Colonization of the Intestinal Mucus Layer by *Campylobacter jejuni*

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ABSTRACT

Campylobacter jejuni is a major cause of bacterial gastroenteritis in the developed world; however, many aspects of its biology remain poorly understood, including its colonization of the mucus layer lining the gastrointestinal tract. In this study, we utilized microarray transposon tracking to compile a list of 195 genes essential for the growth of *C. jejuni in vitro* under microaerophilic conditions. Then we characterized *C. jejuni* growing in an extracted intestinal mucus medium. We found that *C. jejuni* will grow efficiently in a medium comprised of either chick and piglet intestinal mucus, and that these media have a dramatic impact on its transcriptome. Within the genes identified as differentially expressed during growth in a mucus medium, we identified a single operon, (*cj0481-cj0490*), which we have subsequently characterized as being responsible for both the uptake and metabolism of L-fucose. This represents the first observation of carbohydrate metabolism by the otherwise asaccharolytic *C. jejuni*. We further found that the inability to utilize L-fucose puts *C. jejuni* at a competitive disadvantage when colonizing the piglet intestine, but not the chick cecum. Finally, we examined *C. jejuni*'s ability to utilize mucins as a carbon source while growing within the mucus layer. We found that despite mucins being a major source of L-fucose and amino acids within the intestine, *C. jejuni* has a minimal ability to degrade and utilize mucins on its own. However, close proximity to mucolytic bacteria within the microbiota of the intestine, allows for increased *C. jejuni* growth. Together, this paints the picture of an organism that is well adapted to survival within the mucus lining of the intestine and establishing itself as part of the intestinal microbiota.

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List of Abbreviations

CAC = Citric Acid Cycle

VNTR = Variable Number Tandem Repeat

PTS domain = Proline/Threonine/Serine domain

SEA domain = Sea urchin sperm protein, Enterokinase and Agrin domain

LOS = lipooligosaccharide

IBD = Inflammatory Bowel Disease

DSS = Dextran Sulfate Sodium

SCFA = Short-Chain Fatty Acids

MH = Mueller-Hinton medium

CM = Chloramphenicol

MATT = Microarray Transposon Tracking

ERI = Evolutionary Retention Index

FBA = Flux Balance Analysis

MEM = Minimal Essential Medium

FBS = Fetal Bovine Serum

HBSS = Hanks Buffered Saline Solution

CPS = Capsular Polysaccharides

ABA = Anaerobic Basal Agar

FAB = Fastidious Anaerobe Broth

LB = Luria-Bertani medium

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CHAPTER 1

Introduction

1.1. Objectives of this study

Campylobacter jejuni is a gram-negative, microaerophilic bacterium that is one of the most common causes of bacterial gastroenteritis in the industrialized world (CDC). Despite being a common human pathogen, our understanding of its mechanisms of pathogenesis has so far been limited. This understanding has been hampered by our lack of knowledge regarding its basic internal biology and its growth within its natural habitat, the intestinal mucus layer. While some information is known about *C. jejuni's* nutrient requirements and factors involved in colonization of the host's intestine, we really do not fully understand how it interacts with the environment of the intestine. Furthermore, a mystery that has remained regarding *C. jejuni* is why it demonstrates significant differences in pathogenicity between different hosts; causing gastroenteritis in humans, but being a benign commensal in most other animals.

With the studies described herein, our objective is to gain a better understanding of how *C. jejuni* establishes itself within the mucus layer along the epithelial lining, and in turn, how that mucus layer influences the behaviour and pathogenesis of *C. jejuni.* We hypothesize that *C. jejuni* is able to derive nutrients from the mucus layer of the intestine, allowing for its establishment within the intestinal microbiota. We equally hypothesize that differences in the mucus layer lead to differences in the pathogenesis of *C. jejuni* with regards to its different hosts.

1.2. *Campylobacter* Infection

1.2.1. History and taxonomy

Theodor Escherich first observed *Campylobacter* in 1886, when he described the presence of *vibrio*-like organisms in the stool of infants with diarrhea in Germany (44). It was subsequently identified as a cause of infectious abortion in cattle and sheep, but the human diarrheal disease caused by *Campylobacter* was not described until Elizabeth King isolated it in 1957 (123). The name *Campylobacter* was not given to the organism (previously known as *Vibrio fetus)* until 1963 when Sebald and Véron suggested that the observed organism differed from other *Vibrios*, and should be reclassified (213). Its new name, *Campylobacter*, is Greek for "curved rod" and describes the helical rod shape of the cell (217). In 1973, Butzler *et al.* (28) isolated a "related *vibrio*," that would later be known as the now-common *Campylobacter jejuni*. *Campylobacters* are now classified as part of the class *ε-Proteobacteria* under the *Proteobacteria* phylum. The ε-Proteobacteria encompass all of the bacteria of the genera *Campylobacter* and *Helicobacter* as well as the less well known genera *Wolinella, Acrobacter, Thiovulum* and *Sulfurospirillum* (44). This is an extremely diverse group of bacteria, found in a wide variety of environments, but the most well known are the *Campylobacters* and *Helicobacters,* both of which are causes of human disease (169).

1.2.2. Pathology

In humans, *C. jejuni* causes gastroenteritis, characterized by diarrhea (sometimes bloody), abdominal pain and fever. Symptoms usually occur 1-10 days after infection, although some infected individuals may remain asymptomatic. The disease is usually self-limiting, lasting only 2-5 days, although more severe cases may last several weeks and

require hospitalization (69). *C. jejuni* infection has also been linked to a number of post-infection complications such as Guillain-Barré syndrome (GBS), an acute, acquired peripheral neuropathy (86). The link between *C. jejuni* and GBS is thought to be due to lipo-oligosaccharides (LOS) on the bacteria's outer membrane, which have the potential to mimic certain human gangliosides on peripheral nerve axons (259). These similarities trigger an autoimmune reaction against the peripheral nervous system, causing demyelization of motor and sometimes sensory neurons in the peripheral nervous system. In more serious cases, nerves controlling the respiratory system can be affected, requiring the patient to be intubated. Fortunately, this autoimmune response is usually temporary, and the patient recovers with little lasting damage (86).

1.2.3. Epidemiology

C. jejuni is the most common cause of gastroenteritis in the developed world. The CDC estimates that in the United States, the infection rate is 13 per 100,000 people (CDC, 2010), with a total of 2.4 million infections per year if estimates of undiagnosed or unreported cases are included. In Canada, 28.4 infections per 100,000 people were reported in 2008 (Public health agency of Canada, 2008), with the number of infections likely to be much higher due to many cases being unreported. In the United Kingdom, a rate of 105.1 per 100,000 people was reported in England and Wales in 2009, with the number of infections increasing every year since 2004 (Health Protection Agency, 2009). This makes *Campylobacter* one of the most common causes of bacterial gastroenteritis (8). Out of these, over 90% of isolates are found to be *C. jejuni*, with the remaining being mostly *C. coli*, although the ratio of prevalence of different species varies by region (68). In developing nations, adult infection rates are similar to developed nations, but pediatric *Campylobacter*

infections remain a serious problem with infection rates being as high as 40-60,000 per 100,000 population in children under the age of 5 (34). Worldwide, there are an estimated 400-500 million infections per year, making *Campylobacter* an exceedingly common gastrointestinal illness worldwide (200).

The most common sources of *C. jejuni* for human infection are contaminated poultry products (216). *C jejuni* will colonize the intestinal tract of poultry in a commensal fashion to high levels without eliciting an immune response. It is due to this commensal colonization that they establish themselves in the flocks, which often serve as a reservoir for human infection (138). Poultry meat is easily contaminated during processing, and if the resulting meat is not properly prepared, *C. jejuni* can cause infection in a human with a dose as small as a few hundred bacterial cells (198). Other known sources of infection are from contaminated water supplies and the consumption of contaminated raw milk (216). The prevention of *Campylobacter* contamination of poultry flocks has been a focus for preventing human infections, however, the numerous sources of contamination make the process difficult. The initial contamination of a flock has been linked to contaminated water, exposure from wild birds, and contamination by insects such as flies (51). Once a flock is exposed, the coprophagic behaviour of the chickens quickly spreads the contamination amongst the entire flock (167).

1.2.4. Treatment and antibiotic resistance

Campylobacteriosis has been readily treatable by antibiotics, but resistance has been rising rapidly amongst *C. jejuni* isolates, making antibiotic resistance a growing concern. Antibiotics that have been typically used to treat *C. jejuni* are tetracycline, fluoroquinolones

such as ciprofloxacin, and macrolides such as erythromycin and azithromycin (69); however, resistance to each of these drugs has been widely described among *Campylobacter* isolates.

Fluoroquinolone resistance, although rare before 1992, has recently been found to have risen to between 19-47% among isolates in the USA and Canada (155). In places such as Spain, Thailand and Hong Kong, resistance to fluoroquiolones has reached the astoundingly high rates of 80-100% (33, 103, 203). The rapid rise in resistance to this class of drug has been linked to their high use in agriculture (155). A single point mutation in DNA-gyrase A (GyrA) is sufficient to substantially reduce fluoroquinolone susceptibility, making resistance an easily acquired trait (182). In addition to this, this mutation does not reduce, and may even increase the fitness of *Campylobacter*, allowing the mutation to persist, even in the absence of the selection pressure from this antibiotic (156).

Tetracycline resistance in *Campylobacter* is also rising due to the acquisition of the *tetO* resistance gene (13), and macrolide resistance is slowly increasing due to the acquisition of point mutations in the target 23S rRNA sequence. Fortunately, this resistance to macrolides is more difficult to acquire and results in a fitness reduction in the resistance strains, but it has never-the-less been increasing in recent years (30).

1.3. *Campylobacter* biology

Compared to other gastrointestinal pathogens such as *Escherichia coli* and *Salmonella*, *C. jejuni* remains poorly understood as far as its biology and pathogenesis. Early characterizations of *C. jejuni* found that some of its key characteristics were that it was a gram-negative, microaerophilic organism, requiring oxygen for growth, but also being unable to survive at atmospheric levels of oxygen. Further, it was thought to be completely

asaccharolytic, meaning it was not able to utilize glucose or other carbohydrates as a substrate for growth (180).

The genome of the strain *C. jejuni* NCTC11168 was first sequenced in 2000 (180), and since then, dozens of strains and species of *Campylobacter* have had their genomes sequenced and published (24, 37, 63, 67, 94, 180, 183, 187, 188, 227, 228). The full genome sequence of NCTC11168 confirmed many of the previous observations regarding *C. jejuni* biology and pathogenesis, while creating new questions. Although it possesses a complete set of enzymes necessary for the synthesis of glucose-6-phosphate via gluconeogenesis (240), it lacks any form of glucokinase to phosporylate extracellular glucose, as well as 6-phosphofructokinase, necessary for the irreversible step of phosporylating fructose-6 phosphate to fructose-1,6 diphosphate during glycolysis. Interestingly, it still possesses a pyruvate kinase, an enzyme required for another irreversible step of glycolysis, but not gluconeogenesis (240). The lack of a 6-phosphofructokinase prevents the metabolism of glucose, while the absence of other previously known carbohydrate metabolizing pathways seemingly confirmed the asaccharolytic phenotype. It was only the research described here and published by Muraoka *et al.* (171) and Stahl *et al.* (222), that identified an exception to its asaccharolytic nature by identifying a functional L-fucose metabolic pathway.

The identification of a complete citric acid cycle (CAC) supported the observation that it can metabolize CAC intermediates and amino acids that can be fed into the pathway (180). Conversely few identifiable pathogenesis genes were found. The previously characterized cytolethal distending toxin (*cdtABC*) and other known virulence genes such as *ciaB*, are not necessary for colonization and may not play a central role in pathogenesis. (128, 189). For example, not all *C. jejuni* strains produce cytolethal-distending toxin (1) and Δ*cdtABC* mutants are not substantially impaired for colonization (189). Similarly, *ciaB* is

often absent from *C. jejuni* strains (56), while the *ciaB* knockout mutants are impaired for both cell invasion and colonization, the absence of *ciaB*, only delays the onset of diarrhea in colonized piglets (128, 129). This has led to a greater appreciation for the importance of *Campylobacter's* basic cellular biology, colonization factors and metabolic needs as a determinant for pathogenesis, rather than searching for individual toxins or pathogenicity factors (232).

To be successful in colonization, *C. jejuni* must possess the ability to survive in a wide variety of hosts, from its commensal avian host, to its human host. In between, it must also survive in the environment, in water or food, before being ingested. This requires a certain degree of metabolic diversity, to make sufficient use of what is available. Among the primary nutrient sources for *C. jejuni,* are a handful of amino acids that it can acquire while growing within the gut. It will also draw on available amino acids in a preferential manner, with serine, aspartate, asparagine and glutamate being preferred, followed by proline, which it utilizes only after other nutrients have begun to become exhausted (255). It should also be noted that serine, aspartate, glutamate and proline make up some of the most common amino acids found in chick excreta, perhaps explaining why they play such a central role in *C. jejuni* metabolism (181).

1.3.1. Serine

Serine metabolism is achieved thanks to two proteins, SdaC and SdaA (163, 241). SdaC acts as a transporter for serine into the cell, while SdaA is a L-serine dehydratase. Work by Velayudhan *et al.* (241) found that the SdaC transport protein acts as a high capacity, low affinity L-serine transporter. Deletion mutants into this gene completely impaired serine uptake into the cell, indicating that SdaC is the sole transporter capable of

transporting significant amounts of L-serine into the cell. Additionally, due to SdaC's low specific affinity for L-serine, SdaC also exhibits some ability to transport other amino acids into the cell as well, particularly L-threonine, raising the possibility this transporter plays a broader role in amino acid uptake (241).

In the same study, the SdaA protein was confirmed to be an L-serine dehydratase and was necessary for the metabolism of L-serine (241). Interestingly, unlike most other L-serine dehydratases, the protein found in *C. jejuni* was not pyridoxal-5'-phosphate dependent, but rather utilized a [4Fe-4S] cluster in a manner more reminiscent of many anaerobic bacteria (241). This enzyme is one among many enzymes in key metabolic pathways that contain oxygen sensitive iron-sulfur complexes (Figure 1.1). It has been proposed that the presence of these iron-sulfur containing enzymes is one of the key reasons for *C. jejuni*'s sensitivity to oxygen, as molecular oxygen or reactive oxygen species, in sufficient quantity will oxidize and damage the iron-sulfur complexes (59).

L-serine utilization has also been found to be absolutely critical for host colonization, with mutants in SdaC or SdaA being completely impaired for colonization in chicks (241). However, these same mutants were able to successfully grow *in vitro*. This result suggests that while L-serine is not essential for *C. jejuni* growth, it is necessary in the environment of the gut, where L-serine usage must provide *C. jejuni* with a key carbon source while establishing itself within the intestinal microbiota (241).

1.3.2. Aspartate/glutamate

Aspartate and glutamate metabolism are very similar to each other. Both amino acids are taken up through the Peb1 system, composed of the proteins Peb1a, Cj0919c, Cj0920c, and PebC. Work by Leon-Kempis *et al.* (141) has confirmed the function of Peb1A as the

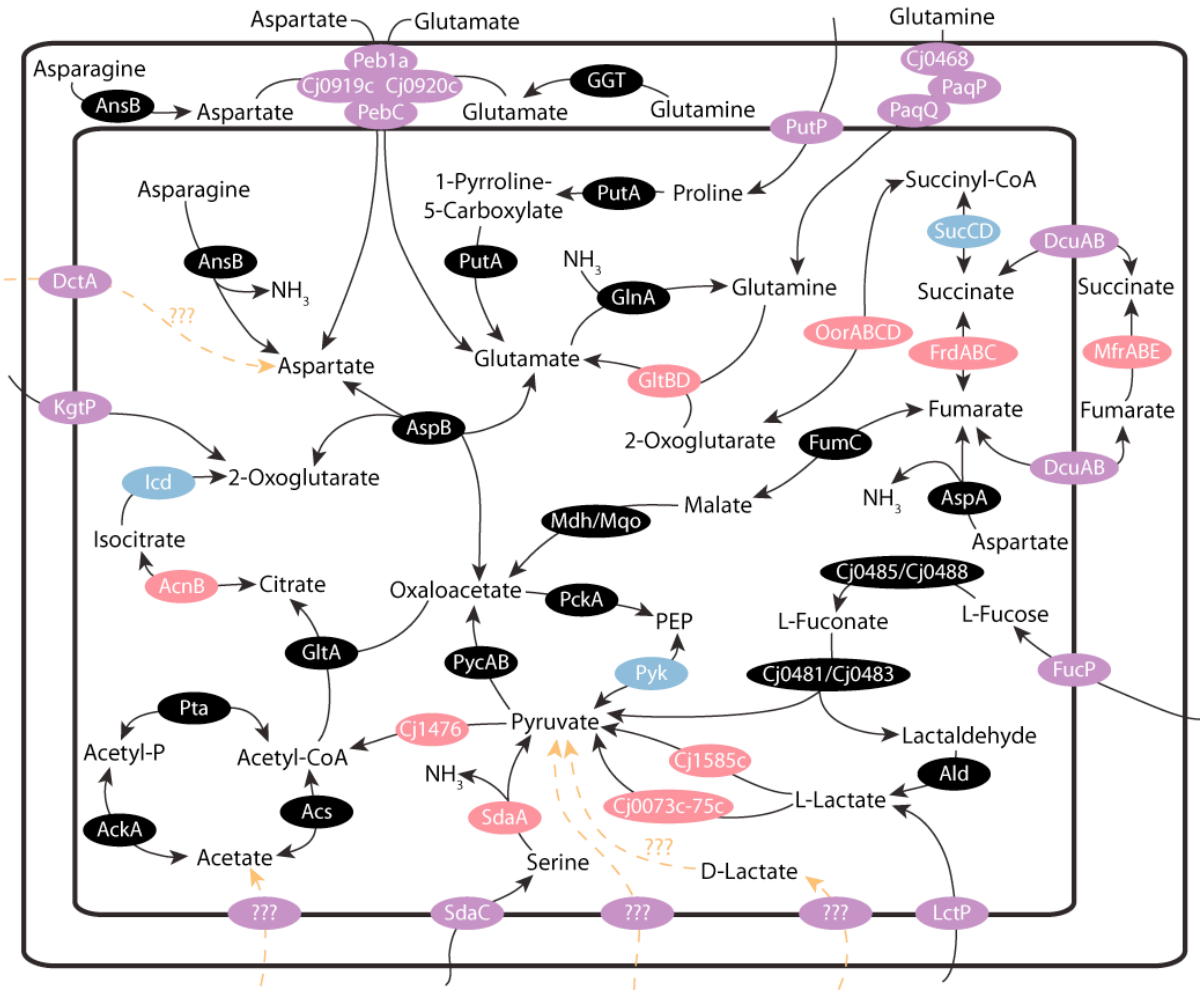

Figure 1.1: **Central metabolism in *C. jejuni*:** Schematic of the transporters and anaplerotic reactions that make up the central metabolic pathways of *C. jejuni*. The function and experimental evidence for each reaction is explained in the text. Note the central role that pyruvate plays in *C. jejuni* central metabolism; as serine, lactate and L-fucose metabolism results in the production of pyruvate. Glutamate and aspartate also play key roles in the production of citric acid cycle intermediates. Enzymes or enzyme complexes containing iron-sulfur complexes are indicated in pink, magnesium containing enzymes are indicated in blue, transporters are denoted as purple and hypothetical reactions are indicated in orange (Figure prepared by James Butcher).

transporter responsible for most, if not all, of the uptake of aspartate and glutamate into the cell. The Peb1a protein itself had been previously identified by Fauchere *et al.* (55) as linked to cell adhesion and further work using mutational analysis also found that mutants in Peb1A were significantly impaired for adhesion to epithelial cells (184). Leon-Kempis *et al.* (141) confirmed that this protein was found in the periplasm, and mutants in the gene for Peb1a were mostly impaired for L-aspartate uptake and were completely impaired for L-glutamate uptake. The remaining uptake of L-aspartate in the mutant strain was attributed to the function of the C4-dicarboxylate transporters (DctA, DcuA and DcuB), but was too minimal to allow for growth utilizing L-aspartate or L-glutamate as a sole carbon source (141).

Once glutamate has been taken up into the cell, the enzyme AspB, allows for the transamination of glutamate to aspartate. One molecule of glutamate and oxaloacetate are used to produce aspartate and 2-oxoglutarate in a reversible reaction (78). The aspartate can then be deaminated by the aspartase AspA into ammonia and fumarate, which can be fed into the citric acid cycle (78).

1.3.3 Proline

Proline has been found to be a less preferred amino acid compared to serine, glutamate and aspartate, but is still utilized by most strains of *C. jejuni* (255). As a major constituent of mucins, along with L-serine and L-threonine, it is a plentiful nutrient source within the intestine. Proline uptake and metabolism has been linked to the proteins PutP and PutA. PutP acts as a sodium/proline symporter, while PutA is a bifunctional proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase. The enzyme works to consume L-proline and FAD, producing 1- pyrroline-5-carboxylate, while reducing the

electron acceptor FAD to $FADH_2$. PutA subsequently catalyzes the second step of the pathway, by acting as a 1-pyrroline-5-carboxylate dehydrogenase, and oxidizes 1-pyrroline-5-carboxylate to glutamate with the concomitant reduction of NAD to NADH. Glutamate can then be further metabolized to aspartate and subsequently fumarate as described above. Although neither PutP or PutA has been specifically studied in *C. jejuni,* they are closely homologous to the well-studied PutA and PutP proteins in *E. coli* (229, 262) and *Helicobacter* (131, 173). In *Helicobacter*, PutA has been found to be key for stomach colonization and swarming motility (173). Whether it may have similar importance in *C. jejuni* has yet to be described.

1.3.4. Asparagine

While most strains of *C. jejuni* possess an asparaginase gene (*ansB*), *C. jejuni* does not possess any identifiable asparagine transporters, and indeed many strains that possess *ansB* are unable to grow on asparagine as a primary carbon source. Nevertheless, certain strains, including 81-176, possess an *ansB* gene with an additional ~40bp Sec-dependent secretion signal peptide (93). The AnsB protein is normally found exclusively in the cytoplasm, however with this secretion signal peptide, AnsB is transported to the periplasm, where it would be capable of deaminating asparagine to aspartate within the periplasm. Thus, strains that lack an asparagine transporter can utilize asparagine since the aspartate formed by AnsB can be transported to the cytoplasm by the Peb1 complex and fed to the citric acid cycle (CAC) by AspA (93).

Interestingly, it was found that there are two alternate transcriptional start sites in the *ansB* gene of 81-176, allowing it to produce both the cytoplasmic and periplasmic versions of AnsB. It has been hypothesized by Hofreuter *et al.* (93) that the cytoplasmic AnsB might

still serve a purpose to metabolize asparagine originating from small peptides. Although little research has been done to identify small-peptide transporters in *C. jejuni*, *cstA* has been annotated as a small peptide transporter and could possibly serve this purpose. Mutants in the *ansB* gene and/or its signal peptide are still capable of colonizing the intestine in a comparable fashion to the wild-type strain, however, it was found that strains lacking the periplasmic AnsB suffered a significant defect for liver colonization in infected mice. This suggests a tissue specific value for asparagine metabolism (93).

1.3.5. Glutamine

The ability of *C. jejuni* to utilize glutamine has remained somewhat controversial. Based on gene annotations and experimental evidence, it contains a functional glutamine uptake system, encoded by the genes cj0468, *paqP*, and *paqQ* (148). Additionally, it contains the genes coding for glutamate dehydrogenase (*gltBD*), capable of converting glutamine and 2-oxoglutarate into two molecules of glutamate. Finally, *C. jejuni* also contains GlnA for the reverse reaction of converting glutamate to glutamine in an ATP-dependent manner. This reaction also serves as a means to incorporate free ammonia, a major route of nitrogen assimilation for *C. jejuni*. However, despite having the biochemical means of transporting and converting glutamine to glutamate via glutamate dehydrogenase, the NCTC11168 strain exhibits little ability to grow on glutamine as a primary carbon source (93), unlike the other previously discussed amino acids. The exception to this appears to be strains such as 81-176 and 81116, which contain γ-glutamyl transpeptidase (GGT) (94). GGT is secreted to the periplasm, and acts to hydrolyze glutamine to glutamate and a free ammonia ion, or glutathione to glutamine and a γ-glutamylcysteine. This whole process takes place within the periplasm of the cell. While *C. jejuni* has not been shown to grow on γ-

glutamylcysteine, the glutamate produced from glutathione or glutamine is readily taken up by the Peb1 complex, where it can be metabolized by AspA and AspB as previously discussed (93).

The presence of the GGT enzyme in certain strains appears to have a significant effect on colonization. Knockouts of the GGT enzyme impaired the colonization of MyD88 -/- deficient mice by the 81-176 strain (93), while a GGT knockout in the 81116 strain was defective for chick colonization (15). Although both of these knockouts showed considerable colonization disadvantages relative to their wild-type strains, other strains of *C. jejuni* lacking GGT are perfectly capable of colonizing these two hosts. This suggests that any individual strain may have varying metabolic requirements, making certain pathways dispensable for them. The lack of GGT in a strain such as NCTC11168 may be compensated for the presence of other pathways, such as the recently discovered L-fucose acquisition and metabolic pathway (222). Overall, the diversity of available mechanisms for amino acid acquisition and utilization between strains may allow different *C. jejuni* strains to co-colonize and to colonize multiple niches or different hosts, thereby expanding the versatility of the species.

1.3.6. Citric acid cycle intermediates

C. jejuni depends heavily on the citric acid cycle for its energy needs. All of the aforementioned pathways produce pyruvate, fumarate, oxaloacetate, or 2-oxoglutarate, each of which is fed directly into the citric acid cycle. In addition to this, *C. jejuni* is capable of transporting several of the citric acid cycle intermediates, and using them directly as nutrient sources. These include 2-oxoglutarate, for which it has the permease KgtP; and succinate, fumarate and malate, which can be transported by the C4-dicarboxylate transporters, DcuA

and DcuB (78). The putative C4-dicarboxylate transporter DctA may also play a minor role in the uptake of both aspartate and CAC intermediates (78, 141), however, this has not been conclusively demonstrated. Additionally, there exists the gene *cj0025c*, annotated as a putative sodium:dicarboxylate family transmembrane symporter (180). However, no research has been done to characterize this transporter as of yet, so its possible role as a dicarboxylate transporter has yet to be described. Many strains also possess a citrate transporter (*cj0203* in *C. jejuni* NCTC11168), allowing for the acquisition of citrate (180). *C. jejuni* is known to utilize pyruvate as a primary carbon source while it is present in the growth medium (240), however, no pyruvate transporters have been identified or studied in *C. jejuni*, leaving the mechanism by which it transports pyruvate into the cell currently unknown.

The dicarboxylate transporters DcuA and DcuB may play an important role in *C. jejuni* thanks to the prominent role of both fumarate and succinate as electron donors and acceptors respectively. *C. jejuni* possesses the cytoplasmic fumarate reductase FrdABC, capable of oxidizing succinate to fumarate, as well as the reverse reaction. Although *C. jejuni* was initially annotated as possessing succinate dehydrogenase (*sdhABC*), it was later found that these genes did not code for succinate dehydrogenase (248), but rather for a periplasmic methylmenaquinol: fumarate reductase (*mfrABE*) (77). This complex was found to be in the periplasm, and reduced fumarate, crotonate and mesaconate to succinate, butyrate, and 2-methylsuccinate respectively under low-oxygen conditions. The cytoplasmic-periplasmic membrane transport of succinate and fumarate necessary for these processes is believed to be facilitated by the DcuA and DcuB transporters (77).

1.4. Colonization of the host

When a potential host is exposed to *Campylobacter* infection, *C. jejuni* will first pass through the stomach. Unlike the closely related *Helicobacters*, *C. jejuni* does not colonize the stomach, although resistance to acidic conditions allows it to persist for long periods at a pH as low as 4.5 and survive for brief periods at a pH even lower (193). This allows it to survive exposure to stomach acid and successfully pass through the stomach into the small intestine. In the upper small intestine (duodenum), *C. jejuni* is resistant to the exposure to bile due to the *cmeR* regulon. CmeR controls, among other genes, the *cmeABC* efflux pumps, which contributes significantly to the tolerance of *C. jejuni* to bile (150). *C. jejuni*'s preferred niches are the cecum and colon of its host, where it will establish itself in high numbers. Here, it will use its high motility and helical shape to penetrate into the mucus layer of the intestine where it will establish itself within this layer and often in close proximity to the epithelial layer (142). Growth within this mucus layer provides both a favourable environment to *C. jejuni* as well as special challenges.

1.4.1. The intestinal mucus layer

The mucus lining serves as a protective layer, protecting the intestinal epithelium from both infections from potential pathogens, but also from physical damage caused by material passing through the gut (36). The intestinal lining consists primarily of columnar epithelial cells that are responsible for absorbing most of the nutrients that are made available through the digestion of material in the intestines. These epithelial cells are constantly being replaced by pluripotent stem cells located at the bottom of the intestinal crypts (122). These valuable stem cells are protected from infection and other damages by the paneth cells at the base of the crypts, which produce large amounts of antimicrobial

peptides to keep the crypts clear of foreign bacteria (23). The mucus layer, produced by goblet cells, which are scattered throughout the intestinal epithelium (122), provides a non-specific defense against infection of the epithelium (47).

The mucus layer of the intestine is a dynamic, semi-permeable, gel-like layer coating the interior lining of the intestine and other mucousal epithelial tissues. Its structure is maintained by interlinking glycoproteins known as mucins. These mucins divide into two general types, gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC6) and cell-associated mucins (MUC1, MUC3, MUC12, MUC13, MUC16, and MUC17) (36, 47, 161). The cell-associated mucins remain anchored to the cell surface via a transmembrane domain. These mucins form a tight glycocalyx along the surface of the cell for the purpose of preventing the attachment of intestinal bacteria (110, 111). Gel-forming mucins, primarily MUC2, although similar in structure to cell-associated mucins, lack a transmembrane domain and are secreted into the lumen of the intestine, where they form a loose, dynamic outer mucus layer and an inner, more-firm, sterile mucus layer (6, 109-111), outlined in figure 1.2.

The individual mucin proteins consist of a protein core, which is heavily glycosylated to the point where glycans make up as much as 50-85% of the total mass of the glycoprotein (6). More specifically, mucins can be defined by the presence of a serine and threonine rich tandem repeat domain, a proline/threonine/serine (PTS) rich domain and cysteine rich domains. For example, the MUC2 protein (Figure 1.2b), which makes up the primary gel-forming mucin in the human intestine, contains a 2300 a.a. variable number tandem repeat (VNTR) domain consisting of a repeating 23 a.a. sequence of serine and threonine residues. Separated from it by a 148 a.a. cysteine-rich domain is a 347 a.a. PTS domain. At the N-terminal and C-terminal ends exist respectively a 1400 a.a. and 984 a.a. cysteine-rich domain. This brings the Muc2 protein to a total of over 5100 a.a. (6).

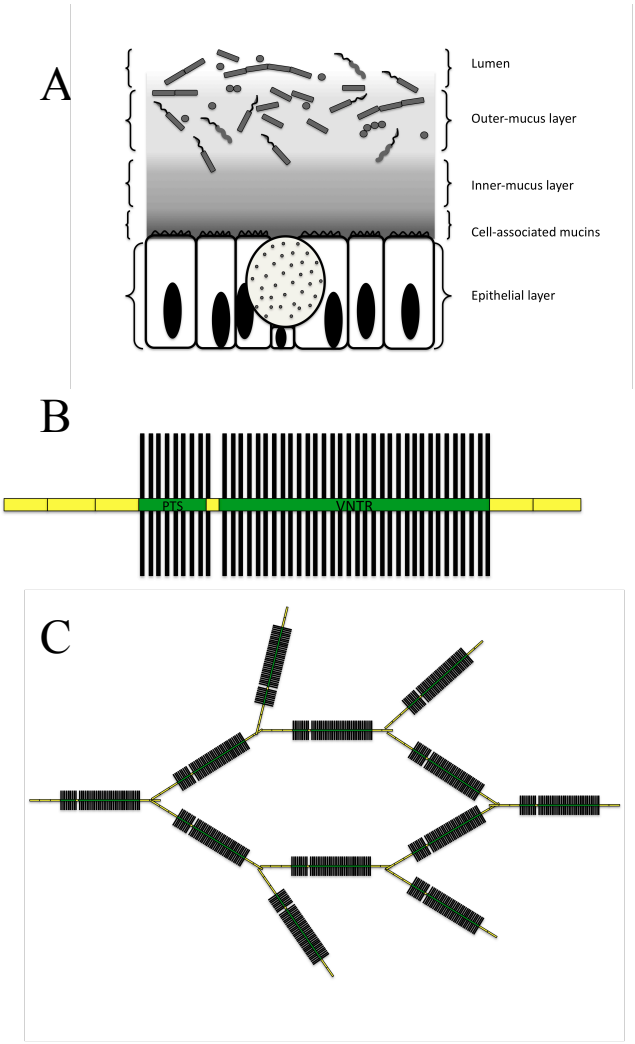

Figure 1.2: Diagram of the intestinal mucus layer. (A) Members of the intestinal microbiota are primarily found within the loose, outer-mucus layer comprised of secreted mucins. The inner-mucus layers are normally sterile, and the cell-associated mucins form a tight layer along the epithelial cell layer. (B) The structure of the secreted mucin MUC2. The serine, proline and threonine rich PTS and VNTR domains are shown in green. Cysteine rich regions are shown in yellow. The extensions from the protein core represent O-linked glycans attached to the PTS and VNTR domains. (C) Interlocking trimers of MUC2 proteins connected by disulfide bonds formed between cysteine residues. Networks of mucin polymers can extend several μm, forming the gel-like structure of the mucus layer.

The VNTR and PTS domains are common amongst all mucin types and are heavily O-glycosylated by glycans made up of 1-20 sugar residues. The cysteine-rich domains allow for the interlinking of mucin monomers into polymers by disulfide bonds between the cysteines of two or three individual proteins. This allows the mucins to link together into long chains reaching lengths of several μm as shown in figure 1.2c (9, 76).

The MUC2 mucins are the main constituent of the outer mucus layer, which is constantly being replaced, as the mucins are degraded through the action of digestive enzymes and bacteria, or are sheared off through the movement of material through the intestinal lumen (110). The rapid loss of the outer layers of the mucus layer is matched by the rapid production of mucins by intestinal goblet cells. This leads to a rapid turnover of the mucus layer, which serves to keep larger material and potentially harmful bacteria away from the epithelium.

Beneath the layer of secreted mucins are cell-associated mucins containing a trans-membrane domain, anchoring them to the cell membrane, with their highly glycosylated threonine and serine rich domains extending away from the cell surface to create a glycoprotein layer surrounding the cell. If a foreign cell or virus particle should pass through the outer layer of secreted mucins, they will adhere to the membrane bound mucins, thereby halting their advance (152, 202). Although much more stable than the outer layer, the membrane bound mucins are still subject to rapid turnover. As foreign particles become bound to the glycans of the mucin, they become detached from the cell surface due to the action of a characteristic SEA domain located just above the transmembrane domain of the mucin. These domains undergo auto-proteolysis in response to mechanical stress to the

protein, thereby cleaving the mucin protein from the transmembrane domain, releasing it from the cell and taking the bound foreign particle with it (143, 158).

The actual mechanism by which mucins will bind to bacterial cells remains poorly understood. The glycans often terminate with a negative charge, due to sulfate or carboxyl groups. This can explain its binding to some targets that contain a positive charge such as many viral particles (34). The negative charge will not however aid in binding bacteria, which usually carry a negative charge on their exterior cell wall or membrane due to teichoic acids or LOS. To bind bacteria, the glycans on the mucins are believed to mimic cell surface glycans thereby providing false-binding targets for bacterial adhesion (152, 202). It has been demonstrated that *C. jejuni* will adhere to certain glycan motifs, particularly those of fucosylated glycans found on both cell membranes and mucin glycans (42). This same observation has been made in other bacteria as well, where they will bind to glycan structures present on both cell surfaces and mucins, making a possible function for mucins to act as a decoy for bacterial adhesion (95, 102, 256).

In order to bypass the mucus layer, pathogens such as *C. jejuni* employ several different mechanisms. Bacteria such as *Vibrio cholerae* (215, 226) or *E. coli* (87), among others, will secrete mucin-degrading proteases and glycosidases, allowing them to degrade mucins creating a path through the layer to the epithelium below. Alternatively or additionally, bacteria such as *Salmonella typhi* (126) or *Shigella flexneri* (245) will avoid mucins entirely by invading through M-cells, where the mucus layer is the thinnest (112, 208). Many toxins may also be able to travel through the mucus layer ahead of the bacteria, disrupting the underlying epithelial layer and mucus production (161). Once in the mucus layer, the production of toxins can be triggered by exposure to mucins as has been demonstrated in *C. jejuni,* where upon exposure to MUC2, it up-regulates several

pathogenicity factors including cytolethal distending toxin and vacuolating toxin (234). Finally, certain bacteria such as *Campylobacter* and *Helicobacter* use their high motility, helical shape and rotating movement to physically force their way through the mucus layer (58, 142).

1.4.2. The intestinal microbiota

Despite the role of the mucus lining as a barrier to bacterial and viral infection, for bacteria adapted to survive under those conditions, it becomes a plentiful environment to colonize. Although the precise numbers vary significantly between individuals, the human intestinal microbiota is made up of as much as 100 trillion cells comprising hundreds of bacterial species (50, 64, 145, 185, 190). The two most represented phyla are the *Firmicutes* and *Bacteroidetes,* representing approximately 51% and 48% of the microbial population (50). Several studies have however found significant variation in the proportion of these two phyla between individuals and especially in diseases such as IBD (64, 185) and celiac's disease (207). Of the *Firmicutes*, most (~95%) belong to the *Clostridia* class, while smaller numbers belong to the *Mollicutes* and *Bacilli* classes. In terms of diversity though, despite being only 51% of the total number of bacteria, the *Firmicutes* represented 76% of the total identified phylotypes in a study by Eckburg *et al.* (50). In contrast, the representatives of the phylum *Bacteroidetes*, despite almost matching the *Firmicutes* in total number, were much less diverse, with 31% of them being *Bacteroides vulgatus* (15% of total bacteria), and 12% *Bacteroides thetaiotaomicron* (6% of total bacteria), making those two individual species the most prevalent bacteria within the gut (50, 144). Other bacterial phyla found in the above studies included *Proteobacteria, Actinobacteria, Verrucomicrobia, Fusobacteria*, and a single species from the *Archaea* domain, *Methanobrevibacter smithii* (50, 206).

Interestingly, most common gastrointestinal pathogens such as *Campylobacter* or *E. coli* are members of the *Proteobacteria*, which is only a relatively small portion of the normal microbiota. Metagenomic studies of the microbiota during infection, dextran sulfate sodium (DSS)-induced inflammation or following disruptions such as with antibiotic treatments, find dramatic shifts in certain types of bacteria, but such shifts are generally temporary and the microbiota will usually return to its previous composition over time (92, 157).

1.4.3. The intestinal ecosystem

It is into this complex community of organisms that *C. jejuni* must establish itself in order to colonize its host. It has been proposed that in order for a new bacterial species to establish itself into a complex ecosystem such as the gut, it must find at least one nutrient source that it can utilize better than existing bacteria. This hypothesis was first put forward by Freter *et al.* in 1983 (66), where they found that *E. coli* needed an open ecological niche to colonize the mouse intestine. The basic principle behind this hypothesis is simply that of survival of the fittest, where an organism, in order to establish itself, must either find an unoccupied niche or successfully outcompete an organism currently occupying it's target niche.

The mucus layer itself, upon which this complex microbial community establishes, is more than simply the mucin complexes described above. The cells of the intestinal epithelium release numerous antimicrobial peptides and immunoglobulins into the lumen of the gut to control the microbial population of the intestine and keep it away from the epithelium (23). Food is constantly passing through the intestine, being degraded by not only enzymes released from the host, but also through the digestive actions of the microbiota

as well. The microbiota will act on macromolecules, especially the mucins, produced by the epithelium, degrading them into their individual amino acids and glycans for their own benefit. *Bacteroides thetaiotaomicron* alone, one of the most common single species in the gut, contains as many as 172 different glycosidases, and is capable of degrading most glycans present in both insoluble foodstuffs in the intestine, as well as on mucins in the mucus layer (35).

Beyond the simple degradation of larger macromolecules, the microbiota also creates an intricate, interdependent metabolic network. The metabolic byproducts from one organism are subsequently utilized by the host or other bacteria within the ecosystem (18). The best example of this is the production of short-chain fatty acids (SCFA) such as acetate, lactate, propionate, and butyrate. Significant research has been done to elucidate the interconnected relationships between bacteria producing SCFA. The first step of the process is done by carbohydrate-fermenting bacteria, with members of the *Clostridia*, *Bacteroidia* or *Actinobacteria* classes being prime examples. These bacteria possess enzymes, first for the degradation of larger carbohydrates such as starches, breaking them down into individual mono and disaccharides (17, 18, 61). These sugars can then be fermented anaerobically, producing large amounts of acetate, lactate and some propionate and ethanol as byproducts (17, 154). Studies into the SCFA content in feces, find that particularly large amounts of acetate are present, presumably produced for the most part as a product of bacterial fermentation in the colon (48).

Next, extracellular acetate can be converted to butyrate using one of several variants of a butyrate-producing pathway. The mechanisms have been well studied and have been found in *Roseburia* spp. *Eubacterium* spp. *Coprococcus* spp. and *Faecalibacterium prausnitzii* to name a few (154). Most of these inhabit the colon and together make up as

much as 3% of the total bacteria found there (154). The production of butyrate itself has an important function within the colon as it serves as one of the primary energy sources for the nutrition of the colonocytes that make up the epithelium of the colon (85, 154), and a lack of butyrate has been linked to both colon cancer and inflammation of the colon (85, 162). To the bacteria of the microbiota, the production of these SCFA adds to the repertoire of potential carbon sources available to the microbial population with different species of bacteria often taking-up and metabolizing acetate, lactate, propionate and butyrate (17, 18, 48, 154).

This is also the point where *C. jejuni* likely enters into the picture. Although there are bacteria that will make use of all of these molecules, known pathways and experimental evidence in *C. jejuni* suggest that it only has the capacity to transport and metabolize both acetate and lactate (231, 255). Acetate is initially produced and secreted as a byproduct in several of *C. jejuni's* primary metabolic pathways such as L-serine and pyruvate metabolism (163). However, in later stages of growth, when more preferable sources are exhausted, acetate can also be used as a carbon source for *C. jejuni* (255). Although a transporter for acetate has not been identified in *C. jejuni*, with the exception of an annotated acetate permease (ActP) in *C. jejuni subsp. doylei* (178), it has been experimentally demonstrated *in vitro* that *C. jejuni* has the ability to use acetate as a primary carbon source (255).

The production and excretion of acetate comes as the result of the need to recycle CoA. The enzyme Pta produces acetyl-phosphate from acetyl-CoA, freeing the CoA for reuse. The enzyme acetate kinase (AckA) then produces acetate from acetyl-phosphate, which is excreted and builds up in the surrounding medium. As other sources of nutrients become scarcer, a switch in the direction of the pathway allows for the uptake of acetate into

the cell, and the synthesis of acetyl-CoA from acetate by Acetyl-CoA synthase (Acs), or by reversing the direction of the AckA-Pta pathway (240, 255).

Lactate is also produced in large quantities in the colon as a byproduct of carbohydrate fermentation by numerous varieties of bacteria commonly found in the gut. However, despite its rapid production, overall levels of lactate within the gut remain relatively low, as high rates of production are matched by rapid consumption of lactate by other bacteria (17). A recent study by Thomas *et al.* (231) has well documented the usage of lactate by *C. jejuni*, although several points still require further research.

While lactate is a common byproduct of fermentation in some bacteria, there is no evidence of lactate excretion by *C. jejuni*. Moreover, while *C. jejuni* possesses an annotated L-lactate permease (LctP), a mutant into the *lctP* gene was still capable of some L-lactate transport, suggesting the existent of a second, as of yet unidentified lactate permease (231). Once inside the cell, L-lactate can be catabolized into pyruvate through one of several pathways. The first involves three genes, *cj0073c*, *cj0074c*, and *cj0075c*. These three genes code for a non-flavin iron-sulfur containing oxidoreductase complex that has been shown to convert L-lactate to pyruvate (231). A second, flavin and iron-sulfur containing enzyme Cj1585c, is also capable of converting L-lactate to pyruvate, although the reason for this redundancy is not immediately clear, and indeed, several strains of *C. jejuni* such as the 81-176 strain, lack homologues to *cj1585c*. A lactate dehydrogenase (Ldh) was also annotated in the *C. jejuni* genome; however, Thomas *et al.* were unable to find a role for this enzyme in lactate metabolism (231). Knocking out *ldh* did not impede lactate utilization, while a double knockout in the other two pathways completely eliminated lactate utilization, suggesting that this gene has been either misannotated, or is used under only specific conditions not found in

their study (231). *C. jejuni* has also been found to grow on D-lactate, however, no transporters or pathways have been identified as responsible for this phenotype (231).

1.5 The importance of studying the basic biology of *C. jejuni*

As mentioned above, there are very few individual toxins or virulence factors that can be solidly attributed to *C. jejuni* virulence (232). This makes the understanding of its biology more imperative. Louis Pasteur once aptly described the human body as a culture vessel (26), and this appears to be particularly relevant in the case of *C. jejuni*. To colonize a human host, bacteria must find an environment where it is supplied with the nutrients necessary to grow and spread. When *C. jejuni* is in its human host, it associates closely with the tissues surrounding the intestine, triggering an immune reaction. When it is in its commensal host, it does not appear to invade into the host tissues to any significant extent. In both hosts however, the key target for its colonization is not within the tissues of the host, but rather in the mucus lining of the intestine. This is the highly competitive environment described above, where the ecological competitiveness of *C. jejuni* takes on a new importance. To approach this, a deeper understanding of the basic biology of *C. jejuni* is necessary.

CHAPTER 2

Mapping the Essential Genes of *C. jejuni*

2.1. Introduction

When studying the basic biology of an organism such as *C. jejuni,* knowledge of the core genes that are essential for growth is important for an understanding of how an organism survives in its environment. Despite the increasing number of fully sequenced genomes from *C. jejuni* and other organisms, a comprehensive understanding of many of its most fundamental genes is still lacking. This led to an increased interest in assembling information as to what were the "core" genes of the organisms, and which ones were essential and which ones were replaceable or amenable to mutation or deletion.

A number of experimental approaches and genome-wide surveys to identify essential genes have been previously reported. Extensive lists of essential and dispensable genes have been generated for *Acinetobacter baylyi* (43), *Escherichia coli* (11, 72, 211, 233), *Helicobacter pylori* (205), *Francisella novicida* (70, 130), *Haemophilus influenzae* (3), *Mycobacterium tuberculosis* (133, 209), *Bacillus subtilis* (125), *Pseudomonas aeruginosa* (146), *Mycoplasma genitalium* (75), *Mycoplasma pulmonis* (65), *Staphylococcus aureus* (62, 108), *Streptococcus pneumoniae* (220, 230), *Salmonella typhimurium* (124) and recently in *Campylobacter jejuni* by Stahl et al. (221) and Metris et al. (165), including the research described herein. The methods employed include systematic single-gene deletion mutants (43, 125), comprehensive transposon mutant library (3, 65, 70, 75, 130, 146, 165, 205, 209, 221), antisense RNA technology (108), trapping lethal insertions (124) and/or using *in silico* approaches (165).

To date, the complete genome sequences of more than a dozen isolates of *Campylobacter jejuni* subsp. *jejuni* have been obtained (24, 37, 63, 67, 94, 180, 183, 187, 188, 227, 228), plus numerous unfinished genome sequences. *C. jejuni* NCTC11168 possesses a genome of approximately 1.6 Mb which is predicted to encode 1643 proteins (81) and other strains harbor a genome comparable in size, although with small variations. In addition to *C. jejuni* species, increasing numbers of other species of *Campylobacter* and related organisms have been sequenced, including *C. coli*, *C. lari*, *C. upsaliensis* (63), *C. fetus* subsp. *fetus* and *venerealis* (168), and numerous others. As more genomes are sequenced, we have gained a better understanding as to which genes are conserved across the plethora of *Campylobacter* strains and species, and we have begun to assemble both "pan" and "core" genomes (139, 252). Pan genomes are the full complement of genes found in the species. While few if any individual strains would contain all of these genes, they represent the gene reservoir available to the species as a whole. The core genome on the other hand, is the set of conserved genes amongst all strains and species (139). While it may be assumed that these comprise an essential gene set, this is not necessarily the case, as pathway redundancies and compensatory mutations render even important genes dispensable.

Defining the concept of gene essentiality can be both difficult and misleading. Although it is tempting to assume that an essential gene encodes a protein absolutely critical for growth, it must be considered that essentiality should be viewed as contextual (45). Indeed, growth conditions can significantly affect the list of essential genes. For example, 119 genes from *E. coli* were found to be essential for growth on glycerol minimal medium while the corresponding mutants were viable on rich medium (115). Genes encoding proteins involved in general metabolic processes are likely to be no more than conditionally

essential and incorporating the metabolic product(s) from the defective pathways in the growth medium could readily revive a lethal phenotype.

Consequently, it is tempting to speculate that the number and the list of essential or conditionally essential genes may vary significantly between structurally, metabolically and ecologically-distinct microorganisms. In agreement with this, the set of essential genes can differ significantly between microorganisms. Sassetti *et al.* have found that only 47% of the genes found to be essential in *H. influenzae* are required for the growth of *Mycobacteria* (209). Clearly, essential pathways are not universally conserved, preventing the sole identification of essential genes by bioinformatic approaches and warranting their characterization in diverse microorganisms. Here we sought to identify the *C. jejuni* NCTC 111168 genes necessary for growth in a rich beef-extract medium (Mueller-Hinton) under microaerophilic (8% O_2, 4% H_2, 10% CO_2, 78% N_2) conditions.

To accomplish this, we employed a transposon-based mutagenesis combined with a microarray mapping approach to identify *C. jejuni* dispensable and essential genes. Cross comparison of essential genes of *C. jejuni* to other species revealed a common set of core genes as well as *Campylobacter* specific essential genes. This study was the first assessment of gene essentiality in *C. jejuni* and was published by Stahl *et al.* in 2011 (221).

2.2. Materials and Methods

2.2.1. *C. jejuni* strains and growth conditions

C. jejuni NCTC 11168 was grown on Mueller-Hinton (MH) agar plates at 37°C under microaerophilic conditions (83% N_2, 8% O_2, 4% H_2 and 5% CO_2) in a MACS-VA500 workstation (Don Whitley, West Yorkshire, England). Chloramphenicol (20 μg/ml) was added to the MH medium when required.

2.2.2. Transposon mutagenesis and construction of a transposon library of *C. jejuni* NCTC 11168

A transposon library of *C. jejuni* NCTC 11168 was generated using the EZ-Tn5 transposase (Epicentre Biotechnologies, Madison, WI) and the EZ-Tn5-Cm transposon vector previously constructed by cloning the Cm^R cassette from pRY111 into the EZ-Tn5 pMOD-3 <R6Kγori/MCS> transposon construction vector (Epicentre) (194). The transposon library was constructed by carrying out *in vitro* transposition according to the manufacturer's instructions. Briefly, the transposon was PCR amplified from the EZ-Tn5-Cm transposon vector using the manufacturer-designed primers pMOD forward and reverse. The *in vitro* transposition insertion reaction consisted of 1 μl of 10x transposase buffer, 1 μl of transposase, 2 μg of chromosomal DNA and 1 μg of amplified transposon, in a final reaction volume at 10 μl. Following 2 hours incubation at 37°C, the reaction was stopped by adding 1 μl of 10x stop solution and incubating at 70°C for 10 min. The transposed genomic DNA was purified using a Qiagen PCR purification kit, eluted into 44 μl sterile water, and repaired by adding 5 μl of 10 x T4 DNA ligase buffer from New England Biolabs (NEB), 0.5 μl of 10 mM dNTPs and 2.5 units of T4 polymerase (NEB) and incubating for 20 min at 11°C followed by 15 min at 75°C. Next, the DNA was subsequently ligated overnight at 16°C by the addition of 5 μl of 10x T4 DNA ligase buffer and 600 units of T4 DNA ligase (NEB) in a 100 μl final volume. The repair transposed genomic DNA was purified using the Qiagen PCR purification kit and transformed into *C. jejuni* NCTC 11168 as previously described (193). Transformants were selected on MH-agar plates containing 20 μg/ml chloramphenicol. Each transformant was individually grown again on selective MH-agar

plates to ensure chloramphenicol resistance. Next, transformants were inoculated into 300 µl of MH broth in 96 well plates and grown under microaerophilic conditions for 18 hours. Twenty µl of dimethyl sulfoxide was then added to each well and the plates were stored at -80°C.

2.2.3. Transposon mutant growth conditions and DNA extraction

The mutants for each 96-well plate were divided into two groups of 48 and each of the 48 mutants was grown individually in separate patches on a single MH-agar plate containing 20 µg/ml chloramphenicol. These plates were incubated at 37°C under microaerophilic conditions for two days. The 48 mutants from each plate were pooled by resuspending them in 3 ml MH broth. Finally, genomic DNA was extracted from this suspension using a standard phenol:chloroform extraction method.

2.2.4. Microarray transposon tracking (MATT) using a PCR product microarray

(i) Experimental approach and DNA template:

A modification of MATT (205) was used to identify the transposon insertion sites and is outlined in figure 2.1. Briefly, the flanking sequences of the transposon insertion sites were PCR amplified using a 2-steps semi-degenerate PCR approach. The first step consisted of a low stringency amplification that generated PCR products containing fragments corresponding to either end of the transposon extremity and the corresponding flanking chromosomal DNA. The second step consisted in labeling the obtained transposon flanking regions with fluorescent dyes followed by hybridization on the microarray slide. Total genomic DNA was extracted from 150 pools of 48 mutants each (representing our library of

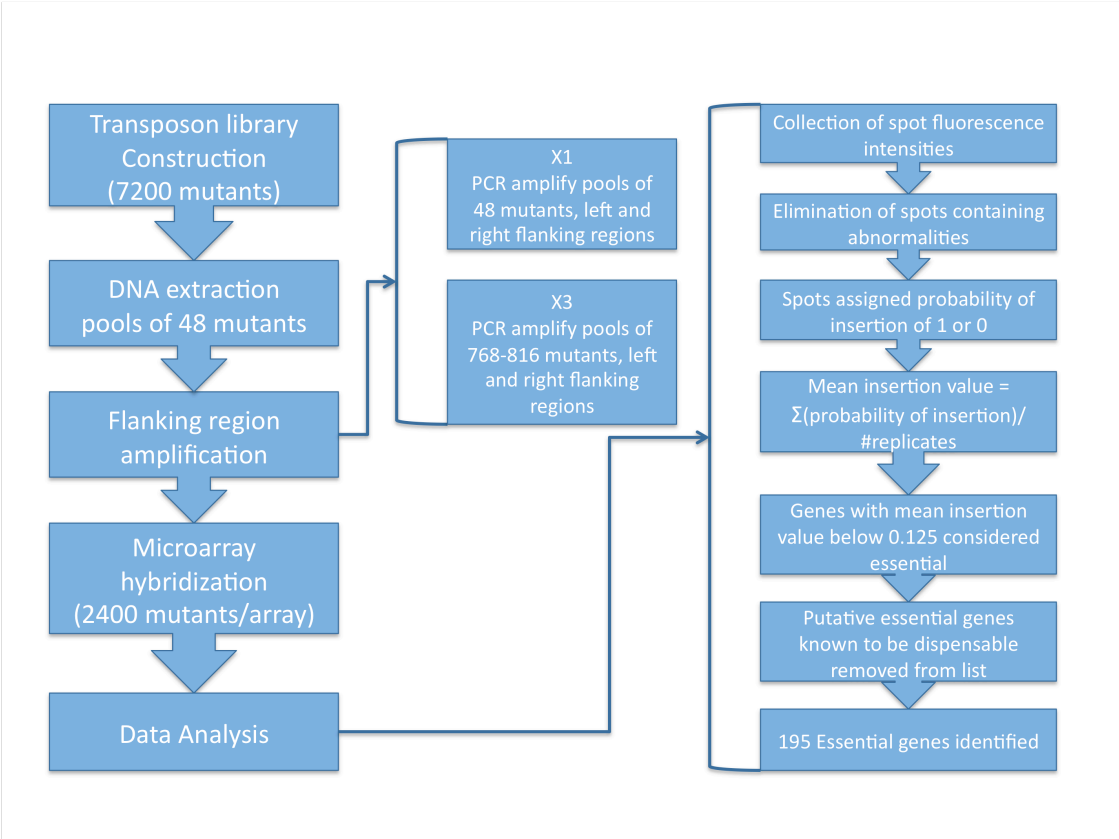

Figure 2.1: **Flowchart of the experimental approach of the microarray transposon tracking and data analysis**. The transposon mutant library was divided into pools for PCR amplification and microarray hybridization. Ensuing data was normalized to the background fluenscence intensity, was assigned a binomial value of 1 or 0 denoting its presence or absence from each array, and its mean insertion value was calculated.

7,200 mutants). Two approaches were employed to PCR amplify the 7,200 transposon flanking regions. In the first approach, the transposon flanking sequences from each pool of 48 insertions were amplified using a two-step PCR and 50 reactions were combined together before labeling for microarray hybridization (representing 2,400 insertions). A total of 3 microarray experiments were performed to screen the entire collection of 7,200 insertions. In the second approach, 16 to 17 pools of genomic DNA were combined together (representing 768 to 816 insertions) prior to the two-step PCR. Next the PCR products representing 2,400 insertions were pooled and labeled for microarray hybridization. A total of 3 microarray experiments were carried out to cover the 7,200 insertions.

(ii) Transposon insertion site labeling:

Fifty ng of template DNA was added to a reaction mixture containing 2 μM of the primer AR54 or CM2 (both primers bind within the transposon and are oriented outwards on either end of the transposon), 1 μM of each degenerate primer MS1 and MS3 (Appendix I), 0.5 mM dNTPs, and 4.4 mM $MgCl_2$ in 1 x PCR reaction buffer (Invitrogen) with 1 U of taq polymerase (Invitrogen). The PCR conditions used were the following: 94°C for 2 min; followed by 6 cycles at 94°C for 30 s, 42°C for 30 s and 72°C for 3 min, with 1°C lowering of the annealing temperature (42°C) on each cycle; and 25 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 3 min. The PCR products of this first amplification were purified using the PCR purification kit from Invitrogen, suspended into 50 μl of sterile deionized water, and 2 μl was used as a template for the second PCR amplification. This second step was designed to specifically label the transposon-flanking region by incorporating aminoallyl-dUTP for subsequent labeling with fluors. This second PCR mixture contained 0.5 mM each of dGTP, dATP, and dCTP, 0.34 mM aminoallyl-dUTP, 0.4 μM of the transposon nested primer SqFP

or SqRP (that binds at either end of the transposon; Appendix I), 0.4 μM of primer MS2 (primer complementary to the conserved tail sequence of MS1 and MS3), 4.4 mM $MgCl_2$, 1 x PCR reaction buffer, with 1 U of taq polymerase (Invitrogen), in a final reaction volume of 100 μl. The PCR amplification of the transposon DNA flanking region was performed by 30 cycles at 94°C for 30s, 56°C for 30s and 72°C for 2 min. The resulting PCR products were fluorescently labeled as previously described. Briefly, the aminoallyl-labeled PCR products were purified from free dNTPs and aminoallyl-dUTP by adding 350 μl of water and spinning through a Microcon YM-30 filter (Millipore) for 8 min at 10000 rpm, followed by four washings with sterile deionized water. Following the last wash, the sample was concentrated to a volume less than 9 μl under vacuum using a SpeedVac (Eppendorf). Next, the volume was adjusted to 10 μl using 1 μl of 1 M sodium carbonate pH 9.0 and water. Finally, the PCR products were fluorescently labeled by adding 10 μl of dimethyl sulfoxide containing monoreactive fluor indocarbocyanine (Cy3 dye) or indodicarbocyanine (Cy5 dye) and incubating in the dark for 1 h at room temperature. The PCR products obtained using primers AR54 and SqFP were labeled with Cy3 dye and those obtained using CM2 and SqRP were labeled with Cy5 dye. The labeled PCR products were purified following the addition of 55 μl of water and 35 μl of 100 mM NaOAc pH 5.2, and using the PCR purification kit from Invitrogen.

(iii) Microarray hybridization and washing:

The Cy3 and Cy5 fluorescently-labeled PCR products (originating from the same pool of mutants) were combined, concentrated under vacuum using a SpeedVac, and resuspended into 15.14 μl of water. The *C. jejuni* NCTC 11168 microarray slides used in this study harbored PCR products representing each open reading frame from *C. jejuni*

genome and were constructed as described in Stintzi (224). The microarray slides were prehybridized by incubation in 100 ml hybridization buffer (25% formamide, 5XSSC, 0.1% SDS, and 1% BSA) for 45 min at 42°C. Prior to hybridization, the slides were rinsed with water and dried by spinning. The combined labeled PCR products were hybridized to the microarray slides overnight in a solution containing 25% formamide, 5x SSC (1x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% sodium dodecyl sulfate, and 0.7 μg/μl salmon sperm DNA at 42°C in a humidity chamber (Arrayit) as previously described (175). Following hybridization, the slides were washed for 5 min in 100 ml of 2 X SSC and 0.1 % SDS at 42°C, 10 min in 100 ml 0.1 X SSC and 0.1 % SDS at room temperature, and 4 times for 1 min in 400 ml 0.1X SSC. Finally, the slide was rinsed in deionized water, spun dry and scanned at 10 μm resolution using the ScanArray Gx from PerkinElmer.

2.2.5. Microarray data analysis

The collection of the fluorescence intensities was performed using the ScanArray Express software (Perkin Elmer). Because this software is prone to misidentification of spots, each spot was visually inspected to confirm its presence or absence. Spots within slide abnormalities were excluded. The Cy3 and Cy5 channels were individually analyzed. Spots with fluorescent mean intensities greater than 2 standard deviations from the background were considered present and assigned a probability of insertion of 1. Spots with fluorescent mean intensities less than 2 standard deviations from the background were considered absent and assigned a probability of insertion of 0. The data from all microarrays were collated and the mean of the probability of insertion computed. Insertions were only considered to be present within the library if they appeared as present within multiple microarray experiments. A mean insertion value of 0.125 was empirically determined based on expected essential

genes as an appropriate cutoff to identify most candidate essential genes while minimizing false positives. Next, known dispensable genes were removed from the obtained list of candidate essential genes.

2.2.6. PCR confirmation of predicted essential genes

The insertion or absences of insertion were confirmed in 15 genes by PCR as follows. A pair of forward and reverse primers was designed to amplify each specific gene. Each primer was combined with the transposon specific primer SqFP and the 2 pairs of primers were used to detect the presence of an insertion by PCR using the pool DNA extracted from the mutant library as PCR template. The PCR reaction mixtures consisted of: 0.5 µl of 10 mM dNTPs, 1 µl of 20 µM of SqFP and gene specific primer (forward or reverse primer), 2.2 µl of 50 mM $MgCl_2$, 2.5 µl of the 10X reaction buffer from Invitrogen, 0.5 µl of template DNA, 2.5 units of Taq polymerase in a final volume 25 µl. The PCR conditions used were the following: 94°C for 2 min followed by 35 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 2 min; and finishing with 72°C for 4 min. Finally, the PCR products were analyzed by agarose gel electophoresis.

2.2.7. Identification of putative orthologs of *C. jejuni* genes in various sets of microbial genomes and determination of the evolutionary retention indexes (ERIs)

Putative orthologs of *C. jejuni* genes were identified by comparing each individual protein sequence to the sequences in the NCBI COG database using a modified BLAST tool designed to search orthologous group data named BlastO (261). The NCBI COG database consists of 4,873 clusters of orthologous proteins delineated from the proteins encoded in the genome of 66 microorganisms (50 eubacteria, 13 archaea, and 3 unicellular eukaryotes).

These microorganisms are *Agrobacterium tumefaciens*, *Brucella melitensis*, *Caulobacter crescentus*, *Mesorhizobium loti*, *Sinorhizobium meliloti*, *Rickettsia conorii*, *Rickettsia prowazekii*, *Buchnera sp*, *Escherichia coli K12*, *Escherichia coli O157:H7*, *Escherichia coli O157:H7 EDL933*, *Salmonella typhi*, *Yersinia pestis*, *Haemophilus influenzae*, *Pasteurella multocida*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Xylella fastidiosa*, *Neisseria meningitidis MC58*, *Neisseria meningitides Z2491*, *Ralstonia solanaraceum*, *Campylobacter jejuni*, *Helicobacter pylori 26695*, *Helicobacter pylori J99*, *Bacillus halodurans*, *Bacillus subtilis*, *Clostridium acetobutilicum*, *Lactococcus lactis*, *Listeria innocua*, *Mycoplasma genitalium*, *Mycoplasma pneumoniae*, *Mycoplasma pulmonis*, *Ureaplasma urealyticum*, *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Corinebacterium glutamicum*, *Mycobacterium tuberculosis H37Rv*, *Mycobacterium tuberculosis CDC1551*, *Thermotoga maritima*, *Aquifex aeolicus*, *Mycobacterium leprae*, *Synechocystis sp.*, *Nostoc sp.*, *Borrelia burgdorferi*, *Treponema pallidum*, *Chlamydia trachomatis*, *Chlamydophila pneumoniae*, *Deinococcus radiodurans*, *Fusobacterium nucleatum*, *Archaeoglobus fulgidus*, *Methanocaldococcus jannaschii*, *Methanothermobacter autotrophicus*, *Methanopyrus kandleri*, *Methanosarcina acetivorans*, *Pyrococcus abyssi*, *Pyrococcus horikoshii*, *Thermoplasma acidophilum*, *Thermoplasma volcanium*, *Halobacterium sp.*, *Aeropyrum pernix*, *Pyrobaculum aerophylum*, *Sulfolobus solfataricus*, *Saccharomyces cerevisiae*, *Encephalitozoon cuniculi*, and *Schizosaccharomyces pombe.* A BlastO query was computed for each the 1,643 *C. jejuni* genes and the number of microorganisms that contain an ortholog (N_o) was recorded. Next, the evolutionary retention index (ERI) was calculated by dividing the N_o of each gene by the total number of microorganisms in the database (equal to 66). An ERI of 1 would correspond to a gene

conserved across the 66 microorganisms, while an ERI of O would indicate a gene unique to *C. jejuni*.

2.2.8. Calculation of the theoretical probability of transposon insertion

Assuming random distribution of transposon insertions in the genome of *C. jejuni*, the probability *P* of detecting an insertion in a given gene is based on the following formula: $P = 1 - (1 - (l/g))^n$, where l is the length of the gene in bp, g the size of *C. jejuni* genome in bp (1,641,481), and n the number of mutants in our library (7,200).

2.3. Results

2.3.1. Library construction

In order to identify genes that are required for the growth of *C. jejuni*, we constructed a library of transposon insertion mutants in *C. jejuni* NCTC 11168. The approach used for the construction of this mutant collection has been previously described and is based on a Tn5 transposon containing a chloramphenicol-resistant cassette (194, 221). Briefly, the mutagenesis strategy comprised the following steps: (1) *in vitro* transposition reactions with purified chromosomal DNA from *C. jejuni* NCTC 11168 (extracted from culture grown to stationary phase in order to prevent preferential transposition near the origin of replication); (2) natural transformation of transposed chromosomal DNA into *C. jejuni* NCTC 11168; (3) selection of transposon mutants on selective MH agar plates at 37°C under microaerophilic conditions; and (4) growth of individual mutants in 96-well microtiter dishes containing MH broth. A total of 7,201 viable mutants were obtained, representing 4.48 x coverage of the entire genome. Forty-eight of these mutants were grown individually and sequenced using transposon nested primers to confirm random insertion around the chromosome.

2.3.2. Identification of the transposon insertion sites

We used a modification of the microarray transposon tracking method (MATT) to identify the transposon insertion sites (205). This method was previously devised to monitor the presence of *H. pylori* transposon mutants in a library using DNA microarray (205). The MATT method relies on the amplification of the transposon flanking regions using a 2-step PCR approach (Figure 2.2). The first PCR step uses a combination of transposon-specific and semi-degenerated primers. The specific primers anneal to sequences within the transposon while the semi-degenerated primers contain a four-nucleotide unique anchor preceded by 10 degenerate nucleotides and a unique tail sequence. The PCR products from this process are then hybridized to a microarray slide, allowing for the identification of the PCR amplified regions neighbouring transposon insertion sites.

2.3.3. Identification of essential gene candidates

In total, we analyzed 7,201 transposon mutants to determine the location of the transposon insert. Essential genes were identified as genes that cannot tolerate transposon insertion, corresponding to genuine essential genes or genes that upon mutation affect the expression of a distal gene whose product is essential. A total of 283 genes were determined as potential essential genes using our cutoff. Next, the obtained catalog of essential gene candidates was cross-referenced to a list of known dispensable genes in *C. jejuni* (obtained through literature searches, unpublished mutant data from Stintzi, and a list of 255 mutants from the *Campylobacter* Resource Facility at the London School of Hygiene and Tropical Medicine (http://crf.lshtm.ac.uk/). This additional filtering step removed approximately 1/3 of the previously identified essential gene candidates, indicating a relatively high rate of false positives and yielding a final list of 195 essential gene candidates.

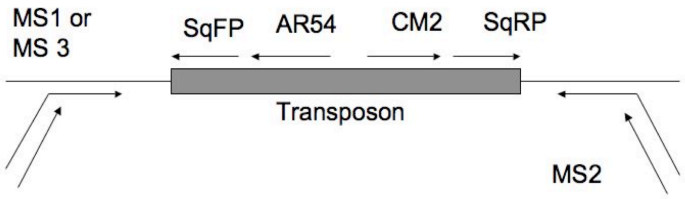

Figure 2.2: **Schematic diagram of the semi-degenerated PCR strategy**. Nested primers AR54 or CM2 and the degenerate primers MS1 or MS3 are used for the first PCR round. The resulting amplicon is purified and used as a template for the second PCR amplification using the nested primers SqFP or SqRP and the primer specific to the tail sequence, MS2.

Analysis of the relative abundance of genes as a function of gene length revealed an over-representation of essential gene candidates with a length less than 500 bp. The probability of transposon insertion within any individual gene increases with the gene length and therefore transposon insertion is more likely to occur in longer genes than shorter genes. In fact, a gene shorter than 500 bp will have less than 90% probability of being disrupted by transposon insertion. Our list of essential gene candidates contains 45.6% of genes with a length less than 500 bp as compared to 23% for all the predicted open readings frames of *C. jejuni*. Assuming a similar distribution of gene length for essential and dispensable genes, approximately half of the short genes (44 genes) are not mutated because of their small size rather than because they are essential. Taking the number of likely false positives genes into account, we estimated the true number of essential genes in *C. jejuni* to be between 150 and 195.

The genes that were identified as essential gene candidates were grouped into functional classes based on the second annotation of the genome of *C. jejuni* NCTC 11168 (81) (Appendix II), and updated with the most recent gene annotations available from genbank (http://www.ncbi.nlm.nih.gov/genbank/). Most of the essential genes are involved in core metabolic pathways, macromolecule metabolism or cellular processes, including energy metabolism (9 genes), amino-acid biosynthesis (6 genes), nucleic acid biosynthesis (4 genes), cofactors biosynthesis (12 genes), macromolecule synthesis and modification (30 genes), cell envelope (45 genes), and cell processes (19 genes). Interestingly, 49 genes encode conserved hypothetical proteins or unknown proteins.

2.3.4. Validation of predicted essential and non-essential genes

In order to validate our list of essential genes, we assessed by PCR the presence or absence of a transposon insertion within 4 predicted essential genes, *dnaA, dnaN, miaA* and *pdxA* and 11 predicted dispensable genes, *Cj0025, Cj1257, ftsZ, cdtA, rplE, eno, nrdA, waaV, ftsA, dnaG* and *dnaE*. The PCR was performed using the combined pools of genomic DNA or the pools that were predicted to contain an insertion in the gene tested (in case of dispensable gene) and using primers annealing at the 5' and 3' ends of the gene and a transposon specific primer. No amplification product should be seen for a gene lacking an insertion, while one PCR band should be obtained for every transposon insertion into any given gene. As figure 2.3 illustrates for 3 of the genes, *dnaN* shows no appreciable amplification, *ftsZ* indicates a single transposon insertion within the gene, and *eno* shows multiple transposon insertions within the gene and possibly in neighboring genes as well. In summary, all eleven of the predicted dispensable genes produced one or more PCR products indicating in some cases numerous transposon insertions within the same gene. While no PCR product was obtained for the three predicted essential genes *dnaA, dnaN, and miaA* in agreement with their essentiality, one PCR product was obtained with *pdxA,* suggesting that this gene might be incorrectly classified as essential. Overall, 14 out of 15 genes were confirmed by PCR (3/4 predicted essential genes and 11/11 non-essential genes).

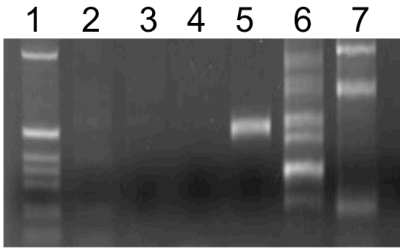

Figure 2.3: Validation of the presence or absence of transposon insertions within the genes *dnaN*, *ftsZ*, and *eno* by PCR analysis. A gene-specific forward or reverse primer was used in combination with the transposon specific primer SqFP to detect the presence of insertions by PCR using as a PCR template the pool DNA extracted from the mutant library. Lane 1 shows the 1 kb DNA ladder. Lanes 2 and 3 indicate the absence of a PCR product with primers SqFP and *dnaN*-forward and primers SqFP and *dnaN*-reverse, respectively. Lane 4 shows the absence of a PCR product with primers SqFP and *ftsZ*-forward. Lane 5 shows a PCR product with primers SqFP and *ftsZ*-reverse, indicating a single transposon insertion. Lanes 6 and 7 shows the amplification of multiple PCR products with primers SqFP and *eno*-forward and primers SqFP and *eno-reverse*, respectively.

2.3.5. Chromosomal distribution of the dispensable and candidate essential genes

The distribution of the identified *C. jejuni* essential genes is depicted along the *C. jejuni* chromosome in figure 2.4. In contrast to other bacteria for which the essential genes were found to be uniformly distributed (70, 72, 133, 209), 3 main regions of *C. jejuni* genome appear to lack essential genes and constitute relatively large dispensable regions (DSP) of 83 kb, 47 kb, and 164 kb for DSP1, DSP2, and DSP3 respectively. Interestingly, the DSP2 and DSP3 regions correspond to two of the largest hypervariable plasticity regions of the *C. jejuni* genome (49, 67). These particular regions contain genes involved in the production and modification of surface structures, including lipooligosaccharides and extracellular polysaccharides (67, 179). The absence of essential genes within hypervariable regions validates our results, as essential genes would be expected to be conserved among various isolates of the same species of *C. jejuni*. However, as the essential genes were compared across bacterial species, it was found that while the predicted essential genes in *C. jejuni* are highly conserved within the species, there is little conservation across the bacterial kingdom compared to dispensable genes (221).

Figure 2.4: Chromosomal mapping of *C. jejuni* essential genes

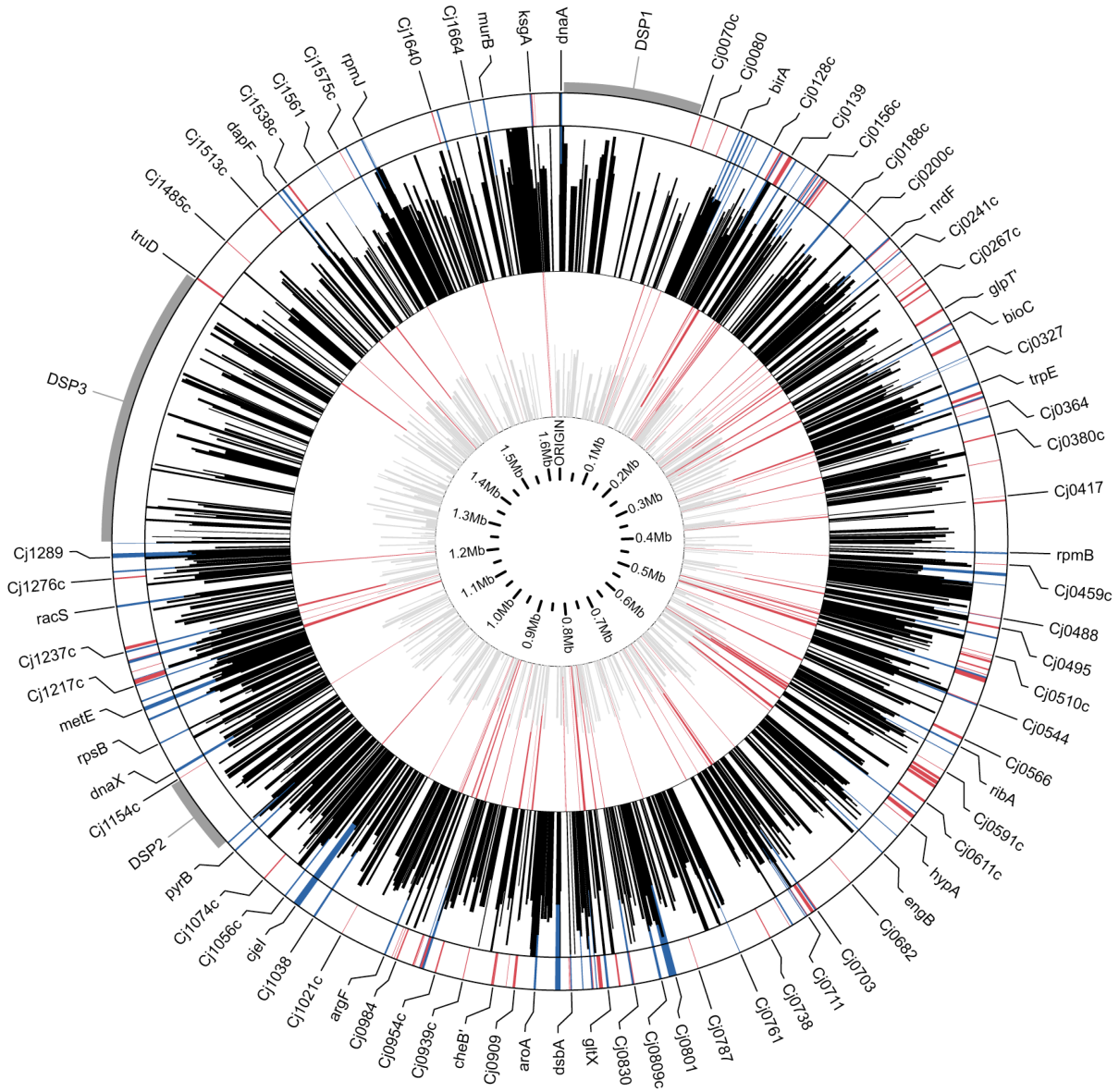

Figure 2.4: Chromosomal mapping of *C. jejuni* essential genes. Genes found to be essential were mapped to the *C. jejuni* NCTC11168 genome. The outer track denotes the genomic location of each gene identified to be essential along with the three dispensable regions (DSP) identified in this study. The two inner tracks represent the ERI value of each gene within the *C. jejuni* NCTC11168 genome. The ERI values are represented as histograms with genes containing an ERI >0.5 denoted in black on the middle track and genes with an ERI<0.5 denoted in grey on the inner-most track. Essential genes are highlighted in either blue or red for ERI values >0.5 or <0.5 respectively. The origin of replication is located at 12 o'clock. Figure was created by James Butcher (221) using Circos v0.52 (132).

2.3.6. Evolutionary conservation of essential genes

In a previous report, essential *E. coli* genes have been shown to be preserved across the bacterial kingdom (72). In order to similarly evaluate the evolutionary conservation of *C. jejuni* essential genes, we analyzed the occurrence of orthologs of *C. jejuni* essential and dispensable genes within 66 complete genomes of phylogenetically diverse microorganisms and computed the evolutionary retention index (ERI) for each *C. jejuni* gene as described by others (72) (see the Materials and Methods section). The ERI of a gene corresponds to the number of microorganisms carrying a putative ortholog of this gene divided by 66, the total number of microorganisms queried. An ERI value of 1 indicates a gene conserved across the 66 microorganisms, while a value of 0 indicates a gene unique to *C. jejuni*. Figure 2.4 represents the distribution of essential and dispensable genes and their ERIs along the *C. jejuni* chromosomes. In contrast to the study in *E. coli* (72), *C. jejuni* gene essentiality was not found to positively correlate to the ERI values. This result indicates that *C. jejuni* essential genes are not preferentially conserved across the bacterial kingdom as compared to dispensable genes.

2.3.7. Comparison with the essential gene pools of other bacteria

The list of essential genes has been previously determined for 13 bacterial species, namely *Acinetobacter baylyi* (43), *Escherichia coli* (11, 72, 211), *Helicobacter pylori* (205), *Francisella novicida* (70, 130), *Haemophilus influenzae* (3), *Mycobacterium tuberculosis* (133, 209), *Bacillus subtilis* (125), *Pseudomonas aeruginosa* (146), *Mycoplasma genitalium* (75), *Mycoplasma pulmonis* (65), *Staphylococcus aureus* (62, 108), *Streptococcus pneumoniae* (220, 230), and *Salmonella typhimurium* (124). Essential genes encode proteins indispensable to sustain life and therefore their functions would be expected to be conserved

across bacterial species with similar growth requirements. To identify the set of essential genes shared by *C. jejuni* and other bacterial species, we searched for homologous essential genes in the catalog of essential genes for each of the 13 bacterial species using the BLAST tool in the database of essential genes (with the following criteria of significance: BLAST E score $\leq 10^{-4}$ and amino-acid identity $\geq$ 20% over 80% of the sequence length). It should be noted that the conservation of an essential gene across bacterial species could be misleading and should be taken with caution, as the list of essential genes in any given organism is dependent on the methodology and the growth medium used to identify the essential genes. Of the 195 genes in our list, 51 were found to be essential in *A. baylyi*, 50 in *E. coli*, 44 in *H. pylori*, 41 in *F. novicida* and *H. influenzae*, 37 in *M. tuberculosis*, 33 in *B. subtilis* and *P. aeruginosa*, 31 in *M. genitalium*, 27 in *M. pulmonis*, 23 in *S. aureus*, 15 in *S. pneumoniae*, and 14 in *S. typhymurium*. Overall, more than half of the genes predicted to be essential in *C. jejuni* (99 out of 195 candidate essential genes) were also found to be essential in at least one of the 13 bacterial species queried (Figure 2.5). Up to 25% of *C. jejuni* essential genes are conserved in more than 4 bacterial species. A large majority of these genes encode proteins involved in the biosynthesis of cofactors, such biotin (BioC and BirA), thioredoxin (TrxA), heme (HemC and HemH), folic acid (PabA and FolD), menaquinone (IspA) and riboflavin (RibA), proteins involved in ribosomal protein synthesis and modification (RpmG, Cj1280, RpmF, RplS, RpmB, RpmH, RpmJ, RpmI, RpsO, RplT, and RpsB), aminoacyl tRNA synthetases (MiaA, GlyQ, CysS and GltX), proteins in fatty acid biosynthesis (AccB, AccC, and AcpP2), and proteins involved in DNA replication (DnaA, DnaX, and Cj0722c). Genes with unknown function were mainly found to have essential homolog genes in *H. pylori*, which is the closest relative to *C. jejuni*. Most of the essential genes found to be unique to *C. jejuni* encode membrane proteins and hypothetical proteins.

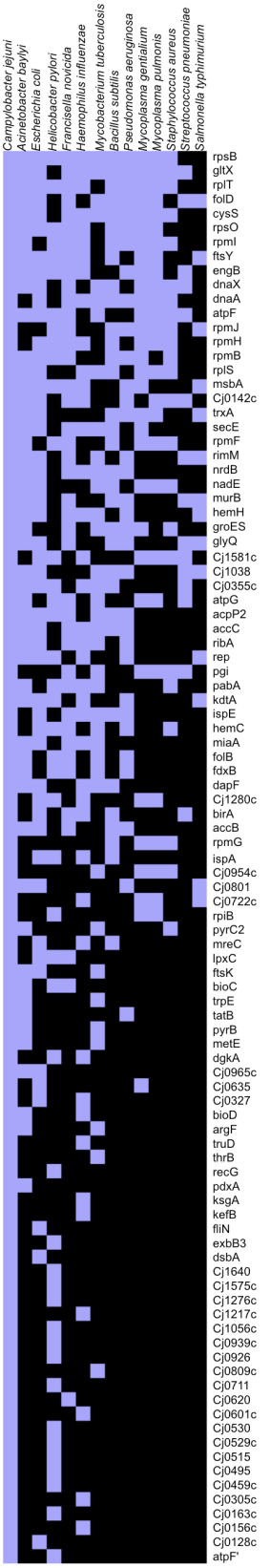

Figure 2.5: Conservation of *C. jejuni* essential genes other bacteria. Only the genes that were reported to bebe essential in at least one other bacterium are listed. blue color indicates the presence of a homologous essential gene in the given bacterium. A black color indicates that the corresponding homologous gene was reported as dispensable or absent in the given bacterium.

2.4. Discussion

We created a large collection of transposon insertion mutants in *C. jejuni* NCTC 11168, consisting of 7,201 individual mutants and mapped the locations of the transposon insertion sites using a DNA microarray-based approach previously described by others (205). Essential gene candidates were identified as genes that could not tolerate transposon disruption without a lethal effect. A total of 283 genes were initially identified as potential essential genes. However, due to a high false positive rate, the true number of essential genes in *C. jejuni* was estimated to be between 150 and 195. This high false positive rate was deemed to result from two main factors. First, the use of a DNA microarray constructed using PCR products may fail to identify transposon insertion sites directly adjacent to the probe on the microarray. Second, the probability of transposon insertion into genes shorter than 500 bp was found to be below 90% (221), thus resulting in an over-representation of short genes in our list of essential gene candidates. We experimentally confirmed our predictions for 14 genes and thirteen of them were found to be absent as expected, thereby validating our experimental approach. Moreover, two of the identified essential gene candidates were previously known to be essential in *C. jejuni*, *cosR* (Cj0355c) and *ribB*. *CosR* encodes a two-component regulator, which exhibits an amino-acid identity of 60% with an essential gene of *H. pylori*, HP1043 (170). The role of CosR has been linked to the regulation of the oxidative stress response in *C. jejuni* to paraquat (71) and the regulation key oxidative stress response genes including *ahpC* and *sodB* (101). Previous work has confirmed this gene to be essential for the growth of *C. jejuni* (71). The *ribB* gene (Cj0572) codes for a protein involved in riboflavin biosynthesis and the construction of an isogenic *ribB* mutant has been previously shown to be dependent on the presence of exogenous riboflavin (39). Therefore, a *ribB* mutant would not be obtained under the growth conditions

used in our study. To further validate our data, we also compared our list of essential gene candidates to similar lists from other bacterial species, finding that over half were also found to be essential in at least one other species. This data clearly shows that the essential gene set varies between species, especially between more distantly related organisms, suggesting that bioinformatic approaches may be of limited usefulness in evaluating the essential genes of an organism.

The 195 essential gene candidates could be grouped into 5 main functional categories: small molecule metabolism, macromolecule metabolism, cell processes, broad regulatory function, and proteins of unknown function. The small molecule metabolism genes encode proteins involved in energy metabolism, central intermediary metabolism, and the biosynthesis of amino acids, nucleosides, nucleotides, cofactors, prosthetic groups, and fatty acids. Most of these genes are associated with the basic cellular functions and were also found to be essential in other bacterial species (73). The identity of these genes provides information on the metabolic requirement of *C. jejuni* while growing on MH medium under microaerophilic conditions at 37°C. In particular, the biosynthesis of riboflavin, biotin, folic acid, thioredoxin, heme, pyridoxine, and menaquinone appeared to be required for *C. jejuni* growth on MH medium. As expected, many genes encoding ribosomal proteins, tRNA synthetases, and enzymes of DNA replication were found to be essential. For example, the essential DNA replication genes include *dnaX* (DNA polymerase III), *recG* (helicase), *dnaA* (replication initiator protein), *cjeI* (restriction modification enzyme), *cj0722c* (putative DNA methylase), and *cj0139* (putative endonuclease). However, it is important to note that several genes known to encode key enzymes of DNA replication were found to be dispensable, such as *polA* (DNA polymerase I), *ligA* (DNA ligase), and *dnaG* (DNA primase). This observation may indicate essential genes missed by our study.

Along with these genes found to be non-essential in *C. jejuni,* we identified a number of genes associated with metabolic functions. These included genes involved in the glycolysis and gluconeogenesis pathways, the pentose phosphate pathway, and the citric acid cycle and some parts of the electron transport chain. For example, the phosphopyruvate hydratase gene (*eno*) and the transketolase gene (*tkt*), which are essential in many microorganisms, were found to be dispensable. This is not surprising considering that these glycolytic pathways are known to not be functional in *C. jejuni* (240). The lack of essential genes in major metabolic pathways also suggests that multiple sources of nutrients metabolized through a variety of pathways are likely to be available in the nutrient rich MH growth medium, making any individual metabolic pathways non-essential. The essential metabolic genes were found only at key points within *C. jejuni* metabolism where alternate enzymes would not be available. These include subunits of ATP synthase (AtpGFF') and ferredoxin (FdxB) to name a few, both necessary for the proper functioning of the *C. jejuni* electron transport chain.

More significant to the nutrient requirements for *C. jejuni* are the biosynthetic pathways containing identified essential genes. These include the synthesis of biotin (BirA, BioCD), folate (FolBD), riboflavin (RibA), and fatty acids (AccBC, AcpP2); indicating that *C. jejuni* is incapable of acquiring these types of molecules from the medium while growing in MH broth. Also interesting to note is the essential nature of the genes *pgi, rpiB* and *rep*. These genes are isomerases and epimerases necessary for the interconversion between D-xylulose-5P, D-ribulose-5P and D-ribose-5P (*rep* and *rpiB* respectively); and D-fructose-6P, and α and βD-glucose-6P (*pgi*). Since *C. jejuni* does not catabolize these sugars for energy as other bacteria do, their essential role may be more closely linked to the biosynthesis of sugar phosphates for the construction of larger glycans for glycoproteins and the capsule.

A second study of the essential genes of *C. jejuni* was published by Metris *et al.* in November, 2011 (165). This study employed a dual approach using bioinformatic analyses to identify essential genes based on "Flux balance analysis" (FBA), complemented with a MATT approach, similar to the method described in this study. Their *in silico* and *in vitro* approaches identified 176 and 233 essential genes respectively, however, only 43 genes were common between the two methods. Equally, although our *in vitro* transposition method found 195 essential genes, we identified only 34 genes in common with the Metris et al. transposition study, and 24 with their in bioinformatic study (165). The substantial differences between the two transposition studies likely represent a certain degree of error inherent with the MATT approach as well as different growth conditions used, underlining the dependence of genes essentiality on the precise growth conditions. Their transposon mutants were grown using blood agar base no. 2 (165), a medium substantially different from the MH medium used in our study. It equally highlights the unreliability of bioinformatic approaches to gene essentiality, as neither transposon study significantly matched the results predicted by the flux balance analysis.

Despite these differences, the points of overlap between the three lists of essential genes are of substantial interest. Eight genes were indentified by all three methods, strongly supporting the conclusion that these genes are essential, even under disparate growth conditions. These are *nrdF* (*cj0231c), hemC (cj0545*), *kdtA (cj0707*), *nadE (cj0810*), *folD (cj0855*), *aroA (cj0895c), ispA* (*cj1644*), and *murB (cj1676)*. While these genes are each from different pathways, they are all involved in the biosynthesis of key molecules including: ribonucleotides, heme, capsule polysaccharides, nicotinamide adenine dinucleotide, folate, aromatic amino acids, lipids, and peptidoglycan. As Metris et al. (2011) (165) demonstrated in their study using phenylalanine and tyrosine biosynthesis as an

example, when the results from all three approaches are combined, nearly complete pathways are assembled, supporting our conclusions regarding the essential nature of several of the above mentioned biosynthetic pathways.

Together, these results also paint a conflicting view of *C. jejuni*. While many common biosynthetic pathways were identified to be essential, as would be expected, many genes commonly identified as essential in other bacteria were not identified in this study. Some margin of error is present within this type of study due to the difficulty of creating a sufficiently large transposon mutant library to include very short genes and the possibility of erroneously including or excluding some genes during the mapping process. However, many of the differences in the essential genes of *C. jejuni* and other organisms likely quite simply lies in the many differences that exist between the metabolism and basic cell function between *C. jejuni* and many of the other commonly studied bacteria. As the growth environment of a bacterium changes (with *C. jejuni* becoming adapted to its niche within the low oxygen, highly competitive environment of the gut) the genes necessary for growth would be subject to substantial change compared to bacteria adapted to other environments. Even the essentiality of what would be considered many core housekeeping genes may be subject to change, as would be indicated by the results of this study. Overall, these results will help to identify which genes and pathways are key for growth under common laboratory conditions and where further study can help identify the inner workings of *C. jejuni*.

CHAPTER 3

Growth of *C. jejuni* in Extracted Intestinal Mucus

3.1. Introduction

When *C. jejuni* colonizes its host, it establishes itself in the mucus lining of the intestinal tract. In most of the hosts which *C. jejuni* inhabits, colonization is asymptomatic (242). In poultry and other birds, *C. jejuni* will colonize to high levels (10^7 CFU/gram of cecal contents or higher) (242), and will generally remain in the intestinal tract for the bird's lifetime (149). In numerous other hosts, including the mice commonly used in research, colonization is generally transient, with high levels of colonization for a relatively brief period of time, followed by complete clearance (25, 107). Although immunocompromised mice, or mice with an altered intestinal microbiota may exhibit symptoms as a result of colonization by *C. jejuni*, a healthy animal will generally be asymptomatic (32, 160, 247). Humans remain one of the few animals that display the symptoms of gastroenteritis in response to *C. jejuni* infection. These symptoms vary significantly between individuals, range from very mild to severe, and generally include abdominal pain, diarrhea, which may be bloody, and fever (69, 105). The infection itself commonly clears within a few days or weeks, with *C. jejuni* being completely eradicated from the GI tract, but post-infection complications such as Guillain-Barré syndrome are also known to occur on rare occasions (69).

A lack of models that accurately reflect the symptoms of human colonization and infection by *C. jejuni* has long hampered researcher advances. Young ferrets and non-human primates have been successfully used to replicate the disease (19, 104), but most researchers

lack access and the necessary facilities to work with these animals. Mice do not normally exhibit symptoms from *C. jejuni* colonization (25, 107), however immunodeficient mice such as MyD88 (247) or IL-10 (160) knockouts have been used, but the relevancy of studying colonization in such an immunologically-impaired model can be questionable, since the bacteria will not be exposed to a normal immune response. Altering the intestinal microbiota of mice has also been explored as a means to increase mouse susceptibility to *C. jejuni* infection. Germ free mice or mice with limited enteric biota have been found to be susceptible to *C. jejuni* infection (21, 32). Additionally, a study by Bereswill *et al.* found that if the natural mouse microbiota is eradicated, and replaced with a complete human microbiota, these "humanized" mice display significantly increased susceptibility to *C. jejuni* (21), although, the robustness of this model still remains to be demonstrated.

Likewise, a rabbit ileal loop model has been used with some success (52, 225). In this model, *C. jejuni* is injected directly into the rabbit ileum, and has been shown to replicate the histopathological lesions associated with human infection (225). However, since this model bypasses the normal entry process into the host, there are limitations to the use of this model as well. Recent work with a *Galleria melonella* model (31) has also been conducted, but the vast differences between human and insect biology limit the applicability of the insect model. Colostrum-deprived, neonatal piglets when infected with *C. jejuni* exhibit many of the symptoms of human infection (12, 172) and constitute the primary animal model of *Campylobacter* pathogenesis used in the study. Commensal colonization has been widely studied using newly hatched chicks (250). In chicks, *C. jejuni* does not generally elicit any substantial immune response, nor does it cause any symptoms of gastroenteritis (250).

In both commensal and pathogenic models, along with human infections, relatively small numbers of *C. jejuni* are sufficient to successfully infect and colonize the intestinal

tract (198). *C. jejuni* will embed itself in the mucus lining of the intestine where it will proliferate to high numbers. In the infection models, *C. jejuni* will become closely associated with the epithelial cell layer and will often translocate into the surrounding tissues (237, 238). Although the links between cell invasion and *C. jejuni* are not entirely clear (128), researchers have often used cell invasion assays as a means to gauge *C. jejuni* pathogenesis as more invasive species have been considered to be more pathogenic in humans (127).

Another approach to model *in vivo* conditions has been recently established using *ex vivo* tissues. This model was successfully developed by Byrne *et al*. to study cell invasion of intestinal epithelial tissues by *C. jejuni* in the presence of either chicken or human intestinal mucus (29). This study led to the intriguing finding that layering epithelial cells with chicken mucus inhibited *C. jejuni* cell invasion, while significantly more cell invasion took place in the presence of human intestinal mucus. This occurred regardless of the origin of the epithelial cells, and *C. jejuni* proved perfectly capable of invading chicken cells as long as chick intestinal mucus was absent (29). This discovery leads to the hypothesis that the mucus layer of the intestine plays a significant role in determining whether pathogenic, or commensal colonization occurs. Here we attempt to characterize the nature of *C. jejuni'*s growth in the mucus layer, in both the piglet model for human colonization and the chick commensal colonization model.

3.2. Materials and Methods

3.2.1. Strains used and growth media

C. jejuni strains NCTC 11168, sequenced by the Sanger Centre in 2000 (180); 81-176, an isolate from an outbreak of *Campylobacter* in raw milk (94); and RM1221, an isolate

recovered from a chicken carcass (179); were used in this study. Routine growth of *C. jejuni* was carried out on Mueller-Hinton (MH) agar plates, MH biphasic flasks, or MH liquid cultures. Cultures were incubated at 37°C in MACS-VA500 microaerophilic incubators with a gas concentration of 8% O_2, 4% H_2, 5% CO_2, and 83% N_2.

3.2.2. Mucus extraction and growth assay

Mucus samples were extracted as described previously (29, 166, 238). Briefly, piglet or chick cecal/intestinal segments were cut longitudinally to expose the inner lumen. Luminal contents were washed away with 0.9% sodium chloride or removed with tweezers. The mucus layer was scraped from the epithelium with a scalpel and collected in microcentrifuge tubes. The sample was emulsified with 15% v/v PBS buffer and separated from the debris by two, 30-minute centrifugations at 18,000 x g. The resulting mucus was collected, sterilized under UV light and stored at -20°C. A growth medium was prepared from each mucus sample by pooling the mucus from at least three animals, measuring the protein content using a Bradford assay, and diluting each sample in PBS buffer to 2 mg of protein content/ml.

3.2.3. Determining the iron content of mucus/mucin

The iron content of the porcine gastric mucin (Sigma) as well as the chick and piglet mucus samples was determined using desferrioxamine (DFO) to chelate iron from the mucus and mucin samples. A standard curve was prepared using predetermined concentrations of $FeSO_4$ in dH_2O (from 10 μM to 10 mM). One, 2, 5, 10, and 20 mg/ml concentrations of mucin in dH_2O were prepared for testing. To each standard and sample concentration was

added 40 μM DFO. Each mixture was vortexed thoroughly and incubated at room temperature for 30 minutes. Due to the turbidity of the mucus/mucin solutions, the undissolved material was removed by centrifuging at 18,000 x g for 5min. The iron concentration was determined by measuring the absorbance of iron bound DFO at 425nm of each sample and standard as described in (53). The curve of the standards was used to calculate the concentration of iron per mg/ml of added mucin or mucus samples.

3.2.4. Cell invasion/adherence assays

Caco-2 cells were maintained in $25cm^3$, tissue culture flasks, and grown with MEMα medium containing glutamine and 10% fetal bovine serum and incubated at 37°C and 5% CO_2. Cells were inoculated into 24-well hanging insert pates with 1μm PET membranes (Millipore). These cultures were allowed to grow for 3 days, with fresh media being added each day. At the end of 3 days, the cell monolayer was inspected by microscope to confirm the presence of a solid cellular monolayer in the well. Before adding an inoculum to each well, the media was drained, and 100 μl of the 2 mg/ml piglet or chick intestinal mucus medium was added. Control wells contained no added mucus. On top of the mucus layer, and into each well below the hanging insert was added MEMα medium with 10% FBS. An inoculum of approximately 10^6 cells from overnight cultures of *C. jejuni* 81-176 or NCTC11168 were added to each well, containing approximately 10^5 Caco-2 cells, and were incubated for 3 hours at 37°C and 5% CO_2.

Following incubation, samples from each well and receiver well, were serially diluted and plated to count the relative number of bacteria in the original well and those that translocated to the receiver well. The cells were then washed with Hanks buffered saline

solution (HBSS) and incubated for 1 hour with fresh media containing 250 μg/ml gentamicin. Finally, the cells were washed a second time, resuspended in HBSS buffer with 0.1% tween 20, serially diluted and plated to determine the relative number of internalized cells.

3.2.5. Mucus/mucin growth curves

Growth on the extracted mucus medium was measured for 12 hours under microaerophilic conditions by inoculating 2ml of the selected medium with approximately 10^7 CFU/ml of *C. jejuni* NCTC11168. Ten μl samples were taken every hour, serially diluted, and plated for colony counting.

MEMα medium (Gibco 41061) containing glutamine, but no phenol red, was supplemented with 20 μM $FeSO_4$ and 1 or 2 mg/ml of UV-sterilized porcine gastric mucin (Sigma-Aldrich). Control medium was prepared with $FeSO_4$ but no mucin. The *C. jejuni* strains NCTC11168, 81-176 and RM1221 were grown overnight in MH biphasic cultures. These cultures were used to inoculate the growth medium at an OD_{600} of 0.01. Aliquots of these suspensions (300 μl) were dispensed into honeycomb 100 well plates designed for use with a Bioscreen C incubator/plate reader. Each growth condition was replicated in 10 wells each, as were blanks containing only MEMα medium. The plates were incubated for 1 hour, at 37°C in a MACS-VA500 workstation, under microaerobic conditions, and were sealed shut with airtight polyethylene sealing tape (Micronova mfg.). The plates were then placed in a Bioscreen C plate reader and incubated at 37°C with moderate, continuous shaking for 36 hours with wideband (420 nm-580 nm) O.D. readings every 15 minutes. The results were displayed as the maximum O.D. obtained during 36 hours of growth, normalized against the

background O.D. of the medium. Statistical significance was determined using Students *t*-test with a p value ≤ 0.05.

3.2.6. RNA extraction

RNA was extracted from *C. jejuni* NCTC 11168 grown into several media; media consisting of 2mg/ml chick cecal, chick small intestinal and porcine small intestinal mucus; as well as MH medium, UV-sterilized MEMα medium containing 2 mg/ml porcine gastric mucin (Sigma) and MEMα medium containing 20 mM sodium pyruvate and 20 μM $FeSO_4$. Cultures were grown to an O.D. at 600 nm of 0.2, representing mid-log phase, at which point the RNA quality was preserved by adding a 1/1 volume of RNAlater (Ambion) to the culture. The cultures were pelleted by centrifugation at 6,000 x g at 4°C for 15 minutes. The pellets were resuspended and centrifuged again in 50% RNAlater/PBS buffer. Total RNA was extracted using a hot-phenol extraction method as described previously (175, 176, 193). Briefly, pellets were resuspended and lysed in 2ml of denaturing buffer (4M guanidium thiocyanate, 25mM sodium citrate, 0.5% N-laurylsarcosine, and 1% N-acetyl cysteine, 0.1M 2-mercaptoethanol), and divided into 500μl aliquots, to which was added 4μl of 1M sodium acetate (pH 5.2). Five hundred μl of buffer saturated phenol (pH 4.3) was added to each sample, and incubated at 64°C for 10 minutes, with frequent mixing. The phenol was separated from the solution by the addition of 1ml of chloroform to the solution, followed by a 15 minutes incubation on ice, and a 18,000 x g centrifugation, at 4°C for 30 minutes. RNA was precipitated from the aqueous layer with 1/10 volume 3M sodium acetate, 500mM DEPC treated EDTA and 2 volumes of cold ethanol and incubated at -80°C overnight. Next, the RNA was pelleted at 4°C by centrifugation, washed 4 times with 80% ethanol and

resuspended in 100µl of RNAse free dH_2O. Genomic DNA was removed by two Dnase I (Epicentre) treatments, and the RNA was cleaned using RNeasy columns (Qiagen). The absence of genomic DNA was confirmed by PCR and final RNA quality and quantity was ascertained using the BioRad's Experion StandardSensRNA system following the manufacturer's protocols.

3.2.7. Microarray hybridization and analysis

The *C. jejuni* NCTC 11168 microarray was constructed as described elsewhere (175, 224), and covers approximately 98% of the ORFs of the genome. Microarray slides and labeled cDNA probes were prepared and hybridized as described previously (175, 176, 193). Briefly, 10µg of total RNA from each sample was reverse transcribed to cDNA using superscript II (Invitrogen) and a dNTP mixture of 0.5 mM each of dGTP, ATP, and CTP; 0.16 mM dTTP; and 0.34 mM aminoallyl-dUTP. Free amines and unincorporated aminoallyl-dUTP were removed by centrifugation in Microcon YM-30 filters (Millipore). cDNA from the control cultures were then labeled with indocarbocyanine [Cy3], and experimental samples were labeled with indodicarbocyanine [Cy5]. The samples were purified using PCR purification columns. The arrays were prehybridized with 25% formamide, 5× SSC buffer, 0.1% SDS, and 1% bovine serum albumin and incubated for 45 minutes at 42°C. The fluor-labeled cDNA mix was dried under vacuum with a SpeedVac and resuspended in 15.14 µl of water, to which the following was added: 2.5 µl of salmon sperm DNA (10 mg/ml), 9 µl of 20× SSC (1× 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. The probes were denatured for 2 minutes at 99°C, and applied to the microarray slide underneath a coverslip. The slides were placed in a humidified chamber (ArrayIt), and incubated in the dark

overnight at 42°C. Following hybridization, the slides were washed in 2× SSC-0.1% SDS for 5 min at 42°C, 0.1× SSC-0.1% SDS for 10 min at room temperature, and four times in 0.1× SSC for 1 min at room temperature. Slides were then rinsed with distilled water and dried by centrifugation.

The arrays were scanned with a laser-activated confocal scanner (ScanArray Gx, Perkin Elmer) at a resolution of 10μm. The signal intensities of each spot were collected using ScanArray software. Spots were inspected individually and those exhibiting hybridization anomalies were excluded from the analysis. The raw median fluorescence intensity values were background subtracted, and spots with background-subtracted values less than three times the standard deviation of the local background in both channels were also excluded from further analysis. Subsequently, the background-subtracted fluorescent intensity in each wavelength (channel 1 for Cy3 and channel 2 for Cy5) was normalized using the MIDAS software (available from TIGR, http://www.tigr.org/software/) and by applying a locally weighted linear regression (Lowess), as previously described (175). Microarray data was collected from three independent biological replicates and three technical replicates using the RNA extracted from each biological replicate for a total of nine microarray slides for each condition. Each slide contained triplicates of each probe, yielding a total of 27 replicate spots for analysis. Finally, the ratio of channel 2 to channel 1 was converted to $\log_2$, and the data were statistically analyzed using the empirical Bayes method, as previously described (175).

3.3. Results

3.3.1. *Campylobacter jejuni* grows on extracted intestinal mucus *in vitro*

A previous study suggested that *C. jejuni* is capable of efficient growth on a medium composed of extracted intestinal mucus (238). This study observed efficient *C. jejuni* growth in a chick intestinal mucus medium. To validate this observation and to assess if this conclusion also stands with mucus extracted from porcine intestine, we prepared a medium consisting of 2mg protein of intestinal mucus, suspended in 1 ml of sterile PBS buffer. These mucus samples were extracted from porcine small intestine, chick small intestine and chick cecum. Cultures of *C. jejuni* NCTC11168 grown under microaerophilic conditions, in 1ml aliquots of this mucus-based medium, attained a maximum density of 10^9-10^{10} CFUs/ml over a 48-hour growth period, similar to a comparable culture in MH broth (Figure 3.1).

3.3.2. *C. jejuni* exhibits increased growth in minimal essential medium supplemented with porcine gastric mucin.

While *C. jejuni* is capable of growing efficiently in extracted mucus as a whole, it is less clear which nutrients it is able to utilize within this complex medium. One of the primary constituents of intestinal mucus are mucin glycoproteins, therefore, we examined *C. jejuni's* ability to grow in a medium supplemented with porcine gastric mucin (Sigma). One and two mg/ml porcine gastric mucin was suspended in MEMα medium and used as a growth medium. MEMα medium contains few carbon sources for *C. jejuni*; however, it contains many of the essential micronutrients (metals, cofactors, etc), not otherwise provided by solely adding mucin as a carbon source. The addition of porcine gastric mucin may

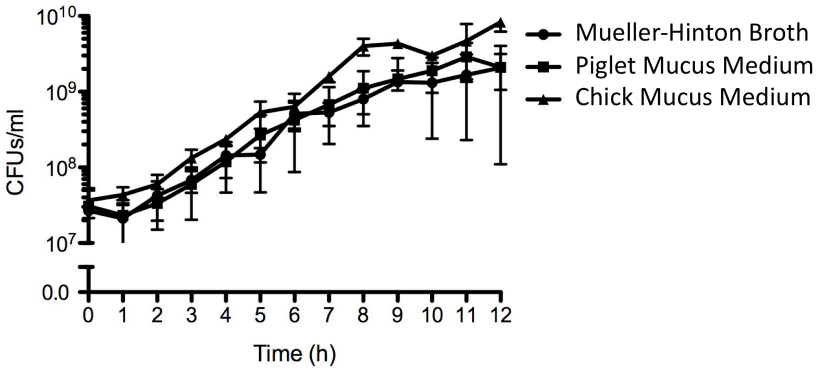

Figure 3.1: Growth of *C. jejuni* NCTC11168 in extracted intestinal mucus medium. *C. jejuni* NCTC11168 in media comprised of 2mg protein/ml of extracted intestinal mucus suspended in PBS buffer. The intestinal mucus was extracted from piglet small intestinal samples, and chicken small intestinal samples. As a reference, the growth in mucus medium is compared to growth in MH medium. Error bars represent standard deviation, n = 3.

however add significant amounts of iron to the medium, a critical nutrient for *C. jejuni* growth. Indeed, quantification of the iron content indicated that 6.22 nmoles of iron was added along with the addition of 1 mg of mucins to the otherwise iron restricted MEMα medium (1mg mucin/ml). To compensate, 20μM $FeSO_4$ was added to the control MEMα medium, thereby removing iron as a limiting factor for growth. When the *C. jejuni* strains NCTC11168, 81-176 and RM1221, were grown on mucin-supplemented media, a statistically significant, concentration dependent increase in growth was observed for each strain (Figure 3.2).

3.3.3. Intestinal mucus modulates *C. jejuni* cell invasion and tissue translocation *in vitro*

Previous research has suggested that the mucus layer has the potential to modulate cell invasion and tissue translocation by *C. jejuni* (5, 29). These studies found a link between human intestinal mucus and increased cell invasion, while chick mucus had an inhibitory effect. We sought to determine if porcine mucus would exhibit a similar effect as human mucus in relation to cell invasion. Porcine and chick intestinal mucus samples were layered onto monolayers of Caco-2 cells grown on a membrane in a hanging-well insert plate (Figure 3.3), and we compared both cell invasion, and translocation of *C. jejuni* NCTC11168 and 81-176 across the membrane in the presence of either mucus samples. Relative to control samples without any added mucus, the Caco-2 cultures layered with chick mucus exhibited a significant decrease in *Campylobacter* cell invasion and translocation (1.45 to 2.38 fold decrease respectively, with a p value <0.01; Figure 3.4). In contrast, porcine mucus increased *Campylobacter* cell invasion and translocation (1.49 to 1.96 fold increase respectively, with a p value <0.01; Figure 3.4). The exception to this observation was the NCTC11168 strain, which exhibited differential translocation, but little difference in cell invasion. These results

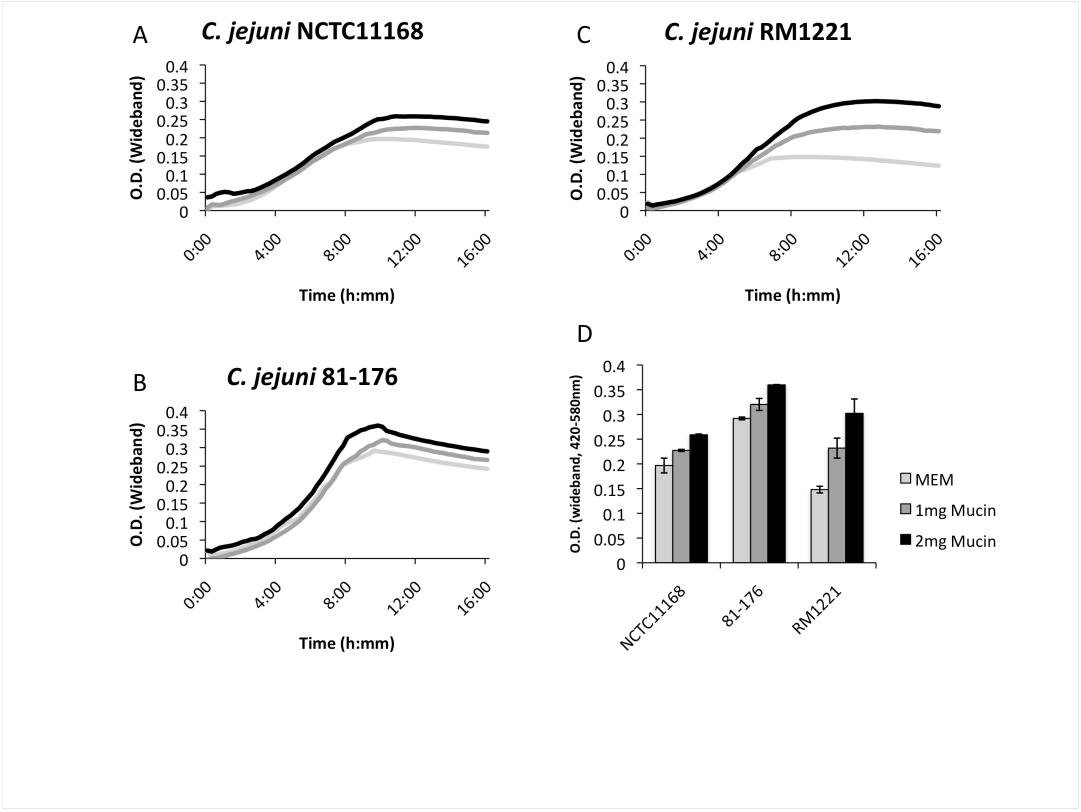

Figure 3.2: Growth of *C. jejuni* in MEMα supplemented with porcine gastric mucin. *C. jejuni* NCTC11168, 81-176 and RM1221 over 16 hours in MEMα supplemented with 20μM $FeSO_4$ and with or without 0, 1, or 2mg/ml porcine gastric mucin (panels A, B and C respectively). Panel D represents the maximum O.D. obtained by each strain in each mucin concentration.

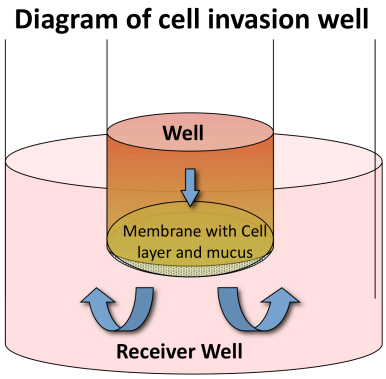

Figure 3.3: Diagram of the hanging well insert model used in the cell invasion and translocation assays. A confluent, polarized cell layer of Caco-2 cells is grown along a hanging membrane insert. An inoculum of *C. jejuni* is added to the well and after three hours, the number of internalized cells is determined via a gentamycin protective assay, and the translocation efficiency through the cell layer is determined by sampling the number of bacteria that pass from the well to the receiver well.

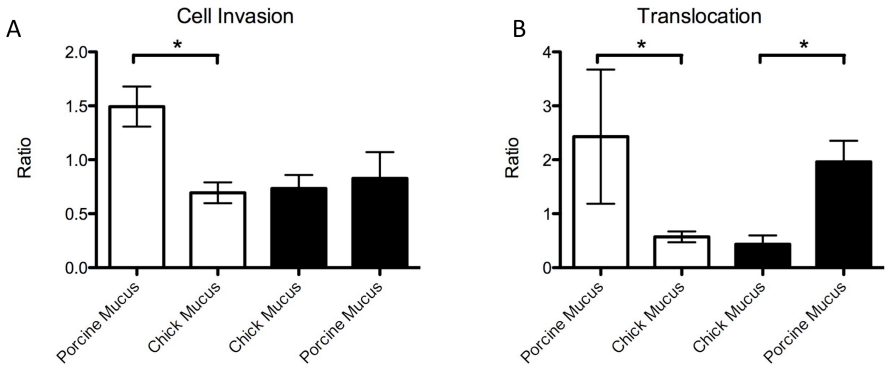

Figure 3.4: **Cell invasion and translocation by *C. jejuni* in the presence of mucus.** (A) Cell invasion as determined by a gentamicin protective assay. The y-axis displays the ratio between the numbers of internalized cells in the mucus-layered sample relative to the number of internalized cells in the non-mucus control wells. White bars denote *C. jejuni* 81-176 and black bars denote *C. jejuni* NCTC11168. (B) Translocation of bacterial cells across the cell layer and membrane into the receiver well. The y values display the ratio of the number of bacteria recovered from the receiver well from the mucus-layered samples relative to the non-mucus controls. *$p<0.01$, $n=4$.

are in agreement with the previous findings using chick anf human mucus samples (29). The similarities in the cell invasion and translocation phenotypes between *C. jejuni* in the presence of human and porcine mucus support our use of piglets as a model for human disease.

3.3.4. Expression profile of *C. jejuni* in mucus medium, and mucin supplemented medium

We determined the gene expression profile of *C. jejuni* NCTC11168 while growing in mucus medium derived from porcine small intestinal samples, chick small intestinal samples and chick cecal samples, relative to a control culture in MH medium. We equally determined the gene expression profile of *C. jejuni* while growing in MEMα medium supplemented with 2mg/ml porcine gastric mucin, relative to control MEMα medium. Overall, using a cutoff of 1.5-fold and $p<10^{-4}$, we observed decreased abundance in 270 transcripts and increased level in 193 transcripts from cultures in the porcine mucus medium, relative to a control culture grown in MH medium (Table 3.1). From the chick cecal and intestinal mucus medium, we observed decreased abundance in 55 and 51 transcripts respectively, and increased level in 67 and 68 transcripts respectively. The full transcriptome results are displayed in the appendices tables, 3, 4, 5 and 6 and selected results are displayed in table 3.2.

Table 3.1	**Number of transcripts with differential level**	
Condition	Increased abundance	Decreased abundance
Porcine small intestinal mucus medium	193	270
Chick cecal mucus medium	67	55
Chick small intestinal mucus	68	51
Mucin-supplemented MEMα	186	193

Table 3.1: Number of transcripts with differential level. The total number of genes demonstrating a significant fold-change in expression, using a threshold of threshold of $p<10^{-4}$, 1.5 fold difference.

Table 3.2	***C. jejuni* transcriptional state**							
Gene	Piglet small intestinal mucus		Chick small intestinal mucus		Chick cecal mucus		Porcine gastric mucin	
	Fold change*	p value	Fold change*	p value	Fold change*	p value	Fold change*	p value
Oxidative stress								
katA	3.26	2.54E-06	-0.19	0.27	-1.24	1.94E-06	-5.63	0
ahpC	0.81	0.00046	-0.77	8.26E-14	-1.78	0	-3.72	3.91E-14
sodB	0.91	1.15E-06	-1.35	0	-1.16	0	-1.04	2.28E-06
cj0358	0.0034	0.98	1.04	0	1.6	1.81E-09	5.21	0
tpx	0.29	0.012	-1.21	2.22E-16	-1.44	0	-2.09	0
Metabolism								
putA	1.20	1.06E-08	0.26	7.02E-05	2.65	0	2.93	0
putP	1.16	1.79E-09	0.22	7.65E-05	2.44	0	3.06	0
peb1a	2.29	5.26E-11	0.032	0.53	1.86	0	3.12	0
aspA	0.66	0.00032	1.68	0	2.00	0	0.23	0.042
aspB	1.05	2.22E-16	1.17	6.14E-10	0.072	0.29	1.58	0
glnA	1.41	1.02E-08	-1.17	7.82E-05	-1.37	2.00E-15	0.91	0
gltD	0.80	4.96E-06	-0.87	0.0029	-1.09	1.11E-16	0.53	2.09E-05
cj0073c	1.11	9.73E-09	-1.05	0	1.48	2.84E-14	4.58	0
Capsule								
kpsM	-0.84	4.82E-10	0.06	0.14	-0.22	0.00054	-0.91	1.67E-11
kpsF	-1.11	2.26E-09	-0.26	1.47E-05	-0.43	2.16E-10	-0.30	6.51E-05
cj1440	-1.11	1.93E-09	-0.16	4.01E-04	-0.15	0.0091	-0.54	5.79E-11
cj1442	-1.61	2.39E-10	-0.20	4.37E-04	-0.28	0.00019	-0.36	1.46E-06
Chemotaxis								
cetA	-0.46	3.07E-06	-1.82	0	-0.99	1.73E-13	-2.21	0
cetB	0.06	0.64	-1.45	0	-0.71	7.65E-10	-1.75	0
Bile response								
cmeA	1.81	0	2.09	0	-0.43	5.23E-08	-0.27	0
cj0561c	2.87	0	2.48	0	-0.0075	0.90	0.81	2.04E-05
Unknown								
cj770c	1.65	2.92E-08	-0.63	3.33E-16	-0.11	0.12	0.63	1.09E-06
cstA	1.56	4.44E-16	-0.21	0.0017	1.90	0	2.04	0
cj0025c	0.59	0.0037	-2.27	0	-3.03	0	-2.14	1.10E-10
cj1500	-1.74	2.52E-06	-2.72	0	-2.68	2.91E-08	-3.45	0

Table 3.2: *C. jejuni* transcriptional State. Select genes from the transcriptiome results for *C. jejuni* grown in Piglet and chick intestinal mucus and mucin-supplemented MEMα medium. The fold changes displayed in $\log_2$. The p value was derived using an empirical Bayes method. A threshold of $p<10^{-4}$ and 1.5 fold difference (0.58 $\log_2$) was used to denote a significant difference.

Table 3.3	**List of Gene Clusters**			
Cluster	Cluster characteristics			Number of genes
	Porcine mucus medium	Chick cecal mucus medium	Chick intestinal mucus medium	
Cluster 1	Unchanged	Lower	Lower	29
Cluster 2	Higher	Unchanged	Higher	12
Cluster 3	Unchanged	Higher	Higher	13
Cluster 4	Lower	Unchanged	Unchanged	152
Cluster 5	Higher	Higher	Unchanged	31
Cluster 6	Higher	Unchanged	Unchanged	64

Table 3.3: List of gene clusters. Transcriptomic results of *C. jejuni* from each of the mucus-medium cultures were filtered using a 2-fold difference cutoff and hierarchically clustered into 6 separate clusters.

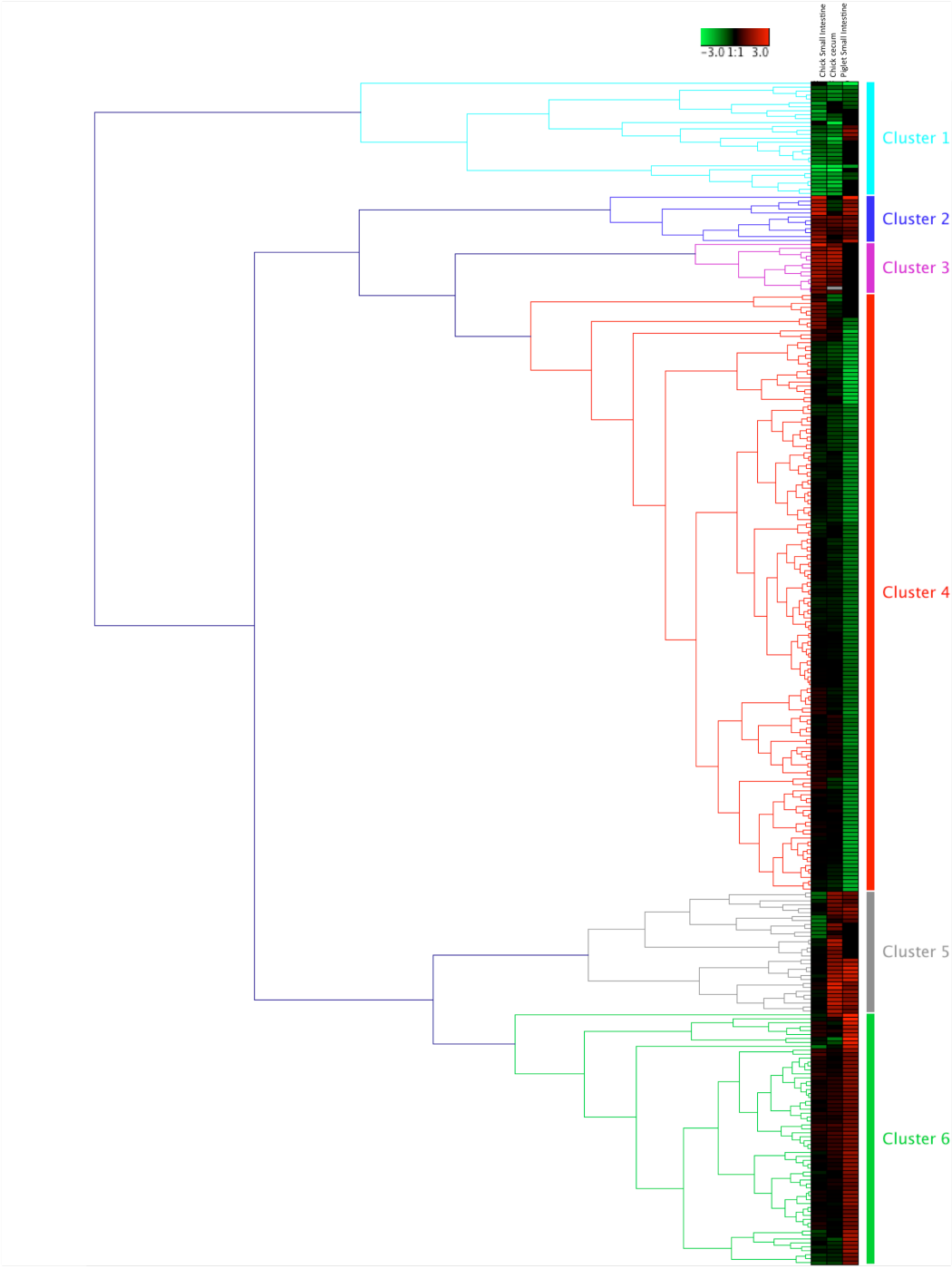

Figure 3.5: Hierarchical clustering of *C. jejuni* transcriptomes in intestinal mucus medium

Figure 3.5: Hierarchical clustering of *C. jejuni* transcriptomes in intestinal mucus medium. This figure displays the hierarchical clustering of the microarray transcriptomic data of *C. jejuni* while growing in porcine small-intestinal mucus medium, chick cecal mucus medium, and chick small-intestinal mucus medium. Figure was prepared using genesis software (Institute for Genomics and Bioinformatics, Graz University of Technology). Red colouring denotes up-regulation relative to the control culture, while green denotes down-regulation relative to the control culture.

Hierarchical clustering analysis of the transcriptomic data from the three mucus-medium conditions identified 6 distinct clusters (Table 3.3, Figure 3.5). Clustering was performed on transcripts exhibiting at least 2-fold differential abundance with a bayesian p-value below 10^{-4} as opposed to the previously used 1.5-fold cutoff so as to focus on the genes exhibiting the most significant difference between conditions.

Cluster 1 comprises transcripts with substantial lower abundance in the two chick-mucus cultures, while there is no substantial difference in the piglet mucus culture. Twenty-nine genes were contained within this cluster, representing a diverse group of genes, including *cj0025c (*encoding a putative transporter), *fdhABC* (coding for the formate dehydrogenase), *thiC* (encoding a thiamine biosynthesis protein), *nifSU* (encoding Fe-S cluster formation proteins), *ahpC* (the alkylhydroperoxide reductase gene), and *cetAB* and *cj0262c* (chemotaxis-related genes).

Cluster 2 represents the 12 genes stimulated in the two small-intestinal mucus medium cultures, but not the chick cecal mucus medium culture. This cluster contains *cj0561*, and *cmeABC*, all of which are part of the CmeR regulon, and are known to be up-regulated in response to bile (83, 150, 151).

Cluster 3 represents the 13 genes highly expressed within *C. jejuni* when grown in the chick mucus cultures. These are *dcuAB and mfrABE* (formerly annotated as *sdhABC*), involved in using fumarate as an electron acceptor under low-oxygen conditions (77); and *nrfAH*, involved in the use of nitrite as an electron acceptor (186).

Cluster 4 represents 152 genes, half of the total number included for clustering. These are genes exhibiting decreased transcript abundance when *C. jejuni* was grown in the piglet mucus medium, but no change was observed in the two other media. This cluster consists of a large variety of genes, many of which are involved in biosynthesis of amino

acids, RNA, proteins and other macromolecules. Also within this cluster are three genes, *napAGH,* which exhibit increased transcript levels in the chick small intestinal mucus medium, unchanged in the chick cecal mucus medium, but slightly decreased in the piglet mucus medium. These three genes are all involved in nitrate reduction for the purpose of using it as a final electron acceptor in the electron transport chain (186).

Cluster 5 is comprised of 31 genes more highly expressed in samples from the piglet and chick cecal mucus medium as compared to the control. Within this cluster we identified a group of genes with identical expression pattern, suggesting an operonic structure (*cj0481-cj0490*). While most of these genes have an unknown function, the gene *cj0486* has been annotated as a putative sugar transporter (180), whose role in L-fucose uptake and metabolism will be discussed in more detail in chapter 4.

Cluster 6 represents the 64 genes more highly expressed by *C. jejuni* cultured in piglet mucus medium, and relatively unchanged in both chick mucus cultures compared to MH broth. These include several uncharacterized NLPA lipoproteins genes (*cj0770c-72c*), the glutamine and glutamate synthase genes (*glnA* and *gltBD*), and the catalase gene (*katA*).

Since the primary component of the intestinal mucus layer are mucin glycoproteins, we also sought to examine the transcriptomic state of *C. jejuni* grown in MEMα medium supplemented with 2mg/ml porcine gastric mucin as compared to cells grown in MEMα medium supplemented with pyruvate. Under these conditions, we observed decreased expression in 193 genes and increased expression in 186 genes in a mucin containing medium relative to a control culture grown in MEMα medium. This data was not clustered with the previous conditions due to substantial differences between the mucin-supplemented MEMα medium and the three mucus media, making a direct comparison by hierarchical clustering misleading.

Among genes found to be differentially expressed between the mucin-supplemented and control medium, were *katA, sodB, ahpC, cj0358* and numerous other genes within the PerR and Fur regulons; as well as numerous metabolic (*peb1a*, *putAP*, *gltBD*, *aspB, cj0073c-75c*), chemotaxis (*cetAB*), and capsule (*kpsMF*, *cj1440c*, *cj1442*) related genes, many of which exhibited expression patterns similar to those observed in *C. jejuni* from the mucus medium cultures.

3.4. Discussion

Previous studies have suggested an important role for the mucus layer in modulating the pathogenicity of *C. jejuni* within its different hosts (5, 29). We sought to further explore this phenomenon, by layering Caco-2 cells with extracted intestinal mucus from chick or piglet small intestinal samples. We observed decreases in invasion and translocation in the presence of chicken mucus, and increases in invasion and translocation in the presence of piglet mucus, relative to *C. jejuni* invasion and translocation in Caco-2 cells alone. Our results with porcine and chicken mucus are consistent with previous findings from Byrne *et al.* (29). Their study also observed decreased cell invasion in cells layered with chick intestinal mucus *in vitro.* Additionally, using human mucus samples, they observed a hyper-invasive phenotype with *C. jejuni*; similar to our results using extracted porcine intestinal mucus.

Byrne *et al.* were not able to offer conclusions as to why mucus was having this effect, although they suggested some role for mucus composition (29). We found that *C. jejuni* grew efficiently on both mucus- and mucin-based media, consistent with results reported by others (234, 238). Consequently neither mucus nor mucin samples exhibit bacteriocidal or growth inhibitory effects on *C. jejuni*. Work by Alemka *et al.* into the role

of mucins on *C. jejuni* cell invasion found that purified chicken mucins, when layered onto HCT-8 ileocaecal adenocarcinoma cell lines grown *in vitro*, had a similar effect as the chicken-derived mucus samples (5). Based on the properties of fucosylated or sialilated glycans to bind foreign bacteria such as *C. jejuni* (42), thereby inhibiting adhesion and invasion, they treated the mucins with fucosidases and sialylases. However, this treatment had no influence on the mucin's protective effect (5). On the other hand, pretreatment of chicken mucins with sodium metaperiodate to remove the entirety of their glycan structures eliminated their inhibitory potential, supporting the hypothesis that mucin glycans bind target bacteria, thereby preventing them from accessing the HCT-8 cell surface. However, despite these insights, it remains unclear as to how differences between human, piglet and chick mucins may alter *C. jejuni* invasiveness, and numerous factors from the mucus samples may also play a role in influencing *C. jejuni*'s invasiveness.

To further explore how mucus composition may be affecting the *C. jejuni* invasion and translocation phenotypes, we sought to compare its transcriptome while growing in our porcine and chick mucus media. These transcriptomic studies revealed a number of significant differences and similarities in genes expression in *C. jejuni* when growing in a medium derived from porcine intestinal mucus, chick cecal mucus and chicken intestinal mucus.

The transcriptomic results for *C. jejuni* grown in three mucus-medium types, chick small intestine, chick cecum, and piglet small intestine, were organized using hierarchical clustering resulting in 6 separate clusters, based on similarities and differences in transcript levels between the three conditions. Of these, several groups of genes are of particular interest. Firstly, we observed substantial differences in the expression of a number of genes related to *C. jejuni's* oxidative stress response. These include the catalase (*katA*),

alkylhydroperoxide reductase (*ahpC*), superoxide dismutase (*sodB*), and thioperoxidase (*tpx*) genes, all of which were more highly expressed in *C. jejuni* grown in the piglet small intestinal mucus-based medium, relative to the MH medium control culture. Conversely, expression of all of these oxidative stress-response genes was substantially decreased in *C. jejuni* grown in the two chick-mucus based medium cultures, as well as from the mucin-supplemented medium, relative to the control. Most of these genes belong to the PerR regulon (176). PerR has been shown to regulate a number of genes, including *katA*, *ahpC* and *tpx*, in both an iron and H_2O_2 dependent manner (176). While iron is known to affect the expression level of PerR-regulated genes (176), our results indicated comparable iron concentrations between the chick and piglet mucus samples (18.7 and 18.09 nmoles/ml respectively), and thus would suggest that this is not the case. In agreement with this conclusion, iron acquisition and other iron-regulated genes were expressed at similar levels between the different samples and controls. Therefore, the above mentioned PerR-regulated genes are likely induced in response to true oxidative stress in the growth medium containing porcine mucus. Alternatively, manganese may also impact PerR regulation (98, 137), and we do not have any indication as to whether manganese concentrations vary between these mucus samples.

We also observed the increased expression of a number of genes from the CmeR regulon in *C. jejuni* grown in the two small-intestine derived mucus media. Previous research has found that in the presence of bile, repression by CmeR is inhibited, allowing for increased expression of the genes otherwise repressed by CmeR (83, 150, 151). These include the CmeABC multidrug efflux pump, involved in both antibiotic and bile resistances, as well as the unknown gene *cj0561*, all of which were observed to be more highly expressed in our experiments.

Woodall *et al*., recently reported *C. jejuni*'s transcriptome while colonizing the chick cecum *in vivo* (253). Their transcriptomic analysis identified numerous genes known to be required for growth in a low oxygen environment, as well as metabolic genes and others, which bear striking similarities to those identified in our transcriptomic studies, especially our *ex vivo* cecal mucus-medium data. These include the up-regulated genes *dcuAB*, *mfrABE*(*sdhABC*), *frdAB*, and *napAB*, all of which were linked to growth in a low-oxygen environment (253). It is interesting to note that these genes were similarly regulated in our culture, even though the same microaerophilic conditions were used for our mucus-medium and control cultures, indicating that the regulation of these genes are independent of actual oxygen levels. Genes down-regulated in Woodall's study and ours include *cj0414*/*15*, *nifSU*, *thiC, cj0025c*, *omp50*, and *tpx,* among others. Overall, 48 of the 68 genes identified in Woodall's study were also identified in at least one of our experimental conditions (253). This suggests that our *in vitro* transcriptome results from cultures based on extracted intestinal mucus medium, to a certain extent, reflects the *in vivo* growth of *C. jejuni*.

Clearly, our transcriptome results likely reflect *C. jejuni*'s environment while it is growing within the intestine. A substantial decrease in the transcription of *thiC* while growing in mucus medium suggests a readily available source of thiamine is available, either present in the mucus from consumed food by the chick or piglet, or synthesized by the intestinal microbiota (254) prior to mucus extraction. Increased expression of *dcuAB*, *mfrABE*(*sdhABC*), *frdAB*, *nrfAH* and *napAB,* while not necessarily indicating a lack of oxygen in the environment, do indicate that *C. jejuni* is using molecules other than oxygen as final electron acceptors, either fumarate (*mfrABE/frdAB*) (77), nitrite (*nrfAH*) or nitrate (*napAB*) (186). Increased expression of the *peb1* complex, along with *glnA*, *gltBD*, *aspA* and

aspB suggests that *C. jejuni* is using glutamate as a major carbon source in this environment, while the increased expression of *putA* and *putP,* may indicate the same for proline.

We also observed substantial similarities between the transcriptomes of *C. jejuni* from the mucus-medium cultures, and cultures grown in MEMα supplemented with porcine gastric mucin. Although substantial differences in the base medium make it difficult to do a direct comparison between these transcriptomic data and the data obtained from the mucus medium cultures, some marked similarities between certain genes in both conditions are of interest. Most notably are the oxidative stress response genes *katA*, *ahpC*, *sodB* and *tpx*. They are all expressed at low levels relative to the control in both the mucin supplemented medium and the chick mucus medium data, suggesting that mucins within the mucus may be involved in the changes in expression of the oxidative stress response genes observed in the mucus based medium.

Additionally, metabolic genes involved in proline metabolism (*putAP*), glutamate metabolism (*peb1a*, *aspB*) and many of the other above-mentioned metabolism genes, exhibited increased expression, similar to the expression from the mucus medium cultures. Indeed there are numerous similarities between the transcriptome profiles of *C. jejuni* grown in mucus and mucin supplemented media. Up-regulated genes include many genes whose role with mucin is not immediately apparent, comprising the above-mentioned oxidative stress response genes, thiamine biosynthesis (*thiC*), capsule-related genes (*kpsM*), lactate metabolism (*cj0073c-75c*), genes involved in the use of non-oxygen electron acceptors (*dcuAB*, *mfrABE*, *frdAB*, *nrfAH*), chemotaxis (*cetAB*) and outer membrane proteins (*omp50*). Together, our data suggest that not only is mucin playing a substantial role in shaping the transcriptome of *C. jejuni* while in a mucus medium, but may also play a signaling role in regulating the expression of genes beyond pathways directly related to mucin utilization.

CHAPTER 4

L-fucose utilization by *C. jejuni*

4.1. Introduction

Our transcriptomic study of *C. jejuni* grown in porcine or chick intestinal mucus-based medium revealed the increased expression of the genes *cj0481* to *cj0490*. While none of these genes had identifiable functions, they were annotated as dehydrogenases, transporters, and hydrodrolases, suggesting their involvement in an uncharacterized metabolic pathway. The presence of a gene encoding a potential L-fucose permease within this operon raised the possibility that these genes might metabolize L-fucose. This hypothesis was explored further by the studies discussed below, and published by Muraoka *et al.* (171) and Stahl *et al*. (222).

The importance of fucose to the intestinal microbiota is well established (38, 97, 196). Fucosylated glycans are common within the digestive tract where they are found on cell surfaces and are typically located in terminal positions on the extensively modified mucin glycoproteins (16, 196). These fucosylated glycans coating the mucins are known to act as adherence targets for many intestinal bacteria (including *C. jejuni* (42)) potentially contributing to their function of mucin as a barrier to intestinal pathogens (36). Fucose monomers and fucosylated glycans can serve as a chemoattractant (100) and carbon source for many species within the gut (212). Being so common within the intestine, fucose is an integral part of the intestinal ecology. For example, the presence of the ubiquitous commensal *Bacteroides thetaiotaomicron* in the gut triggers an increase in the production of fucosylated mucins by the intestinal epithelium, which is important for gut maturation (27).

These bacteria then secrete fucosidases to release fucose residues from the oligosaccharides and transport the sugar into the cell for metabolism and surface presentation (95, 97). Even bacteria such as *E. coli,* which do not express fucosidases, use this same pathway for fucose transport (82) and colonization of the bovine rectum (219).

Despite the lack of an annotated fucose metabolic pathway, fucose has long been described as influencing the behavior of *C. jejuni.* In 1988, Hugdahl *et al*. reported *C. jejuni* chemotaxis towards L-fucose, as well as towards intact mucins, which would have included fucosylated glycans (100). Also, fucosylated milk oligosaccharides were used in an elegant experiment to competitively inhibit the specific attachment of *C. jejuni* to intestinal H-antigens (201). More recently, Korolik e*t al.* have made use of glycan microarrays to demonstrate increased *C. jejuni* adherence to fucosylated glycans such as those found on mucin and cell surfaces (42); structures that were suggested to be significant factors in the ability of *C. jejuni* to closely adhere to the cell surface and invade intestinal epithelial cells.

Here we investigate the role of a previously unidentified pathway in the metabolism of L-fucose in an organism that was previously thought to be asacharolytic. Furthermore, L-fucose appears to have a role in the colonization of certain hosts with potential implications for *C. jejuni* pathogenesis.

4.2. Methods and Materials

4.2.1. Strains and growth media used

The *C. jejuni* strains NCTC 11168, 81-176 and RM1221 were grown on MH agar plates, MH biphasic flasks, or MH liquid cultures; supplemented with chloramphenicol (20 μg/ml), and/or kanamycin (10 μg/ml) as needed. Cultures were incubated at 37°C in MACS microaerophilic incubators with a gas concentration of 8% O_2, 4% H_2, 5% CO_2, and 83% N_2.

Genetic manipulations were conducted using *Escherichia coli* strain DH5α grown on Luria-Bertani (LB) agar plates or LB broth supplemented with ampicillin (100 μg/ml), chloramphenicol (20 μg/ml) and/or kanamycin (10 μg/ml) as needed and incubated at 37°C. Fifty-three *C. jejuni* environmental isolates from Iceland were provided by Ruff Lowman (Canadian Food Inspection Agency). Included with these samples were FlaA types and the precise source of each strain.

4.2.2. Mutant construction

Mutants in the genes *cj0481, cj0483, cj0486, cj0487*, and *cj0490* were constructed using a mutagenesis and gene-replacement strategy. The target gene was PCR amplified using the primers listed in Appendix I and the genomic DNA of NCTC 11168 was used as the template. All primers used for these cloning reactions were designed for use with the In-fusion™ Dry down PCR cloning kit (Clontech, CA). The PCR products were cloned into *Bam*HI-digested pUC19 plasmids using the manufacturer's protocols. Once recombinant plasmids were obtained, they were used as a template for an inverse PCR reaction, so as to delete regions within the gene, and create a site for the insertion of a chloramphenicol-resistance cassette (*cat*). The primers used for the inverse PCR reaction are listed in Appendix I. The *cat* gene was amplified from pRY111 using Pfx polymerase (Invitrogen) and the primers indicated in appendix I. The *cat* gene was then cloned into the inverse PCR product using the Clontech kit as above. Constructs were confirmed by sequencing and the recombinant plasmids were naturally transformed into motile *C. jejuni* NCTC 11168 as described previously (80, 193) by selecting for chloramphenicol resistance on MH agar. The exception to this protocol was the mutation of *cj0486,* which was created by Lorna Friis

(University of Alberta). In this case, the *C. jejuni cj0486* mutant was created by blunt insertion of the *Sma*I-cut 1488 bp kanamycin cassette from pWM2 into the *Spe*I-cut *C. jejuni cj0486* cloned into pBR322 described above. Electroporation of 1 μg of this plasmid into *C. jejuni* NCTC 11168 was performed as described elsewhere (117), selecting for kanamycin resistant *C. jejuni* transformants. Each mutant was confirmed to have comparable motility to the wild-type using standard motility assays on soft agar plates (194). Constructs were confirmed by PCR and sequence analysis of the recombinant gene.

4.2.3. *C. jejuni* mutant complementation

The plasmid used to construct CjWM227a was kindly provided by Qijing Zhang (171). This construct contained the amplified region *cj0486-cj0490,* cloned into the pRRK vector (175) as described in by Muraoka *et al*. (171). This construct was transformed into the *Δcj0487* mutant to create the *cj0486-cj0490* complemented *Δcj0487*strain. To construct the *Δcj0486+ (cj0486-cj0490)* strain, the pRRKCj0486-cj0490 plasmid was digested with *Mfe*I-HF to remove the kanamycin resistance cassette and replace it with a chloramphenicol resistance cassette. The resultant plasmid pRRC486-490 was transformed into the *Δcj0486* mutant.

The *cj0481-cj0490* genomic island was also added to the *C. jejuni* 81-176 strain using the pCE111 plasmid using an approach previously described by Larsen et al. (134) and conducted by Dr. Harald Nothaft (University of Alberta).

4.2.4. Growth on L-fucose

MEMα medium (Gibco 41061) containing glutamine, but no phenol red, was supplemented with 20 μM $FeSO_4$ and 25 mM L-fucose (Sigma-Aldrich) and filter sterilized (0.2 μm pore size). The wild-type or mutant strains were grown overnight in MH biphasic cultures. These were used to inoculate the growth medium at an OD_{600} of 0.01. Aliquots of this culture (300 μl) were injected into honeycomb 100 well plates designed for use with a Bioscreen C incubator/plate reader as described in the materials and methods of chapter 2.

4.2.5. Radioactive L-fucose uptake and ATP-dependent L-fucose phosphorylation and GTP-dependent L-fucose activation.

Radioactive L-fucose uptake was conducted by Dr. Lorna Friis and Dr. Harald Nothaft (University of Alberta) and the methods and results were published and described by Stahl *et al*. (222).

4.2.6. Competitive assays

The ability of the *cj0486* mutant to compete against the NCTC 11168 wild-type strain was tested in both *in vitro* and *in vivo* conditions. For the porcine competitive colonization assay: male, specific pathogen-free neonatal colostrum-deprived piglets were acquired from the Canadian Food Inspection Agency. The piglets were allowed to acclimatize to their environment overnight and four piglets were inoculated with approximately 10^7 cells of a 1:1 mixture of wild-type *C. jejuni* NCTC 11168 and the *cj0486* mutant. After three days, they were euthanized and samples were taken from the duodenum, jejunum, ileum, cecum, and colon. The tissue samples were homogenized, serially diluted in MH broth and plated onto *Campylobacter* agar base (Oxoid CM935) containing *Campylobacter*–selective Karmali

supplements (Oxoid SR167E). The mutant strains were isolated using selective plates also containing kanamycin. The plates were incubated at 37°C, under microaerophilic conditions for 48 hours. Statistical significance was determined using a one-way ANOVA.

A similar approach was used for competitive assays using the chick model. Forty-three chicks were acquired from the Canadian Food Inspection Agency's specific pathogen free flock. These chicks were inoculated with approximately 10^4 cells of a 1:1 mixture of NCTC 11168 and *Δcj0486*. At 3, 6, 10 and 14 days post inoculation, 10 or 11 chicks were euthanized by cervical dislocation, and their ceca were collected and weighed. The cecal contents were resuspended into MH broth, serially diluted and plated onto Karmali selective plates as described above. The bacteria counts for all the *in vitro* and *in vivo* competitive assays were determined and are represented as the $\log_{10}$ ratio of *cj0486* CFU/ wild-type CFU. Twenty additional chicks were syringe fed 17mg L-fucose supplements, 3 times daily, at 8-hour intervals. They were inoculated with a 1:1 mixture of *Δcj0486* mutant to wild-type as described above, following their second fucose supplement. Nine and eleven chicks were euthanized 3 and 6 days post inoculation respectively. Statistical significance was determined by a Student's *t*-test.

An *in vitro* competitive growth assay was carried out in filter-sterilized MEMα medium supplemented with 20 μM $FeSO_4$, both with and without 25mM L-fucose, as well as in MH medium alone. As above, the media was inoculated with a 1:1 mixture of the *Δcj0486* mutant and wild-type and grown for 36 hours with samples taken and plated at 6 hour intervals.

4.2.7. Total RNA extraction for real-time PCR and microarray studies

Cultures of *C. jejuni* NCTC 11168 were inoculated into filter-sterilized MEMα medium containing 20 μM $FeSO_4$ and 25 mM L-fucose, or control medium of MEMα containing 20 mM sodium pyruvate and 20 μM $FeSO_4$. Cultures were grown to an O.D. at 600 nm of 0.2, representing mid-log phase, at which point the RNA quality was preserved adding a 1/10-volume stop solution of 10% buffer saturated phenol (pH 4.3) and ethanol. The cultures were pelleted by centrifugation at 6000rpm, at 4°C for 5 minutes. Total RNA was extracted using a hot-phenol extraction method as described previously in chapter 3 (175, 176, 193). The RNA was treated twice with Dnase I (Epicentre) and the absence of genomic DNA was confirmed by PCR. Final RNA quality and quantity was ascertained using BioRad's Experion StdSensRNA system using the manufacturer's protocols. Microarray hybridization and analysis was also conducted as previously described by Stahl et al. (222), Reid et al. (193), Palyada et al. (175, 176) and in chapter 3.

4.2.8. Confirmation of an operonic structure

The organization of the genes *cj0481* to *cj0490* was confirmed to be in an operonic structure by PCR amplification of regions spanning neighboring genes using cDNA as a template. Total RNA extracted from cultures containing 25mM L-fucose was reverse transcribed to cDNA using Superscript II reverse transcriptase (Invitrogen), following the manufacturer's protocols. To amplify the regions in between genes, the primers previously used for the inverse PCR step of mutant construction were employed (Appendix I). PCR

products from the cDNA template were compared against products from a control genomic DNA template.

4.2.9. Quantitative RT-PCR

Quantitative RT-PCR was conducted using the Applied Biosystems 7300 DNA analyzer and the Qiagen Quantitect SYBR Green RT-PCR kit using the manufacturer's protocols and as described previously (193). Each sample used total RNA extracted from cultures of *C. jejuni NCTC11168, Δcj0481, Δcj0486, Δcj0487*, and the complemented strains: *Δcj0486+(cj0486-cj0490)* and *Δcj0487+(cj0486-cj0490)*; grown in MEMα supplemented with 25mM L-fucose. RNA expression relative to the wild type control was determined for the genes *cj0480c, cj0481*, *cj0486*, and *cj0487* with *rpsL* acting as an endogenous control. The qRT-PCRs were carried out at least in triplicate and the extent of induction of gene expression was obtained using the comparative threshold cycle (ΔΔCτ) method, using the Applied Biosystems 7300 sequence detection software versions 1.3.1.

4.2.10. Cell invasion/adherence assays

Caco-2 cells were maintained in 25cm^3, tissue culture flasks, and grown with MEMα medium containing glutamine and 10% fetal bovine serum (FBS), and were incubated at 37°C and 5% CO_2. Cells were inoculated into a 24-well plate and allowed to grow for 3 days, replacing the media each day. After 3 days, the cell monolayer was inspected by microscope to confirm the presence of a solid cellular monolayer in the well. *C. jejuni* cultures of the strains 81-176, NCTC11168 and *Δcj0486* were grown in MH biphasic flasks overnight and adjusted to an O.D. 600nm of 1 in MEMα medium. Cell invasion by the wild-

type strains NCTC11168 and 81-176 in the presence of L-fucose was compared using MEMα medium containing 1% FBS was prepared and filter sterilized with L-fucose added to concentrations of 25mM, 10mM, 5mM, and a control medium was prepared with no supplemented fucose. The *C. jejuni* strains 81-176 and NCTC11168 were each inoculated into MEMα medium with each of the four concentrations of fucose, to an inoculation O.D.600nm of 0.01. The cell invasion potential of the *Δcj0486* mutant was compared to the wild-type NCTC11168 strain in MEMα medium containing 1% FBS, but no added fucose. Approximately 10^6 cells from each strain were added to each well of a 24 well plate and the cultures were incubated at 37°C and 5% CO_2 for 3 hours.

Cell invasion was measured using the same approach as described previously in chapter 3, and adherence to the cell layer was determined with a similar method, without treating the cells with gentamicin. The experiment involving the strains 81-176 and NCTC11168 in varying concentrations of free L-fucose was repeated 5 times, with 3 technical replicates of each condition per biological replicate. This number was done for both the invasion assays and adherence assays. The comparison between the *Δcj0486* mutant and wild-type NCTC11168 was repeated three times with up to 12 technical replicates per experiment, for both the invasion and adherence assays. Statistical significance was determined using Student's t-test.

4.3. Results

4.3.1. Fucose up-regulates expression of genes linked to fucose usage

The previously described microarray experiments using chick and porcine intestinal mucus, as well as porcine gastric mucin identified a group of genes numbered *cj0481-cj0490* as

being up-regulated in all the conditions, except for chick small-intestinal mucus (Table 4.1). Although these genes did not contain any experimentally confirmed functions, the presence of a putative L-fucose permease suggested their potential involvement in L-fucose metabolism. Microarray experiments were used to characterize the transcriptomic profile of *C. jejuni* NCTC 11168 during growth in MEMα medium supplemented with 25 mM L-fucose. This led to the identification of 74 up-regulated genes, and 52 down-regulated genes (Table 4.1). The most notable change from the list of up-regulated genes was the putative operon between *cj0481* and *cj0490*, which exhibited 6-9 fold up-regulation. The putative regulator *cj0480c* did not show any significant change in expression. In addition to the *cj0481-cj0490* operon, cultures grown in the presence of L-fucose also exhibited an up-regulation of metabolic genes, including genes involved in the citric acid cycle, proline metabolism (*putAB*), and aspartate/glutamate transport and metabolism (*peb1A*, *cj0919, cj0920*, *pebC, aspB*). Notable groups of genes exhibiting down-regulation included genes encoding proteins involved in oxidative stress response (*ahpC, sodB, tpx*), the electron transport chain (*cydAB, petABC*), chemotaxis (*cetAB, tlp4*) and the outer membrane protein (*omp50*). Complete results are displayed in Appendix VII.

4.3.2. Identification of a genomic island required for fucose utilization

None of the genes *cj0480c-cj0490* have any previously confirmed function, however *cj0486* bears homology to the fucose permease gene (*fucP*) of other bacteria (82). This operon has been identified previously in RM1221 and NCTC 11168 as a genomic island spanning from *cj0480c* to *cj0490* (179) and nestled in between the genes *rpoC* and *fusA* (Figure 4.1) of some strains. Notably this region is absent in the strain 81-176. Although this

Gene	Growth Medium				
	25mM L-fucose	Porcine Small Intestinal Mucus	Porcine Gastric Mucin	Chick Cecal Mucus	Chick Small Intestinal Mucus
Cj0480c	-0.18	-	-0.05	0.09	-0.06
Cj0481	4.36	2.34	1.86	1.91	-0.24
Cj0483	4.36	2.22	1.39	1.84	-0.25
Cj0484	3.75	1.72	1.09	1.33	0.01
Cj0485	3.38	2.43	0.98	1.48	0.03
Cj0486	3.69	2.35	1.21	1.57	.002
Cj0487	3.14	1.97	0.77	1.43	0.11
Cj0488	2.02	1.24	0.49	0.38	0.13
Cj0490	2.99	1.57	0.37	0.92	0.04

Table 4.1: Table comparing the transcription of the L-fucose metabolic pathway. Microarray experiments were conducted from RNA extracted from mid-log phase cultures grown in MEMα supplemented with 25mM L-fucose or 2mg/ml porcine gastric mucin; or 2mg/ml intestinal mucus derived from the porcine small intestine, chick small intestine, or chick cecum. The expression of each gene is displayed as the $\log_2$ of the fold change between the sample and control.

region is not universally conserved within strains of *C. jejuni*, it was later linked to *C. jejuni* hyper-invasiveness (56, 106).

In a comparison of the amino acid sequence of Cj0486 among sequenced *Campylobacter* genomes, *C. jejuni* NCTC 11168, RM1221, CF93-6 and 84-25, and *C. jejuni*, subsp. *doylei* 269.97; all exhibited greater than 98% identity, while *C. coli* RM2228 and *C. fetus* 82-40 displayed 93% and 59% identity respectively. All of these strains contain the whole genomic island in the same location on the chromosome, with the exception of *C. fetus,* which contained a similar group of genes elsewhere in the chromosome. Furthermore, we used PCR, and *cj0486* specific primers (Appendix I) to screen a library of 53 environmental isolates of *C. jejuni* for the presence or absence of *cj0486*. The gene *cj0486* was found in 24 of the 53 isolates, with 20 of the 24 being conserved within the same FlaA type (A04101) (Table 4.2). Despite this link, no correlation was observed between the presence of the *cj0486* gene and the source of the isolate.

FlaA type	Total isolates	cj0486+ isolates	Percentage
A09798	15	3	20%
A13926	5	1	20%
A04101	20	20	100%
A14118	13	0	0%
Total	53	24	45.3%

Table 4.2: Prevalence of *cj0486* in environmental isolates. The number of *cj0486* positive strains amongst 53 environmental and clinical isolates of *C. jejuni*. (strains provided by Dr Ruff Lowman, CFIA).

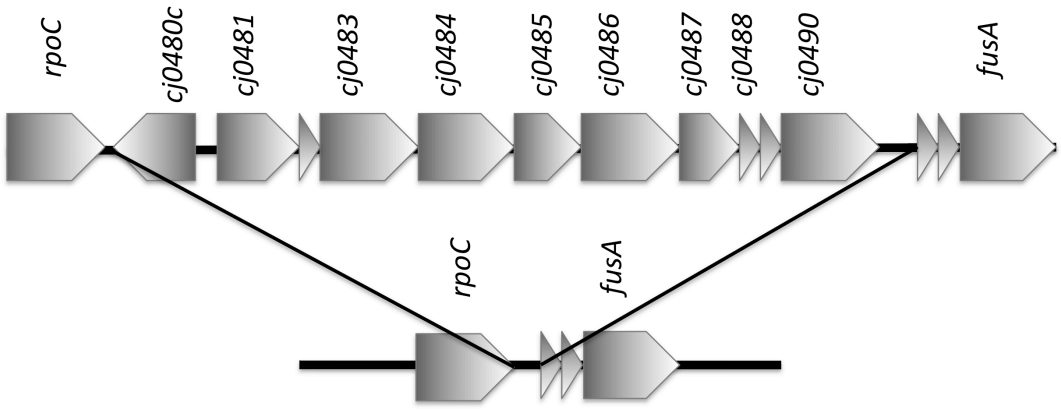

Figure 4.1: Genomic region surrounding the putative fucose permease (*fucP, cj0486*). The top strand is the region as it appears in *C. jejuni* strains NCTC 11168, RM1221, CF93-6, 84-25, *C. jejuni* subsp. *doylei* 269.97, and *C. coli* RM2228. The lower strand is the region as it appears in the strains lacking *fucP: C. jejuni* 81-176, CG8486, HB93-13, 260.94 and 81116.

4.3.3. The genes *cj0481-cj0490* exist as a single operon

We used PCR of sections spanning neighboring genes, and cDNA derived from extracted total RNA to confirm that the genes *cj0481-cj0490* are expressed as a single transcript. PCR products were obtained from regions spanning each pair of genes *cj0481-cj0483, cj0483-cj0484, cj0484-cj0485, cj0485-cj0486, cj0486-cj0487* and *cj0487-cj0490*. Each of these products was of equal size to the product obtained from a genomic DNA template. The exception to this was the region in between *cj0480c* and *cj0481*. As these genes are oriented in opposite directions, they should not be transcriptionally linked. A product was obtained from the genomic DNA template, but none from the cDNA template.

4.3.4. L-fucose enhances *C. jejuni* growth

With *C. jejuni* demonstrating positive chemotaxis toward both L-fucose and fucosylated mucins (100) and our observation of L-fucose uptake, we sought to determine if *C. jejuni* has the capacity to utilize L-fucose for growth. Three commonly studied strains of *C. jejuni*, NCTC 11168, 81-176 and RM1221, were grown in minimal essential medium (MEMα) with and without 25 mM L-fucose. Two of the three strains, NCTC 11168 and RM1221, demonstrated significant increases in growth in response to the availability of L-fucose as a nutrient source ($p<0.05$) (Figure 4.2a and b). The third strain, 81-176, while growing better than the other two strains (likely due to its ability to use glutamine in the growth medium (93)), did not show increased growth in the presence of L-fucose. However, when the operon *cj0481 – cj0490* was added to the 81-176 strain on the pCE111 plasmid, the 81-176 + cj0481-cj0490 strain exhibited increased growth in the presence of L-fucose (Figure 4.2c).

Each of the previously constructed mutants (and *cj0490*) was grown on MEMα medium, with and without 25mM L-fucose, and growth was compared to wild-type NCTC 11168. Similar to the wild-type, mutants containing insertions in *cj0483* and *cj0490* (Figure 4.2b) exhibited increased growth in the presence of L-fucose. However, the mutants *cj0481*, *cj0486* and *cj0487* did not show increased growth beyond normal levels in MEMα medium, indicating a loss in their ability to utilize L-fucose. The loss of Cj0486, the putative permease, would impair L-fucose uptake in agreement with our transport experiments. However, the roles of Cj0487, annotated as an amidohydrolase, and Cj0481, annotated as a putative dihydrodipicolinate synthase, are not currently evident.

4.3.5. Cj0486 acts as a fucose permease to transport extracellular fucose

We sought to ascertain whether the *C. jejuni cj0486* gene product was functional as a fucose transporter in *E. coli*. As shown in figures 1 and 2b by Stahl et al. (222), transformation of the *C. jejuni* NCTC 11168 *fucP* gene homologue, *cj0486*, into an *E. coli fucP* mutant successfully restored transport and metabolism of L-fucose in the *E. coli fucP* mutant, indicating that despite low homology between the genes (37.2%), Cj0486 is able to function as an L-fucose transporter in *E. coli*. Further comparison of the *E. coli fucP* and the *C. jejuni Cj0486* protein sequence (Figure 4.3) reveals conservation of Asp46, Glu135 and Asn146, all identified by Dang *et al*. as critical for the active transport of fucose into the cell (40). Cj0484 is annotated as a major facilitator superfamily (MFS) transporter, but does not contain these conserved residues or a significant degree of sequence similarity, suggesting it does not serve as a second L-fucose transporter.

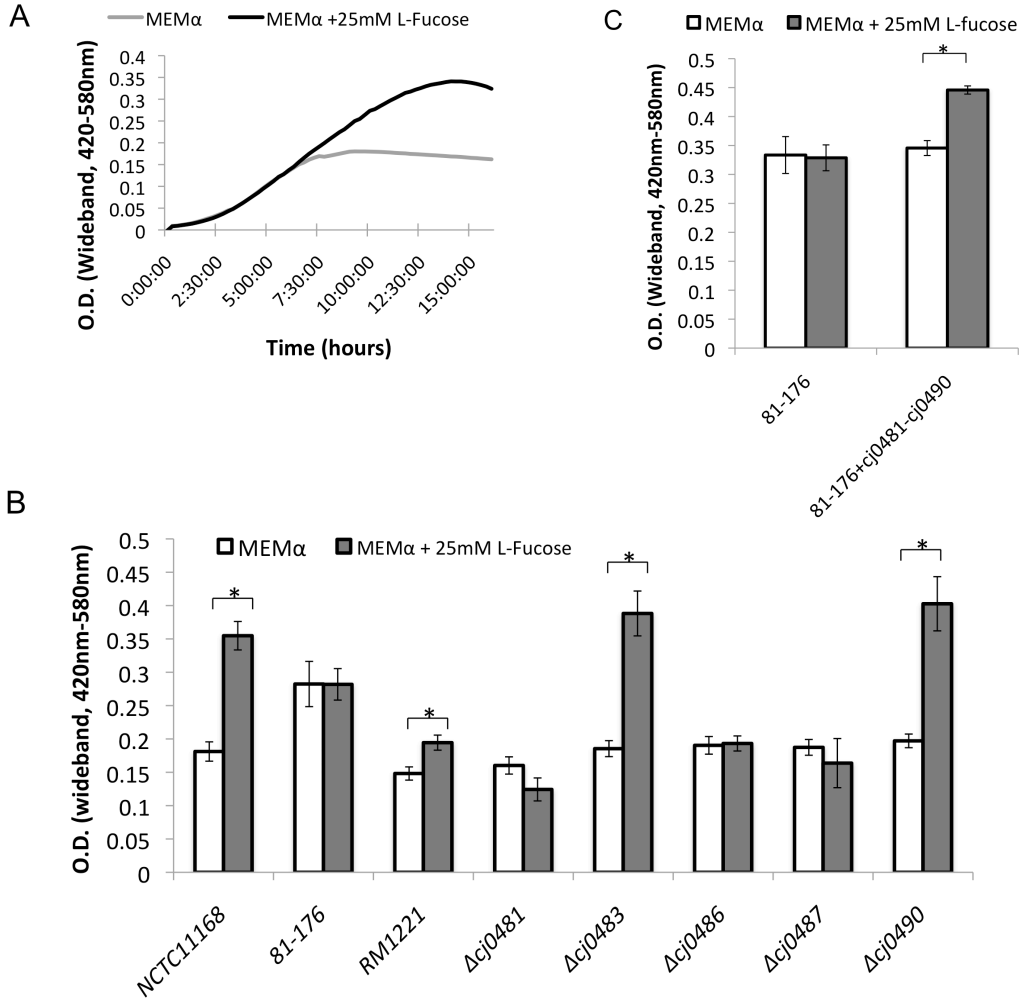

Figure 4.2: *C. jejuni* growth in the presence of L-fucose. (A) Growth of *Campylobacter jejuni* NCTC 11168 in MEMα medium with and without 25 mM L-fucose over 16 hours. (B) Maximum O.D. attained over 36 hours of growth for the reference strains *C. jejuni* NCTC 11168, 81-176 and RM1221, and the mutant strains *C. jejuni* Δ*cj0481*, Δ*cj0483*, Δ*cj0486*, Δ*cj0487*, and Δ*cj0490*. (C) Maximum O.D. attained over 36 hours of growth for the reference strain *C. jejuni* 81-176 and 81-176+*cj0481-cj0490* (*) denotes statistical significance, $p<0.05$ using a paired T-test. Error bars represent the standard deviation, n=3.

Figure 4.3: The sequence aligment between the *E. coli* FucP, and the *C. jejuni* Cj0486 and Cj0484 proteins. The alignment was done using the online program ClustalW2 from EMBL-EBI, http://www.ebi.ac.uk/Tools/msa/clustalw2/. Highlighted are the amino acid residues Asp46, Glu135 and Asn146, known to be highly conserved and essential for function in *E. coli* FucP (40).

Active transport of L-fucose by *C. jejuni* was confirmed in ^{3}H-L-fucose uptake experiments. We found that in wild-type cells grown in the absence of L-fucose, the initial uptake was approximately 3-fold lower than in cells grown in the presence of L-fucose, indicating that L-fucose transport is inducible by its substrate (Figure 4.4). The *Δcj0486* mutant showed no significant L-fucose transport (< 0.2 pmols/min/10^{9} cfu), demonstrating that Cj0486 is an essential component of the active L-fucose assimilation pathway in *C. jejuni*. The mutants *cj0481, cj0483*, and *cj0487* all transported L-fucose at levels comparable to an uninduced wild-type strain, however, uptake was not increased over time, despite the presence of L-fucose, suggesting a degree of feedback-inhibition.

Complementation of *cj0486* showed only partial, but significant, restoration of L-fucose uptake. Quantitative RT-PCR analysis demonstrated that *cj0486* and *cj0487* are expressed at <11% of the wild-type levels in the complemented strains (Figure 4.5), explaining the partial restoration of transport phenotypes. Similarly, complementation of the *E. coli fucP* mutant with *cj0486* was able to partially restore L-fucose uptake (222). Complementation of the *cj0486* and *cj0487* strains did not restore growth, despite the partial restoration of L-fucose uptake (Figure 4.4). Not only were the transcript levels of *cj0486* and *cj0487* in the complements at very low levels, but also *cj0481* was not expressed in any of the mutants or complements (Figure 4.5), indicating an absence of induction of the whole operon, rather than simply disruption of downstream expression. These results suggest tight regulation of this operon by L-fucose induction and show that the low level of fucose assimilation into the complemented *cj0486* and *cj0487* strains is insufficient for induction and transcription by these complemented strains.

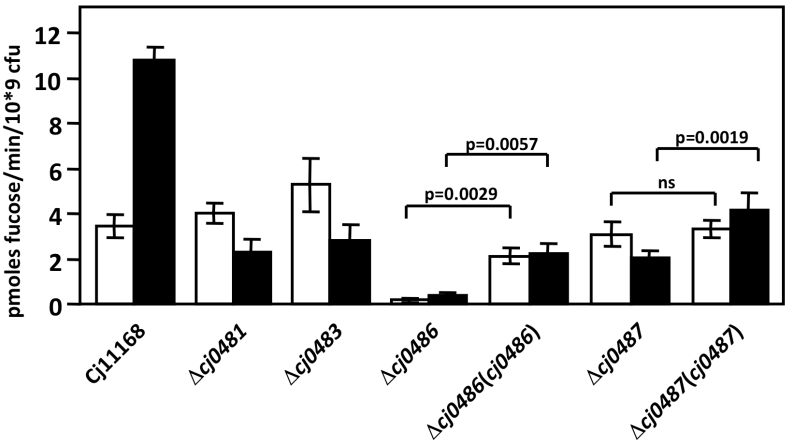

Figure 4.4: Fucose uptake by *Campylobacter jejuni*. The initial velocity of [^{3}H] fucose incorporation, which was linear within the first 120 sec of the assay, is shown for the indicated strains. White bars: grown in the absence of fucose (uninduced); black bars, grown in the presence of fucose (induced). Standard deviation is displayed by error bars. p-values derived from paired T-tests compare the statistical differences in uptake rates between the mutants and their complements. ns, no statistically significant difference ie $p>0.05$ (Figure prepared by Dr. Harald Nothaft, University of Alberta (222)).

4.3.6. Fucose metabolism in *C. jejuni* does not rely on ATP-dependent phosphorylation or GTP-dependent activation of L-fucose

One of the key enzymes of the *E. coli* L-fucose metabolic pathway is the L-fuculose kinase encoded by *fucK* (27). This ATP-dependent kinase phosphorylates L-fuculose to L-fuculose 1-phosphate, allowing for L-fucolose-1-phosphate aldolase to convert it to L-lactaldehyde and dihydroxyacetone phosphate (14). The absence of an annotated L-fucose kinase in the *C. jejuni* genome would suggest that *C. jejuni* does not make use of this pathway. We confirmed this hypothesis using a modified approach outlined by Wang *et al*. (243). As predicted, sugar phosphates were not formed from ^{3}H-L-fucose by *C. jejuni,* whereas they were readily formed by *E. coli* in an L-fuculose kinase-dependent manner (Figure 4.6) (222).

In contrast, *Bacteroides* species activate L-fucose through a bifunctional L-fucokinase/GDP-fucose pyrophosphorylase (FKP) pathway which requires ATP to generate a fucose-1-phosphate intermediate which is then activated with GTP to generate GDP-fucose (Figure 4.6) (38). Again, since *C. jejuni* does not possess FKP pathway homologues, ^{3}H-modified fucose was not detected, but high levels of fucose intermediates were detected in *B. thetaiotaomicron* extracts incubated with ATP (which could then be converted with endogenous GTP) and augmented with added GTP (Figure 4.6) (222).

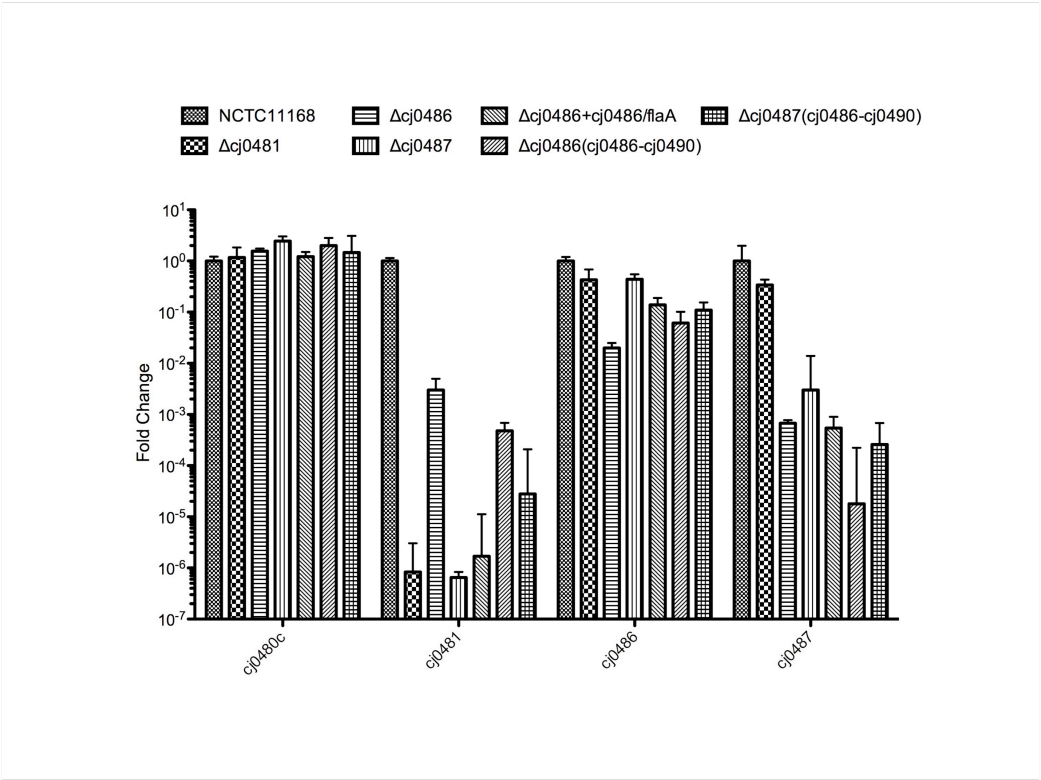

Figure 4.5: Gene expression of *cj0480c, cj0481, cj0486* and *cj0487*. Expression of the genes *cj0480c, cj0481, cj0486* and *cj0487* as determined by real-time PCR from total RNA extracts from *C. jejuni NCTC11168, Δcj0481, Δcj0486, Δcj0487*, and the complemented strains: *Δcj0486+(cj0486-cj0490)* and *Δcj0487+(cj0486-cj0490)*. Results are displayed as fold change relative to the *C. jejuni* NCTC11168 wild-type.

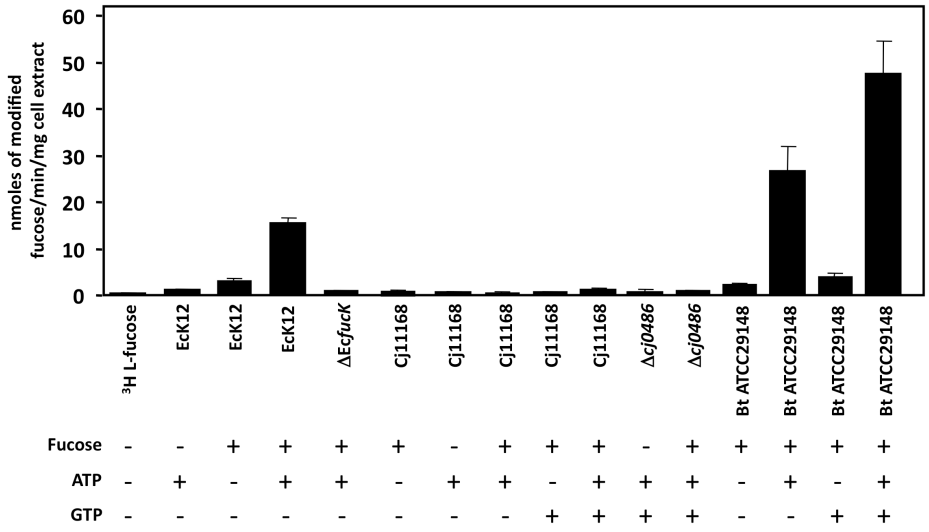

Figure 4.6: ATP-dependent ^{3}H-L-fucose phosphorylation and GTP-dependent ^{3}H-L-fucose activation. Whole cell lysates (100 μg) of the indicated strains grown in the presence or absence of [^{3}H]L-fucose (as indicated) for 24 h and assayed for their potential to fucose under the indicated assay conditions. Ec, *E. coli* K12 wild-type; ΔEc*fucK*, *E. coli* K12 *fucK* mutant; Cj, *C. jejuni* NCTC11168 wild-type; Δ*cj0486*, *C. jejuni* NCTC11168 Δ*0486*; Bt, *Bacteroides thetaiotaomicron* wild-type. The presence or absence of ATP and GTP are indicated by (+) and (-). Formation of modified ^{3}H-L-fucose was found to be linear over the first 10 min of the assay. Standard deviations are displayed by error bars. ^{3}H-L-fucose alone did not show any significant binding to the column material.

4.3.7. Fucose transport provides *C. jejuni* with a competitive advantage *in vitro* and *in vivo*

To assess the advantage of fucose transport to *C. jejuni in vivo,* we carried out competitive colonization assays. Wild-type NCTC 11168 and the *cj0486* mutant were inoculated at a 1:1 ratio into either the pathogenic piglet or the commensal chicken model. After three days post-infection of neonatal piglets, samples from each section of the intestine (duodenum, jejunum, ileum, cecum and colon) were collected and the numbers of colonizing bacteria were enumerated. The mutant to wild-type ratio recovered from the intestinal sections ranged from 0.37 (2.7 fold reduction) to 4.10^{-4} (2,500 fold reduction), which corresponds to an average 15 to 100-fold reduction ($p<0.002$) in the mutant strain relative to the wild-type strain in each intestinal region (Figure 4.7). Thus, in the pathogenic model, the ability to transport fucose provides a strong competitive advantage for colonization.

Two-day-old chicks were inoculated to establish whether this phenomenon also occurred in a commensally colonized host. On days 3, 6, 10 and 14 post-inoculation, ceca were extracted from the chicks and the mutant to wild-type ratio was determined. As shown in Figure 4.7b, the mean of each day showed very little change above or below a competitive index of 1 (despite variability between chicks), indicating that neither the wild-type nor the mutant had a competitive advantage in this model system ($p=0.82$). To determine whether L-fucose supplementation was capable of promoting a competitive advantage for *C. jejuni* wild-type growth in our chick model, we repeated the colonization experiment by orally feeding chicks with L-fucose three times daily at 8-hour intervals. The competitive index of the *cj0486* mutant was 0.11 (9 fold reduction) and 0.19 (5 fold reduction) at 3 and 7 days post-inoculation respectively (Figure 4.7). While statistically significant for only day 3 ($p<0.05$), this result indicates a notable decrease in the competitiveness of the mutant

compared to wild-type, suggesting a role for fucose utilization in the early stages of colonization. The competitive index of the *cj0486* mutant grown in MH medium or MEM medium supplemented with 25 mM L-fucose (for 18 hours) was 0.94 and 0.5 (2-fold reduction) respectively. These results indicated that L-fucose confers a fitness advantage to *C. jejuni* both *in vitro* and *in vivo*, and the up to 50-fold difference between the *in vitro* and *in vivo* piglet competitive indices indicates an improved competitiveness of the wild-type strain in the piglet infectious model possibly through enhanced virulence or induced pathogenic traits.

4.3.8. Cj0486 is important for *C. jejuni* cell invasion

Previous research by Javed *et al.* (106) on the *C. jejuni cj0480c-cj0490* genomic island indicated that this region is essential for a hyper-invasive phenotype. The addition of 5, 10 and 25mM L-fucose did not enhance *C. jejuni* invasion or adherence (Figure 4.8a, b). However, by comparing the invasion and adherence potential of the Δ*cj0486* mutant to the wild-type NCTC 11168, we have confirmed that the *C. jejuni* Δ*cj0486* mutant, unable to transport fucose, is also defective in its ability to invade Caco-2 intestinal epithelial cells (Figure 4.8b). A 4-fold reduction in invasion ($p<0.05$) but no significant difference in the ability to adhere to the cell surface was observed (Figure 4.8c). The inability to transport fucose, therefore, was linked solely to invasion, and not to adherence.

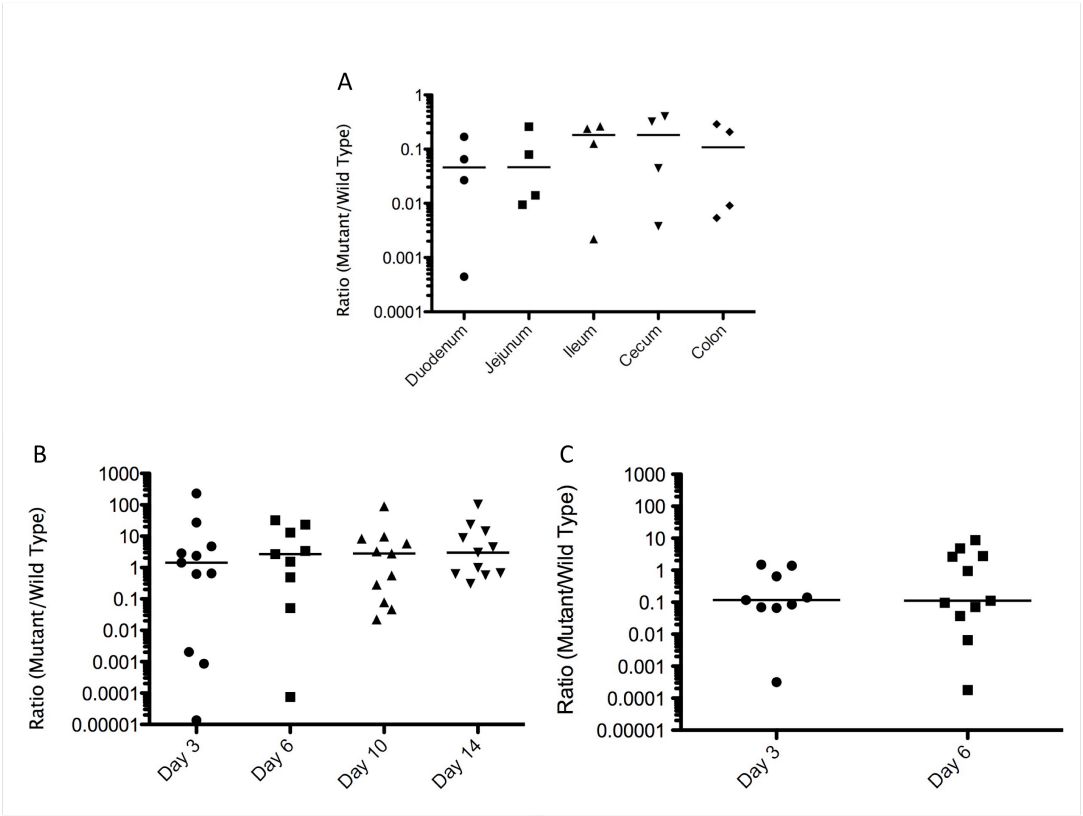

Figure 4.7: Competitive *C. jejuni* colonization assays. (A) Competitive *C. jejuni* colonization assay in neonatal piglets. The points represent the ratio of Δ*cj0486* mutant/wild-type NCTC 11168 recovered from each intestinal segment indicated, three days post inoculation. Values are adjusted relative to the ratio of the inoculum of 0.927. A line denotes the mean for each data set where n=4. Statistical significance was determined by a one-way ANOVA, $p<0.002$. (B) Competitive *C. jejuni* colonization assay of chicks over a two-week period post inoculation. Each point is the ratio of mutant to wild-type recovered from the cecum of a single chick. A line denotes the mean for each day, n = 9 to 11 per day. Statistical significance was determined by a one-way ANOVA, $p=0.82$. (C) Competitive *C. jejuni* colonization assay in chicks receiving an L-fucose dietary supplement over a six-day period post inoculum. Day 3, n=9 and day 6, n=11. Statistical significance was determined by a Student's *t*-test for each day, $p<0.05$ for day 3.

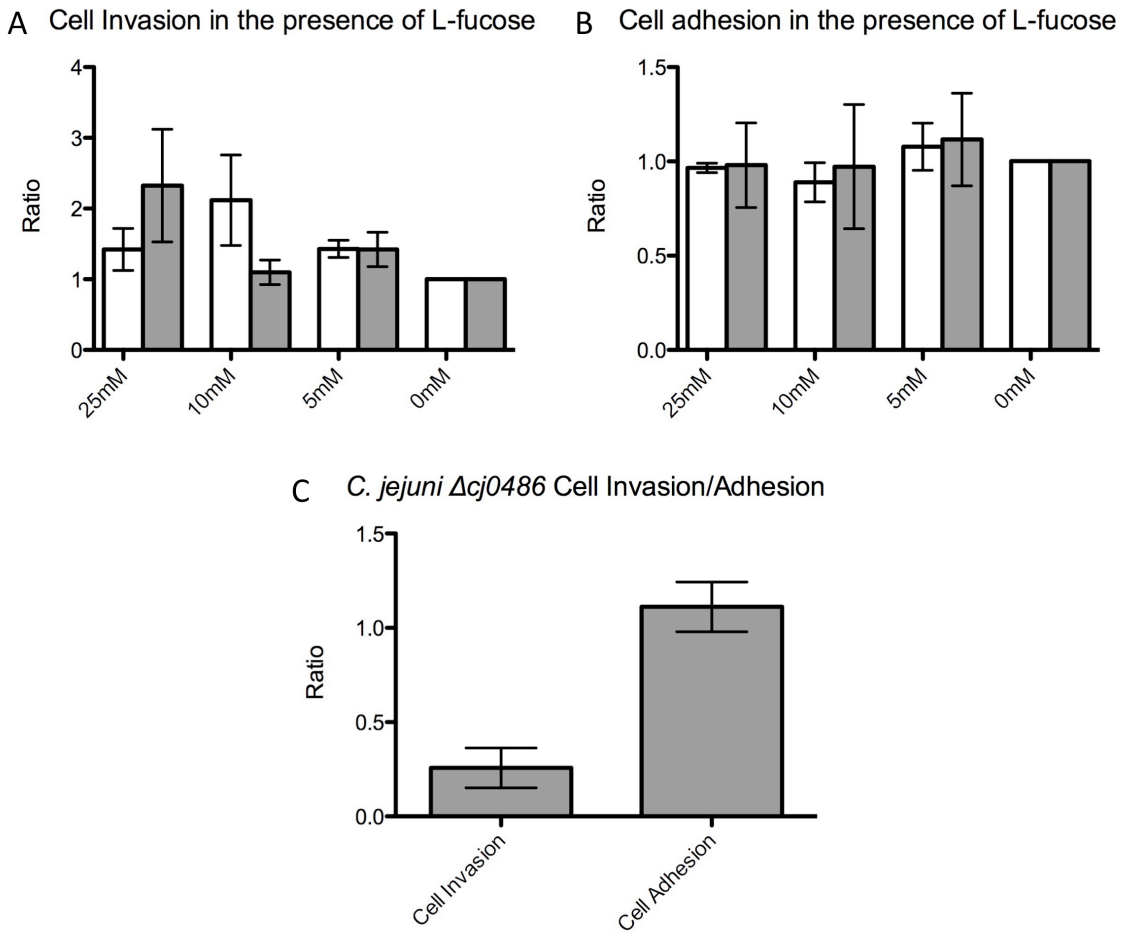

Figure 4.8: **Cell invasion by *C. jejuni* in the presence of L-fucose.** Cell invasion (A) and cell adhesion (B) by *C. jejuni* NCTC11168 (white) and 81-176 (grey) in the presence of 25mM, 10mM and 5mM concentrations of L-fucose in MEMα medium. Values are denoted as the ratio between cell invasion in the presence of L-fucose compared to a control of MEMα with no added L-fucose. (C) Cell invasion and adhesion by the *Δcj0486* mutant compared to the NCTC11168 wild-type strain in Caco-2 cells with MEMα medium with no added L-fucose. Statistical significance was determined by student's *t* test, $p<0.05$.

4.4. Discussion

Since early studies describing *C. jejuni* as an asaccharolytic organism, it was believed that *C. jejuni* relied completely on other molecules such as amino acids as substrates for growth (118). This was further confirmed when the first *C. jejuni* genome was sequenced and no known carbohydrate metabolic pathways were annotated (180). Contrary to this current dogma, we demonstrated that some strains of *C. jejuni* exhibit substantially increased growth in the presence of L-fucose (222). We also identified a key functional protein in this pathway, Cj0486, which acts as a fucose permease to transport L-fucose into the bacterial cell. This observation is consistent with results obtained by Muraoka *et al*. (171), who observed the reduction of tetrazolium with a phenotypic microarray, and increased growth in minimal media by *C. jejuni* NCTC 11168 in the presence of L-fucose.

The use of L-fucose as a nutrient source is common to bacteria, especially amongst the gut microbiota. Organisms such as *B. thetaiotaomicron* and *E. coli* use well-studied pathways for fucose metabolism (Figure 4.9) (27). Typically, a regulator (*fucR*), a permease (*fucP*), an isomerase (*fucI*), a kinase (*fucK*) and an aldolase (*fucA*) are required to produce lactaldehyde and dihydroxyacetone phosphate, which is fed into the glycolytic pathway. The lactaldehyde can then be converted into lactate under aerobic conditions by lactaldehyde dehydrogenase (*ald*), or 1,2-propanediol under anaerobic conditions by *fucO* (14). However, with the exception of *cj0486* (a homologue of *fucP*), and *ald* (*cj0490*), *C. jejuni* does not contain any identifiable homologues of this pathway. Instead, surrounding these two genes is a variety of other genes with largely unknown functions. These include an *iclR*-type regulator (Cj0480c), a putative dihydrodipicolinate synthase (Cj0481), a putative altronate hydrolase (Cj0483), an MFS transport protein (Cj0484), a short chain dehydrogenase (Cj0485), a putative amidohydrolase (Cj0487), and a hypothetical protein (Cj0488).

Our experiments confirmed that unlike in *E. coli* or *Bacteroides* species, *C. jejuni* does not phosphorylate or GDP-activate L-fucose for metabolism, which would be consistent with the absence of an annotated *fucK* or FKP encoding gene. This could suggest that *C. jejuni* metabolizes L-fucose via an alternate pathway. The genes identified here bear some resemblance to a fucose metabolic pathway recently identified in *Xanthomonas campestris* (258). This pathway utilizes an L-fucose dehydrogenase to form L-fuconolactone, followed by an L-fuconolactonase to form L-fuconate. Further steps can catabolize L-fuconate to smaller molecules such as pyruvate and L-lactate that can be used by other pathways of the cell via the mechanism described in *X. campestris* (Figure 4.9) (258), or the previously described L-rhamnose pathways (246). How closely *C. jejuni* may follow this model cannot be determined from the available information, and further research is necessary to elucidate the precise mechanisms employed by this pathway.

Experiments published by Stahl *et al.* (222) demonstrated that *cj0486* complements *E. coli fucP,* enabling the transport and subsequent metabolism of L-fucose. In experiments described here, we observed inducible, Cj0486-dependent uptake of ^{3}H-L-fucose by *C. jejuni* NCTC 11168 providing further support that Cj0486 functions as a fucose permease (222). Three amino acid residues in the *E. coli* FucP (Asp46, Glu135 and Asn162) have previously been found to be critical for the proper functioning of FucP. These three residues are also conserved within the sequence of Cj0486, supporting the observation that Cj0486 serves as a fucose permease.

The fact that *C. jejuni* NCTC 11168 receives a growth benefit from L-fucose supplementation suggests that *C. jejuni* is transporting L-fucose into the cytoplasm for

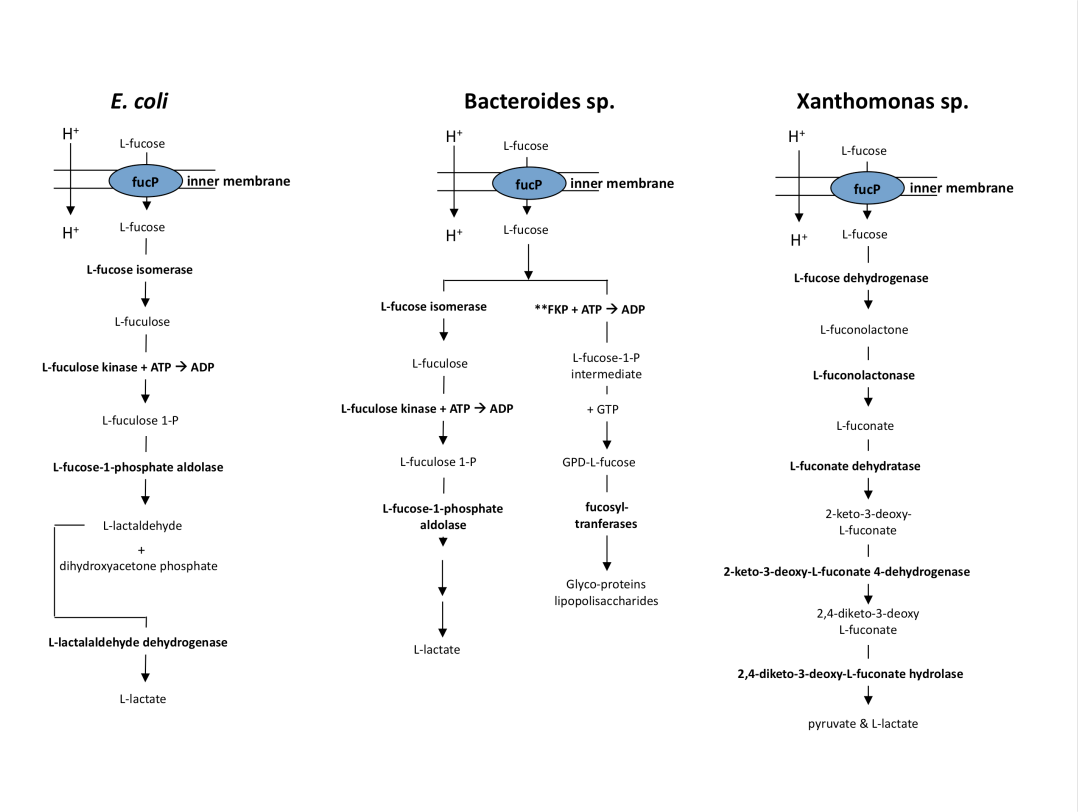

Figure 4.9: L-fucose metabolic pathways. General pathways for fucose uptake and utilization in *E. coli*, *Bacteroides* sp., and *Xanthomonas* sp. are depicted. **FKP: bifunctional L-fucokinase/GDP-fucose pyrophosphorylase. Adapted from (222).

metabolism. Although Liu *et al*. recently showed that the related organism, *Helicobacter pylori* can attach host-derived L-fucose to the bacterial outer surface (153); we do not currently have data demonstrating that this occurs for *C. jejuni*. An explanation for the down-regulation of the capsular polysaccharide (CPS) putative transferases *cj1434c* and *cj1438c* (Appendix VII) in the presence of L-fucose is also not immediately clear. However, cross-talk between glycoconjugate biosynthetic pathways has been shown previously (22, 79) and it could be speculated that L-fucose provides a cue to *C. jejuni* to downregulate CPS production.

Within the gut, the primary sources of L-fucose will be the host itself, where both host-secreted and cell surface-associated mucins (particularly in the small intestine and cecum) are heavily fucosylated. Previous studies into the fucose content of pig ileal digesta have found that as much as 74% of the fucose present is mucin-derived (147). Our microarray results from *C. jejuni* cultures grown in the presence of porcine gastric mucin as well as procine and chick intestinal mucus (Table 4.1) found a significant up-regulation of the genes *cj0481-cj0490*, similar to when *C. jejuni* is grown with L-fucose alone, demonstrating that purified mucin and intestinal mucus also triggers this operon. Interestingly, the chick small intestinal mucus did not trigger an up-regulation of this operon, and even in the chick cecal mucus, it proved less up-regulated relative to the porcine sample, suggesting a host difference in the intestinal mucus.

While mass spectrometry analyses described by Stahl *et al.* (222) indicate similar fucosylation levels in both chick and porcine mucins, it was observed that the fucosylated *O*-glycan mucin structures from chicks are more sulfated compared to their pig counterparts (222). The sulfation of mucin oligosaccharides may decrease the accessibility of fucose by mucin-degrading fucosidases (197) and has been demonstrated to decrease the intestinal

barrier permeability to pathogens, including *C. jejuni* (41). When an excess of free L-fucose was added to our chick colonization experiment, we observed a slight competitive advantage of wild-type *C. jejuni* over the *fucP* mutant further suggesting that the availability of fucose in the intestine influences bacterial fitness. The precise impact that mucin sulfation may have on the degradation of intestinal mucins and on nutrient availability in the gut remains largely unknown and deserves further study.

We demonstrated that a functional L-fucose permease provides a distinct competitive advantage for *C. jejuni* while colonizing the porcine intestine, not found during normal colonization of the chick cecum. A 15 to 100-fold reduction in the colonization of the *cj0486* mutant compared to the wild-type was observed in the piglet pathogenic model, while no significant difference was observed in the chick commensal model in the absence of added L-fucose. Differences in the microbial ecosystems between the two animals may alter the competition for these niches, making the ability to draw upon L-fucose valuable for *C. jejuni* colonization in the piglet, but less relevant in the chick. When the chicks were given an L-fucose supplement into their diet, the ratio of colonizing mutant and wild-type showed a distinct shift in favour of the L-fucose utilizing wild-type strain during early stages of colonization. The results point to the conclusion that if significant amounts of L-fucose are available in the environment, the ability to acquire and utilize L-fucose provides a competitive advantage for *C. jejuni.*

Even though we have now demonstrated *C. jejuni's* ability to utilize L-fucose, its importance *in vivo,* and we have hypothesized that mucin derived L-fucose represents the primary source of L-fucose to *C. jejuni* in its natural environment, several questions remain unanswered. Most bacteria that would be suspected of making use of glycans derived from intestinal mucins, contain fucosidases or other forms of glycosidases, however, there are no

obvious candidates for this role in *C. jejuni*. Therefore we decided to investigate how *C. jejuni* acquire L-fucose from mucin glycoproteins. The results from this study are presented in the following chapter.

CHAPTER 5

C. jejuni and the Intestinal Microbiota

5.1. Introduction

When colonizing its host, *C. jejuni* will proliferate within the mucus lining of the intestine. Although it can be found along the entire length of the intestinal tract, the highest levels of bacteria are recovered from the cecum and colon of the host (32). While *C. jejuni* will begin to infiltrate into the intestinal epithelium, most of the bacteria will be found in the outer (loose) mucus layer, or in some cases, in the inner mucus layer, along the epithelial wall, and into the intestinal crypts (110, 136). In the environment of the mucus layer, they must successfully compete and proliferate amongst the normal intestinal microbiota. To do this, according to Freter's nutrient niche hypothesis, they must successfully utilize one or more nutrients present within this medium in order to successfully colonize (66). Previous results here and elsewhere have described *C. jejuni*'s ability to utilize several nutrient sources including amino acids and L-fucose (93, 221, 241). Previous results in chapter 3 have described *C. jejuni*'s growth in extracted intestinal mucus, as well in media containing mucins. However, precisely how it can make use of these nutrient sources in the context of the intestinal environment remains poorly understood.

The gastrointestinal tract itself is a very complex ecosystem with as many as several hundred to over 1,000 different species of resident bacteria, although only 30-40 species make up 99% of the total population (54). The two largest single species present are *Bacteroides vulgatus* and *Bacteroides thetiotaomicron* (50), both belonging to the phylum *Bacteroidetes*. The *Bacteroidetes* are gram-negative, obligate anaerobes. One of their

notable properties is their ability to degrade a large variety of different polysaccharides in the gut, making them a cornerstone of the gut ecosystem (35, 257). Genomic and proteomic analysis of *Bacteroides thetaiotaomicorn* has found that it possesses 172 glycosylhydrolases, with 11% of these being located on the outer membrane, or released extracellularly (35). Together, the *Bacteroidetes* phylum generally comprises slightly less than half of the bacteria found in the intestine, although there is wide variation between individuals (50).

With roughly equal numbers to the *Bacteroidetes*, are the *Firmicutes*, which are predominantly represented by the class *Clostridia* in the gut (50, 74, 235). These comprise most of the gram-positive bacteria found in the intestine, and make up as much as half of the total number of bacteria, although they also represent most of the diversity in terms of bacterial species (50). The *Proteobacteria, Actinobacteria, Verrucomicrobia,* and *Fusobacteria* all together make up only a small percentage of the remaining bacteria of the microbiota (50, 74). Together, these bacteria form a highly complex and integrated ecosystem within the gut (190).

Despite the complexity and relative stability of the human adult microbiota, in early childhood, the intestinal microbiota undergoes rapid change. The initial colonization of the neonatal intestinal tract in the day following birth has been found to be primarily by aerotolerant bacteria such as *Streptococcus spp.*, *Enterobacter* spp., *Lactococcus lactis* and *Leuconostoc citreum* (177, 204). Most of these bacteria only transiently colonize, and in the ensuing few days of life are quickly followed by high numbers of aerobic *Proteobacteria* such as *E. coli*, *Enterococcus faecalis*, and *Enterobacter* spp. (54, 174, 177). Within several weeks or months, the anaerobic *Bifidobacterium* of the phylum *Actinobacteria* establishes itself as a major component of the intestinal microbiota and the dominance of the *Proteobacteria* within the microbiota begins to fade (119, 159, 174). It is not until the

introduction of solid food post-weaning that the anaerobic *Firmicutes* and *Bacteroidetes* establish themselves as the dominant component of the human microbiota, and the composition of the microbiota begins to resemble that of an adult (54, 119, 159, 174).

While this process varies considerably between individuals, and depends on many factors such as diet, genetics and environment, the general characteristics of this microbial succession are consistent between individuals (54, 159, 174). It is believed that there are two main factors driving the slow development of this ecosystem to the adult microbiota. Firstly, the gastrointestinal tract is not initially an anaerobic environment (159). Swallowed oxygen, and diffusion from the surrounding tissues create an environment that is microaerophilic and possibly not very suitable for the strict anaerobes that make up the adult microbiota (88, 210). It has been hypothesized that the early dominance of aerotolerant bacteria such as the *Proteobacteria* and *Bacilli* help to establish a reduced environment more suitable to anaerobic bacteria such as the *Clostridia* and *Bacteroidetes* (159, 223). It is also hypothesized that this succession of species helps to slowly develop the complex interdependent metabolic and immunoregulatory relationship between the different bacteria of the microbiota and the host (96, 119, 174, 199).

C. jejuni will generally be colonizing individuals with a more mature microbiota comprised mostly of obligate and facultative anaerobes. Despite this, from what we know of its metabolic needs, it is an obligate microaerobe, not able to grow in a strictly anaerobic environment (214), and it utilizes a relatively limited number of amino acids, citric acid cycle intermediates and in some cases L-fucose to survive (120, 222). Despite these limitations, it must compete and survive within the intestinal microbiota.

It has been previously observed that *B. thetaiotaomicron* will induce increased fucosylation of mucins, where it can then use a α-1, 2 fucosidase to free L-fucose monomers

for its own use (97, 263). This same species as well as many other species of bacteria are known to degrade mucins or other ingested macomolecules such as starches or insoluble polysaccharides to provide themselves with plentiful sources of sugars or amino acids (60, 61, 140). While it is known that *C. jejuni* will utilize the amino acids serine, aspartate, glutamate, and proline, and the sugar L-fucose, all of which are present in mucins, no candidate extracellular glycosidases or proteases have been characterized that would indicate how *C. jejuni* might acquire these molecules from large mucin glycoproteins (180). If *C. jejuni* does not possess any of these enzymes itself, then it is possible that *C. jejuni* might benefit from the degradation of these molecules by the other plentiful bacteria that inhabit the mucus along side *C. jejuni.*

This study seeks to demonstrate how *C. jejuni* integrates itself into the intestinal microbiota and establishes itself within the ecological niche of the intestine. Within the environment of the mucus layer, mucins provide one of the most prominent sources of the nutrients *C. jejuni* needs to survive, but the question remains whether it can directly utilize these nutrients, or whether it relies on the metabolic activities of the plentiful bacteria from the host microbiota.

5.2. Materials and Methods

5.2.1. Bacterial strains and Growth conditions

The *C. jejuni* strains NCTC11168, 81-176 and RM1221 were used in this study. Routine growth of *C. jejuni* was carried out on MH agar plates, MH biphasic flasks, or MH liquid cultures. Cultures were incubated at 37°C in MACS microaerophilic incubators with a gas concentration of 8% O_2, 4% H_2, 5% CO_2, and 83% N_2. The anaerobic bacteria *Bacteroides thetaiotaomicron* ATCC 29148*, Bacteroides vulgatus* ATCC 8482*, Clostridium*

ramosum, Clostridium perfringens, Clostridium difficile and *Fusobacterium nucleatum* were routinely grown on anaerobic basal agar (ABA) plates (Oxoid), or in cooked meat broth (Oxoid) at 37°C under anaerobic conditions (10% CO_2, 5% H_2, 85% N_2). The aerobic bacteria *Pseudomonas aeruginosa, Escherichia coli* K12, and *Enterococcus faecalis* ATCC 19433 were routinely grown on Luria-Bertani (LB) agar plates or LB broth at 37°C under standard aerobic conditions.

5.2.2. Growth on whole or degraded mucins

We compared the growth of *C. jejuni* on whole mucins to mucins previously degraded by proteinases or hydrolyzed by sodium hydroxide. One ml aliquots of 10 mg/ml porcine gastric mucin were suspended in dH_2O. For proteinase degradation: 15 μg/ml of proteinase K was added to the 10 mg/ml mucin aliquot and incubated for 3 hours at 37°C. For sodium hydroxide degradation: 10 M NaOH was added to the 10mg/ml mucin aliquot to a final concentration of 0.3 M. After 1-hour incubation at room temperature, the pH was neutralized with a molar equivalent addition of 5M acetic acid. The 10 mg/ml mucin aliquot was added to 9ml of MEMα medium supplemented with 20 μM $FeSO_4$, for a final concentration of 1 mg/ml whole or degraded mucin, or 1 ml of sterile dH_2O was used as a negative control. Growth assays were conducted using a Bioscreen C instrument as described previously in chapters 3 and 4.

5.2.3. Mucin plate degradation assays

Mucin plate degradation assays were carried out following previously published approaches (57, 84, 260), but with several key differences in the growth medium. In order to allow for the growth of all the desired *C. jejuni*, aerobic and anaerobic bacteria, fastidious

anerobe broth (FAB) (LabM) was used as a base medium, supplemented with 0.5% porcine gastric mucin and solidified with 1.5% agarose. Ten µl from an overnight culture of the desired bacteria grown under their appropriate routine growth conditions were spotted on agar-plates. Plates were briefly allowed to dry, and then incubated for 48 hours under microaerophilic, aerobic or anaerobic conditions depending on the requirements of the inoculated bacteria. Finally, the plates were stained with 0.1% Amido Black (Sigma) in 3.5 M acetic acid for 2 hours and destained for 2 hours with 1.2 M acetic acid.

5.2.4. Mucin and Gelatin Zymogram

Zymograms were conducted using 12% polyacrylamide SDS-PAGE gels, containing 1% gelatin or porcine gastric mucin (with 4% polyacrylamide stacking gel). Bacterial cultures grown overnight in FAB medium supplemented with 0.5% porcine gastric mucin were centrifuged to pellet the bacterial cells, and 40 µl of the supernatant was added into each well. The same culture was used to load both gelatin and mucin-containing gels so as to test both substrates for proteolytic activity. Control lanes consisted of 20 µl of 0.25% trypsin. The SDS-PAGE gels were run at 90V for approximately 45 minutes. The SDS was then removed from the gels with two 20-minute washes with sterile dH_2O containing 2.5% triton-X-100. The gels were then incubated for 48 hours in incubation buffer (50 mM Tris-HCl (pH 8) and 5 mM $CaCl_2$) at 37°C. Finally, gels were washed in sterile dH_2O, stained with coomassie blue, destained (50% methanol, 10% acetic acid), washed with 10% acetic acid and photographed.

5.2.5. Coculture Growth Assay

C. jejuni, *B. thetaiotaomicron, B. vulgatus, C. perfringens* and *F. nucleatum* were grown overnight in MH (*C. jejuni*) or FAB (anaerobes) broth under microaerophilic or anaerobic conditions respectively. A 10^6 CFU incoculum of *C. jejuni* was used to inoculate 75ml of FAB, supplemented with 0.5% porcine gastric mucin, and allowed to grow overnight for 9 hours under microaerophilic conditions. Next, 10^7 CFUs of one of the four anaerobic bacteria was added to the *C. jejuni* culture, and growth was allowed to continue for 48 additional hours under microaerophilic conditions, with CFU counts every 12 hours. For each time-point, 1ml aliquot was removed from the culture, serially diluted, and plated on MH and ABA plates and incubated at 37°C under microaerophilic or anaerobic conditions. After 48 hours, colonies were enumerated and statistical significance was determined by Student's *t*-test.

5.2.6. Gnotobiotic mouse mono and co-colonization

Eighteen female Swiss Webster germ-free mice were housed in pairs, in gnotobiotic isolators, where they were fed autoclaved standard chow diets. Fecal samples were collected, resuspended in fastidious anaerobe broth, and incubated under aerobic, anaerobic and microaerobic conditions to confirm sterility. The mice were divided into groups of six and inoculated with a single gavage of approximately 10^7 CFUs of *C. jejuni* NCTC 11168, *B. vulgatus* ATCC 8482, or an equal mixture of both strains. The mice were maintained under sterile conditions for 14 days. Every two days, the mice were weighed and fresh stool samples were collected, serially diluted, plated, incubated under anaerobic and microaerobic conditions and the numbers of *C. jejuni* and *B. vulgatus* per gram of fecal contents were determined. Fourteen days post-inoculation, the mice were euthanized, weighed, and their

ileum, cecum and colon were immediately collected and processed to determine the level of bacterial colonization and cytokine production as follows:

i) Bacterial Colonization

Samples from the ileum, cecum and colon of each mouse, were collected, weighed, cut longitudinally, homogenized, serially diluted in FAB and plated on MH and ABA plates. The plates were incubated for 48 hours under anaerobic or microaerophilic conditions, and the resulting colonies were enumerated. The numbers of colonies were calculated per gram of intestinal content.

ii) Cytokine production

Tissues samples from the cecum and colon from each mouse were cut longitudinally to reveal the inner epithelium. Samples were washed with sterile PBS buffer to remove luminal contents and cut with a sterile scalpel into $1cm^2$ sections. Each section was submersed in 1ml RPMI medium containing 1% FBS, 50μg/ml gentamicin, and incubated at room temperature for 30 minutes with gentle shaking. The medium was then replaced with 1ml of fresh RPMI medium containing 1% FBS, 50μg/ml gentamicin, 1X Antibiotic, Antimycotic Solution (Sigma, A5955); and they were incubated for 24 hours, at 37°C. The supernatants were then collected and stored at -20°C until needed. The production of TNFα, IL-10 and IFNγ within the supernatants was measure in duplicate using the mouse cytokine ELIZA kits (Pierce) and following the manufacturer protocols.

5.3. Results

5.3.1. *C. jejuni* benefits from the exogenous degradation of mucin

In the experiments previously described in chapter 3, figure 3.2, *C. jejuni* showed a small, but statistically significant increase in growth, when grown in MEMα supplemented with 1 or 2 mM porcine gastric mucin. However, this increase in growth was much smaller than expected given the amount of mucin proteins being added to the medium. Considering that mucins are exceedingly large glycoproteins that need to be degraded into short peptides or individual amino acids or glycan before they can be transported into the cell, we hypothesized that a lack of protein degradation may explain the limited increase in *C. jejuni* growth. When this mucin was degraded prior to inoculation with *C. jejuni*, through sodium hydroxide hydrolysis, or incubation with proteinase K, growth was significantly increased. Overnight incubation with proteinase K increased the O.D. obtained by 20.0% ($p<0.05$) (Figure 5.1). Incubation with NaOH, which will hydrolyze the mucins into individual monosaccharides and amino acids, increased the O.D. obtained by 45.7%, ($p<0.05$), (Figure 5.1). These results indicated that the exogenous degradation of the mucin proteins substantially increases the growth of *C. jejuni,* suggesting that *Campylobacter* has little to no ability to degrade mucin on its own.

5.3.2. *Campylobacter* does not display any mucolytic activity *in vitro*

To confirm that *C. jejuni* does not display any mucolytic activity when growing in a medium containing mucin, a mucin plate degradation assay was performed on *C. jejuni* and several other representative species of bacteria. *Pseudomonas aeruginosa* has been previously described as having mucolytic properties (90), and was used as a positive control, while *Bacteroides thetaiotaomicron, Bacteroides vulgatus, Clostridium difficile, Clostridium*

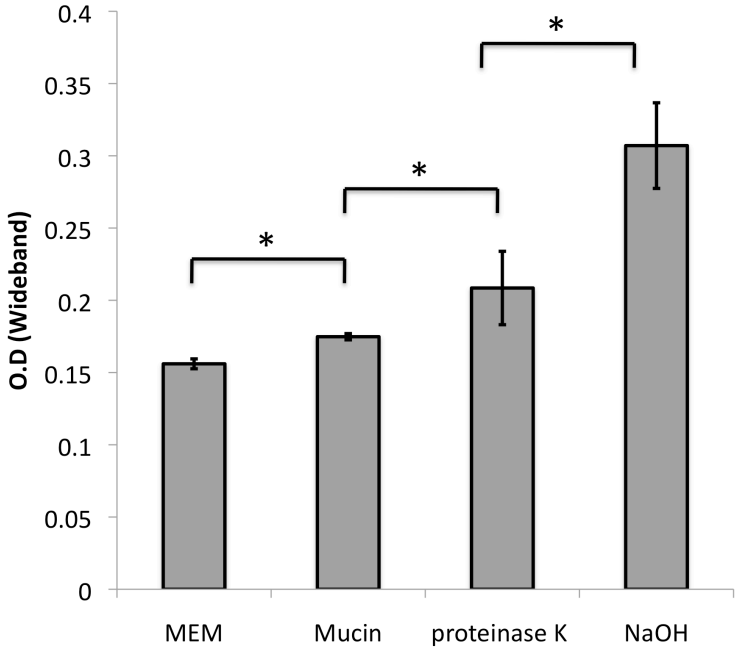

Figure 5.1: Growth of *C. jejuni* on whole, or degraded porcine gastric mucin. Maximum O.D. obtained during 36 hours of growth by *C. jejuni* NCTC11168, while growing in MEMα supplemented with whole, or porcine gastric mucin treated with proteinase K or NaOH. Statistical significance was determined using a *t*-test, *$p<0.05$, n=40.

perfringens, *Clostridium ramonsum, Enterococcus faecalis, Fusobacterium nucleatum,* and *E. coli* were used as representative members of the intestinal microbiota. For *C. jejuni*, three strains were tested, NCTC11168, 81-176 and RM1221. The results are summarized in table 5.1 and figure 5.2. As expected *P. aeruginosa* displayed significant clearing around the colony, indicating mucolytic activity (Figure 5.2d). Likewise, *C. perfringens and B. vulgatus* showed significant clearing (Figure 5.2e, f). The mucolytic activities of *B. thetaiotaomicron, C. ramosum, C. difficile, E. coli* and *E. faecalis* were found to be lower, but still distinctly visible areas of clearing around their colonies were observed (Figure 5.2h-k). In contrast, *F. nucleatum* showed no visible mucolytic activity (Figure 5.2l), consistent with a previously observed lack of glycolytic or proteolytic activity in the organism (251). The three *C. jejuni* strains tested did not show any visible mucin clearing around their colonies, supporting the hypothesis that they do not exhibit any mucolytic activity (Figure 5.2a-c).

To validate these findings, we assessed the mucolytic activity of *C. jejuni* and the above-mentioned bacteria by zymographic analysis. The gels containing either gelatin or mucin as a substrate were loaded with culture supernatant from *C. jejuni* cultures, the cultures of the bacteria listed above or 0.25% trypsin as a positive control for proteolytic activity. Bands of clearance in the gel following 2 days of incubation at 37°C indicate the presence of active proteolytic enzymes. Consistent with the results from the mucin degradation plates, *P. aeruginosa, C. perfringens* and *B. vulgatus* showed significant clearance (Figure 5.3). The results were also consistent between samples on both the mucin and gelatin-loaded gels, however only the gelatin-loaded gels are displayed in figure 5.3 due to very light staining in the mucin-loaded gels making visualization difficult. Although the size of the cleared area prevented any accurate counting of the number of bands,

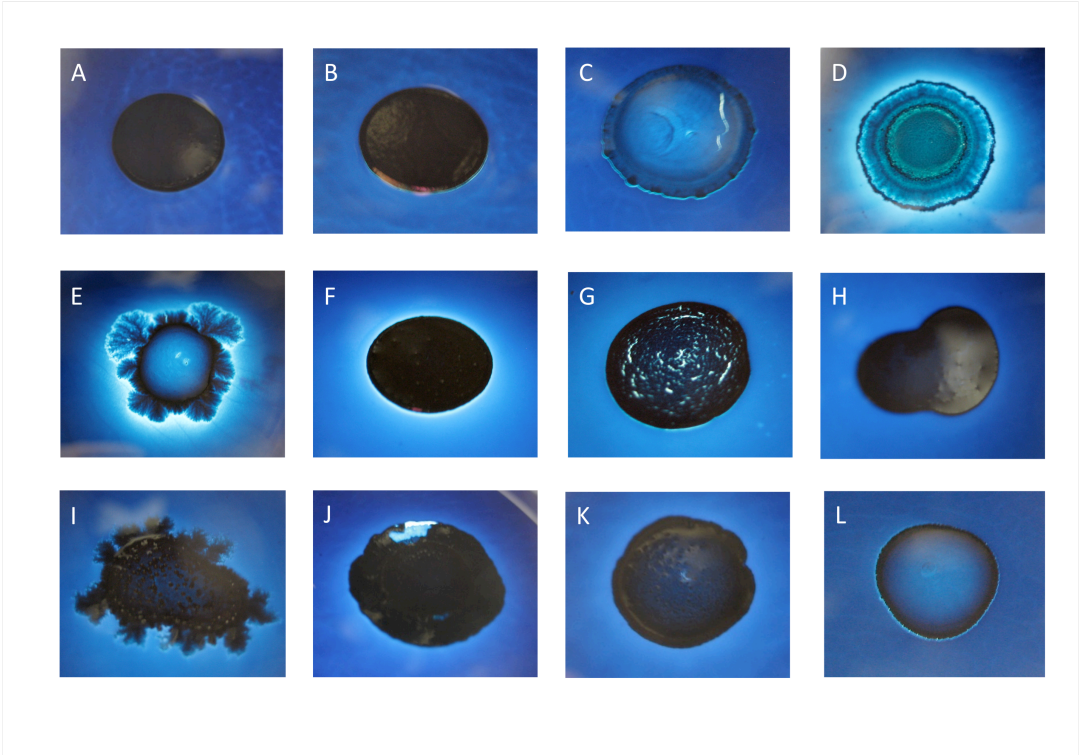

Figure 5.2: Mucin plate degradation assays.(A) *C. jejuni* NCTC11168, (B) *C. jejuni* 81-176, (C) *C. jejuni* RM1221, (D) *Pseudomonas aeruginosa*, (E) *Clostridium perfringens*, (F) *Bacteroides vulgatus*, (G) *Bacteroides thetaiotaomicron*, (H) *Clostridium ramosum*, (I) *Clostridium difficile*, (J) *Escherichia coli* K12, (K) *Enterococcus faecalis*, (L) *Fusobacterium nucleatum*. Plates were stained with amido black and photographed in front of a light source.

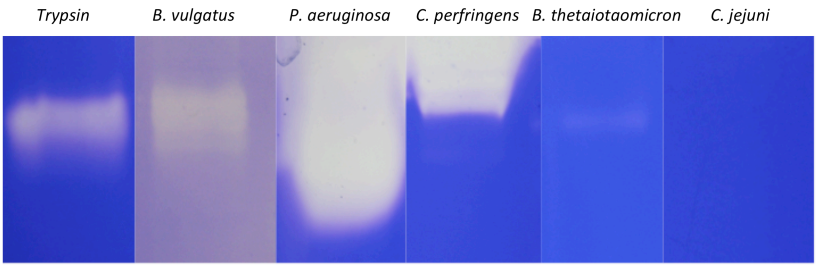

Figure 5.3: Gelatin zymograms loaded with culture supernatants. Compilation of representative zymograms, containing 1% gelatin as a substrate protein, and loaded with the indicated sample supernatant. Trypsin serves as a reference control for proteolytic acitivity. To note, the lanes shown were not from identical gels, and size cannot be inferred from the bands.

Species	Mucin degradation (plate)	Zymogram
C. jejuni NCTC11168	None	None
C. jejuni 81-176	None	None
C. jejuni RM1221	None	None
Pseudomonas aeruginosa	++	++
Enterococcus faecalis	+	None
Escherichia coli K12	+	None
Clostridium perfringens	++	++
Clostridium difficile	+	NA
Clostridium ramosum	+	NA
Bacteroides thetaiotaomicron	+	+
Fusobacterium nucleatum	None	None
Bacteroides vulgatus	++	++

Table 5.1: Summary of the results of the plate degradation assays, and mucin/gelatin zymogram assays. The ranking of the proteolytic activity of each organism was based on a visual characterization of the intensity of the halo or band. ++ = significant proteolytic activity, + = minimal proteolytic activity, none = no proteolytic activity, NA = not assayed.

P. aeruginosa, B. vulgatus and *C. perfringens* appeared to produce multiple bands, indicating the presence of multiple proteolytic enzymes. *B. thetaiotaomicron* culture supernatant produced a single, faint band on the gel. No other culture supernatant tested produced any visible bands. This included the three *C. jejuni* strains, which once again displayed no evidence of proteolytic activity.

5.3.3. Coculture with *C. jejuni* permits the growth of anaerobes under microaerophilic conditions

Our previous results would support the hypothesis that despite living in an environment rich in mucin proteins, *C. jejuni* itself possesses no appreciable ability to degrade mucins on its own. However, when colonizing the intestine of its host, it is likely to be in close proximity to bacteria comprising the intestinal microbiota capable of degrading mucins. This does present an apparent contradiction since many of the mucolytic bacteria found in the intestine are strict anaerobes, while *C. jejuni* requires low levels of oxygen to survive. We therefore hypothesize that the intestine consists of a microaerophilic environment and that *C. jejuni* has the capacity to consume excess oxygen from this medium, thereby creating an anaerobic environment and contributing to the survival of obligate anaerobes.

When cultures of *C. jejuni* and anaerobic bacteria (*C. perfringens, B. vulgatus. B. thetaiotaomicron,* or *F. nucleatum*) were grown together in mucin-containing FAB medium, under microaerophilic conditions, the coculture with *C. jejuni* enables the growth of the strict anaerobic bacteria in the presence of 8% O_2 (Figure 5.4). When the anaerobic bacteria were grown in the same conditions under monoculture, the cultures did not demonstrate any growth above the inoculum, and no viable bacteria were recovered from the cultures at 24

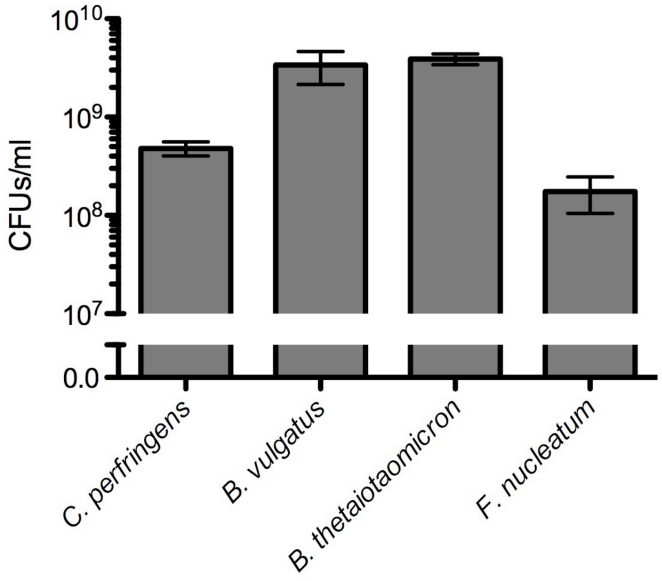

Figure 5.4: Growth of anaerobic bacteria under microaerophilic conditions in the presence of *C. jejuni.* The number of colony forming units (CFU) per ml of FAB supplemented with 0.5% porcine gastric mucin, following 24 hours of growth under microaerobic (8% O_2) conditions. Each culture was grown in coculture between the indicated anaerobic bacteria and *C. jejuni* NCTC11168. No viable bacteria were recovered from comparable cultures of the anaerobes grown in monoculture under microaerobic conditions following 24 hours, n=3.

hours (data not shown). A resazurin indicator in the FAB medium indicated that *C. jejuni* growth contributed to anaerobiosis of the medium. This commonly used oxygen-indicator turns pink when exposed to oxygen, while it stays clear under anaerobic conditions (116). After overnight growth, flasks with *C. jejuni* or co-cultures had little-to-no visible oxidized resazurin (yellow colour), indicating low oxygen tension. In contrast, the flask inoculated with the anaerobic bacteria continued to contain oxidized resazurin (red-brown colour), indicating the continued presence of oxygen (Figure 5.5).

5.3.4. Coculture with mucolytic bacteria enhances *C. jejuni* growth

While coculture of strict anaerobes with *C. jejuni* allows for the growth of the anaerobic bacteria in the presence of oxygen, there is also the potential for a reciprocal relationship between these bacteria. When grown in FAB medium containing 0.5% mucin as described above, *C. jejuni* also shows a significant 5-10 fold increase in growth while growing in the presence of the mucolytic bacteria, *C. perfringens*, *B. thetaiotaomicron* and *B. vulgatus* (Figure 5.6). This boost of growth was not observed when *C. jejuni* was grown in presence of the non-mulcolytic bacterium *Fusabacterium nucleatum* highlighting the importance of the mucolytic activity.

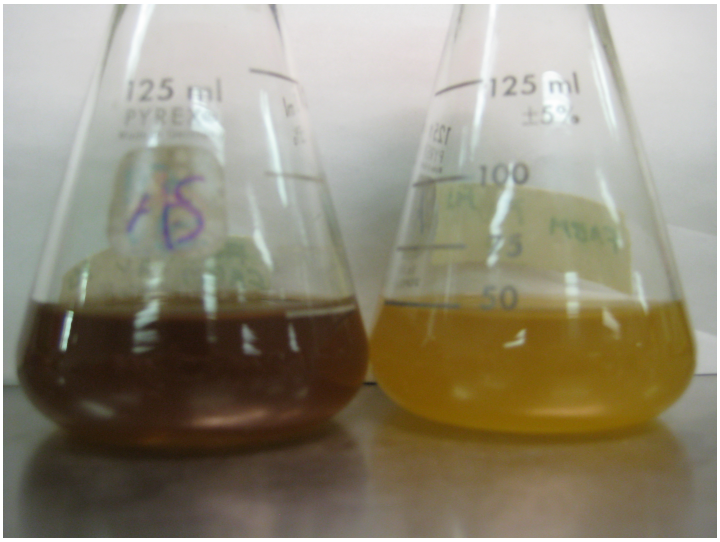

Figure 5.5: **Oxygen content of co-culture and mon-culture flasks under microaerophilic conditions.** Representative example of anaerobic bacteria (*B. vulgatus)*, inoculated into a flask of resuzurin-containing FAB medium supplemented with porcine gastric mucin, and incubated for 24hours at 37°C under microaerophilic conditions (8% 0_2, 4% H_2, 10% CO_2) (left), and in coculture with *C. jejuni* (right). The resazurin present within the FAB medium produces a redish-brown colour in the presence of oxygen.

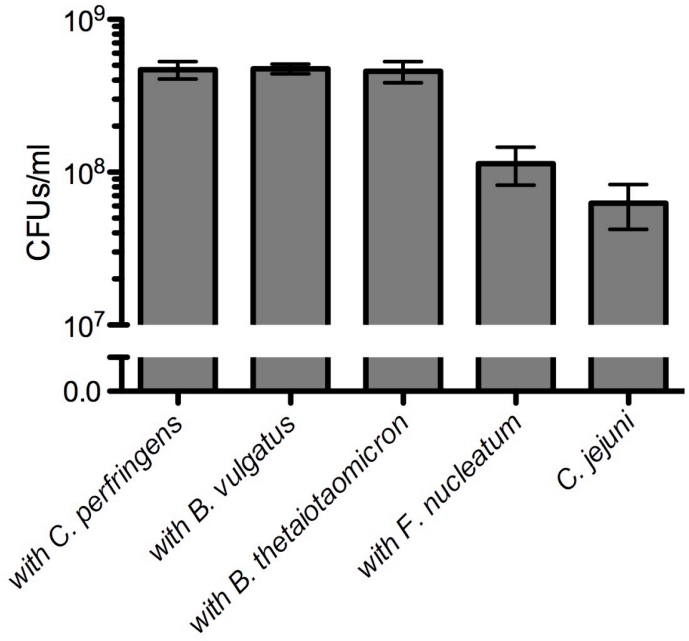

Figure 5.6: **Growth of *C. jejuni* alone and in coculture with select anaerobic bacteria**. Results are displayed as the number of CFUs recovered/ml following 24 hours growth under microaerobic conditions. Statistical significance determined using a 1-way ANOVA ($p<0.0001$), with Bonferroni post-test. *C. jejuni* with *C. perfringens, B. vulgatus, and B. thetaiotaomicron* show statistically significantly ($p<0.05$) higher growth than with *F. nucleatum*, or alone. The difference between growth with *F. nucleatum* and alone is not significant ($p>0.05$).

5.3.5. Co-inoculation with *B. vulgatus* boosts *C. jejuni* growth *in vivo*

Three-week-old germ-free mice were either mono- or dual-associated with *C. jejuni* and/or *B. vulgatus*. Every other day, the mice were weighed and fecal samples were collected to assess intestinal bacterial colonization. Two weeks post incoculation, the mice were euthanized, and intestinal samples were collected to determine the relative colonization level of dual and mono-associated mice. Mono or dual-associated mice had no apparent clinical sign of colitis or disease. Gained weight varied significantly between mice during the first 5 days post-inoculum (0-15% change per measurement) with no significant trend for any group. From days 7 to 14, gained weight was steady (5-10% per measurement), with no difference between groups.

Fecal samples were used to monitor the colonization by *C. jejuni* and *B. vulgatus* during the course of the experiment. *C. jejuni* recovered from the fecal samples of the dual-associated mice was slightly, but consistently ($p=0.02$) higher than from the mono-associated mice (Figure 5.7a). Post-mortem, samples of the ileum, cecum and colon were extracted, and the number of bacteria per gram of intestine was determined. Slightly higher numbers of *C. jejuni* were recovered per gram of intestine from the cecum and colon of dual-associated mice (Figure 5.8b,c), however the difference was not statistically significant based on a Mann-Witney test. In contrast, higher numbers of *C. jejuni* were recovered from the ilea of mono-associated mice, compared to dual-associated mice ($p=0.025$).

In contrast to *C. jejuni*, no substantial differences in the number of *B. vulgatus* was recovered from the fecal or intestinal samples of the mono and dual-associated mice (Figure 5.7b, Figure 5.8d-f). The exception to this was a substantial drop in the numbers of *B. vulgatus* recovered from the fecal samples of the mono-associated mice on days 9 and 11 (Figure 5.7b), which was not observed in the corresponding dual-associated mice, however,

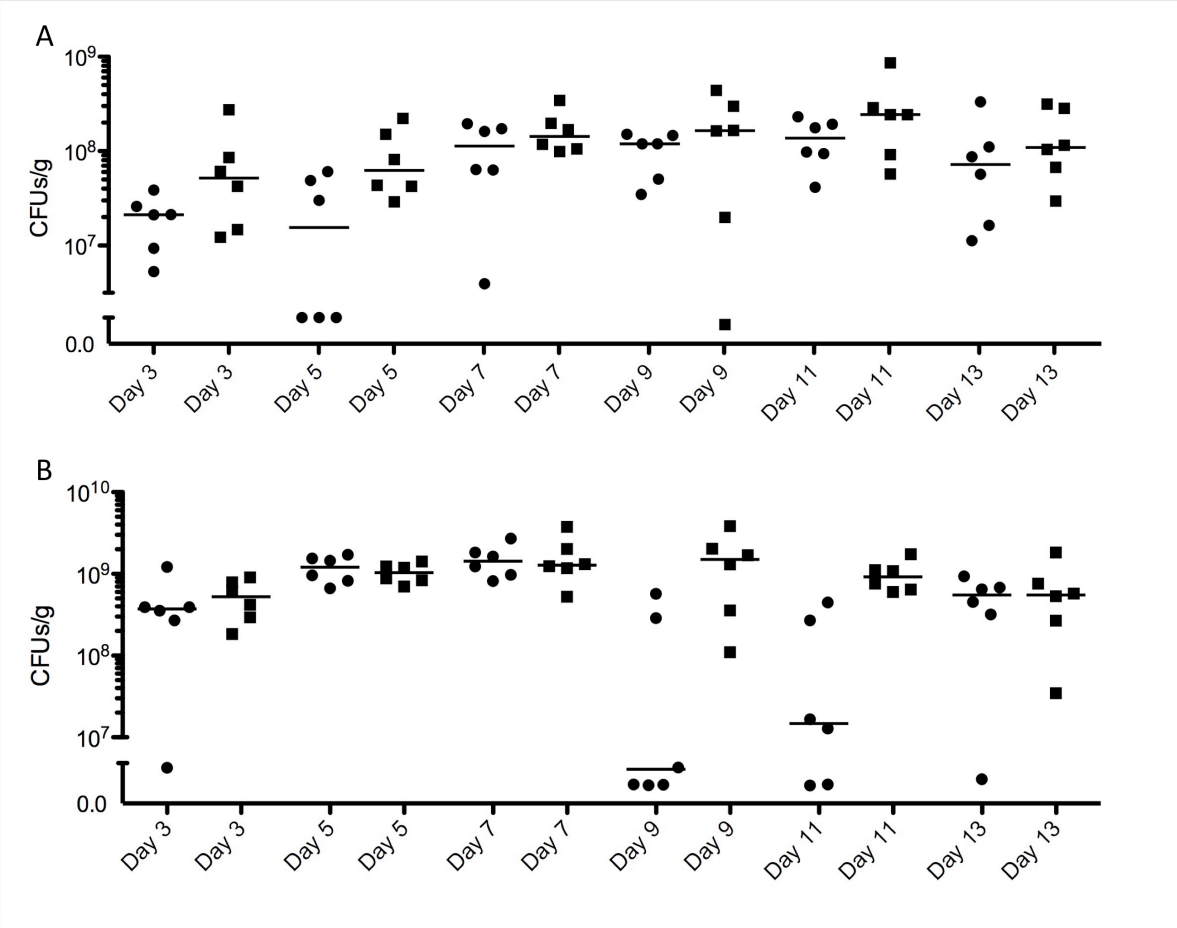

Figure 5.7: Bacterial enumeration from mouse fecal samples. The number of CFUs of *C. jejuni* (A) and *B. vulgatus* (B) recovered from the feces of each mouse. Each circle indicates a sample from a mono-associated mouse, while each square represents a sample from a dual-associated mouse. The number of CFUs was normalized against the grams of fecal material. A 1-way ANOVA showed a statistically significant difference between the mono and dual-associated mice for *C. jejuni* (A)($p=0.02$).

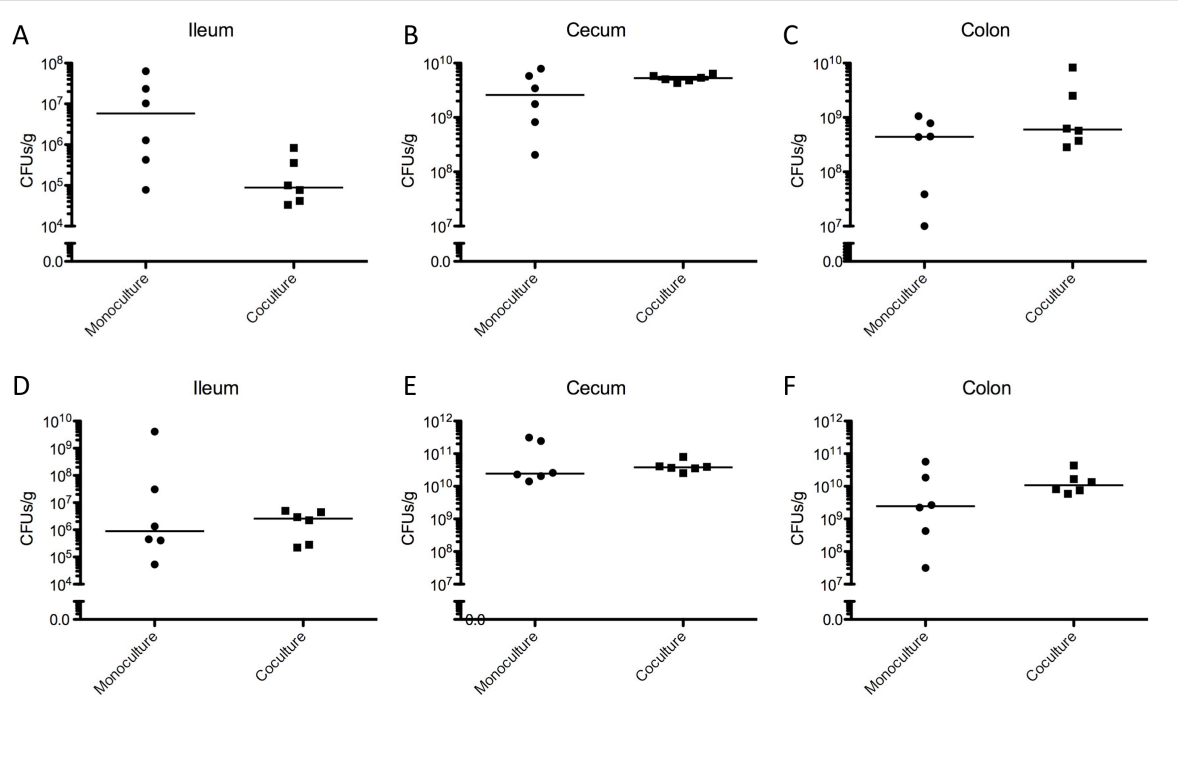

Figure 5.8: Bacterial enumeration from mouse intestinal samples. The number of CFUs recovered per gram of intestine from germ-free mice inoculated with monocultures of *C. jejuni*, *B. vulgatus*, or a 1-1 mixture. Circles indicate samples from mono-associated mice, while squares indicate samples from dual-associated mice. (A-C) the number of *C. jejuni* recovered, (D-F) the numbers of *B. vulgatus* recovered.

the numbers returned to previous levels, by day 13, and no substantial difference was observed in the intestinal samples.

5.3.6. Cytokine assay

In addition to assess differences in the numbers of *C. jejuni* colonizing the mouse intestine between mono and dual-associated mice, we also sought to determine if mono- or co-colonization by *C. jejuni* and/or *B. vulgatus* would trigger an inflammatory response by measuring cytokine production in the mouse intestine. Immediately after euthanazia, intestinal segments were taken from the cecum and colon from each mouse. These were cut into equal 0.25cm^2 squares and cultured overnight in RPMI medium to collect cytokines produced by the intestinal tissue. ELIZA kits (Pierce) were used to quantify TNF-α, IL-10 and INFγ collected in the supernatant (Figure 5.9). Low levels of TNFα were observed in the mice mono-associated with *C. jejuni*, while little to no TNFα was found in the other two groups. Notably higher levels of IL-10 and INFγ were observed in dual associated mice than in the mice mono-associated with *C. jejuni* or *B. vulgatus*. It should also be noted that levels of INFγ in the *C. jejuni* samples correlated very closely with the numbers of *C. jejuni* recovered from each mouse ($R=0.90$, $p=0.013$). The possible correlation is also present in the co-colonized samples ($R=0.72$, $p=0.10$), although at a lower statistical significance.

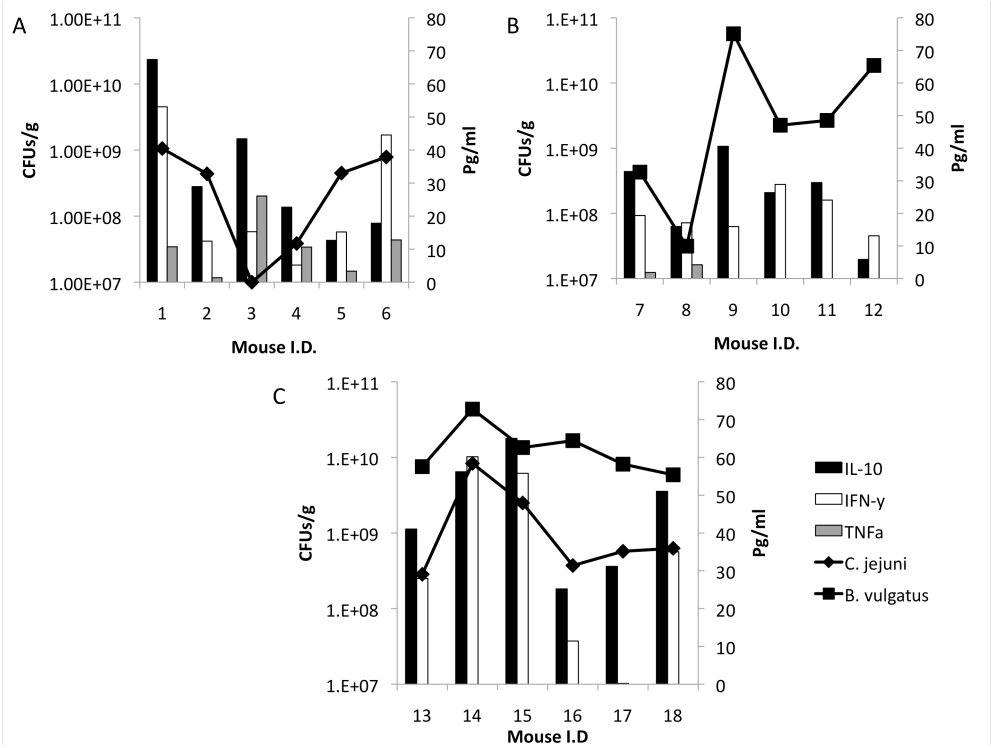

Figure 5.9: Cytokine production from whole-organ tissue culture. The levels of TNFα, IFNγ and IL-10 recovered from the supernatants of whole–organ cell-cultures, plotted against the number of CFUs of bacteria/g of intestinal tissue for each mouse. Mice 1-6 (A) were mono-associated with *C. jejuni*, mice 7-12 (B) were mono-associated with *B. vulgatus* and mice 13-18 (C) were dual-associated.

5.4. Discussion

When colonizing the gastrointestinal tract, *C. jejuni* establishes itself within the mucus lining of the intestine. While *C. jejuni* has the capacity to establish itself close to the epithelial layer, most of the *C. jejuni* cells will inhabit the outer layers of the mucus layer (135), where it will be dispersed amongst the normal intestinal microbiota. Being able to compete in this environment is key to establish itself within the intestine. *C. jejuni*, along with other commensals and pathogens, must have the capacity to find an ecological niche within the gut where it can establish itself as part of the intestinal microbiota (66).

As we discussed in the previous chapter, we have found that *C. jejuni* is able to metabolize L-fucose, which confers a competitive advantage when L-fucose is available, such as in the piglet infection model (222). Previous research has also found that serine is essential for *C. jejuni* colonization, even when it is not necessary for in vitro growth, suggesting it has a prominent role in *C. jejuni's* ecological niche within the gut (241). While little information is know as to the relative importance of *C. jejuni*'s other nutrient sources, we do know it must reliably acquire these nutrients *in vivo* in order to not only survive, but thrive within the gut.

C. jejuni is able to utilize serine, glutamate, aspartate, proline and L-fucose, all of which are present within mucins (2, 147), but there has not been any previous research to suggest as to how it might access these nutrients *in vivo*. Research presented here and elsewhere (234, 238) has found that *C. jejuni* is able to both grow on media containing mucin, but a lack of prospective secreted glycosidases or proteases (180) begs the question as to whether *C. jejuni* has any capacity to degrade this large macromolecule. When *C. jejuni* was grown in MEMα containing supplemented porcine gastric mucin, a small increase in growth was observed. However, prior degradation of mucin with proteinase K or NaOH

increased *Campylobacter*'s growth on mucin substantially, suggesting that *C. jejuni* lacks the ability to degrade mucin. This absence of mucolytic activity was confirmed using zymographic analyses and mucin plate degradation assays.

Despite *C. jejuni*'s own lack of mucolytic ability, it is well known that many bacteria within the intestine, including members of the phyla *Bacteroidetes* (20)*, Firmicutes* (46), *Proteobacteria* (89)*, Actinobacteria* (121) and *Verrucomicrobia* (239, 244), efficiently degrade mucins and other macromolecules within the gut. We hypothesized that *C. jejuni* may work in concert with many of these bacteria, and take advantage of the amino acids and glycans released through their catabolic activities. This is not without precedent. Recent work by Flint and colleagues has sought to describe the complex relationships between members of the intestinal microbiota regarding short chain fatty acids and polysaccharide degradation (17, 18). These close relationships are the result of prolonged co-evolution, resulting in very specific adaptations to one another, their environment and their host. With this in mind, it is reasonable to suspect similar forms of evolution from *C. jejuni*.

We have shown here that coculture of *C. jejuni* with mucolytic bacteria (*C. perfringens, B. vulgatus* and *B. thetaiotaomicron*) significantly enhanced *C. jejuni*'s growth in a mucin-containing medium compared to a control monoculture. This increase was almost non-existent when *C. jejuni* was grown with the non-mucolytic bacteria *F. nucleatum*. While the observed increase in growth might not be solely due to the degradation of mucins within the medium, our observations clearly suggest a role of mucin degradation in *Campylobacter*'s growth enhancement. Indeed, in addition to mucin, there are other factors that may contribute to the observed increase in *C. jejuni* growth. As a by-product of sugar fermentation, many bacteria, including those being studied here, will produce SCFAs (18). These include acetate and lactate, which can then be utilized by *C. jejuni* as a carbon source

(231, 255). Other factors may also be involved, but we cannot hypothesize at this time what these may be with our current knowledge.

Beyond this observed phenotype *in vitro*, we sought to determine if this mutually beneficial growth effect was also present *in vivo.* To limit the complexity of a mature microbiota, we inoculated germ-free mice with *C. jejuni* and *B. vulgatus* individually and in combination. We observed a similar increase in colonization by *C. jejuni* in gnotobiotic mice co-colonized with *B. vulgatus,* similar to the *in vitro* experiments, although the observed increase was smaller and less consistent than in the *in vitro* model, likely due to the complexity of a living system. In contrast to the observed higher *C. jejuni* colonization in the cecum and colon, it was notably less in the ileum of the co-colonized mice. Although for both *C. jejuni* and *B. vulgatus*, colonization levels are typically much lower in the small intestine (195).

The other notable finding from the *in vitro* coculture experiments was the observation that *C. jejuni* creates an anaerobic environment, capable of supporting the growth of strict anaerobic bacteria, even in the presence of 8% oxygen. While *C. jejuni* does not have the same tolerance to higher oxygen levels of other bacteria such as *E. coli*, it has the unique characteristic of being both sensitive to oxygen, and dependent on it at the same time. The presence of excessive molecular oxygen, or reactive oxygen species will damage iron-sulfur complexes in key metabolic enzymes in *C. jejuni* (10), however, it will still use oxygen as a final electron acceptor (249) and requires oxygen for the proper functioning of the ribonucleotide reductase NrdAB involved in DNA synthesis (114, 214). Even in presence of other electron acceptors, the oxygen requirement of NrdAB is absolutely essential for growth (214). The oxygen consumption by *C. jejuni* is sufficient for the growth of the anaerobic bacterium *B. vulgatus in vitro*. However, the high and similar colonization levels of *B.*

vulgatus in mono- and dual-associated mice indicate that any difference in luminal oxygen levels between the mono and dual-associated mice have no substantial impact on *B. vulgatus* colonization.

One significant aspect to the bacterial colonization of the intestine is the effect on the health of the host. When *C. jejuni* colonizes a host, whether it is humans, or immunocompromised mice, an inflammatory reaction is usually observed, characterized by the increased production of the pro-inflammatory cytokines TNFα, INFγ, as well as the regulatory, anti-inflammatory cytokine IL-10 (7, 99, 105, 192), typical of a T_H1 response.

The pro-inflammatory TNFα was detected at low levels in the *C. jejuni* mono-associated mice only, while higher levels of IL-10 and IFNγ were detected on average in the dual-associated mice. Higher levels of TNF alpha and a relatively close correlation between the pro-inflammatory cytokine IFNγ and the colonization levels of *C. jejuni* may be a consequence of increased cell internalization with *C. jejuni* and likely indicative of tissue inflammation (113, 218). Additionally, higher levels of both IL-10 and IFN-γ may also be indicative of an IL-12 induced T_H1 response. Interestingly, IL-10 production was induced to a higher level in co-associated mice as compared to mono-associated mice. Based on these observations, it is tempting to speculate that the presence of commensal bacteria, such as *B. vulgatus* in our model, are required to prime the immune system enabling production of the anti-inflammatory cytokine IL-10 upon pathogens' attack. IL-10 deficient mice have been linked to severe colitis with *C. jejuni* (160), therefore production of IL-10 likely plays an important in resolving *C. jejuni* infection.

All together, we can draw several conclusions regarding the relationship between *C. jejuni* and both mucins and the intestinal microbiota. Firstly *C. jejuni* itself has no observable ability to degrade mucins. Although this may appear to be a limitation for an

organism living primarily the intestinal mucus layer, a mutually beneficial relationship appears to exist between *C. jejuni* and certain members of the intestinal microbiota, whereas *C. jejuni* can take advantage of the degradation of mucins by certain bacteria, while consuming excess oxygen from the environment. Together this demonstrates the degree to which *C. jejuni* has adapted itself specifically to its natural environment within the intestine.

CHAPTER 6

General Discussion

6.1. Conclusions.

The research presented here has sought to describe how *Campylobacter jejuni* colonizes the gastrointestinal tract of its host. We employed a microarray transposon-tracking (MATT) approach to assemble a list of genes essential for growth under laboratory growth conditions; we used a microarray-based approach to characterize the transcriptome of *C. jejuni* while growing in extracted intestinal mucus as a growth medium; we characterized *C. jejuni*'s ability to utilize L-fucose as a carbon source while growing within the gut; and we described how members of the intestinal microbiota within the mucus layer may improve the growth of *C. jejuni* by degrading larger proteins into usable nutrients of *C. jejuni*.

Our analysis of the essential genes of *C. jejuni* provided several key insights to the biology of *C. jejuni*. While the essentiality of a gene may change based on the growth medium of the cell, the characterization of essential genes required for growth in a rich growth medium, where nutrients are plentifully available, helps to illustrate which genes are essential under most conditions to which the bacteria will be exposed. Among others, we described many of the basic housekeeping genes of the cell, as well as several key biosynthetic pathways contributing to the biosynthesis of folate, heme, aromatic amino acids, and other molecules critical to the cell. Importantly, the essential genes have the potential to serve as novel targets for antibiotics, since they are necessary for cell survival. It also provides insight into which genes may comprise the core genome of *Campylobacter*, and

which pathways have become irreplaceable for the species as it has adapted and evolved to proliferate in the environment of the gut.

While the essential genes are of significant interest for studying the survival of *C. jejuni* within the intestine, there are limits to our knowledge as to how *C. jejuni* interacts with its environment. Therefore, it becomes necessary to look at the more dynamic transciptome of the organism as it grows within different media and in different hosts in order to examine how it interacts with its environment under each of these conditions. We conducted microarray experiments, using RNA extracted from *C. jejuni* grown in a medium composed of extracted intestinal mucus from both a porcine model used to replicate human disease, as well as a chick model, which represents a commensal host for *C. jejuni*. These transcriptomes revealed genes that were regulated in a similar fashion between different mucus media compared to growth in MH growth medium, but also genes that appeared to be differentially regulated between the samples. These included genes such as those in the CmeR regulon, which were differentially expressed in the small intestine, likely in response to bile, but not in the cecum. These also include genes that appeared to be regulated in a host-specific manner, showing differential expression, dependent on whether the mucus was extracted from a porcine or poultry source. These included metabolic genes, the oxidative stress response, and capsule related genes, among others. Together these indicate that as *C. jejuni* colonizes its different hosts, it encounters significantly different environments and reacts differently as a result.

Among the genes indentified as being transcriptionally regulated in response to mucus colonization were a group of previously uncharacterized genes, which we successfully linked to *C. jejuni's* ability to utilize L-fucose as a substrate for growth. This observation in itself was novel, as *C. jejuni* had never previously been observed to

metabolize a carbohydrate. Furthermore, a mutant in the L-fucose permease Cj0486 was significantly impaired for colonization of the piglet model relative to the wild-type strain. However, the same mutant was not significantly disadvantaged in the chick model unless L-fucose was supplemented into the chicks' diet.

This observation that L-fucose utilization plays a role in our pathogenic porcine model, while not being important for growth in the chick commensal model may indicate that there is a significant role for L-fucose in *C. jejuni* virulence, however, it remains unclear exactly what that importance is. From the transcriptomic data of *C. jejuni* while growing in a medium supplemented with L-fucose, there is no indication that any genes previously linked with pathogenicity (such as *ciaB* or *cdtABC*) are up-regulated in response to L-fucose. Indeed, the addition of L-fucose to the medium, does not seem to affect cell adhesion or invasion, although the *fucP* mutant seemed mildly impaired for cell invasion, a factor not linked to the presence of fucose in the medium. Instead, the importance of L-fucose may come more as a result of inflammation. The intestinal mucus layer itself is rich in nutrients, and under normal circumstances, L-fucose may not be enough of a factor to substantially influence *C. jejuni* colonization. However, as outlined in figure 6.1, in the presence of exudative intestinal inflammation, the flushing of the intestinal epithelium substantially restricts nutrient availability. With the intestinal contents removed, the remaining bacteria rely on what's left of the intestinal mucus layer for their nutritional needs. Under these nutrient-starved conditions, even a small advantage, such as the ability to utilize any available L-fucose may provide and substantial competitive advantage to *C. jejuni* as it attempts to persist in the face of the host's immune response. Precisely why and when L-fucose utilization becomes advantageous to *C. jejuni* is something that warrants further research. If it can be proven that it provides a substantial advantage at certain times of

infection, such as in response to nutrient depletion caused by diarrhea, then targeting these key nutrient acquisition and metabolic pathways may have therapeutic potential in the treatment of *Campylobacter* infection.

We further explored the nutrient availability to *C. jejuni* within the mucus layer, by assessing whether *C. jejuni* was capable of degrading intestinal mucins, thereby freeing not only L-fucose, but also numerous amino acids, which can be used by *C. jejuni.* We did not find any evidence that *C. jejuni* itself was capable of the exogenous degradation of mucins; however, we hypothesized that *C. jejuni*'s close proximity to mucolytic bacteria within the intestinal microbiota might allow it to scavenge off mucins degraded by other bacteria. When *C. jejuni* was grown in the presence of mucolytic bacteria, both *in vitro* and *in vivo*, its growth was increased, suggesting a beneficial relationship between *C. jejuni* and members of the intestinal microbiota.

The potential importance of the intestinal microbiota in *C. jejuni* colonization has been demonstrated in several recent publications (4, 21, 91, 164). Aside from several studies showing species such as *Lactobacillus* prevent adhesion of *C. jejuni* to intestinal cells (4, 164), Bereswill et al. (21) published the study that really highlights the importance of the microbiota. This study found that although *C. jejuni* will not persistently colonize a normal mouse intestine, if the microbiota of these mice is eliminated and replaced by a microbiota of human origin, then *C. jejuni* is able to colonize and can cause some symptoms of disease normally associated with human infection. The only difference between these two phenotypes is the microbiota composition, making them a prime target as a pathogenicity determinant. Exactly what the differences are that lead to this increased *C. jejuni* colonization and pathogenesis remains unknown. The research presented here has

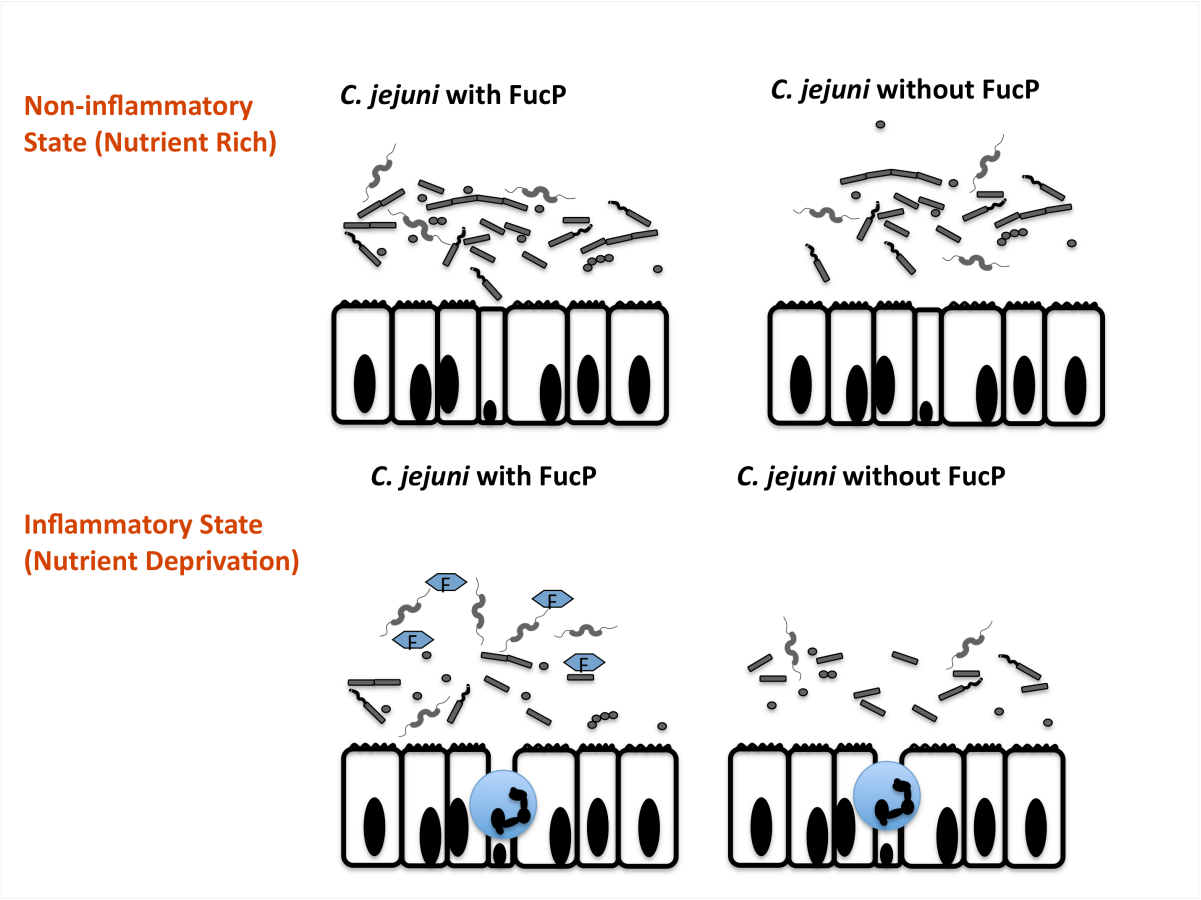

Figure 6.1: A proposed role for L-fucose in an inflamed intestine. In a nutrient rich environment (top panels), a plethora of nutrient sources reduced the importance of any individual nutrient source, such as L-fucose. Therefore, the numbers of *C. jejuni* with and without FucP will be comparable. However, in a nutrient deprived environment (bottom panels), such as under inflammatory conditions, as immune cells and fluid infiltrate the epithelium and mucus layer, the ability to utilize L-fucose becomes a competitive advantage as available nutrients become depleted, allowing for increased numbers of C. jejuni able to transport and metabolize L-fucose.

found that certain bacteria commonly associated with the human microbiota, such as *B. vulgatus,* can enhance *C. jejuni* growth in a mucin-containing medium. This could suggest that the difference may rest in nutrient availability, and which ecological niches are available in the mouse or human intestine, but further research is needed to fully describe the intricacies of *C. jejuni's* relationship with the resident microbiota and how it impacts pathogenicity.

6.2. Future directions

One of the major findings in this research has been the identification of a novel pathway allowing for the utilization of L-fucose by *C. jejuni* and involving proteins coded by the genes *cj0481-cj0490*. Despite the identification of the general function of these genes, and some evidence suggesting a mechanism similar to a pathway identified in *Xanthomonas campestris*, insufficient information is currently available to confirm the mechanism by which this pathway operates. Further work to elucidate the mechanisms of this novel metabolic pathway would be necessary in order to understand how *C. jejuni* is able to metabolize L-fucose. Specifically, although our results would suggest that there is no kinase activity within this pathway, the absence of a kinase does not necessarily prove the presence of an L-fucose dehydrogenase, although the similarities to the *X. campestris* pathway would suggest this is the case. To further prove that this is the case, experiments to detect L-fucose dehydrogenase activity via the production of NADH or NADPH would need to be undertaken using purified enzyme and L-fucose as a substrate. Using purified enzymes and mass spec to track the consumption and production of each intermediate within the pathway would also serve to elucidate the exact mechanisms used by the putative L-fucose metabolic pathway.

A second point of interest that deserves further research is the role of the transporter Cj0484. We successfully demonstrated the role of the permease Cj0486 in the transport of L-fucose; however, the function of Cj0484 still remains a mystery. As shown in figure 4.3, it does not bear any significant similarities to an L-fucose permease, but its annotation indicates that it is a major facilitator superfamily (MFS) type transporter, with some similarities to tartrate or sugar transporters. Whether this has a role in transporting L-fucose, byproducts from L-fucose metabolism, or perhaps a broad-spectrum sugar transporter remains unknown and is in need of further study.

Finally, the regulation of this pathway still remains largely unknown. The data presented in this study, along with results published by Muraoka et al. (171) would suggest that the operon is up-regulated in response to the presence of L-fucose, and in its absence, it is not significantly expressed at all. This regulation is presumably under the control of its associated regulator, Cj0480c, annotated as an iclR family regulator. The most likely mechanism of action for this regulator would be that of a repressor whose binding is inhibited in the presence of L-fucose, although this has yet to be experimentally demonstrated. It would be of significant interest if it can be shown through gel-shift assays, or other approaches, that the DNA-binding repressor activity of the Cj0480c regulator can be inhibited through the binding of L-fucose to the protein. This would have implications not just in the understanding of L-fucose metabolic regulation in *C. jejuni*, but it would have the potential to serve as a useful tool in *Campylobacter* research. A gene under the control of a regulator, and whose expression can be turned on or off in response to the presence of a single metabolite, could serve as a useful tool. If placed in front of a gene of interest, it could allow for the controlled expression of a target gene in a manner not unlike the classical lac operon, extensively used in other bacteria, but not function in *C. jejuni*.

The role of the intestinal microbiota in the colonization of *C. jejuni* provides perhaps the future avenue of research with the most significant potential implications to not just the study of *Campylobacter,* but in the study of gastrointestinal health in general. In recent years, research into the role of the microbiota in gastrointestinal health has exploded. While in the past, the relationship in between host and pathogen were largely taken in isolation, we now realize that the wider environment of the gut can have significant implications on human health. In the context of the host-pathogen relationship, neither the host nor the pathogen exists on their own, but the host-defence and the pathogen's ability to infect both rely on many levels with the state of the intestinal microbiota. We know how the microbiota can modulate the host's immune response, making it better able to quickly respond to potential pathogens. Further, the ecology of the gut is of critical importance if a pathogen is to establish itself and cause disease. As Freter et al. found; if there is no open niche, then there can be no infection (66).

How this relates to *Campylobacter* will rest on finding the details of the ecological niche in which *C. jejuni* inhabits. The research presented here, as well as elsewhere has found that in addition to several core metabolic pathways such as the utilization of aspartate, glutamate, serine and proline, the ability to utilize glutamine, asparagine and L-fucose varies in between strains. The implication of this is that depending on the repertoire of pathways present within any individual *C. jejuni* strain, it may be able to establish itself into different ecological niches, perhaps altering its range of hosts, and allowing multiple *Campylobacter* species or strains to co-inhabit a single host. Furthermore, as a niche changes, so may change the inclination of *C. jejuni* to infiltrate into surrounding tissues, with possible implications of infection and immunity.

The study of the specific nutrient requirements of *C. jejuni* within different hosts (chicks, piglets, mice and humans), and how those requirements can be altered is an important way forward in this avenue of research. If mutants in certain metabolic pathways are affected for the fitness in some hosts but not others, then we can narrow our focus for *C. jejuni's* ecological niche. If this can be done, then we can focus on the intestinal microbiota so examine if a probiotic or other alteration to the intestinal microbiota can block this niche and either inhibit *Campylobacter* colonization, or reduce its pathogenicity in humans.

The research presented in this thesis represents an effort to characterize *C. jejuni*, while it is growing in its natural habitat or the intestinal mucus layer. We characterized the genes essential for its growth in an in vitro environment and proceeded to characterize its transcriptome in extracted intestinal mucus from sources that represent both commensal and pathogenic models of colonization. This transcriptomic data led to the characterization of a novel pathway for the metabolism of L-fucose and shed light on the nature of *C. jejuni* growth in the intestinal mucus layer. This also led to further characterization of the mutually beneficial relationships that exist in between *C. jejuni* and the resident microbiota of the intestine. Together, we can conclude that *C. jejuni* is well adapted to the environment of the intestine, and further research into its growth within this environment has the potential to yield significant discoveries to *Campylobacter* research.

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Contributions of Collaborators

Dr. Christine Szymanski, Dr. Lorna Friis, and Dr. Harald Nothaft: Work regarding the characterizing of *cj0486* and the rest of the L-fucose utilization operon was done in collaboration with Dr. Christine Szymanski, Alberta Ingenuity Centre for Carbohydrate Science, Department of Biological Sciences, of the University of Alberta. Dr. Harald Nothaft and Dr. Lorna Friis from the Szymanski lab undertook the experiments involving L-fucose uptake, initial construction of the *Δcj0486* mutant, and the *C. jejuni* 81-176 + pCE111+cj0481-cj0490 complemented strain, as well to experimental design and the interpretation of results.

James Butcher and Annika Flint: James Butcher (Stintzi lab, University of Ottawa), contributed to the preparation of figures 1.1 and 2.4, and both he and Annika Flint (Stintzi lab, University of Ottawa) assisted in the maintenance of neonatal piglets and newly hatched chicks.

Dr. Alain Stintzi: Dr. Alain Stintzi (Department of Biochemistry, Microbiology and Immunology, University of Ottawa), contributed both as a supervisor to the project as a whole, and directly contributed to the data analysis for the identification of the essential genes, and other microarray data, and assisted with the maintenance, euthanasia, and necropsies for all experiments involving neonatal piglets, newly hatched chicks and germ-free mice.

Appendix I.
Primer List

Primer Name	Sequence (5'-3')
Chapter 1	
pMOD forward	ATTCAGGCTGCGCAACTGT
pMOD reverse	GTCAGTGAGCGAGGAAGCGGAAG
MS1	GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT
MS2	GGCCACGCGTCGACTAGTAC
MS3	GGCCACGCGTCGACTAGTACNNNNNNNNNNAGAG
AR54	AGAGCTTAGTACGTTAAACATGA
CM2	GGAACTATAGGGCGCAGGAT
SqFP	GCCAACGACTACGCACTAGCCAAC
SqRP	GAGCCAATATGCGAGAACACCCGAGAA
Chapter 3	
Gene Cloning	
cj0481L	CGGTACCCGGGGATCCAAAAGGAACTTTACCGGCTTTA
cj0481R	CGACTCTAGAGGATCCTTGCTTCATCAAAGCGAGTG
cj0483L	CGGTACCCGGGGATCCGGAGCAATGCCAATGTTTTT
cj0483R	CGACTCTAGAGGATCCGCTCATCACGCACTTCTTCTT
cj0487L	CGGTACCCGGGGATCCCACCTTTGGGATTTGGAAAA
cj0487R	CGACTCTAGAGGATCCCACTGGATAATTGCTCCCAAA
cj0490L	ATGGCAAGAGATGAAAGCACAC
cj0490R	CCATCAGCTCCGCCTATGC
CfucP_F	CGGGATCCTGGGATGAAAACTAAGGAGTA
CfucP_R	ACGCGTCGACAAAGGTGTAGATGAGCATCAA
Inverse PCR	
cj04811	GAACTAAAGGGCGCAGCATAGGTGGAGTTTTTCCAG
cj04812	GAACACCGCCGAGCAACTTGCTCTTCCACGCTCAA
cj04831	GAACTAAAGGGCGCATTTATGAATACACCAGGCAATGA
cj04832	GAACACCGCCGAGCATGCAGCACTTTCAAGCATTT
cj04871	GAACTAAAGGGCGCAGGTTTTAAATCATCTAGGAAGTCCA
cj04872	GAACACCGCCGAGCACGCTAAACAAAACAAAAGATTATGAA
cj04901	AGTGTGCAAAGATGCGGATA
cj04902	TAAAGCAGGTGCCATCTTCC
Chloramphenicol cassette	
catSE	TGCTCGGCGGTGTTCCTTTCCAAG
catAS	TGCGCCCTTTAGTTCCTAAAGGGT

Appendix II.
Essential gene candidates in *Campylobacter jejuni* NCTC 11168

Category	Subcategory	Gene	Gene Size	Protein function
Broad regulatory functions [2/52 genes]	Signal transduction [2/26 genes]	*racS*	1236 bp	Two-component sensor (histidine kinase)
		Cj0355c	672 bp	Two-component regulator
Small molecule metabolism [36/337 genes]	Energy metabolism [9/107 genes]	*pgi*	1221 bp	Glucose-6-phosphate isomerase
		rpiB	438 bp	Ribose 5-phosphate isomerase
		hypF	2190 bp	Carbamoyltransferase
		hypA	345 bp	Hydrogenase expression/formation protein
		fdxB	246 bp	Ferredoxin
		Cj1664	489 bp	Putative periplasmic thioredoxin
		atpG	885 bp	F0F1 ATP synthase subunit gamma
		atpF'	425 bp	F0F1 ATP synthase subunit B'
		atpF	513 bp	F0F1 ATP synthase subunit B
	Central intermediary metabolism [2/30 genes]	*rep*	648 bp	Ribulose-phosphate 3-epimerase
		ald	1181 bp	Putative aldehyde dehydrogenase
	Amino acid biosynthesis [6/66 genes]	*argF*	921 bp	Ornithine carbamoyltransferase
		thrB	879 bp	Homoserine kinase
		metE	2265 bp	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase
		dapF	750 bp	Diaminopimelate epimerase
		trpE	1251 bp	Putative anthranilate synthase component I
		aroA	1287 bp	3-phosphoshikimate 1-carboxyvinyltransferase
	Purines, pyrimidines, nucleosides and nucleotides [4/40 genes]	*purS*	246 bp	Phosphoribosylformylglycinamidine (FGAM) synthase
		pyrC2	1179 bp	Dihydroorotase
		pyrB	888 bp	Aspartate carbamoyltransferase catalytic subunit
		nrdB	1023 bp	Ribonucleotide-diphosphate reductase subunit beta
	Biosynthesis of cofactors, prosthetic groups and carriers [12/64 genes]	*birA*	654 bp	Biotin-protein ligase
		bioD	606 bp	Putative dethiobiotin synthetase
		bioC	687 bp	Putative biotin synthesis protein
		trxA	315 bp	Thioredoxin
		ispA	846 bp	Geranyltranstransferase
		hemH	912 bp	Ferrochelatase
		hemC	924 bp	Porphobilinogen deaminase
		pabA	567 bp	Para-aminobenzoate synthase glutamine amidotransferase component II
		folD	849 bp	Methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase
		pdxA	1095 bp	4-hydroxythreonine-4-phosphate

				dehydrogenase
		nadE	741 bp	NH(3)-dependent NAD(+) synthetase
		ribA	1020 bp	Bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II protein
	Fatty acid biosynthesis [3/21 genes]	*acpP2*	231 bp	Putative acyl carrier protein
		accC	1332 bp	Biotin carboxylase
		accB	456 bp	Putative biotin carboxyl carrier protein of acetyl-CoA carboxylase
Macromolecule metabolism [76/612 genes]	Synthesis and modification of macromolecules [30/215 genes]	*dgkA*	357 bp	Diacylglycerol kinase
		rpsO	252 bp	30S ribosomal protein S15
		rpsB	792 bp	30S ribosomal protein S2
		rpmJ	114 bp	50S ribosomal protein L36
		rpmI	192 bp	50s ribosomal protein L35
		rpmH	135 bp	50S ribosomal protein L34
		rpmG	159 bp	50S ribosomal protein L33
		rpmF	146 bp	50S ribosomal protein L32
		rpmB	237 bp	50S ribosomal protein L28
		rplT	354 bp	50S ribosomal protein L20
		rplS	357 bp	50S ribosomal protein L19
		Cj1280c	969 bp	Putative ribosomal pseudouridine synthase
		truD	1119 bp	tRNA pseudouridine synthase D
		rimM	540 bp	Putative 16S rRNA processing protein
		ksgA	801 bp	Dimethyladenosine transferase
		trmA	1074 bp	tRNA (uracil-5-)-methyltransferase
		miaA	870 bp	tRNA delta(2)-isopentenylpyrophosphate transferase
		glyQ	864 bp	Glycyl-tRNA synthetase subunit alpha
		gltX	1296 bp	Glutamyl-tRNA synthetase
		cysS	1389 bp	Cysteinyl-tRNA synthetase
		recG	1824 bp	ATP-dependent DNA helicase
		dnaX	1530 bp	DNA polymerase III subunits gamma and tau
		dnaA	1323 bp	Chromosomal replication initiator protein
		cjeI	4020 bp	Restriction modification enzyme
		Cj0722c	816 bp	Putative DNA methylase
		Cj0635	384 bp	Holliday junction resolvase-like protein
		Cj0139	2352 bp	Putative endonuclease
		dsbD	1704 bp	Putative thiol:disulphide interchange protein
		dsbA	642 bp	Putative protein disulphide isomerase
		Cj1154c	207 bp	Putative cytochrome oxidase maturation protein cbb3-type
	Degradation of macromolecules [1/28 genes]	Cj0327	351 bp	Putative endoribonuclease L-PSP family protein
	Cell envelope [45/369 genes]	*pal*	498 bp	Peptidoglycan associated lipoprotein (omp18)
		Cj1276c	807 bp	Putative integral membrane protein
		Cj1074c	648 bp	Putative lipoprotein

		Cj0989	207 bp	Hypothetical protein Cj0989
		Cj0986c	189 bp	Putative integral membrane protein
		Cj0926	333 bp	Hypothetical protein Cj0926
		Cj0851c	504 bp	Putative integral membrane protein
		Cj0830	417 bp	Putative integral membrane protein
		Cj0801	1452 bp	Putative integral membrane protein (MviN homolog)
		Cj0598	1443 bp	Hypothetical protein Cj0598
		Cj0591c	222 bp	Putative lipoprotein
		Cj0544	390 bp	Putative integral memnbrane protein
		Cj0523	276 bp	Hypothetical protein Cj0523
		Cj0421c	957 bp	Putative integral membrane protein
		Cj0352	798 bp	Putative transmembrane protein
		Cj0267c	531 bp	Putative integral membrane protein
		Cj0232c	411 bp	Putative integral membrane protein
		Cj0167c	564 bp	Putative integral membrane protein
		Cj0158c	426 bp	Putative haem-binding lipoprotein
		Cj0090	369 bp	Putative lipoprotein
		Cj0080	270 bp	Putative membrane protein. Functional classification-Membranes, lipoproteins and porins
		lpxC	885 bp	UDP-3-O-
		kdtA	1158 bp	3-deoxy-D-manno-octulosonic-acid transferase
		fliN	309 bp	Flagellar motor switch protein
		fliF	1683 bp	Flagellar MS-ring protein
		fliE	297 bp	Flagellar hook-basal body protein FliE
		murB	777 bp	UDP-N-acetylenolpyruvoylglucosamine reductase
		Cj1513c	192 bp	Possible periplasmic protein
		Cj1486c	222 bp	Putative periplasmic protein
		Cj1485c	102 bp	Putative periplasmic protein
		Cj1289	816 bp	Possible periplasmic protein
		Cj1240c	603 bp	Putative periplasmic protein
		Cj1219c	2541 bp	Putative periplasmic protein
		Cj1021c	192 bp	Putative periplasmic protein
		Cj0969	132 bp	Pseudogene (putative periplasmic protein)
		Cj0964	1191 bp	Putative periplasmic protein
		Cj0911	561 bp	Putative periplasmic protein
		Cj0910	453 bp	Putative periplasmic protein
		Cj0909	420 bp	Putative periplasmic protein
		Cj0876c	288 bp	Putative periplasmic protein
		Cj0854c	348 bp	Putative periplasmic protein
		Cj0530	2571 bp	Putative periplasmic protein
		Cj0515	1197 bp	Putative periplasmic protein
		Cj0200c	348 bp	Putative periplasmic protein
		Cj0168c	168 bp	Putative periplasmic protein
Cell processes [19/231 genes]	Transport/binding proteins [10/154 genes]	*kefB*	1626 bp	Putative glutathione-regulated potassium-efflux system protein

		Cj0522	504 bp	Putative Na+/Pi cotransporter protein
		Cj0241c	402 bp	Putative iron-binding protein
		Cj0072c	602 bp	Pseudogene (putative iron binding protein)
		Cj1538c	996 bp	Putative anion-uptake ABC-transport system ATP-binding protein
		msbA	1743 bp	Lipid export ABC transport protein
		exbB3	555 bp	Putative MotA/TolQ/ExbB proton channel family protein
		Cj1581c	711 bp	Putative peptide ABC-transport system ATP-binding protein
		Cj0601c	1344 bp	Putative sodium-dependent transmembrane transport protein
		Cj0142c	855 bp	Putative ABC transporter ATP-binding protein
	Chaperones, chaperonins, heat shock [2/16 genes]	*groES*	261 bp	Co-chaperonin GroES
		Cj0954c	771 bp	Putative DnaJ-like protein
	Cell division [3/9 genes]	*mreC*	750 bp	Homolog of *E. coli* rod shape-determining protein
		ftsK	2841 bp	Putative cell division protein
		Cj1038	1164 bp	Putative cell division/peptidoglycan biosynthesis protein
	Chemotaxis and mobility [1/9 genes]	*cheB'*	555 bp	Putative MCP protein-glutamate methylesterase
	Protein and peptide secretion [3/20 genes]	*tatB*	417 bp	Sec-independent translocase
		secE	180 bp	Preprotein translocase subunit SecE
		ftsY	867 bp	Putative signal recognition particle protein
Other	Conserved hypothetical proteins	*engB*	597 bp	GTPase EngB
[62/260 genes]	[27/122 genes]	Cj1712	537 bp	Hypothetical protein Cj1712
		Cj1640	552 bp	Hypothetical protein Cj1640
		Cj1589	798 bp	Conserved hypothetical protein Cj1189
		Cj1575c	228 bp	Hypothetical protein Cj1575c
		Cj1225	249 bp	Conserved hypothetical protein Cj1225
		Cj1217c	681 bp	Conserved hypothetical protein Cj1217c
		Cj0984	741 bp	Conserved hypothetical protein Cj0984
		Cj0963	609 bp	Hypothetical protein Cj0963
		Cj0939c	381 bp	Hypothetical protein Cj0939c
		Cj0916c	198 bp	Conserved hypothetical protein Cj0916c
		Cj0849c	2160 bp	Conserved hypothetical protein Cj0849c
		Cj0787	249 bp	Conserved hypothetical protein Cj0787
		Cj0761	342 bp	Hypothetical protein Cj0761
		Cj0711	243 bp	Hypothetical protein Cj0711
		Cj0706	717 bp	Conserved hypothetical protein Cj0706
		Cj0703	537 bp	Hypothetical protein Cj0703
		Cj0620	648 bp	Conserved hypothetical protein Cj0620
		Cj0510c	291 bp	Conserved hypothetical protein Cj0510c

		Cj0496	375 bp	Conserved hypothetical protein Cj0496
		Cj0488	318 bp	Conserved hypothetical protein Cj0488
		Cj0459c	267 bp	Hypothetical protein Cj0459c
		Cj0305c	612 bp	Conserved hypothetical protein Cj0305c
		Cj0272	1092 bp	Conserved hypothetical protein Cj0272
		Cj0251c	156 bp	Highly acidic protein
		Cj0156c	657 bp	16S rRNA m3U1498 methyltransferase
		Cj0135	258 bp	Conserved hypothetical protein Cj0135
	Unknown [22/113 genes]	Cj1714	99 bp	Small hydrophobic protein
		Cj1514c	714 bp	Hypothetical protein Cj1514c
		Cj1232	333 bp	Hypothetical protein Cj1232
		Cj0972	324 bp	Hypothetical protein Cj0972
		Cj0971	393 bp	Hypothetical protein Cj0971
		Cj0873c	213 bp	Hypothetical protein Cj0873c
		Cj0837c	942 bp	Conserved hypothetical protein Cj0837c
		Cj0740	165 bp	Hypothetical protein Cj0740
		Cj0739	174 bp	Hypothetical protein Cj0739
		Cj0738	165 bp	Hypothetical protein Cj0738
		Cj0724	186 bp	Hypothetical protein Cj0724
		Cj0682	243 bp	Hypothetical protein Cj0682
		Cj0600	876 bp	Hypothetical protein Cj0600
		Cj0566	1467 bp	Hypothetical protein Cj0566
		Cj0417	147 bp	Hypothetical protein Cj0417
		Cj0395c	291 bp	Hypothetical protein Cj0395c
		Cj0380c	786 bp	Hypothetical protein Cj0380c
		Cj0364	252 bp	Hypothetical protein Cj0364
		Cj0350	423 bp	Hypothetical protein Cj0350
		Cj0170	745 bp	Hypothetical protein Cj0170
		Cj0163c	498 bp	Hypothetical protein Cj0163c
		Cj0070c	159 bp	Hypothetical protein Cj0070c
	Misc [13/152 genes]	*ispE*	768 bp	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
		folB	318 bp	Putative dihydroneopterin aldolase
		Cj1237c	975 bp	Putative phosphatase
		Cj1056c	777 bp	Putative carbon-nitrogen hydrolase family protein
		Cj0965c	375 bp	Putative acyl-CoA thioester hydrolase
		Cj0962	480 bp	Putative acetyltransferase
		Cj0809c	597 bp	Putative hydrolase
		Cj0611c	1377 bp	Putative acyltransferase family protein
		Cj0529c	1002 bp	Putative aminodeoxychorismate lyase family protein
		Cj0495	702 bp	Putative methyltransferase domain protein
		Cj0188c	1371 bp	Putative kinase
		Cj0160c	744 bp	Putative radical SAM domain protein
		Cj0128c	723 bp	Putative inositol monophosphatase family protein

Appendix III.
Transcriptome data from from piglet small intestinal mucus medium

Name	Gene	Fold Change (log_2)	Bayes.p	Protein
Cj1537c	*acs*	3.46	0	acetyl-CoA synthetase
Cj1385	*katA*	3.26	2.54E-06	catalase
Cj0759	*dnaK*	3.20	NA	molecular chaperone DnaK
Cj0561c	Cj0561c	2.87	0	putative periplasmic protein
Cj1508c	*fdhD*	2.65	0	formate dehydrogenase accessory protein
Cj0303c	*modA*	2.54	0	putative molybdate-binding lipoprotein
Cj0485	Cj0485	2.44	1.10E-14	short chain dehydrogenase
Cj0486	*fucP*	2.35	0	putative sugar transporter
Cj0806	*dapA*	2.35	0	dihydrodipicolinate synthase
Cj0921c	*peb1A*	2.29	5.26E-11	bifunctional adhesin/ABC transporter aspartate/glutamate-binding protein
Cj0987c	Cj0987c	2.23	1.30E-11	putative MFS (Major Facilitator Superfamily) transport protein
Cj0482	*uxaA′*	2.23	0	putative altronate hydrolase N-terminus
Cj0720c	*flaC*	2.04	2.24E-12	flagellin
Cj0487	Cj0487	1.98	1.22E-15	putative amidohydrolase
Cj0170	Cj0170	1.93	NA	hypothetical protein Cj0170
Cj0012c	*rrc*	1.87	1.33E-15	non-haem iron protein
Cj1687	Cj1687	1.82	6.08E-12	putative efflux protein
Cj0771c	Cj0771c	1.81	3.11E-10	putative NLPA family lipoprotein
Cj0367c	*cmeA*	1.81	0	periplasmic fusion protein CmeA (multidrug efflux system CmeABC)
Cj0874c	Cj0874c	1.78	3.85E-08	putative cytochrome C
Cj0484	Cj0484	1.72	1.33E-15	putative MFS (Major Facilitator Superfamily) transport protein
Cj1507c	Cj1507c	1.68	2.99E-12	putative regulatory protein
Cj1619	*kgtP*	1.65	2.31E-09	alpha-ketoglutarate permease
Cj0770c	Cj0770c	1.65	2.92E-08	putative NLPA family lipoprotein
Cj0034c	Cj0034c	1.62	3.17E-05	putative periplasmic protein
Cj1222c	*dccS*	1.62	NA	two-component sensor (histidine kinase)
Cj0490	*ald′*	1.58	0	putative aldehyde dehydrogenase C-terminus
Cj0917c	*cstA*	1.56	4.44E-16	putative integral membrane protein (CstA homolog)
Cj1199	Cj1199	1.53	1.69E-05	putative iron/ascorbate-dependent oxidoreductase
Cj0011c	Cj0011c	1.52	4.26E-09	putative non-specific DNA binding protein
Cj0008	Cj0008	1.50	2.78E-06	conserved hypothetical protein Cj0008
Cj1200	Cj1200	1.45	5.54E-06	putative NLPA family lipoprotein
Cj1466	*flgK*	1.45	2.81E-08	flagellar hook-associated protein FlgK
Cj1464	*flgM*	1.44	7.97E-08	hypothetical protein Cj1464
Cj1167	*ldh*	1.44	2.07E-08	L-lactate dehydrogenase
Cj1201	*metE*	1.43	6.82E-05	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
Cj1356c	Cj1356c	1.43	1.07E-07	putative integral membrane protein
Cj0689	*ackA*	1.41	2.25E-12	acetate kinase
Cj0699c	*glnA*	1.41	7.82E-05	glutamine synthetase
Cj0333c	*fdxA*	1.41	3.58E-12	ferredoxin
Cj0572	*ribA*	1.39	1.13E-09	bifunctional 3,4-dihydroxy-2-butanone 4-phosphate synthase/GTP cyclohydrolase II protein
Cj0664c	*rplI*	1.38	2.79E-10	50S ribosomal protein L9
Cj0366c	*cmeB*	1.37	1.11E-16	inner membrane efflux transporter CmeB (multidrug efflux system CmeABC)
Cj0021c	Cj0021c	1.36	9.92E-13	putative fumarylacetoacetate (FAA) hydrolase family

				protein
Cj0719c	Cj0719c	1.32	1.57E-10	conserved hypothetical protein Cj0719c
Cj0922c	*pebC*	1.31	1.68E-13	ABC-type amino-acid transporter ATP-binding protein
Cj0835c	*acnB*	1.30	4.46E-14	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
Cj0612c	*cft*	1.28	9.50E-12	ferritin
Cj0045c	Cj0045c	1.28	4.50E-06	putative iron-binding protein
Cj0865	*dsbB*	1.27	2.38E-07	putative disulfide oxidoreductase
Cj0653c	Cj0653c	1.26	7.41E-10	putative aminopeptidase
Cj0864	Cj0864	1.26	1.73E-08	putative periplasmic protein
Cj0370	*rpsU*	1.25	1.92E-06	30S ribosomal protein S21
Cj0772c	Cj0772c	1.25	4.25E-11	putative NLPA family lipoprotein
Cj0488	Cj0488	1.24	4.36E-10	conserved hypothetical protein Cj0488
Cj0229	Cj0229	1.22	3.44E-07	putative acetyltransferase
Cj1727c	*metY*	1.22	3.71E-07	putative O-acetylhomoserine (thiol)-lyase
Cj0688	*pta*	1.20	4.00E-10	putative phosphate acetyltransferase
Cj1503c	*putA*	1.20	1.06E-08	putative proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase
Cj0573	Cj0573	1.20	3.85E-07	putative GatB/Yqey family protein
Cj0985c	*hipO*	1.19	4.83E-11	hippurate hydrolase
Cj0440c	Cj0440c	1.19	4.35E-08	putative transcriptional regulator
Cj0069	Cj0069	1.18	5.52E-08	hypothetical protein Cj0069
Cj0044c	Cj0044c	1.17	6.45E-08	hypothetical protein Cj0044c
Cj1726c	*metA*	1.16	2.78E-07	homoserine O-succinyltransferase
Cj0980	Cj0980	1.16	2.82E-10	putative peptidase
Cj0365c	*cmeC*	1.16	8.88E-16	outer membrane channel protein CmeC (multidrug efflux system CmeABC)
Cj0665c	*argG*	1.16	1.13E-08	argininosuccinate synthase
Cj1450	Cj1450	1.16	2.23E-07	putative ATP/GTP-binding protein
Cj0654c	Cj0654c	1.16	9.06E-08	probable transmembrane transport protein pseudogene
Cj1502c	*putP*	1.16	1.79E-09	putative sodium/proline symporter
Cj0391c	Cj0391c	1.15	1.55E-06	hypothetical protein Cj0391c
Cj0604	Cj0604	1.14	6.85E-08	putative polyphosphate kinase
Cj1534c	Cj1534c	1.14	4.10E-07	putative bacterioferritin
Cj0022c	Cj0022c	1.13	NA	putative ribosomal pseudouridine synthase
Cj1660	Cj1660	1.13	6.17E-08	putative integral membrane protein
Cj0998c	Cj0998c	1.12	5.77E-14	putative periplasmic protein
Cj0603c	*dsbD*	1.12	3.96E-06	putative thiol:disulphide interchange protein
Cj0203	Cj0203	1.12	5.80E-10	putative citrate transporter
Cj0860	Cj0860	1.12	2.81E-07	putative integral membrane protein
Cj0073c	Cj0073c	1.11	9.73E-09	conserved hypothetical protein Cj0073c
Cj1345c	Cj1345c	1.10	1.49E-09	putative periplasmic protein
Cj0687c	*flgH*	1.09	2.25E-11	flagellar basal body L-ring protein
Cj0200c	Cj0200c	1.07	4.44E-06	putative periplasmic protein
Cj0762c	*aspB*	1.05	6.14E-10	aspartate aminotransferase
Cj0920c	Cj0920c	1.03	9.16E-12	putative ABC-type amino-acid transporter permease protein
Cj1459	Cj1459	1.02	4.80E-08	hypothetical protein Cj1459
Cj1225	Cj1225	1.02	1.23E-05	conserved hypothetical protein Cj1225
Cj0914c	*ciaB*	1.02	1.12E-08	CiaB protein
Cj0715	Cj0715	1.01	1.04E-07	transthyretin-like periplasmic protein
Cj0075c	Cj0075c	1.01	1.49E-11	putative oxidoreductase iron-sulfur subunit
Cj0776c	Cj0776c	1.00	2.76E-07	putative periplasmic protein
Cj0574	*ilvI*	1.00	2.73E-09	acetolactate synthase 3 catalytic subunit
Cj1682c	*gltA*	0.99	1.45E-11	citrate synthase
Cj0663c	*hslV*	0.99	2.47E-06	ATP-dependent protease peptidase subunit

Cj1037c	*pycA*	0.97	1.69E-06	acetyl-CoA carboxylase
Cj0007	*gltB*	0.96	6.10E-07	glutamate synthase (NADPH) large subunit
Cj1307	Cj1307	0.95	8.83E-07	putative amino acid activating enzyme
Cj1138	Cj1138	0.93	NA	putative glycosyltransferase
Cj1339c	*flaA*	0.93	2.23E-06	flagellin
Cj0282c	*serB*	0.91	1.15E-06	putative phosphoserine phosphatase
Cj1451	*dut*	0.91	1.78E-06	dUTPase
Cj1293	*pseB*	0.89	3.99E-07	UDP-GlcNAc-specific C4,6 dehydratase/C5 epimerase
Cj0559	Cj0559	0.89	1.14E-09	putative pyridine nucleotide-disulphide oxidoreductase
Cj1523c	Cj1523c	0.89	2.39E-05	putative CRISPR-associated protein
Cj0004c	Cj0004c	0.88	6.24E-06	monohaem cytochrome C
Cj0736	Cj0736	0.88	NA	hypothetical protein Cj0736
Cj0349	*trpA*	0.87	1.47E-08	tryptophan synthase subunit alpha
Cj0005c	Cj0005c	0.87	2.49E-07	molydopterin containing oxidoreductase
Cj1228c	*htrA*	0.86	5.51E-08	serine protease (protease DO)
Cj0392c	*pyk*	0.86	5.30E-09	pyruvate kinase
Cj0369c	Cj0369c	0.86	3.11E-07	putative ferredoxin domain-containing integral membrane protein
Cj0924c	*cheB'*	0.84	1.22E-06	putative MCP protein-glutamate methylesterase
Cj0345	*trpE*	0.84	4.95E-05	putative anthranilate synthase component I
Cj1250	*purD*	0.83	1.50E-06	phosphoribosylamine--glycine ligase
Cj0428	Cj0428	0.82	7.10E-09	hypothetical protein Cj0428
Cj0977	Cj0977	0.81	2.10E-06	hypothetical protein Cj0977
Cj1316c	Cj1316c	0.81	1.50E-05	pseudaminic acid biosynthesis PseA protein
Cj0525c	*pbpB*	0.81	NA	putative penicillin-binding protein
Cj1589	Cj1589	0.80	6.30E-07	conserved hypothetical protein Cj1189
Cj1000	Cj1000	0.80	9.51E-05	putative transcriptional regulator (LysR family)
Cj0876c	Cj0876c	0.80	9.32E-07	putative periplasmic protein
Cj1008c	*aroB*	0.80	5.78E-09	3-dehydroquinate synthase
Cj0143c	Cj0143c	0.80	2.31E-07	putative periplasmic solute binding protein for ABC transport system
Cj0548	*fliD*	0.80	7.45E-05	flagellar capping protein
Cj0721c	Cj0721c	0.79	2.24E-07	putative integral membrane protein.
Cj0527c	*flgC*	0.79	1.14E-07	flagellar basal body rod protein FlgC
Cj0449c	Cj0449c	0.79	8.63E-08	conserved hypothetical protein Cj0449c
Cj0854c	Cj0854c	0.78	1.05E-06	putative periplasmic protein
Cj0965c	Cj0965c	0.78	3.12E-07	putative acyl-CoA thioester hydrolase
Cj0872	*dsbA*	0.78	7.96E-08	putative protein disulphide isomerase
Cj0379c	Cj0379c	0.78	3.30E-05	putative sulfite oxidase subunit YedY
Cj1050c	*npdA*	0.78	1.05E-05	NAD-dependent deacetylase
Cj0943	Cj0943	0.78	1.39E-07	outer-membrane lipoprotein carrier protein
Cj0434	*pgm*	0.77	9.98E-06	phosphoglyceromutase
Cj0727	Cj0727	0.77	NA	putative periplasmic solute-binding protein
Cj0447	Cj0447	0.76	3.02E-06	putative NUDIX hydrolase family protein
Cj0716	Cj0716	0.76	3.29E-06	putative phospho-2-dehydro-3-deoxyheptonate aldolase
Cj1220	*groES*	0.76	5.37E-08	co-chaperonin GroES
Cj0246c	Cj0246c	0.75	NA	putative MCP-domain signal transduction protein
Cj0686	*gcpE*	0.75	2.87E-07	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase
Cj0348	*trpB*	0.74	6.75E-09	tryptophan synthase subunit beta
Cj1141	*neuB1*	0.74	3.86E-06	sialic acid synthase (N-acetylneuraminic acid synthetase)
Cj0821	*glmU*	0.74	4.28E-06	UDP-N-acetylglucosamine pyrophosphorylase
Cj0873c	Cj0873c	0.74	3.90E-08	hypothetical protein Cj0873c
Cj0057	Cj0057	0.74	2.56E-06	putative periplasmic protein
Cj0539	Cj0539	0.73	1.16E-05	hypothetical protein Cj0539
Cj0575	*ilvH*	0.72	8.49E-08	acetolactate synthase 3 regulatory subunit
Cj1533c	Cj1533c	0.71	2.34E-05	putative helix-turn-helix containing protein

Cj1719c	*leuA*	0.71	5.90E-08	2-isopropylmalate synthase
Cj1729c	*flgE2*	0.70	8.92E-06	flagellar hook protein FlgE
Cj1656c	Cj1656c	0.70	6.72E-06	hypothetical protein Cj1656c
Cj1194	Cj1194	0.70	6.66E-06	possible phosphate permease
Cj0253	Cj0253	0.70	8.44E-07	hypothetical protein Cj0253
Cj1717c	*leuC*	0.70	1.51E-09	isopropylmalate isomerase large subunit
Cj1718c	*leuB*	0.70	5.50E-07	3-isopropylmalate dehydrogenase
Cj1347c	*cdsA*	0.70	3.93E-05	phosphatidate cytidylyltransferase
Cj0725c	*mog*	0.69	2.66E-06	molybdenum cofactor biosynthesis protein
Cj0171	Cj0171	0.69	1.81E-08	merged with Cj0170
Cj0260c	Cj0260c	0.68	1.42E-06	small hydrophobic protein
Cj1240c	Cj1240c	0.67	4.11E-06	putative periplasmic protein
Cj1553c	*hsdM*	0.67	1.08E-06	putative type I restriction enzyme M protein
Cj1609	Cj1609	0.67	1.40E-07	putative sulfate adenylyltransferase
Cj0606	Cj0606	0.67	9.43E-05	putative secretion protein HlyD
Cj0834c	Cj0834c	0.67	6.45E-09	ankyrin repeat-containing putative periplasmic protein
Cj0552	Cj0552	0.67	7.37E-07	hypothetical protein Cj0552
Cj0453	*thiC*	0.67	5.96E-07	thiamine biosynthesis protein ThiC
Cj0898	Cj0898	0.67	2.04E-06	putative histidine triad (HIT) family protein
Cj1139c	*wlaN*	0.66	7.32E-07	beta-1,3 galactosyltransferase
Cj0489	*ald'*	0.66	7.34E-06	putative aldehyde dehydrogenase N-terminus
Cj1176c	*tatA*	0.66	6.06E-06	Sec-independent protein translocase (TatA/E homolog)
Cj0065c	*folK*	0.66	NA	putative 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase
Cj1548c	Cj1548c	0.65	4.81E-06	putative NADP-dependent alcohol dehydrogenase
Cj0176c	Cj0176c	0.65	1.98E-06	putative lipoprotein
Cj0697	*flgG2*	0.64	6.17E-07	flagellar basal-body rod protein
Cj0350	Cj0350	0.64	1.37E-07	hypothetical protein Cj0350
Cj0698	*flgG*	0.64	1.55E-06	flagellar basal body rod protein FlgG
Cj0384c	*kdsA*	0.64	8.64E-06	2-dehydro-3-deoxyphosphooctonate aldolase
Cj1237c	Cj1237c	0.64	6.82E-05	putative phosphatase
Cj0058	Cj0058	0.63	5.46E-05	putative peptidase C39 family protein
Cj0043	*flgE*	0.63	3.56E-06	flagellar hook protein
Cj0734c	*hisJ*	0.63	2.24E-06	histidine-binding protein precursor
Cj0833c	Cj0833c	0.62	2.90E-09	putative oxidoreductase
Cj1034c	Cj1034c	0.62	9.85E-05	adenylosuccinate lyase
Cj1280c	Cj1280c	0.61	4.87E-05	putative ribosomal pseudouridine synthase
Cj0912c	*cysM*	0.61	1.39E-06	cysteine synthase
Cj1531	*dapF*	0.61	5.93E-07	diaminopimelate epimerase
Cj0419	Cj0419	0.61	1.07E-06	putative histidine triad (HIT) family protein
Cj1403c	*gapA*	0.61	6.09E-07	glyceraldehyde 3-phosphate dehydrogenase
Cj1062	Cj1062	0.61	4.24E-05	putative CinA-like protein
Cj0752	Cj0752	0.60	5.44E-06	probable IS element transposase pseudogene,
Cj1598	*hisD*	-0.60	1.77E-05	histidinol dehydrogenase
Cj1026c	Cj1026c	-0.60	7.82E-06	putative lipoprotein
Cj1676	*murB*	-0.60	8.04E-05	UDP-N-acetylenolpyruvoylglucosamine reductase
Cj1509c	*fdhC*	-0.60	9.37E-05	putative formate dehydrogenase, cytochrom B subunit
Cj1566c	*nuoN*	-0.61	7.15E-05	NADH dehydrogenase I chain N
Cj1291c	*accB*	-0.61	6.77E-05	putative biotin carboxyl carrier protein of acetyl-CoA carboxylase
Cj0498	*trpC*	-0.61	4.58E-05	indole-3-glycerol phosphate synthase
Cj1125c	*pglA*	-0.62	3.24E-06	GalNAc transferase
Cj0342c	*uvrA*	-0.62	2.20E-06	excinuclease ABC subunit A
Cj1432c	Cj1432c	-0.63	8.51E-09	putative sugar transferase
Cj0273	*fabZ*	-0.63	9.68E-06	(3R)-hydroxymyristoyl-ACP dehydratase
Cj1634c	*aroC*	-0.63	5.68E-07	chorismate synthase

Cj0881c	Cj0881c	-0.63	1.25E-07	hypothetical protein Cj0881c
Cj1487c	*ccoP*	-0.64	1.92E-07	cb-type cytochrome C oxidase subunit III
Cj0589	*ribF*	-0.64	NA	bifunctional riboflavin kinase/FMN adenylyltransferase
Cj0712	*rimM*	-0.65	4.42E-07	putative 16S rRNA processing protein
Cj1439c	*glf*	-0.66	6.54E-07	UDP-galactopyranose mutase
Cj0556	Cj0556	-0.67	3.78E-06	putative amidohydrolase family protein
Cj0067	Cj0067	-0.67	2.19E-05	chlorohydrolase
Cj0936	*atpE*	-0.68	2.60E-06	F0F1 ATP synthase subunit C
Cj0405	*aroE*	-0.68	1.45E-05	shikimate 5-dehydrogenase
Cj0625	*hypD*	-0.68	2.81E-06	hydrogenase isoenzymes formation protein
Cj0500	Cj0500	-0.68	NA	tRNA 2-selenouridine synthase
Cj0652	*pbpC*	-0.69	7.06E-07	penicillin-binding protein
Cj0312	*pth*	-0.69	9.13E-06	peptidyl-tRNA hydrolase
Cj1084c	Cj1084c	-0.69	7.93E-05	putative ATP/GTP-binding protein
Cj0331c	Cj0331c	-0.69	5.43E-05	hypothetical protein Cj0331c
Cj1498c	*purA*	-0.69	3.39E-06	adenylosuccinate synthetase
Cj1252	Cj1252	-0.69	1.08E-05	putative periplasmic protein
Cj1082c	*thiD*	-0.69	3.98E-07	phosphomethylpyrimidine kinase
Cj1643	Cj1643	-0.71	4.70E-06	putative periplasmic protein
Cj1015c	*livG*	-0.71	1.97E-05	branched-chain amino-acid ABC transport system ATP-binding protein
Cj1684c	Cj1684c	-0.71	1.28E-05	putative transmembrane transport protein
Cj1013c	Cj1013c	-0.71	1.59E-05	putative cytochrome C biogenesis protein
Cj0777	Cj0777	-0.72	3.03E-06	putative ATP-dependent DNA helicase
Cj0311	Cj0311	-0.72	1.28E-06	50S ribosomal protein L25/general stress protein Ctc
Cj1260c	*dnaJ*	-0.73	6.46E-07	chaperone DnaJ
Cj1269c	*amiA*	-0.73	5.18E-05	N-acetylmuramoyl-L-alanine amidase
Cj1568c	*nuoL*	-0.73	1.32E-06	NADH dehydrogenase subunit L
Cj0601c	Cj0601c	-0.74	1.12E-05	putative sodium-dependent transmembrane transport protein
Cj1027c	*gyrA*	-0.74	9.42E-07	DNA gyrase subunit A
Cj1457c	*truD*	-0.74	2.55E-06	tRNA pseudouridine synthase D
Cj0896c	*pheT*	-0.75	5.35E-07	phenylalanyl-tRNA synthetase subunit beta
Cj0956c	*thdF*	-0.75	5.19E-07	tRNA modification GTPase TrmE
Cj1696c	*rplX*	-0.75	5.07E-06	50S ribosomal protein L24
Cj0006	Cj0006	-0.75	5.71E-08	putative Na+/H+ antiporter family protein
Cj1414c	*kpsC*	-0.75	5.88E-06	capsule polysaccharide modification protein
Cj1166c	Cj1166c	-0.76	5.68E-10	putative integral membrane protein
Cj1253	*pnp*	-0.76	9.58E-05	polynucleotide phosphorylase/polyadenylase
Cj0412	Cj0412	-0.76	1.30E-06	putative ATP/GTP binding protein
Cj0905c	*alr*	-0.77	2.13E-07	alanine racemase
Cj0536	*oorA*	-0.77	9.72E-05	2-oxoglutarate-acceptor oxidoreductase subunit OorA
Cj0090	Cj0090	-0.77	7.44E-08	putative lipoprotein
Cj0178	Cj0178	-0.78	NA	putative TonB-denpendent outer membrane receptor
Cj1510c	*fdhB*	-0.78	2.79E-06	putative formate dehydrogenase iron-sulfur subunit
Cj0855	*folD*	-0.79	2.71E-05	methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase
Cj1123c	*pglI*	-0.80	7.97E-05	acetyltransferase
Cj1567c	*nuoM*	-0.80	8.19E-06	NADH dehydrogenase I chain M
Cj1180c	Cj1180c	-0.80	6.17E-07	putative ABC transporter ATP-binding protein
Cj1303	*fabH2*	-0.81	4.09E-05	3-oxoacyl-(acyl carrier protein) synthase III
Cj1018c	*livK*	-0.82	1.27E-05	branched-chain amino-acid ABC transport system,periplasmic binding protein
Cj0316	*pheA*	-0.83	4.63E-07	chorismate mutase/prephenate dehydratase
Cj0827	*truA*	-0.83	7.14E-06	tRNA pseudouridine synthase A
Cj1241	Cj1241	-0.83	3.32E-05	putative MFS (Major Facilitator Superfamily) transporter

				protein
Cj1051c	*cjeI*	-0.83	5.13E-05	restriction modification enzyme
Cj1599	*hisB*	-0.83	8.50E-06	imidazole glycerol-phosphate dehydratase/histidinol phosphatase
Cj0551	*efp*	-0.84	4.21E-05	elongation factor P
Cj1187c	*arsB*	-0.84	2.14E-09	arsenical pump membrane protein
Cj1448c	*kpsM*	-0.84	4.82E-10	capsule polysaccharide export system inner membrane protein
Cj0124c	Cj0124c	-0.84	4.68E-06	hypothetical protein Cj0124c
Cj0555	Cj0555	-0.84	7.45E-08	putative dicarboxylate carrier protein MatC
Cj0892c	Cj0892c	-0.85	1.49E-07	putative periplasmic protein
Cj1606c	*mrp*	-0.85	1.88E-07	putative ATP/GTP-binding protein (Mrp protein homolog)
Cj0112	*tolB*	-0.85	2.47E-07	translocation protein TolB
Cj1596	*rplQ*	-0.87	5.28E-05	50S ribosomal protein L17
Cj1191c	Cj1191c	-0.87	5.42E-06	putative PAS domain containing signal-transduction sensor protein
Cj1204c	*atpB*	-0.87	2.14E-09	F0F1 ATP synthase subunit A
Cj0764c	*speA*	-0.87	9.04E-05	arginine decarboxylase
Cj1286c	*upp*	-0.88	9.01E-07	uracil phosphoribosyltransferase
Cj1438c	Cj1438c	-0.88	3.20E-07	putative sugar transferase
Cj0508	*pbpA*	-0.88	3.75E-06	penicillin-binding protein
Cj1601	*hisA*	-0.89	3.47E-07	1-(5-phosphoribosyl)-5-
Cj0375	Cj0375	-0.89	9.08E-05	putative lipoprotein
Cj0545	*hemC*	-0.89	2.99E-07	porphobilinogen deaminase
Cj0696	*ftsZ*	-0.90	1.01E-07	cell division protein FtsZ
Cj0116	*fabD*	-0.90	2.21E-06	malonyl CoA-acyl carrier protein transacylase
Cj0543	*proS*	-0.90	6.13E-08	prolyl-tRNA synthetase
Cj1188c	*gidA*	-0.90	6.77E-07	tRNA uridine 5-carboxymethylaminomethyl modification enzyme GidA
Cj0321	*dxs*	-0.90	1.97E-06	1-deoxy-D-xylulose-5-phosphate synthase
Cj1481c	Cj1481c	-0.90	1.45E-06	putative helicase
Cj0415	Cj0415	-0.90	4.30E-09	putative GMC oxidoreductase subunit
Cj1437c	Cj1437c	-0.91	1.23E-07	aminotransferase
Cj0381c	*pyrF*	-0.91	3.58E-07	orotidine 5'-phosphate decarboxylase
Cj1284	*ktrA*	-0.91	2.05E-06	putative K+ uptake protein
Cj1602	Cj1602	-0.91	8.44E-05	conserved hypothetical protein Cj1602
Cj0414	Cj0414	-0.92	1.27E-07	putative oxidoreductase subunit
Cj0611c	Cj0611c	-0.92	3.68E-07	putative acyltransferase family protein
Cj1474c	*ctsD*	-0.92	2.69E-09	putative type II protein secretion system D protein
Cj0546	*ubiD*	-0.92	5.03E-07	putative 3-octaprenyl-4-hydroxybenzoate carboxy-lyase
Cj1600	*hisH*	-0.92	4.83E-07	imidazole glycerol phosphate synthase subunit HisH
Cj1073c	*lon*	-0.93	7.80E-05	ATP-dependent protease La
Cj1482c	Cj1482c	-0.93	6.60E-09	hypothetical protein Cj1482c
Cj0259	*pyrC*	-0.94	6.47E-06	dihydroorotase
Cj0197c	*dapB*	-0.95	1.52E-05	dihydrodipicolinate reductase
Cj1302	Cj1302	-0.96	3.43E-05	putative HAD-superfamily phosphatase, subfamily IIIC
Cj0709	*ffh*	-0.96	1.59E-05	signal recognition particle protein
Cj0134	*thrB*	-0.96	1.50E-05	homoserine kinase
Cj0173c	*cfbpC*	-0.97	NA	putative iron-uptake ABC transport system ATP-binding protein
Cj0970	Cj0970	-0.97	NA	hypothetical protein Cj0970
Cj1708c	*rpsJ*	-0.97	6.24E-06	30S ribosomal protein S10
Cj1455	*prfB*	-0.97	1.22E-05	peptide chain release factor 2
Cj0208	Cj0208	-0.97	1.01E-05	putative DNA modification methylase (adenine-specific methyltransferase)
Cj1706c	*rplD*	-0.98	1.39E-07	50S ribosomal protein L4

Cj1030c	*lepA*	-0.99	4.87E-08	GTP-binding protein LepA
Cj0515	Cj0515	-0.99	3.00E-08	putative periplasmic protein
Cj1071	*ssb*	-0.99	4.98E-06	single-strand DNA-binding protein
Cj0262c	Cj0262c	-1.00	5.42E-11	putative methyl-accepting chemotaxis signal transduction protein
Cj0413	Cj0413	-1.00	1.81E-07	putative periplasmic protein
Cj1283	*ktrB*	-1.00	4.33E-09	putative K+ uptake protein
Cj1690c	*rpsE*	-1.00	1.92E-06	30S ribosomal protein S5
Cj0713	*trmD*	-1.00	9.81E-09	tRNA (guanine-N(1)-)-methyltransferase
Cj0714	*rplS*	-1.02	9.19E-10	50S ribosomal protein L19
Cj0274	*lpxA*	-1.02	8.96E-06	UDP-N-acetylglucosamine acyltransferase
Cj1425c	*hddA*	-1.03	2.96E-06	putative D-glycero-D-manno-heptose 7-phosphate kinase
Cj0091	Cj0091	-1.03	1.94E-08	putative lipoprotein
Cj1427c	Cj1427c	-1.04	2.54E-09	putative sugar-nucleotide epimerase/dehydratease
Cj0780	*napA*	-1.04	1.52E-13	nitrate reductase
Cj0231c	*nrdF*	-1.04	8.36E-07	ribonucleotide-diphosphate reductase subunit beta
Cj1603	*hisF*	-1.05	4.81E-07	imidazole glycerol phosphate synthase subunit HisF
Cj0474	*rplK*	-1.06	7.24E-07	50S ribosomal protein L11
Cj0621	Cj0621	-1.06	1.41E-06	hypothetical protein Cj0621
Cj0503c	*hemH*	-1.06	NA	ferrochelatase
Cj1205c	*radA*	-1.06	2.14E-10	DNA repair protein RadA
Cj0622	*hypF*	-1.08	4.11E-08	carbamoyltransferase
Cj0128c	Cj0128c	-1.08	6.42E-05	putative inositol monophosphatase family protein
Cj0293	*surE*	-1.09	3.95E-08	stationary phase survival protein SurE
Cj1093c	*secD*	-1.10	2.08E-07	preprotein translocase subunit SecD
Cj0891c	*serA*	-1.10	1.96E-09	D-3-phosphoglycerate dehydrogenase
Cj1440c	Cj1440c	-1.11	1.93E-09	putative sugar transferase
Cj1443c	*kpsF*	-1.11	2.26E-09	D-arabinose 5-phosphate isomerase
Cj0196c	*purF*	-1.12	2.90E-11	amidophosphoribosyltransferase
Cj1691c	*rplR*	-1.12	1.54E-06	50S ribosomal protein L18
Cj0991c	Cj0991c	-1.12	7.21E-06	putative oxidoreductase ferredoxin-type electron transport protein
Cj0183	Cj0183	-1.12	2.53E-05	putative integral membrane protein with haemolysin domain
Cj0245	*rplT*	-1.13	4.22E-06	50S ribosomal protein L20
Cj1428c	*fcl*	-1.14	1.79E-07	GDP-L-fucose synthetase
Cj1612	*prfA*	-1.14	1.69E-05	peptide chain release factor 1
Cj0507	*maf*	-1.14	2.71E-05	Maf-like protein
Cj0882c	*flhA*	-1.14	1.11E-06	flagellar biosynthesis protein FlhA
Cj0514	*purQ*	-1.16	2.59E-09	phosphoribosylformylglycinamidine synthase subunit I
Cj0518	*htpG*	-1.16	2.53E-07	heat shock protein 90
Cj1014c	*livF*	-1.16	7.86E-09	branched-chain amino-acid ABC transport system ATP-binding protein
Cj0856	*lepP*	-1.17	9.24E-08	signal peptidase I
Cj1479c	*rpsI*	-1.18	1.14E-06	30S ribosomal protein S9
Cj0108	*atpC*	-1.18	1.98E-10	F0F1 ATP synthase subunit epsilon
Cj1092c	*secF*	-1.18	6.63E-07	preprotein translocase subunit SecF
Cj1707c	*rplC*	-1.19	8.79E-06	50S ribosomal protein L3
Cj0283c	*cheW*	-1.19	8.34E-05	chemotaxis protein
Cj0107	*atpD*	-1.19	1.73E-08	F0F1 ATP synthase subunit beta
Cj1429c	Cj1429c	-1.19	1.20E-05	hypothetical protein Cj1429c
Cj0060c	*fliM*	-1.19	7.26E-08	flagellar motor switch protein FliM
Cj0626	*hypE*	-1.20	2.65E-08	hydrogenase isoenzymes formation protein
Cj0106	*atpG*	-1.20	9.98E-07	F0F1 ATP synthase subunit gamma
Cj0105	*atpA*	-1.21	3.84E-09	F0F1 ATP synthase subunit alpha
Cj1593	*rpsK*	-1.22	7.56E-07	30S ribosomal protein S11

Cj1605c	*dapD*	-1.22	1.03E-05	putative 2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase
Cj1186c	*petA*	-1.23	6.38E-10	putative ubiquinol-cytochrome C reductase iron-sulfur subunit
Cj1644	*ispA*	-1.23	2.06E-07	geranyltranstransferase
Cj1371	Cj1371	-1.23	5.00E-10	putative periplasmic protein (VacJ homolog)
Cj0284c	*cheA*	-1.23	4.46E-06	chemotaxis histidine kinase
Cj0542	*hemA*	-1.24	3.58E-07	glutamyl-tRNA reductase
Cj0353c	Cj0353c	-1.24	6.12E-08	phosphatase
Cj1441c	*kfiD*	-1.24	1.61E-11	UDP-glucose 6-dehydrogenase
Cj0782	*napH*	-1.25	3.97E-12	quinol dehydrogenase membrane component
Cj1017c	*livH*	-1.26	1.20E-07	branched-chain amino-acid ABC transport system permease protein
Cj1290c	*accC*	-1.27	5.94E-08	biotin carboxylase
Cj1038	Cj1038	-1.27	2.88E-09	putative cell division/peptidoglycan biosynthesis protein
Cj1702c	*rplV*	-1.28	7.62E-06	50S ribosomal protein L22
Cj0089	Cj0089	-1.29	1.08E-08	putative lipoprotein
Cj1434c	Cj1434c	-1.29	1.21E-09	putative sugar transferase
Cj0958c	Cj0958c	-1.29	1.76E-06	putative inner membrane protein translocase component YidC
Cj0781	*napG*	-1.29	6.57E-13	quinol dehydrogenase periplasmic component
Cj1645	*tkt*	-1.30	1.17E-06	transketolase
Cj0992c	*hemN*	-1.30	2.17E-08	coproporphyrinogen III oxidase
Cj1700c	*rplP*	-1.30	4.17E-06	50S ribosomal protein L16
Cj0504c	Cj0504c	-1.30	1.19E-06	putative oxidoreductase
Cj0479	*rpoC*	-1.31	3.07E-06	DNA-directed RNA polymerase beta' chain
Cj0707	*kdtA*	-1.31	3.02E-10	3-deoxy-D-manno-octulosonic-acid transferase
Cj0954c	Cj0954c	-1.33	4.72E-07	putative DnaJ-like protein
Cj0230c	Cj0230c	-1.33	4.73E-07	nicotinate phosphoribosyltransferase
Cj0281c	*tal*	-1.34	1.88E-08	transaldolase
Cj0093	Cj0093	-1.34	1.54E-09	putative periplasmic protein
Cj0636	Cj0636	-1.34	9.16E-09	NOL1/NOP2/sun family protein
Cj0947c	Cj0947c	-1.35	5.33E-06	putative carbon-nitrogen hydrolase
Cj0276	*mreB*	-1.36	2.61E-06	homolog of *E. coli* rod shape-determining protein
Cj1436c	Cj1436c	-1.36	3.28E-09	aminotransferase
Cj0633	Cj0633	-1.41	6.86E-06	putative periplasmic protein
Cj1052c	*mutS*	-1.42	5.05E-09	recombination and DNA strand exchange inhibitor protein
Cj0475	*rplA*	-1.42	9.49E-08	50S ribosomal protein L1
Cj0118	Cj0118	-1.44	7.33E-07	conserved hypothetical protein Cj0118
Cj0117	*pfs*	-1.44	5.75E-07	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase
Cj0092	Cj0092	-1.45	1.57E-12	putative periplasmic protein
Cj1693c	*rpsH*	-1.48	2.58E-06	30S ribosomal protein S8
Cj1362	*ruvB*	-1.48	1.27E-05	Holliday junction DNA helicase B
Cj0478	*rpoB*	-1.49	9.71E-08	DNA-directed RNA polymerase subunit beta
Cj0477	*rplL*	-1.50	1.54E-09	50S ribosomal protein L7/L12
Cj1016c	*livM*	-1.50	1.17E-07	branched-chain amino-acid ABC transport system permease protein
Cj0269c	*ilvE*	-1.52	1.37E-11	branched-chain amino acid aminotransferase
Cj0206	*thrS*	-1.52	2.00E-08	threonyl-tRNA synthetase
Cj0895c	*aroA*	-1.52	5.72E-07	3-phosphoshikimate 1-carboxyvinyltransferase
Cj1172c	Cj1172c	-1.53	2.08E-10	hypothetical protein Cj1172c
Cj0190c	Cj0190c	-1.53	2.92E-06	conserved hypothetical protein Cj0190c
Cj1181c	*tsf*	-1.53	1.19E-08	elongation factor Ts
Cj0442	*fabF*	-1.54	2.47E-08	3-oxoacyl-(acyl carrier protein) synthase II
Cj0473	*nusG*	-1.55	1.65E-08	transcription antitermination protein NusG

Cj1595	*rpoA*	-1.56	1.44E-06	DNA-directed RNA polymerase subunit alpha
Cj1480c	*rplM*	-1.57	6.33E-07	50S ribosomal protein L13
Cj0265c	Cj0265c	-1.58	8.08E-06	putative cytochrome C-type haem-binding periplasmic protein
Cj0328c	*fabH*	-1.60	4.70E-07	3-oxoacyl-(acyl carrier protein) synthase III
Cj1442c	Cj1442c	-1.61	2.39E-10	putative sugar transferase
Cj0267c	Cj0267c	-1.61	1.73E-07	putative integral membrane protein
Cj0329c	*plsX*	-1.63	2.69E-06	putative glycerol-3-phosphate acyltransferase PlsX
Cj0039c	*typA*	-1.63	5.98E-10	GTP-binding protein TypA homolog
Cj0098	*fmt*	-1.63	7.10E-08	methionyl-tRNA formyltransferase
Cj1701c	*rpsC*	-1.64	7.55E-06	30S ribosomal protein S3
Cj1689c	*rplO*	-1.65	2.40E-06	50S ribosomal protein L15
Cj1659	*p19*	-1.66	3.13E-14	periplasmic protein p19
Cj0492	*rpsG*	-1.66	2.10E-10	30S ribosomal protein S7
Cj0082	*cydB*	-1.66	2.23E-14	cytochrome bd oxidase subunit II
Cj0443	*accA*	-1.67	2.91E-07	acetyl-CoA carboxylase carboxyltransferase subunit alpha
Cj0275	*clpX*	-1.67	8.47E-07	ATP-dependent protease ATP-binding subunit
Cj0264c	Cj0264c	-1.71	4.59E-08	molybdopterin containing oxidoreductase
Cj1592	*rpsM*	-1.73	1.25E-06	30S ribosomal protein S13
Cj0226	*argB*	-1.74	2.37E-09	acetylglutamate kinase
Cj0470	*tuf*	-1.74	9.75E-09	elongation factor Tu
Cj1500	Cj1500	-1.74	2.52E-06	putative inner membrane protein
Cj1698c	*rpsQ*	-1.78	3.27E-07	30S ribosomal protein S17
Cj0953c	*purH*	-1.79	3.39E-09	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
Cj0023	*purB*	-1.79	2.34E-09	adenylosuccinate lyase
Cj1710c	Cj1710c	-1.80	4.29E-08	putative metallo-beta-lactamase family protein
Cj0268c	Cj0268c	-1.84	6.16E-06	putative transmembrane protein
Cj0704	*glyQ*	-1.84	1.99E-07	glycyl-tRNA synthetase subunit alpha
Cj1594	*rpsD*	-1.84	6.17E-06	30S ribosomal protein S4
Cj1182c	*rpsB*	-1.84	1.41E-08	30S ribosomal protein S2
Cj1695c	*rplE*	-1.87	1.81E-06	50S ribosomal protein L5
Cj1704c	*rplB*	-1.91	2.14E-06	50S ribosomal protein L2
Cj1711c	*ksgA*	-1.92	7.39E-11	dimethyladenosine transferase
Cj0433c	*mraY*	-1.93	7.62E-10	phospho-N-acetylmuramoyl-pentapeptide-transferase
Cj0705	Cj0705	-1.93	1.65E-08	conserved hypothetical protein Cj0705
Cj0109	*exbB3*	-1.94	2.52E-10	putative MotA/TolQ/ExbB proton channel family protein
Cj1692c	*rplF*	-1.95	4.34E-07	50S ribosomal protein L6
Cj0955c	*purL*	-1.96	1.79E-11	phosphoribosylformylglycinamidine synthase II
Cj1694c	*rpsN*	-1.96	1.10E-06	30S ribosomal protein S14
Cj0081	*cydA*	-1.98	3.55E-15	cytochrome bd oxidase subunit I
Cj0277	*mreC*	-2.08	NA	homolog of *E. coli* rod shape-determining protein
Cj1184c	*petC*	-2.16	1.51E-09	putative ubiquinol-cytochrome C reductase cytochrome C subunit
Cj0024	*nrdA*	-2.17	7.12E-09	ribonucleotide-diphosphate reductase subunit alpha
Cj0016	Cj0016	-2.24	4.68E-13	putative transcriptional regulatory protein
Cj1688c	*secY*	-2.25	1.00E-06	preprotein translocase subunit SecY
Cj0493	*fusA*	-2.28	4.30E-10	elongation factor G
Cj1185c	*petB*	-2.29	1.99E-11	putative ubiquinol-cytochrome C reductase cytochrome B subunit
Cj0701	Cj0701	-2.32	1.12E-09	putative protease
Cj0154c	Cj0154c	-2.33	2.19E-12	putative tetrapyrrole methylase family protein
Cj0224	*argC*	-2.35	1.27E-11	N-acetyl-gamma-glutamyl-phosphate reductase
Cj0505c	Cj0505c	-2.42	1.36E-10	putative aminotransferase (degT family).
Cj1170c	*omp50*	-3.07	3.33E-16	50 kda outer membrane protein precursor

Appendix IV.
Transcriptome data from chick small intestinal mucus medium

Gene	Name	Fold Change (log_2)	Bayes.p	Protein
Cj1357c	*nrfA*	2.65	0	putative periplasmic cytochrome C
Cj0561c	Cj0561c	2.48	0	putative periplasmic protein
Cj0874c	Cj0874c	2.45	0	putative cytochrome C
Cj0367c	*cmeA*	2.09	0	periplasmic fusion protein CmeA (multidrug efflux system CmeABC)
Cj0366c	*cmeB*	2.00	0	inner membrane efflux transporter CmeB (multidrug efflux system CmeABC)
Cj1358c	*nrfH*	1.94	0	putative periplasmic cytochrome C
Cj0437	*mrfA*	1.76	0	succinate dehydrogenase flavoprotein subunit
Cj0365c	*cmeC*	1.69	0	outer membrane channel protein CmeC (multidrug efflux system CmeABC)
Cj0087	*aspA*	1.68	0	aspartate ammonia-lyase
Cj0876c	Cj0876c	1.67	0	putative periplasmic protein
Cj0671	*dcuB*	1.65	0	anaerobic C4-dicarboxylate transporter
Cj0088	*dcuA*	1.56	0	anaerobic C4-dicarboxylate transporter
Cj0438	*mrfB*	1.54	0	putative succinate dehydrogenase iron-sulfur protein
Cj0439	*mrfE*	1.48	0	putative succinate dehydrogenase subunit C
Cj1183c	*cfa*	1.40	1.11E-16	cyclopropane-fatty-acyl-phospholipid synthase
Cj0346	*trpD*	1.33	2.23E-11	anthranilate synthase component II
Cj0448c	Cj0448c	1.32	0	putative MCP-type signal transduction protein
Cj1687	Cj1687	1.25	0	putative efflux protein
Cj0781	*napG*	1.20	0	quinol dehydrogenase periplasmic component
Cj0037c	Cj0037c	1.17	0	putative cytochrome C
Cj0345	*trpE*	1.16	7.19E-14	putative anthranilate synthase component I
Cj0782	*napH*	1.15	0	quinol dehydrogenase membrane component
Cj0449c	Cj0449c	1.14	1.11E-16	conserved hypothetical protein Cj0449c
Cj0780	*napA*	1.11	0	nitrate reductase
Cj1360c	Cj1360c	1.08	2.22E-16	putative proteolysis tag for 10Sa_RNA
Cj0035c	Cj0035c	1.05	0	putative efflux protein
Cj0358	Cj0358	1.04	0	putative cytochrome C551 peroxidase
Cj0348	*trpB*	1.01	0	tryptophan synthase subunit beta
Cj1540	Cj1540	1.00	0	putative periplasmic protein
Cj1324	Cj1324	0.94	0	hypothetical protein Cj1324
Cj0757	hrcA	0.92	1.11E-16	heat-inducible transcription repressor
Cj0005c	Cj0005c	0.92	1.86E-11	molydopterin containing oxidoreductase
Cj0755	*cfrA*	0.90	1.33E-09	ferric enterobactin uptake receptor
Cj0440c	Cj0440c	0.85	1.11E-16	putative transcriptional regulator
Cj0171	Cj0170	0.84	6.03E-08	merged with Cj0170
Cj0759	*dnaK*	0.79	5.72E-13	molecular chaperone DnaK
Cj0349	*trpA*	0.78	2.98E-11	tryptophan synthase subunit alpha
Cj1228c	*htrA*	0.77	1.89E-12	serine protease (protease DO)
Cj0069	Cj0069	0.74	7.13E-14	hypothetical protein Cj0069
Cj0778	*peb2*	0.74	0	major antigenic peptide PEB2
Cj0998c	Cj0998c	0.73	2.55E-15	putative periplasmic protein
Cj1000	Cj1000	0.73	2.59E-10	putative transcriptional regulator (LysR family)
Cj0758	*grpE*	0.73	1.25E-10	heat shock protein GrpE
Cj0999c	Cj0999c	0.72	3.32E-10	putative integral membrane protein
Cj1719c	*leuA*	0.72	1.77E-10	2-isopropylmalate synthase
Cj0168c	Cj0168c	0.70	1.70E-06	putative periplasmic protein

Cj0039c	*typA*	0.69	0	GTP-binding protein TypA homolog
Cj1326	Cj1325	0.68	1.11E-16	merged with Cj1325
Cj0014c	Cj0014c	0.68	7.03E-14	putative integral membrane protein
Cj1193c	Cj1193c	0.65	3.56E-09	putative periplasmic protein
Cj0783	*napB*	0.64	6.80E-12	periplasmic nitrate reductase small subunit (cytochrome C-type protein)
Cj0731	Cj0731	0.63	3.40E-04	putative ABC transport system permease
Cj0175c	*cfbpA*	0.63	4.52E-13	putative iron-uptake ABC transport system,periplasmic iron-binding protein
Cj0903c	Cj0903c	-0.61	3.35E-12	putative amino-acid transport protein
Cj0770c	Cj0770c	-0.63	3.33E-16	putative NLPA family lipoprotein
Cj0827	*truA*	-0.64	2.92E-04	tRNA pseudouridine synthase A
Cj0204	Cj0204	-0.65	2.91E-13	putative oligopeptide transporter, OPT family
Cj0831c	*trmA*	-0.66	6.33E-07	tRNA (uracil-5-)-methyltransferase
Cj0607	Cj0607	-0.66	8.96E-11	ABC-type transmembrane transport protein
Cj0379c	Cj0379c	-0.68	1.34E-12	putative sulfite oxidase subunit YedY
Cj0281c	*tal*	-0.68	7.77E-16	transaldolase
Cj0501	Cj0501	-0.70	1.47E-07	probable ammonium transporter pseudogene
Cj0817	*glnH*	-0.70	1.41E-14	putative glutamine binding periplasmic protein
Cj1727c	*metB*	-0.73	1.96E-13	putative O-acetylhomoserine (thiol)-lyase
Cj0334	*ahpC*	-0.77	8.26E-14	alkyl hydroperoxide reductase
Cj0008	Cj0008	-0.78	1.47E-09	conserved hypothetical protein Cj0008
Cj1666c	Cj1666c	-0.78	3.66E-15	putative periplasmic protein
Cj1375	Cj1375	-0.83	3.26E-14	putative multidrug efflux transporter
Cj0415	Cj0415	-0.85	2.41E-07	putative GMC oxidoreductase subunit
Cj0846	Cj0846	-0.86	1.22E-04	putative metallophosphoesterase
Cj0009	*gltD*	-0.87	1.11E-16	putative oxidoreductase
Cj1110c	Cj1110c	-0.88	0	putative MCP-type signal transduction protein
Cj0262c	Cj0262c	-0.89	0	putative methyl-accepting chemotaxis signal transduction protein
Cj1376	Cj1376	-0.93	0	putative periplasmic protein
Cj1625c	*sdaC*	-0.93	0	amino acid transporter
Cj0076c	*lctP*	-1.04	0	L-lactate permease
Cj0073c	Cj0073c	-1.05	0	conserved hypothetical protein Cj0073c
Cj0075c	Cj0075c	-1.05	0	putative oxidoreductase iron-sulfur subunit
Cj0074c	Cj0074c	-1.06	0	putative iron-sulfur protein
Cj0343c	Cj0343c	-1.06	0	putative integral membrane protein
Cj1624c	*sdaA*	-1.06	0	L-serine dehydratase
Cj0553	Cj0553	-1.07	1.11E-16	putative integral membrane protein
Cj0556	Cj0556	-1.07	3.07E-10	putative amidohydrolase family protein
Cj0203	Cj0203	-1.08	7.11E-15	putative citrate transporter
Cj0414	Cj0414	-1.09	1.81E-09	putative oxidoreductase subunit
Cj0699c	*glnA*	-1.17	2.00E-15	glutamine synthetase
Cj0552	Cj0552	-1.18	0	hypothetical protein Cj0552
Cj0453	*thiC*	-1.19	0	thiamine biosynthesis protein ThiC
Cj0779	*tpx*	-1.21	2.22E-16	thiol peroxidase
Cj0987c	Cj0987c	-1.28	5.29E-11	putative MFS (Major Facilitator Superfamily) transport protein
Cj0169	*sodB*	-1.35	0	superoxide dismutase (Fe)
Cj1189c	*cetB*	-1.45	0	bipartate energy taxis response protein cetB
Cj0239c	Cj0239c	-1.70	0	NifU protein homolog
Cj0240c	*iscS*	-1.73	0	cysteine desulfurase (NifS protein homolog)
Cj0555	Cj0555	-1.73	9.08E-14	putative dicarboxylate carrier protein MatC
Cj1513c	Cj1513c	-1.77	1.11E-16	possible periplasmic protein
Cj1510c	*fdhB*	-1.81	0	putative formate dehydrogenase iron-sulfur subunit
Cj1190c	*cetA*	-1.82	0	bipartate energy taxis response protein cetA

Cj1509c	*fdhC*	-1.84	0	putative formate dehydrogenase, cytochrom B subunit
Cj1511c	*fdhA*	-1.90	0	putative formate dehydrogenase large subunit (Selenocysteine containing)
Cj0289c	*peb3*	-1.90	0	major antigenic peptide PEB3
Cj1514c	Cj1514c	-2.03	0	hypothetical protein Cj1514c
Cj0025c	Cj0025c	-2.27	0	putative sodium:dicarboxylate family transmembrane symporter
Cj1500	Cj1500	-2.72	0	putative inner membrane protein

Appendix V.
Transcriptomic data from chick cecal mucus medium

Gene	Name	Fold Change (log_2)	Bayes.p	Protein
Cj1502c	*putP*	2.65	0	putative sodium/proline symporter
Cj1037c	*pycA*	2.44	0	acetyl-CoA carboxylase
Cj1444c	*kpsD*	2.15	0	capsule polysaccharide export system periplasmic protein
Cj0087	*aspA*	2.00	0	aspartate ammonia-lyase
Cj0929	*pepA*	1.97	0	leucyl aminopeptidase
Cj0481	*dapA*	1.92	0	putative dihydrodipicolinate synthase
Cj0081	*cydA*	1.90	0	cytochrome bd oxidase subunit I
Cj0438	*mrfB*	1.89	2.00E-09	putative succinate dehydrogenase iron-sulfur protein
Cj0671	*dcuB*	1.88	0	anaerobic C4-dicarboxylate transporter
Cj0919c	Cj0919c	1.86	0	putative ABC-type amino-acid transporter permease protein
Cj0778	*peb2*	1.86	0	major antigenic peptide PEB2
Cj0483	*uxaA'*	1.85	3.21E-14	putative altronate hydrolase C-terminus
Cj0007	*gltB*	1.80	0	glutamate synthase (NADPH) large subunit
Cj0021c	Cj0021c	1.78	1.89E-15	putative fumarylacetoacetate (FAA) hydrolase family protein
Cj0358	Cj0358	1.62	1.81E-09	putative cytochrome C551 peroxidase
Cj0920c	Cj0920c	1.61	0	putative ABC-type amino-acid transporter permease protein
Cj0486	*fucP*	1.58	5.24E-14	putative sugar transporter
Cj0439	*mrfE*	1.50	1.96E-08	putative succinate dehydrogenase subunit C
Cj0073c	Cj0073c	1.48	2.84E-14	conserved hypothetical protein Cj0073c
Cj0485	Cj0485	1.48	9.57E-11	short chain dehydrogenase
Cj0487	Cj0487	1.44	6.49E-10	putative amidohydrolase
Cj1537c	*acs*	1.37	4.36E-13	acetyl-CoA synthetase
Cj0075c	Cj0075c	1.36	2.78E-15	putative oxidoreductase iron-sulfur subunit
Cj0484	Cj0484	1.34	5.12E-11	putative MFS (Major Facilitator Superfamily) transport protein
Cj0074c	Cj0074c	1.28	3.28E-14	putative iron-sulfur protein
Cj0942c	*secA*	1.26	6.21E-10	preprotein translocase subunit SecA
Cj0802	*cysS*	1.25	0	cysteinyl-tRNA synthetase
Cj0088	*dcuA*	1.22	0	anaerobic C4-dicarboxylate transporter
Cj0834c	Cj0834c	1.18	0	ankyrin repeat-containing putative periplasmic protein
Cj0762c	*aspB*	1.17	2.22E-16	aspartate aminotransferase
Cj1542	Cj1542	1.12	0	putative allophanate hydrolase subunit 1
Cj1167	*ldh*	1.10	2.22E-12	L-lactate dehydrogenase
Cj1357c	*nrfA*	1.06	1.08E-10	putative periplasmic cytochrome C
Cj1358c	*nrfH*	1.05	2.57E-08	putative periplasmic cytochrome C
Cj0835c	*acnB*	1.02	0	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
Cj0347	*trpF*	1.02	3.94E-05	N-(5'-phosphoribosyl)anthranilate isomerase
Cj1544c	Cj1544c	1.00	4.44E-16	putative integral membrane protein
Cj1543	Cj1543	0.99	0	putative allophanate hydrolase subunit 2
Cj0175c	*cfbpA*	0.98	1.23E-08	putative iron-uptake ABC transport system,periplasmic iron-binding protein
Cj0798c	*ddl*	0.94	7.81E-08	D-alanyl-alanine synthetase A
Cj0490	*ald'*	0.92	1.18E-11	putative aldehyde dehydrogenase C-terminus
Cj1324	Cj1324	0.89	1.11E-16	hypothetical protein Cj1324
Cj0069	Cj0069	0.88	1.54E-07	hypothetical protein Cj0069
Cj0833c	Cj0833c	0.87	0	putative oxidoreductase

Cj0393c	*mqo*	0.85	2.65E-14	putative malate:quinone oxidoreductase
Cj1380	Cj1380	0.84	3.37E-12	putative periplasmic protein
Cj0992c	hemN	0.82	3.33E-16	coproporphyrinogen III oxidase
Cj0420	Cj0420	0.78	6.33E-08	putative periplasmic protein
Cj0437	*mrfA*	0.78	2.30E-11	succinate dehydrogenase flavoprotein subunit
Cj0440c	Cj0440c	0.78	2.78E-14	putative transcriptional regulator
Cj0448c	Cj0448c	0.72	6.27E-08	putative MCP-type signal transduction protein
Cj0689	*ackA*	0.71	1.64E-10	acetate kinase
Cj0255c	*exoA*	0.70	4.41E-05	exodeoxyribonuclease
Cj1625c	*sdaC*	0.70	3.71E-08	amino acid transporter
Cj1451	*dut*	0.68	8.02E-05	dUTPase
Cj1718c	*leuB*	0.66	2.82E-08	3-isopropylmalate dehydrogenase
Cj0345	*trpE*	0.66	1.40E-07	putative anthranilate synthase component I
Cj1098	*pyrB*	0.65	4.84E-09	aspartate carbamoyltransferase catalytic subunit
Cj1242	Cj1242	0.65	3.58E-07	hypothetical protein Cj1242
Cj0312	*pth*	0.64	2.00E-08	peptidyl-tRNA hydrolase
Cj0194	*folE*	0.64	2.89E-15	GTP cyclohydrolase I
Cj1182c	*rpsB*	0.64	1.49E-09	30S ribosomal protein S2
Cj1193c	Cj1193c	0.61	1.10E-06	putative periplasmic protein
Cj1243	*hemE*	-0.60	1.87E-09	uroporphyrinogen decarboxylase
Cj0460	*nusA*	-0.61	1.51E-09	transcription elongation factor NusA
Cj1570c	*nuoJ*	-0.61	6.73E-14	NADH dehydrogenase subunit J
Cj1576c	*nuoD*	-0.61	3.63E-12	NADH dehydrogenase subunit D
Cj0595c	*nth*	-0.62	1.79E-11	endonuclease III
Cj1638	*dnaG*	-0.63	2.23E-12	DNA primase
Cj1620c	*mutY*	-0.64	1.43E-09	A/G-specific adenine glycosylase
Cj1577c	*nuoC*	-0.64	7.33E-15	NADH dehydrogenase subunit C
Cj1379	*selB*	-0.65	5.49E-07	putative selenocysteine-specific elongation factor
Cj1567c	*nuoM*	-0.65	8.62E-14	NADH dehydrogenase I chain M
Cj1161c	Cj1161c	-0.68	8.59E-09	putative cation-transporting ATPase
Cj0955c	*purL*	-0.71	2.40E-08	phosphoribosylformylglycinamidine synthase II
Cj1189c	*cetB*	-0.71	7.65E-10	bipartate energy taxis response protein cetB
Cj0082	*cydB*	-0.74	7.22E-10	cytochrome bd oxidase subunit II
Cj0012c	*rrc*	-0.75	2.81E-09	non-haem iron protein
Cj0333c	*fdxA*	-0.77	4.65E-05	ferredoxin
Cj1573c	*nuoG*	-0.78	2.37E-11	NADH dehydrogenase subunit G
Cj0940c	*glnP*	-0.79	6.66E-16	putative glutamine transport system permease
Cj0237	*cynT*	-0.81	4.14E-07	carbonic anyhydrase
Cj0002	*dnaN*	-0.87	3.59E-06	DNA polymerase III subunit beta
Cj1220	*groES*	-0.98	2.36E-13	co-chaperonin GroES
Cj1010	*tgt*	-0.99	4.27E-11	queuine tRNA-ribosyltransferase
Cj1190c	*cetA*	-0.99	1.73E-13	bipartate energy taxis response protein cetA
Cj0017c	*dsbI*	-1.09	2.65E-14	disulphide bond formation protein
Cj0845c	*gltX*	-1.09	4.96E-06	glutamyl-tRNA synthetase
Cj0764c	*speA*	-1.16	0	arginine decarboxylase
Cj0192c	*clpP*	-1.17	1.21E-07	ATP-dependent Clp protease proteolytic subunit
Cj0008	Cj0008	-1.20	3.95E-06	conserved hypothetical protein Cj0008
Cj0379c	Cj0379c	-1.22	2.22E-16	putative sulfite oxidase subunit YedY
Cj0677	*kdpB*	-1.24	1.94E-06	potassium-transporting ATPase subunit B
Cj0343c	Cj0343c	-1.26	0	putative integral membrane protein
Cj1514c	Cj1514c	-1.30	6.35E-14	hypothetical protein Cj1514c
Cj0262c	Cj0262c	-1.37	4.21E-12	putative methyl-accepting chemotaxis signal transduction protein
Cj0817	*glnH*	-1.37	1.02E-08	putative glutamine binding periplasmic protein
Cj0415	Cj0415	-1.41	4.29E-08	putative GMC oxidoreductase subunit
Cj0831c	*trmA*	-1.44	0	tRNA (uracil-5-)-methyltransferase

Cj1510c	*fdhB*	-1.51	0	putative formate dehydrogenase iron-sulfur subunit
Cj0414	Cj0414	-1.61	5.91E-10	putative oxidoreductase subunit
Cj1508c	*fdhD*	-1.68	1.11E-16	formate dehydrogenase accessory protein
Cj1509c	*fdhC*	-1.73	0	putative formate dehydrogenase, cytochrom B subunit
Cj0334	*ahpC*	-1.78	0	alkyl hydroperoxide reductase
Cj1515c	Cj1515c	-1.84	0	putative decarboxylase
Cj1170c	*omp50*	-2.04	9.25E-09	50 kda outer membrane protein precursor
Cj0239c	Cj0239c	-2.27	0	NifU protein homolog
Cj0240c	*iscS*	-2.33	0	cysteine desulfurase (NifS protein homolog)
Cj1082c	*thiD*	-2.36	2.22E-10	phosphomethylpyrimidine kinase
Cj1501	Cj1501	-2.68	2.91E-08	hypothetical protein Cj1501
Cj0025c	Cj0025c	-3.03	0	putative sodium:dicarboxylate family transmembrane symporter

Appendix VI.
Transcriptomc data from mucin supplemented medium

Name	Gene	Fold Change (log_2)	Bayes.p	Protein
Cj0438	*mrfB*	5.44	0	putative succinate dehydrogenase iron-sulfur protein
Cj0358	Cj0358	5.21	0	putative cytochrome C551 peroxidase
Cj0439	*mrfE*	4.79	0	putative succinate dehydrogenase subunit C
Cj0437	*mrfA*	4.78	0	succinate dehydrogenase flavoprotein subunit
Cj0073c	Cj0073c	4.58	NA	conserved hypothetical protein Cj0073c
Cj0075c	Cj0075c	4.30	1.28E-13	putative oxidoreductase iron-sulfur subunit
Cj0074c	Cj0074c	4.22	1.01E-13	putative iron-sulfur protein
Cj1682c	*gltA*	4.03	0	citrate synthase
Cj1357c	*nrfA*	3.86	0	putative periplasmic cytochrome C
Cj0012c	*rrc*	3.43	4.16E-07	non-haem iron protein
Cj1619	*kgtP*	3.18	NA	alpha-ketoglutarate permease
Cj0921c	*peb1A*	3.12	0	bifunctional adhesin/ABC transporter aspartate/glutamate-binding protein
Cj1502c	*putP*	3.06	0	putative sodium/proline symporter
Cj0671	*dcuB*	3.05	0	anaerobic C4-dicarboxylate transporter
Cj1503c	*putA*	2.93	0	putative proline dehydrogenase/delta-1-pyrroline-5-carboxylate dehydrogenase
Cj0782	*napH*	2.80	3.12E-07	quinol dehydrogenase membrane component
Cj0919c	Cj0919c	2.74	0	putative ABC-type amino-acid transporter permease protein
Cj0968	Cj0969	2.73	NA	probable periplasmic protein
Cj0922c	*pebC*	2.66	0	ABC-type amino-acid transporter ATP-binding protein
Cj0834c	Cj0834c	2.66	2.66E-15	ankyrin repeat-containing putative periplasmic protein
Cj0021c	Cj0021c	2.63	0	putative fumarylacetoacetate (FAA) hydrolase family protein
Cj1358c	*nrfH*	2.61	NA	putative periplasmic cytochrome C
Cj1511c	*fdhA*	2.54	NA	putative formate dehydrogenase large subunit (Selenocysteine containing)
Cj0277	*mreC*	2.53	NA	homolog of *E. coli* rod shape-determining protein
Cj0920c	Cj0920c	2.53	0	putative ABC-type amino-acid transporter permease protein
Cj0781	*napG*	2.46	3.39E-07	quinol dehydrogenase periplasmic component
Cj0780	*napA*	2.40	4.15E-07	nitrate reductase
Cj1509c	*fdhC*	2.35	3.33E-16	putative formate dehydrogenase, cytochrom B subunit
Cj0833c	Cj0833c	2.32	NA	putative oxidoreductase
Cj0853c	*hemL*	2.28	4.77E-15	glutamate-1-semialdehyde aminotransferase
Cj0903c	Cj0903c	2.27	0	putative amino-acid transport protein
Cj0333c	*fdxA*	2.17	3.33E-16	ferredoxin
Cj1510c	*fdhB*	2.09	8.88E-16	putative formate dehydrogenase iron-sulfur subunit
Cj0917c	*cstA*	2.04	0	putative integral membrane protein (CstA homolog)
Cj0076c	*lctP*	2.04	1.17E-14	L-lactate permease
Cj0538	*oorC*	1.94	2.15E-14	2-oxoglutarate-acceptor oxidoreductase subunit OorC
Cj1541	Cj1541	1.94	0	hypothetical protein Cj1541
Cj0832c	Cj0832c	1.92	1.11E-16	putative Na+/H+ antiporter family protein
Cj0537	*oorB*	1.90	9.41E-12	2-oxoglutarate-acceptor oxidoreductase subunit OorB
Cj0410	*frdB*	1.89	3.81E-10	fumarate reductase iron-sulfur subunit
Cj1192	*dctA*	1.88	NA	putative C4-dicarboxylate transport protein
Cj0835c	*acnB*	1.88	9.33E-15	bifunctional aconitate hydratase 2/2-methylisocitrate dehydratase
Cj0536	*oorA*	1.88	9.10E-11	2-oxoglutarate-acceptor oxidoreductase subunit OorA

Cj0428	Cj0428	1.87	0	hypothetical protein Cj0428
Cj0481	*dapA*	1.86	0	putative dihydrodipicolinate synthase
Cj0850c	Cj0850c	1.82	1.11E-16	putative MFS (Major Facilitator Superfamily) transport protein
Cj1624c	*sdaA*	1.81	2.02E-14	L-serine dehydratase
Cj0409	*frdA*	1.74	3.04E-08	fumarate reductase flavoprotein subunit
Cj0552	Cj0552	1.73	NA	hypothetical protein Cj0552
Cj0408	*frdC*	1.65	1.86E-07	fumarate reductase cytochrome b-556 subunit
Cj1473c	*ctsP*	1.62	NA	putative ATP/GTP-binding protein
Cj0553	Cj0553	1.59	NA	putative integral membrane protein
Cj0762c	*aspB*	1.58	0	aspartate aminotransferase
Cj0427	Cj0427	1.56	0	hypothetical protein Cj0427
Cj1681c	*cysQ*	1.55	0	CysQ protein homolog
Cj1184c	*petC*	1.51	1.75E-07	putative ubiquinol-cytochrome C reductase cytochrome C subunit
Cj0089	Cj0089	1.48	0	putative lipoprotein
Cj0978c	Cj0978c	1.44	NA	putative lipoprotein
Cj1729c	*flgE2*	1.43	2.56E-08	flagellar hook protein FlgE
Cj1487c	*ccoP*	1.43	4.33E-15	cb-type cytochrome C oxidase subunit III
Cj0535	*oorD*	1.42	1.13E-10	2-oxoglutarate-acceptor oxidoreductase subunit OorD
Cj0393c	*mqo*	1.42	0	putative malate:quinone oxidoreductase
Cj0482	*uxaA′*	1.40	NA	putative altronate hydrolase N-terminus
Cj0689	*ackA*	1.39	0	acetate kinase
Cj1185c	*petB*	1.38	5.20E-07	putative ubiquinol-cytochrome C reductase cytochrome B subunit
Cj1625c	*sdaC*	1.35	2.22E-15	amino acid transporter
Cj0042	*flgD*	1.31	1.46E-12	flagellar basal body rod modification protein
Cj1265c	*hydC*	1.29	8.25E-07	Ni/Fe-hydrogenase B-type cytochrome subunit
Cj1543	Cj1543	1.27	NA	putative allophanate hydrolase subunit 2
Cj0449c	Cj0449c	1.27	4.98E-14	conserved hypothetical protein Cj0449c
Cj1186c	*petA*	1.27	8.83E-07	putative ubiquinol-cytochrome C reductase iron-sulfur subunit
Cj1468	Cj1468	1.25	NA	putative integral membrane protein
Cj0603c	*dsbD*	1.24	NA	putative thiol:disulphide interchange protein
Cj1267c	*hydA*	1.24	4.42E-05	Ni/Fe-hydrogenase small chain
Cj1360c	Cj1360c	1.24	5.77E-15	putative proteolysis tag for 10Sa_RNA
Cj1486c	Cj1486c	1.22	2.22E-16	putative periplasmic protein
Cj0486	*fucP*	1.22	NA	putative sugar transporter
Cj0088	*dcuA*	1.20	4.00E-15	anaerobic C4-dicarboxylate transporter
Cj1153	Cj1153	1.18	8.12E-13	putative periplasmic cytochrome C
Cj0687c	*flgH*	1.17	1.38E-08	flagellar basal body L-ring protein
Cj0448c	Cj0448c	1.17	0	putative MCP-type signal transduction protein
Cj1488c	*ccoQ*	1.16	4.15E-12	cb-type cytochrome C oxidase subunit IV
Cj1462	*flgI*	1.13	8.29E-10	flagellar basal body P-ring protein
Cj0156c	Cj0156c	1.10	NA	16S rRNA m3U1498 methyltransferase
Cj0688	*pta*	1.10	1.11E-16	putative phosphate acetyltransferase
Cj0484	Cj0484	1.09	NA	putative MFS (Major Facilitator Superfamily) transport protein
Cj1356c	Cj1356c	1.09	0	putative integral membrane protein
Cj0982c	*cjaA*	1.08	4.66E-15	putative amino-acid transporter periplasmic solute-binding protein
Cj1266c	*hydB*	1.07	0.000165 977	Ni/Fe-hydrogenase large subunit
Cj0604	Cj0604	1.06	2.42E-14	putative polyphosphate kinase
Cj0910	Cj0910	1.04	NA	putative periplasmic protein
Cj1466	*flgK*	1.03	2.05E-07	flagellar hook-associated protein FlgK

Cj1542	Cj1542	1.02	5.67E-10	putative allophanate hydrolase subunit 1
Cj0041	*fliK*	1.02	NA	putative flagellar hook-length control protein
Cj0887c	*flgL*	1.01	NA	putative flagellin
Cj0370	*rpsU*	1.01	NA	30S ribosomal protein S21
Cj1489c	*ccoO*	1.00	4.08E-11	cb-type cytochrome C oxidase subunit II
Cj0952c	Cj0952c	1.00	6.66E-16	putative HAMP containing membrane protein
Cj0697	*flgG2*	1.00	5.21E-08	flagellar basal-body rod protein
Cj0011c	Cj0011c	1.00	1.22E-07	putative non-specific DNA binding protein.
Cj1537c	*acs*	0.99	1.94E-07	acetyl-CoA synthetase
Cj0485	Cj0485	0.98	NA	short chain dehydrogenase
Cj0854c	Cj0854c	0.98	NA	putative periplasmic protein
Cj1464	*flgM*	0.97	2.07E-12	hypothetical protein Cj1464
Cj0831c	*trmA*	0.97	NA	tRNA (uracil-5-)-methyltransferase
Cj0849c	Cj0849c	0.97	1.21E-14	conserved hypothetical protein Cj0849c
Cj1264c	*hydD*	0.96	NA	putative hydrogenase maturation protease
Cj0865	*dsbB*	0.94	NA	putative disulfide oxidoreductase
Cj0368c	*cmeR*	0.94	NA	transcriptional regulator CmeR
Cj0987c	Cj0987c	0.93	NA	putative MFS (Major Facilitator Superfamily) transport protein
Cj0534	*sucD*	0.92	4.80E-10	succinyl-coA synthetase alpha chain
Cj0043	*flgE*	0.92	7.44E-10	flagellar hook protein
Cj0913c	*hupB*	0.92	8.61E-10	DNA-binding protein HU homolog
Cj0699c	*glnA*	0.91	NA	glutamine synthetase
Cj1719c	*leuA*	0.91	3.27E-11	2-isopropylmalate synthase
Cj1399c	*hydA2*	0.90	NA	putative Ni/Fe-hydrogenase small subunit
Cj0565	Cj0565	0.89	1.22E-07	probable pseudogene
Cj0735	Cj0735	0.88	NA	putative periplasmic protein
Cj0069	Cj0069	0.88	1.72E-11	hypothetical protein Cj0069
Cj0977	Cj0977	0.88	1.36E-08	hypothetical protein Cj0977
Cj0532	*mdh*	0.88	1.31E-08	malate dehydrogenase
Cj0560	Cj0560	0.87	NA	putative MATE family transport protein
Cj0980	Cj0980	0.85	1.71E-07	putative peptidase
Cj0698	*flgG*	0.85	8.83E-09	flagellar basal body rod protein FlgG
Cj0601c	Cj0601c	0.85	NA	putative sodium-dependent transmembrane transport protein
Cj1585c	Cj1585c	0.85	NA	putative oxidoreductase
Cj0013	*ilvD*	0.83	NA	dihydroxy-acid dehydratase
Cj1167	*ldh*	0.82	NA	L-lactate dehydrogenase
Cj0522	Cj0522	0.81	NA	putative Na+/Pi cotransporter protein
Cj0561c	Cj0561c	0.81	2.04E-05	putative periplasmic protein
Cj0783	*napB*	0.80	7.80E-05	periplasmic nitrate reductase small subunit (cytochrome C-type protein)
Cj0734c	*hisJ*	0.79	8.41E-09	histidine-binding protein precursor
Cj1026c	Cj1026c	0.79	3.41E-08	putative lipoprotein
Cj0566	Cj0566	0.78	NA	hypothetical protein Cj0566
Cj1484c	Cj1484c	0.78	NA	hypothetical protein Cj1484c
Cj0487	Cj0487	0.78	NA	putative amidohydrolase
Cj0092	Cj0092	0.78	2.23E-14	putative periplasmic protein
Cj0122	Cj0122	0.77	NA	hypothetical protein Cj0122
Cj0981c	*cjaB*	0.77	2.59E-13	putative MFS (Major Facilitator Superfamily) transport protein
Cj0091	Cj0091	0.76	4.17E-11	putative lipoprotein
Cj1631c	Cj1631c	0.76	NA	conserved hypothetical protein Cj1631c
Cj0373	Cj0373	0.76	3.80E-14	2-hydroxyacid dehydrogenase
Cj1717c	*leuC*	0.75	NA	isopropylmalate isomerase large subunit
Cj0935c	Cj0935c	0.75	NA	putative sodium:amino-acid symporter family protein

Cj1493c	Cj1493c	0.74	NA	putative integral membrane protein
Cj0390	Cj0390	0.73	NA	putative transmembrane protein
Cj0951c	Cj0951c	0.73	NA	putative MCP-domain signal transduction protein
Cj0350	Cj0350	0.73	2.99E-14	hypothetical protein Cj0350
Cj0332c	*ndk*	0.73	4.87E-14	nucleoside diphosphate kinase
Cj0606	Cj0606	0.73	NA	putative secretion protein HlyD
Cj0008	Cj0008	0.72	NA	conserved hypothetical protein Cj0008
Cj1476c	Cj1476c	0.71	0.00044	pyruvate-flavodoxin oxidoreductase
Cj0531	*icd*	0.71	3.62E-09	isocitrate dehydrogenase
Cj0090	Cj0090	0.71	3.33E-16	putative lipoprotein
Cj1463	*flgJ*	0.70	NA	hypothetical protein Cj1463
Cj1490c	*ccoN*	0.69	1.13E-08	cb-type cytochrome C oxidase subunit I
Cj0720c	*flaC*	0.69	4.13E-11	flagellin
Cj0134	*thrB*	0.69	NA	homoserine kinase
Cj1450	Cj1450	0.69	NA	putative ATP/GTP-binding protein
Cj0014c	Cj0014c	0.68	1.76E-13	putative integral membrane protein
Cj0040	Cj0040	0.68	3.59E-09	hypothetical protein Cj0040
Cj0654c	Cj0654c	0.67	2.26E-11	probable transmembrane transport protein pseudogene
Cj0413	Cj0413	0.67	4.48E-11	putative periplasmic protein
Cj0417	Cj0417	0.66	NA	hypothetical protein Cj0417
Cj0533	*sucC*	0.66	1.77E-08	succinyl-coA synthetase beta chain
Cj0328c	*fabH*	0.65	6.12E-10	3-oxoacyl-(acyl carrier protein) synthase III
Cj1461	Cj1461	0.65	NA	putative DNA methylase
Cj1522c	Cj1522c	0.65	NA	putative CRISPR-associated protein
Cj0303c	*modA*	0.63	5.43E-12	putative molybdate-binding lipoprotein
Cj0770c	Cj0770c	0.63	1.09E-06	putative NLPA family lipoprotein
Cj1718c	*leuB*	0.63	1.07E-05	3-isopropylmalate dehydrogenase
Cj1584c	Cj1584c	0.62	NA	putative peptide ABC-transport system periplasmic peptide-binding protein
Cj1523c	Cj1523c	0.62	NA	putative CRISPR-associated protein
Cj1001	*rpoD*	0.62	NA	RNA polymerase sigma factor RpoD
Cj0549	*fliS*	0.62	7.31E-08	flagellar protein FliS
Cj0798c	*ddl*	0.61	2.29E-09	D-alanyl-alanine synthetase A
Cj1025c	Cj1025c	0.61	NA	hypothetical protein Cj1025c
Cj0793	*flgS*	0.61	NA	signal transduction histidine kinase
Cj0965c	Cj0965c	0.61	1.45E-11	putative acyl-CoA thioester hydrolase
Cj1596	*rplQ*	0.61	NA	50S ribosomal protein L17
Cj1573c	*nuoG*	0.61	2.61E-06	NADH dehydrogenase subunit G
Cj0736	Cj0736	0.61	NA	hypothetical protein Cj0736
Cj0574	*ilvI*	0.60	4.13E-13	acetolactate synthase 3 catalytic subunit
Cj0845c	*gltX*	-0.60	NA	glutamyl-tRNA synthetase
Cj0828c	*ilvA*	-0.61	NA	threonine dehydratase
Cj1290c	*accC*	-0.61	4.42E-11	biotin carboxylase
Cj0306c	*bioF*	-0.61	NA	8-amino-7-oxononanoate synthase
Cj0595c	*nth*	-0.61	1.26E-08	endonuclease III
Cj1471c	*ctsE*	-0.62	NA	putative type II protein secretion system E protein
Cj0953c	*purH*	-0.62	1.02E-08	bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
Cj1123c	*pglD*	-0.62	NA	acetyltransferase
Cj1445c	*kpsE*	-0.62	4.84E-09	capsule polysaccharide export system inner membrane protein
Cj1227c	Cj1227c	-0.62	8.55E-09	putative two-component regulator
Cj1248	*guaA*	-0.62	1.31E-13	bifunctional GMP synthase/glutamine amidotransferase protein
Cj0054c	Cj0054c	-0.62	2.60E-08	putative lysine decarboxylase family protein
Cj1250	*purD*	-0.63	5.84E-11	phosphoribosylamine--glycine ligase

Cj1310c	Cj1310c	-0.63	9.82E-13	hypothetical protein Cj1310c
Cj0433c	*mraY*	-0.63	6.65E-09	phospho-N-acetylmuramoyl-pentapeptide-transferase
Cj0954c	Cj0954c	-0.63	NA	putative DnaJ-like protein
Cj1481c	Cj1481c	-0.63	3.29E-08	putative helicase
Cj0241c	Cj0241c	-0.63	5.04E-08	putative iron-binding protein
Cj1325	Cj1325	-0.63	1.45E-13	putative methyltransferase
Cj0609c	Cj0609c	-0.64	NA	possible periplasmic protein
Cj0767c	*coaD*	-0.64	NA	phosphopantetheine adenylyltransferase
Cj0339	Cj0339	-0.64	1.17E-11	putative MFS (Major Facilitator Superfamily) transport protein
Cj1220	*groES*	-0.65	9.04E-09	co-chaperonin GroES
Cj1324	Cj1324	-0.65	NA	hypothetical protein Cj1324
Cj0095	*rpmA*	-0.65	2.09E-06	50S ribosomal protein L27
Cj0100	Cj0100	-0.65	1.66E-08	parA family protein
Cj1597	*hisG*	-0.66	2.37E-09	ATP phosphoribosyltransferase
Cj1329	Cj1329	-0.66	NA	putative sugar-phosphate nucleotide transferase
Cj1598	*hisD*	-0.66	9.57E-12	histidinol dehydrogenase
Cj1425c	*hddA*	-0.67	4.58E-11	putative D-glycero-D-manno-heptose 7-phosphate kinase
Cj1641	*murE*	-0.67	1.21E-09	UDP-N-acetylmuramoylalanyl-D-glutamate--2,6-diaminopimelate ligase
Cj0418c	Cj0418c	-0.67	5.90E-13	hypothetical protein Cj0418c
Cj1306c	Cj1306c	-0.67	9.16E-11	hypothetical protein Cj1306c
Cj0035c	Cj0035c	-0.67	NA	putative efflux protein
Cj0847	*psd*	-0.68	8.97E-11	phosphatidylserine decarboxylase
Cj0800c	Cj0800c	-0.68	NA	putative ATPase
Cj1437c	Cj1437c	-0.68	NA	aminotransferase
Cj1150c	*hldE*	-0.68	3.77E-15	D-beta-D-heptose 7-phosphate kinase/D-beta-D-heptose 1-phosphate adenylyltransferase
Cj0636	Cj0636	-0.69	NA	NOL1/NOP2/sun family protein
Cj0420	Cj0420	-0.69	0.0011	putative periplasmic protein
Cj1328	*neuC2*	-0.69	5.70E-14	putative UDP-N-acetylglucosamine 2-epimerase
Cj0652	*pbpC*	-0.69	NA	penicillin-binding protein
Cj0701	Cj0701	-0.70	5.48E-12	putative protease
Cj1157	*dnaX*	-0.70	NA	DNA polymerase III subunits gamma and tau
Cj1245c	Cj1245c	-0.70	NA	hypothetical protein Cj1245c
Cj1494c	*carA*	-0.70	1.68E-09	carbamoyl phosphate synthase small subunit
Cj0709	*ffh*	-0.70	2.76E-07	signal recognition particle protein
Cj0061c	*fliA*	-0.70	NA	flagellar biosynthesis sigma factor
Cj1709c	Cj1709c	-0.70	NA	putative ribosomal pseudouridine synthase
Cj0621	Cj0621	-0.70	1.43E-08	hypothetical protein Cj0621
Cj1288c	*gltX2*	-0.70	1.08E-14	glutamyl-tRNA synthetase
Cj1286c	*upp*	-0.71	5.63E-10	uracil phosphoribosyltransferase
Cj0863c	*xerD*	-0.71	NA	DNA recombinase
Cj1386	Cj1386	-0.71	NA	ankyrin-repeat containing protein
Cj0466	*nssR*	-0.71	NA	transcriptional regulator
Cj0265c	Cj0265c	-0.71	4.33E-06	putative cytochrome C-type haem-binding periplasmic protein
Cj0001	*dnaA*	-0.71	NA	chromosomal replication initiator protein
Cj1352	*ceuB*	-0.71	NA	enterochelin uptake permease
Cj1252	Cj1252	-0.72	NA	putative periplasmic protein
Cj1143	*neuA1, cgtA*	-0.72	NA	two-domain bifunctional protein (beta-1,4-N-acetylgalactosaminyltransferase/CMP-Neu5Ac synthase)
Cj0435	*fabG*	-0.73	6.64E-11	3-ketoacyl-(acyl-carrier-protein) reductase
Cj0576	*lpxD*	-0.73	1.27E-09	UDP-3-O-
Cj1156	*rho*	-0.73	1.24E-11	transcription termination factor Rho
Cj1529c	*purM*	-0.74	9.18E-11	phosphoribosylaminoimidazole synthetase

Cj1115c	Cj1115c	-0.74	1.63E-10	putative phosphatidylserine decarboxylase-related protein
Cj1196c	*gpsA*	-0.74	3.33E-16	NAD(P)H-dependent glycerol-3-phosphate dehydrogenase
Cj1606c	*mrp*	-0.74	NA	putative ATP/GTP-binding protein (Mrp protein homolog)
Cj0182	Cj0182	-0.75	8.52E-05	putative transporter
Cj1530	*coaE*	-0.75	NA	dephospho-CoA kinase
Cj1312	*pseG*	-0.77	7.57E-14	nucleotidase specific for PseC product,UDP-4-amino-4,6-dideoxy-beta-L-AltNAc
Cj1517	*moaD*	-0.77	2.22E-16	putative molybdopterin converting factor,subunit 1
Cj0518	*htpG*	-0.77	1.32E-13	heat shock protein 90
Cj1686c	*topA*	-0.77	1.01E-07	DNA topoisomerase I
Cj0293	*surE*	-0.78	NA	stationary phase survival protein SurE
Cj0024	*nrdA*	-0.78	1.44E-15	ribonucleotide-diphosphate reductase subunit alpha
Cj1376	Cj1376	-0.79	NA	putative periplasmic protein
Cj1407c	Cj1407c	-0.79	NA	putative phospho-sugar mutase
Cj0505c	Cj0505c	-0.79	1.51E-12	putative aminotransferase (degT family).
Cj0914c	*ciaB*	-0.79	3.54E-12	CiaB protein
Cj1444c	*kpsD*	-0.79	3.33E-08	capsule polysaccharide export system periplasmic protein
Cj1713	Cj1713	-0.79	NA	putative radical SAM domain protein
Cj0442	*fabF*	-0.79	2.88E-11	3-oxoacyl-(acyl carrier protein) synthase II
Cj1221	*groEL*	-0.80	5.22E-13	chaperonin GroEL
Cj0610c	Cj0610c	-0.80	NA	putative periplasmic protein
Cj0228c	*pcm*	-0.80	1.79E-11	protein-L-isoaspartate O-methyltransferase
Cj0286c	Cj0286c	-0.80	NA	hypothetical protein Cj0286c
Cj0708	Cj0708	-0.80	NA	putative ribosomal pseudouridine synthase
Cj1519	*moeA2*	-0.80	1.50E-14	putative molybdopterin biosynthesis protein
Cj0707	*kdtA*	-0.81	8.07E-13	3-deoxy-D-manno-octulosonic-acid transferase
Cj0905c	*alr*	-0.81	4.25E-12	alanine racemase
Cj0226	*argB*	-0.81	NA	acetylglutamate kinase
Cj1104	*ispE*	-0.83	NA	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase
Cj1379	*selB*	-0.83	NA	putative selenocysteine-specific elongation factor
Cj1375	Cj1375	-0.83	1.04E-06	putative multidrug efflux transporter
Cj1274c	*pyrH*	-0.83	1.01E-11	uridylate kinase
Cj1532	Cj1532	-0.85	9.21E-15	putative periplasmic protein
Cj0881c	Cj0881c	-0.85	NA	hypothetical protein Cj0881c
Cj1305c	Cj1305c	-0.85	3.44E-15	hypothetical protein Cj1305c
Cj0897c	*pheS*	-0.85	1.50E-14	phenylalanyl-tRNA synthetase subunit alpha
Cj1362	*ruvB*	-0.86	NA	Holliday junction DNA helicase B
Cj0197c	*dapB*	-0.87	1.99E-12	dihydrodipicolinate reductase
Cj0998c	Cj0998c	-0.87	3.40E-12	putative periplasmic protein
Cj0874c	Cj0874c	-0.88	NA	putative cytochrome C
Cj0777	Cj0777	-0.88	0	putative ATP-dependent DNA helicase
Cj0582	*lysC*	-0.89	1.25E-11	aspartate kinase
Cj1531	*dapF*	-0.90	0	diaminopimelate epimerase
Cj1096c	*metK*	-0.90	3.73E-13	S-adenosylmethionine synthetase
Cj1518	*moaE*	-0.91	1.73E-14	putative molybdopterin converting factor,subunit 2
Cj0862c	*pabB*	-0.91	NA	para-aminobenzoate synthase component I
Cj1448c	*kpsM*	-0.91	1.67E-11	capsule polysaccharide export system inner membrane protein
Cj0034c	Cj0034c	-0.91	NA	putative periplasmic protein
Cj0622	*hypF*	-0.93	NA	carbamoyltransferase
Cj1193c	Cj1193c	-0.93	NA	putative periplasmic protein
Cj0298c	*panB*	-0.94	1.15E-07	3-methyl-2-oxobutanoate hydroxymethyltransferase
Cj1163c	Cj1163c	-0.96	NA	putative cation transport protein
Cj1161c	Cj1161c	-0.97	NA	putative cation-transporting ATPase
Cj1629	*exbD2*	-0.97	2.58E-08	putative exbD/tolR family transport protein
Cj0705	Cj0705	-0.97	2.22E-15	conserved hypothetical protein Cj0705

Cj0297c	*panC*	-0.98	6.17E-09	pantoate--beta-alanine ligase
Cj0504c	Cj0504c	-1.01	1.44E-10	putative oxidoreductase
Cj1172c	Cj1172c	-1.01	0	hypothetical protein Cj1172c
Cj0169	*sodB*	-1.04	2.28E-06	superoxide dismutase (Fe)
Cj0263	*zupT*	-1.05	2.56E-07	zinc transporter ZupT
Cj1638	*dnaG*	-1.05	NA	DNA primase
Cj0594c	Cj0594c	-1.07	0	putative DNA/RNA non-specific endonuclease
Cj1183c	*cfa*	-1.09	NA	cyclopropane-fatty-acyl-phospholipid synthase
Cj0322	*perR*	-1.09	9.03E-13	peroxide stress regulator
Cj0946	Cj0946	-1.12	1.44E-11	putative lipoprotein
Cj1711c	*ksgA*	-1.12	NA	dimethyladenosine transferase
Cj0023	*purB*	-1.13	4.89E-11	adenylosuccinate lyase
Cj1031	*cmeD*	-1.15	NA	outer membrane component of efflux system (multidrug efflux system CmeDEF)
Cj1283	*ktrB*	-1.15	NA	putative K+ uptake protein
Cj0692c	Cj0692c	-1.21	NA	hypothetical protein Cj0692c
Cj0616	*pstB*	-1.21	NA	phosphate transport ATP-binding protein
Cj1378	*selA*	-1.26	0	selenocysteine synthase
Cj0173c	*cfbpC*	-1.26	NA	putative iron-uptake ABC transport system ATP-binding protein
Cj0718	*dnaE*	-1.28	1.11E-16	DNA polymerase III subunit alpha
Cj1226c	Cj1226c	-1.29	0	putative two-component sensor (histidine kinase)
Cj0611c	Cj0611c	-1.31	0	putative acyltransferase family protein
Cj0414	Cj0414	-1.33	8.36E-06	putative oxidoreductase subunit
Cj0018c	*dba*	-1.33	NA	disulphide bond formation protein
Cj0415	Cj0415	-1.33	2.98E-05	putative GMC oxidoreductase subunit
Cj1384c	Cj1384c	-1.37	9.56E-10	hypothetical protein Cj1384c
Cj0181	*tonB1*	-1.37	NA	TonB transport protein
Cj1516	Cj1516	-1.37	2.22E-16	putative periplasmic oxidoreductase
Cj1246c	*uvrC*	-1.39	NA	excinuclease ABC subunit C
Cj0224	*argC*	-1.41	1.97E-12	N-acetyl-gamma-glutamyl-phosphate reductase
Cj0262c	Cj0262c	-1.44	1.11E-16	putative methyl-accepting chemotaxis signal transduction protein
Cj0817	*glnH*	-1.44	0	putative glutamine binding periplasmic protein
Cj1710c	Cj1710c	-1.45	5.27E-06	putative metallo-beta-lactamase family protein
Cj0239c	Cj0239c	-1.46	1.79E-11	NifU protein homolog
Cj1628	*exbB2*	-1.48	NA	putative exbB/tolQ family transport protein
Cj1382c	*fldA*	-1.54	0	flavodoxin FldA
Cj1228c	*htrA*	-1.58	3.33E-15	serine protease (protease DO)
Cj1133	*waaC*	-1.61	NA	heptosyltransferase I
Cj0179	*exbB1*	-1.61	NA	biopolymer transport protein
Cj1189c	*cetB*	-1.75	NA	bipartate energy taxis response protein cetB
Cj0453	*thiC*	-1.81	2.52E-12	thiamine biosynthesis protein ThiC
Cj1660	Cj1660	-1.93	NA	putative integral membrane protein
Cj0753c	*tonB3*	-2.01	1.74E-09	TonB transport protein
Cj0174c	*cfbpB*	-2.05	NA	putative iron-uptake ABC transport system permease protein
Cj1355	*ceuE*	-2.08	0	enterochelin uptake periplasmic binding protein
Cj1725	Cj1725	-2.09	0	putative periplasmic protein
Cj0779	*tpx*	-2.09	NA	thiol peroxidase
Cj1353	*ceuC*	-2.12	NA	enterochelin uptake permease
Cj0025c	Cj0025c	-2.14	1.10E-10	putative sodium:dicarboxylate family transmembrane symporter
Cj0343c	Cj0343c	-2.16	0	putative integral membrane protein
Cj1190c	*cetA*	-2.21	0	bipartate energy taxis response protein cetA
Cj0240c	*iscS*	-2.28	0	cysteine desulfurase (NifS protein homolog)

Cj0178	Cj0178	-2.37	NA	putative TonB-denpendent outer membrane receptor
Cj0017c	*dsbI*	-2.41	0	disulphide bond formation protein
Cj0912c	*cysM*	-2.52	0	cysteine synthase
Cj0177	Cj0177	-2.63	NA	putative iron transport protein
Cj1613c	Cj1613c	-2.67	4.59E-09	putative pyridoxamine 5'-phosphate oxidase
Cj1663	Cj1663	-2.79	2.15E-13	putative ABC transport system ATP-binding protein
Cj1615	*chuB*	-2.79	NA	putative haemin uptake system permease protein
Cj0379c	Cj0379c	-2.99	0	putative sulfite oxidase subunit YedY
Cj0146c	*trxB*	-3.04	NA	thioredoxin reductase
Cj1661	Cj1661	-3.05	NA	possible ABC transport system permease
Cj1170c	*omp50*	-3.12	NA	50 kda outer membrane protein precursor
Cj1500	Cj1500	-3.45	0	putative inner membrane protein
Cj0175c	*cfbpA*	-3.51	NA	putative iron-uptake ABC transport system,periplasmic iron-binding protein
Cj1617	*chuD*	-3.53	NA	putative haemin uptake system periplasmic haemin-binding protein
Cj1662	Cj1662	-3.59	NA	putative integral membrane protein
Cj0334	*ahpC*	-3.72	3.91E-14	alkyl hydroperoxide reductase
Cj1658	Cj1658	-3.75	NA	putative iron permease
Cj1616	*chuC*	-3.88	NA	putative haemin uptake system ATP-binding protein
Cj1383c	Cj1383c	-5.21	NA	hypothetical protein Cj1383c
Cj1659	*p19*	-5.29	1.12E-14	periplasmic protein p19
Cj1614	*chuA*	-5.42	NA	haemin uptake system outer membrane receptor
Cj0755	*cfrA*	-5.61	NA	ferric enterobactin uptake receptor
Cj1385	*katA*	-5.63	NA	catalase

Appendix VII
C. jejuni transcriptome in medium containing 25mM L-fucose

Gene	Name	Fold Change (log_2)	Bayes.p	Protein
Cj0481	*dapA*	4.37	3.05E-11	putative dihydrodipicolinate synthase
Cj0483	*uxaA*	4.36	4.40E-13	putative altronate hydrolase
Cj0484	Cj0484	3.75	2.32E-11	putative MFS (Major Facilitator Superfamily) transport protein
Cj0486	*fucP*	3.69	1.97E-11	putative sugar transporter
Cj0485	Cj0485	3.38	4.27E-11	putative oxidoreductase
Cj0487	Cj0487	3.15	4.22E-10	putative amidohydrolase
Cj0490	*ald*	2.99	6.14E-10	putative aldehyde dehydrogenase
Cj1503c	*putA*	2.65	2.78E-11	putative proline dehydrogenase
Cj1502c	*putP*	2.54	1.03E-10	putative sodium/proline symporter
Cj0917c	*cstA*	2.41	2.50E-09	putative integral membrane protein
Cj1537c	*acs*	2.32	3.43E-10	acetyl-coenzyme A synthetase
Cj0488	Cj0488	2.02	3.53E-08	conserved hypothetical protein
Cj0482	*uxaA*	1.92	1.82E-09	putative altronate hydrolase
Cj0921c	*peb1A*	1.88	4.81E-08	aspartate/glutamate-binding ABC transporter protein
Cj0919c	Cj0919c	1.81	8.53E-10	putative ABC-type amino-acid transporter permease protein
Cj1682c	*gltA*	1.71	1.81E-09	citrate synthase
Cj0922c	*pebC*	1.57	1.42E-08	ABC-type amino-acid transporter ATP-binding protein
Cj0920c	Cj0920c	1.53	3.99E-08	putative ABC-type amino-acid transporter permease protein
Cj1541	Cj1541	1.50	3.13E-11	conserved hypothetical protein
Cj0021c	Cj0021c	1.44	3.69E-09	putative fumarylacetoacetate (FAA) hydrolase family protein
Cj1158c	Cj1158c	1.43	1.42E-05	small hydrophobic protein
Cj0489	*ald*	1.41	4.90E-07	putative aldehyde dehydrogenase
Cj0074c	Cj0074c	1.38	2.01E-10	putative iron-sulfur protein
Cj0075c	Cj0075c	1.36	1.57E-09	putative oxidoreductase iron-sulfur subunit
Cj0437	*mrfA*	1.36	1.42E-06	succinate dehydrogenase flavoprotein subunit
Cj0438	*mrfB*	1.21	8.08E-06	putative succinate dehydrogenase iron-sulfur protein
Cj1619	*kgtP*	1.21	2.21E-05	alpha-ketoglutarate permease
Cj0073c	Cj0073c	1.18	8.59E-08	conserved hypothetical protein
Cj0835c	*acnB*	1.16	1.85E-10	aconitate hydratase
Cj0833c	Cj0833c	1.12	1.83E-09	putative oxidoreductase
Cj0689	*ackA*	1.09	5.11E-09	acetate kinase
Cj1681c	*cysQ*	1.05	8.37E-11	CysQ protein homolog
Cj1192	*dctA*	1.03	1.09E-09	putative C4-dicarboxylate transport protein
Cj0533	Cj0553	1.02	5.59E-09	putative integral membrane protein
Cj0832c	Cj0832c	0.98	4.56E-13	putative Na+/H+ antiporter family protein
Cj0853c	*hemL*	0.97	3.07E-09	glutamate-1-semialdehyde 2,1-aminomutase
Cj0688	*pta*	0.97	2.66E-08	putative phosphate acetyltransferase
Cj0522	Cj0552	0.95	1.45E-08	putative membrane protein
Cj1625c	*sdaC*	0.92	3.00E-10	amino acid transporter
Cj0439	*mrfE*	0.91	0.00012	putative succinate dehydrogenase subunit C
Cj0850c	Cj0850c	0.91	5.03E-12	putative MFS (Major Facilitator Superfamily) transport protein
Cj1388	Cj1388	0.89	1.19E-05	putative endoribonuclease L-PSP
Cj0076c	*lctP*	0.88	1.58E-08	L-lactate permease
Cj1542	Cj1542	0.87	9.77E-08	putative allophanate hydrolase subunit 1
Cj0427	Cj0427	0.84	4.65E-09	hypothetical protein

Cj0203	Cj0203	0.83	1.92E-12	putative citrate transporter
Cj0903c	Cj0903c	0.81	1.33E-06	putative amino-acid transport protein
Cj0982c	*cjaA*	0.80	1.36E-11	putative amino-acid transporter periplasmic solute-binding protein
Cj0762c	*aspB*	0.79	1.35E-06	aspartate aminotransferase
Cj0393c	*mqo*	0.79	4.02E-09	putative malate:quinone oxidoreductase
Cj0834c	Cj0834c	0.79	5.66E-08	ankyrin repeat-containing putative periplasmic protein
Cj1543	Cj1543	0.77	3.90E-10	putative allophanate hydrolase subunit 2
Cj1624c	*sdaA*	0.73	1.28E-07	L-serine dehydratase
Cj1389	Cj1389	0.71	7.42E-07	pseudogene (putative C4-dicarboxylate anaerobic carrier)
Cj0534	*sucD*	0.71	2.65E-11	succinyl-coA synthetase alpha chain
Cj0554	Cj0554	0.69	2.00E-06	hypothetical protein
CJ0533	*sucC*	0.67	1.57E-10	succinyl-coA synthetase beta chain
Cj1200	Cj1200	0.64	2.86E-05	putative NLPA family lipoprotein
Cj0531	*icd*	0.63	4.58E-09	isocitrate dehydrogenase
Cj1719c	*leuA*	0.63	1.63E-09	2-isopropylmalate synthase
Cj0373	Cj0373	0.61	2.23E-10	putative D-2-hydroxyacid dehydrogenase
Cj1008c	*aroB*	0.60	1.59E-09	3-dehydroquinate synthase
Cj0555	Cj0555	0.60	2.30E-09	putative dicarboxylate carrier protein MatC
Cj0069	Cj0069	0.59	7.86E-08	hypothetical protein
Cj0981c	*cjaB*	0.59	2.96E-07	putative MFS (Major Facilitator Superfamily) transport protein
Cj1717c	*leuC*	0.58	1.42E-08	3-isopropylmalate dehydratase large subunit
Cj0374	Cj0374	0.58	6.82E-07	conserved hypothetical protein
Cj0987c	Cj0987c	0.57	1.36E-07	putative MFS (Major Facilitator Superfamily) transport protein
Cj0348	*trpB*	0.57	3.04E-06	tryptophan synthase beta chain
Cj0536	*oorA*	0.56	1.46E-11	OORA subunit of 2-oxoglutarate:acceptor oxidoreductase
Cj0346	*trpD*	0.53	6.20E-05	anthranilate synthase component II
Cj0374	Cj0374	0.53	2.98E-08	conserved hypothetical protein Cj0374
Cj0375	Cj0375	0.51	6.32E-10	putative lipoprotein
Cj0532	*mdh*	0.50	5.74E-09	malate dehydrogenase
Cj1185c	*petB*	-0.50	2.42E-06	putative ubiquinol-cytochrome C reductase cytochrome B subunit
Cj0912c	*cysM*	-0.50	0.00012	cysteine synthase
Cj1220	*groES*	-0.50	4.72E-05	10 kD chaperonin (cpn10)
Cj0613	*pstS*	-0.51	3.20E-06	putative periplasmic phosphate binding protein
Cj1659	*p19*	-0.52	6.47E-08	periplasmic protein p19
Cj1688c	*secY*	-0.53	4.35E-06	preprotein translocase subunit
Cj1001	*rpoD*	-0.55	9.37E-07	RNA polymerase sigma factor (sigma-70)
Cj0224	*argC*	-0.55	4.95E-06	N-acetyl-gamma-glutamyl-phosphate reductase
Cj0515	Cj0515	-0.55	2.24E-08	putative periplasmic protein
Cj0118	Cj0118	-0.56	2.52E-05	conserved hypothetical protein
Cj0012c	*rrc*	-0.56	3.25E-10	non-haem iron protein
Cj0281	*tal*	-0.56	9.98E-07	putative transaldolase
Cj1511c	*fdhA*	-0.57	8.23E-07	putative formate dehydrogenase large subunit (Selenocysteine containing)
Cj1221	*groEL*	-0.58	7.97E-08	60 kD chaperonin (cpn60)
Cj0082	*cydB*	-0.59	3.96E-05	cytochrome bd oxidase subunit II
Cj0343c	Cj0343c	-0.60	8.88E-05	putative integral membrane protein
Cj0081	*cydA*	-0.63	4.68E-05	cytochrome bd oxidase subunit I
Cj1190c	*cetA*	-0.63	0.00026	bipartate energy taxis response protein
Cj0328c	*fabH*	-0.63	4.43E-06	3-oxoacyl-[acyl-carrier-protein] synthase
Cj1438c	Cj1438c	-0.63	4.58E-07	putative sugar transferase
Cj1181c	*tsf*	-0.64	8.15E-08	elongation factor TS
Cj1184c	*petC*	-0.64	8.63E-09	putative ubiquinol-cytochrome C reductase cytochrome C

				subunit
Cj1180c	Cj1180c	-0.65	2.54E-09	putative ABC transporter ATP-binding protein
Cj1514c	Cj1514c	-0.65	4.48E-09	hypothetical protein
Cj0134	*thrB*	-0.66	2.90E-07	putative homoserine kinase
Cj1434c	Cj1434c	-0.67	1.24E-07	putative sugar transferase
Cj0475	*rplA*	-0.68	6.65E-10	50S ribosomal protein L1
Cj1474c	*ctsD*	-0.69	1.11E-07	putative type II protein secretion system D protein
Cj0117	*pfs*	-0.69	2.35E-08	5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase
Cj1612	*prfA*	-0.71	2.08E-07	peptide chain release factor 1
Cj1596	*rplQ*	-0.71	1.14E-10	50S ribosomal protein L17
Cj0343c	Cj0343c	-0.72	4.91E-05	putative integral membrane protein
Cj1500	Cj1500	-0.72	3.37E-06	putative integral membrane protein
Cj0025c	Cj0025c	-0.74	5.74E-06	putative sodium:dicarboxylate family transmembrane symporter
Cj0190	Cj0190c	-0.77	2.60E-05	conserved hypothetical protein
Cj1189c	*cetB*	-0.77	6.90E-06	bipartate energy taxis response protein
Cj0264c	Cj0264c	-0.78	4.05E-08	molybdopterin containing oxidoreductase
Cj0265c	Cj0265c	-0.81	2.87E-09	putative cytochrome C-type haem-binding periplasmic protein
Cj0370	*rpsU*	-0.82	2.71E-12	30S ribosomal protein S21
Cj1153	Cj1153	-0.85	2.36E-11	putative periplasmic cytochrome C
Cj0779	*tpx*	-0.87	1.93E-06	thiol peroxidase
Cj0169	*sodB*	-0.91	6.42E-09	superoxide dismutase (Fe)
Cj0379c	Cj0379c	-0.98	1.33E-07	putative molybdenum containing oxidoreductase
Cj0262c	Cj0262c	-1.04	8.48E-11	putative methyl-accepting chemotaxis signal transduction protein
Cj1169c	Cj1169c	-1.16	1.17E-08	putative periplasmic protein
Cj0239c	Cj0239c	-1.32	2.18E-07	NifU protein homolog
Cj0334	*ahpC*	-1.36	4.18E-13	alkyl hydroperoxide reductase
Cj0415	Cj0415	-1.37	1.12E-06	putative GMC oxidoreductase subunit
Cj0240c	*iscS*	-1.38	4.98E-07	cysteine desulfurase (NifS protein homolog)
Cj0414	Cj0414	-1.55	1.57E-07	putative oxidoreductase subunit
Cj0453	*thiC*	-1.63	0	thiamin biosynthesis protein
Cj0017c	*dsbI*	-1.72	6.61E-10	disulphide bond formation protein
Cj1170c	*omp50*	-1.82	1.07E-08	50 kda outer membrane protein precursor

Martin Stahl

curriculum vitae

Personal

Born:	February 17th, 1984
Citizenship:	Canada
Languages:	Bilingual (English, French)
	Intermediate written and spoken German

Education

PhD — University of Ottawa, Ottawa, ON, Canada
Microbiology, Expected early 2012

Thesis: Colonization of the Intestinal Mucus Layer by *Campylobacter jejuni*
Supervisor: Dr. Alain Stintzi

B.A *cum laude* — Concordia College, Moorhead, MN, USA
Biology, 2006

Honours and Awards

2006-2012 University of Ottawa Admission Scholarship
2009-2010 Ontario Graduate Scholarship in Science and Technology

Research Experience

2006-2012 Graduate Student**:** Department of Biochemistry, Microbiology and Immunology, University of Ottawa.
Laboratory of Dr. Alain Stintzi

- Doctoral research in bacterial genetics, pathogenesis, and metabolism.
- Experience with:
  - Molecular biology techniques (cloning, RNA, DNA and protein work)
  - Transcriptomics
    - Microarrays

        - High-throughput sequencing
    - Animal work (piglets and chicks)
    - Growth and manipulation of bacteria under specialized environments and growth conditions (microaerophilic and anaerobic)
    - Cell culture work

Teaching Experience

2007-2010 Lab supervisor/demonstrator
- BCH3356 Molecular Biology Lab (English)
- BCH3756 Laboratoire de Biologie Moléculaire (French)

2008-2011 Marker/Proctor
- BCH3520 Métabolism (French)

2003-2006 Tutor and Mentor for the Concordia College Academic Enhancement Center.
- Certified level 2 tutor and level 1 mentor.

Publications

Martin Stahl, James Butcher, and Alain Stintzi. Nutrient Acquisition and Metabolism by *Campylobacter jejuni* *Front. Cell. Inf. Microbio.* 2012. Accepted.

Martin Stahl, Lorna Friis, Harald Nothaft, Xin Liu, Jianjun Li, Christine Szymanski, Alain Stintzi. L-fucose utilization provides *Campylobacter jejuni* with a competitive advantage. *Proc Natl Acad Sci U S A*. 2011 Apr 26;108(17):7194-9

Martin Stahl, Alain Stintzi. Identification of essential genes in *C. jejuni* genome highlights hyper-variable plasticity regions. *Funct Integr Genomics.* 2011 Jun; 11(2):241-57

James Butcher, Annika Flint., **Martin Stahl**, and Alain Stintzi. *Campylobacter* Fur and PerR regulons. 2010. In *Iron Uptake in Microorganisms*. Horizon Scientific Press. *Pierre Cornelis and Simon Andrews* (eds.).

Conference Presentations

Oral Presentations
- **Stahl M**, "The World of *Campylobacter jejuni:* Uncovering the Mechanisms of Pathogenesis." Ottawa Regional Microbiology Symposium. April 2010

- **Stahl M**, Szymanski C, and Stintzi A. "Characterization of *Campylobacter jejuni* Growth in the Mucus Lining of the Intestinal Epithelium". 3rd Congress of European Microbiologists, Gothenburg Sweden. June 2009. (Seminar and Poster presentation)

- **Stahl M,** Stintzi A. "The Identification of the Essential genes of *Campylobacter jejuni*." 2008 Canadian *Campylobacter* Conference, Montreal, Canada. September 2008.

Poster presentations

- **Stahl M,** Stintzi A. "Coculture with mucolytic commensal bacteria improve the growth of *Campylobacter jejuni* in vitro" 2011 CHRO meeting. Vancouver, Canada, September 2011
- **Martin Stahl,** Lorna Friis, Harald Nothaft, Christine Szymanski, Alain Stintzi. "The Presence of an L-Fucose Metabolic Pathway Provides *Campylobacter jejuni* with a Competitive Advantage in vivo". 2011 CHRO meeting. Vancouver, Canada, September 2011
- **M.S.H.Stahl**, A. Stintzi "The use of mucin as a substrate for growth by *Campylobacter jejuni"* 2010 ASM General Meeting, San Diego, California. May 2010

- **Stahl M**, Szymanski C, and Stintzi A. "Growth of *Campylobacter jejuni* within the Intestinal Mucus Lining." Progress in Systems Biology: the Brain and Mind. Ottawa, Canada. April 2009

Volunteer Experience

2010-2012 VP finance, University of Ottawa. BMI Graduate Students Association

- Managing the income and expenditures of the BMIGSA

2009-2010 VP Internal, University of Ottawa, BMI Graduate Students Association

- Attending departmental and faculty meetings as the graduate student representative.
- Editor of the BMI Bulletin, news bulletin for the students and faculty of the BMI department.

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