inner membranes (Salton, 1953). The outer membrane of most Gram negative bacteria is composed of phospholipids and Lipopolysaccharides (LPS) that stabilizes the outer membrane structure, helps bacterial attachment to surfaces, and creates a permeability barrier by restricting the entry of bile salts and harmful hydrophobic substances (Perkins 1963). The LPS consists of a lipid part (Lipid A) that is the main toxic section and a polysaccharide part that varies in length and composition (Willey et al., 2007). Lipid A is referred to as endotoxin while LPS is the trigger of the host immune response due to the presence of the O side chain on its chemical structure (Rothfield and Horecker, 1964). In Gram positive bacteria the peptidoglycan layer is composed mainly of murein and teichoic acids. The teichoic acids bind the peptidoglycan layer to the cytoplasmic membrane (Boix and Nogues., 2007).

The presence of teichoic acid in Gram positive and LPS in Gram negative bacteria render the bacterial cell wall negatively charged (Willey et al., 2007). The host takes advantage of this common bacterial characteristic to eliminate the invading pathogens by secreting positively charged antimicrobial peptides as a component of the innate immunity system (Ganz et al., 1985).

1.4.3. *C. jejuni* variable outer membrane

C. jejuni outer membrane is composed of lipooligosaccharides, LOS (Adeyeye et al., 1989) instead of LPS like other Gram negative bacteria. Structural variability is a crucial feature of this cellular component. This variability is due to different modifications such as the addition of different monosaccharide or the altered linkage between the sugar units or the derivatization of the monosaccharides with non-carbohydrate moieties (Parker et al., 2008). Eight LOS biosynthesis locus classes were defined (Parker et al., 2008). Three classes (A, B, and C) encode for the sialylated LOS, while the remaining five (D, E, F, G, and H) lack the sialic acid transferase. Recently, another 11 classes were identified (Parker et al., 2008). These new loci were found to result from the recombination of the original eight classes. The highly variable LOS structure in *C. jejuni* plays different roles, including adherence and invasion of eukaryotic cells (Guerry et al., 2002). In addition, the LOS resemblance to human carbohydrate moieties of gangliosides can induce autoantibody production against nerve gangliosides leading to Guillain-Barré syndrome, or GBS (Perera et al., 2007; Yuki et al., 2004). Interestingly, a recent study has demonstrated the role of LOS in antimicrobial resistance in *C. jejuni* 11168 (Jeon et al., 2009). In this study, the authors were able to show that disrupting the LOS structure, by constructing a knockout in the *waaF* gene, affects the bacterial surface hydrophobicity and alters the sensitivity of the mutant strain to antimicrobial drugs such as erythromycin. It is worth noting that the sensitivity to polymyxin B, which is an antimicrobial peptide, was not different between the wild-type strain and the LOS knockout mutant.

Proteins are another variable component of *C. jejuni* outer membrane. Interestingly, a recent study has shown a difference in outer membrane protein expression between poor and robust chicken colonization by *Campylobacter* isolates (Hiett et al., 2008). The study emphasizes the importance of the *Campylobacter* outer membrane in adaptation to a variety of hosts and environments by its ability to modify the membrane structures accordingly (Hiett et al., 2008).

Lastly, flagellin is another highly variable surface structure in *Campylobacter.* Variability is due to the alterations in flagellin glycosylation pattern that can affect the virulence of *C. jejuni* 81-176 (Guerry et al., 2006). Moreover, although FlaA, which is a flagellar filament protein, has 19 sites of *O*-linked glycosylation (Logan et al., 2002), researchers have identified new flagellin carbohydrate modifications in *C. jejuni* 11168 (Logan et al., 2009). The structure of these newly identified carbohydrates was confirmed to be a dimethylated derivative of pseudaminic acid.

The above mentioned structural variations are important for *C. jejuni* to evade the host immune system and to be able to adapt to various environmental changes.

1.5. Antimicrobial peptides

One of the stresses that bacteria encounter upon entering a host are antimicrobial peptides. These molecules are part of the innate immune system, and they are found in all biological kingdoms (Ganz et al., 1985). In human, different peptides have been isolated from different organs in recent years and researchers classified them in different groups according to their size and structure (Ganz et al., 1985). Although these categories have

different biophysical parameters and chemical structures, recent reports have indicated the presence of a shared common motif mainly among the molecules with cysteine residues, such as defensins in human and gallinacins in chicken (Yeaman et al., 2007). This motif was named the γ-core signature which consists of three residues (glycine-X-cysteine), where X is any amino acid. Additionally, the authors were able to identify a positive charge distributed to the pole of the motif. Interestingly, variations of this motif also exist in other families of antimicrobial peptides. In some cases, the presence of this motif is enough to confer an antimicrobial activity to these peptides. However, not all the molecules with similar motif have the same mechanism of action (Yount and Yeaman., 2004; Yeaman et al., 2007).

Since *Campylobacter* spp. colonize the gastrointestinal tract of chicken and humans, only the antimicrobial peptides that are constitutively secreted or induced upon infection in the intestine of these two hosts will be discussed below in further details.

1.5.1. Antimicrobial peptides of the human gastrointestinal tract

The following peptides have been identified in the human intestinal tract (Dann and Eckmann, 2007):

I- Defensins: alpha and beta-defensins

II- Cathelicidins

III- Resistin-like molecule β (RELMβ)

IV- Bactericidal/permeability-inducing protein (BPI)

V- Antimicrobial lectins

1.5.1.1 Defensins

Defensins are considered the principal antimicrobial peptide group in the small intestine (Cunliffe, 2003; Wehkamp and Stange, 2006). They are characterized by three disulfide bonds and a β-sheet structure (Ganz et al., 1990; Lehrer et al., 1991). It is important to note that neither the exact number of the genes coding for these molecules in humans nor their functions and/or mode of antimicrobial action are fully characterized. Six alpha-defensins have been discovered so far (Ganz et al., 1985). Human neutrophils peptides (HNP-1 to HNP-4), are expressed by neutrophils, while Human Defensins HD-5 and HD-6 are expressed in Paneth cells in the crypts of the small intestine (Porter et al., 1997; Mallow et al., 1996). These peptides are synthesized as propeptides and become active following trypsin cleavage (Ganz, 2003). In addition to the alpha-defensins, 4 beta-defensins have been identified (Wehkamp and Stange, 2006). Human beta-defensin-1 (HBD-1) is constitutively expressed by the intestinal epithelial cells, human beta-defensin-2 (HBD-2) is induced upon infection or intestinal inflammation, and human beta-defensin-3 and -4 (HBD-3, HBD-4) are inducible and expressed by paneth cells in the crypts of the small intestine as well as in the colon (Wehkamp and Stange, 2006). Generally, these peptides are active at a very low concentration (1-10 μg/ml) (Ganz, 2003), and they are more potent against bacteria that are metabolically active rather than bacteria in stationary phase or in nutrient deprived medium (Ganz, 2003).

1.5.1.2. Cathelicidins

The LL-37-human cationic protein 18KD (hCAP18) is expressed and stored as an inactive precursor in various cells such as neutrophils, mast cells, stomach, ileum, and the colon epithelial cells (Agerberth and Gudmundsson, 2006). Benefiting from the fact that cathelicidin expression is up-regulated in response to short fatty acid products (Schauber et al., 2003), Raqib and colleagues were able to improve the clinical symptoms of *Shigella*-infected dysenteric rabbits by oral administration of butyrate (Raqib et al., 2006). This finding indicates the usefulness of these peptides in controlling and treating infectious diseases.

1.5.1.3. Resistin-like molecule β (RELMβ)

RELMβ is expressed by goblet cells in the intestine (He et al., 2003). It protects the host from helminth infections by altering the parasite niche instead of killing the parasite like other known antimicrobial peptides (Wang et al., 2005). In addition, it plays a regulatory role in the innate intestinal response to pathogens by enhancing mucus secretion (Krimi et al., 2008) and macrophages production of tumor necrosis factor, TNF-alpha (McVay et al., 2006).

1.5.1.4. Bactericidal/permeability-inducing protein (BPI)

BPI is constitutively expressed in the intestinal epithelial cells (Dann and Eckmann, 2007). BPI was found to be selective against Gram negative bacteria because of its affinity to the lipid A of the LPS (Weiss, 2003). Its effects involve cytolysis, endotoxin neutralization, and bacterial opsonization (Weiss, 2003).

1.5.1.5. Antimicrobial lectins

Antimicrobial lectins are constitutively expressed in the intestinal epithelium and the endocrine cells in the colon. In addition, they are induced by Gram positive bacteria and possess mitogenic and antiapoptotic effect on various cell lines (Dann and Eckmann, 2007).

1.5.2. Antimicrobial peptides of the chicken gastrointestinal tract

Although several chicken antimicrobial peptides have been discovered, their role in the chicken digestive tract has not been studied (Van Dijk et al., 2007). The chicken gastrointestinal CAMPs belong to three main categories:

I- Cathelicidins

II- Liver-expressed antimicrobial peptide (LEAP)

III- Defensins

1.5.2.1. Chicken cathelicidins

Cathelicidins are expressed at moderate levels in the gizzard, small and large intestine (Lynn et al., 2004). However, researchers have recently reported the expression of previously unidentified cathelicidin-B1 by the mucosal M cells (Goitsuka et al., 2007). These cells are present in the intestinal Peyer's patches and they are considered the transporter of microorganisms into the lymphoid structures to initiate the immune response (Neutra 1998). Cathelicidin-B1 has bactericidal activity against Gram negative (*E .coli* and *P. aeruginosa*) and Gram positive (*S. aureus*) bacteria (Goituska et al, 2007). Interestingly, homologs of this peptide in humans intestinal mucosa have not been identified yet.

1.5.2.2. Chicken Liver-expressed antimicrobial peptide (LEAP)

LEAP is present at high levels in the chicken small intestine and liver (Lynn et al., 2004). Of note, this peptide was found to be up-regulated in response to *Salmonella enterica* infection in 4 day old chicks (Townes et al., 2004).

1.5.2.3. Chicken Defensins

Avian beta-defensins play a crucial role in birds innate immune defense system because avian heterophils lack oxidative defense mechanism such as hydrogen peroxide, and they rely mainly on heterophil lysozyme and cationic peptides (Harmon 1998). Interestingly, these antimicrobial peptides, such as the chicken heterophil peptide (CHP-1) and turkey heterophil peptide (THP-3), exhibited bactericidal activity against *Campylobacter jejuni* (Evans et al., 1995). In addition, a new defensin, gallinacin-6, was found to be expressed in the chicken digestive tract (Van Dijk et al., 2007). The authors were able to show that this peptide has a wide bactericidal spectrum against food-borne pathogens including *C. jejuni* NCTC 11168.

1.5.3. Mechanism of action of antimicrobial peptides

The electrostatic interaction between the positively charged antimicrobial peptides and the negative charges on the microbial cell walls is the driving force for these molecule effects. The nonspecific electrostatic interactions allow the peptides to associate with the bacterial membrane. Then the peptides are able to disrupt and penetrate the lipid bilayer of the target microorganism (Izadapanah et al., 2005; Yeaman et al., 2003). Permeabilization is the main step in antimicrobial activities and it is essential for bacterial killing (Ganz, 2003).

It is worth noting that CAMPs (Cationic Antimicrobial Peptides) mechanisms of action were based on the interaction of antimicrobial peptides with phospholipids in model membranes. Basically, antibacterial agents were incubated with the membrane to determine the type of induced damage (Brogden 2005). Three hypotheses have been proposed to explain how CAMPs achieve their antimicrobial effects:

1- The barrel-stave hypothesis:

Peptides are imbedded in a barrel-like shape in the membrane forming an aqueous pore. The hydrophobic surface of the peptide faces towards the outside of the barrel while the hydrophilic residues line the inner surface of the pore (Mak and Webb, 1995). Pore formation affects membrane permeability and leads to cell lysis.

2- The toroid pore or wormhole hypothesis:

In this model, peptides with alpha-helical structure are located initially in parallel to the membrane. Their interaction with the membrane exerts a force that further destabilizes the membrane integrity. When the peptide:lipid ratio reaches a threshold, the peptides orient themselves perpendicularly to the membrane to form a pore like structure (Yang et al., 2000). Finally, when the formed pore breaks, a few peptide molecules move to the plasma membrane. This step allows CAMPs to reach their potential intracellular targets (Matsuzaki et al., 1999).

3- The carpet hypothesis:

This mechanism is described by the presence of a high concentration of peptides that accumulate on the bacterial membrane leading to its disintegration and disruption. For instance, detergents exert this effect on the membrane (Yeaman et al., 2003).

It is important to differentiate the CAMPs mechanisms of action from their modes of action. As mentioned above, they all affect the membrane as their site of first interaction, but their mode of action differs. This phenomenon is known as a "multi-hit process" (Zhang et al., 2000). The cellular pathways and processes that are affected by CAMPs could include DNA, RNA, and protein synthesis (Brogden 2005). For instance, Arginine-rich peptides can translocate across the cellular membrane and once in the cytoplasm they can affect septum formation and inhibit cell-wall synthesis, which lead to bacterial cell elongation and growth arrest (Subbalakashmi and Sitaram, 1998). In another example, alpha-defensins were shown to block thymidine, uridine, and leucine uptake in *E. coli* inhibiting nucleic acids and protein synthesis (Lehrer et al., 1989).

The mammalian cell membrane is generally not affected by CAMPs for various reasons (Yeaman et al., 2003). The composition of the eukaryotic membrane differs from the composition of the prokaryotic membrane by the presence of phosphatidylcholine and phosphatidylethanolamine that normally do not bear a net negative charge. Additionally, the transmembrane potential of mammalian cell is between -90 and -110 mV while in bacteria, this potential varies between -130 to -150 mV (Yeaman et al., 2003). Researchers have suggested that this difference in transmembrane potential might play a role in the selectivity of CAMPs towards the bacterial rather than the mammalian cell walls. In spite of these variations and selectivity, antimicrobial peptides can become toxic to mammalian cells at concentrations higher than their physiological levels (Kagan et al., 1994).

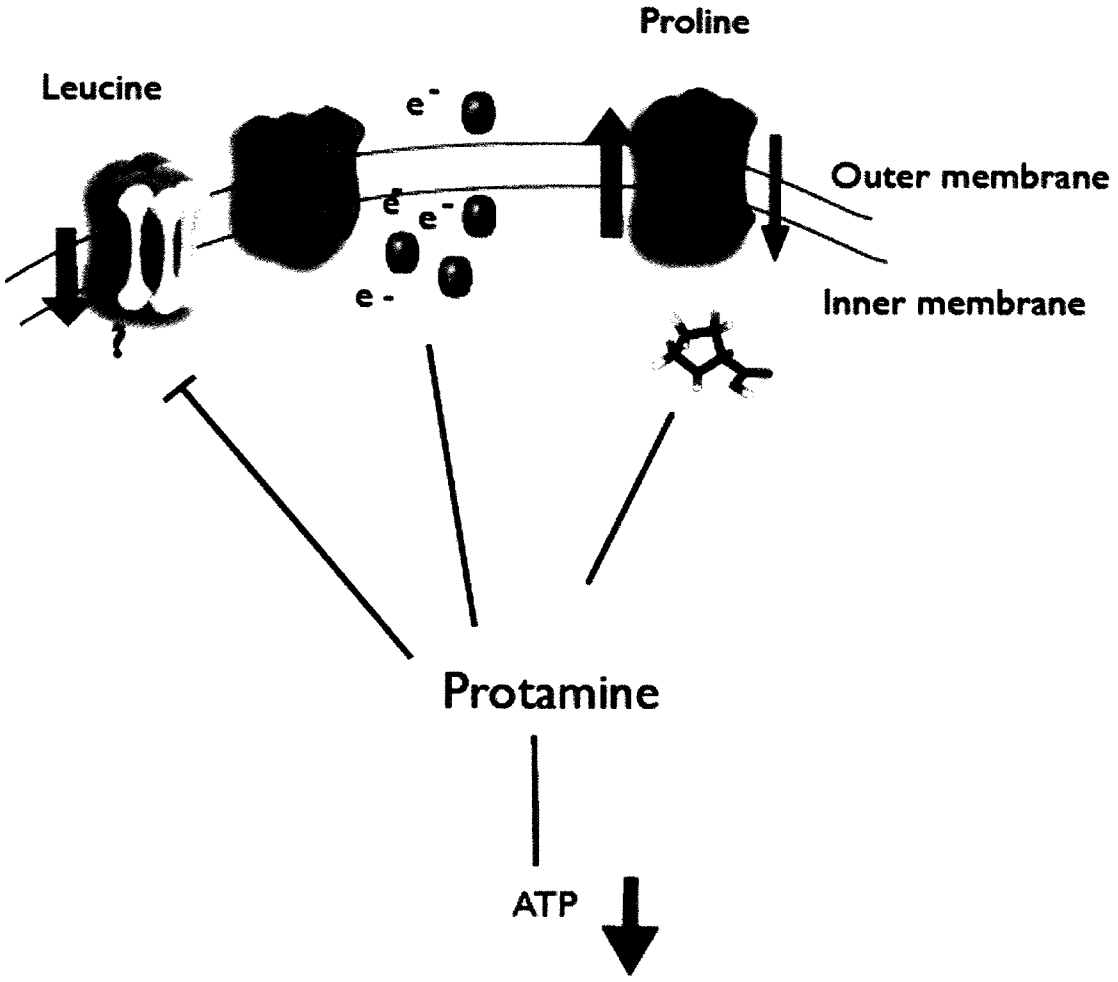
1.5.4. Protamine sulfate

Protamine is the most prevalent cationic antimicrobial peptide identified in nature so far. It consists of 30 amino acids, 20 of which are arginine (Sautiere et al., 1984). This peptide is widely used in industry as a preservative, and it can be obtained from the spermatic cells of mammals, fish, and birds (Potter et al., 2005). In 1996, Aspedon and Groisman unraveled the mechanism of action of protamine (Aspedon and Groisman, 1996) (Figure 1). Using *S. typhimurium* as their bacterial model, the authors were able to show that protamine leads to the inhibition of proline uptake and efflux from the cells, to reduction of the cell's ATP content, leucine dissipation, and to inhibition of protein synthesis. In addition, they demonstrated that protamine disrupts the energy transduction of the bacterial cytoplasmic membrane and that protamine is more effective against respiring cells than non-respiring cells (Aspedon and Groisman, 1996).

The protamine total positive charge affects its antimicrobial activity. Reducing this charge decreases the killing ability of protamine (Potter et al., 2005). Similarly, when the charge of the bacterial surface becomes more negative, due to structural modifications at the membrane level, bacterial susceptibility to protamine increases (Potter et al., 2005).

Figure 1. Mechanism of action of protamine sulfate

Protamine sulfate affects different pathways in *S. typhimurium.* These pathways include general protein synthesis, proline transport system, leucine uptake, membrane potential, and bacterial cell ATP reservoir. Protamine affects proline transport system by increasing its efflux out of the cells and by inhibiting its uptake. Leucine uptake is inhibited by protamine. Together, these two mechanisms lead to inhibition of protein synthesis. Protamine can also disrupt energy transduction of the bacterial cytoplasmic membrane and deplete the cells from ATP. Yellow, blue, green bars are different transporters.


Proline
Leucine
e -
e -
e -
e -
Outer membrane
Inner membrane
Protamine
ATP

1.5.5. Resistance to antimicrobial peptides

Interestingly, many pathogens are able to survive in their host despite the presence of CAMPs. Researchers have defined two tactics that are used by microorganisms to resist the effects of these peptides: constitutive (passive) and inducible (adaptive) resistance (Table 2).

1.5.5.1. Constitutive resistance

Constitutive resistance encompasses basic characteristics of the bacterial niche or the microorganism species which conveys decreased sensitivity to antimicrobial compounds. The intrinsically responsible genes are expressed independently from the presence of the antimicrobial peptides. For instance, *Serratia, Proteus,* and *Burkholderia* are resistant to a broad range of CAMPs (Viljanen and Vaara, 1984). Several mechanisms were implicated in their resistance, including reduced outer membrane negative charge, altered bacterial membrane energetic, capsule formation and electrostatic shielding (Friedrich et al., 1999). Bacterial constitutive resistance could also be due to the infected host. In this niche-specific resistance, bacteria benefit from the special characteristics of the host's colonization site. A good example includes the presence of *Pseudomonas aeruginosa* in abnormal lung tissues that have high osmotic and ionic strength, which reduce the bactericidal effect of CAMPs (Smith et al., 1996).

Table 2. Mechanisms of resistance to antimicrobial peptides.

The table shows various resistance mechanisms against antimicrobial peptides, the microorganism in which the mechanism was identified, and the factor that confers this bacterial resistance.

Table 2. Mechanisms of resistance to antimicrobial peptides

Resistance mechanism	Microorganism	Involved Factors	Reference
Passive resistance	*Burkholderia*	WaaF, LytB, dehydrogenase / (HldA, HldD)	Burtnick and Woods, 1999. Loutet et al., 2006
Niche specific	13 *Staphylococcus spp.*	(lysyl-PG)	Nahaie et al., 1984
Extracellular degradation	*Salmonella enterica*	PgtE	Guina et al., 2000
Anionic capsule shielding	*Pseudomonas aeruginosa*	Alginic acid	Friedrich et al., 1999
Lipid A modification	*Salmonella typhimurium*	Aminoarabinose	Gunn et al., 1998
Cell membrane phospholipids modification	*Bacillus anthracis*	Lysylphosphatidylglycerol	Samant et al., 2009
Cell wall modification	*Lactobacillus lactis*	d-alanyl esters, thickness	Kramer et al., 2008
Intracellular target modification	*E. coli*	DNA gyrase B subunit	DelCastillo et al., 2001
Peptide efflux	*Neisseria gonorrhoeae* *Yersinia enterocolitica*	MtrCDE RosA/RosB	Shafer et al., 1998 Bengoechea and Skurnik,2000
Coordinate adaptation	*Salmonella typhimurium*	*phoQ/phoP* regulon	Gunn and Miller, 1996

1.5.5.2. Inducible resistance

Bacteria have evolved various resistance mechanisms in response to host antimicrobial peptides. One of these mechanisms include alteration of the outer membrane or cell wall composition. For instance, *Pseudomonas aeruginosa* can modify the structure of its outer membrane by inserting an aminoarabinose moiety into the lipid A. This addition reduces the membrane negative charge and consequently the initial interaction with the peptides (Ernst et al., 1999). In another example, *Staphylococcus aureus* is able to add an alanine group on teichoic acid by expressing the *Dlt* gene, to reduce the electronegativity of its cell wall (Peschel et al., 1999). A second resistance mechanism involves the adjustment of the bacterial membrane hydrophobicity. This adjustment can alter peptide-bacteria interaction. For example, in *Staphylococcus aureus* and under iron-deprived conditions, the expressed IsdA (iron-regulated surface determinant) protein reduces the cell wall hydrophobicity. This reduction confers resistance against antimicrobial peptides such as nisin (Clarke et al., 2007). Another resistance mechanism includes the secretion of proteases. For instance, the PgtE protein degrades cathelicidin-LL-37, in *Salmonella* (Guina et al., 2000).

Strikingly, some bacteria have been found to control the expression of the host's antimicrobial peptides including cathelicidin-LL-37 and human beta defensin HBD-1 (Islam et al., 2001). *Shigella* spp. are able to manipulate the host signaling pathways that are required for antimicrobial peptides synthesis resulting in the down-regulation of their expression. This mechanism allows *Shigella* to survive when it encounters the intestinal epithelium (Islam et al., 2001).

Finally, bacterial efflux pumps were reported to take part in microbial resistance to antimicrobial peptides. An example includes *Neisseria's* Mtr energy-dependent efflux system that is responsible for *Neisseria* resistance against protegrins, which are antimicrobial peptides from pig neutrophils (Shafer et al., 1998).

1.6. Bacterial efflux pumps

To minimize the concentration of toxic compounds in their cytoplasm, bacterial cells can alter the permeability of their membrane and/or can force out hazardous molecules through efflux pumps (Cattoir, 2004). These pumps are ubiquitous in prokaryotes (Cattoir, 2004) and eukaryotes (Schinkel and Borst, 1991). Efflux pumps can be specific to certain substrates and their related compounds such as the Mef exporters for macrolides, or unspecific, with unrelated substrates being extruded by the same pump, like the NorA-like transporter in Gram positive bacteria (Marquez, 2005; Poole, 2007).

Conventionally, bacterial transporters are divided into five main superfamilies: the major facilitator (MF), the ATP-binding cassette (ABC), the resistance-nodulation-division (RND), the small multidrug resistance (SMR), and the multidrug and toxic compound extrusion (MATE) (Cattoir, 2004; Poole, 2007). The five superfamilies belong to two different classes according to their energy resources: (1) primary system, which uses ATP, and (2) the secondary system, which uses proton and/or sodium motive force for the intrusion or extrusion of solutes (Bolhuis et al. 1996; Van Veen et al., 1996). In the primary system one ATP molecule is required for each transport event by the ABC pumps and it is considered more energetically costly for the cell than the secondary transporters (Konings,

2006). The extrusion process occurs in sequential events described as follows. The toxic compound interacts with the membrane. Next it is transferred to the inner leaflet of the membrane, then the transporter picks up the substrate and pumps it out (Bolhuis et al., 1996). The slowest and the most critical step in resistance is the substrate's turn over because it keeps the inner membrane free from the toxic compounds and prevents the excess toxin from penetrating into the cytoplasm and reaching its targets (Bolhuis et al., 1996).

Like other microorganisms, *C. jejuni* NCTC 11168 expresses different types of transporters to adapt and survive in various environments (Lin et al., 2002; Ge et al., 2005; Lin and Martinez, 2006). All the five described transporter superfamilies exist in *C. jejuni,* even though the substrates for some of these transporters are not known yet. Examples include, Cj0141c, Cj0142c, and Cj0143c (ABC transporter), Cj1173 and Cj1174 from the drug/metabolite transporter superfamily (DMT), Cj0035c (MFS), Cj0619 and Cj0560 (MATE), Cj0366c, and CmeABC (RND). The latter is extensively studied in *Campylobacter* and is involved in multidrug resistance (Lin et al., 2002). Moreover, it confers resistance to bile salts, enhancing *Campylobacter* colonization and adaptation to the host intestinal environment (Lin and Martinez, 2006).

1.7. Hypothesis

Interestingly, *C. jejuni* can reside in the chicken gastrointestinal tract without causing a disease. However, in humans this microorganism is a leading cause of foodborne illness in developed countries. Both *C. jejuni* hosts secrete antimicrobial peptides that were found to be effective against *Campylobacter.* This observation suggests that *C. jejuni* has developed resistance mechanisms to evade the innate immune system, especially CAMPs, to colonize the gastrointestinal (GI) tracts of chickens and humans. The response of *Campylobacter* to antimicrobial peptides has not been studied yet. Therefore, a genome-wide transcriptome analysis approach was followed in this study in order to identify candidate genes that play a role in *C. jejuni* resistance to CAMPs. As transcriptional profiles would not identify genes involved in constitutive resistance, we also screened a transposon insertion library of *C. jejuni* NCTC 11168 to identify mutants that were affected in their ability to survive protamine exposure. Finally, several genes of interest were then selected for further characterization using *in vitro* and *in vivo* assays.

CHAPTER TWO: GENERAL MATERIAL AND METHODS

2.1. Bacterial strains and growth conditions

Campylobacter jejuni NCTC 11168 was obtained from the National Collection of Type Cultures (NCTC). All *Campylobacter* strains in this study were grown under microaerophilic conditions (N_2: 83%, O_2: 8%, H_2: 4%, and CO_2: 5%) at 37°C in a Modular Atmosphere Controlled System (MACS-VA500, Don Whitley Scientific Limited). Bacterial strains were conserved in glycerol 15% at -80°C. Fusion-Blue™ *Escherichia coli* K12 Competent Cells were obtained from Clontech (catalogue # 636758) and were grown at 37°C in LB medium.

The media used in this study were: Minimum Essential Medium-Alpha (MEM-alpha, Invitrogen Corporation GIBCO™ catalogue # 11900-024); Mueller Hinton broth (Difco™ catalogue # 275730); BBL™ Mueller Hinton II Broth, cation adjusted (CAMH, Becton Dickinson, catalogue # 212322); Mueller Hinton (MH) with agar mixed (Difco™ catalogue # 225220); and Luria Bertani (LB) broth medium (Difco™ catalogue # 244610). Agar was purchased from Fisher Scientific BioReagent® catalogue # BP-1423. Karmali agar medium (Oxoid code # SR0205)

C. jejuni was grown in broth using a biphasic culture method. Briefly, 8 ml MH agar was added to a cell culture flask, which was kept at room temperature in a horizontal position until the medium was solidified. The prepared flasks were placed at 4°C. When needed, 8 ml MH broth was added to the flask and the bacterial colonies were suspended in the broth.

The antibiotics used in this study were chloramphenicol (Cm; Alfa Aesar catalogue # B20841; stock solution of 34 mg/ml in ethanol), kanamycin sulfate (Km; OmniPur EMD™ catalogue # 5880; stock solution of 10 mg/ml in water), and ampicillin (Amp; VWR catalogue # VW1507-01; stock solution of 100 mg/ml in water). All solutions were filter sterilized using PES (polyethersulfone) 0.2 μm filter. Antibiotic solutions were used at final concentrations of 10, 50, and 10 μg/ml for Cm, Km, and Amp respectively.

2.2. Minimum inhibitory concentration (MIC)

Protamine sulfate was purchased from Invitrogen® (catalogue # P3369). Stock solutions were prepared at a concentration of 16 mg/ml in water, filter sterilized using 0.2 μm PES filter (Whatman Schleicher & Schuell catalogue #6896-2502) and stored at -20°C in 1.5 ml polypropylene microcentrifuge tubes until needed. The solution was thawed once before use. To study the sensitivity of *C. jejuni* strains against antimicrobial peptides, 3 different methods were performed: (1) the microdilution method; (2) the macrodilution method; and (3) the agar dilution method.

2.2.1. Microdilution method

The MIC was determined according to the CLSI (Clinical and Laboratory Standards Institute) guidelines as follows. The assay was performed using polypropylene 96 well plates (Corning Incorporation catalogue # 3790). *C. jejuni* strains (wild-type and mutants) were grown on MH/CAMH agar plates for two days. Then, four to five single colonies were picked to inoculate a biphasic culture containing 8 ml of MH/CAMH broth. This pre-culture

was placed in the microaerophilic station at 37°C. At mid-log phase, the pre-culture was used to prepare the bacterial inoculum. The wells of 96 well plate contained a total volume of 100 µl divided as follows. First, 50 µl of the appropriate medium (MH, CAMH) was added to all wells except the first one, which contained 100 µl of protamine sulfate at a concentration of 800 µg/ml. Then, a serial dilution of protamine was prepared by transferring 50 µl from the first well to the second well and so on. The last well contained only 50 µl of the medium and was considered as a positive control. Finally, a 50 µl bacterial inoculum was added to have approximately 10^6 CFU in each well. The lowest protamine concentration in the assay was 0.78 µg/ml. The plate was put in a moist chamber and placed in the microaerophilic station at 37°C for 24 to 48 hours. The experiment was repeated at least three times for each medium (MH, CAMH). The MIC was assessed visually by observing a pellet in the wells below the MIC and in the positive control.

2.2.2. Macrodilution method

The MIC was determined according to the CLSI (Clinical and Laboratory Standards Institute) guidelines in MH, CAMH, and MEM media using glass tubes (Fisher Scientific catalogue # 14-961-27) as follows. The different bacterial strains were grown in a biphasic MH culture until they reached mid-log phase. For testing in CAMH and MEM media, the appropriate volume from the initial biphasic culture was centrifuged, the supernatant was discarded, and the pellet was resuspended in CAMH and MEM, respectively. These pre-cultures were used to inoculate the test tubes to a final bacterial concentration of approximately 10^6 CFU. A serial dilution of protamine sulfate was prepared with a highest

concentration of 1600 μg/ml and a lowest concentration of 1.56 μg/ml. The final volume in the test tubes was 1 ml composed of 500 μl of protamine from the prepared serial dilutions and 500 μl of the prepared bacterial inoculum. The final protamine concentrations in the test tubes were between 800 and 0.78 μg/ml. The tubes were covered with aluminum foil and placed in the microaerophilic station for 24 hours and the MIC was determined visually based on the turbidity of the culture in the tubes. The first clear tube without bacterial growth was considered the MIC.

2.2.3. Agar dilution method

Briefly, *C. jejuni* NCTC 11168 was grown on MH plates. Next, colonies were re-streaked or patched on new MH plates to which protamine sulfate was added to final concentrations ranging from 200 μg/ml to 800 μg/ml. The plates were placed in the microaerophilic station and checked for bacterial growth after 24 and 36 hours. The MIC was assessed visually by determining the protamine concentration at which no bacterial growth was observed.

2.3. Survival assay

The survival assay was performed in MEM-α medium as follows. *C. jejuni* was grown in MH biphasic medium until mid-log phase (O.D. = 0.7 at 600 nm). This pre-culture was used to inoculate 50 ml of MEM-α medium in two 125 ml Erlenmeyer flasks with a starting O.D. of 0.05. The cultures were incubated and stirred in the MACS-VA workstation until mid-log phase (approximately O.D. = 0.2). At time zero, 1 ml of the broth was

withdrawn from each flask, serially diluted, and 10 µl from each dilution was spotted on MH agar plates. Then, protamine sulfate was added to the flasks at a final concentration of 25 µg/ml. Five minutes following the addition of protamine, a 1 ml of the broth was withdrawn, serially diluted, and 10 µl spotted on MH agar plates. The plates were placed in the microaerophilic station for 24 to 48 hours and the number of colonies was enumerated. A two sample *t-test* was used to statistically analyze the difference between the sensitivity of the wild-type strain and the sensitivity of the mutant to protamine sulfate. The confidence interval was set at 95%.

For the strain that overexpresses Cj0355c, the survival assay was performed in MH medium as follows. Four to 5 colonies of the wild-type and the constructed strain were selected to inoculate a separate MH biphasic cultures. These pre-culture were placed in the microaerophilic station until mid log phase and were used to inoculate the final MH broth (25 ml) to an O.D. of 0.05 in two 125 ml Erlenmeyer flasks. The flasks were placed in the MACS-VA station on a stirrer until the culture reached mid log phase. A one ml sample was withdrawn from the flask (time zero), serially diluted, and 100 µl was plated on MH plates for enumeration. Each dilution was plated in triplicate. Thereafter, protamine sulfate was added to a final concentration of 50 µg/ml. After five minutes, a 1 ml sample from each flask was withdrawn, serially diluted, and plated on MH agar plates. The same procedure was followed for time points 10, 20, 40, and 60 minutes. The plates were placed in the microaerophilic station for two days before counting the number of bacterial colonies. The mean of the three replicates was calculated and a two sample *t-test* was used to determine

whether the difference between the wild-type strain and the strain that overexpresses Cj0355c was significant.

The ability of *C. jejuni* to survive protamine exposure was also estimated using the Bioscreen C reader (Oy Growth Curves Ab Ltd., Finland). Briefly, *C. jejuni* strains were grown on MH agar plates for two days. Four colonies were picked to inoculate 8 ml MH biphasic cultures, which were incubated in the MACS-VA workstation overnight until they reached mid-log phase. The cultures were diluted to an O.D. of 0.05 in 20 ml MH broth. Then, the appropriate volume was transferred to each well of 100-well Honeycomb plates (Oy Growth Curves Ab Ltd., Finland). MH broth was added to 3 wells as blank controls. The plate was placed in the MACS-VA workstation for an hour, then sealed with a tape and placed in the Bioscreen C chamber. The plate was set on continuous shaking at 37°C and measurements (O.D. 620 nm) were taken every 15 minutes. At mid log phase the plate was removed from the Bioscreen C incubator and protamine sulfate was added to a final concentration of 25 μg/ml except for 3 wells, which were kept as positive controls. The plate was sealed again and placed back in the Bioscreen C incubator and the incubation was continued for another 24 hours.

2.4. RNA extraction

Briefly, *C. jejuni* strain NCTC 11168 was grown for two days on a MH agar plate from which four colonies were picked to inoculate a biphasic culture containing 8 ml MH broth. The biphasic culture was incubated in the microaerophilic incubator until mid-log phase (O.D. 600 nm ~ 0.7). This pre-culture was used to inoculate a 100 ml MEM-α medium

in an Erlenmeyer glass flask at a starting O.D of 0.05. This culture was stirred until mid-log phase (O.D. = 0.2) in the MACS-VA incubator. After reaching mid-log phase, the 100 ml culture was split into two 50 ml cultures. One was considered as a control to which sterile water was added, while different concentrations of protamine sulfate were added to the other 50 ml culture. Each tested protamine concentration was repeated twice. Five minutes after protamine addition, the total RNA was extracted according to a hot phenol-chloroform protocol (Tao et al., 1999). Briefly, an RNA degradation stop solution (10% phenol [pH 4.2] in ethanol 99%) was added to both cultures while still in the incubator, then they were quickly put on ice and spun down for 20 minutes at 4°C. The bacterial pellet was resuspended in 800 µl TE buffer (50 mM Tris-Cl [pH 8], 1 mM EDTA) and 80 µl 10% SDS in order to lyse the cells. To extract the RNA from the lysate, 1 ml phenol (pH = 4.2) was added, centrifuged at 12,000 rpm (revolutions per minute) for 10 minutes at 4°C. The aqueous layer was transferred to a new tube, to which 750 µl chloroform was added, mixed and centrifuged at 12,000 rpm for 10 minutes at 4°C. The aqueous layer was then separated from the chloroform and split into two new tubes. Finally, 15.2 µl of 25 mM EDTA, 1/10 volume of 3M sodium acetate, and 2.5 volume of cold ethanol (99%) were added and the tubes were put at -80°C overnight. The tubes were then thawed and centrifuged at 4°C for 25 minutes. The total RNA was then washed with 80% ethanol three times and resuspended in 100 µl Diethylpyrocarbonate (DEPC) treated water. All the reagents used were treated with DEPC (0.01 %). After the extraction, all samples were DNase treated to eliminate any residual chromosomal as follows; 0.5 µl RNase inhibitor (RNase out, Boehringer Mannhein, catalogue # 799017), 4 µl DNase I (Epicentre, catalogue # D9902K) and 10 µl of the DNase

buffer were added to each tube. The tubes were incubated for 30 minutes at 37°C. The DNase treatment was repeated four times for every sample. After this treatment, the RNA was purified using the Qiagen RNeasy Mini Kit (catalogue # 74106) according to the manufacturer's guidelines, and total RNA was eluted in a final volume of 100 µl RNase free water. The absence of DNA in the samples was confirmed by PCR with primers amplifying the gene Cj0340. Moreover, the integrity of the RNA was checked using the Experion™ system (Bio-Rad laboratories Inc., catalogue # 700-7103). This technology provides an accurate analysis of the quality and quantity of total RNA. Only non-degraded RNA samples were used in the microarray, qRT-PCR, and the one step RT-PCR assays.

2.5. Microarray hybridization, analysis, and hierarchical clustering

Sixteen micrograms of total RNA were used for the labeling reaction. Briefly, total RNA from each condition was converted to cDNA in the presence of aminoallyl-dUTP with Superscript II (Invitrogen) at 42°C. The reverse transcription reaction included: 16 µg of total RNA plus 10 µg of random hexamers in a 34.35 µl reaction mixture containing 8 µl of 5x Superscript II reverse transcriptase buffer and 2 µl of 0.1 M dithiothreitol. After a 5 min incubation at 65°C, the reaction mixture was brought to a final volume of 40 µl by adding (final concentrations): 0.5 mM each of dGTP, ATP, and CTP; 0.16 mM dTTP; 0.34 mM aminoallyl-dUTP; and 2 µl of Superscript II. Reaction mixtures were incubated at 42°C for 120 min. At the end of the first-strand synthesis, the reaction was stopped and the RNA was hydrolyzed by adding 4 µl of 50 mM EDTA and 2 µl of 10 N NaOH and by incubation at 65°C for 20 min. This reaction was then neutralized by adding 4 µl of 5 M acetic acid. The

aminoallyl-labeled cDNA was purified from free amines and unincorporated aminoallyl-dUTP by adding 450 µl of water and spinning through a Microcon YM-30 filter (Millipore) for 8 min at 10,000 rpm. The washing step was repeated three times. After the last wash, the aminoallyl-labeled probes were concentrated to less than 8 µl under vacuum in a SpeedVac and adjusted to a final volume of 10 µl by adding 1 µl of 1 M sodium carbonate (pH 9.0) and water. The resulting aminoallyl-labeled cDNA was coupled to monoreactive fluors (Amersham) by adding 10 µl of dimethyl sulfoxide containing one-sixth of one reaction vial FluoroLink cyanine-3 (cy-3, indocarbocyanine monofunctional NHS-ester) for the control or cyanine-5 (cy-5, indodicarbocyanine monofunctional NHS-ester) dye for the protamine-treated samples. Incubations were performed for 60 min at room temperature in the dark. The labeled cDNA probes were then purified using Qiagen PCR purification kit (catalogue # 28106) or Invitrogen PureLink PCR purification kit (catalogue # K3100-02) according to each manufacturer's instructions. The fluor-labeled cDNA mix was dried under vacuum using SpeedVac and resuspended in 15.14 µl of water, to which 2.5 µl of salmon sperm DNA (10 mg/ml), 9 µl of 20x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7), 0.36 µl of 10% sodium dodecyl sulfate (SDS), and 9 µl of formamide were added. Prior to hybridization, microarray slides were prehybridized at 42°C for 45 min in prehybridization buffer (25% formamide, 5x SSC buffer, 0.1% SDS, and 1% bovine serum albumin), rinsed with water, and dried by spinning. The probe was denatured by boiling for 2 min, followed by cooling to 42°C. The probe was then applied to the slide's coverslip. The slide was placed in a humid chamber and incubated at 42°C overnight. After hybridization, slides were washed in 2x SSC-0.1% SDS for 5 min at 42°C, 0.1x SSC-0.1% SDS for 10 min at room

temperature, and four times in 0.1x SSC for 1 min at room temperature. Slides were then rinsed with distilled water and dried by centrifugation (Palyada et al. 2004). The slides were scanned using Perkin Elmer G_x scanner and the generated data were analyzed using ScanArray Express software version 3.0.1 (PerkinElmer LAS, Inc.). The PCR products of the 1,654 open reading frames (ORF) of the *C. jejuni* NCTC 11168 genome were previously spotted on the microarray slide. Each slide has 3 meta columns as technical replicates of the printed spots. The columns contain 12 subarrays in which blanks and nonspecific binding genes were added. Two technical replicates for each biological replicates were performed. The intensities of the included spots were normalized by applying locally weighted linear regression (Lowess) using MIDAS software (TIGR, http://www.tigr.org/software/). Finally, the $\log_2$ of the normalized ratio of the two channels was calculated and the data were analyzed as previously described (Palyada et al., 2004) using the empirical Bayes method (Long et al., 2001). After analyzing the data, genes were selected according to the following criteria: (1) 1.5-fold increase or decrease in expression, and (2) Bayesian *P* value < 0.0001. Finally, the selected genes were clustered using Genesis software (Graz University of Technology, http://genome.tugraz.at; Sturn et al., 2002).

2.6. Quantitative RT-PCR

To confirm and validate the gene expression data obtained from the microarray analysis, four over-expressed genes (Cj0200c, Cj1004, Cj1721c, Cj0550) and one down-regulated gene (Cj0355c) were selected. Five hundred ng of the extracted RNA was used as template. The *ilvC* gene was used as the endogenous control in all experiments because its

expression does not change between the tested conditions. Primers (0.5 μM was used in each reaction tube) were specially designed for this experiment according to Qiagen guidelines and are listed in Table 3. A negative control, without reverse transcriptase, was run in parallel for each assay. The Qiagen QuantiTect SYBR Green RT-PCR kit (catalogue # 204243) was used and the reactions were performed using the Applied Biosystems 7300 machine. The PCR program consisted of the following steps: (1) reverse transcription for 30 minutes at 50°C; (2) Initial polymerase activation for 15 minutes at 95°C; (3) Denaturation for 15 seconds at 94°C; (4) Annealing for 30 sec at 55°C; and (5) Extension for 30 sec at 72°C. The last 3 steps were repeated for 40 cycles. The critical threshold (C_T) value corresponds to the PCR cycle at which there is the first detectable increase in fluorescence associated with the exponential growth of the PCR products. In addition, at the end of the main run, a melting curve analysis was performed to check either for primer dimers or secondary PCR products formation. The generated data was analyzed using 7300 System SDS software version 1.3.1 and the fold change in gene expression was calculated based on the ΔCt obtained from the software final output. Quantitative values were obtained with the comparative threshold cycle (C_T) method, as recommended by Applied Biosystems.

The transcript level from each RNA sample was assayed six times, and the mean C_T value was used for further analysis. For data analysis, the induction of the specific genes was calculated as $2^{-\Delta\Delta C_T}$, where $C_T = C_{T\,(C2)} - C_{T\,(C1)}$. C_T condition 1 [$C_{T\,(C1)}$] and C_T condition 2 [$C_{T\,(C2)}$] are obtained by subtracting the mean C_T value of the specific gene from the mean C_T value of the reference gene (*ilvC*) of the control or the treated sample.

Table 3. Primers used in this study

F, forward primer, R reverse primer. IF, inverse forward primer, IR, inverse reverse primer. RT, in parenthesis, indicates that these primers were used in the qRT-PCR or one-step RT-PCR assays.

Table 3. Primers used in this study

	Primer	Sequence 5' - 3'
Primers for mutants construction	CAT F	TGCTCGGCGGTGTTCCTTTCCAAG
	CAT R	TGCGCCCTTTAGTTCCTAAAGGGT
	cj0560 F	CGGTACCCGGGGATCTTTGGCATTTTATTAGCGGTTT
	cj0560 R	CGACTCTAGAGGATCAACCTATCAAATTGTCCTAACTGG
	cj0560 IF	GAACTAAAGGGCGCAGCAGCTTTGATGCTTTTTGCTT
	cj0560 IR	GAACACCGCCGAGCAATCAAGGCTAGTGCCGCTAA
	cj1004 F	CGGTACCCGGGGATCAAAGCTGCCAATAAGCTCCA
	cj1004 R	CGACTCTAGAGGATCGGTTCTAGAGCCATGAGATTGA
	cj1004 IF	GAACTAAAGGGCGCATGGAGCTTGGAAGGAAATTG
	cj1004 IR	GAACACCGCCGAGCAACGATCCCTATAGGCGCTTT
	cj0200c F	CGGTACCCGGGGATCTCAAAGCTCCTTTTAATGAAGG
	cj0200c R	CGACTCTAGAGGATCGGTTGAAAGCTGTGTATGAGAGTG
	cj0200c IF	GAACTAAAGGGCGCATGGAACACCTGCTACACCAA
	cj0200c IR	GAACACCGCCGAGCAATAGCCATTTGTCCGCCATA
	cj0550 F	CGGTACCCGGGGATCCCCGCAAAAACTTATTGAA
	cj0550 R	CGACTCTAGAGGATCGAGGAAAGTCCGAGCTGCTA
	cj0550 IF	GAACTAAAGGGCGCATTAGCCAAAGAATCGCCTTC
	cj0550 IR	GAACACCGCCGAGCATGCAAGTTTAAAATCACTGAGC
	cj1721c R	CGACTCTAGAGGATCTGGAGGTTGAAATCCTACCG
	cj1721c F	CGGTACCCGGGGATCTCCACTCTGTGTTTTCGCTCT
	cj1721c IF	GAACTAAAGGGCGCAAAGAGCTGTTGCAGGTGGAT
	cj1721c IR	GAACACCGCCGAGCAAAAAGCCGAGCTTGAAAACA

	Primer	Sequence 5' - 3'
Primers for construction of the strain that overexpresses Cj0355	cj0355c F	GATTTAGATGTCTAGTGGTATATTAGATTTCGAAAGA AGGAA
	cj0355c R	GGGGAAGCTTTCTAGCAGGCAGGCTCTGAAAAATC
	ak233	GCAAGAGTTTTGCTTATGTTAGCAG
	ak234	GAAATGGGCAGAGTGTATTCTCCG
	ak235	GTGCGGATAATGTTGTTTCTG
Primers for operon mapping	cj1721c	CAAAGCCGAGCTTGAAAAC
	cj1722c	AATATGCTGTGCCACCACAA
	cj1723c	AACCATACGCTTCCCAATCC
	cj1724c	CAAACTCAAGCCAAAATGGA
Primers for locating Tn5	AR56	CATCCTCTTCGTCTTGGTAGC
Primers for qRT-PCR	cj1004 F (RT)	GCCTATAGGGATCGTGCAAA
	cj1004 R (RT)	CAATTTCCTTCCAAGCTCCA
	cj0200c F (RT)	TTCGCATCAGCACTTTTAGC
	cj0200c R (RT)	TAGCCATTTGTCCGCCATA
	cj0550 F (RT)	GCTTGGAGAGAAGTGCATAACA
	cj0550 R (RT)	TGAAGGCGATTCTTTGGCTA
	cj1721c F (RT)	GTTTTCAAGCTCGGCTTTTG
	cj1721c R (RT)	AACAGCACCGGTTTGATTG
	cj0355c F (RT)	TTGAAAGCTGGAGCTGATGA
	cj0355c R (RT)	ATAACATTGGTTCCGCCAAG

2.7. One step reverse transcriptase PCR

To confirm that Cj1721c, Cj1722c, Cj1723c, and Cj1724c are co-transcribed, a one step reverse transcriptase PCR kit from Qiagen (catalogue # 210210) was used. Primers are listed in Table 3. They were designed and used according to Qiagen's recommendations (0.6 μM from each primer). *C. jejuni* NCTC 11168 chromosomal DNA was used as a positive control. Two negative controls were run in parallel as well, one without the reverse transcriptase enzyme and the other without DNA or RNA template. The PCR products were generated from pairs of primers with each one in the pair is annealing to a different gene in the operon. For instance, Cj1721c and Cj1723c primers were used to determine that the two corresponding genes are co-transcribed. The thermal cycler program consisted of the following steps: (1) Reverse transcription for 30 minutes at 50°C; (2) Initial polymerase activation step for 15 minutes at 95°C; (3) Denaturation for 1 min at 94°C; (4) Annealing for 1 min at 55°C; (5) Extension for 1 min at 72°C; and (6) Extension step for 10 minutes at 72°C. Steps (3, 4, 5) were repeated 30 times. The reactions products were run on a 0.7% agarose gel to assess product sizes and confirm the absence of contaminants.

2.8. Construction of the deletion mutants

Deletion mutants in Cj0560 and Cj1721c were constructed using the In-Fusion™ PCR cloning kit, from Clontech Laboratories Inc., U.S.A. Primers used are listed in Table 3 (Cj0560 F/R-IF/IR, Cj1721c F/R-IF/IR, and CAT F/R). An extension of 15 base pairs were added to the primers amplifying the gene of interest. The primers extension recombines to the nucleotides flanking the BamHI restriction site on the pUC19 vector. The appropriate

ratio (1:2) of the amplified gene was mixed with that of the BamHI-restricted pUC19 plasmid and added to the provided tube containing the recombination mixture. Fusion-Blue™ *E.coli* K12 Competent Cells (Clontech, catalogue # 636758) were added to the cloning reactions. Then, the cells were plated on LB agar plates to which Amp 50 µg/ml, 40 µl of X-gal 2% plus 7 µl IPTG 20% were added. The plates were incubated at 37°C for 18 hours and the white colonies were then selected and patched on fresh LB agar plates containing Amp 50 µg/ml. A PCR reaction was performed to confirm that the gene of interest was recombined into pUC19 by using the primers that were designed to amplify the corresponding gene. Bands corresponding to the right sizes of the deleted genes Cj1721c and Cj0560 indicated the successfulness of the recombination reaction. The cloned plasmid from the previous step was extracted and purified from the competent cells using the Qiagen Plasmid Mini Kit (catalogue # 12123). Next, an inverse PCR product was generated for each gene (Cj1721c and Cj0560) using the extracted plasmid and the correspondent primers that were marked IR, IF (Table 3). 322 bp within the Cj1721c gene (892 bp) and 500 bp from within the Cj0560 gene (1329 bp) were deleted. The primers for the inverse PCR reactions were designed with extra 15 base pairs that are homologous and recombine to the nucleotides flanking the chloramphenicol resistance cassette (CAT). To enhance the yield of the recombination reaction, the inverse PCR reaction was digested first at 37°C for 1 hour with 1 µl DpnI restriction enzyme and 2 µl REACT 4 buffer to eliminate methylated and/or hemi-methylated DNA. On the other hand, the CAT cassette from the pRY111 plasmid was amplified using CAT F and CAT R primers (Table 3). Next, the inverse PCR product and the CAT cassette were mixed at 2:1 molar ratio and recombined using the In-Fusion PCR

cloning Kit after ethanol co-precipitation as follows. The total volume of the mixed reaction was brought to 50 µl after adding 5 µl sodium acetate 3M and 1 µl glycogen as a carrier and distilled water. Three volumes of cold ethanol 99% (-20°C) were added (150 µl) and then centrifuged for 10 minutes at 4°C. The pellet was washed with 70% ethanol and centrifuged for 10 minutes at 4°C. Then the pellet was air dry for 15 minutes. Distilled water (10 µl) was added to dissolve the pellet and the mix was used for the cloning reaction.

Fusion-Blue™ *E.coli* K12 Competent Cells (Clontech, catalogue # 636758) were used to transfer the constructed plasmid. The cloning reaction mixture was added to the thawed bacterial cells on ice following the manufacturer's guidelines. Next, the cells were plated on LB agar plates, containing Amp 20 µg/ml plus Cm 10 µg/ml, and incubated at 37°C for 18 hours. The plasmid of the constructed clones was extracted using the Qiagen MiniPrep Kit and the mutated genes were amplified using the original primers (Cj0560 F-R, Cj1721c F-R) to validate the construction. Moreover, to confirm the absence of point mutations, the plasmids were sequenced with the primers that were used to amplify the original gene. Finally, the purified plasmid with the deleted gene was transferred (natural transformation) to *C. jejuni* NCTC 11168 as follows. *C. jejuni* NCTC 11168 was grown in MH biphasic medium to mid log phase. Extracted plasmid was added to the broth culture and kept for an additional 7-8 hours in the microaerophilic station. Different volumes from the final broth culture were plated on selective MH agar plates containing Cm 10 µg/ml.

2.9. Construction of the strain that overexpresses Cj0355c

For the construction of the overexpressing Cj0355c strain, the Cj0355c gene was amplified by PCR using primers Cj0355c F and Cj0355c R (Table 3). The position of the forward primer (Cj0355c F) was within *folB* gene upstream of Cj0355c. This will ensure that the Cj0355c promoter region is intact and the gene is expressed via its own promoter. The PCR product was purified using the Qiagen purification kit and then cloned into pRRK-1 plasmid (Reid et al., 2008), which was previously cut with XbaI restriction enzyme. The cloning step was achieved using the Clontech In-Fusion™ PCR cloning kit. Briefly, the primers were designed with 15 bp extension that recombines to the nucleotides flanking the XbaI restriction site on the pRRK-1 vector. The prepared plasmid was transformed into Fusion-Blue competent cells by incubating the cells with the cloning mixture. Next, the cells were plated on LB agar plates containing Km 50 μg/ml. The cloned plasmid with the Cj0355c gene was extracted from the grown competent cells, purified and sequenced to confirm the absence of point mutations. The plasmid was then naturally transformed into *C. jejuni* NCTC 11168 as described above using MH agar plates containing 10 μg/ml Km. Selected grown colonies were stored at -80°C in 15% glycerol. The level of Cj0355c transcripts in the obtained construct was determined by performing a qRT-PCR. This assay showed that the number of transcripts in the constructed strain was double (1.99) that in the wild-type strain.

2.10. Growth curves

The growth curves of the wild-type strain, Cj1721c and Cj0560 deletion mutants, and the strain that overexpresses Cj0355c were generated by using the Bioscreen automated temperature controlled growth curves machine from Oy Growth Curves Ab Ltd. Finland. Briefly, the strains were grown on MH agar plates for 2 days and 4 to 5 colonies were selected to inoculate a 8 ml MH biphasic broth culture. The biphasic cultures were incubated in the MACS-VA workstation until mid-log phase, and used to inoculate the Honeycomb plate wells to a total volume of 300 µl per well with a starting O.D. of 0.05. At least six wells were inoculated with the same strain. For the growth curve in MEM medium, the inoculum was spun down at maximum speed (12,000 rpm at room temperature for 3 minutes) and the pellet was resuspended in MEM medium to a starting O.D. of 0.05. The plates were incubated in the MACS-VA workstation for 1 hour before being sealed with a tape. The sealed plate was then placed in the Bioscreen C chamber of the growth curve instrument. The plates were set for continuous shaking and the O.D. (620 nm) measurements were taken every 15 minutes for a total of 24 hours at 37°C. The mean of the technical replicates in the same plate was calculated. In addition, MH or MEM medium were added to three wells as blanks. The readings from these blank wells were used to normalize the readings from the actual samples by subtracting the sample readings from the blank readings.

2.11. Motility assay

C. *jejuni* NCTC 11168, ΔCj1721c, and the strain that overexpresses Cj0355c were grown in MH biphasic medium until mid log phase. The O.D. (600 nm) was adjusted to

0.7 in 1 ml microcentrifuge tubes. A 10 µl plastic loop was then used to prick the centre of a motility plate (0.4% agar in MH medium). The plates were placed in MACS-VA workstation for up to 48 hours. The motility behavior of each strain was monitored by measuring the diameter of growth following 24 and 48 hours incubation. The swarming motility for the strain that overexpresses Cj0355c was evaluated on 2% MH agar plates with (200 µg/ml) and without protamine sulfate. The plates were stabbed with a 10 µl plastic loop of the prepared inoculum (O.D. adjusted to 0.7) and then placed in the microaerophilic incubator for 48 hours. The assay was repeated three times.

2.12. Pellicle formation assay

To study the biofilm formation ability of the Cj1721c mutant and the strain that overexpresses Cj0355c, a pellicle formation assay was conducted as described by others (Kalmokoff et al. 2006). *C. jejuni* strains were grown in an MH biphasic medium overnight until mid-log phase and then the O.D. was adjusted to 0.7. Four µl of the bacterial suspension were used to inoculate one well of a 24 wells plate (Nunclon ™ Surface, catalogue # 143982, Denmark). Each well contained 2 ml of MH broth. Each plate had three wells inoculated with the same strain as technical replicate. Three wells containing uninoculated MH broth were used as negative control. The plate was incubated in the MACS-VA workstation for 3 days. Pellicle formation was visualized at day 3 using multiimage™ light cabinet camera, Alpha Innotech Corporation.

2.13. Biofilm assay

Bacterial strains were grown on MH plates for 2 days. Four to five colonies were selected to inoculate a MH biphasic broth culture which was placed in the microaerophilic workstation until it reached an O.D. of 0.3. This pre-culture was used to prepare a 10 ml MH broth with a starting O.D. of 0.05. From this culture a 1 ml aliquot was transferred to borosilicate tubes that were then placed in the microaerophilic station for 24, 48, and 72 hours (without shaking). In addition, a tube containing only MH medium was used as a blank. At the selected time points, 250 µl of 1% crystal violet in 95% ethanol was added to each tube and incubated for 15 minutes at room temperature (McLennan et al. 2008). The tubes were rinsed with distilled water and photographed with a Canon SD 750 digital camera. Next, 1.5 ml of DMSO was added to the tubes to quantify the formed biofilm. The tubes were incubated for another 24 hours and 200 µl from each tube was transferred to a polystyrene 96 well plate and the absorbance was measured at 574 nm using Tecan Spectra Shell spectrophotometer, and winSeLecT software (Tecan U.S., Inc). The O.D. readings from the different samples were deducted from the O.D. reading of the blank. A two-sample *t*-test was used to compare the tested strains with a confidence interval of 95%.

2.14. Screening of a transposon mutants library

The transposon mutant library used in this study was previously constructed as follows (Reid et al., 2008). Briefly, a chloramphenicol cassette was amplified from pRY111 plasmid and cloned into EZ-Tn5 pMOD-3 transposon, (Epicenter Biotechnologies), which was digested with a restriction enzyme BamHI, to obtain EZ-Tn5-Cm transposon. Then the

transposition reaction was performed *in vitro* by adding the constructed transposon to *C. jejuni* chromosomal DNA and EZ-Tn5 transposase. Next, the transposed DNA was naturally transformed to *C. jejuni* NCTC 11168. Finally, the cells containing the EZ-Tn5-Cm were selected on MH agar plates containing 20 μg/ml Cm, inoculated into MH broth, grown overnight under microaerophilic conditions and stored at -80°C in 7% DMSO.

The library was screened in order to identify genes that could play a role in intrinsic resistance to protamine sulfate and possibly to other antimicrobial peptides. First, the MIC on agar plates was determined as follows. In each plate 25 ml medium was added and *C. jejuni* NCTC 11168 was streaked on plates containing concentrations of protamine sulfate ranging from 100 to 800 μg/ml. The minimum concentration at which no growth was observed, was 450 μg/ml. Next, the transposon mutant library was screened against two concentrations of protamine sulfate (200 and 500 μg/ml) as follows. Two microlitres from each mutant suspension were spotted on selective CAMH agar plates containing 20 μg/ml Cm. The plates were incubated in the MACS-VA workstation for two days. Each mutant was consecutively patched from the original plate onto new CAMH agar plates containing a sub-inhibitory concentration of protamine (200 μg/ml), an inhibitory concentration of protamine (500 μg/ml) and to a control CAMH plate (no protamine). The plates were incubated in MACS-VA workstation for 24 hours before identifying protamine-sensitive and protamine-resistant mutants. In total, 2112 mutants were screened in triplicate.

Transposon mutant growth curves were generated in the same way as described above. In addition, the doubling time was calculated in each well for each mutant by plotting the initial growth curves on a semilogarthmic scale ($\log_2$). The slope from the best linear fit

was calculated and the inverse of the slope was considered as the doubling time. The mean of the technical replicates was compared to the mean of the wild-type in the three biological replicates independently using Bonferroni test at a significance value of 0.05.

A P value < 0.05 indicates that the mutant's generation time is significantly different from that of the wild-type strain.

2.15. Mapping of the identified transposon mutants

To identify the transposition site, the chromosomal DNA of each transposon mutant (from a total of 27) was extracted as follows. Briefly, a mid-log phase bacterial culture was spun down at 12,000 rpm and the pellet was resuspended in 100 µl of TE buffer (10 mM Tris and 1 mM EDTA). Next, an extraction buffer (50 mM Tris, 100 mM EDTA, 0.5% SDS) containing RNase (10 mg/ml) and proteinase K (20 mg/ml) was added to this suspension and incubated for 3 hours at 42°C. One ml of phenol (pH, 6.5) was added to the cell lysate, incubated at 37°C for 15 minutes, and then centrifuged at 12,000 rpm for 5 minutes. The aqueous phase was transferred to a new microcentrifuge tube, and an equal volume of chloroform (1 ml) was added. Next, the tubes were centrifuged at 12,000 rpm for 5 minutes. Finally, the DNA was precipitated by adding 0.2 volume of 10 M ammonium acetate and 2 volumes of 99% ethanol. Pasteur pipettes were used to recover the isolated DNA, which was washed with 70% ethanol and dried at room temperature for 10 minutes. The DNA was resuspended in 100 µl distilled water and the concentration was determined using a spectrophotometer. To identify the transposon insertion site, the flanking region on the

genomic DNA was directly sequenced using a primer, AR 56, annealing within the transposon (Table 3).

2.16. Piglet model and competitive assay

Piglets were chosen as animal model due to the similarity between the pathogenicity of *C. jejuni* in piglets and humans. Colostrum-deprived piglets from CFIA were maintained in cages in a controlled temperature room with heating lamps. The control piglets were separated from the infected piglets from day 0. Piglets were fed milk-replacer four times a day. On day 2, the piglets were orally challenged with a 10 ml 1:1 ratio mix of the *C. jejuni* wild-type strain and the strain overexpressing Cj0355c, in MH medium, approximately 2 hours after their last meal. To prepare the inoculum, bacterial strains were grown on MH agar plates for two days then 4 to five colonies were picked to inoculate a biphasic culture, which was placed in the microaerophilic station overnight. The optical densities of the original cultures were adjusted to 1 (at 600 nm) to ensure approximately identical CFU number between the two tested strains. Twelve ml from the suspension of each bacterial strain were pooled and centrifuged for 5 minutes at room temperature. The pellets were resuspended by pipetting up and down. Appropriate volume was used to obtain a final O.D. of 0.8 in 30 ml fresh MH medium. In order to determine the inoculum size 100 µl was taken from the prepared inoculum, serially diluted, and 10 µl from each dilution was spotted in quadruplicate on MH agar plates with and without Km (10 µg/ml). Colonies were counted on both selective (MH plates + Km) and non-selective plates (MH plates). Next, the number of colonies on the selective plates was subtracted from the number of colonies on the regular

plate to calculate the number of wild-type CFUs. The input ratio was then calculated by dividing the CFU of the Cj0355c overexpressing strain by the wild-type CFU amount. Piglets were sacrificed two days post-inoculation and necropsies were immediately performed. Five to 7 centimeters from 5 different segments of the piglets' gastrointestinal tracts (duodenum, jejunum, ileum, cecum, and colon) were harvested. Each segment was weighed, and the mucus layer was scraped and homogenized in 5 ml MH broth. Serial dilution was prepared from each homogenized section and 100 µl was plated on Karmali agar plates with and without Km (10 µg/ml). The plates were kept at room temperature to dry, then transferred to the microaerophilic incubator for 2 days. The number of colonies on the selective Karmali plates with Km (representing solely the strain that overexpresses Cj0355c) was subtracted from the number of colonies on the Karmali agar plates (representing the wild-type and the strain that overexpresses Cj0355c). The output ratio was obtained by dividing the CFU of the Cj0355c overexpressing strain by the CFU of the wild-type strain. The competitive index was calculated by dividing the output ratio *(in vivo)* by the input ratio *(in vitro)*.

To confirm that the growth rate of the strain that overexpresses Cj0355c is comparable to that of the wild-type strain *in vitro* and the obtained results are not due to growth defect in the constructed strain, a mixture of 1:1 ratio of both strains was grown in a biphasic culture with a starting O.D. of 0.1. The flask was placed in MACS-VA workstation. Samples were withdrawn at three different time points, serially diluted, and plated on MH agar plates with and without Km (10 µg/ml). The competitive index was calculated as described above.

2.17. Acid stress assay

C. jejuni NCTC 11168 was grown in 8 ml biphasic growth medium until mid-log phase. One ml was withdrawn from the culture and spun down at 12,000 rpm for 2 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml MH broth at either pH 7 or pH 4 (adjusted using HCl 12 N). After 4 minutes, the bacterial suspension was centrifuged for 10 minutes at maximum speed at room temperature. The pellet was resuspended in 10 ml of a fresh MH medium at pH 7. At time zero, a 100 µl aliquot was withdrawn from this bacterial suspension, serially diluted, and 10 µl from each dilution was spotted in quadruplicates on MH agar plates for CFU determination. Next, protamine sulfate was added to the bacterial suspension at a final concentration of 50 µg/ml. Five minutes after protamine addition, the number of viable bacteria was enumerated as described above. The MH agar plates were incubated in the MACS-VA workstation for 24 hours before CFU counting.

CHAPTER THREE: IDENTIFICATION AND CHARACTERIZATION OF GENES THAT ARE POTENTIALLY INVOLVED IN *C. JEJUNI* NCTC 11168 RESPONSE TO PROTAMINE SULPHATE

3. Introduction

In microbiology, DNA microarray has been used to study gene regulation, bacterial response to environmental changes, and genome organization (Ehrenreich, 2006). DNA microarray technique was used by various groups to study the bacterial response to antimicrobial peptides. Therefore, this analytical method was chosen in our study to elucidate the overall response of *C. jejuni* NCTC 11168 to protamine sulfate.

A recent paper by Gooderham and colleagues shows the up-regulation of *psrA*, which encodes a transcriptional regulator in *P. aeruginosa*, upon adding indolcin, a bovine antimicrobial peptide. Furthermore, by using microarray analysis, the authors were able to identify the previously unknown genes that are regulated by PsrA. These genes were found to be involved in type III secretion system, adhesion and motility, metabolism and energy, and outer membrane permeability (Gooderham et al., 2008). In another example, microarray analysis was used to identify factors conferring nisin resistance to *Lactococcus lactis* (Kramer et al., 2006). The collected data highlight the involvement of proteins coding for cell wall biosynthesis, energy metabolism, fatty acid and phospholipid metabolism, regulatory functions, and metal/peptide transport. Finally, genome-wide transcriptional profiling was used to study the response of *E. coli* to proline-rich antimicrobial peptides (Tomasinsig et al., 2004). Using this method, the authors were able to demonstrate that the

antimicrobial peptide, Bac7, could interact with bacterial components affecting different biological processes such as metabolic activities.

The main advantage of DNA microarray is its ability to generate comprehensive views of the transcriptional response of the bacterial genome to an endogenous or exogenous stimulus (Wildsmith and Elcock, 2001). However, the limitation of the technique is that the transcriptome does not correspond, in some cases, to protein levels. Moreover, the technique does not provide any data about the post-transcriptional events that take place (Ehrenreich, 2006). And finally, bacterial resistance could be due to the constitutive expression of certain gene(s) independently from the stimulus. For example, *Serratia* is intrinsically resistant to various antimicrobial peptides (Viljanen and Vera, 1984).

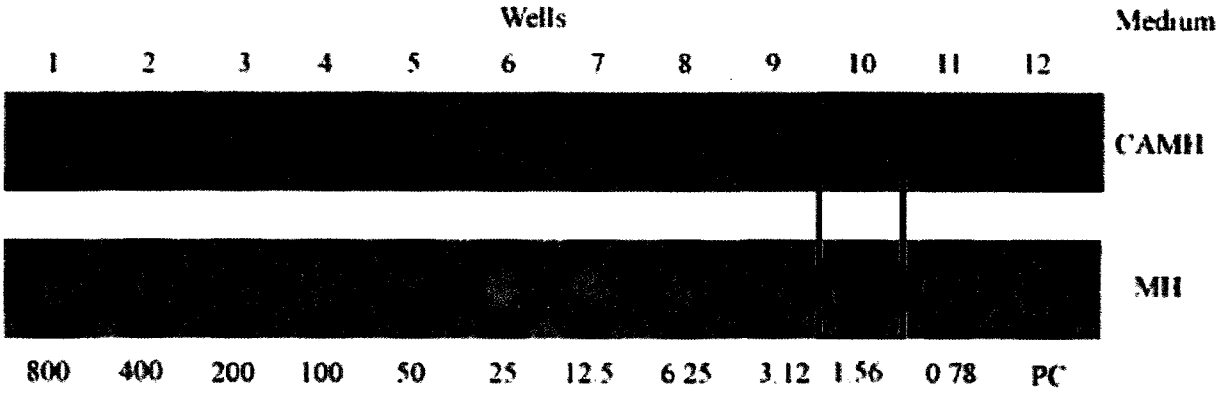
3.1. Results

3.1.1. Minimum inhibitory concentration (MIC) of protamine sulfate against *C. jejuni* NCTC 11168

The MIC of protamine sulfate against *C. jejuni* NCTC 11168 was determined according to the CLSI guidelines (Clinical and Laboratory Standards Institute) using macrodilution and microdilution methods (Wiegand et al., 2008). As shown in figure 2 for the microdilution assay, bacterial growth (pellets) could only be observed in wells containing the lowest peptide concentration (0.78 μg/ml) and in the negative control well to which no protamine had been added. This data indicates an MIC of 1.56 μg/ml independently of the

Figure 2. Minimum Inhibitory Concentration (MIC) of protamine against *C. jejuni* NCTC 11168 using the microdilution method.

The test was performed using two different media; Cation Adjusted Mueller-Hinton (CAMH) and Mueller-Hinton (MH). Serial dilution of protamine ranging from 800 μg/ml (well 1) to 0.78 μg/ml (well 11) were prepared. Well 12 is a positive control containing the bacterial strain without the addition of protamine. The MIC was determined to be the lowest concentration of protamine that results in the absence of bacterial growth (visualized by the absence of a bacterial pellet as observed in well 10). The box indicates the MIC, 1.56 μg/ml. PC, positive control without protamine sulfate.



Protamine concentration (μg/ml)

growth medium used, CAMH or MH. To note, the same MIC was obtained using the macrodilution method.

3.1.2. Survival assay of C. jejuni NCTC 11168

To test the effect of protamine on *C. jejuni* cell viability, we determined the susceptibility of the wild-type NCTC 11168 strain to protamine sulfate. The wild-type strain was exposed to 3 different concentrations of protamine (3.12, 12.5, and 25 μg/ml) for 5 minutes. The cells were grown in MEM medium before adding the protamine. As expected, figure 3 shows that the number of viable cells decreases upon increasing protamine concentrations (51.4, 3.1, 0.2 survival percentage respectively). The analysis of variance (ANOVA) indicates that the difference between the three tested concentrations is significant ($P < 0.0001$).

3.1.3. Transcriptome analysis

In order to identify protamine responsive genes, a genome-wide transcriptional analysis of *C. jejuni* NCTC 11168 was performed following 5 minutes exposure to 3 different concentrations of protamine sulfate (3.12, 12.5, and 25 μg/ml). The goal of this transcriptional study was to identify genes that might play a role in adaptive resistance, not only to protamine, but possibly to other antimicrobial peptides of the innate immune system. The data from all the biological and technical replicates (12 arrays) were statistically analyzed by using a Bayesian empirical method and clustered using Genesis software (Sturn et al, 2002). Based on gene expression fold change and calculated Bayesian *p* value, a total

Figure 3. Percentage of bacterial cells viable after protamine sulfate exposure.

Bacteria were grown in MEM medium to mid-log phase and different concentrations of protamine sulfate were added directly to the bacterial cultures. A 1 ml sample was withdrawn from the cultures before and after 5 minutes of protamine addition. Bacterial counts were enumerated by plating serial dilutions on MH-agar plates and following 24 hours incubation under microaerophilic conditions at 37°C. The assay with 50 μg/ml protamine concentration (yellow bar) was performed only once. Other protamine concentrations (3.12, 12.5, 25 μg/ml) were tested in triplicate. *P* values were 0.001, 0.02, 0.001 respectively. Analysis of variance ANOVA of the three concentrations was < 0.0001.

* Indicates significant difference from the untreated control sample.

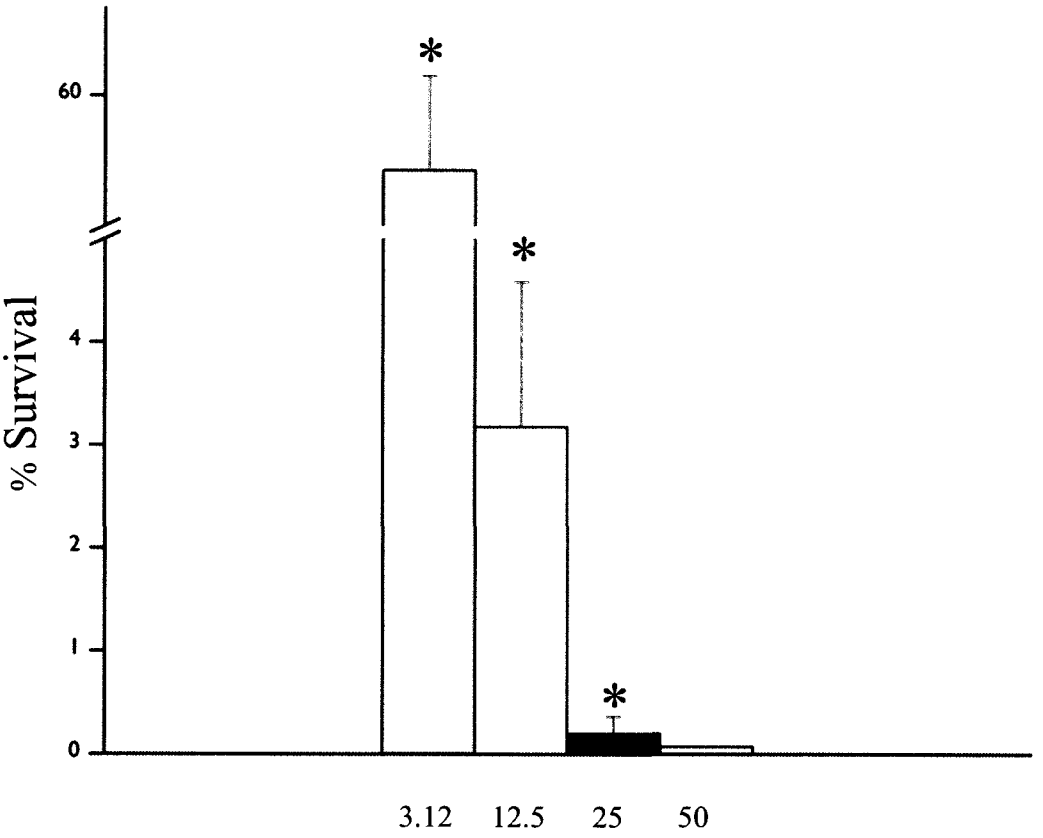
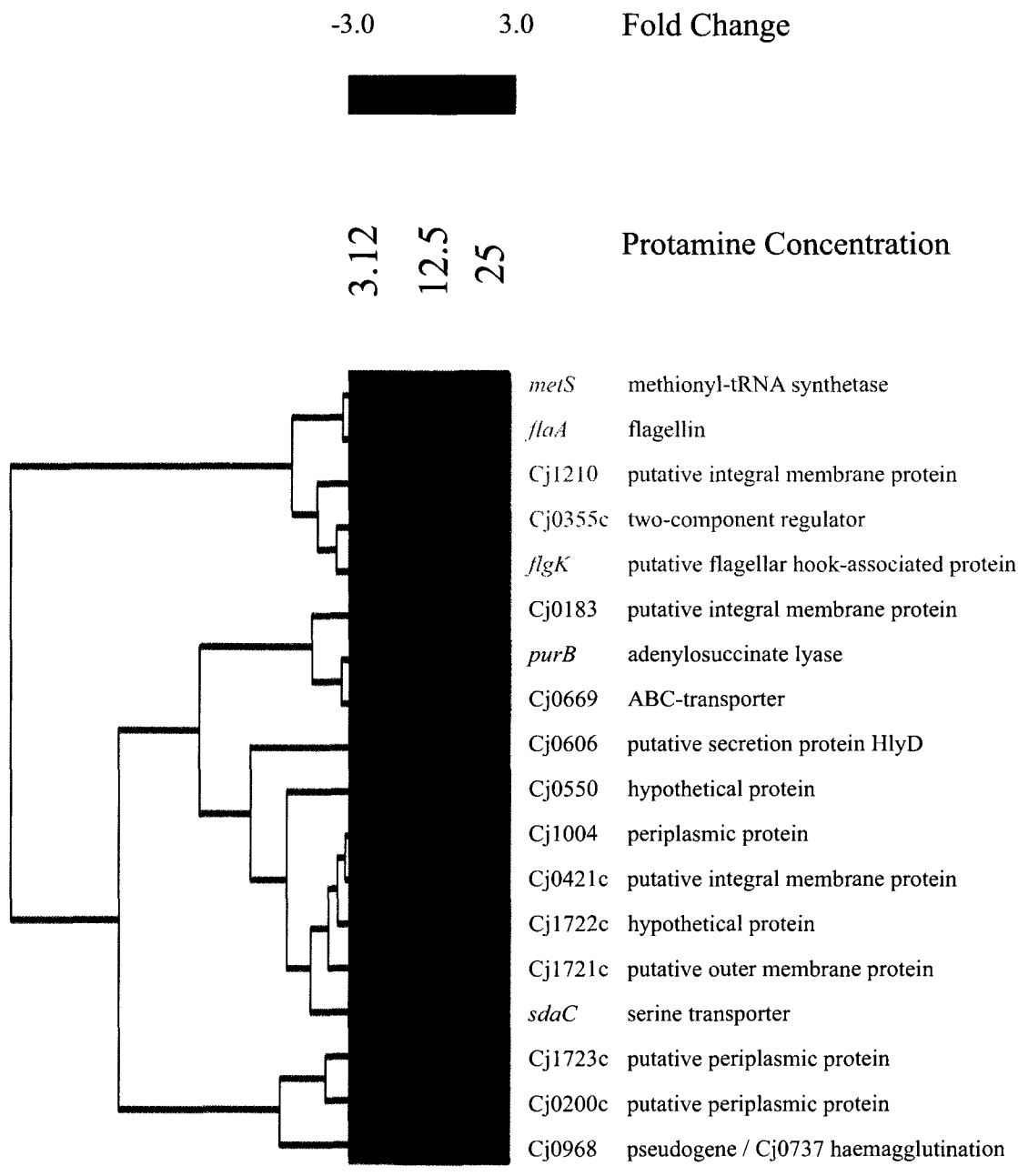

% Survival
60
4
3
2
1
0
*
*
*
3.12
12.5
25
50
Protamine concentrations µg/ml

Figure 4. Hierarchical cluster analysis of genes found to be significantly up- or down-regulated.

From left to right, the columns represent the transcriptome changes after 5 min at different protamine concentrations, 3.12, 12.5, and 25 μg/ml. The intensity of the color is proportional to the fold change of the transcript level (ranging between -3 and +3). Red colour represents up-regulated genes. Green represents down-regulated genes. Missing data are in gray. The length of the lines on the left corresponds to the value of the distance between the genes measured using Euclidian coefficient.


-3.0
3.0
Fold Change
3.12
12.5
25
Protamine Concentration
metS methionyl-tRNA synthetase
flaA flagellin
Cj1210 putative integral membrane protein
Cj0355c two-component regulator
flgK putative flagellar hook-associated protein
Cj0183 putative integral membrane protein
purB adenylosuccinate lyase
Cj0669 ABC-transporter
Cj0606 putative secretion protein HlyD
Cj0550 hypothetical protein
Cj1004 periplasmic protein
Cj0421c putative integral membrane protein
Cj1722c hypothetical protein
Cj1721c putative outer membrane protein
sdaC serine transporter
Cj1723c putative periplasmic protein
Cj0200c putative periplasmic protein
Cj0968 pseudogene / Cj0737 haemagglutination

of 18 genes were identified as differentially expressed among the three tested concentrations (Figure 4). Five of these genes were down-regulated and 13 were up-regulated. Interestingly, the number of differentially expressed genes was relatively small compared to *C. jejuni* responses to other stimuli such as temperature (Stintzi, 2003) or iron (Palyada et al., 2004).

Significantly down-regulated genes include Cj1210, *flaA, flgK, metS,* and Cj0355c. Cj1210, is a putative integral membrane protein with similarity to DedA protein family. DedA protein has been shown to play a role in selenite uptake and resistance in *E.coli* (Ledgham et al., 2005). The *flaA* and *flgK* encode two flagellum genes. And *metS (*methionyl t-RNA synthetase) is essential for bacterial protein synthesis. Cj0355c encodes a two component system regulator, which is thought to be essential in *C. jejuni* NCTC 11168 (Stahl et al., unpublished data; Raphael et al., 2005). Based on bioinformatic analysis this gene exhibits 60% similarity with a *Helicobacter pylori* gene HP1043 (Raphael et al., 2005) that regulates genes related to growth phase (Delany et al., 2002). Similar to the HP1043 in *H. pylori,* the Cj0355c is an orphan regulator, because a corresponding histidine kinase sensor has not yet been identified (Delany et al., 2002). Interestingly, a recent report showed that Cj0355c protein was down-regulated upon 1 hour exposure to oxidative stress caused by paraquat suggesting a potential role for Cj0355c in oxidative stress adaptation (Garénaux et al., 2008).

Up-regulated genes could be divided in three subgroups. The first group includes genes with no assigned function Cj1721c, Cj1722c, and Cj1723c. The second group includes genes encoding putative and hypothetical proteins such as Cj1004, Cj0200c, Cj0550 and Cj0606. The last group covers genes encoding proteins with known functions: Cj0669 (ABC

transporter), *sdaC* (serine transporter), and *purB* (adenylsuccinate lyase). None of these genes have previously been implicated in resistance to protamine or to any other antimicrobial peptides in *C. jejuni*.

3.1.4. Microarray data confirmation

Quantitative reverse transcriptase real time PCR (qRT-PCR) was used to confirm the gene expression data obtained from the microarray experiment. Four genes were selected: Cj0200c, Cj0550, Cj1004, and Cj1721c. As shown in Figure 5, the fold changes in gene expression obtained from this assay were in agreement with the expression from the microarray data, except for the Cj0550 gene.

3.1.5. Cj1721c - Cj1724c in operonic structure

The genomic organization of the three differentially expressed genes (Cj1721c, Cj1722c, and Cj1723c) suggests that they could be in an operonic structure. In addition, the location and orientation of the uncharacterized Cj1724c indicated that it might be part of the same operon. To verify this operonic structure, primers annealing to flanking regions between two adjacent genes were designed as shown in Figure 6. Using a one-step reverse transcriptase PCR, three PCR products (114, 482, 526 bp) were obtained, which indicates that the genes are co-transcribed and confirms that the four genes are in an operonic structure. This group of genes constitutes an independent transcriptional unit since the downstream gene, Cj1720, is oriented in the opposite transcriptional direction.

Figure 5. Confirmation of microarray data by qRT-PCR.

The expression of Cj0200c, Cj0550, Cj1004, and Cj1721c was confirmed by qRT-PCR. The extracted RNA from the exposure to 3.12 μg/ml of protamine was used. The assay was repeated twice. Two-sample *t*-test shows that the expression of all genes, except Cj0550, was significantly different from the endogenous control gene, *ilvC* ($p = 0.04, 0.18, 0.003, 0.08$ respectively).

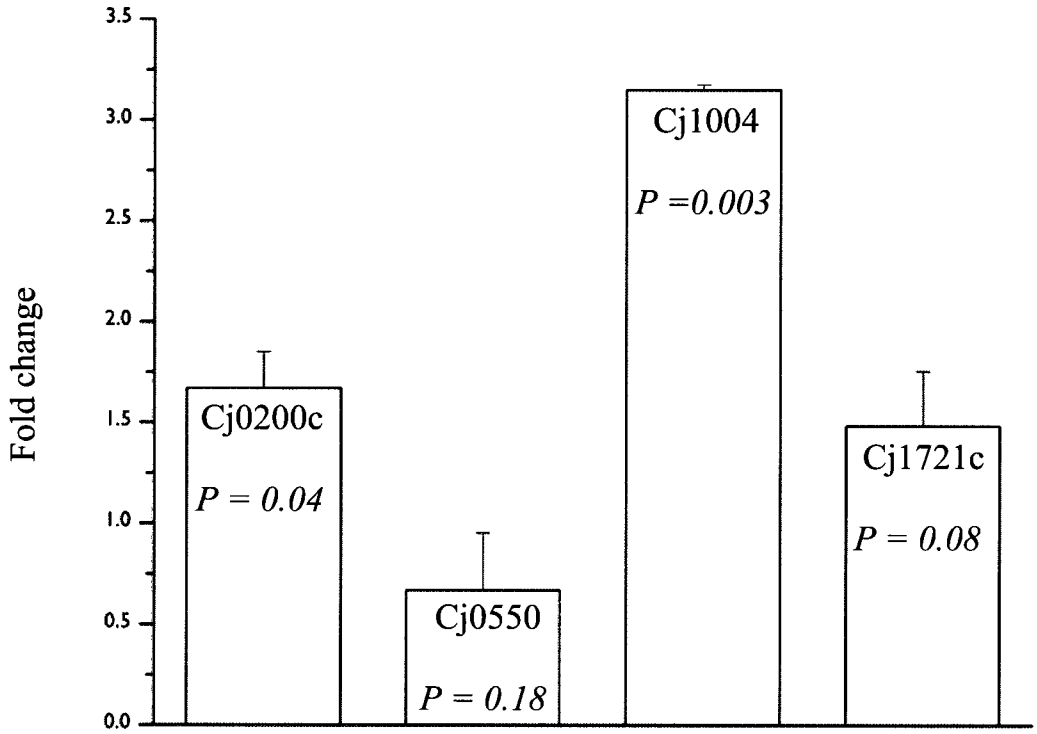
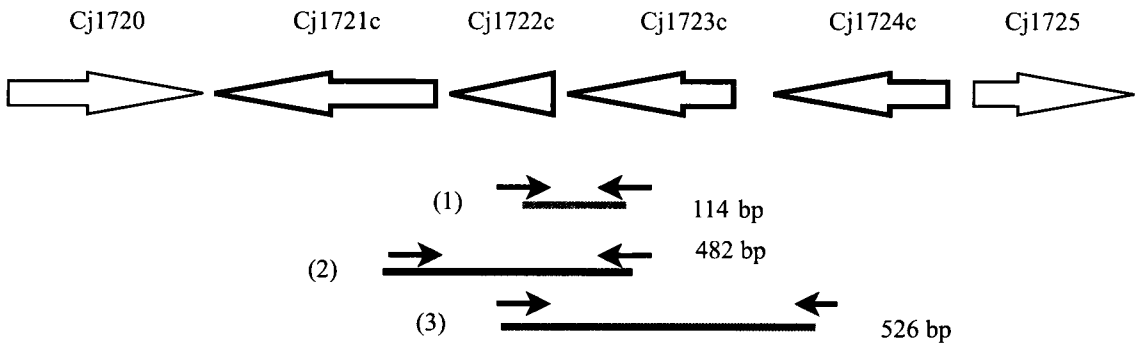

Fold change
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Cj0200c
P = 0.04
Cj0550
P = 0.18
Cj1004
P =0.003
Cj1721c
P = 0.08

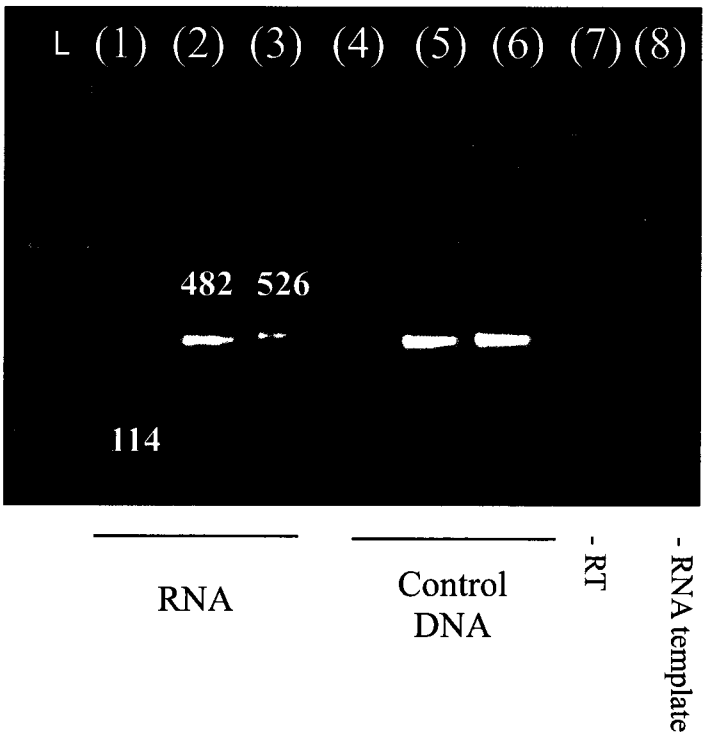
Figure 6. Operon mapping by one step RT-PCR analysis of Cj1721c-Cj1724c genes.

The template RNA was purified from bacteria that were grown in MEM medium and exposed to protamine sulfate for 5 minutes. The genes in the same operon are represented in open red arrows. Predicted RT-PCR fragments with gene names are labeled 1, 2, and 3. The gel lanes match the RT-PCR fragment labels. Lanes 1, 2, and 3 show the generated PCR products from the RNA template. Lanes 4, 5, and 6 show the generated PCR products using the control *C. jejuni* genomic DNA template. Lane (L) is a 1Kb DNA ladder. Lane (7) represents a control sample without the reverse transcriptase enzyme. Lane (8) represents a negative control with no RNA template added. The black solid arrows represent the primers used to generate the PCR products and their approximate position on the genome.

A)


Cj1720
Cj1721c
Cj1722c
Cj1723c
Cj1724c
Cj1725
(1)
114 bp
(2)
482 bp
(3)
526 bp

B)


L (1) (2) (3) (4) (5) (6) (7) (8)
482 526
114
RNA
Control DNA
- RT
- RNA template

3.1.6. Construction of the Cj1721c deletion mutant

A deletion mutant of the Cj1721c gene was constructed in which a chloramphenicol resistance cassette (CAT) was inserted by allelic recombination. 322 bp of the total 892 bp original gene length were replaced by the CAT insertion. It is unlikely that the knockout could affect the expression of other genes in the operon because they are encoded on the complement DNA strand and no genes are encoded downstream of Cj1721c. The construction of the mutant was confirmed by sequencing the knockout gene using the genomic DNA from the mutant and the primers Cj1721c-F and Cj1721c-R (Table 3).

3.1.7. Characterization of the Cj1721c deletion mutant

Different phenotypic assays were performed to characterize the Cj1721c deletion mutant and to investigate its potential role in protamine sulfate resistance. These assays will be discussed below.

3.1.7.1. Growth curve

The growth curves were generated using the Bioscreen system as described in the materials and methods section. Compared to the wild-type NCTC 11168, the ΔCj1721c mutant did not present any significant growth defect. The same results were obtained in MH (Figure 7) and in MEM media (Figure 8). It is important to note that, as previously observed, *C. jejuni* reached a lower optical density in MEM medium than in MH medium (Palyada et al., 2004).

Figure 7. Growth curves of *C. jejuni* NCTC 11168 and Cj1721c deletion mutant in MH medium.

O.D. measurements (620 nm) were taken every 15 minutes for 24 hours in Mueller Hinton (MH) medium. The growth curves of ΔCj1721c and the wild-type strain NCTC 11168 are represented in green and in blue respectively.

MH

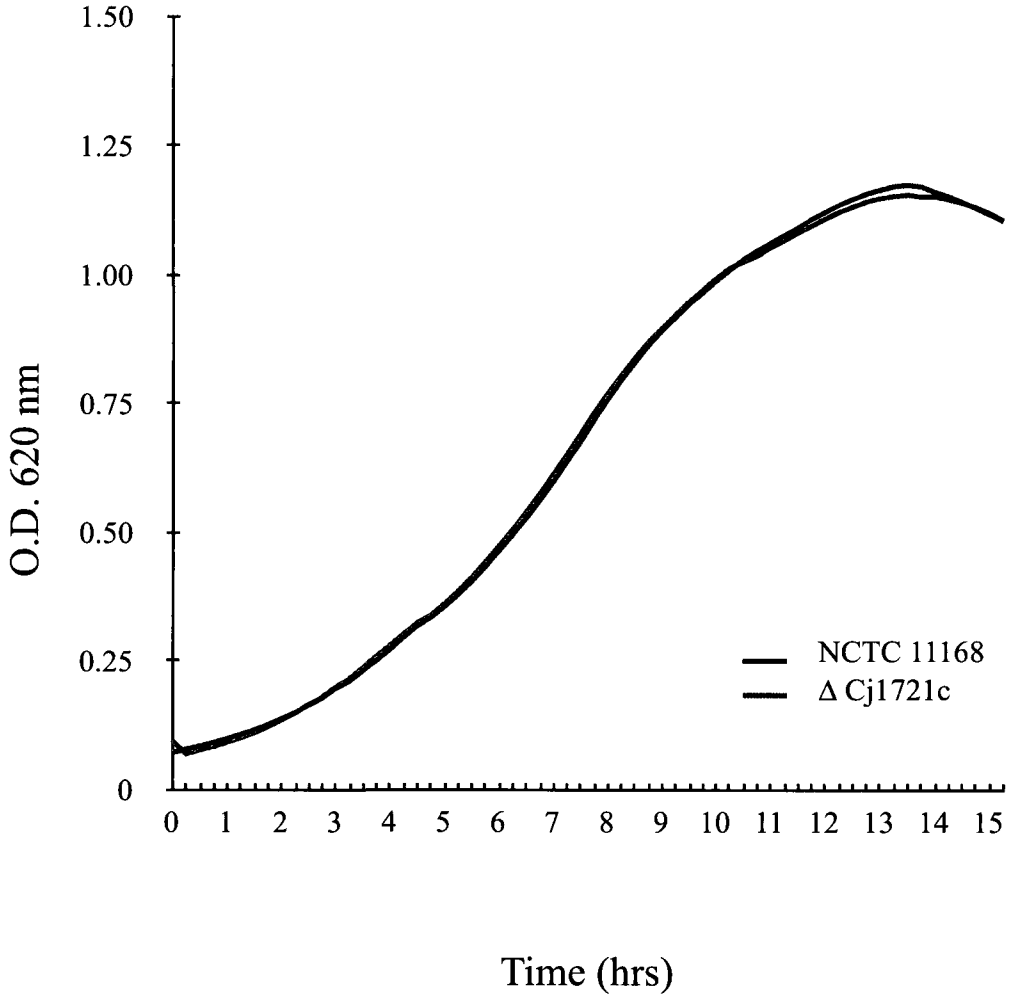
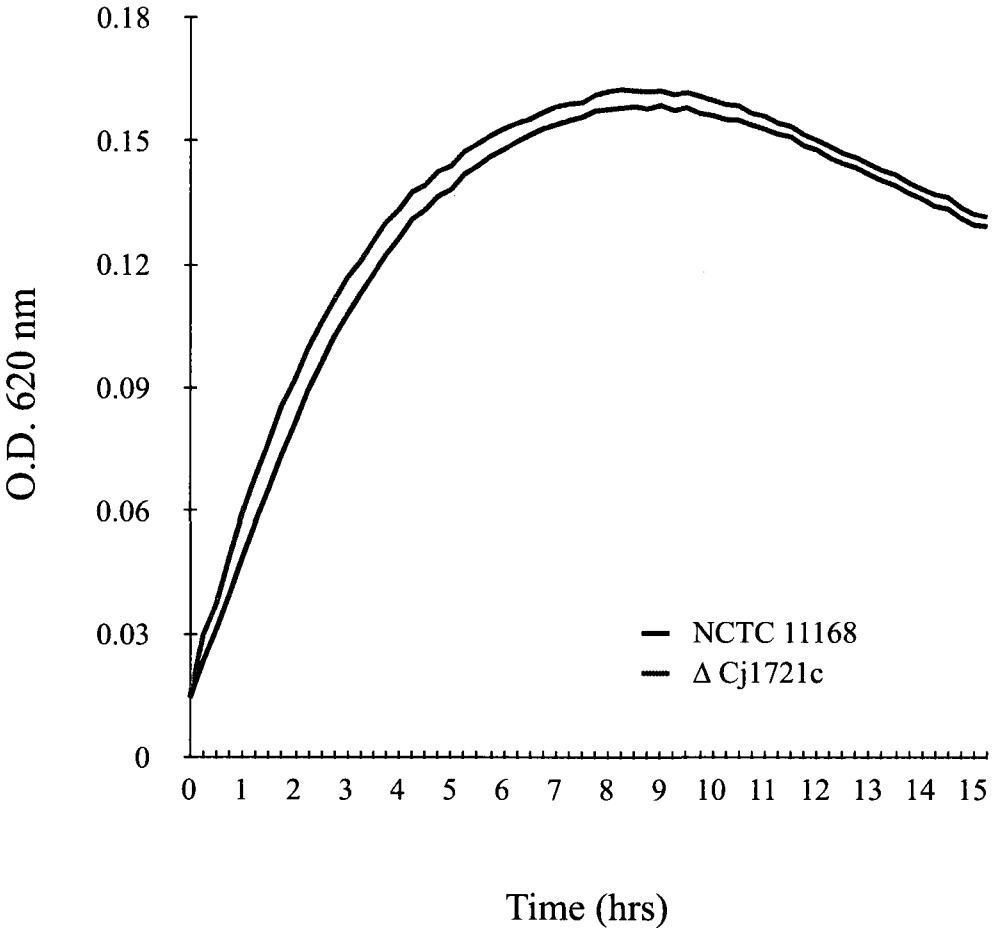

1.50
1.25
1.00
0.75
0.50
0.25
0
O.D. 620 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
NCTC 11168
Δ Cj1721c
Time (hrs)

Figure 8. Growth curves of *C. jejuni* NCTC 11168 and Cj1721c deletion mutant in MEM medium.

O.D. measurements (620 nm) were taken every 15 minutes for 24 hours in Minimum Essential Medium (MEM). ΔCj1721c and the wild-type strain growth curve are shown in green and blue respectively

MEM


0.18
0.15
0.12
0.09
0.06
0.03
0
O.D. 620 nm
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Time (hrs)
NCTC 11168
Δ Cj1721c

3.1.7.2. Minimum inhibition concentration (MIC)

In order to test the susceptibility of the Cj1721c deletion mutant toward protamine, its MIC in MH broth was determined following the same methods used for the wild-type strain. The Cj1721c deletion mutant exhibited the same MIC as the wild-type *C. jejuni* strain NCTC 11168 (1.56 μg/ml) independently of the growth medium (MH, MEM or CAMH) in which the assay was performed.

3.1.7.3. Survival assay

The protamine survival assay was performed to further test the phenotype of the ΔCj1721c mutant. The number of colony forming units (CFU) was counted following the exposure of the mutant and the wild-type strains to 25 μg/ml protamine sulfate in MEM medium. As shown in Figure 9, the ΔCj1721c mutant was more resistant to protamine than the wild-type strain after five minutes exposure to protamine. The cell survival percentage was 38.8% for the ΔCj1721c mutant compared to 6.5% for the wild-type strain ($p = 0.006$).

3.1.7.4. Biofilm formation ability

The biofilm-forming ability of the mutant was compared to that of the wild-type strain. Biofilm formation was assessed at 24, 48, and 72 hours by measuring the absorbance of crystal violet at 574 nm (McLennan et al. 2008). The capacity of the ΔCj1721c mutant to form biofilm was not significantly different from the wild-type strain after 24 hours but reduced significantly after 48 and 72 hours ($p = 0.009$, and 0.01 respectively) (Figure 10).

Figure 9. Survival percentage of ΔCj1721c and wild-type strain following exposure to protamine sulfate

Bacterial strains were grown in MEM medium to mid-log phase. Protamine sulfate (25 μg/ml) was added for 5 minutes. Samples were taken before and after the addition of protamine and the CFU was determined by spotting 10 μl of bacterial culture on MH agar plates. The plates were then placed in the microaerophilic station for 24 hours. Two-sample *t*-test indicates that the difference between the ΔCj1721c and the wild-type strain is significant ($p = 0.006$)

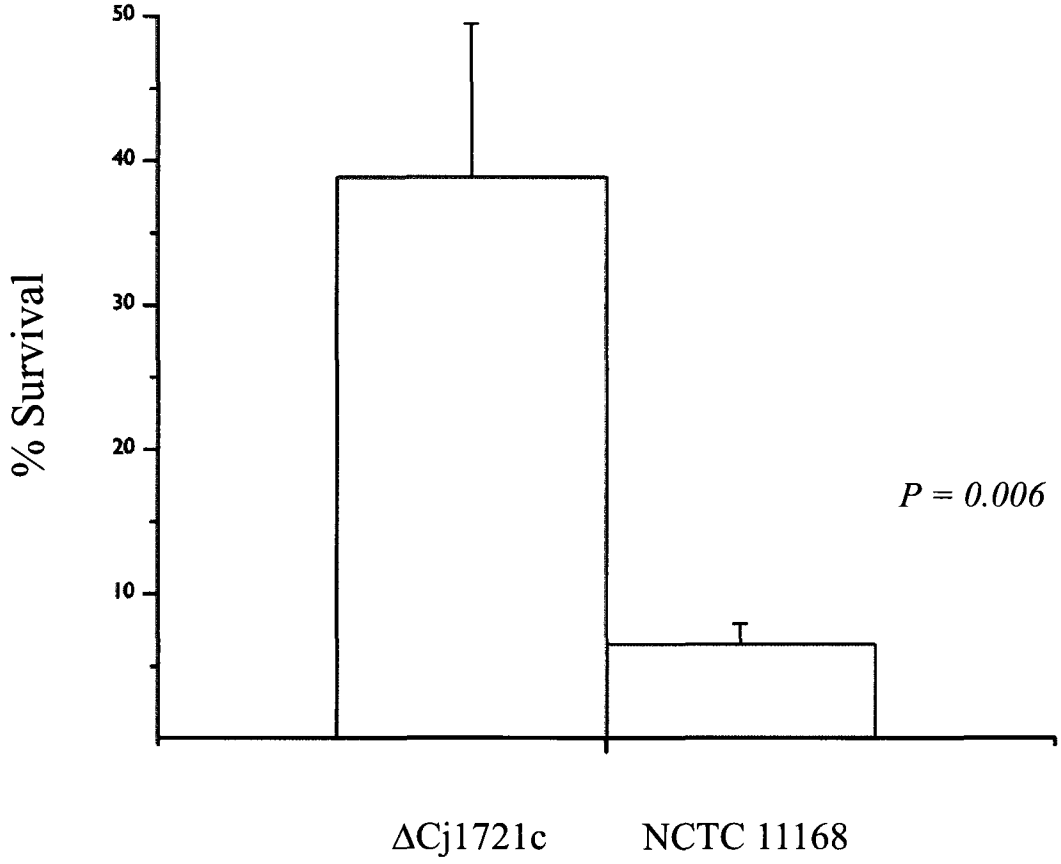
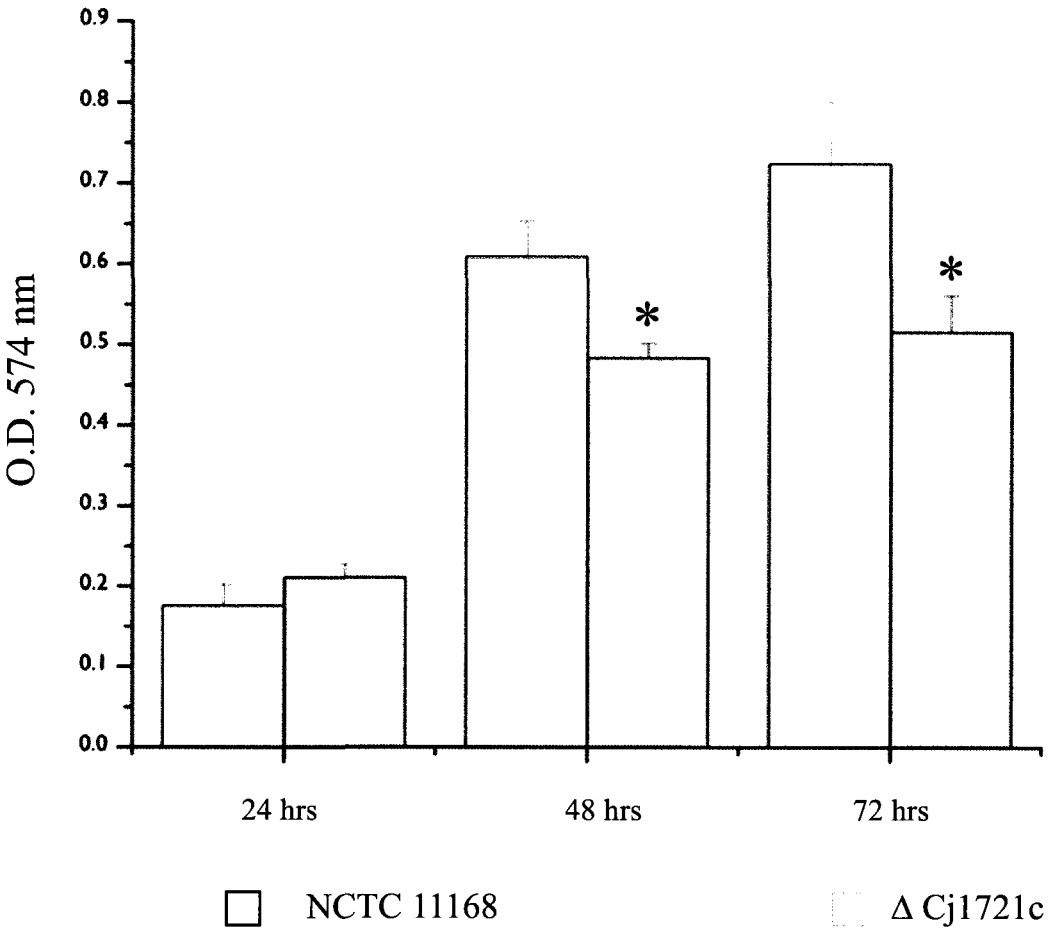

50
40
30
20
10
% Survival
P = 0.006
ΔCj1721c
NCTC 11168

Figure 10. Biofilm formation ability of the wild-type strain and ΔCj1721c mutant.

Crystal violet absorbance (574 nm) was measured at 24, 48, and 72 hours. The *p* values were 0.125, 0.009, 0.01 respectively at the different time points using two-sample *t*-test .

Asterisk (*) represents statistically significant difference.


O.D. 574 nm
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
24 hrs
48 hrs
72 hrs
NCTC 11168
Δ Cj1721c

3.1.7.5. Pellicle formation assay

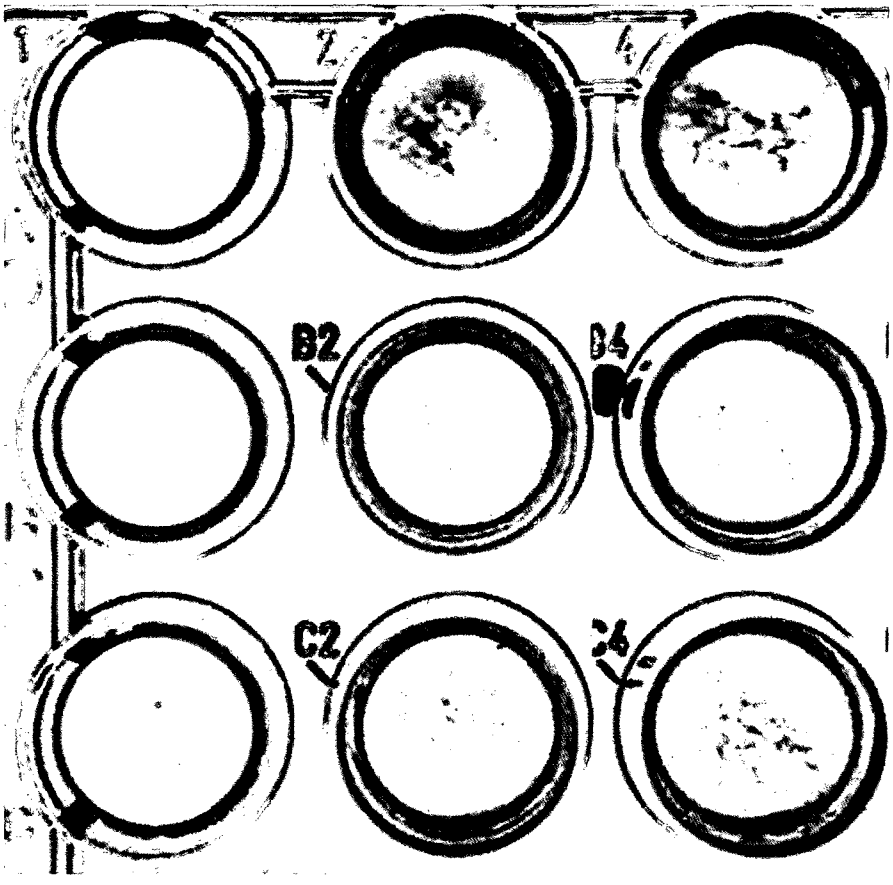
The ability of the ΔCj1721c mutant to form a biofilm at the gas-liquid interface was compared to that of the wild-type strain by using a pellicle formation assay. The experiment was repeated three times and in each time three wells were inoculated with the same strain. Collected data was interpreted qualitatively as shown in Figure 11, which indicates that the ΔCj1721c mutant had a higher ability to form pellicles than the wild-type strain. This result supports the data from the biofilm formation assay in which the ΔCj1721c mutant was affected in its ability to form biofilms compared to the wild-type strain (Figure 10).

3.1.7.6. Motility assay

The motility of the ΔCj1721c mutant was assessed using MH motility agar plates (0.4% agar). After 24 hours, the mean growth diameter was 27 ± 2.64 mm (mean ± SD) and 20 ± 0.57 mm for the ΔCj1721c mutant and the wild-type strain, respectively. Compared to the wild-type strain, the ΔCj1721c mutant was significantly more motile in MH medium ($p = 0.01$). The hypermotility of the ΔCj1721c mutant was observed even after 48 hours and the mean growth diameter was 54.7 ± 2.51 mm compared to 44.3 ± 3.21 mm for the wild-type strain ($p = 0.01$).

Figure 11. Pellicle formation at the gas-liquid interface of the wild-type strain and ΔCj1721c mutant.

The assay was performed in three biological replicates and each replicate included three technical replicates. The figure represents one biological replicate. The negative control well contains MH medium only. Four μl of mid-log phase bacterial culture (O.D. 0.7) were inoculated in the wells. The plate was placed in the microaerophilic station for 3 days.



Negative control NCTC 11168 Δ Cj1721c

3.1.8. Transposon mutant library screening

Two thousand and twelve mutants were screened thrice against two concentrations of protamine sulfate (200 μg/ml and 500 μg/ml) on CAMH agar plates to identify both protamine-sensitive and protamine-resistant mutants. Only the mutants that were shown to be affected in all three replicates were selected for detailed analysis. Eleven mutants were found to be protamine-sensitive and 16 were found to be protamine-resistant (Table 4).

The growth curve and the doubling time of each mutant were determined in CAMH. Each mutant growth curve was repeated at least three times with three technical replicates. Using Bonferroni statistical method, table 5 shows that the growth of only one resistant mutant was significantly different from the growth of the wild-type strain.

To identify the transposon insertion site, the flanking region on the genomic DNA was directly sequenced using a primer annealing within the transposon. Table 4 shows the identified resistant mutants and their annotated function. Direct sequencing of the genomic DNA from the sensitive mutants was unsuccessful.

A microarray approach might be an alternative to locate the transposon insertion site in these mutants. However, unsuccessful sequencing could be explained by the insertion of transposon at multiple locations on *C. jejuni* chromosome.

Table 4. Transposon (Tn5) insertion sites in the identified resistant mutants

The transposon mutant screening assay allows the identification of 11 protamine-sensitive and 16 protamine-resistant mutants. From the 16 resistant mutants, 8 transposon insertion sites were identified. These sites are 2 oxidoreductases (Cj0415, Cj0485), Cj0044c, Cj0486, CRISPR (clustered regularly interspaced short palindromic repeats), *rloH, hsdS,* and a 16 S ribosomal RNA protein. The remaining 10 mutants insertion sites were in the same Cj0415 gene. Unfortunately, we were not able to identified the insertion sites of the protamine-sensitive mutants.

Table 4. Transposon (Tn5) insertion sites in the identified resistant mutants

Affected Gene	Function
Cj0044c	Hypothetical protein
Cj0415c	Putative oxidoreductase
Cj0485	Putative oxidoreductase
Cj0486	Putative sugar transporter
Ribosomal RNA	16S ribosomal RNA
rloH (*Cj1550c*)	Putative ATP/GTP binding protein
hsdS (*Cj1551c*)	Putative type I restriction enzyme S protein
Cj1521, Cj1522c, Cj1523c	Putative CRISPR associated protein

Table 5. Doubling time of the identified transposon mutants.

The table shows the doubling time of *C. jejuni* wild-type strain and the identified transposon mutants. The mean and the standard deviation (SD) from the biological (2) and technical replicates (6) were calculated. Bonferroni statistical method shows that the growth of one resistant mutant (#15) was significantly different from the wild-type strain ($p < 0.05$).
NS, not significant, S*, significant, WT, wild-type.

Table 5. Doubling time of the identified transposon mutants

Strain	Mean	SD	ANOVA ($p = 0.05$)
	NCTC 11168		
WT	3.18	0.40	
	Resistant strains		
1	3.58	0.27	NS
2	3.37	0.44	NS
3	3.48	0.49	NS
4	3.77	0.72	NS
5	3.19	0.17	NS
6	3.31	0.73	NS
7	4.10	0.42	NS
8	3.81	0.91	NS
9	3.50	0.32	NS
10	3.95	1.03	NS
11	3.64	0.63	NS
12	3.69	0.54	NS
13	3.80	0.48	NS
14	3.86	0.52	NS
15	**4.57**	**1.62**	**S***
16	3.55	0.58	NS
	Sensitive strains		
1	2.35	0.26	NS
2	3.46	0.22	NS
3	2.42	0.07	NS
4	2.99	0.55	NS
5	3.43	0.47	NS
6	3.81	0.71	NS
7	3.43	0.31	NS

3.1.9. The role of the Cj0560 MATE efflux pump

Although *C. jejuni* NCTC 11168 genome-wide transcriptome analysis did not reveal any role of efflux pumps in response to protamine, the MATE efflux pump was selected for further study for two main reasons. First, this pump is known to play a role in resistance to cationic drugs like antimicrobial peptides in other microbes (Rouqette-Loughlin et al., 2003). Second, the function of this pump has not been investigated in *Campylobacter*. Therefore, a Cj0560 deletion mutant was created as described in the materials and methods. It is worth noting that *C. jejuni* NCTC 11168 genome contains two genes (Cj0560 and Cj0619) that have been identified so far encoding MATE efflux pumps. The *Cj0560* knockout phenotype was studied in further detail.

3.1.9.1. Growth curve

The growth curve was generated using the Bioscreen system. The ΔCj0560 mutant did not exhibit any growth abnormality compared to the wild-type strain in MH and MEM media.

3.1.9.2. Minimum inhibition concentration (MIC)

The MIC of protamine for the Cj0560 mutant was compared to that of the wild-type strain. Both MICs were identical (1.56 μg/ml). To eliminate the possibility of other pump systems compensating for the absence of Cj0560, a general unspecific efflux pumps inhibitor, phenylalanine-arginine β-naphthylamide (PAβN) (Mamelli et al., 2006), was added to the medium and the MIC of protamine was determined again. In this assay, 2 fold increase

in sensitivity was observed between the mutant and the wild-type strain. This finding indicates that the MATE efflux pump system is not involved in resistance to protamine sulfate.

3.1.9.3. Survival assay

In order to test the ΔCj0560 sensitivity to protamine sulfate, the number of CFUs for the Cj0560 mutant and the wild-type strain was counted following 5 and 20 minutes exposure to protamine (12.5 μg/ml). The data showed that the ΔCj0560 susceptibility to protamine is similar to that of the wild-type strain (Figure 12). These results confirm that the MATE efflux pump (Cj0560) does not alter the susceptibility of *C. jejuni* to protamine.

3.1.10. Survival assay after exposure to acid stress

A protamine survival assay was performed after exposing *C. jejuni* NCTC 11168 to acid stress in order to mimic the passage of the bacterium through the stomach and to investigate if this passage could induce resistance to the innate immune system, specifically for antimicrobial peptides. Exposing *C. jejuni* NCTC 11168 to an acidic environment (pH = 4) for 10 minutes caused the stressed population to become more resistant to 50 μg/ml of protamine sulfate as compared to the unexposed population ($p = 0.001$). The survival percentages were 37% and 79% in the unstressed versus the stressed population respectively (Figure 13).

Figure 12. Survival percentage of the wild-type strain and the Cj0560 deletion mutant following protamine exposure.

Bacterial strains were grown in Mueller-Hinton medium to mid log phase before adding protamine sulfate (12.5 μg/ml). Samples were withdrawn for CFU count following 5 and 20 minutes exposure to protamine sulphate. Two-sample *t*-test indicates that the difference between the two strains is not significant (NS) ($p = 0.13$ and 0.59 for the 5 and 20 minutes time points respectively).

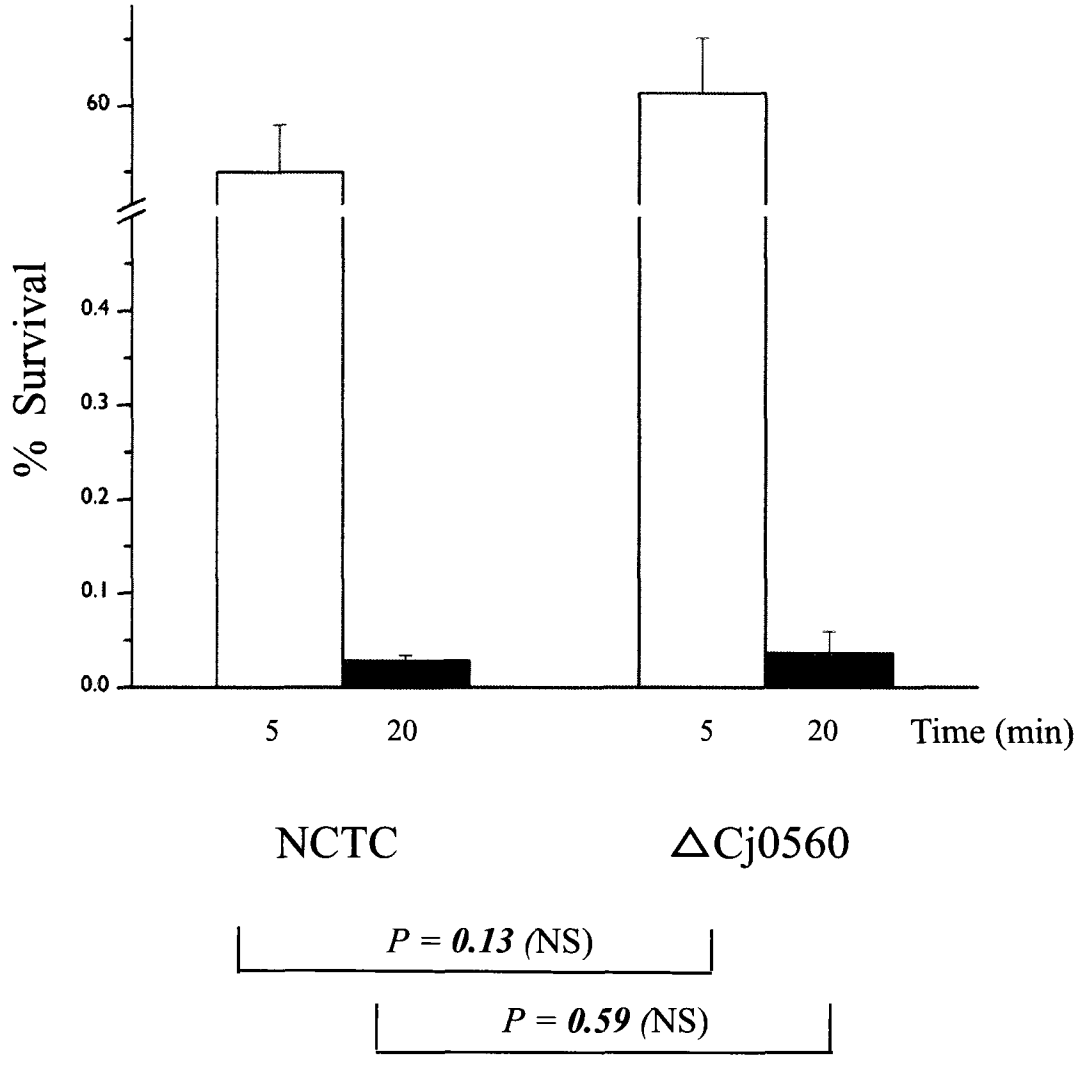
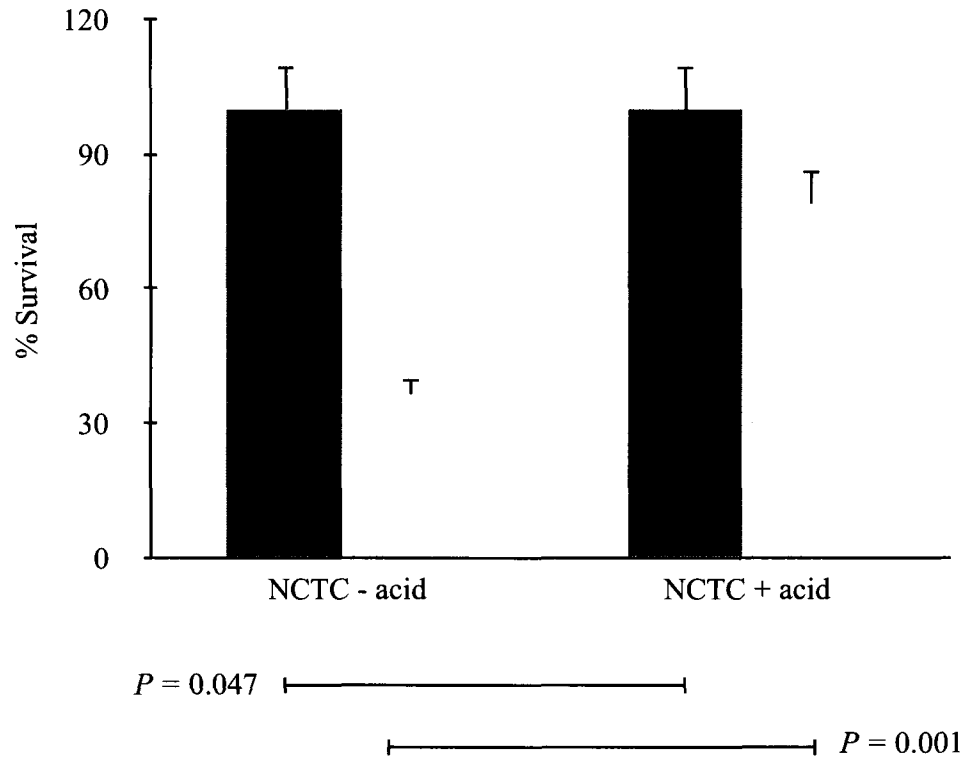

% Survival
60
0.4
0.3
0.2
0.1
0.0
5
20
5
20
Time (min)
NCTC
ΔCj0560
P = 0.13 (NS)
P = 0.59 (NS)

Figure 13. Survival percentage of *C. jejuni* NCTC 11168 with and without exposure to acid stress (pH = 4 for 10 minutes).

Campylobacter jejuni NCTC 11168 was exposed to acid stress (pH=4) for 10 minutes before adding protamine sulfate (50 μg/ml) for 5 minutes. Survival of the stressed and unstressed populations was assessed by CFU count after incubating the plates under microaerophilic conditions.
Two-sample *t*-test indicates that the difference between the two populations is significant ($p = 0.001$).


120
90
60
30
0
% Survival
NCTC - acid
NCTC + acid
P = 0.047
P = 0.001
Percentage of the starting bacterial population
Percentage of cell survival after protamine exposure

3.2. Discussion

3.2.1. Minimum inhibition concentration (MIC) and survival assay of the *C. jejuni* NCTC 11168

The MIC was determined in three different growth media (MH, CAMH, and MEM) to examine if the medium affects protamine sulfate activity. Previous studies have shown that divalent cations, magnesium and calcium, could antagonize the effect of certain antimicrobial peptides (Carrasco et al., 1981). Therefore, CAMH medium was selected because it has higher concentrations of magnesium and calcium compared to the MH medium. MEM medium was chosen to mimic the physiological environment that *C. jejuni* NCTC 11168 encounters upon entry into the gastrointestinal tract. The determination of the MIC in MEM medium was not successful using the microdilution method due to the limited growth of *C. jejuni* in this medium. To overcome this problem, the macrodilution method was used and the data shows that the MIC in MEM was identical to the one in MH and CAMH. In conclusion, the iron deprived medium did not alter *C. jejuni* sensitivity to protamine and the obtained data indicate that the MIC was not affected by the various growth media (1.56 μg/ml). However, this finding does not rule out that other antimicrobial peptides might be affected under these stringent conditions.

The percentage of surviving cells showed a concentration-dependent bactericidal activity of protamine against *C. jejuni*. Increasing the protamine concentration led to a decrease in the number of the viable bacterial cells following five minutes exposure to 3 different concentrations of protamine (3,12, 12.5, and 25 μg/ml).

3.2.2. Genome-wide transcriptome analysis

To identify genes that are involved in *Campylobacter* resistance to antimicrobial peptides, the transcriptome profiles of the wild-type strain with and without protamine were compared. The selection of protamine as a prototype molecule in this study was based on various factors. First, protamine shares the same mechanism of action as other antimicrobial peptides via interacting with the bacterial membrane (Aspedon and Groisman, 1996). Second, protamine is used as a preservative in chicken food industry (Ueno et al., 1989; Potter et al., 2005).

Although protamine has not been shown to be secreted in the gastrointestinal tract of chickens, *Campylobacter* is exposed to similar antimicrobial peptides in the GI tract of chicken, which secrete a wide range of other antimicrobial peptides that might prime *Campylobacter* before colonizing its human host (Lynn et al., 2004; Van Dijk et al., 2007; Evans et al., 1995). Therefore, performing similar transcriptome analysis with other peptide families, especially the ones that are secreted in chicken and human, can provide a global view of the resistance to these molecules and could demonstrate that the same genes which are involved in protamine resistance are also involved in the resistance to other antimicrobial peptides.

Our transcriptome study identified 18 genes that were differentially expressed upon exposure to protamine. Five genes were down-regulated and 13 were up-regulated.

Among the up-regulated genes, 3 were in tandem, Cj1721c, Cj1722c, and Cj1723c. The overexpression of these genes increased as the protamine concentration increased with the highest level of fold change at 25 μg/ml. The genomic organization of Cj1721c - Cj1723c

suggests that these genes might be in an operonic structure. A one step reverse transcriptase PCR showed that they are indeed in operonic structure. Since Cj1724c is in the same transcriptional direction as the Cj1721c - Cj1723c genes, the test was expanded to include this gene. The results demonstrated that Cj1724c is also in the same operon. However, this gene was not found to be differentially expressed in response to protamine exposure, suggesting that the microarray approach failed to identify this gene and/or that the Cj1724c mRNA is being degraded by active endogenous RNases (Jain, 2000). The function of this operon is not known although it is likely to be a transporter because of the predicted organization of the encoding proteins in the bacterial membrane. Cj1721c is a putative outer membrane protein. Cj1722c is a hypothetical protein. Cj1723c is a putative periplasmic protein and Cj1724c is a putative GTP cyclohydrolase I. Recently, Cordwell et al. have shown that Cj1721c transcript was >2 fold more abundant in a virulent *Campylobacter* strain compared to avirulent strain (Cordwell et al., 2008), indicating that this protein might play a role in enhancing *Campylobacter* virulence.

Another up-regulated gene was Cj0669. This gene codes for an ABC transporter ATP-dependent binding protein and is part of an operon including Cj0667, Cj0668, Cj0669, and *rpoN* (Malik-Kale et al., 2007). This operon is involved in flagellum biogenesis and its downregulation was found to affect the virulence of *Campylobacter* strains isolated from different hosts (Malik-Kale et al., 2007).

The other up-regulated genes in this study such as Cj0200c, Cj0606, and Cj1004 were previously predicted to be regulated by the two-component system DccRS (diminished capacity to colonize) (MacKichan et al., 2004). The authors demonstrated that this DccRS

system regulates the expression of bacterial membrane proteins and it is important for *in vivo* colonization. The function of Cj0200c (putative periplasmic protein) is unknown, and no homology to other proteins was found (MacKichan et al., 2004). Cj0606 is a putative secretion protein alpha-hemolysin, HlyD, which forms with Cj0607 and Cj0608 a putative operonic structure. Based on its periplasmic location, the Cj0606 protein could be the bridge between the cytoplasmic and the outer membrane protein, through which hemolysin is being transported. Only Cj0606 gene in this operon was above the statistical cutoff. Hemolytic assays are required to confirm that hemolysin is indeed secreted by this system upon exposure to protamine. This could be done on blood agar plate to which protamine sulfate is added and the hemolytic effect of the bacterial strain is observed. The involvement of this operon in virulence was described earlier in other Gram-negative bacteria (Welch, 1991). Accordingly, *in vivo* colonization assays of the deletion mutant could provide additional information on the role of this operon in Campylobacteriosis.

Another up-regulated gene is *purB,* which codes for an adenylosuccinate lyase. This gene is involved in purine ribonucleotide biosynthesis, like adenosine monophosphate (AMP), a precursor for the biosynthesis of ATP. Moreover, the PurB protein can bind the glutamyl-tRNA synthetase increasing its affinity to glutamate and ATP (Gendron et al., 1992). This function coordinates *de novo* AMP and protein biosynthesis, which are both affected by the addition of protamine (Gendron et al., 1992). Taking these two functions into account, PurB appears to be an essential protein that indirectly controls replication and metabolism (Toth et al., 2000) and its overexpression could counterbalance the decreased ATP level resulting from protamine exposure (Aspedon and Groisman, 1996)

The serine transporter, *sdaC,* was also present among the over-expressed genes. In *E.coli,* SdaC was found to be the receptor for Colicin V, which is an antimicrobial peptide (Gerard et al., 2005). Similarly, this suggests that protamine might bind to *C. jejuni* SdaC affecting serine transport. Serine is an important amino acid for *Campylobacter* growth and colonization of 3-week-old chickens (Velayudhan et al., 2004). Therefore, the overexpression of SdaC could counteract protamine deactivation of serine transport.

The down-regulated genes included the flagellum genes *flgK* and *flaA,* one putative two-component system regulator Cj0355c, one putative integral protein with unknown function Cj1210, and *metS* encoding a methionyl t-RNA synthase. The flagellum genes were previously shown to play a role in motility and colonization (Kalmokoff et al., 2006). The *flaA* knockout resulted in a nonmotile phenotype (Kalmokoff et al., 2006). The *flgK* knockout was found to be less motile than the wild-type strain and exhibited an attenuated cecal colonization ability in chicken (Fernando et al., 2007). The downregulation of the methionyl-tRNA synthetase *(metS)* suggests that protamine affects protein synthesis as previously described in *Salmonella* by Aspedon and Groisman (Aspedon and Groisman, 1996). Another down-regulated gene was Cj1210, which is a putative integral membrane protein with unknown function. This protein shows a similarity to members of the DedA protein family. Although the function of this family is still unknown, a recent paper suggested a link between DedA protein and selenite resistance (Ledgham et al., 2005). In *Stenotrophomonas maltophilia,* a relation between innate resistance to antimicrobials (kanamycin, tetracycline, gentamycin, nalidixic acid) and tolerance to high levels of heavy metals including selenite has been demonstrated (Pages et al., 2008). Therefore, it is

tempting to speculate that this protein could play a role in *C. jejuni* resistance to protamine and other antimicrobial peptides.

Finally, the Cj0355c two-component system regulator was down-regulated (1.63 fold by qRT-PCR, $p = 0.02$) at the lowest protamine concentration (3.12 μg/ml). The Cj0355c homolog in *H. pylori* (HP 1043) has been shown to play a role in viability and growth phase (Beier and Frank, 2000). The role of this two-component system regulator will be discussed and studied in further details in Chapter 4, page 88.

3.2.3. Phenotypic studies of the selected deletion mutants

Two genes, Cj1721c and Cj0560, were selected for further mutagenesis and phenotypic analysis. The selection of Cj1721c was based on its upregulation upon exposure to protamine. Furthermore, a recent study showed that the expression of Cj1721c protein is an indicator of *Campylobacter* virulence potential (Cordwell et al., 2008). On the other hand, the Cj0560 was chosen because it encodes an efflux pump. The role of this pump in resistance to antimicrobial peptides was not previously investigated in *C. jejuni* but has been shown to contribute to antimicrobial peptides resistance in other species such as in *Treponema denticola* (Brissette and Lukehart, 2007) and in both *Neisseria gonorrheae* and *Neisseria meningitidis* (Rouqette-Loughlin et al., 2003).

3.2.3.1. Characterization of the Cj1721c deletion mutant

Compared to the wild-type strain, the Cj1721c deletion mutant did not show differences in growth and MIC in either MH or MEM media. Since there was no difference

in the MIC between the wild-type strain and the Cj1721c deletion mutant, a survival assay was performed. The aim was to test the response of the constructed mutant to the sudden exposure (5 minutes) to protamine. The Cj1721c gene encodes a putative outer membrane protein with unknown function and since it was up-regulated in the microarray data we expected the mutant to be more sensitive. Surprisingly, the Cj1721c mutant was found to be more resistant than the wild-type strain after five minutes exposure to protamine. This observation could be explained by a loss of function mechanism. The protamine might be binding to the Cj1721c protein blocking its function and the overexpression of its gene might counterbalance this loss of function. Therefore, in the deletion mutant the protamine is no longer preferably binding to this protein and a transient resistance is observed.

Given that Cj1721c is an outer membrane protein, it might be involved in surface attachment. To test this hypothesis the mutant's ability to form a biofilm and a pellicle was investigated. The ability of Cj1721c deletion mutant to form a biofilm was less than that of the wild-type strain indicating a role in biofilm formation. On the other hand, the pellicle formation ability of this mutant was higher than that of the wild-type strain. This observation suggests a role for Cj1721c protein in surface attachment, and thus in the first step of biofilm formation. This function is unlikely to be required for the formation of pellicle at the gas-liquid interface, explaining the ability of the mutant to form pellicles. However, further studies need to be performed *in vitro* and *in vivo* to confirm this function. A previous study (Kalmokoff et al., 2006) has demonstrated a link between motility and the ability to form biofilm and pellicle. An aflagellate mutant had dramatically lower biofilm and pellicle formation capacities. Consequently, the motility of the Cj1721c deletion mutant was

assessed. Interestingly, the Cj1721c deletion mutant was more motile than the wild-type strain even though its ability to form a biofilm was affected. This observation suggests a direct role for Cj1721c in biofilm formation, likely independent of motility behavior. A transcriptome analysis of the Cj1721c deletion mutant might provide more data about the relation between the reduced biofilm formation and the enhanced motility. In addition, complementing the deleted Cj1721c gene is essential to confirm that the observed phenotypes are due to the Cj1721c knockout and not to another effect such as phase variation that have been shown to affect flagellar genes expressions (Hendrixson, 2006). To note, it is unlikely that the mutation in this gene is affecting the expression of downstream genes because they are in opposite transcriptional direction as shown in Figure 6.

3.2.3.2. Characterization of the Cj0560 deletion mutant

Bacteria can use various resistance mechanisms to evade the host defense system. Among these mechanisms is the expulsion of antibiotic molecules through a transporter or a pump (Kraus and Peschel, 2006). In *C. jejuni* NCTC 11168 the gene Cj0560 encodes a potential multidrug and toxic compounds extrusion pump (MATE superfamily) that might confer resistance to cationic drugs and organic compounds (Omote et al., 2006). Based on its possible role, a deletion mutant in the Cj0560 gene was constructed and its sensitivity to protamine was assessed. The MIC of the mutant was found to be similar to that of the wild-type strain (1.56 μg/ml). It is worth noting that *C. jejuni* NCTC 11168 has two genes that belong to this transporter family (Parkhill et al., 2000). As a consequence, the other gene, Cj0619, might be compensating for the loss of the Cj0560. To test this hypothesis, a

nonspecific efflux pump inhibitor, phenylalanine-arginine β-naphthylamide PAβN, was added to the medium and the MIC was reevaluated (Mamelli et al., 2006). Only two fold difference was observed between the MIC with and without the pumps inhibitor, 0.78 versus 1.56 μg/ml respectively. This difference is not significant and it indicates a relatively marginal role of efflux pumps in resistance to protamine sulfate. To investigate further and confirm the obtained MIC data, the percentage of surviving cells of the Cj0560 deletion mutant and the wild-type strain were compared after exposure to protamine for 5 and 20 minutes. The results did not reveal any statistically significant difference between the two strains. This data supports the MIC data and reinforces the hypothesis that protamine is not a substrate for the MATE efflux pump family. Furthermore, to eliminate the possibility that the other MATE gene (Cj0619) is compensating for the loss of the Cj0560, the survival assay was repeated after adding the pump inhibitor phenylalanine-arginine β-naphthylamide (PAβN). The data showed no difference in susceptibility compared to the wild-type strain.

The role of another efflux pumps family (CmeABC) was previously tested (Lin et al., 2002) and this study indicated that this transporter was not involved in *C. jejuni* 81-176 resistance to protamine. Altogether, these data indicate that the efflux pumps are not conferring any significant resistance to *Campylobacter* against protamine sulfate. Nevertheless, these transporters might be used by *Campylobacter* to pump out other antimicrobial peptides. Therefore, testing different CAMPs molecules would be required to determine the phenotype of the Cj0560 mutant.

3.2.4. Screening of the transposon mutants library

To investigate *Campylobacter* intrinsic resistance to protamine, a transposon mutant library was screened. In contrast to the genome-wide transcriptome analysis in response to protamine, this method should permit the identification of non-transcriptionally regulated genes that are contributing to protamine survival. To identify these genes, a *C. jejuni* NCTC 11168 transposon mutants library was screened against two concentrations of protamine sulfate (200 and 500 μg/ml). The concentrations were selected based on the MIC of 450 μg/ml that was determined using the agar dilution method. The growth of the wild-type *C. jejuni* was not affected on CAMH agar plates containing 200 μg/ml of protamine, while no bacterial growth was observed on the 500 μg/ml agar plates. This assay allowed the identification of 11 protamine-sensitive and 16 protamine-resistant mutants. From the 16 resistant mutants, 6 different transposon insertion sites were identified. These sites are: 2 oxidoreductases (Cj0415, Cj0485), Cj0044c, CRISPR (clustered regularly interspaced short palindromic repeats), Cj1550c, and a 16 S ribosomal RNA protein. To note, the insertion site of 10 of the 16 mutants was mapped into the Cj0415 gene.

Cj0415 encodes a putative GMC (glucose-methanol-choline) oxidoreductase protein. This gene was found to be iron-regulated (Palyada et al., 2004). In addition, in *C. jejuni* 81-176 this gene was involved in gluconate-dependent respiration and was required for colonization of avian gut (Pajaniappan et al., 2008). Surprisingly, the MIC of this transposon mutant was identical to that of the wild-type strain (data not shown). The Cj0485 gene encodes a putative oxidoreductase with 31.7 % amino acid identity to FabG, which is involved in fatty acid biosynthesis. In *P. aeruginosa* and *E. coli,* FabG is essential and was

found to be required for lipid A, hemolysin, phospholipids, and homoserine lactones biosynthesis (Hoang et al., 2002). Changes in the bacterial outer membrane structure could alter the affinity of the positively charged protamine to the negatively charged bacterial membrane leading to reduce efficiency.

Cj0044c encodes a hypothetical protein and overlaps with Cj0045c which encodes an iron-binding protein. These 2 genes were over-expressed in a virulent flagellated *C. jejuni* strain (Carrillo et al., 2004). The MIC of this mutant was identical to the wild-type strain. However, unlike the wild-type strain, the pellet of the mutant was not visible until 48 hour of growth instead of 24 hours. Based on protamine's mode of action, the slow growth rate may again explain this result.

CRISPR were recently assigned different roles (Jansen et al., 2002). Mainly, they are thought to constitute a bacterial defense system against bacteriophages (Sorek et al., 2008). In addition, they play a role in DNA repair although the mechanism is not fully understood (Makarova et al., 2006; Makarova et al., 2002). Finally, they were found to regulate endogenous gene expression due to their homology with bacterial chromosomal DNA (Viswanathan et al., 2007). In *C. jejuni* NCTC 11168, one CRISPR element was identified (Fouts et al., 2005). Compared to other *Campylobacter* species, only RM1221 has a similar element. The role of CRISPR in protamine resistance is unclear.

Finally, Cj1550, which codes for a putative ATP/GTP binding protein, was identified among the resistance mutants. This gene is embedded within an operon composed of the restriction-modification system *hsdSR* (Miller et al., 2005). The authors assigned two functions to this operon, DNA methylation and endonuclease. The main function of this

restriction-modification (R-M) system is to provide a barrier against foreign and bacteriophages DNA. It is noteworthy that the two systems involved in resistance to bacteriophages (CRISPR and *hsdSR)* were identified in the transposon mutants screening against protamine. None of these genes was identified in the transcriptome profiling approach. This indicates the importance of intrinsic genes or factors that could confer resistance to the innate immunity system especially, antimicrobial peptides. In addition, the role of the identified genes in resistance to CAMPs has not been previously demonstrated. Further studies are required to elucidate the mechanism by which these proteins can render *C. jejuni* more resistant to CAMPs.

3.2.5. Survival assay after exposure to acid stress

The effect of low pH on sensitivity to protamine was previously tested (Aspedon and Groisman, 1996). In this study Aspedon and Groisman concluded that lowering the membrane potential increased *Salmonella* resistance to protamine. A similar assay was conducted in our study on *C. jejuni* NCTC 11168. The results showed that exposing *Campylobacter* to a pH of 4 increased its resistance to protamine. Since Campylobacteriosis is caused by a foodborne pathogen, this means that *Campylobacter* passes through the acidic environment of the stomach before colonizing the intestine. This passage might confer resistance to the pathogen against antimicrobial peptides. It would be interesting to repeat the experiment using other antimicrobial peptides to investigate if their efficiencies will be affected by the membrane potential and to be able to generalize the acid induced tolerance hypothesis after being exposed to a low pH.

3.3. Conclusion

The data obtained from our experiments highlight several interesting points. First, very few genes (total 18) were found to be differentially expressed upon exposure to protamine by transcriptome profiling study. This suggests that *Campylobacter jejuni* could be intrinsically resistant to the effect of protamine and to other antimicrobial peptides. To test this hypothesis a transposon mutants library was screened against protamine sulfate. Indeed, using this method we identified genes that could play a role in resistance. As expected, these genes were not identified in the microarray data analysis. Second, phenotypic analysis of the Cj1721c deletion mutant showed that the mutant is more resistant to protamine than the wild-type strain, more motile, less able to form a biofilm, and its pellicle formation capacity is higher than the wild-type strain. Moreover, mutagenesis of the Cj0560 gene, which encodes an efflux pump of the MATE family, did not reveal a role of this pump in resistance to protamine.

CHAPTER FOUR: INVESTIGATING THE ROLE OF CJ0355C IN RESISTANCE TO PROTAMINE SULFATE

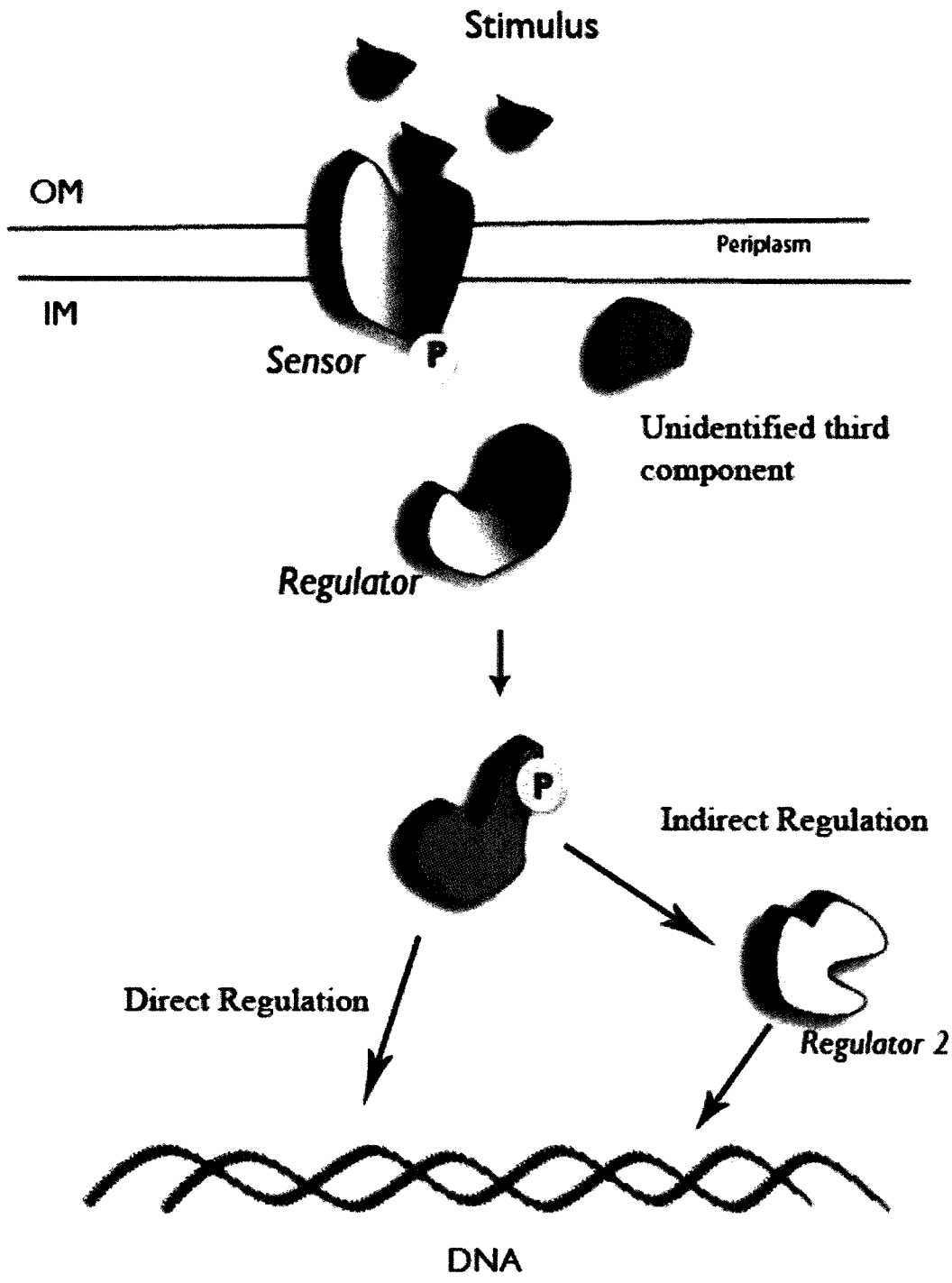
4.1. Introduction to the two-component signal transduction system (TCS)

To survive, organisms have to sense their environments and react appropriately in a way that supports and sustains their growth. Numerous microorganisms have evolved two-component signal transduction systems to perceive their surrounding and transfer the signal to alter genes expression and induce appropriate responses (Mascher et al., 2006). Additionally, these systems provide details about the niches that microorganisms can prosper in since they are able to survive in and interact with these environment (Stock et al., 2000).

The TCS consists of two proteins, the sensor, which is mostly located at the outer membrane, and the regulator, which is located in the cytoplasm (Igo et al., 1990)(Figure 14). In few cases, the sensing domain is present in the cytoplasm, like the Per-Arnt-Sim (PAS) domain that monitors changes in light (Ponting and Aravind, 1997), redox potential, oxygen, and small ligands (Stock et al., 2000). Basically, the sensor, which is a histidine kinase, autophosphorylates upon stimulation and transfers the phosphoryl group to the receiver domain in the response regulator. This phosphorylation promotes conformational changes in the regulator and this alteration facilitates the binding to genomic DNA, causing changes in gene expression, and leading to the physiological responses (Igo et al., 1990) (Figure 14). The DNA-binding domains are divided into three families represented by OmpR (Igo et al., 1990), NarL (Li et al., 1994), and NtrC (Drummond et al., 1986).

Figure 14. Two-component signal transduction system.

A sensor is stimulated first by a specific or unspecific substrate, autophosphorylated, and then the phosphate group is transfered directly or indirectly, through an unidentified third component, to the regulator. After its phosphorylation, the regulator goes conformational changes that facilitate its binding to the DNA. Ultimately, this binding leads to changes in genes expression. In other cases, the activated regulator might affect another regulator that could change genes expression according to the initial stimulus.
OM, outer membrane. P, periplasm. IM, inner membrane.


Stimulus
OM
Periplasm
IM
Sensor
P
Unidentified third component
Regulator
P
Indirect Regulation
Direct Regulation
Regulator 2
DNA

The third family (NtrC) functions solely as an activator while the other two are activators and/or repressors (Stock et al., 2000). TCSs are not only involved in virulence-related but also in non virulence-related phenotypes (Groisman and Mouslim, 2006). For example, the ToxR regulator plays different roles in different bacteria. In *Vibrio cholerae* it is required for toxin synthesis and outer membrane porins regulation (virulence) (Krukonis and DiRita, 2003). However, in *Photobacterium profundum* strain SS9, ToxR is essential for regulating pressure-responsive gene expression (non virulence) (Welch and Bartlett, 1998).

Each bacterium contains several TCSs, indicating that the transferred signal is specific to the stimulus and various stimuli can stimulate different TCSs. This concept is supported by the diversity of the N-terminal sensing domains in different sensors (Stock et al., 2000). Most species contain 20 to 30 pairs of TCSs and some can have up to 200-300 pairs (Skerker et al., 2008). On the other hand, researchers were not able to identify any TCS in *Mycoplasma genitalium* (Stock et al., 2000). The following are few examples of TCSs systems. OxyR is a model for bacterial redox sensors (Green and Paget, 2004), PhoP/PhoQ system recognizes antimicrobial peptides (Groisman and Mouslim, 2006), and CpxRA is activated following envelope stresses such as pH, surface adhesion, and misfolded proteins (Rowley et al., 2006).

Response regulators (RRs) as well as the histidine kinase (HK) have in some cases phosphatase activities (Stock et al., 2000). This function allows the regulation of the phosphorylation lifetime within the RRs (seconds to hours). In addition, it permits the sensors to dephosphorylate its cognate RR. These bifunctional activities are essential in pathways where a quick shutdown of that particular response is needed (Stock et al., 2000).

Although the most common TCS consists of one sensor and one regulator, there are cases where one sensor can phosphorylate two regulators such as CheA that phosphorylates CheB and CheY. In other cases several HKs can phosphorylate one or more RRs. For example, NarX and NarQ regulate NarL and NarP (Li et al., 1995). In addition, there are systems that include a third protein, a histidine-containing phosphotransmitter domain, HPt (Takeda et al., 2001). The intermediate protein mediates the transfer of the phosphoryl group from the HK to the RR. This system is known as a phosphorelay system and it is often encountered in eukaryotes (Takeda et al., 2001). A good example of this system in prokaryotes is the *Bacillus subtilis* sporulation system. It consists of Spo0F as HK, Spo0B as HPt, and Spo0A as the transcription regulator. The phosphoryl group transfers from the Spo0F to Spo0A through Spo0B (Varughese et al., 1998).

4.2. Cj0355c ortholog in *Helicobacter pylori*

H. pylori is a Gram negative microorganism that colonizes the human stomach mucosa and it is the causative agent of peptic ulcer disease (Peterson, 1991). This microorganism benefits from five two-component systems to adapt to its niche in the stomach (Beier and Frank, 2000). Interestingly, one of these regulator, HP1043, is an ortholog to Cj0355c in *C. jejuni* with 60% overall identity (Muller et al., 2005). Interestingly, both regulators, HP1043 and Cj0355c, are orphan and essential proteins for the growth of *H. pylori* and *C. jejuni* respectively (Beier and Frank, 2000; Stahl et al., unpublished data; Raphael et al., 2005). To note, the target genes of these two regulators in both strains are unknown (Muller et al., 2005). However, attempts were carried out in *H. pylori* to investigate

the role and the regulation of expression of HP1043 (Delany et al., 2002; Muller et al., 2005). These studies shows that HP1043 regulates its own expression and suggests that its expression is controlled at the post-transcriptional and/or post-translational levels.

4.3. Phenotypic analysis of the strain that overexpresses Cj0355c

We selected Cj0355c gene for further characterization because our microarray data revealed its down-regulation by 1.6 fold upon exposure to protamine at a concentration of 3.12 μg/ml (Fig. 4). This down-regulation was confirmed by qRT-PCR assay (-1.63, p = 0.02). However, the Cj0355c was not found to be differentially expressed by exposing *C. jejuni* to higher concentrations of protamine (12.5 and 25 μg/ml). It is important to note that the Cj0355c two-component signal transduction regulator is considered to be essential in *C. jejuni* (Stahl et al., unpublished data) and attempts to construct a deletion mutant were unsuccessful (Raphael et al., 2005). Therefore, in order to investigate the function of Cj0355c, we constructed a strain overexpressing this gene. The gene was amplified and cloned into pRRK-1 plasmid (Reid et al., 2008). Next, the purified plasmid was naturally transformed into *Campylobacter jejuni* NCTC 11168. The right construct was confirmed by determining the location of the inserted gene on the NCTC 11168 chromosome. First, the insert position was determined by amplifying the three possible insertion sites on the chromosome using the ak233, ak234, ak235, and AR56 primers (Table 3). The right PCR product size was detected using the ak234 and AR56 primers, which indicates that the Cj0355c was inserted downstream of Cj0431 gene.

Furthermore, the overexpression of Cj0355c in the constructed strain was confirmed by qRT-PCR, which indicated a 1.99 fold increase of the transcription level as compared to the wild-type strain. This data suggests that in contrast to the HP1043 regulation in *H. pylori*, the Cj0355c mRNA in *C. jejuni* is not regulated at the post-transcriptional level.

4.3.1. Growth curve

The growth curve of the strain that overexpresses Cj0355c was analyzed using the Bioscreen system. No significant difference was observed in MH or in MEM medium by comparison to the wild-type strain.

4.3.2. Minimum inhibitory concentration (MIC)

The MIC of protamine was determined in MH and MEM media using the micro- and macrodilution methods. The MICs of the Cj0355c overexpressing strain and the wild-type strain were identical (1.56 μg/ml).

4.3.3. Survival assay

Because the MICs of protamine for both the overexpression construct and the wild-type strain were the same, a survival assay was conducted. In MH medium, the result showed that the strain that overexpresses Cj0355c was significantly more sensitive to protamine sulfate (50 μg/ml) than the wild-type strain NCTC 11168 after 5 minutes exposure (Fig 15). Survival percentages of the strain that overexpresses Cj0355c and the wild-type strain were 6.1 and 14.9 % respectively and the *p* value was 0.008.

4.3.4. Transcriptome analysis of the strain that overexpresses Cj0355c

The transcriptome profile of the strain that overexpresses Cj0355c was compared to that of the wild-type strain, *C. jejuni* NCTC 11168. The analysis was performed in MH and MEM media. In MH medium 25 genes were up-regulated (Table 6), and 29 genes were down regulated ($p < 0.0001$) (Table 7). In MEM medium, 28 genes were up-regulated (Table 8) and 19 were down-regulated ($p < 0.0001$) (Table 9). Interestingly, only a few genes were found to be commonly differentially expressed in both MH and MEM media. These genes include Cj0190c (conserved hypothetical protein), Cj1419c (putative methyltransferase), *flgG* (flagellar basal-body rod protein), Cj0075c (putative oxidoreductase iron-sulfur subunit), *acnB* (aconitate hydratase), and *gpsA* (Glycerol-3-phosphate dehydrogenase [$NAD(P)^+$].

Figure 15. Survival percentage of the wild-type strain and the strain that overexpresses Cj0355c.

Bacterial strains were grown in Mueller-Hinton to mid log phase before adding protamine sulfate (50 μg/ml) for 5 minutes. 1 ml samples were withdrawn before and after protamine addition. Serial dilutions were plated on MH agar plates. Bacterial counts were enumerated after 24 hours incubation under microaerophilic conditions. Two-sample *t*-test shows that the difference between the two strains is significant (p = 0.008).

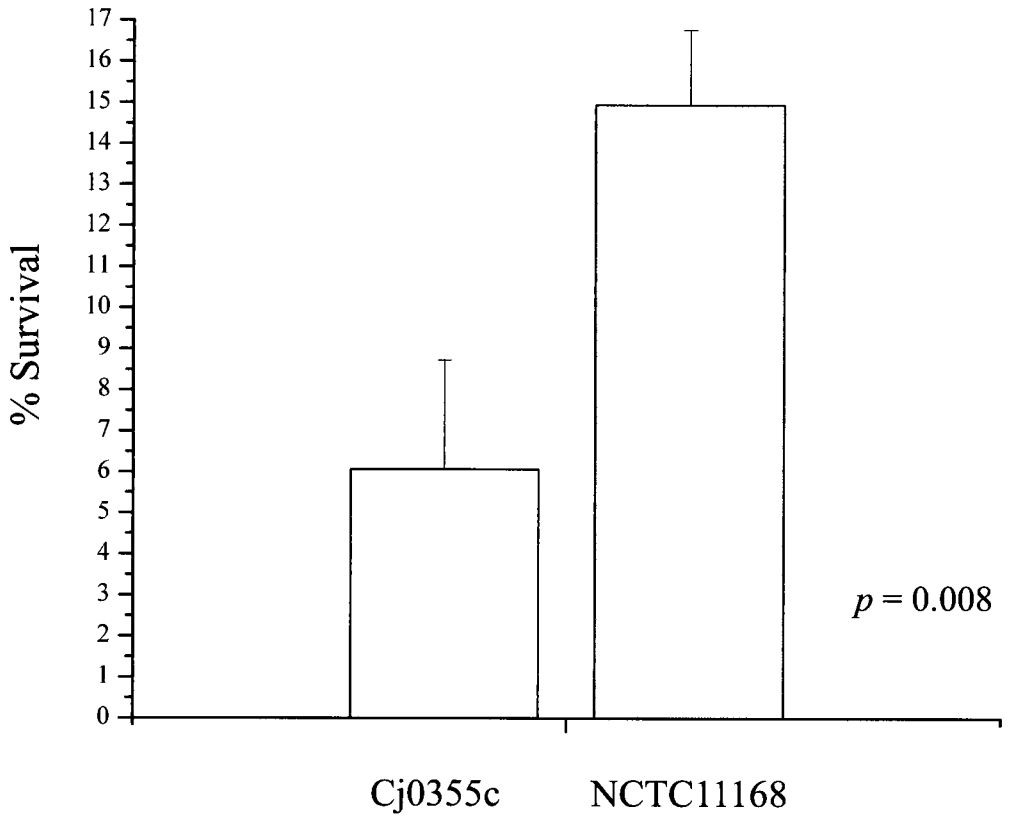

% Survival
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
0
p = 0.008
Cj0355c
NCTC11168

Table 6. Genes up-regulated in MH medium

The table shows the fold change of the identified up-regulated genes in MH medium with a p value < 0.0001

Table 6. Genes up-regulated in MH medium

General function	Gene ID	Annotated Function	Fold Change
Protein synthesis	*hemL*	Glutamate-1-semialdehyde 2,1-aminomutase	1.55
Riboflavin biosynthesis	*ribA*	GTP cyclohydrolase II	1.52
Flagellum	*flgG*	Flagellar basal-body rod protein	1.56
	flgD	Putative flagellar hook assembly protein	1.63
	flaC	Flagellin	3.18
Energy & metabolism	*pyk*	Pyruvate kinase	1.99
	gltA	Citrate synthase	1.87
	gapA	Glyceraldehyde 3- phosphate dehydrogenase	1.73
	Cj0074c	Putative oxidoreductase iron-sulfur subunit	1.78
	Cj0075c		
	acnB	Aconitate hydratase	2.58
Natural transformation	*Cj1473c*	Putative ATP/GTP-binding protein	2.14
Oxidative stress	*Cj0012c*	Non-haem iron protein	2.46
Transporter	*putA*	Putative proline dehydrogenase	1.85
Chaperones	*clpB*	ATP-dependent Clp protease ATP-binding subunit	1.81
Membrane proteins	*Cj0406c*	Putative lipoprotein	1.56
	Cj1419c	Putative methyltransferase	1.91
	Cj1422c	Putative sugar transferase	1.9
	peb4	Major antigenic peptide PEB-cell binding factor	1.63
	glmU	UDP-N-acetylglucosamine pyrophosphorylase	1.44
	Cj0772	Putative NLPA family lipoprotein	1.71
Fatty acid synthesis	*acs*	Acetyl-coenzyme A synthetase	1.72
Hypothetical proteins	*Cj0565*	Pseudogene (conserved hypothetical protein)	2.18
	Cj0980	Putative peptidase	1.76
	Cj0069	Hypothetical protein Cj0069	1.51

Table 7. Genes down-regulated in MH medium

Table 7. Genes down-regulated in MH medium

The table shows the fold change of the identified down-regulated genes in MH medium with a p value < 0.0001

Table 7. Genes down-regulated in MH medium

General function	Gene ID	Annotated Function	Fold Change
Metabolism	*cydAB*	Cytochrome bd oxidase subunit I/II	0.56 - 0.45
	fdhC	Putative formate dehydrogenase, cytochrom B subunit	0.52
	rep	Ribulose-phosphate 3-epimerase	0.58
Oxidative stress	*Cj0017c*	Disulphide bond formation protein	0.57
Transcription	*nusG*	Transcription antitermination protein	0.55
DNA replication	*Cj0190c*	Conserved hypothetical protein	0.42
	DnaQ/X	Exonuclease, DNA polymerase III epsilon/gamma subunit	0.55 / 0.59
	gidA	tRNA uridine 5-carboxymethylaminomethyl modification	0.63
Transporters	*Cj0025c*	Putative sodium:dicarboxylate family transmembrane	0.48
	exbB3	Putative MotA/TolQ/ExbB proton channel family protein	0.97
	Cj0006	Putative Na+/H+ antiporter family protein	0.55
	Cj0501	Pseudogene (ammonium transporter)	0.61
Energy	*atpB/C*	ATP synthase F0 sector A/ F1 sector epsilon subunit	0.55 / 0.64
	guaA	GMP synthase (glutamine-hydrolyzing)	0.63
Fatty acid synthesis	*fabF*	3-oxoacyl-[acyl-carrier-protein] synthase	0.59
	gpsA	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	0.6
Two-component system	*Cj0505c*	Putative aminotransferase (degT family)	0.53
Amino acids synthesis	*trpA*	Tryptophan synthase alpha chain	0.57
	lysC	Aspartokinase, alpha and beta subunits	0.61
	argC	N-acetyl-gamma-glutamyl-phosphate reductase	0.59
Protein synthesis	*fmt*	Methionyl-tRNA formyltransferase	0.59
Signal protein	*ffh*	Signal recognition particle protein	0.55
Membrane proteins	*Cj1295*	Conserved hypothetical protein	0.37
	Cj1170c	Outer membrane protein precursor	0.59
	murC	UDP-N-acetylmuramate--alanine ligase	0.59
	Cj0645	Putative secreted transglycosylase	0.64

Table 8. Genes up-regulated in MEM medium

The table shows the fold change of the identified up-regulated genes in MEM medium with a p value < 0.0001

Table 8. Genes up-regulated in MEM medium

General function	Gene ID	Annotated function	Fold change
Protein synthesis	*rpmA*	50S ribosomal protein	1.63
	Cj1709c	Putative ribosomal pseudouridine synthase	1.55
	pheS	Aminoacyl t-RNA synthase	1.57
	thrS	Aminoacyl t-RNA synthase	1.55
	typA	GTP-binding protein TypA homolog	1.77
DNA replication	*rho*	Transcription termination factor	1.86
	ssb	Single strand binding protein	2.01
	dnaA	Chromosomal replication initiator protein	2.7
	dnaN	DNA polymerase III, beta chain	2.08
	topA	DNA topoisomerase I	1.58
	gyrA	DNA gyrase subunit A	1.64
	gyrB	DNA gyrase subunit B	1.57
Flagellum	*flgG*	Flagellar basal-body rod protein	1.83
	flgG$_2$	Flagellar basal-body rod protein	1.73
Energy & metabolism	*petC*	Putative ubiquinol-cytochrome C reductase	2.12
	purA	Adenylosuccinate synthetase	2.15
	Cj0075c	Putative oxidoreductase iron-sulfur subunit	1.73
	acnB	Aconitate hydratase	1.64
Sigma factors	*fliA*	RNA polymerase sigma factor (flagellar operon)	1.62
Two-component system	*racR*	Two-component regulator	1.82
Transporter	*putA*	Putative proline dehydrogenase	1.54
Chaperones	*lon*	ATP-dependent protease La	1.97
Membrane proteins	*Cj0089*	Putative lipoprotein	1.7
	Cj0093	Putative lipoprotein	2.16
	Cj1419c	Putative methyltransferase	1.94
	Cj1421c	putative sugar transferase	2.1
	Cj0268	Putative transmembrane protein	1.87
	Cj0404	Putative transmembrane protein	1.85

Table 9. Genes down-regulated in MEM medium

The table shows the fold change of the identified down-regulated regulated genes in MEM medium with a p value < 0.0001

Table 9. Genes down-regulated in MEM medium

General function	Gene ID	Annotated Function	Fold change
Transporters	*Cj1040c*	Putative MFS transport protein	0.3
	Cj1660	Putative integral membrane protein	0.45
	Cj0141c	Putative ABC transporter integral membrane protein	0.48
	glnP	Putative glutamine transport system permease	0.47
	Cj1163c	Putative cation transport protein	0.51
	Cj1257c	Putative efflux pump	0.58
	Cj0174c	Putative iron-uptake ABC transport system permease	0.57
	Cj0203	Putative citrate transporter	0.58
	chuB	Putative haemin uptake system permease protein	0.59
	Cj0501	Pseudogene (ammonium transporter)	0.6
	Cj0919c	Putative ABC-type amino-acid transporter permease protein	0.63
Adhesin	*cadF*	Outer membrane fibronectin-binding protein	0.59
Phospholipid synthesis	*gpsA*	Glycerol-3-phosphate dehydrogenase [NAD(P)+]	0.54
	cfa	Cyclopropane-fatty-acyl-phospholipid synthase	0.58
Aerobic metabolism	*ubiA*	Putative 4-hydroxybenzoate octaprenyltransferase	0.58
Shape	*Cj0277*	Homolog of E. coli rod shape-determining protein	0.38
DNA replication	*Cj0190c*	Conserved hypothetical protein	0.38
Colonization	*Cj0454c*	Putative membrane protein	0.46
Oxidative stress	*Cj1383c*	Hypothetical protein Cj1383c	0.46

4.3.5. Biofilm formation ability

A previous study has shown the overexpression of Cj0355c protein in *C. jejuni* NCTC 11168 biofilm (Kalmokoff et al., 2006). This data suggests a potential role for Cj0355c protein in biofilm formation. Consequently, the ability of the wild-type strain and the strain that overexpresses Cj0355c to form a biofilm were assessed after 24, 48, and 72 hours by measuring crystal violet absorbance at 574 nm (McLennan et al. 2008). As shown in Figure 16, the biofilm forming ability of the overexpressing construct is higher than the wild-type strain after 24 and 72 hours ($p = 0.0006$ and 0.02 respectively). However, the difference between the two strains was not statistically different after 48 hours, $p = 0.112$.

4.3.6. Pellicle formation assay

C. jejuni strain NCTC 11168 can exist in three forms when developing a biofilm: attached to a glass surface (biofilm), unattached aggregate (floc), and it can form a pellicle at the liquid-gas interface. These forms were discussed in a previous report (Joshua et al., 2006). Therefore, a pellicle formation assay was performed to further study the pellicle formation characteristics of the strain that overexpresses Cj0355c. Figure 17 indicates that the Cj0355c construct developed less pellicle than the wild-type strain after three days of incubation under microaerophilic conditions. This experiment was repeated three times and identical results were obtained.

Figure 16. Biofilm formation ability of the wild-type strain and the strain that overexpresses Cj0355c.

Crystal violet absorbance (574 nm) was measured at 24, 48, and 72 hours. The *p* values were 0.0006, 0.112, and 0.02 respectively at the different time points using two-sample *t*-test .

Asterisk (*) represents statistically significant difference.

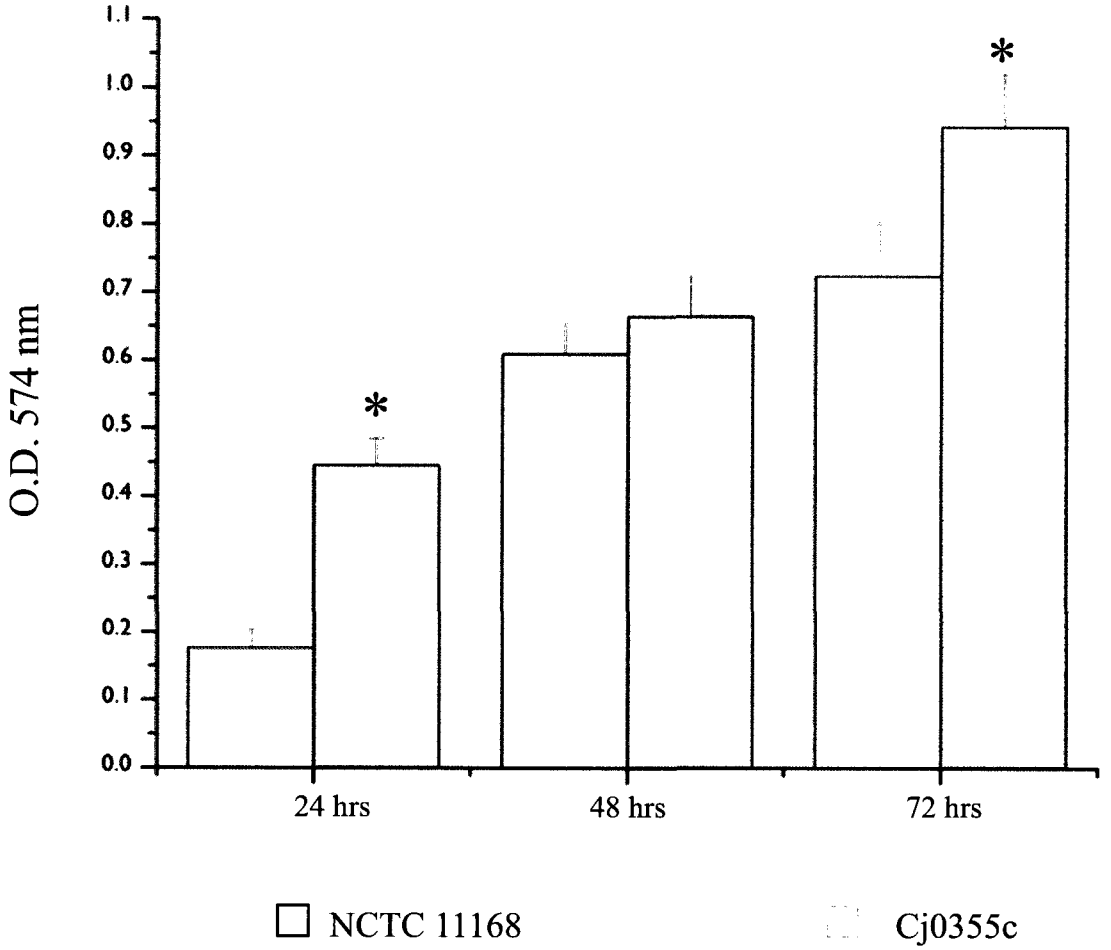
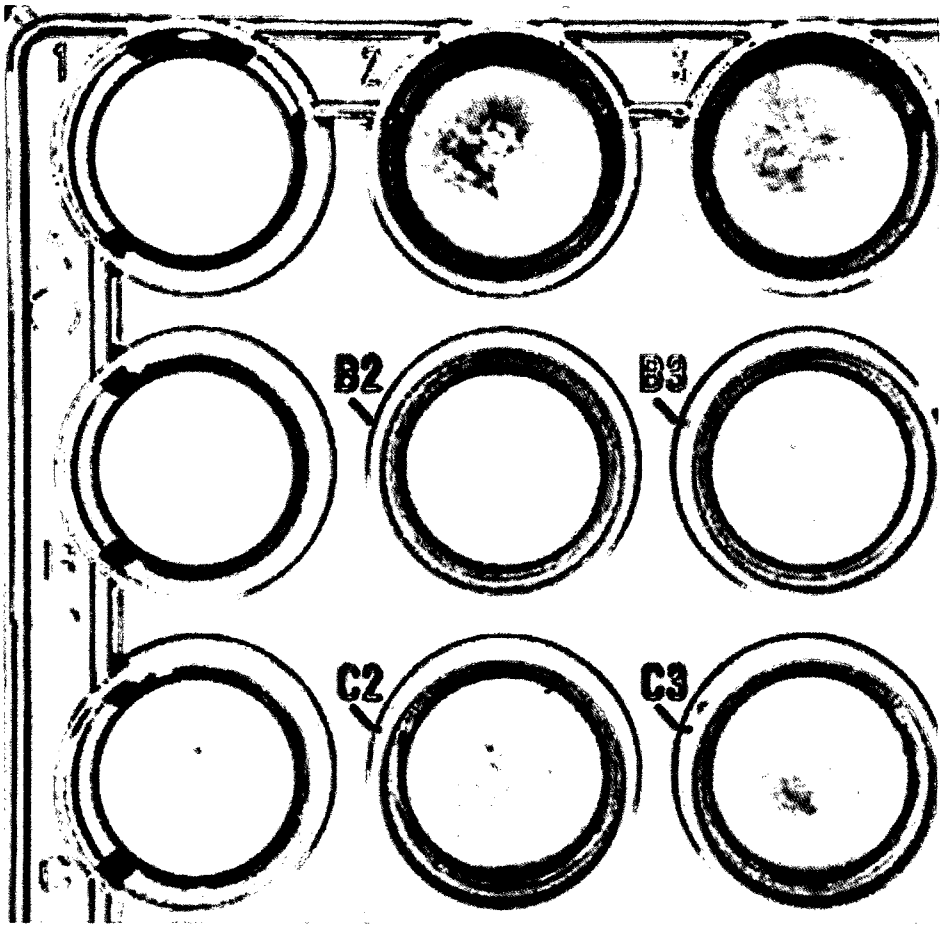

O.D. 574 nm
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
*
24 hrs
48 hrs
72 hrs
NCTC 11168
Cj0355c

Figure 17. Pellicle formation at the gas-liquid interface of the wild-type strain and the strain that overexpresses Cj0355c.

Three biological replicates were performed and each replicate included three technical replicates. The figure represents one biological replicate. The negative control well contains MH medium only. Four μl of mid-log phase bacterial culture (O.D. 0.7) were inoculated in the wells. The plate was placed in the microaerophilic station for 3 days.



Negative control NCTC 11168 Cj0355c construct

4.3.7. Motility assay

The motility of the strain that overexpresses Cj0355c was assessed using MH motility agar plates (0.4% agar). After 24 hours, the mean growth diameter was 19 ± 2 mm (mean ±SD) for the Cj0355c constructs and 20 ± 0.6 mm for the wild-type strain ($p = 0.32$). This indicates a similar motility for both strains.

4.3.8. *In vivo* colonization assay

Newborn piglets were used to assess the potential role of Cj0355c in gut colonization and pathogenesis. The piglets were orally challenged with a 1:1 ratio culture of the wild-type strain NCTC 11168 and the strain that overexpresses Cj0355c. Two days after inoculation the piglets were euthanized. Two out of the three infected piglets in the first batch developed diarrhea and their colon morphology was swollen and enlarged compared to the third piglet. In the second batch only one piglet was tested and it did not develop any gross symptoms. Table 10 shows the competitive ratios for each segment in each biological replicate. The data indicate that the ability of the strain that overexpresses Cj0355c to colonize the duodenum is less than that of the wild-type strain ($p = 0.0001$). The other sections from the gastrointestinal tract of the piglets were not colonized at a sufficient level to permit an accurate determination of the competitive index.

Table 10. Competitive ratio of the strain that overexpresses Cj0355c and the NCTC 11168 strain in the piglet animal model

A 1:1 ratio mixture of the two strains was given to four newborn piglets. The CFU count of both strains was determined in five sections of the piglets' gastrointestinal (GI) tracts (duodenum, jejunum, ileum, cecum, and colon) and the competitive ratio was calculated. One sample *t*-test indicates that the difference between the two strains in the duodenum is significant ($p = 0.0001$). In the other GI sections, not enough data were collected for statistical analysis.

Table 10. Competitive index of the strain that overexpresses Cj0355c and the NCTC 11168 strain in the piglet animal model

	Competitive ratio				Mean
GI[1] Section	Pig 1	Pig 2	Pig 3	Pig 4	
Duodenum	0.002	0.15	0.01	0.01	0.04
Jejunum	-	0.03	1.48	-	0.76
Ileum	-	-	-	0.63	
Cecum	-	-	0.13	-	
Colon	0.001	-	0.16	-	0.08

4.4. Discussion

4.4.1. Survival assay

The strain that overexpresses Cj0355c was more sensitive than the wild-type strain to protamine after five minutes exposure. This result is in agreement with the initial microarray results in which the Cj0355c gene was found to be down-regulated after exposure to protamine. The difference in survival percentage was assessed at other time points as well (10, 20, 40, and 60 minutes). However, the distinction between the two strains was not significant. Because the Cj0355c is a regulator, it is tempting to speculate that it regulates genes involved in protamine sensitivity. It would be interesting to repeat the same experiment in MEM medium and investigate if the strain that overexpresses Cj0355c is more sensitive than the wild-type strain and how the growth environment would affect the regulatory function of the Cj0355c.

4.4.2. Biofilm and pellicle formation assay

Kalmokoff and colleagues showed that the Cj0355c protein, among others, was highly expressed in *C. jejuni* biofilm (Kalmokoff et al., 2006). Based on their data the biofilm and pellicle formation ability of the strain that overexpresses Cj0355c was assessed. Our results indicate that the ability of the strain to form a biofilm was higher than that of the wild-type strain after 24 and 72 hours. It is worth noting that the motility behavior of the Cj0355c overexpressing strain is not affected suggesting a flagellum independent effect on biofilm formation. In contrast, the pellicle formation assay showed that the strain that overexpresses Cj0355c has lower capacity to form a biofilm at the gas-liquid interface than

the wild-type strain. The data from the biofilm assay support the data from the pellicle assay indicating that the enhanced attachment characteristic reduces the strain ability to form a pellicle. This observations suggest that the observed downregulation of the Cj0355c upon protamine exposure might repress the formation of biofilm while enhancing pellicle formation.

4.4.3. *In vivo* colonization assay

To investigate the role of the Cj0355c in pathogenesis, the virulence of the overexpressing construct was assessed in the newborn piglets animal model. This model was selected because of the similarity in pathogenesis between infected piglets and humans. The data showed that the Cj0355c overexpressing construct had a lower ability to colonize the GI tract of newborn piglets than the wild-type strain, suggesting a potential role for Cj0355c in *Campylobacter* pathogenesis.

Since *C. jejuni* is commensal in chickens it would be interesting to study the colonization ability of the strain that overexpresses Cj0355c in the chick animal model to further explore the role of Cj0355c in *Campylobacter* physiology.

4.4.4. Transcriptome profiling of the strain that overexpresses Cj0355c

To elucidate the function of the Cj0355c regulator, the transcriptome profile of the Cj0355c overexpressing strain was compared to that of the wild-type strain NCTC 11168. The comparison was done by growing both strains in MH and MEM media under microaerophilic conditions without applying any stimulus. Our data indicates that the type of the growth medium, MEM or MH, significantly affected the transcriptional profiles between

the wild-type strain and the Cj0355c overexpressing strain, suggesting an important impact of the growth conditions on the Cj0355c regulon. Tables 6, 7, 8, and 9 list all the genes that were found to be differentially expressed in MH and MEM media. Several of the identified genes will be discussed in more details below.

4.4.4.1. Transcriptome analysis of the strain that overexpresses Cj0355c in MH medium

The genome-wide transcriptome analysis showed that 25 genes were >1.5 fold up-regulated and 29 genes were down-regulated.

The up-regulated genes include the flagellum genes *flaC, flgD* and *flgG.* Previous study demonstrated that FlaC protein expression was enhanced in *C. jejuni* biofilm (Kalmokoff et al., 2006). This data is in agreement with the observed phenotype of the strain that overexpresses Cj0355c, which showed a higher biofilm formation ability compared to the wild-type strain. The *flgD* encodes a putative flagellar hook assembly protein. FlgG is a flagellum basal body, which was found to be up-regulated in our microarray data of the MEM medium as well. Another up-regulated gene is Cj0012c that encodes non-heme iron protein, an oxidative stress sensitive protein in *C. jejuni* (Yamasaki et al., 2004). The authors found that the protein concentration decreases with declining viability due to either endogenous or exogenous oxidative stress. Furthermore, a recently published paper showed that Cj0012c could be involved in the response to oxidative stress caused by paraquat (Garénaux et al., 2008). Interestingly, oxidative stress could be generated by bactericidal drugs that are able to increase the formation of endogenous reactive oxygen species (ROS) leading to bacterial death (Kohanski et al., 2007). Cj0021c and *pyk* were also found to be up-regulated. Cj0021c is a putative fumaryl acetoacetate (FAA) hydrolase family protein. This

enzyme catalyzes the last step in the degradation of phenylalanine and tyrosine to form fumarate and acetoacetate, which are substrates in the respiration and metabolism pathways (Arias-Barrau et al., 2004). Pyk is a pyruvate kinase. Interestingly, it has been shown that it contributes to virulence in *Streptococcus iniae* (Bolotin et al., 2007) and that it is an immunogenic surface protein in *S. pyogenes* (Cole et al., 2005). The gene, Cj0772c (encoding a membrane protein) and *acs* (acteyl coenzyme A synthase) were found to be up-regulated. Cj0772c is a putative NLPA family lipoprotein. This family of lipoproteins is antigenic and has been shown to contribute to bacterial virulence in enterotoxigenic *E. coli* (Bodero et al., 2007). *Acs* is involved in membrane fatty acid biosynthesis. Altering the fatty acid composition of the bacterial membrane could be a way to antagonize the effect of the protamine by reducing its interaction with the membrane.

Interestingly, the down-regulated genes included several genes known or predicted to be involved in peptidoglycan biosynthesis and modifications (Cj1295, *MurC,* and Cj0645). Cj1295 is a conserved hypothetical protein that is involved in polysaccharide biosynthesis in *C. jejuni.* This gene is part of the flagellin *O*-linked glycosylation locus (Cj1293-Cj1342). Another crucial down-regulated gene was Cj0017c (DsbI). This gene encodes a disulphide bond formation protein. Disulphide bond formations happen in the oxidative environment of the periplasm and are catalyzed by the Dsb proteins (Raczko et al., 2005). In *Helicobacter pylori* the colonization ability of the *dsbI* mutant was shown to be reduced (Godlweska et al., 2006). Moreover, the *dsbAB* system in *Burkholderia cepacia* was shown to be involved in the production of protease, alkaline phosphatase, motility, metal (Cd^{2+} and Zn^{2+}), and multidrug resistance (Hayashi et al., 2000).

4.4.4.2. Transcriptome analysis of the strain that overexpresses Cj0355c in MEM medium

MEM medium was chosen in order to determine if iron and/or carbon starvation could influence the regulatory function of Cj0355c.

The up-regulated genes in the MEM medium could be divided into several functional groups.

The first group include genes involved in protein synthesis such as *rpsBCDKJ, rplBCMTU, rpmA,* Cj1709c, *pheS, thrS, and typA*. The homolog of Cj1709c, which codes for a putative ribosomal pseudouridine synthase, was shown to be required for ribosomes maturations and modifications in *Bacillus subtilis* (Niu et al., 1999). This gene is located upstream of Cj1710c and *ksgA*, which were also up-regulated in our study. *typA* encodes a GTP-binding protein with an elongation factor motif. The gene is involved in protein translation and modifications. Interestingly, the *typA* homolog in *S. typhimurium* (BipA) was overexpresed more than seven fold upon exposure to bactericidal/permeability increasing protein (BPI), which is an antimicrobial peptide (Qi et al., 1995).

The second group of up-regulated genes includes genes involved in transcription and DNA replication such as the topoisomerase genes *(GyrA, GyrB,* and *TopA)*. GyrA and GyrB are involved in DNA supercoiling, replication, recombination, and transcription (Wang et al., 1985). TopA is required for maintaining the supercoiling state of DNA in *E.coli* and its mutations suppressed bacterial growth (Hsu et al., 2006).

The third group includes genes that provide bacterial cells with energy. The *purA* gene is involved in AMP biosynthesis, *petC* encodes a cytochrome subunit C, and Cj0075c is

a putative oxidoreductase, and *acnB,* an aconitate hydratase, which is considered a major citric-acid-cycle enzyme (Jordan et al., 1999).

The fourth group of up-regulated genes includes genes that are required for motility such as the two flagellar basal-body rod proteins, *flgG* and *flgG*$_2$ and *fliA*. FliA is a sigma factor that regulates flagellum gene expression (Jagannathan et al., 2001). Moreover, in *C. jejuni* 81-176, FliA was shown to regulate the non-flagellated proteins Cj0977, which was also found to be up-regulated in this study.

The fifth group of up-regulated genes includes membrane and periplasmic proteins. The gene *pbpA*, which encodes a penicillin-binding protein, is of particular interest because its homolog knockout in *H. pylori* conferred resistance to amoxicillin that disrupts cell wall synthesis (Paul et al., 2001; Gerrits 2002). Since protamine and other CAMPs disrupt the membrane integrity it is possible that a knockout mutant in this gene could make *C. jejuni* more resistant to antimicrobial peptides. Therefore, the overexpression of PbpA could partially explain the increasing sensitivity to protamine of the strain that overexpresses Cj0355c. The other differentially expressed membrane proteins are Cj1419c and Cj1421c. Both genes belong to the *Campylobacter* capsule locus. However, their function is not precisely known because they might also play a role as coenzyme in metabolism (Gaynor et al., 2005).

Other important genes (Cj1710c, *KsgA, putA, RacR)* were also shown to be up-regulated. Cj1710c and KsgA are responsible for the dimethylation of two adjacent adenosines in the 16S rRNA. Their importance was detected by the inactivation of the ksgA, which led to kasugamycin resistance (Van Buul et al.,1983). Interestingly, kasugamycin, like

protamine, affects protein synthesis in bacteria (Masukawa et al., 1968). Therefore, the downregulation of this operon might be one way to overcome the effect of protamine. Another up-regulated gene is *putA*, which codes for a putative proline dehydrogenase. Together with PutP This protein is involved in proline breakdown and in maintaining the proline level in the bacterial cells (Yamato, 1990). Since protamine mechanism of action involves proline efflux from the cell, the downregulation of PutA by Cj0355c might increase the availability of proline and reduces its depletion. The *RacR,* Reduced Ability to Colonize Regulator, (Bras, 1999), which is a two-component system regulator was up-regulated in our study. This gene was shown to be present in 11 *Campylobacter jejuni* virulent strains that were isolated from different hosts (Datta et al., 2003).

Nineteen genes were down-regulated and their function is discussed in more detail below.

Various transporter proteins were shown to be down-regulated. This includes genes from three iron uptake systems (Cj1660, Cj0174c, and *chuB*) and the glutamine/glutamate transporters (Cj0919c and *glnP*). Interestingly, the iron uptake transporters are Fur (ferric uptake regulator) regulated (Palyada et al., 2004; Holmes et al., 2005; Miller et al., 2008). This data implicate that Cj0355c and Fur could co-regulate genes that are involved in iron acquisition. On the other hand, Cj0919c is a putative ABC-type amino acid transporter permease protein while *glnP* is a putative glutamine transport system permease. The down-regulation of these two glutamine/glutamate transporters indicates the importance of this amino acid as a carbon source when *Campylobacter* is under stress.

Genes that are involved in oxygen tolerance such as Cj0203 and *ubiA* were also found to be down-regulated. Reid and colleagues showed that the putative citrate transporter, Cj0203.was down-regulated *in vivo*, while it was up-regulated *in vitro* upon exposure to pH stress (Reid et al., 2008). UbiA is a putative 4-hydroxybenzoate octaprenyltransferase that is part of a group of key enzymes required in the biosynthetic pathway of ubiquinone, which is crucial for bacterial aerobic metabolism (Brauer et al., 2004).

The Cj1163c gene, which is part of a putative cation transport system, was down-regulated. This system was found to be repressed by a two-component system regulator, CzcR, which shares similarity to Cj0355c (Hayashi et al., 2000). Interestingly, researchers demonstrated a link between the heavy metal resistance (Cd^{2+} and Zn^{2+}) and multidrug resistance. Finally, the down-regulated genes *CadF* and Cj0454c were shown to be involved in host colonization. *CadF* encodes an outer membrane fibronectin-binding protein (Konkel et al., 1997). This protein was shown to be required for the attachment to host cells and bacterial colonization (Moser et al., 1997). Cj0454c encodes a putative membrane protein. A *C. jejuni* transposon mutant of this gene showed reduced motility and colonization ability in *C. jejuni* (Hendrixson and DiRita, 2004).

4.5. Conclusion

The data obtained from the characterization of the strain that overexpresses Cj0355c indicate that this gene is involved in many cellular processes that could contribute to the resistance against protamine. The survival assay determined that the Cj0355c construct is more sensitive to protamine than the wild-type strain NCTC 11168. The transcriptome

profiling of the strain that overexpresses Cj0355c highlights the different cellular functions that could be directly or indirectly affected by this two-component regulator, including protein synthesis, metabolism, DNA replication, transporters, phospholipid synthesis, and oxidative stress. In addition, the strain that overexpresses Cj0355c exhibited a greater biofilm-forming ability than the wild-type strain, while its pellicle formation ability was reduced. Interestingly, *in vivo* colonization assay showed that the strain that overexpresses Cj0355c was affected in its ability to colonize the piglets gastrointestinal tract, suggesting a role for the Cj0355c in *C. jejuni* virulence. Finally, in contrast to HP1043 in H. pylori, we showed that the regulation of Cj0355c expression does not occur at the post-transcriptional level.

CHAPTER FIVE: GENERAL CONCLUSIONS

The response of *Campylobacter jejuni* NCTC 11168 to different concentrations of protamine was studied using genome-wide transcriptome analysis. The collected data identified few differentially expressed genes. Two genes from the obtained list were selected for further analysis, Cj1721c, a hypothetical outer membrane protein, and the Cj0355c, a two-component regulator. The Cj1721c knockout strain was found to be more resistant to protamine, less able to form biofilm, more motile, and its ability to form a pellicle was higher than the wild-type strain. On the other hand, the strain that overexpresses Cj0355c demonstrated higher ability to form biofilm, lower colonization efficiency and it was more sensitive to protamine than the wild-type strain. In addition, the transcriptome profiling of the strain that overexpresses Cj0355c highlighted potential genes that could be regulated by this regulator and that could be involved in resistance to protamine sulfate. Few of the identified genes have been found to confer resistance to antimicrobials in other microorganisms. Others were specific to the timing and growth conditions presented in this study. The list of genes can be grouped in the following functional classes: protein synthesis, energy and metabolism, transporters, membrane proteins, fatty acids and phospholipids biosynthesis, and oxidative stress. The genome analysis was performed in MH and MEM media and surprisingly, few genes were shared between the two profiles. This indicates that the Cj0355c regulator could regulate different genes under different stress or environmental conditions.

Moreover, transposon mutant library screening against protamine identified genes that confer resistance to protamine. Interestingly, none of these genes were identified in the

DNA microarray approach. This indicates that *C. jejuni* might be naturally resistant to the effect of CAMPs. The observed transcriptional response to protamine and the mutants screening indicate that *C. jejuni* has evolved different ways that are synergistically involved in evading the hosts immune system.

Our data suggest that *Campylobacter jejuni* could use different mechanisms to evade the effect of the antimicrobial peptides. These mechanisms could be intrinsic and or induced upon exposure to protamine.

CHAPTER SIX: FUTURE WORK

The work that is presented in this project is the first step in our understanding of the mechanism by which *C. jejuni* can evade the effect of antimicrobial peptides. More detailed studies of the Cj0355c gene will provide a better view of its precise regulatory function. Performing chromatin immunoprecipitation assays could highlight the genes that are directly regulated by this regulator. In addition, analyzing the transcriptome profile of the strain that overexpresses Cj0355c after adding protamine sulfate could be an alternative and complementary experiment to confirm and/or add more information to this regulatory system. Moreover, identifying the sensor, which is still undiscovered in this system, can improve our knowledge about this essential two-component system. The sensor might be only required for antimicrobial peptides sensing or it could be involved in sensing membrane changes. Testing the response of *Campylobacter* to other antimicrobial peptides that are present in humans and chicken could confirm our findings and could support the importance of the identified genes in resistance to the innate immunity system. Colonization assay in chicken as an alternative animal model could highlight the role that the host play in *C. jejuni* virulence.

Regarding the identified, protamine-affected transposon mutants, performing survival assays as described with the other mutants could give us a better view of the role of these genes in susceptibility to protamine and other antimicrobials.

The role of the Cj1721c-Cj1724c operon in resistance could be further explored. Since this operon encodes a possible transporter system, identifying the substrate(s) used by the system could explain how the Cj1721c confers resistance to protamine and/or other

antimicrobials. Using the Biolog phenotype microarray system can help identify the still unknown function of the Cj1721c outer membrane protein and the operon in general. Furthermore, it would be interesting to investigate if the Cj1721c is binding to a host receptor. This binding could be important for *C. jejuni* pathogenesis.

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