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Modified heptose synthesis in the Campylobacter jejuni capsule and its involvement in virulence

Anthony Wong

Modified heptose synthesis in the *Campylobacter jejuni* capsule and its involvement in virulence

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By:

Anthony Wong

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CERTIFICATE OF EXAMINATION

Supervisor

Dr. Carole Creuzenet

Supervisory Committee

Dr. Tom Linn

Dr. Miguel A. Valvano

Examiners

Dr. Peter Cadieux

Dr. Martin McGavin

Dr. Alexander Timoshenko

The thesis by

Anthony Wong

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Date _____

_____
Chair of the Thesis Examination Board

ABSTRACT

Campylobacter jejuni is the leading cause of gastroenteritis. Infections are endemic in developing countries and the emergence of antibiotic resistant isolates in developed countries underline the requirement for new therapeutic targets.

C. jejuni possesses a capsule, which is important in conferring virulence. The capsule of some *C. jejuni* species contains unusual modified heptoses. The genes *cj1427c*, *cj1428c* and *cj1430c* putatively encode enzymes involved in modifying heptose. We hypothesize that modified heptoses play a role in virulence in host-pathogen interactions, and have constructed and phenotypically characterized a *cj1427*::CAT disruption mutant to complete our panel of mutants. Complementation of the mutants was conducted to ensure that the observed phenotypes are gene specific.

The capsular composition of our mutants has been elucidated via NMR, allowing us to correlate capsular structure and the observed phenotypes.

This work will lead to a better understanding of the role of modified heptoses in host-pathogen interactions.

Keywords: *C. jejuni*, enteritis, Capsular polysaccharide (CPS), modified heptoses

CO-AUTHORSHIP

The *cj1427*::CATΔ mutant that is presented in 3.1.2 was constructed by Dirk Lange. The pET1427::CAT Coli construct used for the creation of the new *cj1427*::CAT mutant in 3.1.3 was previously constructed by Dirk Lange. The work presented in 3.1.4; specifically, the expression levels of genes *cj1425c, cj1426c, cj1427c, cj1428c, cj1430c, cj1444c* and *cj1445c* in the *cj1428*::CAT, *cj1430*::CAT, *cj1427*::CATΔ and *KpsM* mutants, were measured by Dirk Lange. The expression of the *cj1429c*, *cj1447c* and *kpsM* genes in the aforementioned strains were carried out as part of the work in this thesis. In addition, the newly constructed *cj1427*::CAT mutant was characterized in this thesis. The work presented in 3.2.3 was conducted in collaboration with Dr. Yuriy Knirel, who performed NMR compositional elucidation of the capsular polysaccharide in various strains of *C. jejuni*. The work presented in 3.5.1 was conducted in part by Xuan Thanh Bui, who constructed the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT complementation constructs and also mobilized these constructs into *E. coli*. As the *cj1427*::CAT complementation construct possessed several deleterious single nucleotide polymorphisms, QuikChange site directed mutagenesis was performed as part of the work in this thesis.

DEDICATION

To my family and friends as well as everyone else that has supported me along the way.

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LIST OF ABBREVIATIONS

bp	base pair(s)
C. jejuni	*Campylobacter jejuni*
CFU	colony forming units
CPS	capsular polysaccharide
DEPC	diethylpyrocarbonate
dNTP	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EtN	ethanolamine
Gal	galactose
GalN	galactosamine
GalNAc	N-acetyl galactosamine
Glc	glucose
GlcA	glucuronic acid
GroN	2-amino-2-deoxyglycerol
Hep	heptose
LB	lysogeny broth (Luria Bertani broth)
LOS	lipooligosaccharide
LPS	lipopolysaccharide
Me	methyl
MeOPN	phosphoramidate
mRNA	messenger Ribonucleic acid

NMR	nuclear magnetic resonance
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rib	ribose
RT	reverse transcriptase
spp.	species
U	unit
WT	wild type

CHAPTER 1 – INTRODUCTION

1.1 Historical perspective

Food is a necessity of life. In the past, mankind secured food through hunting and gathering and agriculture. Today, most of the food consumed by the world is supplied by the food industry; the use of intensive farming and industrial agriculture techniques allows food to be produced on a large scale to meet the increasing needs and demands of mankind.

Due to the large amounts of food being produced in today's society, there is a need for food preservation; a process of treating and handling food in a manner that stops or slows down the spoilage of food to allow for longer storage. Preservation can take a number of different forms including sugaring and canning (i.e. jams), boiling, dehydration, etc. These methods all accomplish the same goal: to prevent the growth of bacteria, yeasts, fungi and other microorganisms.

1.2 Foodborne and waterborne diseases

Foodborne disease is the result of ingesting food that has been contaminated by pathogenic bacteria, viruses, or parasites. In addition, foodborne disease can be caused by ingesting foods contaminated by poisonous chemicals and/or other harmful substances (2). The global burden of foodborne illness is currently unknown, but the World Health Organization (WHO) has estimated that in 2005, 1.8 million people died from diarrheal diseases, largely attributable to contaminated food and drinking water (121). This problem is not restricted to developing countries; foodborne illnesses also occur in developed countries. The Centers for Disease Control and Prevention (CDC) estimates that in the United States alone, 76 million foodborne illnesses occurs each year, resulting in 325,000 hospitalizations and over 5000 deaths (125).

More than 200 microbial, chemical or physical agents can cause illness when ingested (6). Within the last 20 years, foodborne diseases caused by bacteria, parasites, viruses and prions have gained significant public attention (121). Before 1960, the major causes of foodborne illnesses included *Salmonella* spp., *Shigella* spp., *Clostridium botulinum* and *Staphylococcus aureus*. During the 1960s, *Clostridium perfringens* and *Bacillus cereus* were added, and in the 1970s, rotavirus and norovirus. In the 1980s and 1990s, many new pathogens were recognized including *Campylobacter, Yersinia, Listeria monocytogenes,* new strains of *Escherichia coli* such as O157:H7, *Cryptosporidia* and *Cyclospora*, to name a few (121).

Several factors of modern society contribute to the occurrence of foodborne illnesses. These include, but are not limited to: large scale production and wide distribution of food; globalization of the food supply; eating outside the home; the emergence of new pathogens; and a growing population of at risk consumers. Improvements in the safety of food have been driven largely by public demand in response to disease outbreaks. As a result, international standards and legislation have been implemented, decreasing worldwide diarrheal incidences as reported by the WHO (5).

Foodstuffs are not the only source for potential illness; pathogens can also contaminate large water supplies. As of 2006, the CDC estimated that a staggering 1.1 billion people lack access to clean water (1). The burden of unpotable water and poor sanitation and hygienic conditions is mostly concentrated in developing countries, particularly amongst children (4). However, waterborne diseases also occur in developed countries. In past cases of waterborne illnesses, often preceding the pandemic is a period

of heavy rainfall and/or melting snow. The increase in precipitation often leads to run off of surface water into the local water supply. Problems arise when surface run off mixes with animal feces, particularly from farms, resulting in potential contamination of the water supply. Changes in weather climate are thus an important factor to consider when attempting to reduce the impact of waterborne diseases. In addition, it must also be taken into consideration the method in which pathogens are able to move from source to the host. This includes how pathogens may contaminate source water, evade water treatment, etc.

It is clear that despite efforts from scientists, governments and the food industry, foodborne and waterborne illnesses continue to be a major public health problem worldwide, with implications for both the citizens and the economy of nations. Thus, further research must be conducted to prevent and control the spread of the causative agents of foodborne and waterborne illnesses.

1.3 *Campylobacter jejuni*

Theodor Escherich first noted in 1886 non-culturable, spiral shaped bacteria in stool specimens and the large intestinal mucous associated with diarrhea in neonates and kittens. Later, various publications appeared describing the occurrence of "spirilla" shaped bacteria in "cholera-like" and "dysenteric-like" disease (91). These descriptions were likely the first account of *Campylobacter* species. Notably, in 1938, a total of 357 prison inmates were infected by a "spirillum shaped bacteria" (104). It was not until 1963 that the genus was first described, and *Campylobacter* was not isolated until 1972 (119, 145) due to its fastidiousness.

Campylobacteriae are Gram negative bacteria, approximately 0.5 to 8 μm in length, and 0.2 – 0.5 μm wide. They possess a characteristic spiral, or corkscrewed shape (148). Their ability to move rapidly in a corkscrew fashion is attributed to their uni- or bipolar flagella (34, 92). Campylobacters are catalase and oxidase positive, and urease negative. They are microaerophilic, requiring a nitrogen rich atmosphere with low oxygen (5%) and high carbon dioxide (10%) (119). A growth temperature of 42°C, corresponding to the natural temperature of the avian gut, their natural host, allows for optimal growth of Campylobacters, however, growth at 37°C is also tolerated. When stressed, Campylobacters can change into a coccoid form; this form is called the "viable, but non-culturable" form. This occurs when Campylobacters encounter environmental stress such as nutrient starvation, osmotic shock and fluctuations in temperature and pH (75). While in this state, the ability to culture Campylobacters is lost, even though the microorganism is alive and metabolically active (127). There are many different *Campylobacter* species, including *C. jejuni*, *C. coli*, *C. upsaliensis, C. lari, C, concisus*, but approximately 90% of human infection is caused by *C. jejuni*, with *C. coli* accounting for much of the rest (30, 50).

1.3.1 *C. jejuni* pathogenesis

C. jejuni is a commensal bacteria in the intestinal tract of birds and mammals, including domestic chickens (178). Disease often arises from the consumption of contaminated meat, particularly during food processing and preparation. In addition, outbreaks of *Campylobacter* induced gastroenteritis, termed Campylobacteriosis, can be contracted from drinking contaminated water. Most notably, in May of 2000, in the town of Walkerton, Ontario, Canada, after several days of heavy rainfall, an outbreak of *E. coli*

O157:H7 and *Campylobacter* occurred, resulting in the deaths of seven people and approximately 2500 more becoming ill (11). This outbreak was due to a combination of contributing factors including heavy rainfall causing cattle manure from a neighbouring farm to be washed into the water supply, and insufficient chlorination of the water supply. The major role that *Campylobacter* played in this outbreak of water-borne disease was largely ignored by the media and the public.

In developed countries, *C. jejuni* is a leading cause of acute gastroenteritis. With an infectious dose of as low as 500-800 bacteria, *C. jejuni* can produce inflammatory, sometimes bloody, diarrhea or dysentery like syndrome, and often is accompanied by cramps, fever and pain. The organism colonizes the small intestine of the human host early in infection and later moves to the colon, which is the target organ (17, 164). Although *C. jejuni* is generally considered invasive, the level of invasion of intestinal epithelial cells *in vitro* varies among strains (164). The incubation period that precedes acute diarrhea is 2-5 days and although the disease is self-limiting and usually resolved within one week, symptoms can persist for up to two weeks (178).

A long term consequence of *C. jejuni* infection is Guillain-Barré Syndrome (GBS) and its variant, Miller Fisher syndrome. GBS is considered an autoimmune reaction, and causes acute ascending neuromuscular paralysis, characterized by rapidly evolving symmetrical limb weakness, loss of tendon reflexes, the absence of mild sensory signs and autonomic dysfunctions (179). It is thought that the antibodies generated against the bacterial lipooligosaccharide (LOS) cross react with host gangliosides, resulting in their destruction (7, 179). Since the near eradication of poliomyelitis worldwide, GBS ranks as the most frequent cause of acute paralysis; the annual incidence being one or two cases

per 100 000 population (73). In 1982, Rhodes and Tattersfield (140) reported the first case of a patient who developed GBS after *C. jejuni* associated enteritis. Since then, there have been many reports of *C. jejuni* associated GBS cases. In Australia, 21 of 56 patients with GBS had evidence of anti-*Campylobacter* antibodies, while controls had none, showing a strong correlation between *Campylobacter* and subsequent GBS (79). However, it remains difficult to absolutely associate *C. jejuni* with GBS because the bacteria are usually eliminated from the body within 16 days of infection and before the onset of neurological symptoms, which normally begin 10 days to three weeks after the onset of diarrhea (136). As *Campylobacter* is not routinely diagnosed in rural health clinics, many *Campylobacter*-associated GBS cases may go unrecognized because by the time symptoms present themselves, *Campylobacter* is no longer present (72, 111).

In addition to *Campylobacter*-associated GBS, acute bacterial enteritis has been implicated as one of the factors that may incite or exacerbate patients with inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis. These diseases are T cell-mediated and characterized by chronic, relapsing inflammation of the intestinal tract (80). IBD causes lifetime morbidity and in Canada alone, accounts for a financial burden exceeding 1.8 billion dollars per year in economic loss (including greater than 700 million dollars in direct medical costs) (3). The mechanisms by which *Campylobacter* causes inflammatory disorders in the bowel remain obscure. Evidence suggests that *Campylobacter* disrupts intestinal epithelial structure and function and thereby permits the translocation of luminal material, including resident intestinal bacteria, into the subepithelial compartment (74, 80, 99). The loss of intestinal epithelial barrier function

and the subsequent failure to downregulate inflammation following intestinal epithelial injury may lead to IBD (152).

1.3.1.1 *C. jejuni* associated invasion

To establish an infection, *C. jejuni* must bypass the mechanical and immunological barriers of the host gastrointestinal tract. The mucus layer of the GI epithelium serves as the first layer of defense, but *C. jejuni* possesses several virulence factors to circumvent this barrier (178). In particular, motility, combined with the spiral shape, allows the bacteria to effectively "drill" through the mucus lining of the gastrointestinal track (40). Genome sequencing analysis has also shown that the *C. jejuni* genome encodes many of the features found within the *E. coli* chemotaxis system (101, 131). *C. jejuni* displays motility towards amino acids that are found in high levels in the chick gastrointestinal track and towards components of mucus (71).

The invasion of intestinal epithelial cells has been observed in patients and is reproducible in cell lines *in vitro* (163), albeit at very low levels (39, 47, 100, 157). The mechanisms that control invasion are not elucidated, but differences exist between individual strains. It has become clear that microtubule polymerization is required for maximal invasion; some strains also require microfilament polymerization (16, 70, 126). This contrasts the actin-dependent mechanism of entry that is used by many other bacteria.

Similar to the type III secretion system that is employed by *Salmonella typhimurium* and *Shigella flexneri*, whereby effectors are delivered into the host cell, *C. jejuni* also secretes proteins from the flagellar type III secretion system. This is a requirement for maximal invasion of host epithelial cells (29). The proteins that are

synthesized and secreted by *C. jejuni* upon co-cultivation with epithelial cells are termed *Campylobacter* invasion antigens (Cia) (96). Only a few *C. jejuni* secreted proteins have been identified to date due to low levels of protein secretion under *in vivo* conditions (29).

1.3.1.2 Ability of *C. jejuni* to survive within macrophages

C. jejuni survives within murine macrophages (149). The role of monocytes and macrophages in *C. jejuni* infection is unclear because results vary with different cell lines or primary cells (178). Inflammatory cytokines such as NF-κB (nuclear factor kappa B) and interleukin-1β are produced in the presence of *C. jejuni*; however, a significant proportion of monocytes also undergo apoptosis (168). *C. jejuni* is killed within 24 – 48 hours of infection by macrophages. Within 4 – 8 hours, a significant portion of the bacteria turn into the non-culturable coccoid form when exposed to macrophages (174). However, other groups have found that clinical isolates of *C. jejuni* survived for several days inside the murine J774A.1 macrophage cell line (35, 67). Regardless of the contradictory data currently available, the consensus is that the differences observed in *C. jejuni* interactions with macrophages are likely due to strain variation and the use of different macrophage and/or macrophage cell lines.

1.3.2 *C. jejuni* virulence factors

Although *C. jejuni* is a commensal organism in mammals and birds, it has the ability to cause illness in humans. Several virulence factors are expressed by *C. jejuni*, which are discussed below.

1.3.2.1 Glycosylated proteins

C. jejuni expresses two systems of protein glycosylation: *O*-linked glycosylation, which modifies serine or threonine residues on flagellins, and *N*-linked glycosylation, which modifies asparagine residues on many secreted proteins (178).

O-linked protein glycosylation is involved in the glycosylation of *C. jejuni* flagellins. The flagellins in strain 81-176, a human gastric isolate of *C. jejuni*, are glycosylated with pseudaminic acid at up to 19 sites, accounting for approximately 10% of their observed mass (159). Meanwhile, the flagellin of *C. coli* strain VC167 is modified with legionaminic acid, and the genes that encode the proteins that are involved in synthesizing this glycan are shared by many strains of *C. jejuni*, with the exception of the 81-176 strain (112). No consensus sequence has been identified for *O*-linked glycosylation; however, it is required for the proper assembly of the flagellar filament (51). This suggests that *O*-linked glycosylation is required for the interactions of the flagellin subunits with each other, or with another component of the flagellar apparatus. Defects in *O*-linked glycosylation result in loss of motility, a decrease in adherence and invasion of host cells, and decreased virulence in ferrets (57).

Prior to the discovery in *C. jejuni*, the *N*-linked glycosylation pathway had only been observed in eukaryotes and archaea. In *C. jejuni*, *N*-linked glycosylation is encoded by the *pgl* (protein glycosylation) locus. Unlike other surface carbohydrates in *C. jejuni*, such as lipooligosaccharide, capsular polysaccharide, and *O*-linked glycans, *N*-linked glycans appear to be conserved in all *C. jejuni* strains that have been examined to date, and exhibit very little potential for phase variation (158). This conservation implies that *N*-linked glycosylation may have a fundamental role in *C. jejuni* physiology. The site for

N-linked glycosylation, unlike *O*-linked glycosylation, has a consensus sequence: aspartic acid/glutamic acid – X – asparagine – X – serine/threonine, where X can be any amino acid except proline (98, 122). While this sequence is vital for glycosylation, there are other factors, such as tertiary or quarternary structure that also play a role (122). The role of *N*-linked glycosylation in *C. jejuni* is not clear. Mutations in the *pgl* locus have effects on multiple glycosylated proteins; this results in reduced adherence and invasion to intestinal cells, loss of mouse and chick colonization, reduced protein activity to antisera, and defects in natural competence (65, 78, 83, 88, 101, 123, 156).

1.3.2.2 Flagella

C. jejuni flagella and flagellar motility are vital for host colonization, virulence in ferret models, protein secretion and host-cell invasion (178). The motility of *C. jejuni* increases in highly viscous solutions and the speed has been reported to reach 75 μm per second under these conditions (102). This motility may facilitate movement of *C. jejuni* in and through the thick mucus lining of the intestinal tract (40, 102). Black *et al* (17) fed human volunteers mixtures of motile and non-motile phase variants and subsequently, recovered only motile forms from stool samples. This was the first evidence that motility was required for intestinal colonization.

The flagella consists of a major flagellin, FlaA, and a minor flagellin, FlaB, that are highly homologous (55). The *flaA* gene is regulated by a σ^{28} promoter, while *flaB* is regulated by a σ^{54} promoter (54). Mutations in *flaA* result in a severely truncated flagellar filament, with a reduction in motility, while mutants in *flaB*, in contrast, have no significant change in motility and produce a flagellar filament that appears normal (55, 124). Many strains of *C. jejuni* undergo phase variation in flagellin expression by slip

strand mismatch repair (25, 64). The ability of *C. jejuni* to undergo changes in motility and flagellar expression may be beneficial to the organism so that it can adapt to the different environments it encounters within the host.

A connection between flagella and colonization has been established (157). While motility was found to be important for colonization of epithelial cells, genomic studies have also found that *C. jejuni* strains lack a specialized type III secretion system (46). However, there are reports that flagella can function to secrete non-flagellar proteins that may modulate virulence. Konkel *et al* showed that the secretion of at least eight proteins into the supernatant occurs when *C. jejuni* was co-cultured with epithelial cells (96, 97, 143). The secretion of Cia proteins, but not their synthesis, was dependent on growth of *C. jejuni* in the presence of INT407 intestinal cells (96, 97, 143). Thus far, only one Cia protein, CiaB, has been identified. Mutation in *ciaB* results in full motility, but the pathogen is unable to secrete any Cia proteins. Moreover, the mutant was reduced 50-fold in invasion into epithelial cells compared with the parent strain (54, 96). This implies that while motility is not essential for colonization and invasion of the intestinal epithelium, an intact flagellar apparatus is required.

1.3.2.3 Adhesins

C. jejuni produces several adhesins to colonize intestinal epithelial cells. Many microorganisms typically have surface appendages such as pili that facilitate adherence. However, genome annotations of several *C. jejuni* strains do not show obvious pilus or pilus-like open reading frames (43, 131). Despite the lack of pili, several proteins contribute to *C. jejuni* adherence. CadF (*Campylobacter* adherence factor) binds specifically to fibronectin, which is located basolaterally on epithelial cells (95, 116).

CadF is required for maximal binding and invasion by *C. jejuni in vitro*, and *cadF* mutants are greatly reduced in chick colonization compared with wild type (117). While CadF is similar to the OmpA protein in *E. coli* in that it forms membrane channels, its precise function has not been established (109). Another characterized adhesin is JlpA, a surface exposed lipoprotein that is required for binding to Hep-2 epithelial cells (76). Another lipoprotein, CapA, has also been implicated as important for *C. jejuni* adhesion to Caco-2 cells, as CapA deficient mutants show decreased adherence, colonization and persistence within them (8). Some putative *C. jejuni* adhesins are in the periplasm. Peb1 is an adhesin that is required to bind to HeLa cells (90, 133). However, the majority of this adhesin is periplasmic and shares homology to periplasmic binding proteins of amino acid transporters, binding to both aspartate and glutamate with high affinity (103, 132). In addition, while Peb1 does not localize to the inner or outer membrane, some must be surface exposed in order for this protein to act as an adhesin. While it was suggested that Peb1 is anchored to the outer membrane, no evidence has been found, and instead, Peb1 was observed in culture supernatants, suggesting that the protein can be exported across the outer membrane (103).

1.3.2.4 Lipooligosaccharide (LOS)

C. jejuni expresses low molecular weight outer membrane antigens known as lipooligosaccharide (LOS). In many Gram negative bacteria, a set of repeating sugar subunits, the O-chain, is attached to the core polysaccharide, and the entire structure is termed lipopolysaccharide (LPS). However, much like in *Neisseria* and *Haemophilus* sp., the LOS of *C. jejuni* does not possess the long repeating O-antigen polysaccharide chains characteristic of LPS. The pioneering structural work of LOS was done by Aspinall *et al*

(118), and further refined by Szymanski *et al* (158). The LOS is anchored to the membrane via lipid A; attached to the lipid A anchor is the core region of the LOS. The core can be subdivided into the outer core region, proximal to the O-specific chain, and the lipid A proximal inner core region (141, 142). This subdivision exists because of the different sugar composition of the two regions. The outer core consists of hexoses and hexosamines, specifically, galactose and N-acetyl galactosamine, while the inner core consists of sugars such as two molecules of heptose and 3-deoxy-D-*manno*-2-octulosonic acid (Kdo) (118). The Kdo sugar serves as a link between the core oligosaccharide and the lipid A component. Interestingly, *C. jejuni* is one of the few bacteria capable of endogenous synthesis of sialic acid for incorporation into the galactose molecules present in the outer core. The resulting sialylation gives rise to molecular mimicry of host gangliosides, which can result in the development of autoimmune neuropathies such as GBS and Miller Fisher syndrome (58).

1.3.2.5 Capsular polysaccharide (CPS)

Capsules are found on the surface of many Gram positive and Gram negative bacteria. Capsules are considered a virulence factor and can be potentially antigenic. They can either be tightly associated or form a loose, gel-like matrix, and impart a great deal of protection to the bacteria. These include protection from desiccation, bacteriophages, and most hydrophobic toxic materials such as detergents. In addition, capsules provide protection against the complement system by preventing C3 deposition on the surface of the bacteria (166). Capsules also aid in bacteria adherence to surfaces.

Until recently, it was thought that *C. jejuni* strains produced both LOS and a high molecular weight lipopolysaccharide (LPS) (86). *C. jejuni* CPS remained largely

unnoticed until the sequencing of the ATCC 700819 genome where a large cluster of genes with similarities to type II/III CPS transport genes from *Enterobacteriaceae* were found (131). Mutations in these transport genes resulted in a loss of the high molecular weight polysaccharide, as well as the loss of the ability to be typed in the Penner serotyping scheme. A characteristic feature of group II capsular polysaccharides is the presence of a phospholipid anchor. Karlyshev *et al* showed that the high molecular weight polysaccharide was susceptible to phospholipase treatment, thus confirming that what originally was thought to be a high molecular weight LPS was actually a highly variable capsular polysaccharide (CPS) (86).

1.3.2.6 Role of CPS in *C. jejuni* virulence

The CPS of *C. jejuni* is a virulence factor that is important for adherence to and invasion of epithelial cells, chick colonization and virulence in a ferret model (13, 77). A capsuleless *KpsE* mutant in strain ATCC 81116, isolated from a waterborne outbreak in 1982, showed decreased adherence and invasion in intestinal epithelial cells (12) while similarly, a capsuleless *KpsM* mutant in strain ATCC 81-176 shows decreased invasion of intestinal epithelial cells and decreased invasion in a ferret model (13). The same mutant exhibits a decreased ability to colonize the chicken intestine (77). In addition, Keo *et al* created capsuleless mutants in the invasive clinical isolates 84-25 and 84-19 strains of *C. jejuni* and found that they showed a significant increase in serum-dependent killing compared to the wild type (89). These capsuleless mutants showed that CPS was critical for serum resistance and was also responsible for subtle changes in surface charge. However, it is not known whether differences in sugar composition of the capsule among different strains will affect virulence or whether phase variable modifications play a role.

1.3.2.7 Group II and group III capsular groups

Much of the work related to CPS has been performed in *E. coli*, which has over 80 different capsular serotypes. These were originally divided into groups based on their serological properties while later revisions incorporated genetic and biochemical criteria (172). Since this original classification, there now exist four distinct groups (173). *E. coli* group I and IV capsules share a common assembly system, and are fundamentally different than the assembly system used for group II and III (172). While four main capsular groups exist, the *C. jejuni* CPS shows high similarity to the *E. coli* group II and III capsular groups (58, 131) and only these will be described below.

1.3.2.8 Capsular organization

The structural features of the repeating units of *E. coli* group II and III capsules vary extensively; while some contain phosphate residues in their backbone, much like Gram positive teichoic acids, some group II CPS resemble vertebrate glycoconjugates (172).

The chromosomal locus for group II capsule has a generally conserved structure, consisting of three regions (172). This organization is observed not only in *E. coli* but also in other encapsulated bacteria such as *Campylobacter*, *Haemophilus, Neisseria, Pasteurella* and *Actinobacillus* spp. (150). This conservation suggests a common origin for the biosynthetic gene cluster in these species. In fact, Silver *et al* (150) showed that homologues of the *E. coli* transporter from *Actinobacillus pleuropneumoniae, Haemophilus influenzae* and *Neisseria meningitidis* complemented an export defective *E. coli* mutant.

Region 2 is known as the serotype specific central region, and encodes glycosyltransferases as well as many unique sugar nucleotide synthetases required for the production of capsule. As this region is serotype specific, the size and genetic content will vary. In general, the size of region 2 corresponds to the complexity and the number of repeat units of the capsular polysaccharide that is formed (172). Region 2 is flanked by regions 1 and 3, which are generally conserved. Genes in these areas are involved in a range of activities including assembly and subsequent export of the polysaccharide via an ATP-binding cassette (ABC) transporter, consisting of KpsM and KpsT.

1.3.2.9 Capsular biosynthesis

Biosynthesis and assembly of capsular polysaccharides is a complex process. Monosaccharides are the basic building blocks of CPS; once the sugar molecules have been synthesized by the bacterium or obtained from the environment, activation to the sugar nucleotide form occurs. This activation step, whereby the sugars are activated to XTP, XDP or XMP (where X indicates a nucleotide, adenosine, cytosine, thymine or uridine, and TP, DP and MP indicate the triphosphate, diphosphate and monophosphonucleotides, respectively), is required for the final polymerization step catalyzed by glycosyltransferases that transfer the sugars to the correct acceptors (165). Any modifications that occur to the sugar nucleotide precede the final polymerization. After polymerization, the final polysaccharide can be exported to the cell surface where it can be expressed. Each step of this biosynthetic pathway will be introduced below.

Initiation of group II capsular synthesis requires a membrane bound initiator upon which the polysaccharide chain is elongated. The endogenous acceptor has been described as phospholipid, phospholipid-linked 3-deoxy-D-*manno*-octulosonate (Kdo),

"endogenous acceptor" protein, and undecaprenyl phosphate (23, 41, 160, 170). However, to date, the exact nature of the endogenous acceptor remains unknown.

Chain elongation is performed by glycosyltransferases that add additional sugar units to the nascent capsular polysaccharide. This process occurs on the cytoplasmic side of the membrane. A series of acetyl transferases and O-acetyl esterases have been discovered that modify the nascent capsule polysaccharide or its precursors before or during export (154). These modifications suggest that the synthetic components of group II capsule biosynthesis may themselves be associated with the inner membrane, interacting in a super-complex that Steenbergen *et al* have designated the sialisome (155).

The penultimate step to capsular biosynthesis is chain termination, but group II CPS termination of chain polymerization is unsolved. In *E. coli* K1, the majority of the capsular chains terminate with a maximum chain length of 160 – 230 residues, suggesting an active process in size determination (134). A terminal residue on group II capsules could potentially be overlooked in structural analyses (172). The other possibilities for chain termination include the loss of affinity by the glycosyltransferases for the polymer beyond a certain chain length, an abortive chain translocation process within the catalytic site, or an allosteric effect that is mediated by other components of the capsular assembly system (172).

The final step to express the complete capsular polysaccharide on the cell surface is export. This process is driven by ABC transporters at the expense of ATP hydrolysis. KpsM and KpsT were initially identified as the components of an ABC transporter on the basis of their sequences, and their identities were then confirmed by biochemical approaches (150). KpsM is the integral inner-membrane transmembrane domain

component, with six transmembrane helices, and KpsT is the cytoplasmic nucleotide binding domain (172).

Two models of capsular export exist: post-synthetic or direct coupling (165). In the former, as all group II and probably group III capsules bear a terminal reducing phosphatidic acid moiety that anchors them to the outer membrane, this feature could function as a recognition tag for export due to its commonality. In the latter model of capsular export, the glycosyltransferases for polymerization are directed to the exporter by affinity for accessory proteins, essentially coupling synthesis of the capsule to polysaccharide export (165). There is growing evidence that the direct coupling model is the correct model as Steenbergen *et al* showed that in the presence of a depolymerase (capable of cleaving capsular chains greater than or equal to 7 monomers in length), cytoplasmic biosynthesis of the polysaccharide (both synthesis and subsequent export) was protected from degradation. The depolymerase clips nascent chains prior to export; in a post-synthetic transport model, the completed capsular chains would be eliminated before their export, reducing or eliminating surface capsule expression. However, if synthesis and export of the polysaccharide were intimate (as in a directed coupling export model), the polysaccharide would be protected and the capsule expressed regardless of the depolymerase. Results indicated that capsular synthesis was protected during polymerization and export, supporting the directed coupling model (155).

1.3.2.10 The *C. jejuni* capsule and heptose biosynthesis genes

The CPS of *C. jejuni* strain ATCC 700819 is anchored to the outer membrane via a dipalmitoyl-glycerophosphate phospholipid anchor (31). The *C. jejuni* ATCC 700819 capsular backbone contains a β-D-ribose, β-D-Gal*f*NAc (N-acetylgalactosamine in a

furanose conformation), α-D-Glc*p*A6(*N*gro) (a glucuronic acid with 2-amino-2-deoxyglycerol on carbon 6), and a 3,6-*O*-methyl-D-*glycero*-α-L-*gluco* heptose as a side-branch (153). When high-resolution magic-angle spinning nuclear magnetic resonance (HR-MAS NMR) was used to examine intact *C. jejuni* cells, a large variation in capsular structure was found (158). This included a 6-*O*-methyl group on the heptose, an *N*-ethanolamine modification on glucuronic acid instead of the 2-amino-2-deoxyglycerol, and a unique O-methyl phosphoramidate molecule on Gal*f*Nac that had not been previously seen in any other bacteria (158). It is not known whether changes in sugar composition of CPS plays a role in virulence or in interactions with bacteriophages. Other strains of *C. jejuni* are known to possess modifications to heptose. For example, in the 81-176 strain, not only is the heptose in an *altro* conformation, but a dehydration also occurs on carbon 6 (85).

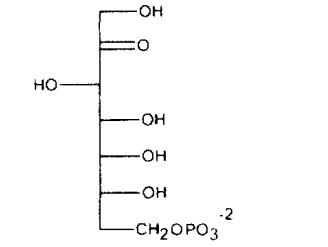
Due to the side-branching nature of the modified heptose as well as the phosphoramidate molecule in *C. jejuni* ATCC 700819, these molecules protrude into the external environment, and become the outermost portion of the CPS that is exposed. These moieties are likely important for bacterial/host interactions as well as pathogenicity of the organism. The enzymes that are involved in the synthesis of these modified heptoses are potential targets for therapeutic agents.

Most Gram negative bacteria contain a single heptose biosynthesis pathway in their LOS or LPS biosynthesis loci, producing ADP-L-*glycero*-β-D-*manno*-heptose, a precursor of the core oligosaccharide (162). Interestingly, *C. jejuni* is an exception to this, as some strains possess additional heptose biosynthesis genes involved in synthesizing GDP-D-*glycero*-α-D-*manno*-heptose which are located within the capsular cluster of

genes (82). The first step of the pathway to create the *manno*-heptose involves converting D-sedoheptulose-7-phosphate to D-*glycero*-α-β-D-*manno*-heptose-7-phosphate via the isomerases GmhA and GmhA2 (Cj1149c and Cj1424c, respectively; **Figure 1**). The D-*glycero*-α-D-*manno*-heptose-1,7-bisphosphate is then formed via the kinase HddA (Cj1425c). GmhB (Cj1152c), encoded from the LOS synthetic pathway, acts next to remove a phosphate, yielding D-*glycero*-α-D-*manno*-heptose-1-phosphate. HddC (Cj1423c) then acts to transfer a guanosine triphosphate (GTP) molecule, yielding GDP-D-*glycero*-α-D-*manno*-heptose (82). In some strains of *C. jejuni*, this *manno*-heptose is further modified before incorporation into the capsule via the heptosyltransferase HddD (Cj1431c). For instance, in ATCC 81-176, the conversion of heptose to deoxyheptose is thought to occur, as a new gene, *wcaG*, was found within the genome (82). The WcaG protein is a homologue of DmhA, known to be involved in conversion of heptose to deoxyheptose in *Yersinia pseudotuberculosis* (24, 69, 130). Karlyshev *et al* report that *cj1427c*, *cj1428c* and *cj1430c* are potential candidates for carbon 3,5 epimerase/carbon 4 reductases. These gene candidates are postulated to be involved in converting the D-*glycero*-α-D-*manno*-heptose to GDP-6-OMe-D-*glycero*-α-L-*gluco*-heptose. The verification of the heptose biosynthetic pathway in *C. jejuni* ATCC 700819 via mutagenesis studies is the main focus of this thesis.

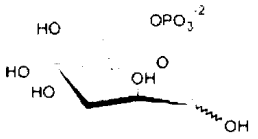
Figure 1. Synthesis of D-*glycero*-α-L-*gluco*-heptose and its subsequent incorporation into the CPS.

The biosynthesis of GDP-6-OMe-D-*glycero*-α-L-*gluco*-heptose in ATCC 700819 from D-sedoheptulose-7-phosphate is shown. The genes *gmhA, gmhA2, hddA, gmhB,* and *hddC* have known functions. The genes *cj1426c, cj1427c, cj1428c,* and *cj1430c* are putatively thought to modify the heptose while *cj1431c* is putatively thought to be a heptosyltransferase.



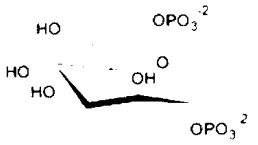
D-sedoheptulose-7-phosphate

GmhA (*cj1149c*)
GmhA2 (*cj1424c*)



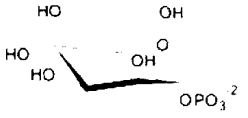
D-*glycero*-D-*manno*-heptose-7-phosphate

HddA (*cj1425c*)



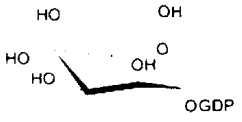
D-*glycero*-D-*manno*-heptose-1,7-bisphosphate

GmhB (*cj1152c*)



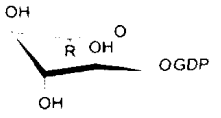
D-*glycero*-D-*manno*-heptose-1-phosphate

HddC (*cj1423c*)

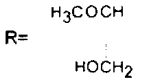


GDP-D-*glycero*-D-*manno*-heptose

cj1426c
cj1427c
cj1428c
cj1430c



GDP-6-OMe-D-*glycero*-α-L-*gluco*-heptose



HddD (*cj1431c*)

CPS

1.4 Rationale and hypothesis

Modified heptoses are relatively unique in bacteria. They have been found in the CPS of several strains of *C. jejuni* and *C. coli*, as well as the LPS of *Yersinia pseudotuberculosis* (28, 49, 69, 81, 82, 162). The CPS plays an important role in pathogenesis, as a capsuleless mutant in *C. jejuni* ATCC 81-176 has reduced adherence and invasion of intestinal epithelial cells as well as reduced virulence in a ferret diarrheal model (13). It is not known whether specific components of the capsule, such as the modified heptose and/or phosphoramidate molecule, play a role in pathogenesis. As these molecules form side-branches away from the main capsular backbone, they are the outermost exposed component of the capsule to the external environment, and may thus play a role in pathogenesis. The capsular structure of *C. jejuni* ATCC 700819 was elucidated and contains 6-O-methyl-D-*glycero*-α-L-*gluco*-heptose (153). To achieve this final conformation from the starting D-*glycero*-α-D-*manno*-heptose, three epimerization steps at carbons 3, 4 and 5 must occur in order to switch from the *manno* to the *gluco* conformation, as well as to switch from the D to the L configuration. These epimerases have not been identified. However, region 2 of the capsular cluster in *C. jejuni* ATCC 700819 contains three genes, *cj1427c*, *cj1428c* and *cj1430c* that are thought to putatively perform these epimerizations on D-*glycero*-α-D-*manno*-heptose. The precise biochemical functions of these enzymes are under investigation in our laboratory.

We believe that the enzymes encoded by *cj1427c*, *cj1428c* and *cj1430c* play important roles in the synthesis of modified heptoses in the capsule of *C. jejuni*, and that specific capsular components, such as modified heptoses, are important for CPS function and the overall virulence of *C. jejuni*.

1.5 Objectives

1) To determine the role of *cj1427c*, *cj1428c* and *cj1430c* in modified heptose biosynthesis.

We have constructed *cj1428*::CAT and *cj1430*::CAT insertional mutants, but lacked a *cj1427*::CAT mutant. To complete our panel of mutants, and to determine the function of these genes of interest, a *cj1427*::CAT mutant was created. The effects on CPS production and composition of these insertional mutants were also investigated.

2) To determine the effect on virulence due to subtle changes in the CPS of *C. jejuni*

Previous laboratory members have characterized phenotypes related with the virulence of *C. jejuni* (i.e. bile salt resistance, serum resistance, survival in macrophages, etc) for the *cj1428*::CAT, *cj1430*::CAT and *KpsM* mutants. However, due to variability observed in the data, particularly for the macrophage assays, these phenotypes were repeated for all our mutants. In addition, with the construction of a new *cj1427*::CAT mutant, characterization of each phenotype must also be investigated to gain a complete understanding of the role of modified heptoses on the virulence of *C. jejuni*.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Bacterial strains and culture conditions

C. jejuni ATCC 700819 (NCTC 11168) as well as *E. coli* DH5α were routinely cultured from -80°C freezer stocks using conditions suitable for each bacterium. *C. jejuni* was grown overnight on Trypticase Soy Agar (TSA; BD, Canada) supplemented with 10 μg/mL vancomycin (Biobasic, Markham, Canada), 5 μg/mL trimethoprim (Sigma-Aldrich, Canada), and 5% sheep blood (Cedarlane, Burlington, Canada). For transformation purposes, *C. jejuni* was grown on either TSA plates supplemented with sheep blood as above or on Mueller Hinton Agar (BD, Canada) plates, containing 10 μg/mL vancomycin, 5 μg/mL trimethoprim, 0.05% pyruvate (Alfa Aesar, USA), and 5% horse serum (Invitrogen, Canada). Routinely, *C. jejuni* was grown for up to 48 hours in a microaerophilic incubator (Nuaire) at 37°C in 5% oxygen, 10% carbon dioxide, 85% nitrogen, and 90% humidity. For experiments, wild type and mutant strains (plates supplemented with appropriate antibiotic(s)) were grown from freezer stocks overnight and then transferred to TSA plates and incubated for a further 20-24 hours (unless otherwise stated) before being used. This ensured that *C. jejuni* was actively growing. Additional antibiotic(s) were added where appropriate at the following concentrations: kanamycin (Biobasic, Markham, Canada) 90μg/mL, chloramphenicol (Fisher Scientific, Canada) 15μg/mL.

E. coli was routinely grown on lysogeny broth (LB) Agar (Bioshop, Canada) in a 37°C incubator. Alternatively, *E. coli* was grown in LB broth (Bioshop, Canada) in a shaking incubator (200 rpm) at 37°C. Antibiotics were supplemented where appropriate at the following concentrations: 100 μg/mL ampicillin (Biobasic, Markham, Canada), 30 μg/mL kanamycin, and 34 μg/mL chloramphenicol. Where indicated, 5-bromo-4-chloro-

3-indolyl-β-D-galactopyranoside (X-gal; Biobasic, Markham, Canada) was used at 4 μg/mL.

2.2 Preparation of calcium chloride competent *E. coli* DH5α

A 3 mL overnight culture of *E. coli* DH5α grown in LB broth was used to inoculate 100 mL LB. The bacteria were incubated for 1-2 hours until an OD_{600} of 0.6 was reached. The bacteria were pelleted at 5000 × *g* (Eppendorf 5415D, Eppendorf) for 10 minutes at 4°C, re-suspended in 20 mL of cold 50 mM calcium chloride solution and incubated on ice for 30 minutes. Following this, the bacteria were pelleted as described above and re-suspended in 5 mL of 50 mM calcium chloride solution and incubated on ice for 5 hours. After this final incubation period, 5 mL of 50% cold glycerol was added, and cells aliquoted before storing at -80°C.

2.3 PCR to verify the inactivation of *cj1427c* in *C. jejuni* ATCC 700819

For the generation of the *cj1427c* mutant, a chloramphenicol resistance gene was inserted into the *cj1427c* gene. This construct was previously constructed in our laboratory (100) and used in this thesis. The construct was amplified in *E. coli* DH5α and was verified using *cj1427* P2 and P3 primers (see appendix for full list of primers). The PCR program used was as follows: Initial denaturation at 94°C for 5 minutes, 25 cycles of: Denaturation at 94°C for 45 seconds, Annealing at 49°C for 45 seconds, Elongation at 68°C for 2 minutes, and Final elongation at 68°C for 7 minutes. DNA was amplified using Expand Long Template (Roche) and was used as per manufacturer's instruction in a total reaction volume of 12.5 μL. Once the construct was verified, it was transformed

into *C. jejuni* (see below for procedure). The inactivation of the *cj1427c* gene was verified using the *cj1427* P2 and P3 primers and the same PCR program as above.

2.4 Creation of *C. jejuni* complementation mutants

The method of Karlyshev *et al* was adapted for the creation of the complementation mutants (87). The 16S – 23S rRNA region was chosen for insertion of the complementation constructs as three copies of these genes exist within the *C. jejuni* genome, and complementation into one of these regions via homologous recombination will not affect the viability of the bacteria. First, the 16S – 23S rRNA region of *C. jejuni* was amplified from wild type genomic DNA using primers 16S rRNA Top – KpnI and 23S rRNA Bottom – *Not*I, respectively. The 16S – 23S rRNA fragment (2024 Kb) was then inserted into the *Kpn*I/*Not*I sites of the pBluescript KS (+) cloning vector. This vector contains an ampicillin resistance gene, as well as a *lacZ* gene for blue and white screening. Positive transformants grew as white colonies on LB agar supplemented with X-gal (4 μg/mL) and ampicillin (100 μg/ mL), indicating that the DNA fragment had been inserted into the vector. The insertion of the fragment was confirmed by PCR using fragment specific primers and DNA sequencing (Robarts Sequencing Facility, University of Western Ontario, London, Ontario).

The complementation constructs were generated using the primer overlap extension method, also known as gene SOE-ing (63). In this method, primers used in PCR contain overlapping sequences of the adjacent DNA fragment to be joined at their 5' end. Overlapping regions were designed to be at least 21 bases in length. Each DNA fragment was first amplified individually using Expand Long Template DNA polymerase (Roche, Canada) and the following PCR program: Initial denaturation at 95°C for 5

minutes, followed by 30 cycles of denaturation at 95°C for 45 seconds, annealing of the primers at the appropriate temperature for 45 seconds, elongation at 68°C for the appropriate duration (1.5 Kb/minute), and a final elongation at 68°C for 7 minutes. The *porA* promoter was amplified using primers ompE For – *Xba*I combined with ompE Rev 1427, ompE Rev 1428, or ompE Rev 1430. The genes to be complemented were amplified using primers 1427 For ompE, 1428 For ompE, or 1430 For ompE, and their corresponding reverse primers 1427 Rev Kan, 1428 Rev Kan, or 1430 Rev Kan. The kanamycin resistance gene was amplified using Kan For 1427, Kan For 1428, or Kan For 1430, combined with the reverse primer AphP3, which contains an *Xba*I restriction enzyme site. For the second and third PCR reactions, 9.6 µL (approximately 200 ng) of each of the fragments to be joined were added to 2.5 µL dNTP (2.5mM), 2.5 µL 10X Expand Buffer 3, 0.5 µL of forward and reverse primers (20 pmol/µL each) and 0.25 µL Expand Long Template DNA polymerase. Between each PCR reaction, the products were run on an agarose gel, and the fragments that corresponded to the correct size were purified using a gel purification kit (Biobasic, Markham, Canada), according to the manufacturer's instructions.

The complementation constructs were then inserted into the multiple cloning site within the pBluescript vector containing the 16S– 23S rRNA fragment to generate the final constructs. Specifically, the restriction endonuclease *Xba*I was used to cut within the inserted 16S – 23S rRNA and also to create sticky ends on the complementation constructs. The vector and the insert was then ligated together using T4 DNA ligase (New England Biolabs). These ligated complementation constructs were then transformed into *E. coli* DH5α and selected on LB agar supplemented with 100 µg/mL

ampicillin and 30 μg/mL kanamycin. These above steps were done by another student in our laboratory, Xuan Than Bui. Positive transformants were confirmed via PCR using OmpE For – *Xba*I and AphP3. The correct order of DNA fragments was confirmed by PCR using primers internal to the constructs. Sequencing of the constructs was performed at the Robarts Sequencing Facility, University of Western Ontario, London, Ontario.

Figure 2. Schematic of the complementation constructs used in this study.

The schematic depicts the complementation constructs that were created for the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT mutants. The 16S rRNA and 23S rRNA areas are present to facilitate homologous recombination of the complementation construct into the *C. jejuni* genome. An outer membrane protein promoter, *porA*, drives the overall expression of the complementation construct. Due to prior nomenclature, primers for the *porA* region have OmpE within their name. Following the promoter is the gene of interest to be complemented. A kanamycin resistance cassette allows for the selection of potential complemented clones. The sizes of each fragment are indicated under their respective regions. Total sizes of the complementation constructs are indicated on the right hand side of the figure.

	16S rRNA	PorA	Gene	KAN	23S rRNA	Total size
cj1427 construct	800 bp	123 bp	942 bp	1400 bp	1224 bp	4489 bp
cj1428 construct			1041 bp			4588 bp
cj1430 construct			546 bp			4093 bp

2.5 Selective methylation of plasmid DNA before transformation of *C. jejuni*

The method outlined by Donahue *et al* was adapted for transforming *C. jejuni* (36). This method protects foreign DNA from restriction modification enzymes by *in vitro* site-specific and strain-specific methylation. To have enough DNA for transformation, *E. coli* harbouring the vector of interest was grown and the plasmid harvested using either a DNA Mini Kit (Qiagen) or an Illustra DNA Midi Kit (GE Healthcare), depending on whether the vector was a high copy or a low copy plasmid, respectively. For each kit, the protocol as recommended by the manufacturer was followed. At least 10 μg of plasmid DNA was treated with a cell-free extract of *C. jejuni* (containing 300-400 μg of protein) in the presence of a methyl donor (200 nM S-adenosyl methionine; Sigma Aldrich) before transformation. Methylation was carried out at 37°C for 1 hour in methylation buffer (20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 5 mM Na_2EDTA, 1 mM dithiothreitol (DTT)) in a total volume of 200 μL. To obtain a cell-free extract, wild type *C. jejuni* was harvested from five TSA plates and resuspended in 4 mL of extraction buffer (20 mM Tris-acetate (pH 7.9), 50 mM potassium acetate, 5 mM Na_2EDTA, 1 mM DTT, protease inhibitor cocktail (Roche, following manufacturer's instructions)). This was passed through a French pressure cell press (Thermo Scientific) at least five times, until the lysate was visibly clearer. The amount of protein in the cell-free extract was quantitated using a Bradford Assay (21) following instructions from the manufacturer (Biorad). Following methylation, phenol:chloroform:isoamyl alcohol (in a 25:24:1 ratio) was used to purify the DNA. Briefly, one volume of phenol:chloroform:isoamyl alcohol was added to the sample and agitated to ensure that the aqueous and organic phases mixed. This was then centrifuged

at maximum speed (16110 × *g*, Eppendorf 5415D, Eppendorf) for one minute to separate the layers. The aqueous phase (top phase) was collected, and the organic phase discarded. An equal volume of phenol:chloroform:isoamyl alcohol was added to the aqueous layer and the above steps repeated until no visible protein layer was observed between the aqueous/organic phase interface. The aqueous phase was collected, and to this, an equal volume of chloroform was added and mixed. This was centrifuged at maximum speed for 1 minute, and the aqueous phase collected. Plasmid DNA was recovered using a sodium acetate/ethanol precipitation method (see below).

2.6 Sodium acetate/ethanol precipitation of DNA

To recover DNA, a sodium acetate/ethanol precipitation method was used. Briefly, 1/10 volume of 3 M sodium acetate (pH 7.0) was added to the DNA sample. To this, 2.5 volumes of ice cold 100% absolute ethanol was added. The sample was spun at maximum speed (16110 × *g*, Eppendorf 5415D, Eppendorf) for 30 seconds to collect all the liquid at the bottom of the tube before being placed into the -20°C freezer for a minimum of 2 hours. The sample was then spun at maximum speed for 30 minutes at 4°C to pellet the DNA. This was followed by a 750 µL 70% ice cold ethanol wash to remove excess salts from the DNA pellet. The sample was spun again at 4°C at maximum speed for 15 minutes. The ethanol was removed, and the DNA pellet allowed to air dry before being resuspended in 50 µL sterile water. Plasmid DNA purified in this manner was stored at -20°C until required.

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments. For most DNA fragments, a 0.7% agarose gel was prepared in TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA), and ethidium bromide was added to a final concentration of 0.01%. For smaller fragments (~200 bp), a 1.4% agarose gel was prepared in a similar manner as above. Samples were mixed with DNA loading buffer (4x TAE buffer, 50% glycerol, 0.01% bromophenol blue) before loading and sizes were compared to a 1 kilobase pair (Kb) DNA ladder standard (Invitrogen, Frogga). Electrophoresis was carried out at 80 volts until proper separation was achieved. DNA bands were visualized using UV light.

2.8 Preparation of *C. jejuni* and electroporation procedure

C. jejuni was grown on TSA supplemented with 5% sheep blood for 24 hours in microaerophilic conditions. On the day of transformation, 4-5 plates of the bacteria were suspended in 1 mL of 15% glycerol/272 mM sucrose and spun down for 2 minutes at 3300 × *g*. The cells were washed three times with 1 mL of cold 15% glycerol/272 mM sucrose before being resuspended in 150 µL ice cold 15% glycerol/272 mM sucrose. The OD_{600} was taken and samples diluted or concentrated to obtain approximately 10^9 cells/mL. An OD_{600} of 1.0 corresponds to approximately 10^9 cells/mL, as determined by a standard curve of colony forming units (CFU) versus OD_{600}. Transformation was done with 70 µL of cells and 2 µg of plasmid DNA in a total volume of 80 µL. A negative transformation control consisted of 70 µL of cells and 10 µL of water. Prior to electroporation, cells were plated on TSA plates to ensure their viability. Cells were then transferred to an ice cold 0.2 cm electroporation cuvette (Gene Pulser) and electroporated at 2.5 kvolts, 600 Ω, 25 µF. Cells were placed back on ice, and 300 µL of Mueller Hinton

(MH) or TSA broth added to the cells. Four spots of 75 μL each were placed onto MH plates or TSA plates supplemented with 5% sheep blood and cells allowed to recover for a minimum of 5 hours under microaerophilic conditions at 37°C. Alternative recovery times of 8 hours and 10 hours were also attempted. After each recovery time period, spots were resuspended in 300 μL of MH broth or TSA broth and 75 μL plated onto MH or TSA agar plates containing selective antibiotics, respectively, using glass beads. Plating onto MH or TSA plates, to ensure viability after electroporation, was also done in parallel. These plates were grown for 2-3 days under microaerophilic conditions at 37°C until colonies were observed.

2.9 Preparation of *C. jejuni* and natural transformation procedure

C. jejuni was grown on TSA supplemented with 5% sheep blood for 24 hours in microaerophilic conditions. On the day of transformation, 2 plates of the bacteria were suspended in 1 mL of TSA broth. The OD_{600} was taken and samples diluted or concentrated to obtain approximately 10^9 cells/mL. Transformation was done with 70 μL of cells and 2 μg of plasmid DNA in a total volume of 80 μL. A negative transformation control consisted of 70 μL of cells and 10 μL of water. The plasmid was mixed with the bacteria, and to this, 300 μL of TSA broth was added to the cells. Four spots of 75 μL each were placed onto TSA plates supplemented with 5% sheep blood and cells allowed to recover for a minimum of 5 hours under microaerophilic conditions at 37°C. Alternative recovery times of 8 hours and 10 hours were also attempted. After each recovery time period, spots were resuspended in 300 μL of TSA broth and 75 μL plated onto TSA agar plates containing selective antibiotics using glass beads. Plating onto TSA

plates, with no antibiotics, to ensure viability after transformation, was also done in parallel. These plates were grown for 2-3 days under microaerophilic conditions at 37°C until colonies were observed.

2.10 Colony PCR

Colony PCR was used to verify potential transformants. Colonies were picked from an agar plate and resuspended in 15 µL of water. 5 µL was added to a PCR reaction with 12.5 µL as the final volume. The PCR reaction mix contained 1X Expand PCR Buffer 2, 0.25 mM dNTP, 20 pmol of each primer, and 1U Expand Long Template. The PCR conditions used for the reaction were as follows: 94°C (initial denaturation) for 10 min, 94°C (denaturation) for 45 seconds, annealing temperature at the appropriate temperature for 45 seconds, 68°C (elongation) for X min, where X is the appropriate duration dependent on the length of the fragment to be amplified) cycled 25 times, followed by 68°C (final elongation) for 7 min. Products were run on a 0.7% DNA agarose gel as described above.

2.11 Preparation of genomic DNA from bacterial pellets

C. jejuni was grown on 1 TSA agar plate for 24 hours under microaerophilic conditions at 37°C and then resuspended in 1 mL of TSA broth. Samples were centrifuged for 5 minutes at 16100 × *g* (Eppendorf 5415D, Eppendorf). The supernatant was removed, and 1 mL of DNAZol (Invitrogen) reagent was added and the sample mixed well. The samples were incubated at room temperature for 60 minutes to allow the cells to lyse. After incubation, samples were spun for 10 minutes at 9300 × *g* to remove any debris. The supernatant was collected to a new 1.5 mL centrifuge tube. Five hundred

μL of ice cold 100% ethanol was added and the tube inverted 5-10 times. Samples were then placed at -20°C for 45 minutes to allow the DNA to precipitate. Following this, the DNA was pelleted via centrifugation for 10 minutes at 16100 × *g* and the DNAzol supernatant removed. Pellets were washed twice with 500 μL 95% ethanol, and spun at 16100 × *g* for two minutes. Following the last wash, the ethanol was removed, and the pellets air dried at room temperature for 30 minutes. The DNA was dissolved in 30-50 μL of water, and left overnight at 4°C. The following day, samples were placed at 37°C for 30 minutes to allow DNA to dissolve further. DNA prepared in this manner was stored at 4°C until further required.

2.12 Preparation of *C. jejuni* RNA and cDNA

C. jejuni was grown for 20-24 hours under microaerophilic conditions at 37°C on one TSA plate. For RNA isolation, no more than 5.0×10^9 cells were resuspended in 200 μL TE buffer, as recommended by the manufacturer, prior to lysis. RNA was isolated using the RNA midi spin kit (GE Health Sciences) as per the manufacturer's instructions. The final elution step was done in 500 μL of RNAse free water, following the manufacturer's instructions. In addition to the on column DNAse I treatment as suggested by the manufacturer, the samples were treated using 60 units of DNAse I (Roche, Canada) for 40 minutes at 37°C. Following the reaction, the DNAse was inactivated via the addition of 8.4 μL 100 mM ethylenediaminetetraacetic acid (EDTA) and heating at 70°C for 10 minutes. The remaining EDTA was titrated via the addition of excess $MgCl_2$ in diethylpyrocarbonate (DEPC)-treated water to a final concentration of 11 mM. The amount of RNA was quantified using a ND-1000 Nanodrop spectrophotometer

(Nanodrop, USA). RNA samples were diluted in DEPC-treated water to a final concentration of 10.5 ng/μL. cDNA was generated using iScript reverse transcriptase (BioRad, Canada). Each reaction contained 30 μL of DNAse-treated RNA, 8 μL 5x iScript reaction mix, and 2 μL of iScript reverse transcriptase. Negative control samples contained 10 μL of the iScript reaction mix and 30 μL of RNA. The reverse transcription reaction was carried out using a BioRad thermocycler (BioRad, Canada) and the program used was as follows: 5 minutes at 25°C for annealing of the random primers, 30 minutes at 42°C for extension, 5 minutes at 85°C for inactivation of reverse transcriptase, and a final 4°C hold. Following the generation of cDNA, the samples were stored at -20°C until use.

2.13 Real-time PCR analysis

Real-time PCR analysis was carried out using the Rotor-Gene 6000 (Corbett Life Science, Canada) to measure the transcription levels of the *cj1425c*, *cj1426c*, *cj1427c*, *cj1428c*, *cj1429c*, *cj1430c*, *cj1444c*, *cj1445c*, *cj1447c* and *kpsM* genes in the wild type and mutant strains. The gene *cj1537c* encoding an acetyl CoA synthetase (a housekeeping gene) was used as a reference for normalization within each strain (intrastrain). *C. jejuni* chromosomal DNA was used as a positive control for the amplification of each fragment. When determining the expression level of each gene of interest, samples were set up in triplicate. For determination of gene expression, 3.75 μL of cDNA were added to 1.34 μL of the appropriate primer mix (0.67 μL of each primer, at 7 pmol/μL (see Appendix for primer sequences)), 7.5 μL SYBR green mix, containing all necessary components for RT-PCR, (BioRad, Canada), and 2.4 μL of water. Negative controls for the RT-PCR contained cDNA but did not contain the SYBR Green mix. An

additional negative control consisted of the SYBR Green mix and RNA which did not undergo reverse transcription. This control was used to ensure there was no chromosomal DNA contamination.

Reactions were carried out as follows: 95°C (initial denaturation) for 5 minutes, 95°C (denaturation) for 45 seconds, 59.5°C (annealing) for 30 seconds, and 72°C (elongation) for 20 seconds. Steps 2-4 were repeated for 40 cycles.

For each primer pair, efficiencies were determined using the dynamic range of chromosomal DNA and amplifying the desired fragments. Following amplification, the data were plotted and a standard curve was generated based on the C_T (the cycle number at which enough amplified product accumulates to yield a detectable fluorescence signal above a set threshold) values for the amplification from the different chromosomal DNA concentrations covering the dynamic range. The primer amplification efficiency was determined from the slope of the standard curve by performing the following calculation: E (efficiency) = $10^{-1/slope}$, which was converted into a percentage using the following: %Efficiency = (E-1) x 100.

The dynamic range of cDNA for downstream real-time PCR analysis was determined using varying concentrations of cDNA. For this, DNA fragments were amplified from serial dilutions of cDNA. The C_T value for each reaction was determined and a standard curve was generated by plotting C_T against the cDNA concentration. The cDNA concentrations yielding the best standard curve (i.e. the C_T values were evenly spaced and a linear standard curve results in a slope indicative of the maximum amplification efficiency (i.e. a doubling of DNA per cycle)) were used in each experiment to determine gene expression.

Two methods of comparison were used to determine differences in gene expression: intrastrain and interstrain comparisons. In intrastrain comparisons, the fold difference of expression of each gene was normalized to an internal housekeeping gene, *cj1537c* (a gene which encodes acetyl CoA synthetase). For interstrain comparisons, the fold expression of each gene was first normalized to the housekeeping gene in each strain as above, and then taken as ratio over the respective genes in the wild type. This was calculated using the Pfaffl equation, which is as follows: Ratio = $(E_{target})^{\Delta CTtarget(calibrator-test)}/(E_{ref})^{\Delta CTref(calibrator-test)}$ (135). The target gene refers to the gene being studied, the reference is *cj1537c,* the calibrator refers to the wild type strain, while the test refers to the knockout mutant of interest. Note that for intrastrain comparisons, a modified Pfaffl equation can thus be used, whereby the equation is simplified to the following: Ratio = $(E_{target})^{(-CTtargetcalibrator)} / (E_{ref})^{(-CTrefcalibrator)}$.

2.14 Growth curves of ATCC 700819 wild type and mutant strains

C. jejuni was grown as described above and re-suspended in MH broth to an OD_{600} of 1.0. A sidearm flask containing 20 mL MH broth and background antibiotics was first saturated with nitrogen by allowing the gas to bubble into the media for approximately 5 minutes through a sterile Pasteur pipette which was inserted into the flask, and partially sealed with a rubber stopper. The MH broth was then inoculated with 1.5 mL of the bacterial suspension to achieve a starting OD_{600} of 0.075. Nitrogen gas was bubbled into the sidearm flask for a further 10 minutes, to allow gas to fill the remaining volume of the flask, creating a microaerophilic environment. The flasks were then sealed and incubated under agitation (120 rpm) at 37°C for up to 24 hours. Growth was monitored using a Klett colony meter (600 nm filter) over 24 hours.

2.15 Motility assay

C. jejuni cells were harvested in MH broth and adjusted to an OD_{600} of 1.0. Motility plates (0.3 % Bacto agar (Difco) in MH broth supplemented with 5% horse serum) were inoculated (via stabbing from equal volumes) in multiple replicates with wild type or mutant strains and incubated for 48 hours under microaerophilic conditions at 37°C. The diameter of the motility halo was measured after 48 hours.

2.16 SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed using either polyacrylamide gels consisting of a 10% separating gel and a 4.8% acrylamide stacking gel or tricine gradient gels (10-20%, Biorad, Canada). For the polyacrylamide gels, the separating and stacking gels were made using 0.8% bis-acrylamide. Gels were run in Tris-glycine running buffer (25 mM Tris pH 8.3, 192 mM glycine, 1% w/v sodium dodecyl sulfate (SDS)) at 12 mA through the stacking gel and at 15 mA through the separating gel. Tricine gradient gels were run in cathode buffer (0.1 M Tris (the pH is not adjusted, but should be approximately 8.25), 0.1 M tricine, and 0.1% SDS) and anode buffer (0.2 M Tris, pH 8.9) at 12 mA through the stacking gel and at 15 mA through the separating gel.

2.17 Western blotting

Crude CPS and LOS samples were prepared by SDS-solubilization as previously described by Hitchcock and Brown (68). Briefly, *C. jejuni* from one TSA plate was resuspended into 1 mL of PBS and pelleted at 16100 × *g* (Eppendorf 5415D, Eppendorf). The resuspended bacteria were diluted to an OD_{600} of 0.375 and 1 mL aliquoted. This aliquot was respun at 16100 × *g* to pellet the bacteria. The bacterial pellet was

resuspended in 200 μL of SDS solubilization buffer (2% SDS, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris pH 6.8, bromophenol blue) and boiled for 10 minutes. To each sample, 5 μL of 20 mg/mL Proteinase K was added and incubated for one hour at 60 °C. Samples were run on a SDS-PAGE gel as described above.

After SDS-PAGE separation, the gel was placed into a BioRad transfer apparatus and transfer occurred for one hour at 180 mA. A PVDF membrane (Biorad) pre-wetted in methanol was used for transfer. Transfer buffer consisted of 192 mM glycine, 25 mM Tris, 20% methanol, and 0.01% SDS. Membranes were blocked overnight with 2.5% skim milk at 4°C. Membranes were incubated with the primary antibody (IgG anti-*Campylobacter*, Santa Cruz, California) (1:100 and 1:200 in TBS; 1:1000 anti-lipid A antibody – kindly provided by Dr. J. S. Lam, University of Guelph) for one hour at room temperature. Membranes were then washed two times for 10 minutes in Tris buffered saline (TBS) plus 0.05% (v/v) Tween 20 and 0.2% (v/v) Triton X-100 and then once in TBS for 10 minutes. The secondary antibody for the anti-*Campylobacter* antibody and for the anti-lipid A antibody was the goat anti-mouse 800 nm (full) antibody (Licor). The membranes were incubated for 30 minutes with the secondary antibody (1:2500 in TBS), followed by three washes with TBS plus 0.05% (v/v) Tween 20 and 0.2% (v/v) Triton X-100. Carbohydrates were visualized using the Licor scanning system.

2.18 Silver staining

The silver staining protocol of Fomsgaard *et al* was followed (42). Briefly, carbohydrates were first separated on a 10% SDS-PAGE and then oxidized in a solution of 0.7% periodic acid, 40% ethanol and 5% acetic acid in reverse osmosis water with shaking for 20 minutes. The oxidation was followed by five washes over 15 minutes in

reverse osmosis water. The gel was then stained with silver nitrate in a staining solution with the following final concentrations: 0.19% (v/v) 10 N NaOH, 1.3% (v/v) ammonium hydroxide, 0.7% (w/v) silver nitrate. Gels were stained for 10 minutes, followed by five washes over 15 minutes in reverse osmosis water. Following the washes, the gels were developed using 0.005% (w/v) citric acid and 0.05% (v/v) formaldehyde (37%) in reverse osmosis water until bands became visible. The gels were then washed several times with reverse osmosis water and scanned.

2.19 Purification of *C. jejuni* CPS

For the purification of CPS from *C. jejuni* ATCC 700819 wild type and *cj1427*::CAT, *cj1428*::CAT, *cj1430*::CAT, *KpsM* and *cj1427*::CATΔ mutants, strains were grown on TSA plates as aforementioned for 24 hours and harvested in saline. To obtain sufficient capsular material for downstream processing, approximately 100 TSA plates were first grown, and then inoculated into 10 L of Brucella broth (BBL Sciences, Canada), containing 7.5% horse serum and 25 mM sodium pyruvate to an OD_{600} of approximately 0.05. After 24 hours of growth (OD_{600} approximately 0.3), the bacteria were spun down at 4200 × *g* (Avanti J-25I, Beckman-Coulter) for 30 minutes. The pellets from 10 L of growth were combined, and then lyophilized to obtain a dry sample. Purification of CPS was performed using the hot water/phenol extraction method (171). Briefly, a dry cell pellet of approximately two grams was re-suspended in 20 mL of MilliQ water pre-heated to 68°C. An equal amount of liquified phenol (Fisher) preheated to 68°C was added to the pellet and sealed in a 50 mL Falcon tube. The samples were incubated in a 68°C water bath for 10 minutes with rapid stirring. The sample was allowed to cool to 10°C on ice and centrifuged for 30 minutes at 6300 × *g* (Eppendorf

5810R, Eppendorf), and 10°C. The aqueous (top) phase was collected, and an equal amount of water was added to the remaining organic phase. The procedure was repeated a total of three times and the aqueous phases were pooled. The aqueous phases were dialyzed (molecular weight cut off 12-14000 Da, Spectra/Por, Spectrum Labs) against running water for 2-3 days until no phenol remained. The sample was lyophilized and re-suspended in double distilled water. Ultracentrifugation of the sample for 30 hours at 4°C and 110000 × *g* (Optima Max-XP Ultracentrifuge, Beckman-Coulter) pelleted most of the LOS, while CPS remained in the supernatant. The sample was lyophilized and re-suspended in 500 µL double distilled water. Treatment with 200 mg of Proteinase K (Biobasic, Markham, Canada) was carried out for two hours at 60°C to degrade any remaining proteins that may not have been removed during the hot water/phenol procedure. CPS samples were stored at -20°C until further required.

2.19.1 Via size exclusion chromatography

Size exclusion chromatography was used in an attempt to separate the smaller LOS molecules away from larger CPS molecules. Hot water/phenol purified samples were run with water as the eluent through a size exclusion column measuring 87.2 cm by 1.6 cm (length x diameter) containing Bio-Gel P6 resin (BioRad – 1000 - 6000 Da fractionation range). Other resins used included the G25 (Sigma Aldrich – 5500 Da exclusion) and the G50 (Sigma Aldrich – 10000 Da exclusion) matrices. Dextran Blue was run to determine the void volume. Fractions were collected for one full column volume, in 1 mL increments. Silver staining was used to verify the presence or absence of LOS in the samples.

2.19.2 Via acetic acid treatment

The method used by Aspinall *et al* (10) was used in an attempt to partially hydrolyze the linkage between Kdo and the lipid A core. Briefly, to a sample of CPS, acetic acid was added to a final concentration of 1% and heated at 100°C for one hour. Any remaining acid was neutralized with 2 M ammonium bicarbonate. Ammonium bicarbonate was added until the solution reached a neutral pH of 7.0. Precipitated lipid A was removed via centrifugation at 5000 × *g* for 5 minutes (Eppendorf 5415D, Eppendorf) and the supernatant lyophilized and resuspended into 500 µL double distilled water. Samples were stored at -20°C until further required.

2.19.3 Via ammonium acetate treatment

Similar to the method used by Aspinall *et al* (10), ammonium acetate is a method that will partially hydrolyze the linkage between Kdo and the lipid A core. However, this method is less harsh than acetic acid, and is less likely to degrade CPS. CPS was treated with 50 mM ammonium acetate at pH 4.5 for 90 minutes at 100°C. Samples were then neutralized with 2 M ammonium bicarbonate. Ammonium bicarbonate was added until the solution reached a neutral pH of 7.0. Precipitated lipid A was removed via centrifugation at 5000 × *g* for 5 minutes (Eppendorf 5415D, Eppendorf) and the supernatant lyophilized and resuspended into 500 µL double distilled water. Samples were stored at -20°C until further required.

2.19.4 Via sodium deoxycholate

The method outlined by Gu and Tsai (1990) was adapted to attempt to purify LOS away from CPS (53). Briefly, raw CPS was processed as above, and the ultracentrifuged

pure samples were resuspended into 500 µL of water. To this, sodium EDTA was added to a concentration of 2 mM, and the pH adjusted to 8.5 with 1 M NaOH. Sodium deoxycholate was added to the mixture to a final concentration of 2% (w/v) and incubated at 37°C for 10 minutes. This was then run on a column containing Sephadex G50 beads using 20 mM Tris, 2 mM EDTA, and 1% sodium deoxycholate (w/v), pH 8.5 as the eluent.

2.20 Nuclear magnetic resonance spectroscopy

Nuclear magnetic resonance was conducted as previously mentioned (69, 94).

2.21 Assessing the susceptibility of *C. jejuni* wild type and mutants to various compounds

2.21.1 Bile salts

C. jejuni cells were washed once in saline and re-suspended to an OD_{600} of 0.1. Bile salts (50%/50% w/v cholate and deoxycholate – Sigma-Aldrich) were diluted to the appropriate concentrations (0, 0.25, 0.5, 0.75. 1.0, and 2.0 g/L) and 90 µL aliquoted into the wells of a 96 well plate. 10 µL of bacteria was added to each well and incubated in microaerophilic conditions at 37°C for 15 minutes. Samples were washed once in TSB media and serially diluted for colony forming unit (CFU) counts. For this, 10 µL of each dilution, over a range of dilutions was taken and spotted onto a square culture plate containing TSA and background antibiotics. Each spot was done in duplicate. The plates were incubated in microaerophilic conditions at 37°C for 36 hours and the CFU counts enumerated. Experimental results were normalized to the CFU counts of bacterial samples that did not receive any bile salt treatment.

2.21.2 SDS

Different concentrations (0.002%, 0.003%, 0.004%, 0.005%, 0.0075%, 0.01%, 0.015%, 0.02% and 0.03%) of SDS were prepared in TSB (+ vancomycin and trimethoprim), using 1% (w/v) SDS as a stock solution. 170 µL of each concentration of SDS was added to an appropriate number of wells of a 96 well plate. *C. jejuni* wild type and mutants were grown as previously described and re-suspended in TSB to an OD_{600} 1.0. To each well, 30 µL of bacterial suspension was added, and controls wells were set up which contained 200 µL TSB. The plates were incubated at 37°C with shaking at 180 rpm in microaerophilic conditions for 15 hours, at which point the OD_{600} was read. Experimental results were normalized to bacteria which did not receive any SDS treatment.

2.21.3 Serum

Fresh rabbit blood (from two rabbits; 6 mL each) was obtained the morning of the experiment from Animal Care and Veterinary Services at the University of Western Ontario. The blood was incubated at room temperature for 1-2 hours, until the blood had visibly clotted. The serum was separated from the clot and centrifuged for 15 minutes at 9300 × *g* at 4°C (Eppendorf 5870R, Eppendorf) to remove remaining blood cells. Half of the serum was inactivated by incubation at 60°C for one hour. Bacteria grown for 24 hours on TSA were washed in saline and re-suspended to an OD_{600} of 0.1. Serum (inactivated or not) was diluted in saline to the appropriate concentrations (0% - 100%) and 90 µL was aliquoted into the wells of a 96 well plate. To the wells, 10 µL of bacteria was added. The plates were then incubated with agitation (100 rpm) for 1.5 hours in microaerophilic conditions at 37°C. Following incubation, 100 µL TSB was added to the

wells and samples were serially diluted and plated for CFU counts. Enumeration of the CFU was done after incubation for 36 hours in microaerophilic conditions.

For time course experiments, bacterial samples were tested at 20% pooled rabbit serum for 1.5 hours.

2.22 Assessing the adhesion and invasion of *C. jejuni* in Caco-2 cells

Caco-2 colorectal epithelial cells (kindly given by D. McKay, University of Calgary) were routinely grown on 75 cm^2 tissue culture flasks in DMEM medium containing high glucose (25 mM) and supplemented with 10% fetal bovine serum (FBS, Gibco), 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 µg/mL streptomycin. Caco-2 cells were grown in a CO_2 incubator containing 5% CO_2 and 37°C until confluent, and passaged every 4-5 days. Passage of Caco-2 cells was carried out by releasing the cells from the flask surface via the addition of 0.25% Trypsin/EDTA (Gibco), followed by incubation in the CO_2 incubator at 37°C for 2-3 minutes. Once released, the trypsin/EDTA was neutralized via the addition of fresh DMEM. Cells were then centrifuged at 200 × *g* (Eppendorf 5702, Eppendorf) for 5 minutes to remove any excess trypsin.

Caco-2 cells were grown for three days until they formed a confluent monolayer (approximately 6.5 x 10^5 cells per well in 24-well plates). The cells were infected for 5 hours with wild type or mutant *C. jejuni*. Approximately 6.5 x 10^7 CFU of *C. jejuni* were added, resulting in a multiplicity of infection of 100. The plates were spun briefly (300 × *g* (Eppendorf 5870R, Eppendorf) for 5 minutes at room temperature) to maximize contact between the bacteria and the cell monolayer. To determine total bacterial cell association (adherent and internalized bacteria), Caco-2 cell monolayers were washed three times,

lysed with 0.1 % Triton X-100 for 10 minutes and viable bacterial counts were determined by plating the serial dilutions.

To determine the number of internalized bacteria, the Caco-2 cell monolayers were treated with 200 μg/mL gentamicin for two hours to kill extracellular bacteria. The cells were then washed and treated as above to determine bacterial viable counts.

2.23 Assessing the interaction of *C. jejuni* with RAW 264.7 macrophages

RAW 264.7 macrophages (ATCC) were grown in 75 cm^2 tissue culture flasks (BD Falcon) in DMEM (Invitrogen) containing high glucose (25 mM), 10% FBS, 0.1 mM non-essential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin in a CO_2 incubator containing 5% CO_2 and at 37°C. They were grown from freezer stocks until 80% confluent and then passaged every 3-4 days to a maximum of 5 passages. Passaging involved washing the macrophages in 1x PBS (Wisent) and detaching them by the addition of 5 mL 0.25% Trypsin/EDTA (Gibco) and incubation in a CO_2 incubator for 2-3 minutes. The cells were detached by allowing 15 mL of fresh DMEM to run over them. Cells were then centrifuged at 200 × *g* (Eppendorf 5702, Eppendorf) for 5 minutes to remove any excess trypsin. To seed new cells, 1 mL of cells was taken and added to 19 mL of fresh DMEM medium in a 75 cm^2 flask and incubated in a CO_2 incubator until needed.

Freezer stocks of the macrophages were made with 45% cells, 45% FBS and 10% dimethyl sulfoxide (DMSO, Sigma-Aldrich). Cells were aliquoted into 2 mL cryotubes and stored at -80°C until required.

For experiments, cells were first counted using a hemocytometer. For this, 10 μL of the cell suspension was loaded onto a hemocytometer and the number of cells was counted in five independent squares. The average number of cells was used.

Cells were then seeded at approximately 2.1×10^5 cells per well of a 24 well plate and incubated overnight in DMEM without antibiotics. *C. jejuni* wild type or mutants were added at a multiplicity of infection (MOI) of 100. The plates were centrifuged for two minutes at 300 × *g* (Eppendorf 5870R, Eppendorf) to synchronize bacteria-macrophage interaction.

For adhesion experiments, the plates were incubated with bacteria at 4°C for 30 minutes, washed with cold PBS five times and the cells were lysed in sterile double distilled water. The bacteria were then serially diluted and plated for CFU counts.

For intracellular survival experiments, cells were seeded as above and infected with bacteria at an MOI of 100 for two hours in a 5% CO_2 incubator. The cells were washed three times using PBS and incubated with fresh DMEM containing 225μg/mL gentamicin for one hour to kill extracellular bacteria. Macrophages were then washed and incubated in fresh DMEM for the times indicated. At each time point, the macrophages were washed three times with PBS and lysed using sterile double distilled water. Samples were serially diluted and plated for CFU counts.

For infection time course experiments, macrophages were seeded as above and infected with bacteria at an MOI of 100 for the times indicated. The macrophages were washed three times in PBS and incubated in fresh DMEM containing 225 μg/mL gentamicin for one hour, followed by lysis as described above and surviving bacteria were enumerated by CFU counts.

2.24 Autoagglutination assay

The agglutination assay was performed as previously described (115). Briefly, 2 mL of sterile PBS was inoculated with *C. jejuni* wild type or mutants at OD_{600} 1.0. At time points 0 hour, 1 hour, and 2 hours, the top 1 mL from each tube was carefully removed and the OD_{600} was read. The value obtained represents bacteria that have not agglutinated, as agglutination leads to the settling of bacterial clumps to the bottom of the tube.

2.25 Statistical analysis

Statistical analysis was carried out using the one-way ANOVA statistical test followed by a Dunnett's multiple comparison test. Results were considered significant if the p value was less than 0.05.

CHAPTER 3 – RESULTS

3.1 THE GENERATION AND GENETIC CHARACTERIZATION OF CPS MUTANTS

At the start of this project, the availability of structural data for *C. jejuni* capsular polysaccharide (CPS) was scarce. It was known that some *C. jejuni* strains possessed modified heptoses, and that these modified heptoses formed a major structural component of the CPS (9, 10). Generation of a random *C. jejuni* ATCC 700819 DNA library and subsequent sequencing revealed protein encoding genes similar to the CPS biosynthetic genes of other bacteria (84). These findings were confirmed when the *C. jejuni* ATCC 700819 complete genome was sequenced, revealing a gene cluster that was similar to the group II and group III CPS biosynthesis genes found in *E. coli*. Inactivation of these genes did not result in the loss of low-molecular weight lipooligosaccharide (LOS) molecules, but instead, the loss of a then unknown high-molecular weight species (86). The combination of these findings led to the realization that these high-molecular weight molecules were indeed CPS, and not LPS, as was originally thought. However, the exact genes that were involved in modifying heptoses remain unknown, even to the present.

3.1.1 The principle of *C. jejuni* mutant generation

Previous work in the Creuzenet laboratory (100) provided *C. jejuni* mutants containing disruptions in *cj1427c*, *cj1428c*, *cj1430c* and *KpsM*. These genes were chosen to be disrupted as they are hypothesized to be involved with either modified heptose synthesis (*cj1427c*, *cj1428c*, and *cj1430c*) or the export of the final capsular polysaccharide (*KpsM*). Briefly, these genes were first inserted into a pET23::MTF vector, while the *KpsM* gene was inserted into a pBluescript KS (+) vector. Subsequent inverse PCR using primers that were internal and divergent to the genes of interest

yielded open pET23::*cj1427*, pET23::*cj1428*, pET23::*cj1430* and pBlueKS::*KpsM*. Into these, either the *C. coli* chloramphenicol resistance gene derived from pRY111 (177) or the kanamycin resistance gene *aph3* from *H. pylori* shuttle vector pHel3 (66) was inserted to generate the disruption construct.

3.1.2 Further analysis of the *cj1427*::CAT mutant reveals multiple deletions

At the outset of this project, real-time PCR data suggested that the previously generated *cj1427*::CAT (hereafter referred to as *cj1427*::CATΔ) mutant possessed additional gene deletions. It was found that the expression levels of the *cj1426c* and *cj1425c* genes in this mutant were zero, which hinted at their potential deletion. To investigate this, conventional PCR was done to determine the presence or absence of genes downstream of the inserted antibiotic resistance cassette. Using primers that annealed to *cj1427c* and *cj1418c*, a large size discrepancy was observed between the wild type and the *cj1427*::CATΔ mutant, indicating that a deletion was present (**Figure 3A**). Following this, primers annealing to *cj1426c* and *cj1425c* were used and it was observed that the *cj1427*::CATΔ mutant did not yield a band of the expected size, indicating that one of these two genes was deleted (**Figure 3B**). Further PCR using primers that annealed to *cj1426c* and *cj1427c* as well as *cj1427c* and *cj1425c* also failed to generate bands of the expected size, confirming that both *cj1426c* and *cj1425c* are deleted in the *cj1427*::CATΔ mutant (**Figure 3C**). A final PCR using primers annealing to *cj1421c* and *cj1418c* also failed to generate a band of the expected size for the *cj1427*::CATΔ mutant, indicating that the genes from *cj1426c* to *cj1421c* are deleted (**Figure 3D**). In all reactions, a *kpsM* gene control was also conducted in parallel to rule out problems with

the chromosomal DNA preparation (i.e. conditions that may lead to inhibitions in the PCR reaction).

To determine the precise location of this deletion, and also to map the junction at which the *C. jejuni* genome was disrupted, PCR samples of the *cj1427*::CATΔ mutant were sequenced (Robarts Research Institute, London, Ontario). It was found that the area between *cj1421c* and *cj1427c* was deleted; specifically, the first 998 bp of the *cj1421c* gene were deleted, leaving only the last 840 bp. The sequence then proceeds directly into the *cj1427c* gene. This deletion of approximately 6.1 Kb agrees with the results of conventional PCR.

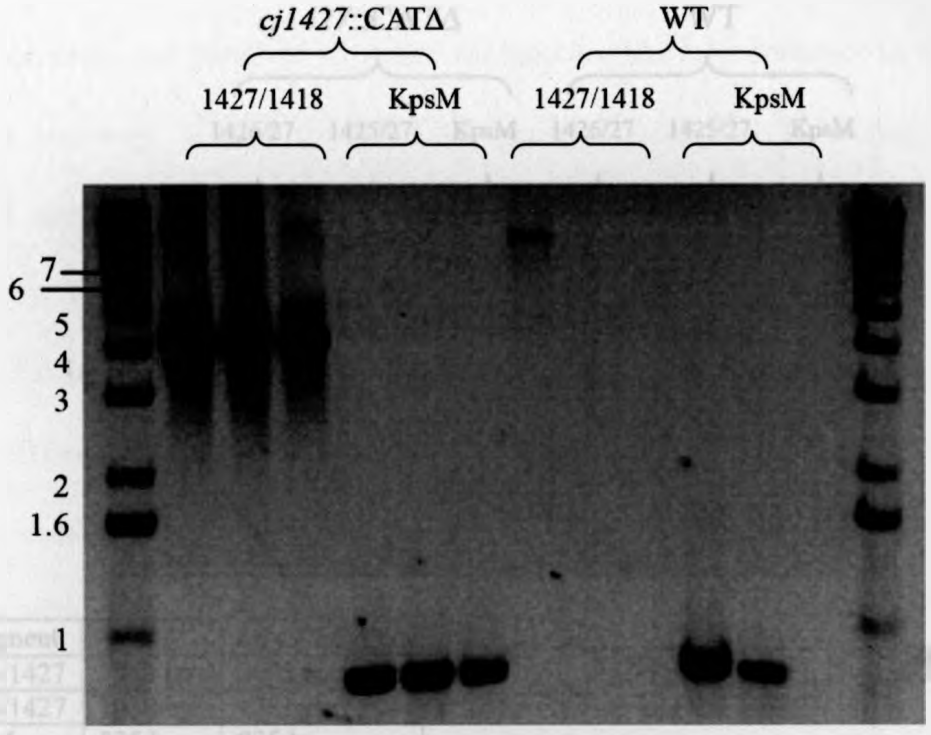
The deletion of these genes will have several profound effects on *C. jejuni*. Firstly, *cj1426c* is a putative methyltransferase involved in the methylation of carbon six on the heptose; its deletion would result in the lack of this modification. However, more importantly is the fact that *cj1425c – cj1423c* are involved in the synthesis of the heptose precursor (GDP-*glycero-manno*-heptose). The deletion of these genes means that the mutant will lack a heptose of any kind, and thus, the deletion of *cj1426c* is redundant, as there is no heptose for the methyltransferase on which to act. Past the genes involved in the synthesis of the heptose precursor are genes *cj1422c* and *cj1421c*. Deletions in these genes will have additional effects on the capsule. Based on results found by McNally *et al* (114), *cj1422c* and *cj1421c* are responsible for transferring the phosphoramidate group to C-4 of D-*glycero*-α-L-*gluco*-heptopyranose and C-3 of the β-D-Gal*f*Nac residue, respectively. In *C. jejuni* strain ATCC 700819, the phosphoramidate group was found only to be attached to the β-D-Gal*f*Nac residue and not the heptose. Since the gene responsible for the transfer of the phosphoramidate moiety is non-functional, not only is

this mutant heptoseless, but it also lacks the capsular attached phosphoramidate. The downstream phosphoramidate synthesis genes (*cj1415c - cj1418c*) are not disrupted, and synthesis of phosphoramidate still occurs. The final result of these deletions is a mutant with a straight-chain capsule; one that is free of both heptose and phosphoramidate side-branches. While this mutant was not the one that was initially desired, it is still of use in our laboratory as it can be used to study if the absence of side-branches in the capsular structure result in changes in *C. jejuni* virulence. This mutant was thus assigned the name *cj1427*::CATΔ, with the Δ to indicate that a large portion of the genes in the capsular cluster had been deleted.

Figure 3. Characterization of the *cj1427*::CATΔ mutant.

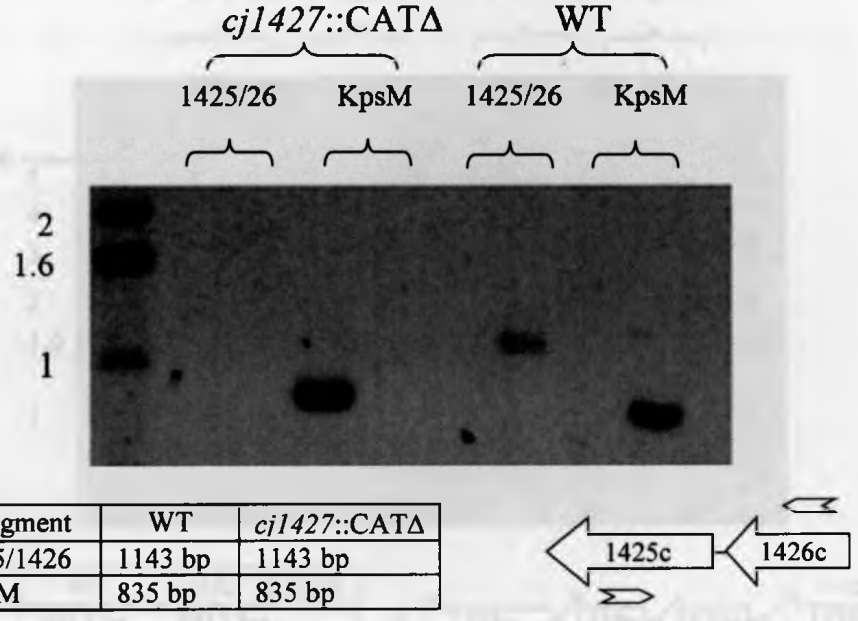
In all panels, a schematic shows the region of the *C. jejuni* genome that was amplified; small arrows above and below the genome represent the primers that were used. The box within the *cj1427c* gene represents the chloramphenicol resistance cassette that was inserted. An inset table shows the sizes of the expected PCR products for both the wild type and the *cj1427*::CATΔ mutant strain. Samples were loaded in multiple replicates with each representing a dilution of template DNA used for PCR. The *kpsM* gene was amplified as a positive control for the chromosomal DNA preparation. The expected size of this band is 835 bp in both wild type and the *cj1427*::CATΔ mutant. **A) A deletion is present in the *cj1427*::CATΔ mutant.** A significant size difference is observed between the *cj1427*::CATΔ strain and the wild type strain in the capsular cluster of genes between *cj1427c* and *cj1418c*. Parts of the *C. jejuni* genome are not shown, and are represented by a double slash (//). **B) One of *cj1425c* or *cj1426c* is not present in the *cj1427*::CATΔ mutant.** No fragment of the expected size is observed for the *cj1427*::CATΔ mutant. However, a fragment of the expected size is observed in the wild type strain. **C) The genes *cj1426c* and *cj1425c* are not present in the *cj1427*::CATΔ mutant.** No fragments of the expected sizes are observed in the *cj1427*::CATΔ mutant, while bands of the expected size are observed in the wild type strain indicating that *cj1426c* and *cj1425c* are deleted in the *cj1427*::CATΔ mutant. **D) The gene *cj1421c* is not present in the *cj1427*::CATΔ mutant.** A fragment of the expected size was not observed for the *cj1427*::CATΔ mutant, while the same band was observed in the wild type strain, indicating that a large fragment of the *C. jejuni* genome, up to and including *cj1421c*, is not present.

A)



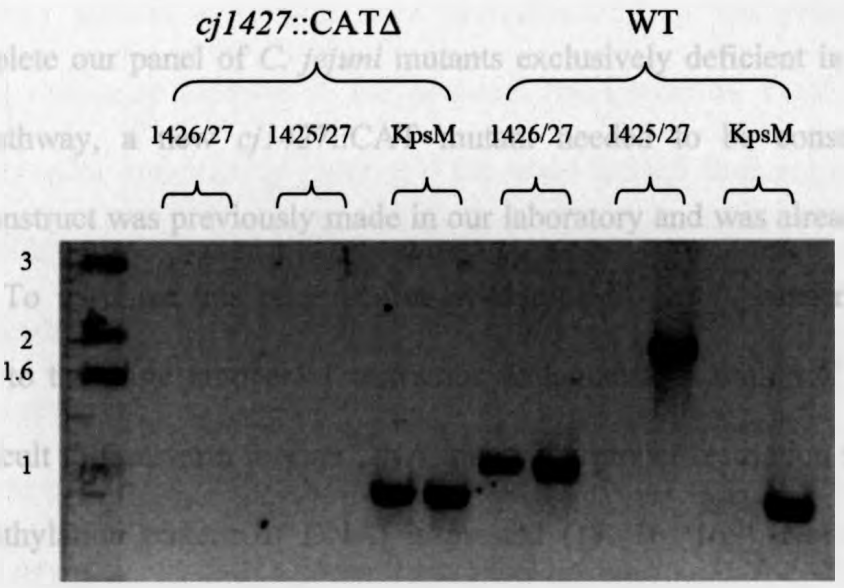
Fragment	WT	*cj1427*::CATΔ
1427/1418	9831 bp	10 574 bp
KpsM	835 bp	835 bp

B)

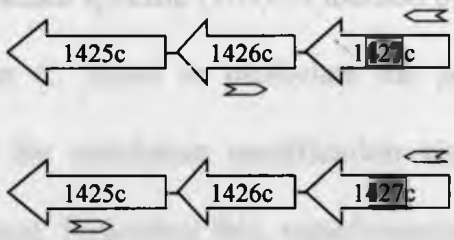


Fragment	WT	*cj1427*::CATΔ
1425/1426	1143 bp	1143 bp
KpsM	835 bp	835 bp

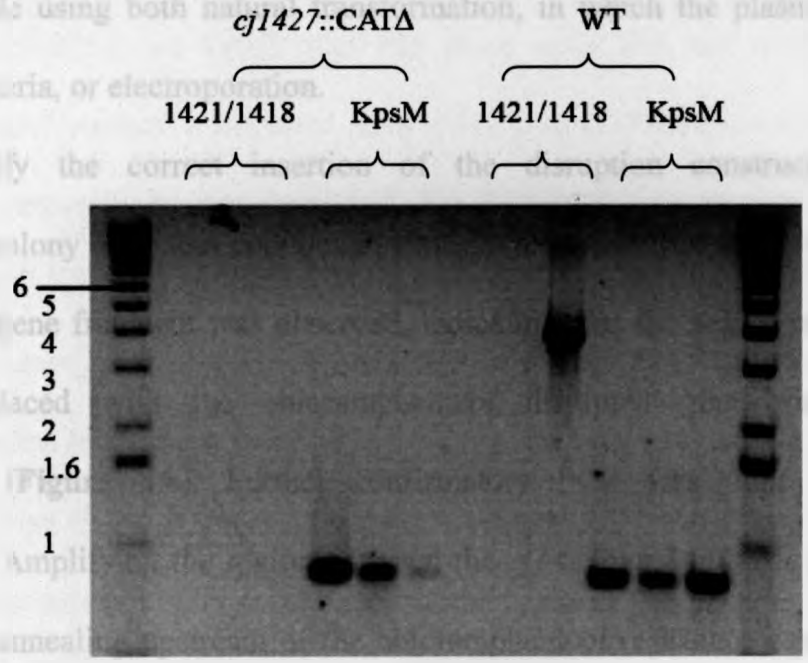
C)



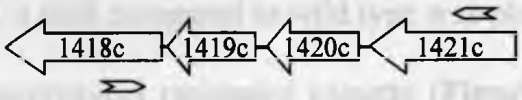
Fragment	WT	*cj1427*::CATΔ
1425/1427	1950 bp	2693 bp
1426/1427	1038 bp	1781 bp
KpsM	835 bp	835 bp



D)



Fragment	WT	*cj1427*::CATΔ
1421/1418	3913 bp	3913 bp
KpsM	835 bp	835 bp



3.1.3 Creation and verification of a new *C. jejuni cj1427*::CAT mutant

To complete our panel of *C. jejuni* mutants exclusively deficient in the heptose modification pathway, a new *cj1427*::CAT mutant needed to be constructed. The *cj1427*::CAT construct was previously made in our laboratory and was already on a pET plasmid (100). To mobilize this plasmid into wild type *C. jejuni*, barriers had to be overcome. Due to the large number of restriction endonucleases within *C. jejuni*, it is often quite difficult to transform foreign DNA unless the proper restriction modification pattern (i.e. methylation pattern of DNA) is present (19, 36, 169). Not only is this methylation pattern species specific, but it is also strain specific (169). A method outlined by Donahue *et al* (36) was adapted for use in *C. jejuni* to methylate the plasmid containing the disrupted *cj1427c* gene so that the restriction modification signature unique to *C. jejuni* ATCC 700819 would be present. Following this, transformation was easily achievable using both natural transformation, in which the plasmid was simply mixed with bacteria, or electroporation.

To verify the correct insertion of the disruption construct in potential transformants, colony PCR was conducted. Using primers specific for *cj1427c*, a shift in the size of the gene fragment was observed, indicating that the wild type *cj1427c* gene had been replaced with the chloramphenicol disrupted gene via homologous recombination (**Figure 4A**). Further confirmatory PCR was done to verify the recombination. Amplifying the region between the *cj1427c* and *cj1426c* genes (primers 1427 RT Top, annealing upstream of the chloramphenicol resistance cassette and 1426 RT Btm; see appendix for primer sequences), a shift compared to wild type was observed, corresponding to the presence of the chloramphenicol resistance cassette (**Figure 4B**).

Similarly, amplifying the area between *cj1427c* and *cj1425c* (1427 RT Top and 1425 RT Btm, respectively) yielded a shift in size corresponding to the presence of the chloramphenicol resistance cassette in the potential transformants. Finally, when the region between *cj1426c* and *cj1425c* (1426 RT Top and 1425 RT Btm, respectively) was amplified, no difference was observed for the fragment when comparing the potential *cj1427*::CAT transformant to wild type, as the amplified region does not contain the chloramphenicol resistance cassette.

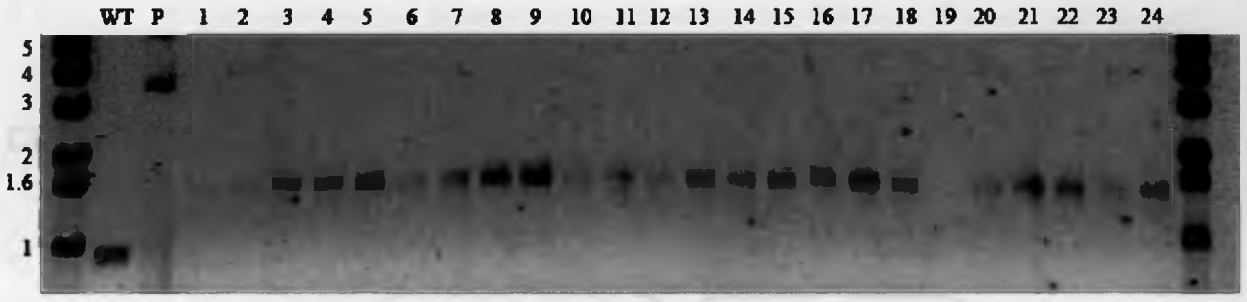
In order to ensure that a double homologous recombination event occurred while constructing the new *cj1427*::CAT mutant, a fragment from *cj1428c* to *cj1426c* (primers 1428 RT Top and 1426 RT Btm, respectively) was amplified (**Figure 4C**). An increase in size of the amplified fragment from the *cj1427*::CAT mutant corresponds to the insertion of the chloramphenicol resistance cassette.

As shown previously, the *cj1427*::CATΔ mutant possesses a large deletion, up to and including *cj1421c*. To verify that the same error did not occur in the new *cj1427*::CAT transformants, a fragment from *cj1425c* to *cj1418c* (primers 1425 RT Top and Cj1418P1, respectively) was amplified. No difference was observed between wild type and *cj1427*::CAT, indicating that no gene deletion event occurred in the *cj1427*::CAT mutant (**Figure 4D**). DNA sequencing was also conducted to verify the correct construction of the new *cj1427*::CAT mutant. Sequencing at the junction where a large deletion of the *C. jejuni* genome occurred in the *cj1427*::CATΔ mutant revealed that there was no such deletion in the newly constructed *cj1427*::CAT mutant.

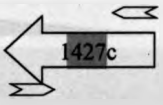
Figure 4. Characterization of the new *cj1427*::CAT mutant via PCR.

In all panels, a schematic shows the region of the *C. jejuni* genome that was amplified; small arrows above and below the genome represent the primers that were used. The box within the *cj1427c* gene represents the chloramphenicol resistance cassette that was inserted. An inset table shows the sizes of the expected PCR products for both the wild type and the *cj1427*::CAT mutant strain. In panels B, C and D, a representative colony of the *cj1427*::CAT transformants is shown. Similar results were observed for other potential transformants that were screened via PCR. **A) Colony PCR screen of the potential *cj1427*::CAT mutants**. Colony PCR of the clones reveals that they all possess a larger *cj1427c* gene, corresponding to the insertion of the chloramphenicol resistance cassette. Numbers at the top indicate the various transformants screened. P = pET1427 CAT::Coli plasmid was run to ensure no potential degradation of the plasmid occurred. The three bands represent the supercoiled, coiled and linear forms of the plasmid. **B) The *cj1427*::CAT mutant possesses the disrupted *cj1427c* gene and does not have any further deletions.** PCR of the regions between *cj1427c* and *cj1426c* and *cj1425*c and *cj1427c* show a size increase in the *cj1427*::CAT mutant, corresponding to the expected size increase due to the insertion of a chloramphenicol resistance cassette. The region between *cj1425c* and *cj1426c* does not show a size difference for the *cj1427*::CAT mutant compared to wild type, as expected. **C) The region upstream of *cj1427c* is not deleted in the *cj1427*::CAT mutant.** PCR of the region between *cj1428c* and *cj1426c* shows a size increase in the *cj1427*::CAT mutant corresponding to the expected size increase due to the insertion of a chloramphenicol resistance cassette. Black triangles indicate the relative amounts of DNA template used for PCR reactions **D) The region between *cj1425c* and *cj1418c* is not deleted in the *cj1427*::CAT mutant.** PCR of the region between *cj1425c* and *cj1418c* shows no difference in size compared to wild type. This indicates that no gene deletion is present in the new *cj1427*::CAT mutant. Parts of the *C. jejuni* genome are not shown, and are represented by a double slash (//). Black triangles indicate the relative amounts of DNA template used for PCR reactions.

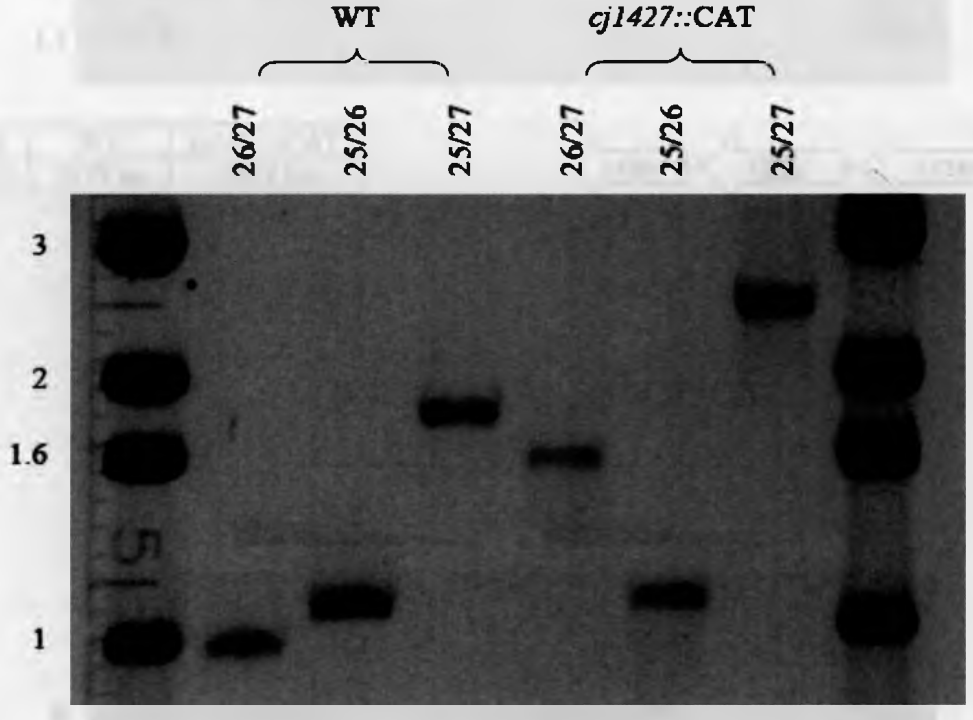
A)



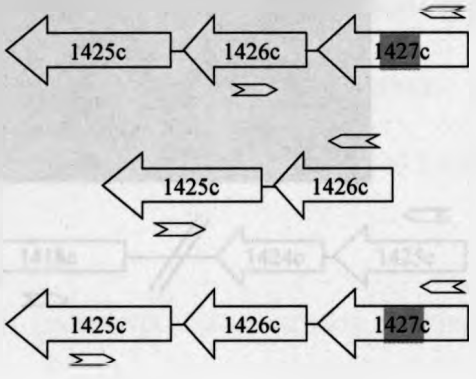
Fragment	WT	*cj1427*::CAT
cj1427c	942 bp	1605 bp



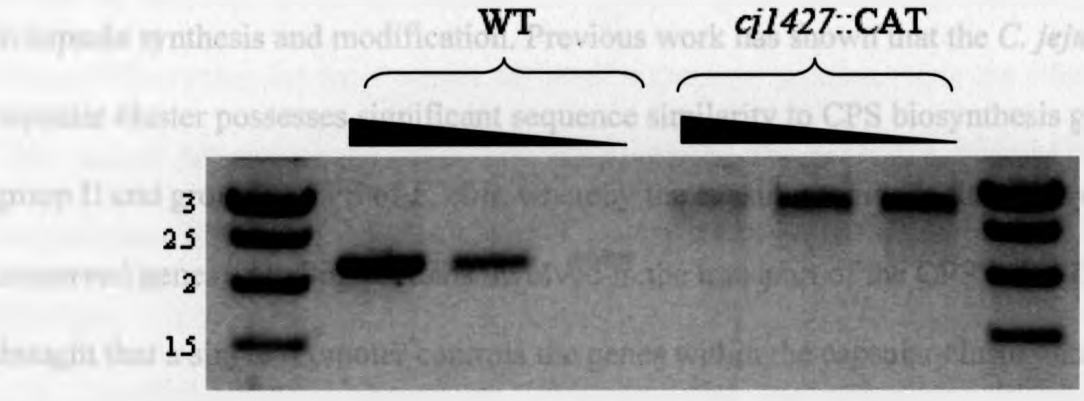
B)



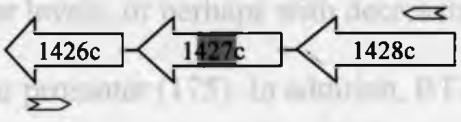
Fragment	WT	*cj1427*::CAT
1426/1427	1039 bp	1702 bp
1425/1426	1144 bp	1144 bp
1425/1427	1951 bp	2614 bp



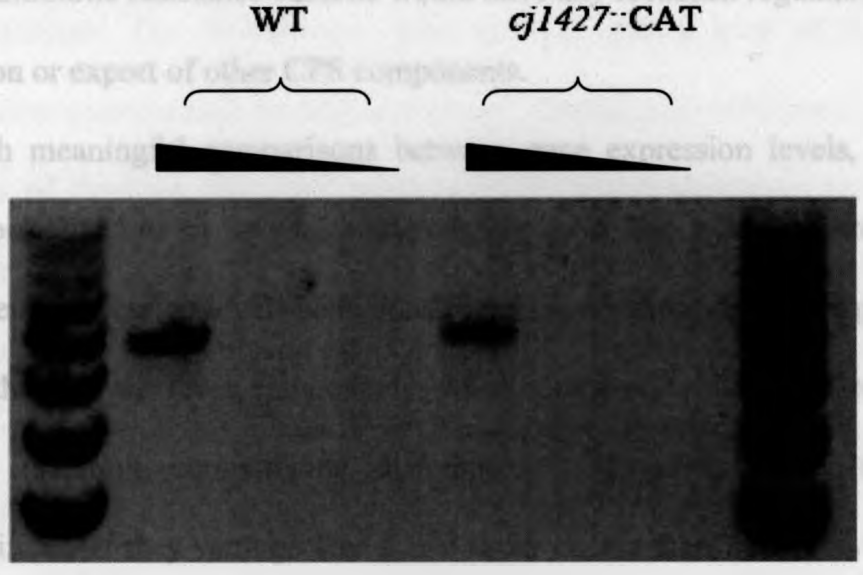
C)



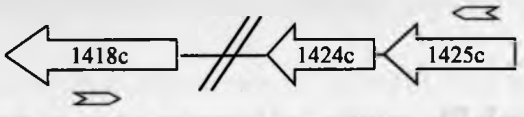
Fragment	WT	*cj1427*::CAT
1428/1426	2179 bp	2842 bp



D)



Fragment	WT	*cj1427*::CAT
1425/1418	8119 bp	8119 bp



3.1.4 Determination of gene transcription in the wild type and mutant strains via reverse transcription and quantification via real-time PCR

Real-time PCR was used to investigate the expression levels of the genes involved in capsule synthesis and modification. Previous work has shown that the *C. jejuni* capsular cluster possesses significant sequence similarity to CPS biosynthesis genes for group II and group III CPS of *E. coli*, whereby the capsular region is flanked by conserved genes encoding proteins involved in the transport of the CPS (84, 173). It is thought that a single promoter controls the genes within the capsular cluster, and as a result, expression of these genes would be at similar levels, or perhaps with decreasing levels proportional to the distance from the capsular promoter (175). In addition, RT-PCR was used to test whether the inactivation of the heptose modification pathway via the insertion of an antibiotic resistance cassette would have any feedback regulatory effects on the production or export of other CPS components.

To reach meaningful comparisons between gene expression levels, each gene must first be normalized to an internal housekeeping gene. For intrastrain comparisons, differences in gene expression levels were considered significant if they were greater than 5 times or less than 1/5 the level, respectively, when compared to the housekeeping gene (*cj1537c*). For interstrain comparisons, differences in gene expression levels were considered significant if they were greater than 5 times or less than 1/5 the level found in the wild type strain, respectively.

Throughout the calculation of differences in gene expression, primer efficiencies must also be taken into consideration. Primer efficiency is defined as the ability of a primer pair to amplify a given template under specific conditions. Primer pairs may differ

in their ability to amplify their respective products. As a consequence, if primer efficiency was not taken into account, then apparent alterations in gene expression could have been due to differences in cDNA amplification and not to differences in the cDNA levels themselves. When calculating the relative gene expression for each gene, the primer efficiencies for each primer set were taken into account using the Pfaffl method. This method determines the expression ratio between two genes (i.e. gene of interest and the reference (housekeeping) gene) in which the efficiencies of the two primer pairs are different.

Several large differences were observed when comparing the expression of each gene relative to the housekeeping gene within each strain (**Figure 5A**). Low levels of transcripts were detected for *kpsM* and the gene immediately downstream, *cj1447c*. Throughout our studies, the expression levels of *kpsM* were extremely low, making it difficult to quantitate. The downstream gene *cj1445c* had a level of transcription comparable to other genes within the capsular cluster. Similarly, *cj1430c* and *cj1429c* had very low levels of detected transcript, while *cj1428c* and downstream genes showed levels of transcription comparable to the other genes within the capsular cluster. This pattern holds true for all the strains for which RT-PCR data were gathered.

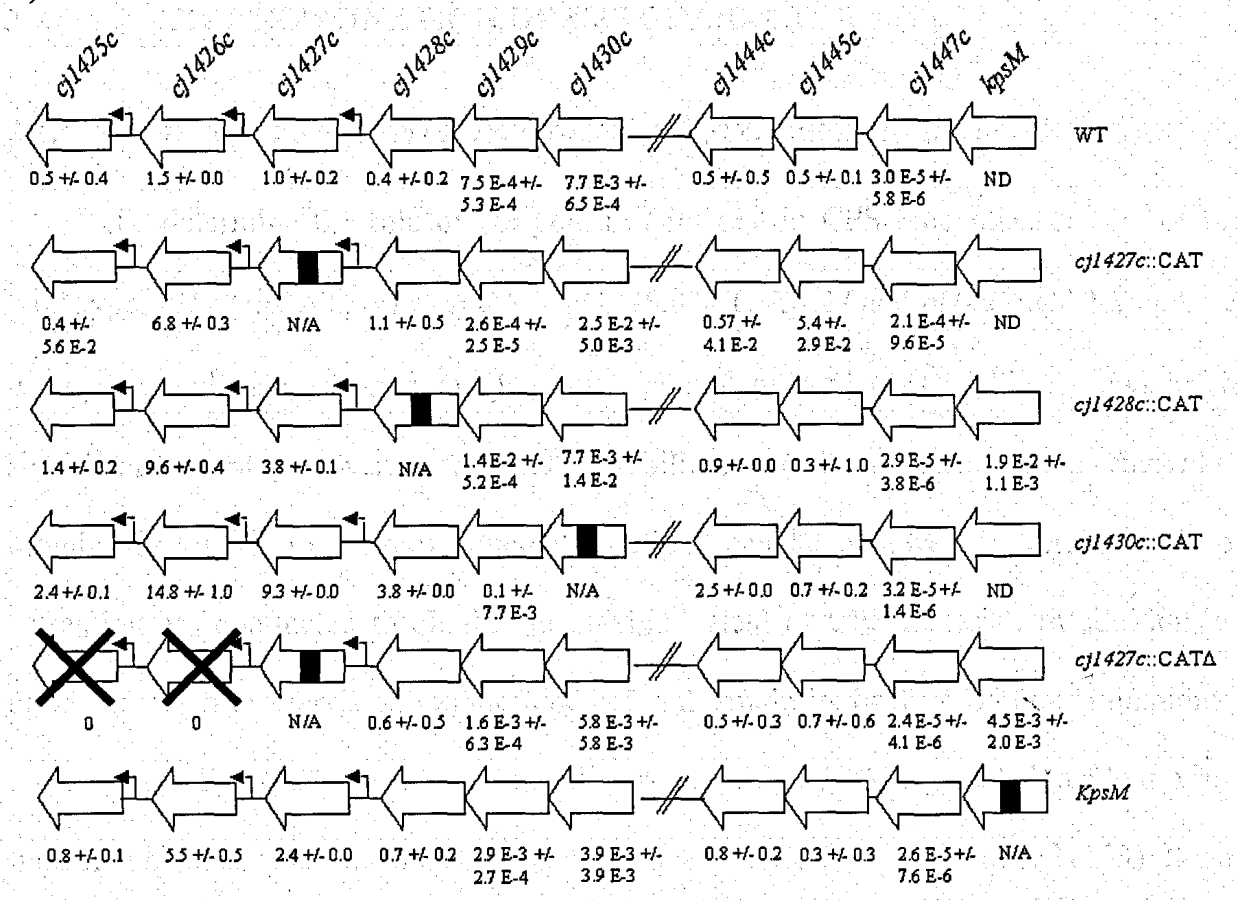
Another objective of the RT-PCR study was to ensure that the genes that were downstream of the inserted antibiotic resistance cassettes were non-polar. To do this, the data from **Figure 5A** (levels of gene expression relative to an internal housekeeping gene) in each strain were taken as a ratio over the respective genes in the wild type strain (**Figure 5B,** interstrain comparison).

The genes that were directly downstream of the chloramphenicol resistance cassette in *cj1427*::CAT and *cj1428*::CAT showed similar levels of transcription compared to wild type, indicating that the insertion of the antibiotic resistance cassette was non-polar (**Figure 5B**). In the *cj1430*::CAT mutant, *cj1429c* is directly downstream of the chloramphenicol resistance cassette. This gene showed significant upregulation, but this upregulation is not due to polarity effects of the chloramphenicol resistance cassette, as previously demonstrated in the *cj1427*::CAT and *cj1428*::CAT strains. More likely, the upregulation in *cj1429c* is due to a feedback regulatory effect of inactivating *cj1430c.* This upregulation in *cj1429c* was also observed in the *cj1428*::CAT mutant, indicating that the loss of either *cj1430c* or *cj1428c* likely generates feedback where, as a result, *cj1429c* expression is significantly upregulated. This same mechanism may also explain why in *cj1428*::CAT and *cj1430*::CAT, expression of the *cj1426c* gene was slightly upregulated. In *KpsM,* similar to the other mutants in our collection, the gene directly downstream of the kanamycin resistance cassette had levels of transcription comparable to the wild type, also indicating that the insertion of this cassette was non-polar.

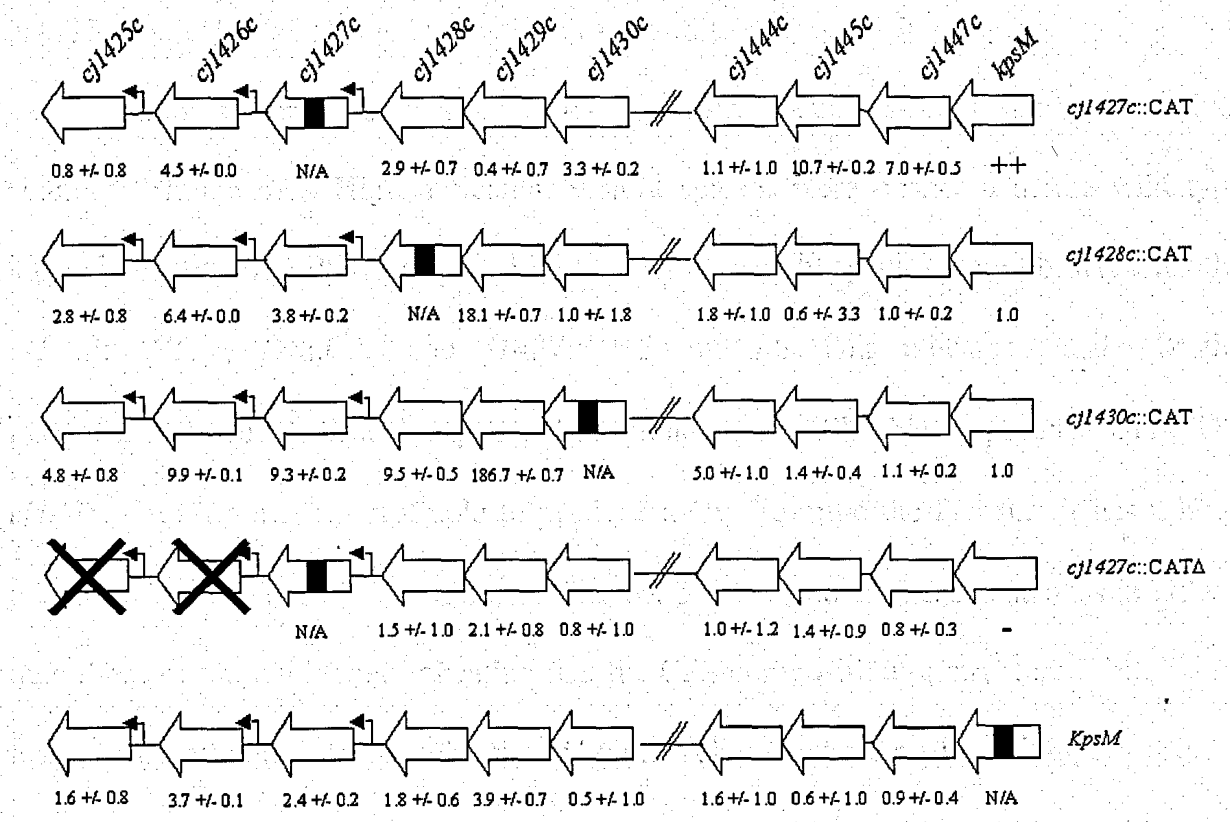
Figure 5. Real-time PCR data in wild type and mutant strains of *C. jejuni* for genes within the capsular cluster.

Due to the naming nomenclature of the genes in the capsular cluster, a *cj1446c* gene is not present. Large Xs in *cj1427*::CATΔ indicate deletions in the particular genes. Additional genes that are also deleted as previously elucidated are not shown. Diagonal lines (//) indicate genes between *cj1430c* and *cj1444c,* exclusive, were not analyzed. Bolded arrows indicate potential cryptic promoter sites. C = Chloramphenicol resistance cassette. K = Kanamycin resistance cassette. N/A = not applicable **A) Intrastrain RT-PCR data for wild type and mutant strains.** Expression levels for the genes tested are shown, relative to the internal housekeeping gene (*cj1537c*). Due to very low levels of transcript for the *kpsM* gene, absolute numbers cannot be presented. As a result, ND indicates none detected. **B) Interstrain RT-PCR data for mutant strains.** The relative gene expression levels for the genes tested are shown, taken as a ratio to the respective genes in the wild type strain. As the absolute transcript abundance for *kpsM* was not determined in **A)**, absolute numbers cannot be presented. Instead, a ++ indicates a much higher level of expression relative to wild type, a – indicates a lower level of expression relative to wild type, while a 1.0 represents a level of transcription similar to wild type levels.

A)


cj1425c
cj1426c
cj1427c
cj1428c
cj1429c
cj1430c
cj1444c
cj1445c
cj1447c
kpsM
WT
0.5 +/- 0.4
1.5 +/- 0.0
1.0 +/- 0.2
0.4 +/- 0.2
7.5 E-4 +/- 5.3 E-4
7.7 E-3 +/- 6.5 E-4
0.5 +/- 0.5
0.5 +/- 0.1
3.0 E-5 +/- 5.8 E-6
ND
cj1427c::CAT
0.4 +/- 5.6 E-2
6.8 +/- 0.3
N/A
1.1 +/- 0.5
2.6 E-4 +/- 2.5 E-5
2.5 E-2 +/- 5.0 E-3
0.57 +/- 4.1 E-2
5.4 +/- 2.9 E-2
2.1 E-4 +/- 9.6 E-5
ND
cj1428c::CAT
1.4 +/- 0.2
9.6 +/- 0.4
3.8 +/- 0.1
N/A
1.4 E-2 +/- 5.2 E-4
7.7 E-3 +/- 1.4 E-2
0.9 +/- 0.0
0.3 +/- 1.0
2.9 E-5 +/- 3.8 E-6
1.9 E-2 +/- 1.1 E-3
cj1430c::CAT
2.4 +/- 0.1
14.8 +/- 1.0
9.3 +/- 0.0
3.8 +/- 0.0
0.1 +/- 7.7 E-3
N/A
2.5 +/- 0.0
0.7 +/- 0.2
3.2 E-5 +/- 1.4 E-6
ND
cj1427c::CATΔ
0
0
N/A
0.6 +/- 0.5
1.6 E-3 +/- 6.3 E-4
5.8 E-3 +/- 5.8 E-3
0.5 +/- 0.3
0.7 +/- 0.6
2.4 E-5 +/- 4.1 E-6
4.5 E-3 +/- 2.0 E-3
KpsM
0.8 +/- 0.1
5.5 +/- 0.5
2.4 +/- 0.0
0.7 +/- 0.2
2.9 E-3 +/- 2.7 E-4
3.9 E-3 +/- 3.9 E-3
0.8 +/- 0.2
0.3 +/- 0.3
2.6 E-5 +/- 7.6 E-6
N/A

B)


cj1425c
cj1426c
cj1427c
cj1428c
cj1429c
cj1430c
cj1444c
cj1445c
cj1447c
kpsM
cj1427c::CAT
0.8 +/- 0.8
4.5 +/- 0.0
N/A
2.9 +/- 0.7
0.4 +/- 0.7
3.3 +/- 0.2
1.1 +/- 1.0
10.7 +/- 0.2
7.0 +/- 0.5
++
cj1428c::CAT
2.8 +/- 0.8
6.4 +/- 0.0
3.8 +/- 0.2
N/A
18.1 +/- 0.7
1.0 +/- 1.8
1.8 +/- 1.0
0.6 +/- 3.3
1.0 +/- 0.2
1.0
cj1430c::CAT
4.8 +/- 0.8
9.9 +/- 0.1
9.3 +/- 0.2
9.5 +/- 0.5
186.7 +/- 0.7
N/A
5.0 +/- 1.0
1.4 +/- 0.4
1.1 +/- 0.2
1.0
cj1427c::CATΔ
N/A
1.5 +/- 1.0
2.1 +/- 0.8
0.8 +/- 1.0
1.0 +/- 1.2
1.4 +/- 0.9
0.8 +/- 0.3
-
KpsM
1.6 +/- 0.8
3.7 +/- 0.1
2.4 +/- 0.2
1.8 +/- 0.6
3.9 +/- 0.7
0.5 +/- 1.0
1.6 +/- 1.0
0.6 +/- 1.0
0.9 +/- 0.4
N/A

3.2 THE EXAMINATION OF CPS CHARACTERISITICS

3.2.1 Involvement of *cj1427c*, *cj1428c*, *cj1430c*, and *kpsM* in capsular synthesis

To determine the role of the genes of interest in CPS biosynthesis, CPS of the wild type, *cj1427*::CAT, *cj1428*::CAT, *cj1430*::CAT, *KpsM* and *cj1427*::CATΔ mutants were extracted using an adapted hot water/phenol method (171). This method offers significant advantages over a crude SDS solubilization (68) as near complete removal of proteins and most LOS from aqueous solutions can be achieved. Several rounds of extraction are conducted to obtain the maximal yield of CPS material. In addition, an ultracentrifugation step is important in pelleting the majority of the LOS contamination.

McNally *et al* have shown that a disruption of the *cj1428c* gene led to a loss of the modified heptose side-branch, leaving a mutant whose CPS was unbranched (153). It was of interest to determine whether inactivation of other heptose modification genes, namely *cj1427c* and *cj1430c*, also led to the lack of a modified heptose side-branch.

Samples were analyzed by SDS-PAGE on 7 cm gels and subsequent silver staining (**Figure 6A**). High molecular weight species were observed in the wild type lanes, indicative of CPS. Similarly, high molecular weight species were observed for the *cj1427*::CAT, *cj1428*::CAT and *cj1427*::CATΔ mutants. This indicates that despite the loss of a heptose modification gene, these mutants are still able to produce CPS. The *cj1427*::CATΔ mutant is still able to produce a CPS, despite the fact that it has a large portion of CPS related genes deleted. The bands observed in these mutants ran slower than those of the wild type, indicating that the CPS composition or structure is altered by the gene disruption. Interestingly, the *cj1430*::CAT mutant showed a strikingly different

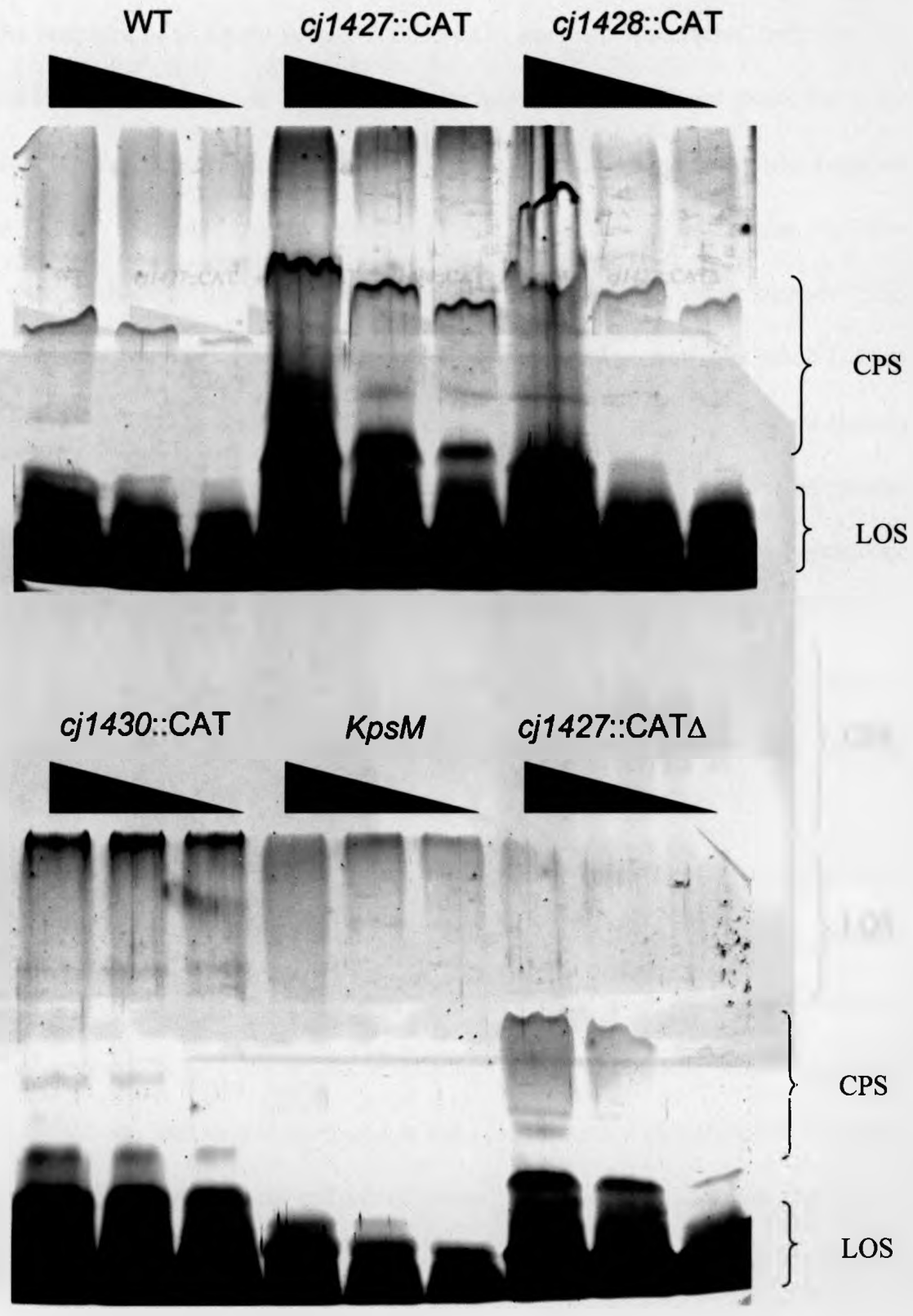
CPS profile than the other mutants, with less CPS produced overall. In addition, the banding pattern of the CPS is significantly different, suggesting that the structure of the CPS is altered. The *KpsM* mutant, in contrast to expectations, showed some degree of staining near the top of the gel; this was thought to be either background staining or high molecular weight material corresponding to the presence of CPS.

To gain more resolution into the CPS of the wild type and mutants and to resolve the potential high molecular weight material observed in the *KpsM* strain, a larger, 13 cm SDS-PAGE gel was used for analysis (**Figure 6B**). This larger format gel offers excellent resolution of the capsule and is desirable for enhancing the banding pattern characteristic of the repeating sugar units that comprise the CPS. With this increase in resolution, the banding pattern of the CPS for the wild type, *cj1427*::CAT, *cj1428*::CAT and *cj1427*::CATΔ mutants became evident. It is clear that CPS synthesis is tightly regulated in the wild type and the mutant strains, as silver staining shows very regular spacing of the bands. Interestingly, the *cj1427*::CAT and *cj1428*::CAT mutants displayed a wider variety of CPS species; CPS molecules of higher and lower molecular weight molecules are present compared to wild type CPS. In contrast, the *cj1427*::CATΔ mutant showed fewer bands than the wild type, indicating that regulation of capsular chain elongation may be disrupted, resulting in fewer species of CPS with multiple sugar units. The *cj1430*::CAT mutant again shows a significantly different CPS staining pattern than the other mutants. With the increase in resolution using a 13 cm gel, the *KpsM* mutant shows the presence of high molecular weight material, apparent as a smeary pattern, suggesting the presence of CPS.

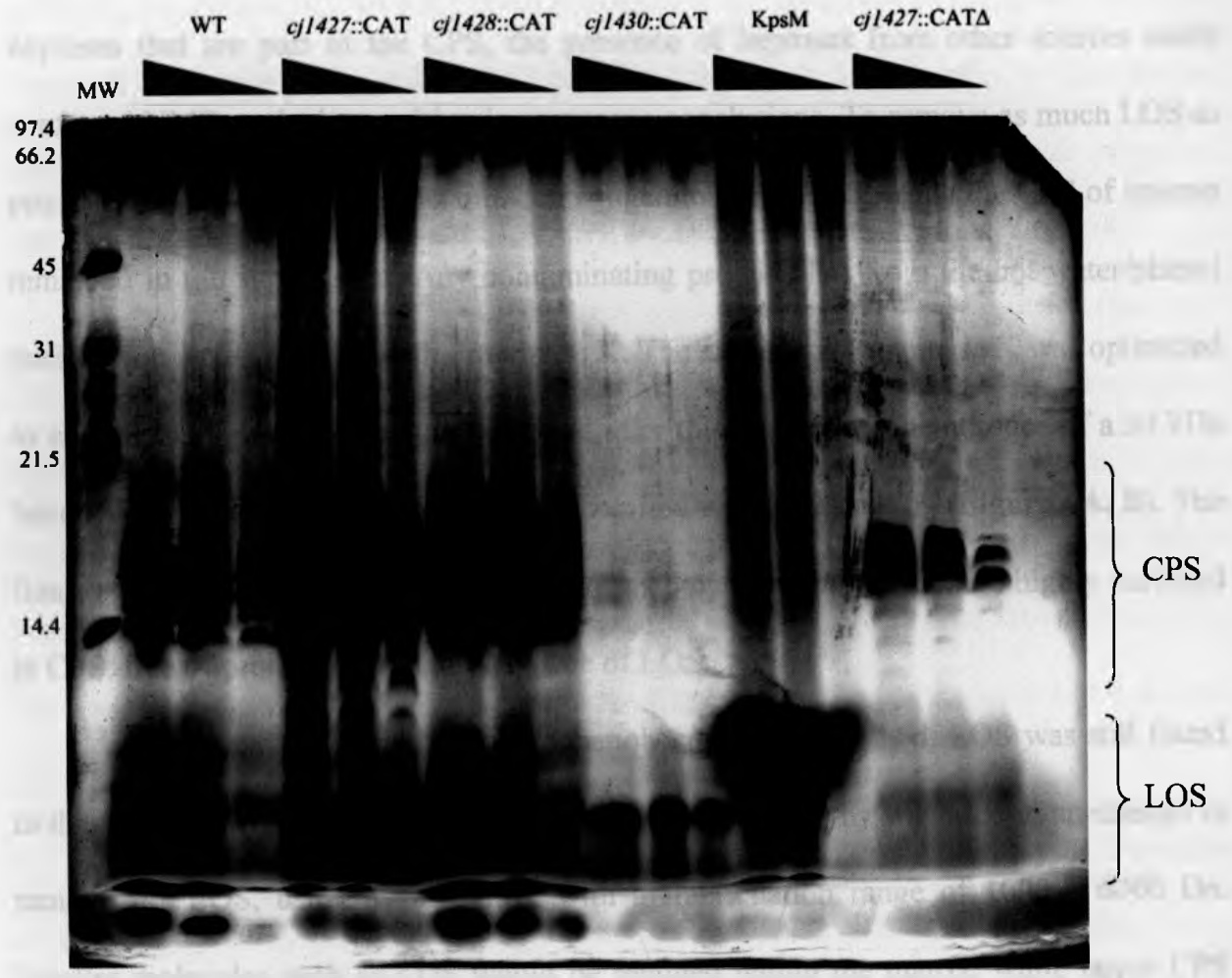
Figure 6. Silver staining of wild type and various *C. jejuni* mutants

Bracketed areas indicate the high molecular weight species thought to be CPS, and the lower molecular weight species thought to be LOS. The relative volumes of each sample loaded are indicated by the triangles. Three dilutions were used so that comparisons between each strain could be made. MW = molecular weight marker. The sizes of the proteins standard in kilodaltons are indicated to the left of the gel. **A) Silver stain of the CPS in wild type and mutant strains on 7 cm SDS-PAGE gels.** Due to the small size of the gel, CPS bands appear as a smear of high molecular weight molecules. Noticeable differences were observed for the mutants' CPS, especially for the *cj1430*::CAT mutant. **B) Silver stain of the CPS in wild type and mutant strains on 13 cm SDS-PAGE gel.** To gain more resolution, a 13 cm SDS-PAGE gel was used. This gel offers more resolution than the 7 cm gels, and the banding nature of CPS is well resolved in all strains. Similar observations as in A) were made regarding the capsular staining patterns.

A)


WT
cj1427::CAT
cj1428::CAT
CPS
LOS
cj1430::CAT
KpsM
cj1427::CATΔ
CPS
LOS

B)


WT
cj1427::CAT
cj1428::CAT
cj1430::CAT
KpsM
cj1427::CATΔ
MW
97.4
66.2
45
31
21.5
14.4
CPS
LOS

3.2.2 Purification of *C. jejuni* CPS from wild type and mutants

The structure of *C. jejuni* ATCC 700819 LOS has been elucidated (158), and it is known that it contains a heptose. Purified CPS samples with no LOS are desirable, as the heptose that may be liberated from LOS could contaminate downstream nuclear magnetic resonance (NMR) analysis. As the purpose of our research is to investigate modified heptoses that are part of the CPS, the presence of heptoses from other sources could confound NMR analysis, resulting in erroneous conclusions. To remove as much LOS as possible, the aqueous phase was ultracentrifuged to pellet LOS, while the CPS of interest remained in the supernatant. Any contaminating proteins left from the hot water/phenol purification were removed with Proteinase K. The amount of Proteinase K was optimized to ensure that a minimal amount remained after this treatment. The absence of a 30 kDa band, corresponding to Proteinase K, was verified via silver staining (**Figure 6A, B**). The final result of this adapted hot water/phenol method are samples that are highly enriched in CPS, free of proteins, and relatively free of LOS.

However, despite the hot water/phenol purification method, LOS was still found in the samples (**Figure 6A, B**). Size exclusion chromatography was used in an attempt to remove the LOS, using a gel matrix with a fractionation range of 1000 – 6000 Da. Smaller molecules such as LOS would be retained within the matrix, while larger CPS molecules would not, and should be found in the void volume. Fractions were collected, and then run on SDS-PAGE gels and silver stained; however, it was found that the gel matrix failed to separate the LOS from the CPS as fractions containing the CPS of interest also showed traces of LOS (**Figure 7A**).

In addition to size exclusion chromatography, another method of removing LOS from mixed LOS and CPS samples was attempted using the method outlined by Aspinall *et al* (10). In this method, LOS molecules were chemically cleaved at the linkage between the lipid A anchor and the Kdo sugar using acetic acid. It was thought that this procedure would reduce the size of the LOS molecules due to the loss of the lipid A anchor, allowing the remaining fragment to be retained by the column matrix. However, it was observed that while acid treatment reduced the amount of LOS present in the samples (compare **Figure 7B**, lane 1 and lane 2, before and after acetic acid treatment, respectively), it did not separate the LOS away from CPS (see fractions 51 and 54).

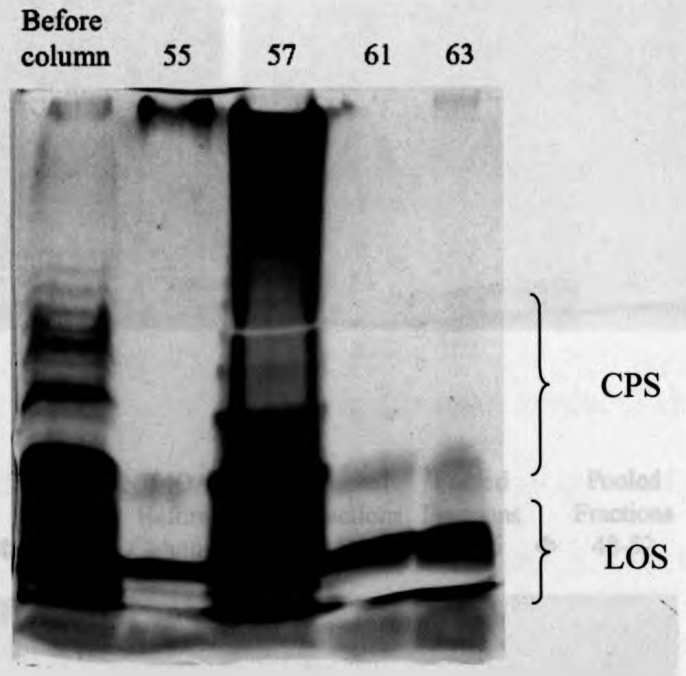
An ammonium acetate treatment was also conducted to compare the effect of a milder acid treatment on LOS (**Figure 7C**). This treatment potentially had an effect on separating LOS from CPS based on the fact that early fractions showed high molecular weight species, indicative of CPS, while later fractions showed low molecular weight species, indicative of LOS (**Figure 7C**). However, a tricine gradient gel showed that there was no LOS degradation after this treatment (**Figure 7D**, compare lanes 1 and 4, before and after ammonium acetate treatment, respectively).

Gu and Tsai (53) reported that lipopolysaccharide in *Neisserial* species were able to be purified away from outer membrane vesicles using sodium deoxycholate. As a similar effect is desired for *C. jejuni*, where LOS separation from CPS is desired, the methodology was adapted and used. While this method failed at achieving the initial goal of separating LOS from CPS, there was an unexpected effect of sharpening the resolution of the CPS bands (**Figure 7E**). The reason for this enhancement of the banding pattern of the CPS is unknown at this time.

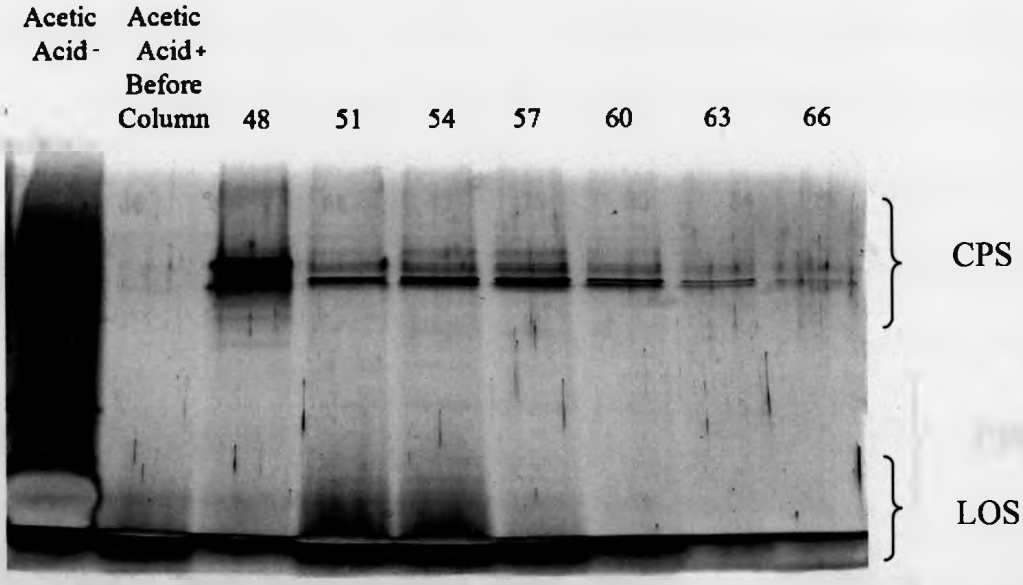
Figure 7. Various methods of purification were attempted to purify LOS away from CPS.

In panels B through E, - and + symbols represent samples that were mock treated or chemically treated, respectively. In all panels, samples were fractionated using size exclusion chromatography, using various gel matrices (panels A – D, Biorad Biogel P-6: fractionation range 1000 – 6000 Da; panel E, Sigma-Aldrich Sephadex G50: fractionation range: 500 – 10000 Da). Numbers above each lane represent the fraction number, corresponding to the *n*th milliliter of the flow through. LOS and CPS regions of interest are bracketed. **A) Silver stain of samples separated by size exclusion chromatography.** Co-elution of both CPS and LOS occurred, resulting in a failure to separate the LOS from CPS. **B) Acetic acid treatment of CPS samples degrades LOS.** The acetic acid treatment degraded LOS, to some degree, without degrading CPS. However, this treatment failed to separate the LOS and CPS molecules. Due to the large concentration of LOS in the non-treated samples, the LOS stains negatively. **C) A mild ammonium acetate treatment appears to separate CPS from LOS.** Fractions 19 – 28 contained CPS while fractions 46 – 52 contained LOS, indicating that the ammonium acetate treatment may have worked to separate the two molecules. **D) Tricine gradient gel analysis of ammonium acetate treated samples**. Analysis by tricine gradient gel analysis shows that ammonium acetate treatment in D) had no effect on LOS as initially hypothesized, as there was no difference in mock treated and ammonium acetate treated samples. A ϕ indicates an empty lane. **E) Sodium deoxycholate fails to separate LOS from CPS**. Sodium deoxycholate did not allow for the separation of LOS from CPS, but had the unexpected effect of sharpening the resolution of CPS banding.

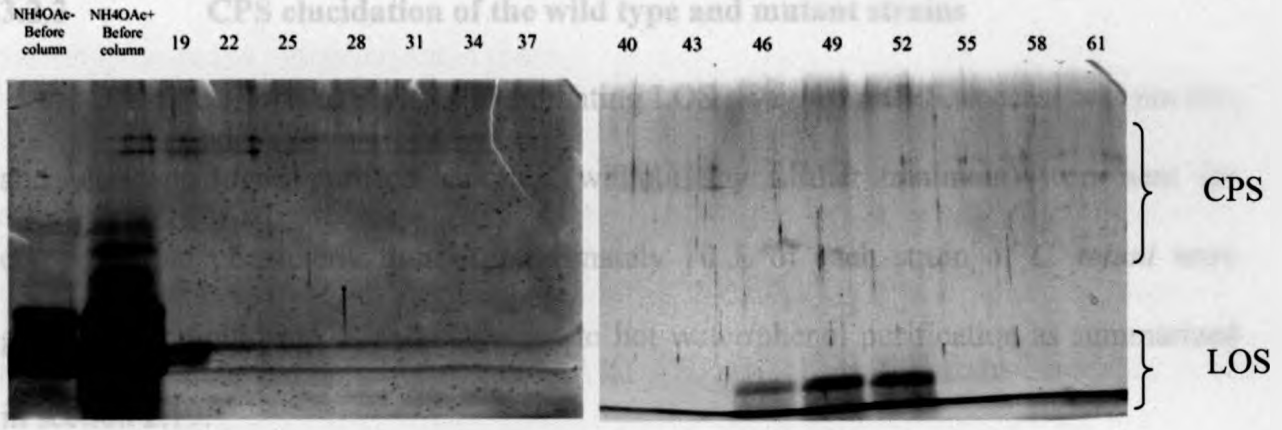
A)


Before column
55
57
61
63
CPS
LOS

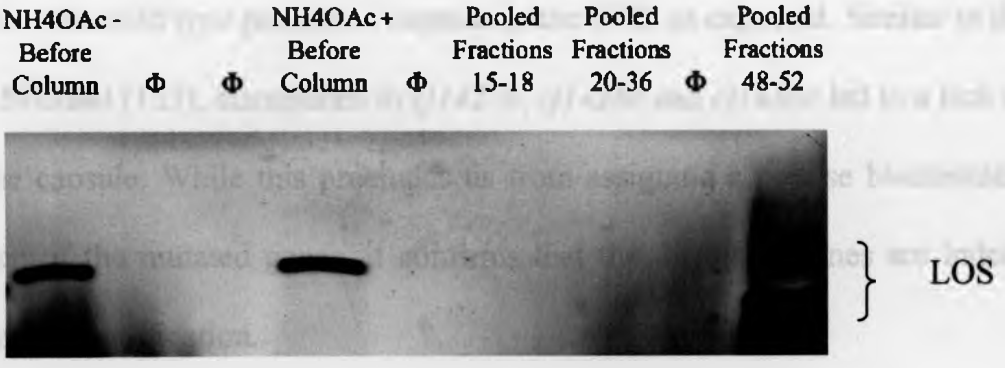
B)


Acetic Acid -
Acetic Acid + Before Column
48
51
54
57
60
63
66
CPS
LOS

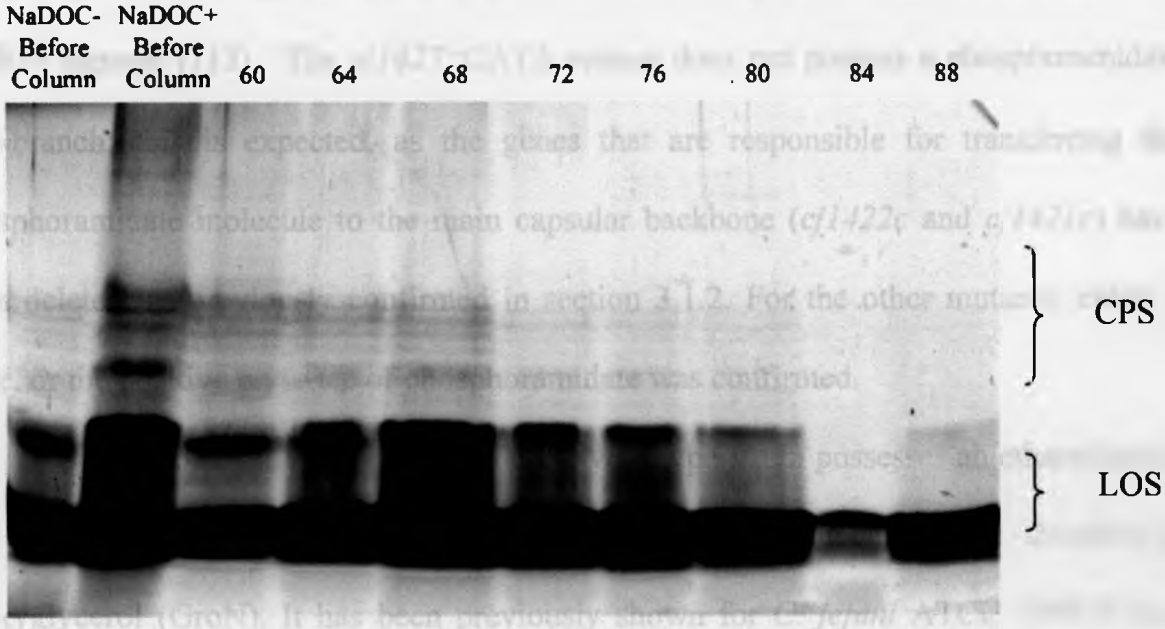
C)


NH4OAc- Before column
NH4OAc+ Before column
19 22 25 28 31 34 37
40 43 46 49 52 55 58 61
CPS
LOS

D)


NH4OAc - Before Column
Φ
Φ
NH4OAc + Before Column
Φ
Pooled Fractions 15-18
Pooled Fractions 20-36
Φ
Pooled Fractions 48-52
LOS

E)


NaDOC- Before Column
NaDOC+ Before Column
60 64 68 72 76 80 84 88
CPS
LOS

3.2.3 CPS elucidation of the wild type and mutant strains

Despite efforts to purify contaminating LOS away from CPS, success was not met, and ultracentrifuged purified samples (without any further treatment) were sent for compositional characterization. Approximately 10 L of each strain of *C. jejuni* were grown up in liquid media and subjected to hot water/phenol purification as summarized in section 2.19.

Several interesting observations were made from the results (**Table 1**). First, it was observed that the wild type possessed heptose in the CPS, as expected. Similar to the findings of St. Michael (153), disruptions in *cj1427c*, *cj1428c* and *cj1430c* led to a lack of a heptose in the capsule. While this precludes us from assigning a precise biochemical function to each of the mutated genes, it confirms that the disrupted genes are indeed involved in heptose modification.

Second, the wild type strain contains no phosphoramidate molecule. This is intriguing, as it is thought that the phosphoramidate moiety is a side-branch of the ATCC 700819 capsule (113). The *cj1427*::CATΔ mutant does not possess a phosphoramidate side-branch; this is expected, as the genes that are responsible for transferring the phosphoramidate molecule to the main capsular backbone (*cj1422c* and *cj1421c*) have been deleted, as previously confirmed in section 3.1.2. For the other mutants, either a trace, or the positive presence of phosphoramidate was confirmed.

It is also worth noting that while the wild type strain possesses an ethanolamine on glucuronic acid, the mutants possessed either ethanolamine or 2-amino-2-deoxyglycerol (GroN). It has been previously shown for *C. jejuni* ATCC 700819 that phenotypic variants are present in a population, despite being grown under the same

conditions (158). This may explain why the mutants showed the presence of both types of substituents on the glucuronic acid moiety.

Sugar analysis data were also obtained via acid hydrolysis; all results were normalized to galactosamine (GalN), a break down product of N-acetyl galactosamine (GalNAc), which is one of the three sugars that make up the repeating trio of sugars in the main capsular backbone of *C. jejuni* ATCC 700819 (114). It was observed that there were varying levels of ribose in each mutant, as well as trace amounts of galactose.

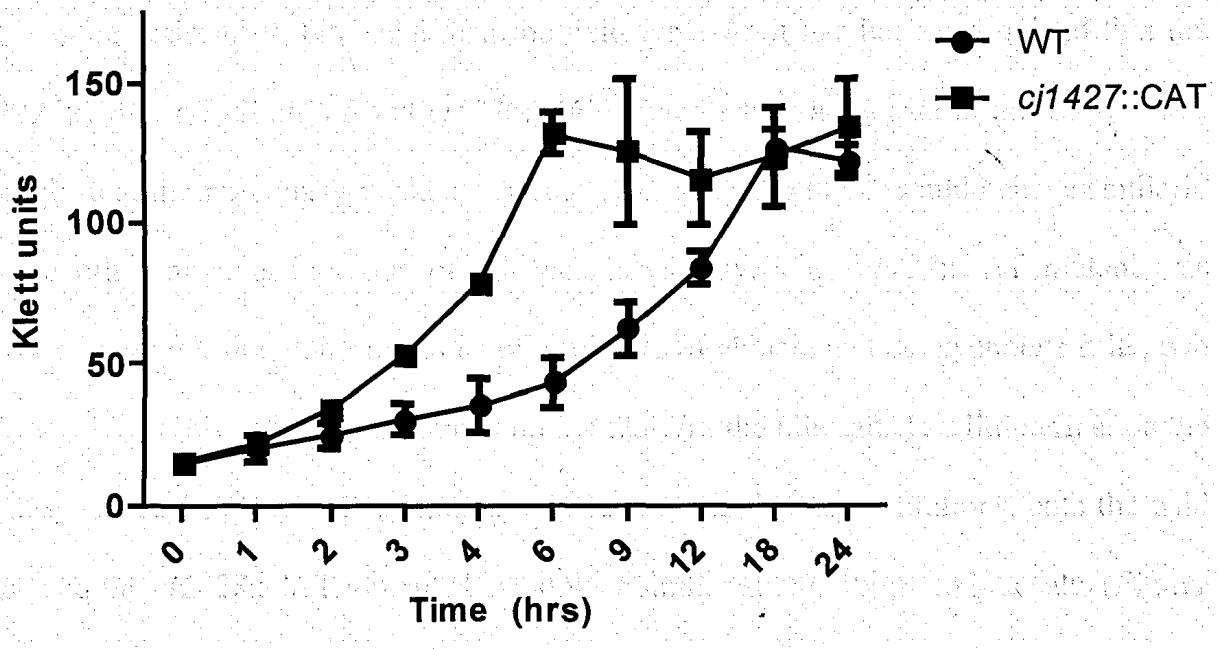
Table 1. Summary of NMR results for wild type and mutant *C. jejuni* strains.

Sugar analysis data was conducted using anion-exchange chromatography after full acid hydrolysis. Numbers in the columns under "Sugar analysis data" represent the amounts of various sugars present, after normalization to GalN. A plus (+) or minus (-) in the NMR spectroscopy data columns represent either the presence or the absence of the specified molecule, respectively. GalN = galactosamine. Rib = ribose. Gal = galactose. Hep = heptose. Me = methyl. MeOPN = phosphoramidate. GalNAc = N-acetyl galactosamine. GlcA = glucuronic acid. EtN = ethanolamine. GroN = 2-amino-2-deoxyglycerol.

	Sugar analysis data			NMR spectroscopy data			
	GalN	Rib	Gal	Hep	8,9-Me_2 on Hep	MeOPN on GalNAc	Substituent on GlcA
WT	1	2.6	0.1	+	+	-	EtN
cj1427::CAT	1	1.2	trace	-	-	+	EtN, GroN
cj1428::CAT	1	3.6	0.15	-	-	trace	EtN, GroN
cj1430::CAT	1	1.4	0.18	-	-	+	EtN, GroN
cj1427::CATΔ	1	0.9	trace	-	-	-	EtN, GroN
KpsM	1	2.7	trace			Not analyzed	

Figure 8. The growth rate of the *cj1427*::CAT mutant is greater than the wild type.

Growth rate experiments were conducted over a period of 24 hours. Density was measured in Klett units. Results shown are the mean and standard errors from three independent experiments. Between 3 and 8 hours, the *cj1427*::CAT mutant was found to grow significantly faster than the wild type indicating it had less of an initial lag phase.

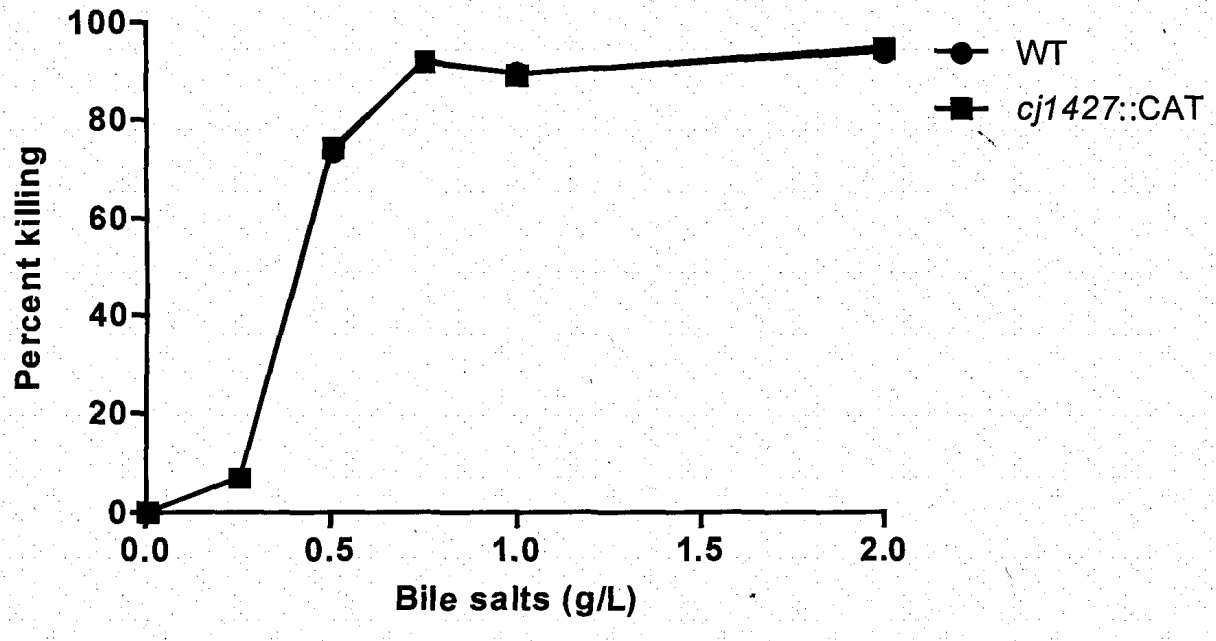

WT
cj1427::CAT
Klett units
150
100
50
0
0
1
2
3
4
6
9
12
18
24
Time (hrs)

3.3.2 Effect of gene disruption on bile salt resistance

C. jejuni has been shown to be resistant to bile salts in the intestine (105, 106). In addition, CPS has also been shown to be involved in resistance to bile salts in other Gram negative bacteria (14, 129). Our laboratory previously found that at low concentrations of bile salts, all mutants behaved similarly to the wild type in that little effect was observed (100). At higher concentrations of bile salts, the *cj1427*::CATΔ and *KpsM* mutants were no more susceptible than wild type. However, the *cj1428*::CAT and *cj1430*::CAT mutant were more resistant to bile salts than the wild type. As it has been determined that the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT mutants lack a heptose in the CPS, it was thought that the previously uncharacterized *cj1427*::CAT mutant would behave similarly to the other mutants in terms of bile salt sensitivity. The *cj1427*::CAT mutant was exposed to increasing concentrations of a mixture of cholate and deoxycholate salts, two components of bile. At concentrations up to 0.25 g/L, the bile salts had little effect on the wild type and the *cj1427*::CAT mutant. However, at higher concentrations, both the wild type and the *cj1427*::CAT mutant showed very similar susceptibility to bile salts (**Figure 9**), contrasting the findings that were previously found for the *cj1428*::CAT and *cj1430*::CAT mutants.

Figure 9. Susceptibility of wild type and the *cj1427*::CAT mutant to bile salts.

Both the wild type and the *cj1427*::CAT mutant were exposed to varying concentrations of bile salts for 15 minutes at 37°C before being serially diluted and plated for CFU counts. Results shown are the mean and standard errors from 3 independent experiments. Both strains showed extremely similar patterns of susceptibility to bile salts. At low concentrations of bile salts, both strains show little susceptibility. At higher concentrations, both strains are susceptible.

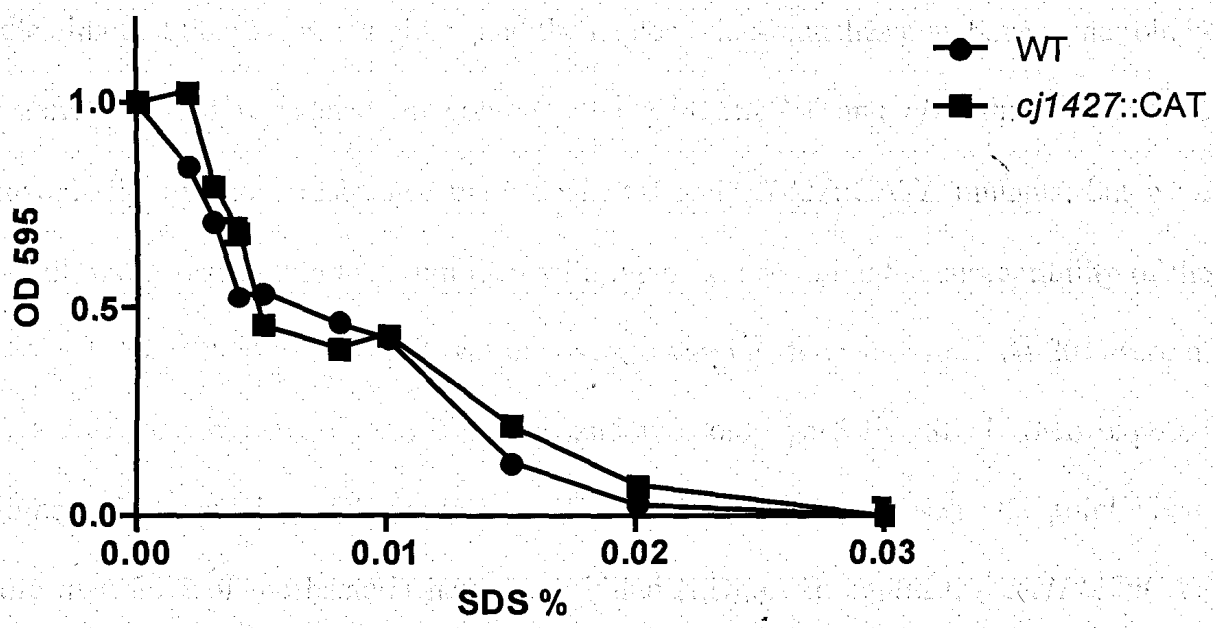

100
80
60
40
20
0
Percent killing
0.0
0.5
1.0
1.5
2.0
Bile salts (g/L)
WT
cj1427::CAT

3.3.3 Effect of gene disruption on SDS resistance

To determine whether *C. jejuni* susceptibility to bile salts was detergent specific, sodium dodecyl sulfate (SDS) susceptibility was also investigated. No significant difference was found between the wild type and the *cj1427*::CAT mutant in terms of susceptibility to SDS (**Figure 10**). This result is similar to what has been previously observed by our laboratory for the other compositional mutants (100).

Figure 10. Susceptibility of wild type and *cj1427*::CAT mutant to SDS.

The wild type and *cj1427*::CAT mutant were exposed to varying concentrations of SDS for 15 hours at 37°C under microaerophilic conditions with shaking to determine their susceptibility. Results shown are the mean and standard errors from two independent experiments. No significant difference was observed between the wild type and the *cj1427*::CAT mutant. With increasing SDS concentrations, more bacteria were killed.


WT
cj1427::CAT
OD 595
1.0
0.5
0.0
0.00
0.01
0.02
0.03
SDS %

3.3.4 Effect of gene disruption on serum resistance

It has been previously shown that CPS plays a role in serum resistance in other Gram negative bacteria (13, 32, 62, 139, 144). In addition, our laboratory has demonstrated that a capsuleless *KpsM* mutant is killed extremely effectively by serum, even at low percentages (5%) (100). This shows that the capsule of strain ATCC 700819 is essential in conveying serum resistance. We have also shown that the *cj1427*::CATΔ mutant behaved very similarly to the *KpsM* mutant, in that it was killed very quickly indicating that the phosphoramidate and the heptose side-branches may have some role in conferring serum resistance. In contrast, the *cj1428*::CAT and *cj1430*::CAT mutants showed slightly more resistance than the *KpsM* and *cj1427*::CATΔ mutants, but were overall, more susceptible to serum than wild type. To determine the susceptibility of the *cj1427*::CAT mutant to serum, it was exposed to varying concentrations. At 20% serum, the wild type strain exhibited resistance and was only partially killed when exposed (**Figure 11A**), agreeing with our previous findings. This value represents the point where more than 50% of the bacteria have been killed (LD_{50}). In contrast, the *cj1427*::CAT mutant, despite having the same capsular composition as the *cj1428*::CAT and *cj1430*::CAT mutants, showed extremely high susceptibility to serum, even at concentrations as low as 10%. To determine whether changes in serum resistance were due to differences in the rate of killing, the wild type and *cj1427*::CAT mutant were exposed to 20% serum over a period of 90 minutes. At this percentage, a significant difference in terms of viability was observed for the wild type and the *cj1427*::CAT mutant even after only 15 minutes (**Figure 11B**). By the end of the timecourse

experiment, the *cj1427*::CAT mutant showed almost complete death, corresponding to the results found in **Figure 11A.**

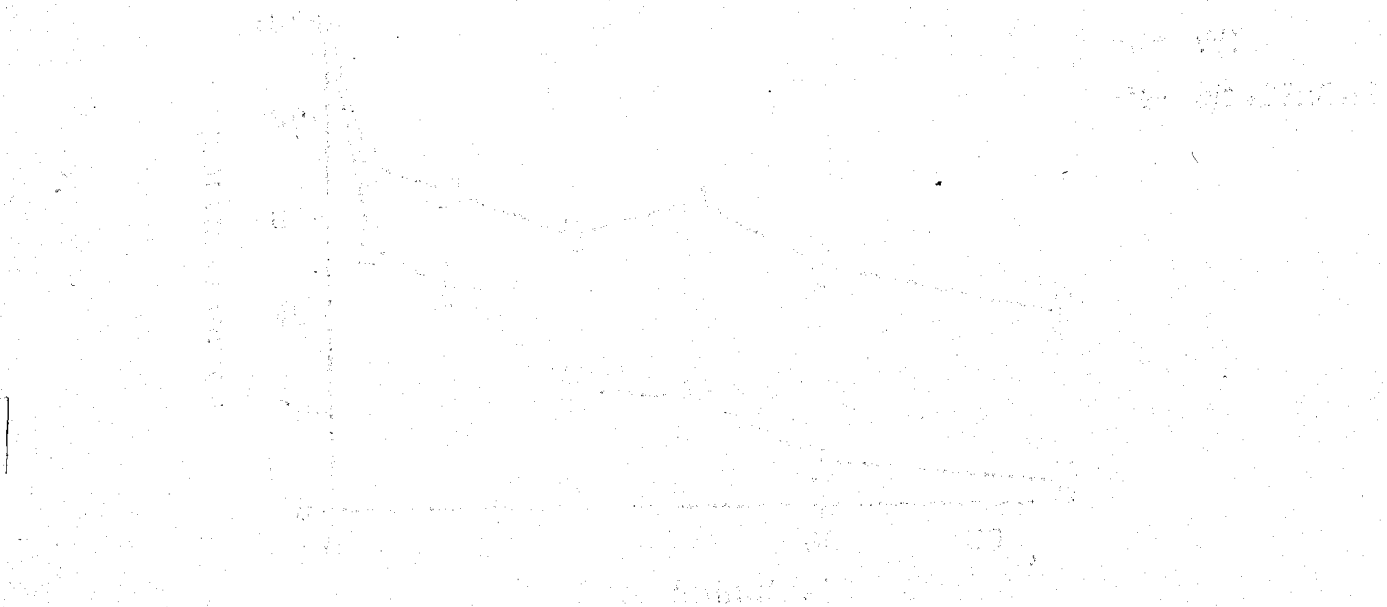
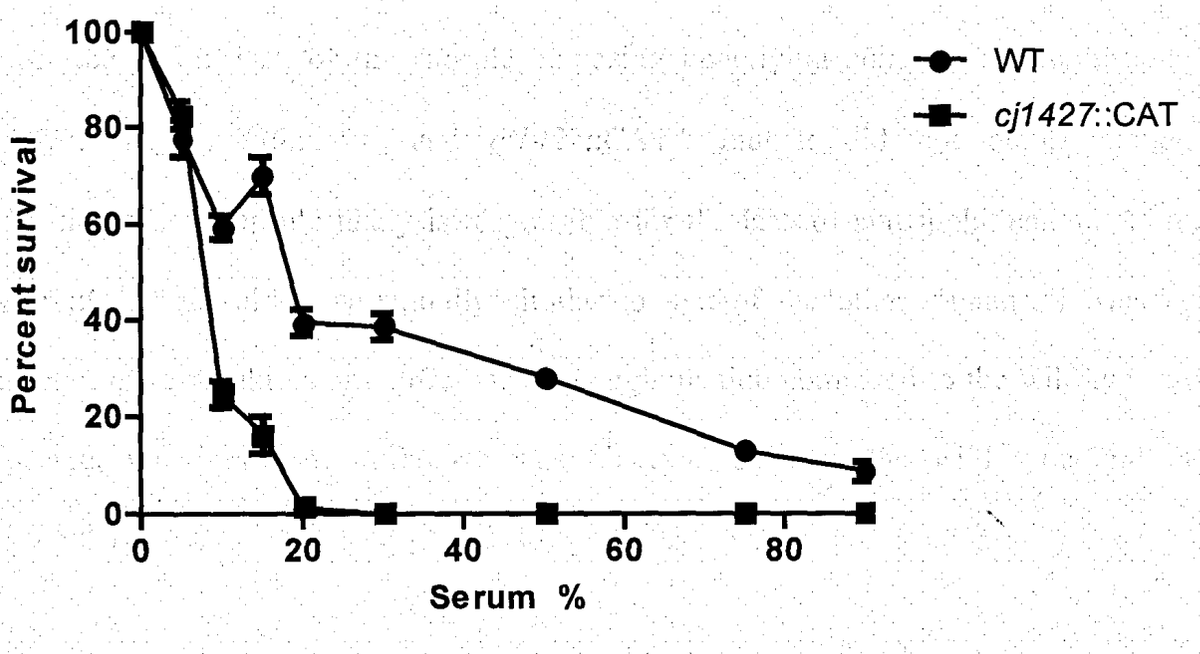


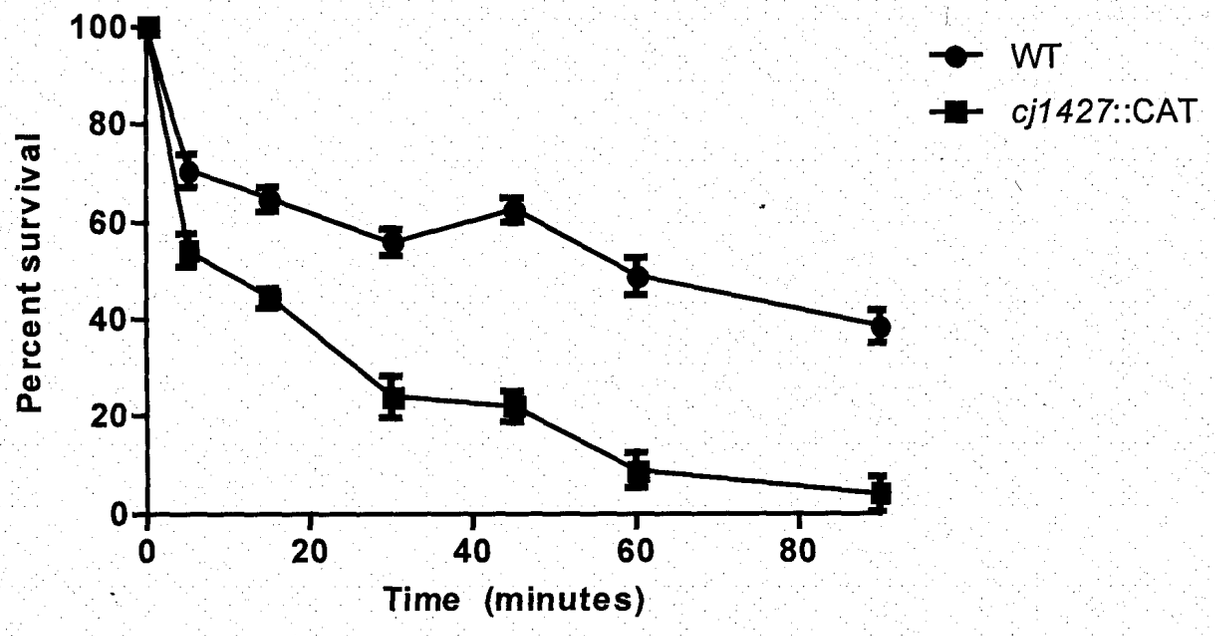
Figure 11. Assessing the susceptibility of wild type *C. jejuni* and the *cj1427*::CAT mutant to serum.

A) The *cj1427*::CAT mutant shows extremely high susceptibility to serum. After incubation with varying serum concentrations for 1.5 hours, the *cj1427*::CAT mutant showed extreme susceptibility to serum; even at low serum percentages (10%), the mutant showed significant loss of viability. At serum concentrations of 20% and above, no growth was observed based on CFU counts. **B) The *cj1427*::CAT mutant dies faster than wild type.** A timecourse experiment using 20% serum shows that the *cj1427*::CAT mutant shows much faster killing kinetics than the wild type strain; more than half the bacteria are killed within the first 15 minutes. Results shown are the mean and standard deviation from two independent experiments.

A)


100
80
60
40
20
0
Percent survival
0
20
40
60
80
Serum %
WT
cj1427::CAT

B)

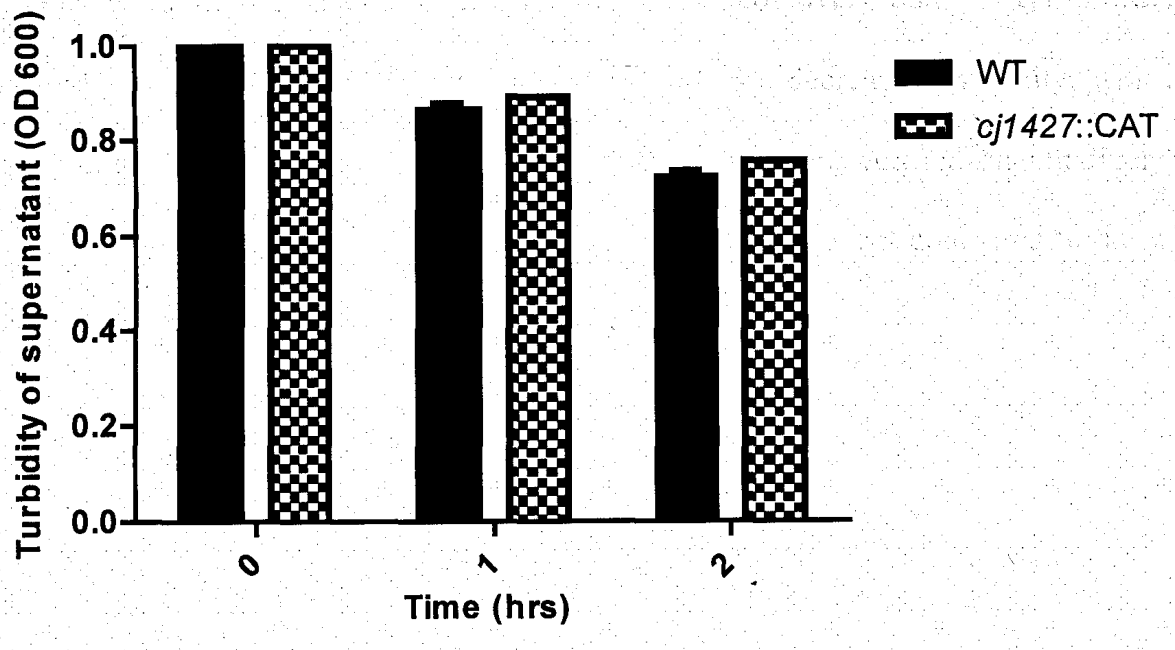

100
80
60
40
20
0
Percent survival
0
20
40
60
80
Time (minutes)
WT
cj1427::CAT

3.3.5 Effect of gene disruption on autoagglutination

Previous work in our laboratory (100) showed that a capsuleless *KpsM* mutant readily agglutinated over time, whereas the wild type bacteria did not. This was thought to be due to the lack of the capsule, exposing underlying adhesins. In contrast, the *cj1428*::CAT, *cj1430*::CAT, and *cj1427*::CATΔ mutant did not autoagglutinate, indicating that a capsule likely is responsible for the lack of autoagglutination. As the *cj1427*::CAT capsule is structurally similar to that of the other mutants, it was not expected there would be any differences in agglutination compared to the wild type. As expected, no significant difference was observed between the wild type and the *cj1427*::CAT over a three hour period (**Figure 12**).

Figure 12. Autoagglutination assay comparing wild type and *cj1427*::CAT.

Both the wild type and the *cj1427*::CAT mutant were inoculated into sterile PBS in a falcon tube and allowed to sit. Over the course of three hours, the top portion of the PBS was carefully removed and the optical density measured to determine whether autoagglutination was occurring. An unchanged optical density would indicate a lack of autoagglutination. Results shown are the mean and standard errors from three independent experiments. No significant differences were observed for all timepoints between the wild type and the *cj1427*::CAT mutant. The slight decrease in optical density over the course of three hours is due to the natural sedimentation of the bacteria.


Turbidity of supernatant (OD 600)
1.0
0.8
0.6
0.4
0.2
0.0
0
1
2
Time (hrs)
WT
cj1427::CAT

3.3.6 Effect of gene disruption on motility

The flagella of *C. jejuni* have been previously shown to be important for virulence (52, 120, 176). As flagella and CPS are both anchored to the outer membrane of the bacteria, we sought to determine whether changes in the CPS due to disruption of the putative heptose biosynthetic genes would affect motility. Motility assays were conducted for the wild type and *cj1427*::CAT mutant. It was found that the *cj1427*::CAT mutant was less motile than the wild type strain when compared visually (**Figure 13**). Previous work in our laboratory has also shown qualitatively that the *cj1428*::CAT, *cj1430*::CAT, *KpsM*, and *cj1427*::CATΔ mutants had a decrease in motility, with the *KpsM* mutant being completely non-motile. These data were verified quantitatively in this thesis; the decrease in motility for all mutants was significant compared to the wild type strain, with p values less than 0.05 (**Table 2**).

Figure 13. Motility assay for wild type and the *cj1427*::CAT mutant.

The *cj1427*::CAT mutant shows significantly less motility than wild type. Both wild type and *cj1427*::CAT were stabbed onto a motility plate consisting of 0.3% agar. Plates were incubated for 48 hours before halos were measured. Shown in this figure is a representative motility plate.

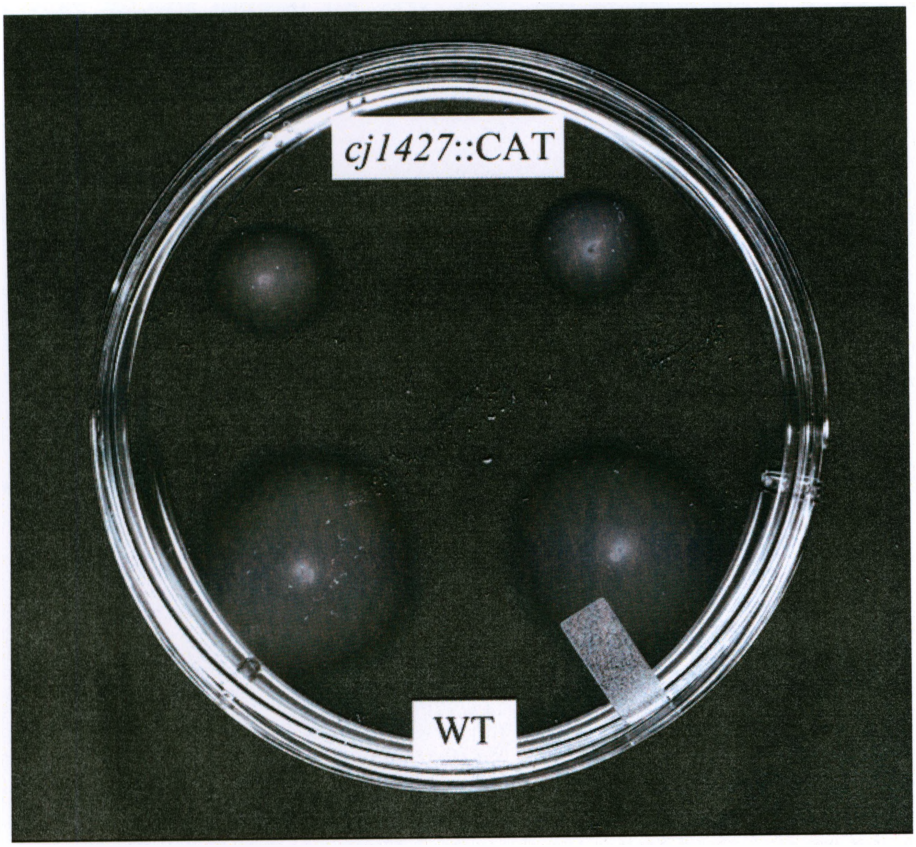

cj1427::CAT
WT

Table 2. A quantitative comparison of motility for wild type and all mutant strains.

The motility of the wild type and mutant strains were measured and compared quantitatively after stabbing into 0.3% agar and incubation for 48 hours under microaerophilic conditions. The values shown are for the diameters of the motility halos, measured in centimetres. The mean and standard errors (SE) of three independent experiments are shown for each strain. In addition, the *p* value, as determined by one-way ANOVA, is shown. Compared to the wild type, all mutant strains were significantly reduced in motility.

C. jejuni strain	Diameter of halo (cm) ± SE	*p* value
WT	3.09 ± 0.09	N/A
cj1427::CAT	2.35 ± 0.08	< 0.01
cj1428::CAT	2.77 ± 0.06	< 0.01
cj1430::CAT	2.73 ± 0.06	< 0.01
cj1427::CATΔ	2.38 ± 0.10	< 0.01
KpsM	0.00 ± 0.00	< 0.01

3.4 THE ROLE OF *C. JEJUNI* CPS ON THE INTERACTION WITH VARIOUS CELL TYPES

3.4.1 Effect of gene disruption on adhesion and invasion of intestinal Caco-2 cells

It is known that the adhesion and invasion of intestinal cells is important to the virulence of *C. jejuni* (17). It has been shown that the CPS of *C. jejuni* strain 81-176 is involved in this process (13). In addition, our laboratory has shown that in ATCC 700819, a capsule is important in modulating the adhesion and invasion of intestinal epithelial cells (100). Our results showed that the capsuleless *KpsM* mutant had a very large increase in adhesion and invasion; this was thought to be due to the exposure of underlying surface adhesins, allowing the bacteria to invade more readily. Interestingly, the *cj1427*::CATΔ mutant was also found to adhere to and invade Caco-2 cells slightly more than the wild type, but not as much as the *KpsM* mutant, indicating that the lack of the heptose and phosphoramidate molecules may facilitate adherence and subsequent invasion (i.e. due to a lack of steric hindrance of underlying adhesins). In contrast, the *cj1428*::CAT and *cj1430*::CAT mutants adhered to Caco-2 cells to the same level as the wild type strain, but failed to invade.

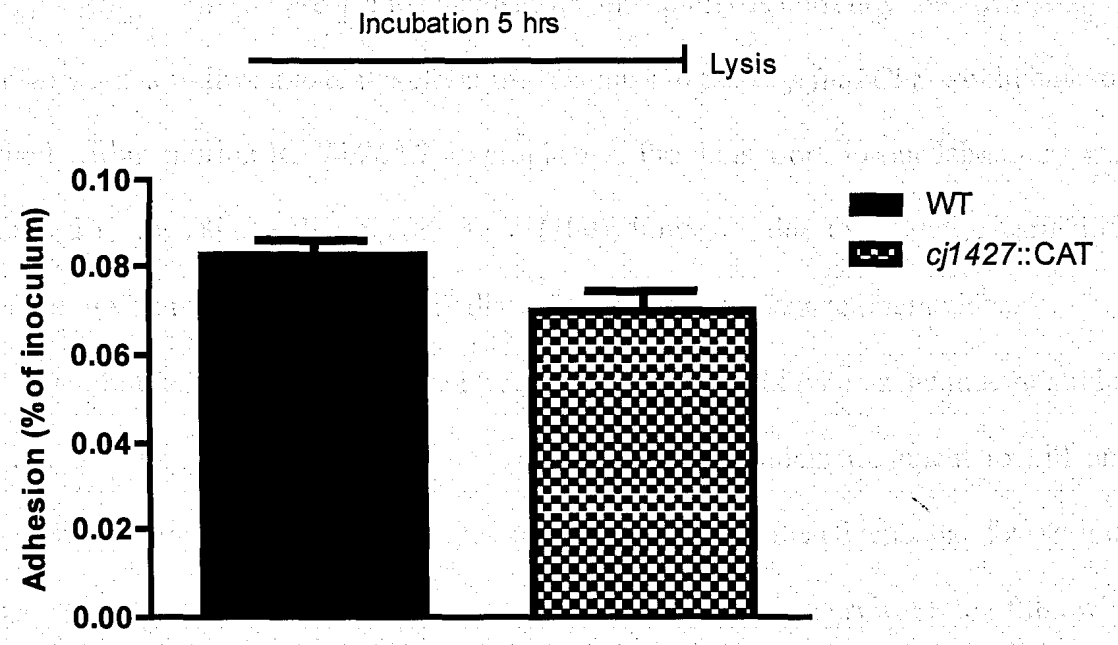
Not surprisingly, the *cj1427*::CAT mutant behaved similarly to the wild type strain in terms of adhesion to epithelial cells (**Figure 14A**). However, contrasting the *cj1428*::CAT and *cj1430*::CAT mutants, there was no significant difference between the *cj1427*::CAT mutant and wild type in terms of invasion of Caco-2 cells, as invasion, albeit at low levels, was observed (**Figure 14B**). The very low levels of adhesion to and

invasion of Caco-2 cells is consistent with what has been previously observed for the wild type strain in the literature (39, 47, 157), as well as in our laboratory (100). Despite these low levels of adhesion and invasion to epithelial cells, *C. jejuni* is still able to cause disease.

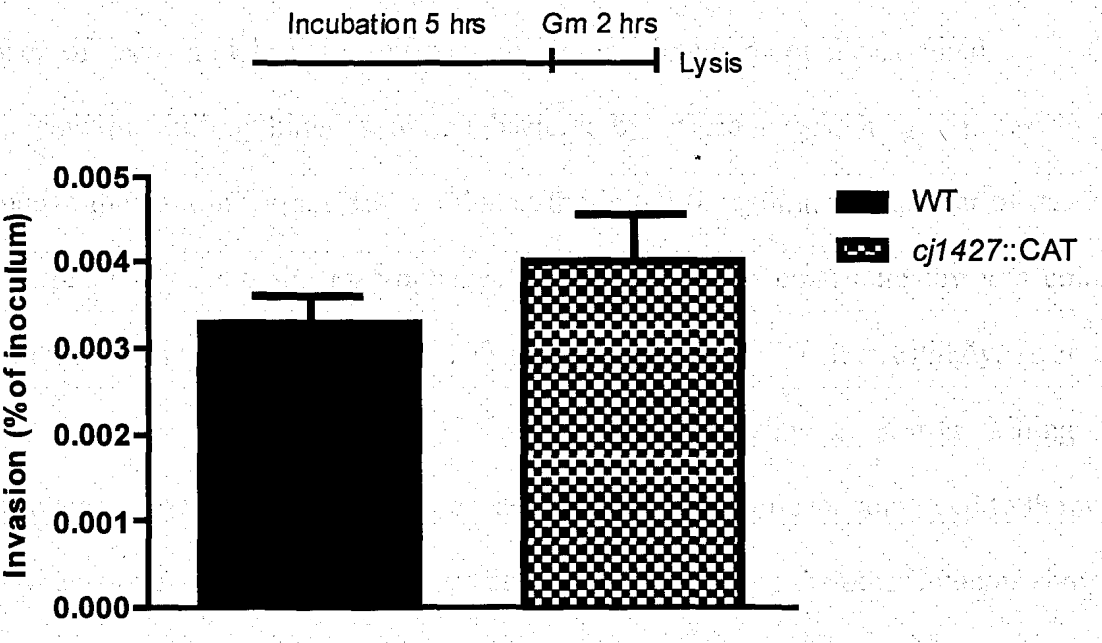
Figure 14. Adhesion and invasion of wild type and *cj1427*::CAT *C. jejuni* to Caco-2 intestinal epithelial cells.

A schematic at the top of the panels indicate the experimental layout. Gm = gentamicin. Results shown are the mean and standard deviation from two independent experiments. **A) Adhesion of wild type and *cj1427*::CAT to Caco-2 cells.** The *cj1427*::CAT mutant behaved very similarly to the wild type strain. Low levels of adhesion were observed for both strains. No statistical significance was found. **B) Invasion of Caco-2 cells by wild type and the *cj1427*::CAT mutant.** Both the wild type and the *cj1427*::CAT were able to invade Caco-2 cell, but at very low levels. No statistical significance was found.

A)


Incubation 5 hrs
Lysis
Adhesion (% of inoculum)
0.10
0.08
0.06
0.04
0.02
0.00
WT
cj1427::CAT

B)


Incubation 5 hrs
Gm 2 hrs
Lysis
Invasion (% of inoculum)
0.005
0.004
0.003
0.002
0.001
0.000
WT
cj1427::CAT

3.4.2 Effect of gene disruption on survival within macrophages

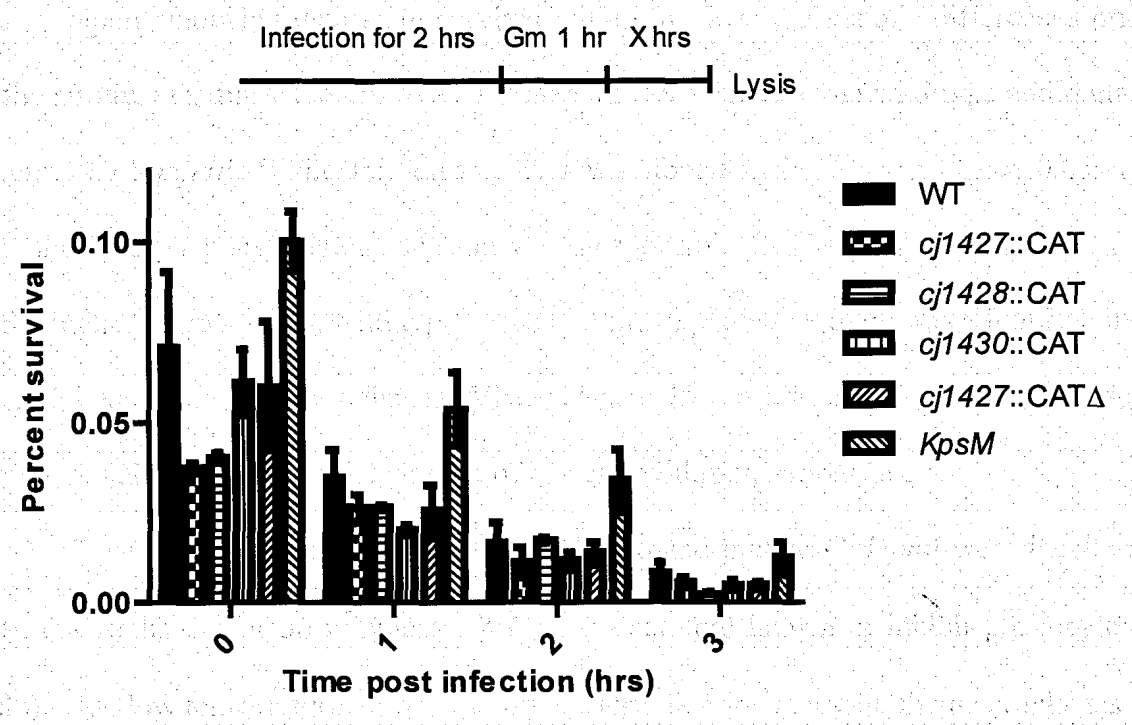
Recently, it has been shown that *C. jejuni* is able to survive within murine macrophages (149). The precise mechanism for this ability is currently unknown, and as such we wanted to investigate the effect that changes in the *C. jejuni* CPS would have on survival within murine RAW 264.7 macrophages. Previous work in our laboratory was conducted to investigate this phenomenon (100); however, due to the large variability observed, it was necessary to carefully dissect *C. jejuni*-macrophage interactions.

To do this, macrophages were infected with either wild type or the mutant strains of *C. jejuni* for two hours followed by a one hour gentamicin treatment to kill any extracellular bacteria. The macrophages and bacteria were then incubated for various timepoints before being lysed for CFU counts. As expected, the survival rate of the wild type and mutant strains decreased with prolonged duration within the macrophages (**Figure 15A**). The *cj1427*::CAT and *cj1428*::CAT mutants appeared to have a lower number of recovered bacteria, even at the initial timepoint of the survival assay. To investigate the killing kinetics of the bacteria by macrophages, it was necessary to normalize the data in **Figure 15A** so that at the initial timepoint, the number of bacteria that were alive within the macrophages (and thus able to contribute towards colony forming units) were deemed to be 100 percent (**Figure 15B**). Interestingly, over the timeline, the *cj1427*::CAT and *cj1428*::CAT mutants showed slower killing by macrophages; however, this difference was deemed non-significant compared to the wild type when a one-way ANOVA test was conducted. Results for the other mutants showed similar killing kinetics to the wild type.

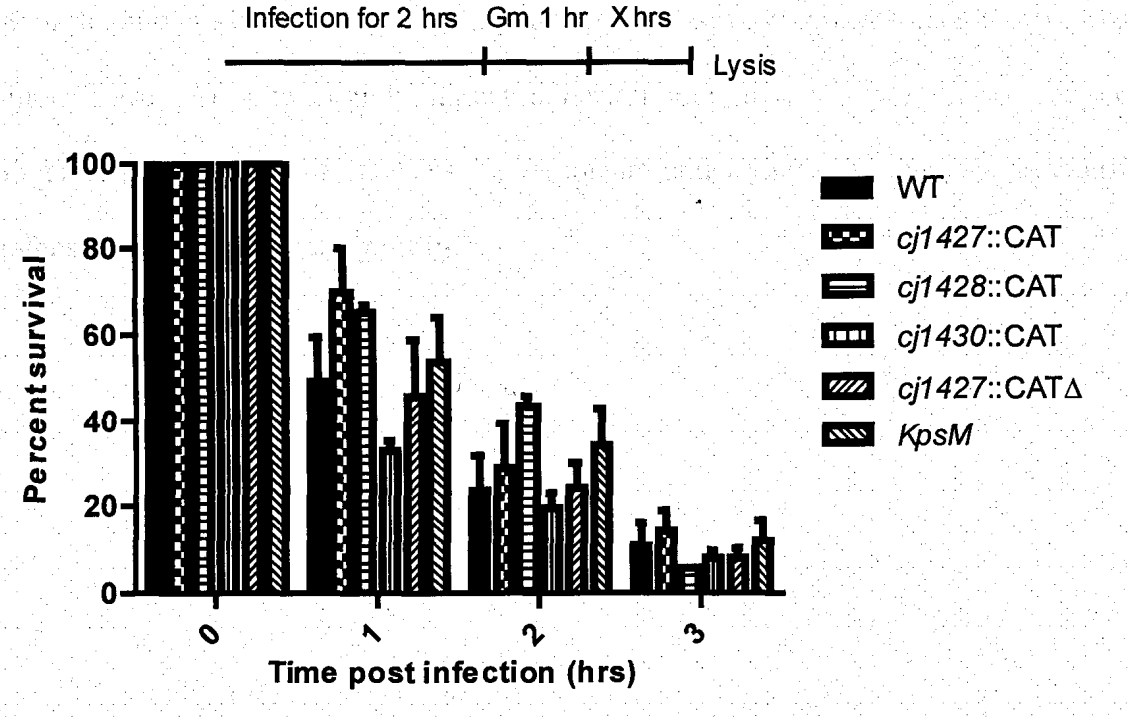
Figure 15. Percent survival of the wild type strain and mutant strains within macrophages.

The survival rate of wild type and the mutant strains was investigated over a time period of three hours. Percent survival was calculated as the number of bacteria recovered from macrophages divided by the number of bacteria recovered under the same growth conditions, in the absence of macrophages. As the length of time post infection increases, the number of bacteria able to survive decreases. Results shown are the mean and standard errors from three independent experiments. The schematic at the top indicates the experimental layout, where X represents the number of hours post infection. Gm = gentamicin. **A) Compared to wild type, the mutant strains have a lower survival rate within macrophages.** With the exception of the *KpsM* mutant, the other mutants appeared to have a slightly lower rate of survival within macrophages. However, there was no statistical difference between the survival rates of the wild type and the mutants when a one-way ANOVA test was conducted with a p value cut off of 0.05. **B) Some mutants appear to have an increased ability to survive within macrophages compared to WT.** The data for each strain at timepoint zero in panel **A)** were normalized to 100 percent survival. The *cj1427*::CAT and *cj1428*::CAT mutants appeared to be killed less slowly than the other mutant strains, when compared to the wild type. However, there was no statistical difference between the killing kinetics of the wild type and the mutants when a one-way ANOVA was conducted.

A)


Infection for 2 hrs
Gm 1 hr
X hrs
Lysis
Percent survival
0.10
0.05
0.00
0
1
2
3
Time post infection (hrs)
WT
cj1427::CAT
cj1428::CAT
cj1430::CAT
cj1427::CATΔ
KpsM

B)


Infection for 2 hrs
Gm 1 hr
X hrs
Lysis
Percent survival
100
80
60
40
20
0
0
1
2
3
Time post infection (hrs)
WT
cj1427::CAT
cj1428::CAT
cj1430::CAT
cj1427::CATΔ
KpsM

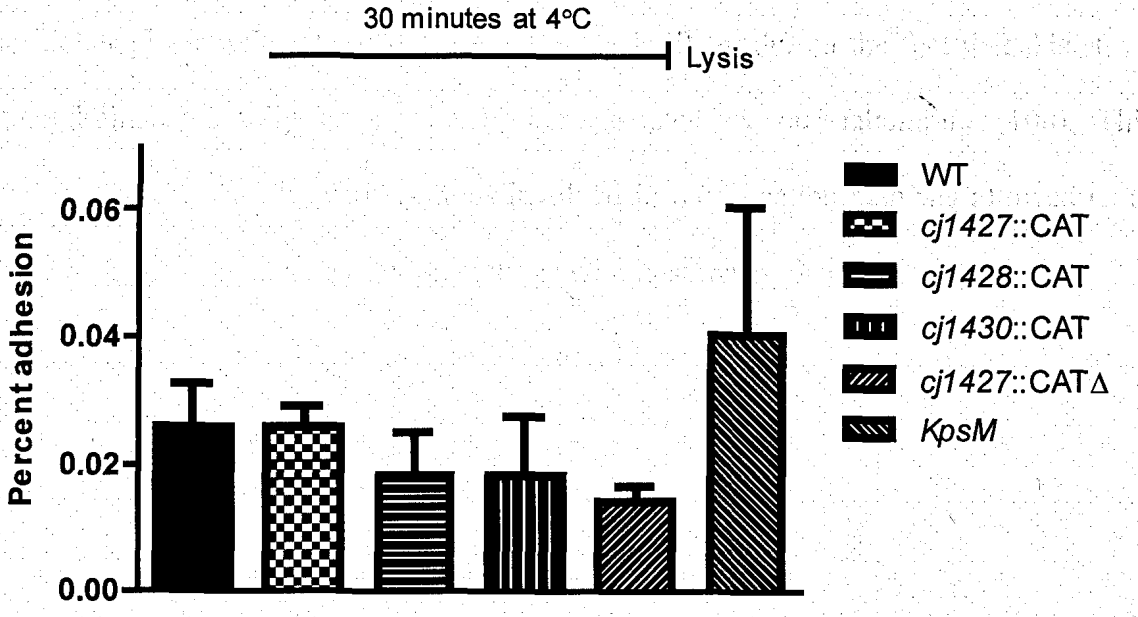
3.4.3 Effect of gene disruption on adhesion to macrophages

Upon analyzing data from survival within macrophages, subtle differences prior to the survival testing were observed at timepoint zero between the wild type and mutant strains of *C. jejuni*. While this discrepancy was found to have a p value > 0.05, we hypothesized phagocytosis or adhesion were responsible for these subtle differences. A higher adhesion rate of the wild type bacteria could explain why there was a higher initial bacterial load within macrophages. Vice versa, a lower adhesion rate of the mutants would explain the lower initial amount of bacteria within macrophages.

For the adhesion assay, both the wild type and mutant *C. jejuni* were incubated with macrophages for 30 minutes at 4°C, a temperature known to inhibit phagocytosis (180). This low temperature allows for any bacteria that interact with the macrophages to be a result of bacterial adhesion, and not as a result of phagocytosis. Results indicate that none of the mutants adhered significantly more or less to macrophages than the wild type strain (**Figure 16**). The slightly higher adhesion rate of the *KpsM* mutant may be attributable to the loss of its capsule, exposing underlying adhesins, as previously hypothesized by our laboratory (100).

Figure 16. Adhesion of the wild type and mutant strains to macrophages.

The adhesion rate of wild type and mutant strains of *C. jejuni* to macrophages was investigated. Percent adhesion was calculated as the number of bacteria recovered from macrophages divided by the number of bacteria recovered under the same growth conditions, in the absence of macrophages. A schematic at the top of the figure represents the experimental layout. Macrophages were exposed to bacteria for 30 minutes at 4°C to allow for bacterial-macrophage interactions to occur before being lysed and plated for CFU counts. Results shown are the mean and standard errors from three independent experiments. No significant differences were found between the wild type and the mutants when a one-way ANOVA test was conducted.

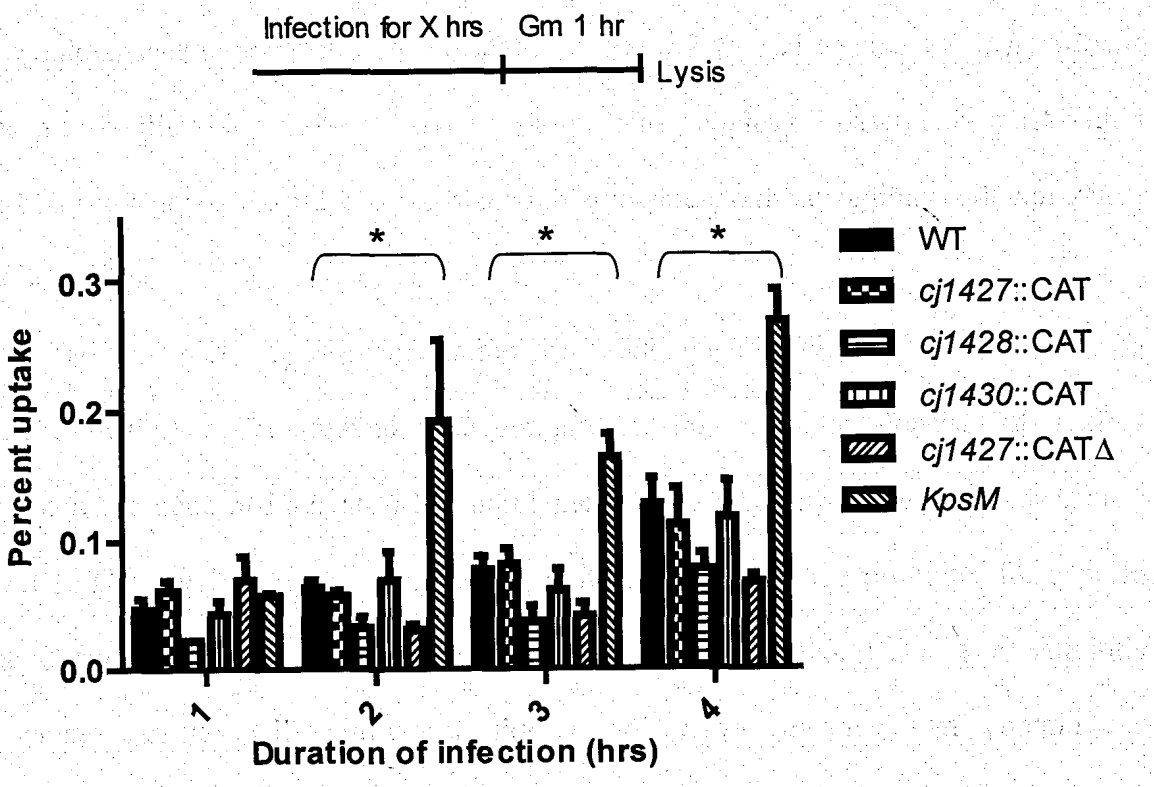

30 minutes at 4°C
Lysis
Percent adhesion
0.06
0.04
0.02
0.00
WT
cj1427::CAT
cj1428::CAT
cj1430::CAT
cj1427::CATΔ
KpsM

3.4.4 Effect of gene disruption on phagocytosis by macrophages.

A crucial aspect of *C. jejuni* interactions with macrophages is phagocytosis. To study phagocytosis of *C. jejuni*, macrophages were incubated with wild type and mutant *C. jejuni* for various durations prior to the gentamicin treatment and subsequent lysis and enumeration of the bacteria via CFU counts. The increase in uptake of the wild type strain over time is intuitive; with a longer infection time, more bacteria are phagocytosed by macrophages (**Figure 17**). The mutant strains, with the exception of the *KpsM* mutant, showed similar degrees of phagocytosis by the macrophages over the same time period. The higher phagocytic rate of the *KpsM* mutant is likely due to the fact that this strain autoagglutinates readily as previously demonstrated by our laboratory (100). This detailed dissection of the various steps involved in the infection pathway allowed us to pinpoint the kinetics of *C. jejuni* interactions with host macrophages.

Figure 17. The uptake rate for the wild type but not the mutant strains increases with time.

The phagocytosis of wild type and mutant *C. jejuni* strains into macrophages was investigated over a period of four hours. Percent uptake was calculated as the number of bacteria recovered from macrophages divided by the number of bacteria recovered under the same growth conditions, in the absence of macrophages. The schematic at the top of this figure indicates the experimental layout, where X represents the infection time. Gm = gentamicin. Results shown are the mean and standard errors from at least three independent experiments. An increase in percent internalization for the wild type strain was observed with longer infection times. The *KpsM* mutant was found to be significantly different ($p < 0.05$) compared to the wild type at the same time point, as per a one-way ANOVA, and is indicated by the asterisks (*).


Infection for X hrs
Gm 1 hr
Lysis
Percent uptake
0.0
0.1
0.2
0.3
*
*
*
1
2
3
4
Duration of infection (hrs)
WT
cj1427::CAT
cj1428::CAT
cj1430::CAT
cj1427::CATΔ
KpsM

3.5 THE GENERATION AND GENETIC CHARACTERIZATION OF COMPLEMENTED CPS MUTANTS

3.5.1 Complementation of disrupted genes via insertion into the 16S rRNA – 23S rRNA intergenic space region

To determine whether the observed phenotypes in the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT mutants are gene specific, complementation must be carried out. The intragenic spacer region between the 16S rRNA and 23S rRNA genes of *C. jejuni* was chosen to facilitate integration into the genome. Three copies of these genes exist within the *C. jejuni* genome, and complementation into one of these regions will not affect viability (87).

To construct the complementation constructs, gene splicing by overlap extension (gene SOE-ing) was employed. The complementation construct consists of a *porA* promoter, the gene of interest to be complemented, followed by an antibiotic resistance cassette. The *porA* promoter was chosen as it is a highly active promoter for a porin protein and will drive expression of the complementation construct. The antibiotic resistance cassette will allow selection of *C. jejuni* mutants that contain the complementation construct, once transformants are obtained.

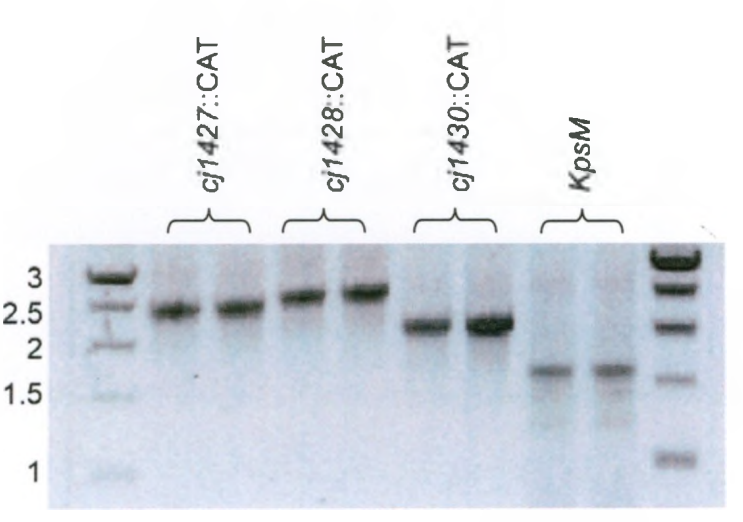
Gene SOE-ing of each construct to complement our mutants (*cj1427*::CAT, *cj1428*::CAT, *cj1430*::CAT and *KpsM*) was done. As previously elucidated, the *cj1427*::CATΔ mutant contains a large area of the genome deleted, and complementation for this mutant was not attempted. Gene SOE-ing of each complementation construct was successful; this was verified via PCR (**Figure 18**).

The complementation constructs were inserted into a pBluescript vector containing the 16S and 23S rRNA regions of *C. jejuni*, constructed by Xuan Thanh Bui, a PhD student in our laboratory. This plasmid was then mobilized into *E. coli*, and transformants selected for and screened. The *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT complementation constructs were successfully mobilized into *E. coli*, and sequenced to ensure the constructs did not have any point mutations that could affect the subsequent function of the protein. Results showed that the *cj1427*::CAT and *cj1428*::CAT mutants had single nucleotide polymorphisms (SNPs), while the *cj1430*::CAT mutant did not. The SNPs in the *cj1427*::CAT mutant were found to be deleterious and were fixed via QuikChange site directed mutagenesis. The SNPs in the *cj1428*::CAT mutant resulted in amino acid changes that were determined to not affect protein function.

At the time of submission for examination, only the *cj1428*::CAT complementation construct was selectively methylated before mobilization into *C. jejuni.* Screening of the potential *cj1428*::CAT transformants was carried out using PCR; results showed that the complementation construct was within *C. jejuni* (**Figure 19A**); however, the complementation construct did not recombine into the 16S – 23S rRNA area of interest (**Figure 19B**). Further PCR screening also revealed that the complementation construct did not integrate into either the *cj1428c* gene area or the *porA* promoter region (**Figure 19C** and **D**, respectively). Despite this setback, this complemented mutant was used in the phenotypic studies that were conducted to determine whether the previously investigated phenotypes were gene specific.

Figure 18. Construction of the complementation constructs via gene SOE-ing.

PCR products showing the successful construction of each complementation mutant is pictured. Each complementation construct was loaded in duplicate. A DNA ladder with the corresponding sizes in kilobase pairs is shown on the left. A schematic shows the primers and the area that was amplified via PCR. The inset table shows the expected sizes for the complementation constructs.



Complementation construct	Expected size
cj1427::CAT complement	2465 bp
cj1428::CAT complement	2564 bp
cj1430::CAT complement	2069 bp
KpsM complement	1647 bp

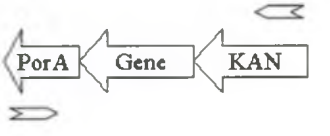
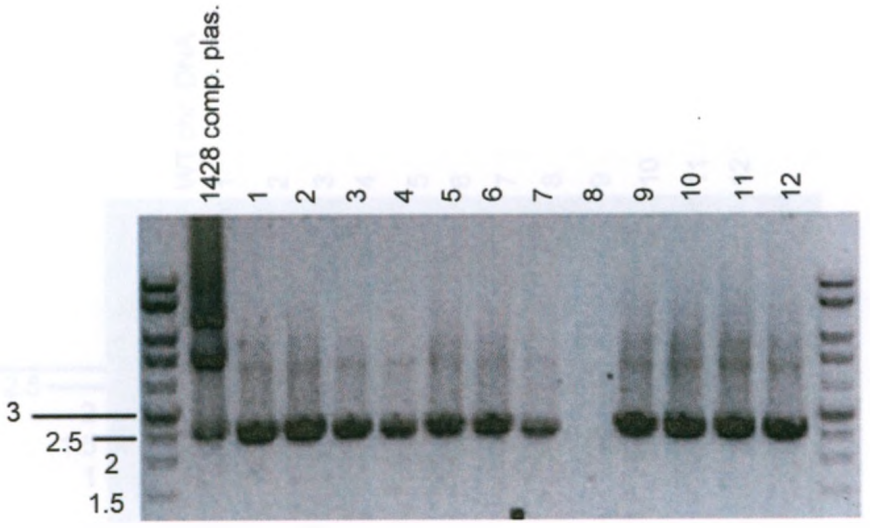


Figure 19. PCR screening of potential *cj1428*::CAT complement transformants in *C. jejuni*.

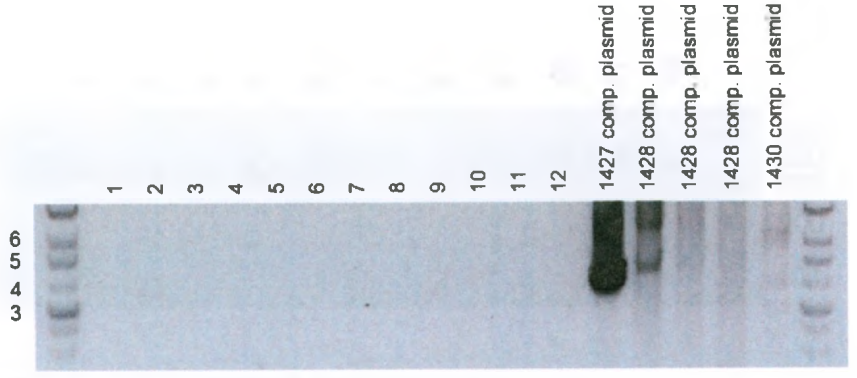
In all panels, a schematic shows the area of interest that was amplified via PCR. An inset table shows the expected sizes of the fragments. Numbers at the top of each gel indicate the various colonies screened. **A) The *cj1428*::CAT complementation construct was successfully transformed into *C. jejuni*.** A PCR showing the complementation construct was successfully introduced into *C. jejuni*. KAN = kanamycin. Comp. plas. = complementation plasmid **B) The *cj1428*::CAT complementation construct did not integrate into the desired area.** PCR using primers between the *porA* and 23s rRNA areas was carried out. The lack of a band indicates that the complementation construct did not integrate into the desired area. KAN = kanamycin. Comp = complementation **C) The *cj1428*::CAT complementation construct did not integrate into the *cj1428c* gene area.** PCR showed that the complementation construct did not integrate into the *cj1428c* gene within the capsular cluster, as a band corresponding to the chloramphenicol disrupted *cj1428c* gene only was observed in all potential transformants. The box within the *cj1428c* gene represents the chloramphenicol antibiotic resistance cassette. Chr = chromosomal. **D) The *cj1428*::CAT complementation construct did not integrate into the *porA* promoter region.** PCR shows that the complementation construct did not integrate into the *porA* gene within the *C. jejuni* genome, as bands of the size corresponding to the wild type *porA* gene size were observed in all potential transformants.

A)

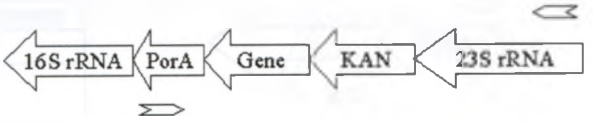


cj1428::CAT complement	Expected size
porA - Kanamycin	2564 bp

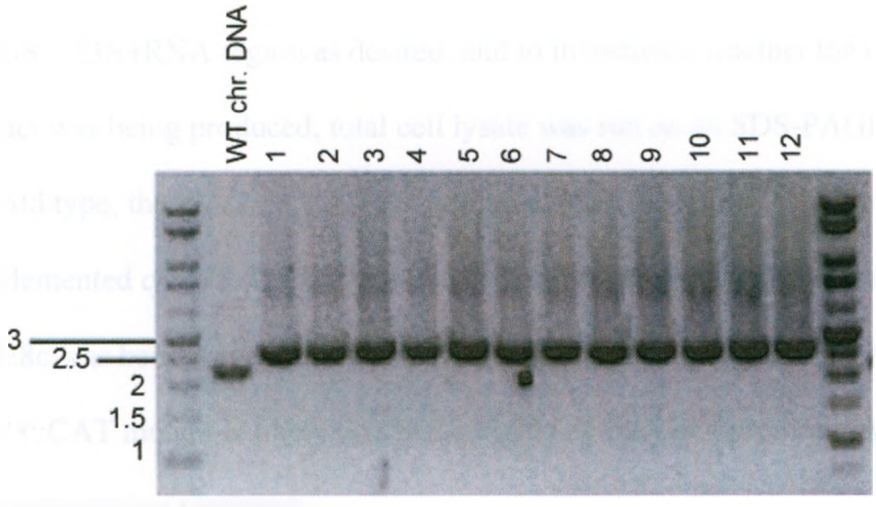
B)



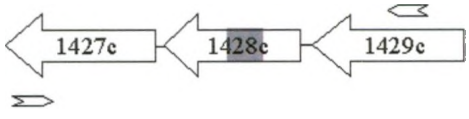
cj1428::CAT complement	Expected size
porA – 23S rRNA	3788 bp



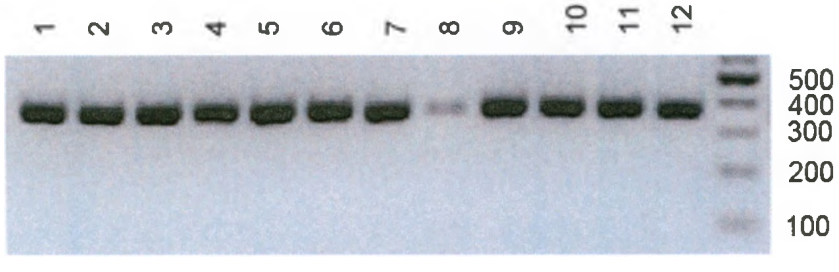
C)



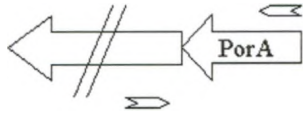
Fragment	WT	*cj1428*::CAT complement
cj1427c – cj1429c	2194 bp	2929 bp



D)



Fragment	*cj1428*::CAT complement
cj1259 – porA	343 bp
porA with complementation construct	2907 bp

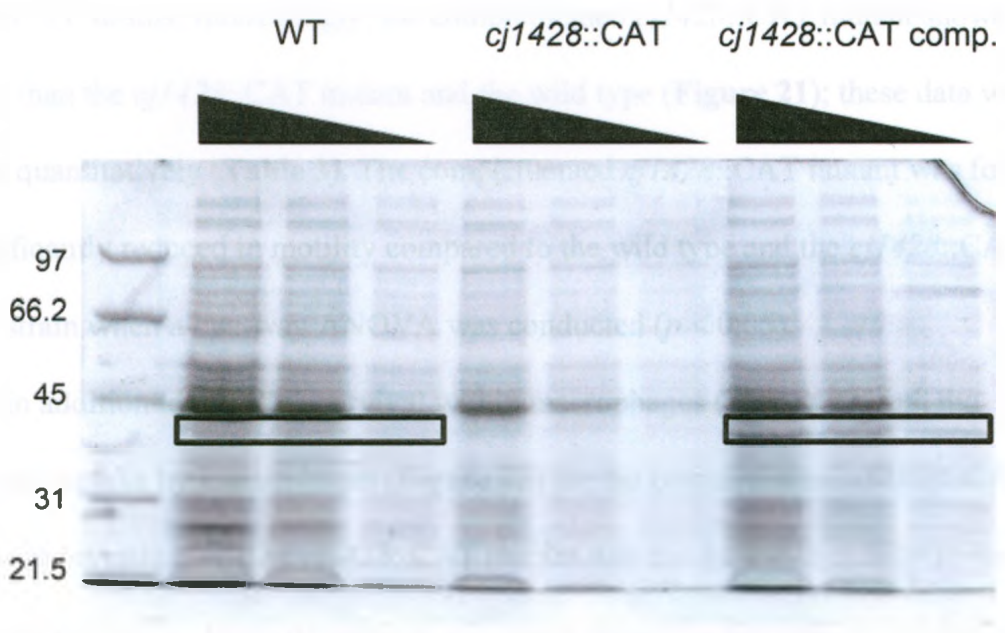


3.5.2 Evidence that the complemented *cj1428*::CAT mutant produces the *cj1428c* gene product

Surprisingly, the *cj1428*::CAT complementation construct did not integrate into the 16S – 23S rRNA region as desired, and to investigate whether the *cj1428c* gene product was being produced, total cell lysate was run on an SDS-PAGE gel. Compared to the wild type, the *cj1428*::CAT mutant did not have a band of the expected size, while the complemented *cj1428*::CAT mutant had a band of the expected size, indicating that Cj1428c was being produced. The higher level that was observed in the complemented *cj1428*::CAT mutant is likely due to the highly active *porA* promoter that is part of the complementation construct.

Figure 20. Coomassie staining of total proteins shows that the *cj1428c* gene product is being produced in the complemented *cj1428*::CAT mutant.

Total proteins of the WT, *cj1428*::CAT and complemented *cj1428*::CAT strains were run on an SDS-PAGE gel. The relative amounts loaded are indicated by the black triangles. The calculated size of the *cj1428c* gene product was 39.4 kDa. Bands of the expected size were observed in the WT and the complemented *cj1428*::CAT mutant (boxed), and was absent in the *cj1428*::CAT mutant. The higher levels of expression of the *cj1428c* gene product in the complemented *cj1428*::CAT mutant is likely due to the higher activity of the *porA* promoter.


WT
cj1428::CAT
cj1428::CAT comp.
97
66.2
45
31
21.5

3.5.3 Analysis of the complemented *cj1428*::CAT mutant phenotypes

Despite the *cj1428*::CAT complementation construct not recombining into the 16S – 23S rRNA region that was desired, studies were carried out to determine whether the phenotypes which previously showed significant differences compared to wild type could be complemented. Restoration of the phenotypes in the complemented *cj1428*::CAT mutant would indicate gene specificity.

Motility assays were conducted for the *cj1428*::CAT and complemented *cj1428*::CAT strains. Interestingly, the complemented *cj1428*::CAT mutant showed less motility than the *cj1428*::CAT mutant and the wild type (**Figure 21**); these data were also verified quantitatively (**Table 3**). The complemented *cj1428*::CAT mutant was found to be significantly reduced in motility compared to the wild type and the *cj1428*::CAT mutant strain when a one-way ANOVA was conducted ($p < 0.05$).

In addition to motility, survival within macrophages (**Figure 22**) and the phagocytic uptake by macrophages (**Figure 23**) for the complemented *cj1428*::CAT strain were investigated. The *cj1428*::CAT mutant was conducted in parallel to serve as an internal control. In terms of survival, all strains showed a decrease in survival with increasing time. The *cj1428*::CAT showed a lower survival rate within macrophages, but similar, or slightly slower killing kinetics compared to the wild type strain. The complemented *cj1428*::CAT mutant showed a significant increase in survival compared to the *cj1428*::CAT mutant and at levels similar to wild type at three timepoints, indicating that partial complementation occurred. A significantly slower killing of the complemented *cj1428*::CAT mutant was observed at one timepoint. This result cannot be rationalized at this time, and will require re-visiting in the future.

For phagocytic uptake by macrophages, as observed previously, an increase in uptake occurred with an increase in infection time for the wild type strain. The *cj1428*::CAT strain was phagocytosed poorly by macrophages, and an increase in uptake was not observed until longer durations of infection were used. The phagocytosis rate of the complemented *cj1428*::CAT mutant appeared to be similar to that of the wild type. When comparisons between the *cj1428*::CAT mutant and the complemented *cj1428*::CAT mutant were made as per a one-way ANOVA, there was no significant difference found between the two, except at the first timepoint. This suggests that only partial complementation of the *cj1428*::CAT mutant occurred.

Figure 21. Motility assay for wild type, *cj1428*::CAT and complemented *cj1428*::CAT mutant.

The motility of the wild type, *cj1428*::CAT and complemented *cj1428*::CAT strains were measured and compared after stabbing into 0.3% agar and incubation for 48 hours under microaerophilic conditions. Plates were incubated for 48 hours before halos were measured. Shown in this figure is a representative motility plate.

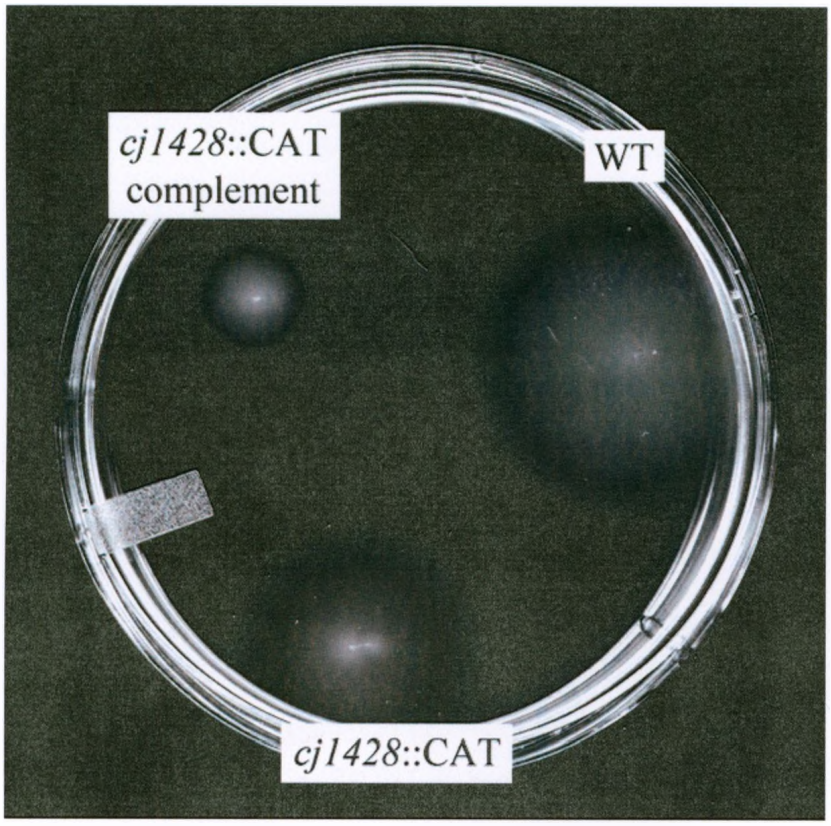

cj1428::CAT
complement
WT
cj1428::CAT

Table 3. Quantitative assessment of the motility of the complemented *cj1428*::CAT mutant.

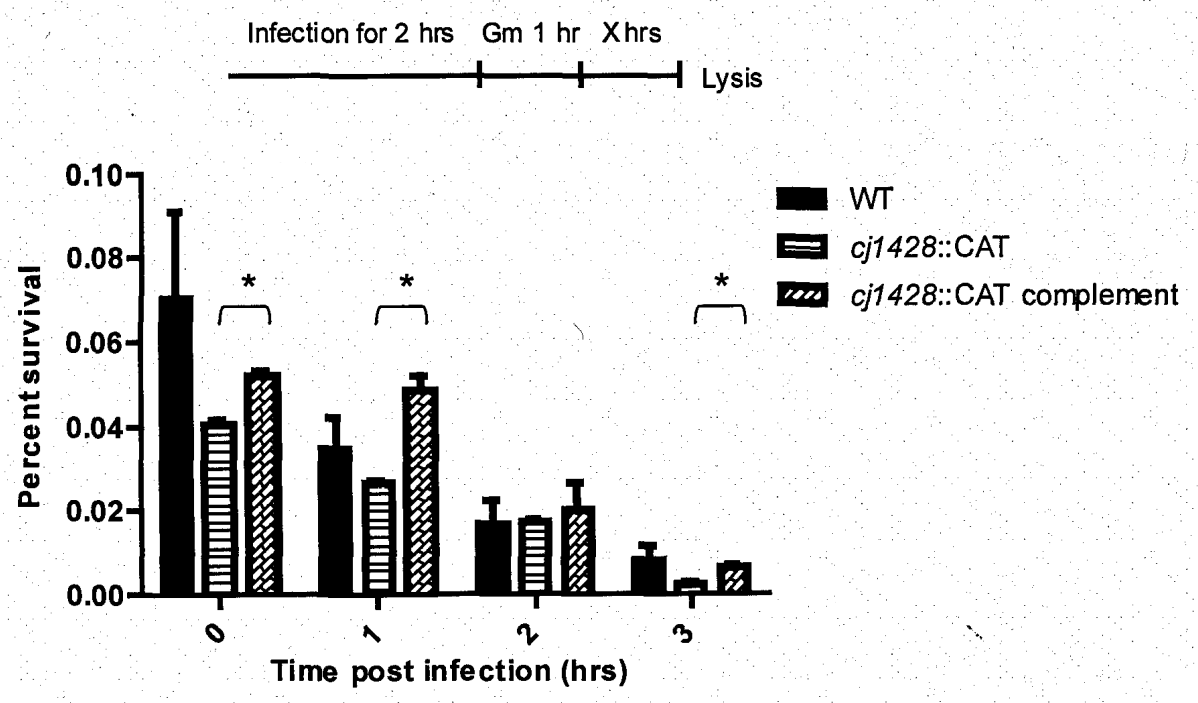
The motility of the wild type, *cj1428*::CAT and complemented *cj1428*::CAT strains were measured and compared quantitatively after stabbing into 0.3% agar and incubation for 48 hours under microaerophilic conditions. The values shown are for the diameters of the motility halos, measured in centimetres. The mean and standard errors (SE) of three independent experiments are shown for each strain. Compared to the wild type, the *cj1428*::CAT and complemented *cj1428*::CAT mutants were significantly less motile.

***C. jejuni* strain**	**Diameter of halo (cm) ± SE**	***p* value (compared to WT)**	***p* value (compared to *cj1428*::CAT)**
WT	3.10 ± 0.10	N/A	N/A
cj1428::CAT	1.90 ± 0.06	< 0.001	N/A
cj1428::CAT complement	1.20 ± 0.05	< 0.001	< 0.001

Figure 22. The ability of *C. jejuni* to survive in macrophages is partially restored in the complemented *cj1428*::CAT strain.

The survival rate of wild type, *cj1428*::CAT and complemented *cj1428*::CAT strain was investigated over a time period of three hours. The schematic at the top of this figure indicates the experimental layout, where X represents the number of hours post infection. Gm = gentamicin. Results shown are the mean and standard errors from three independent experiments. As the length of time post infection increases, the number of bacteria able to survive decreases. The complemented *cj1428*::CAT mutant's ability to survive within macrophages was restored to wild type levels, indicating complementation occurred. **A)** Results are expressed as percent survival; CFUs of bacteria exposed to macrophages were taken as a ratio to the CFUs of bacteria that were incubated under the same conditions, with the exception of not being exposed to macrophages. A one-way ANOVA test showed that there was a significant difference between the survival rate of the *cj1428*::CAT mutant and the complemented *cj1428*::CAT mutant, as indicated by the asterisks (*). **B)** The data for each strain at timepoint zero in panel **A)** were normalized to 100 percent survival. Both the *cj1428*::CAT mutant and the complemented *cj1428*::CAT strain were killed slower than the WT strain. At 1 hour post infection, a significantly slower killing rate was observed between the wild type and the complemented *cj1428*::CAT mutant, as indicated by the asterisk (*).

A)


Infection for 2 hrs
Gm 1 hr
Xhrs
Lysis
Percent survival
0.10
0.08
0.06
0.04
0.02
0.00
*
*
*
0
1
2
3
Time post infection (hrs)
WT
cj1428::CAT
cj1428::CAT complement

B)

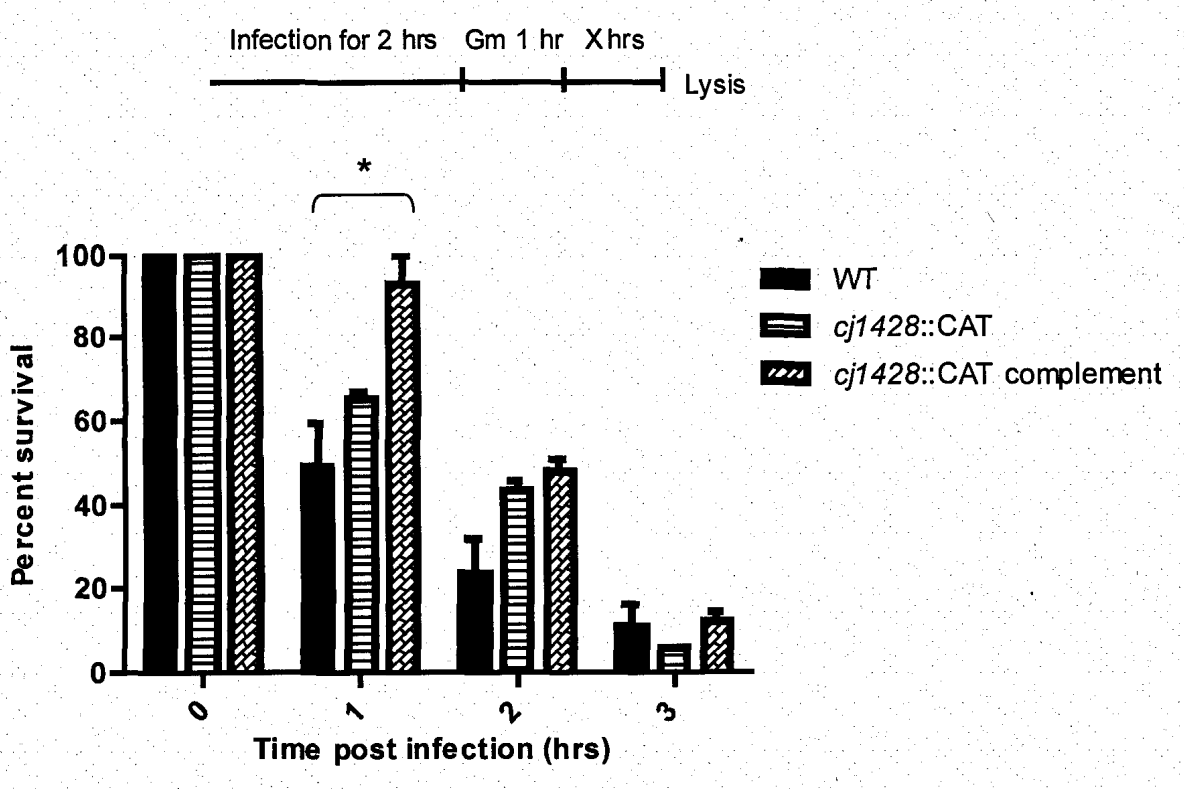
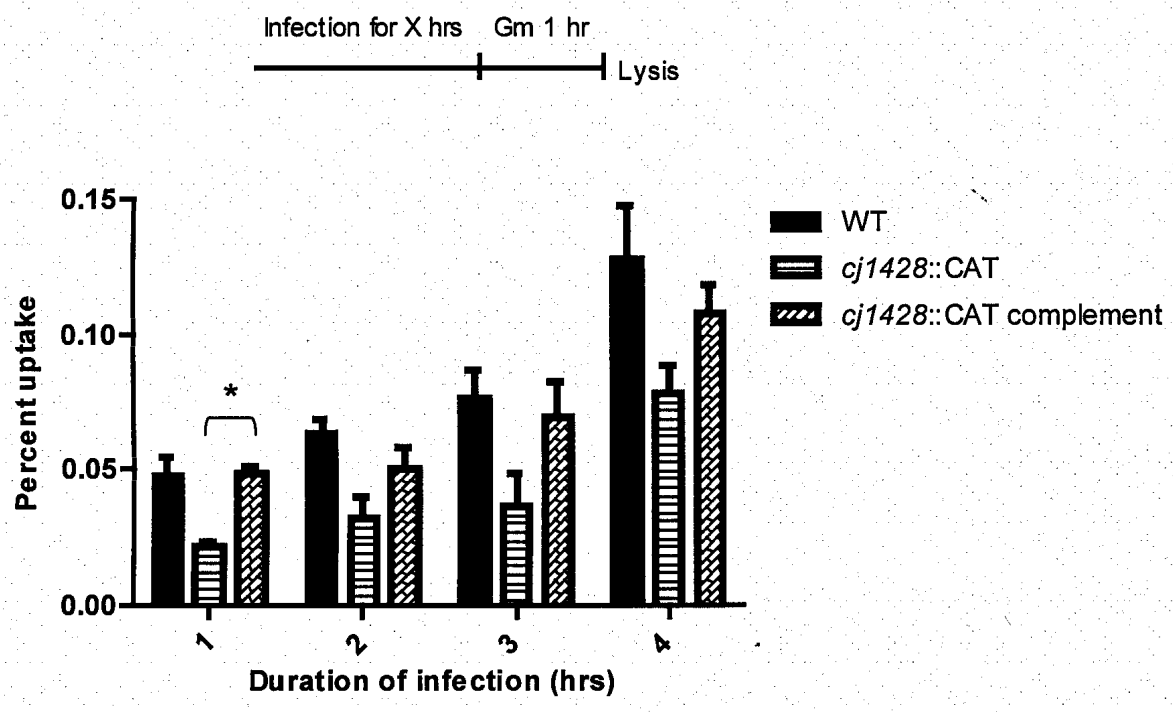

Infection for 2 hrs
Gm 1 hr
Xhrs
Lysis
*
Percent survival
100
80
60
40
20
0
0
1
2
3
Time post infection (hrs)
WT
cj1428::CAT
cj1428::CAT complement

Figure 23. The internalization rate for the complemented *cj1428*::CAT mutant is partially restored to wild type susceptible levels.

The internalization rate of wild type, *cj1428*::CAT and complemented *cj1428*::CAT strains in macrophages was investigated over a period of four hours. Results are expressed as percent internalization; CFUs of bacteria exposed to macrophages were taken as a ratio to the CFUs of bacteria that were incubated under the same conditions, with the exception of not being exposed to macrophages. The schematic at the top of this figure indicates the experimental layout, where X represents the infection time. Gm = gentamicin. Results shown are the mean and standard errors from three independent experiments. For the wild type strain, with an increase in time the bacteria were allowed to infect macrophages, an increase in percent internalization was observed. The internalization rate of the complemented *cj1428*::CAT mutant was restored to wild type levels, particularly when a 1 hour infection was conducted. At this timepoint, a significant difference was observed between the *cj1428*::CAT mutant and the *cj1428*::CAT complemented mutant, as indicated by the asterisk (*) ($p < 0.05$).


Infection for X hrs
Gm 1 hr
Lysis
0.15
0.10
0.05
0.00
Percent uptake
*
1
2
3
4
Duration of infection (hrs)
WT
cj1428::CAT
cj1428::CAT complement

CHAPTER 4 – DISCUSSION

4.1 Creation of a new *cj1427*::CAT mutant

It has been shown that the CPS present on the surface of *C. jejuni* is involved in the virulence of strain ATCC 81-176 (13). Using structural data that was available for *C. jejuni* ATC 700819, our laboratory has previously identified three gene targets in the CPS biosynthesis cluster, *cj1427c*, *cj1428c* and *cj1430c*, that are putatively known to function in the synthesis of a modified heptose. To prove this, we have also previously constructed *cj1428c, cj1430c* and *KpsM* antibiotic insertional knockout mutants. Unfortunately, a *cj1427c* insertional mutant was not achieved, and to complete our panel of mutants, a *cj1427c* mutant was made as part of the work in this thesis.

Prior attempts to create a *cj1427c* insertional mutant in our laboratory were unsuccessful. RT-PCR data revealed that the genes *cj1426c* and *cj1425c* did not have any level of expression in the clones obtained indicating that a gene deletion event likely occurred. Based on these data, PCR was carried out on areas flanking these two genes to confirm that a gene deletion event occurred. It was shown that not only were *cj1426c* and *cj1425c* deleted, but the area extending to, and including a portion of *cj1421c*, was deleted. This deletion was verified via DNA sequencing.

C. jejuni is known to have a genome sequence that possesses very few repeat sequences (131). However, due to the initial method of transforming *C. jejuni* via natural transformation (19, 60, 167, 169), whereby the *cj1427*::CAT disruption construct was ligated to genomic DNA, it is possible that introduction of additional copies of chromosomal DNA lead to homologous recombination in areas that were not of interest. It is likely that an unwanted homologous recombination event occurred during the initial construction of the *cj1427*::CATΔ mutant, resulting in the large deletion of genes in the

capsular cluster. As mentioned previously, the deletion of these genes results in a *C. jejuni* CPS that is significantly altered in structure; not only is the capsule heptoseless, but it is also free of the side-branching phosphoramidate molecule. It has been previously shown that the lack of phosphoramidate on the CPS of *C. jejuni* causes attenuation in virulence (27), and may explain the phenotypic differences that were previously investigated for this mutant (100).

Due to the inability to control for unwanted homologous recombination events, a method outlined by Donahue *et al* (36) was adapted and used for the creation of a new *cj1427*::CAT mutant. As the method involves site-specific and species-specific methylation of foreign plasmid DNA, the advantages are two-fold: 1) there will be no unwanted homologous recombination as additional copies of chromosomal DNA are not being introduced into *C. jejuni* and 2) methylation of the plasmid DNA will result in a methylation signature that is unique to *C. jejuni* ATCC 700819, allowing for extremely efficient transformation.

Following transformation of wild type *C. jejuni* with the disruption construct, all potential transformants were verified using conventional PCR and also DNA sequencing. The resulting *cj1427*::CAT mutant is the one that has been used for all genetic and phenotypic studies conducted in this thesis.

4.2 The expression levels of CPS related genes changes in response to gene disruption

To investigate the transcription levels of genes located downstream of the disrupted genes, RT-PCR was carried out. As expression levels of genes can change drastically depending on the life cycle of the bacteria, a specific time point must be

chosen to harvest the bacteria for RNA isolation. For this, we chose a timepoint 20 – 24 hours post inoculation as *C. jejuni* would be actively growing in the exponential phase, and, based on previous experiments, expressing CPS (**Figure 6A, B**). This timepoint was kept consistent for the harvesting of RNA from all strains. In addition, this timepoint was chosen as phenotypic studies conducted in this thesis were also conducted within the same timeframe.

To gain a better understanding of the expression levels of the capsular cluster of genes, RT-PCR was conducted for each strain in our possession. As mentioned previously, the *C. jejuni* capsular cluster of genes shows similarity to the one observed in *E. coli*, and it was thought that a single promoter would control the genes. It was expected that the genes within the cluster would show similar levels of expression, or perhaps genes with greater distance from the promoter would show lower levels of expression (175). However, against expectations, these data revealed that the level of gene expression across the capsular cluster varied significantly. While most genes are expressed at levels similar to the housekeeping gene, some genes (*kpsM, cj1447c, cj1430c, cj1429c*) appeared to be expressed at lower levels, while *cj1426c* appeared to be expressed at higher levels. KpsM is an autotransporter for capsule and Cj1447c is a probable capsule polysaccharide export ATP-binding molecule, so their low levels of expression may simply be due to a lack of requirement for these catalytic enzymes to be present in high quantities. As the downstream genes *cj1445c* and *cj1444c* are expressed at levels comparable with other genes within the capsular cluster; this may be indicative of promoters that are present either in the intergenic areas between *cj1447c* and *cj1445c* or possibly as part of the end of *cj1447c*. Similarly, *cj1430c* and *cj1429c* show very low

levels of expression, while downstream *cj1428c* shows levels comparable to the other genes within the capsular cluster, again indicating the possibility of promoter sequences within the *C. jejuni* capsular cluster. To investigate this possibility, promoter prediction software was used in an attempt to predict the location of potential cryptic promoter sites. However, currently available software was insufficient for our needs as the foundation of such prediction software is based on *E. coli*. This poses a problem as the *C. jejuni* –35 region was found to be different from the *E. coli* –35 region (175), and thus, software prediction was not suitable. Future potential work may include the investigation of these cryptic promoters using a reporter gene system (i.e. *lac* system (177)) for analysis.

Alternative to the hypothesis of cryptic promoters within the capsular cluster is the possibility that the measured low levels of expression could reflect the inherent instability of the RNA for these genes. It is known that differential mRNA degradation is an important step in the regulation of prokaryotic gene expression (93). KpsM and Cj1447c play a role in transporting CPS and acting as an ATPase, respectively. As these enzymes would be catalytic in nature, it is possible that high levels of these mRNA are not required by *C. jejuni*, and as a result, additional mRNA is quickly degraded by the cell.

Following the investigation of the intrastrain gene expression, an interstrain comparison was performed, whereby the expression levels of the genes in each mutant strain were taken as a ratio over the respective genes in the wild type strain. This was done to determine whether the insertion of the antibiotic resistance cassette had polarity effects on downstream genes. In general, it was found that genes immediately downstream of the resistance cassette in the mutant strains did not significantly differ

from the expression levels of the respective genes in the wild type strain. This is consistent with the fact that the antibiotic resistance cassettes, by design, were non-polar. Interestingly, in the *cj1430*::CAT mutant strain, the downstream *cj1429c* gene showed highly significant upregulation compared to the same gene in the wild type strain. From the other mutant strains in our collection, genes downstream of the resistance cassette do not show significant changes in expression levels and we can conclude the upregulation of *cj1429c* in *cj1430*::CAT is not due to polarity effects of the chloramphenicol resistance cassette. While *cj1429c* is part of the *C. jejuni* capsular cluster (131), its function is currently unknown. The phenotypes attributable to this gene will be further pursued by our laboratory in the future using the same mutagenesis method that was used in this thesis to study the effect of the heptose modification genes.

Another observation from the RT-PCR data was that *cj1426c* was upregulated in the mutant strains. The gene *cj1426c* is a putative methyltransferase and is hypothesized to facilitate the transfer of a methyl group to carbon 6 of D-*glycero*-L-*gluco*-heptose (61, 131). The capsular structure of the wild type and mutant strains has been elucidated via NMR, and based on the data, it was found that none of the mutants produce a heptose. It is puzzling that *cj1426c* is upregulated, as the molecule that requires methylation is no longer present in the capsule of the mutants. It is possible that as a compensatory mechanism, upregulation of *cj1426c* occurs which results in an increase in the measured levels of expression. Precisely why this gene is upregulated is yet unknown, and may be studied in the future.

The *cj1427*::CATΔ mutant was found to lack modified heptose and also the phosphoramidate molecule. In this mutant, genes between *cj1426c* and *cj1421c*, inclusive,

have been deleted. Genes *cj1423c – cj1426c* have been identified as being involved with the synthesis of the modified heptose precursor GDP-*glycero-manno*-heptose, while genes *cj1415c – cj1422c* play a role in the production and transfer of the *O*-methyl phosphoramidate group to the N-acetyl galactosamine of the capsular backbone (112). As the genes for the synthesis of GDP-*glycero-manno*-heptose have been abolished, the *cj1427*::CATΔ mutant will not only lack a modified heptose, but will also lack a normal, non-modified heptose. In addition, *cj1421c* function has been abolished in the *cj1427*::CATΔ mutant, resulting in a linear capsule, free of any side-branching molecules. It is interesting to note that while synthesis of GDP-*glycero-manno*-heptose has been abolished, expression levels of genes responsible for modifying heptose (*cj1428c, cj1430c*) remain unchanged compared to levels in the wild type strain, indicating that there is no feedback to repress the expression of these genes. It is thus puzzling why the disruption of a single gene involved in heptose modification results in feedback within the capsular cluster while deletion of many genes does not.

As previously mentioned, with the exception of the *cj1427*::CATΔ mutant, which possesses a capsule free of any side-branching molecules, all other mutants show either a trace or the positive confirmation of phosphoramidate in the capsule, while the wild type strain does not. In the future, it will be interesting to investigate phosphoramidate related genes to determine possible upregulation of these genes in response to a lack of modified heptose as a compensatory mechanism to decorate the capsule.

4.3 Disruption of *cj1427c*, *cj1428c*, and *cj1430c* affects CPS composition

The CPS present on the surface of *C. jejuni* ATCC 81-176 and ATCC 81116 has been shown to be involved in virulence (12, 13). To determine whether the modified

heptose in the CPS of ATCC 700819 plays a role in virulence as well, we identified three genes, *cj1427c, cj1428c* and *cj1430c* that are putatively involved in modified heptose biosynthesis, and have shown their involvement in CPS synthesis via an antibiotic insertion knockout strategy. Using purified capsular samples and analysis via SDS-PAGE, it was revealed that all the mutants showed a differential mobility of the capsular bands when compared to wild type, suggesting that the enzyme products of the targeted genes affected capsular composition.

The *cj1427*::CAT, *cj1428*::CAT and *cj1427*::CATΔ mutants displayed similar capsular staining patterns when compared to each other (**Figure 6A, B**). Interestingly, the *cj1427*::CAT and *cj1428*::CAT mutants showed a different modality of CPS compared to the wild type strain. A wide range of both lower and high molecular weight species were observed in these two mutants, indicating that the disruption of these genes results in changes in chain elongation of the CPS.

In the *cj1430*::CAT mutant, the pattern obtained was significantly different from that of the other mutants, suggesting that the composition of this mutant's CPS was different from the other mutants. This is puzzling, as both Cj1428c and Cj1430c are thought to be putative epimerases. These two enzymes epimerize different carbons of the heptose, so it may be possible that the different actions of these enzymes would be reflected in the capsular staining pattern. However, the differences in staining pattern cannot be due to differences in capsular structure, as NMR has shown that both the *cj1428*::CAT and *cj1430*::CAT mutants lack a heptose in their capsules. In addition to the differential staining pattern of the *cj1430*::CAT mutant, the amount of CPS that is produced by this mutant is significantly less than the other mutants that were investigated.

It is possible that disruption of this gene, and subsequently the enzyme that it encodes, affects the transport of CPS across the periplasmic space. This would cause an accumulation of CPS within the bacterium. As a result, this mutant behaves much like a *KpsM* mutant, in that both are unable to transport CPS. Initial work in our laboratory has shown that Cj1430c is one of the first enzymes to act in the heptose modification pathway; its disruption could potentially have significant downstream effects leading to a down regulation in the amount of CPS that is produced.

Interestingly, the *KpsM* mutant also showed potential high molecular weight species, suggestive of CPS, despite the fact that this mutant lacks the KpsM transmembrane protein responsible for transport of the CPS (20). It is known that the *KpsM* mutant is acapsular (89), however, while transport of the CPS across the periplasmic space of the bacterium is disrupted, CPS initiation and subsequent elongation still occurs. These molecules of assembled CPS, while not apparent on the cell surface of *C. jejuni*, would be visualized during silver staining.

4.4 CPS purification for downstream analysis

Purification of CPS is important, as one goal of this purification is to perform nuclear magnetic resonance (NMR) analysis so that its composition can be determined. This analysis was able to give a glimpse of the precise composition of the capsule of the wild type and mutant strains. More importantly, we can also determine the effects, if any, of disrupting the *cj1427c*, *cj1428c* and *cj1430c* genes on modified heptose synthesis. However, a barrier to downstream analysis is that two molecules of heptose also exist in the LOS core of *C. jejuni* (158). Thus, to remove LOS, purification of CPS from wild type, *cj1427*::CAT, *cj1428*::CAT, *cj1430*::CAT, *KpsM* and *cj1427*::CATΔ was attempted.

Previous attempts in our laboratory (100) showed that the SDS solubilization method was not effective at removing LOS from capsular preparations. As a result, the hot water/phenol method outlined by Westphal and Jaan was used (171). Part of this method involves dialyzing phenol away from the aqueous phase after the hot water/phenol purification. As the principle of dialysis relies on simple diffusion, it was thought that this extra step may have the added benefit of allowing LOS to diffuse out of the sample. It was previously shown that LOS was significantly smaller than CPS (38), and an appropriately sized dialysis tubing with a molecular weight cut off of 12-14000 Daltons was chosen. With this cut off, molecules that are larger than 14000 Da, such as CPS were retained within the tubing, while smaller molecules, such as LOS, would diffuse out. To further remove LOS, the aqueous phase was then ultracentrifuged to pellet LOS. It was determined that ultracentrifugation was important for the removal of LOS; without this step, LOS contamination remained very high. Various durations were attempted (12, 24, 30, 48 and 72 hours), with increasing amounts of LOS being removed with longer ultracentrifugation spins. However, after 30 hours, any additional LOS that was removed was negligible. While this adapted hot water/phenol method worked well at purifying CPS, a significant amount of LOS still remained within the samples of interest, and additional purification was required.

The first purification method that was attempted after hot water/phenol purification was size exclusion chromatography. In this method, components within a given sample are separated based on their size, and hence the rate at which they move through the gel matrix. The size of LOS is significantly smaller than CPS (38), and appropriate gel matrices capable of resolving such small molecules were chosen. As CPS

molecules are large, they will not be retained by the gel matrix, and will simply flow through the column. However, based on results from silver staining, matrices that were attempted failed to separate the LOS from the CPS. It was hypothesized that the LOS and CPS were forming mixed micelles, resulting in the failure of the gel matrix to separate the LOS from the CPS. Micelles formed in this manner would have a gradient of sizes and separation via size exclusion chromatography would be impossible without their disruption.

The first attempt to disrupt the mixed micelles was based on a method by Aspinall *et al* (10), where LOS molecules are cleaved via an acetic acid treatment with heat. Ideally, this method will cleave the linkage between the lipid A anchor and Kdo sugar, resulting in smaller molecules that can then be retained by the gel matrix, while CPS molecules will not be degraded, as they are heat stable (44, 128). However, after samples were separated via size exclusion chromatography, it was found that while the acetic acid treatment was able to decrease the amount of LOS to some extent, it had no effect on separating LOS from CPS as fractions from size exclusion chromatography showed the presence of both CPS and LOS. It is possible that the LOS/CPS mixed micelles were particularly resistant to acidic treatment, and were not easily separated. In addition, upon removal of the acid via ammonium bicarbonate neutralization, it is possible that the micelles, initially disrupted by the acid treatment, reformed in a random manner.

As acetic acid treatment is quite harsh, and is conducted for a relatively long duration of time, it is conceivable that the CPS molecules may also be degraded. As a result, in parallel, an ammonium acetate treatment was also attempted. Ammonium acetate is also acidic, but much milder than acetic acid, and in principle, its action on

LOS should be the same, while CPS should not be hydrolytically cleaved by this treatment. The ammonium acetate treatment was thought to be successful at first, as LOS molecules and CPS molecules appeared in different fractions. CPS molecules were not retained by the gel matrix, and accordingly, appeared in early fractions. Contrasting this, LOS molecules were well retained by the gel matrix, appearing in later fractions (**Figure 7C**). It was thought that this separation was due to the ammonium acetate treatment, and to determine whether this was indeed the case, a tricine gradient gel was run (**Figure 7D**). Due to the increasing percentage of acrylamide at the bottom of the gel, a gradient gel will be able to resolve the differences in small molecules such as LOS. However, after silver staining of pooled fractions that contained LOS, it was observed that there was no difference between the mock-treated and the ammonium acetate treated samples. Therefore, it was concluded that this treatment had no effect on LOS molecules.

A final method of purification that was attempted was based on the method outlined by Gu and Tsai (53), where *Nesseirial* lipopolysaccharide was separated from outer membrane vesicles via a sodium deoxycholate treatment. This method was adapted for use in *C. jejuni* in an attempt to separate the LOS from the CPS molecules of interest. Sodium deoxycholate is a detergent, and should help to disrupt CPS/LOS mixed micelles. However, upon size exclusion chromatography separation, it was found again that the LOS co-eluted with the CPS, indicating that the sodium deoxycholate treatment failed to disrupt the mixed micelles of LOS and CPS. This may be due to the fact that the critical micelle concentration for the mixed LOS/CPS species is quite low, resulting in micelles that form easily while being difficult to disrupt with sodium deoxycholate. In the future, it may be worth attempting a higher sodium deoxycholate concentration to disrupt the

micellar species. One unexpected effect of the sodium deoxycholate treatment was that the banding pattern of CPS were much more readily observable via silver staining. The reason for this increased resolution of the banding pattern is unknown.

One caveat for all of the purification methods attempted above is that they rely on the assumption that once the LOS/CPS micelles are disrupted, the micelles will remain disrupted. This is not necessarily the case, and one can envision that upon removal of the disrupting agent, (acetic acid, ammonium acetate, sodium deoxycholate) the CPS and LOS molecules will reform into micelles in a random fashion. To prevent the formation of these micelles, it may be necessary to reduce the concentration of CPS and/or LOS to levels below that of the critical micelle concentration, with the caveat that this may make downstream analyses very difficult due to the lower concentrations of sample.

Due to the ineffectiveness of multiple treatments that were attempted, CPS samples which had only undergone the hot water/phenol extraction as well as ultracentrifugation were sent directly for NMR structural analysis.

4.5 CPS composition of the mutants

Nuclear magnetic resonance (NMR) results from Dr. Knirel are summarized in **Table 1**. This table consists of two parts: data that was gathered via acid hydrolysis, and information that was collected via NMR. In the main capsular backbone, one of the three constituent sugars is ribose; ideally, it should be stoichiometrically proportional to galactosamine (GalN). The data in **Table 1** showed that this was not the case. The increase in ribose for all strains (with the exception of *cj1427*::CATΔ) likely indicates that some degree of nucleic acid contamination is present, despite purification. For all strains, small traces of galactose were observed from acid hydrolysis. Acid hydrolysis is

not able to cleave GalNAc to galactose, and thus, the presence of galactose is likely from traces of contaminating LOS, as galactose is known to be a constituent (158). The amount of galactose that is present, however, shows that purification methods were sufficient to significantly reduce the amount of LOS present in samples. Unfortunately, other sugars that are unique to LOS and not CPS, such as Kdo, could not be observed due to the harsh nature of the acid hydrolysis treatment. The presence of Kdo should be minute in quantity, showing that reduction of LOS via purification was successful, resulting in only trace amounts of LOS present in the CPS enriched samples.

It was expected that the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT mutants would lack a modified heptose; however, it was found that these mutants lack even an unmodified heptose in their capsule. Based on silver stains showing the CPS of these mutants (**Figure 6A, B**), the *cj1427*::CAT, *cj1428*::CAT and *cj1427*::CATΔ mutants showed similar capsular staining patterns. However, for *cj1430*::CAT, the pattern obtained was significantly different from that of the other mutants, suggesting that the composition of this mutant's CPS was different from the other mutants, contradicting what was found via NMR. The differences observed in silver staining may suggest that very subtle changes in the cell surface of the mutants may be at play; this is expounded by differences in chick colonization between the *cj1428*::CAT and *cj1430*::CAT mutants (100). While both were able to colonize chicks, *cj1428*::CAT had a much lower log CFU/g of cecum compared to the *cj1430*::CAT mutant (data not shown). The lack of heptose also supports the idea that LOS contamination was very low. The fact that no heptose was detected indicates that the purification methods employed were successful at

reducing the LOS to a level in which NMR results were not confounded by extraneous heptoses from the LOS core.

As the mutants lack even a basic heptose as part of their CPS, this reinforces the notion that the glycosyltransferases responsible for transferring the sugar to the capsular side-branch may be specific, particularly for a modified heptose. Without modification, the transferases are unable to transfer the heptose to the capsule, and thus, will be absent during analysis. Promiscuity of the glycosyltransferases can be eliminated as NMR data has shown that *cj1428*::CAT and *cj1430*::CAT, both with disruptions in putative epimerases of heptose, lack heptose in the capsule, indicating that the correct sugar epimer is required to facilitate its transfer. Importantly, despite the lack of a heptose in the CPS, a capsule is still produced, as shown in silver stains that have been conducted in our lab (**Figure 6A, B**), which is similar to the findings that St. Michael *et al* (153) report for a *cj1428*::CAT mutant.

Another observation from NMR results showed that the wild type strain contained no phosphoramidate molecule. This is intriguing, as it is thought that the phosphoramidate moiety is a normally occurring side-branch of the ATCC 700819 capsule. However, McNally *et al* (114) report that it is possible that a hydroxyl group is attached to the same location as the phosphoramidate moiety, so this may explain the absence of phosphoramidate in the wild type strain. The presence of phosphoramidate on the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT mutants is hypothesized to be due to a compensatory mechanism of the bacteria. As the capsular backbone is missing the normally side-branching heptose, to compensate, the mutants attempt to make another moiety that side-branches from the main capsular backbone, namely, phosphoramidate.

To verify this hypothesis, real-time PCR will be done in the future on the cluster of genes involved in phosphoramidate synthesis (*cj1418c – cj1415c*) to determine whether upregulation of these genes is occurring.

4.6 The *cj1427*::CAT mutant grows better than wild type

Disruption of the putative heptose modification genes of interest, specifically *cj1427c*, led to an increase in growth of the mutant compared to the wild type strain. This is puzzling, as it is normally thought that the wild type CPS would be conducive for optimal growth. The lack of a heptose for the *cj1427*::CAT mutant, as elucidated by NMR, may indicate that there is a certain metabolic cost for maintaining the modified heptose as part of the CPS; once this is abolished via gene disruption, an increased growth rate is observed. The other mutants also lack a heptose, and show an increase growth rate compared to the wild type, lending credibility to this theory (100). Interestingly, only a difference in initial growth rate was observed when the mutants were grown in liquid media; when grown on plates, the mutants and wild type appear to grow to approximately the same final density. The wild type and the mutants also grow to the same final density in liquid media. This may indicate that while the *cj1427*::CAT, *cj1428*::CAT, *cj1430*::CAT and *cj1427*::CATΔ mutants have a faster initial growth rate, they reach final levels of density comparable to wild type.

Other phenotypic studies conducted for the heptoseless mutants also seem to indicate that the role of a modified heptose in *C. jejuni* interactions with host cells is more complex than initially envisioned.

4.7 Changes in the *cj1427*::CAT CPS plays a partial role in sensitivity to insult

As *C. jejuni* is known to colonize the small intestine (178), one of the first host defenses encountered is bile. Bile is a digestive secretion that plays a major role in fat dispersion and absorption. Bile acids, including cholates and deoxycholates, are molecules that act like detergents and act as potent antimicrobials (15, 108); they are secreted by the liver, stored and concentrated in the gall bladder and released into the intestine. It is known that bile primarily exerts its effects on cell membranes, eventually resulting in lysis of potential pathogens. Enzyme assays have also confirmed leakage of intracellular material, implying that bile alters membrane integrity/permeability (45). While information about bile tolerance of Gram negative bacteria is relatively scarce, it is believed that they are inherently more resistant to bile salts than Gram positive bacteria (15, 22); this is why bile salts are often used in the selective enrichment of Gram negative bacteria (i.e. MacConkey agar). *C. jejuni* is considered bile resistant and has been isolated from the gall bladder as well as directly from bile (48, 161). We sought to determine the contribution of alterations in CPS structure to *C. jejuni* bile resistance and tested a range of concentrations of bile salts. The tested concentrations fall within physiological levels of bile salts that have been documented in the intestinal lumen (0.1 – 21 mM) (26). At low bile salt concentrations, the wild type and the *cj1427*::CAT mutant showed resistance, similar to the *cj1428*::CAT and *cj1430*::CAT mutants. However, at concentrations higher than 0.5 g/L (approximately 1.2 mM), both strains showed the same susceptibility. This contrasted the findings for the *cj1428*::CAT and *cj1430*::CAT mutants, which were more susceptible to bile at these higher concentrations. As all mutants lack a heptose in their

CPS, another factor, aside from the heptose, is playing a role in bile resistance. It is known that bile salt resistance in *C. jejuni* is attributable to the CmeABC and CmeDEF multidrug resistance pump (15, 105, 137). Inactivation of the pump confers exquisite sensitivity to bile salts (105). As disruption of *cj1427c* does not result in a change in these efflux pump systems, it is not a surprise that the susceptibility pattern for the *cj1427*::CAT mutant is unaltered compared to wild type. However, the increased susceptibility in the *cj1428*::CAT and *cj1430*::CAT mutants remains to be elucidated.

In addition to bile salts, we also sought to determine whether the susceptibility of *C. jejuni* was specific to bile salts, or whether changes in CPS composition also conferred resistance to other types of detergents. We chose sodium dodecyl sulfate (SDS) as it has been shown that *C. jejuni* possesses resistance to this detergent (137). Exposing the mutant to increasing concentrations of SDS showed that there was no discernable effect due to the lack of a heptose in the CPS. This was reflected in the fact that both the wild type and all our mutants showed similar levels of susceptibility to SDS. The CmeDEF multidrug efflux pump is responsible for resistance to SDS, and as the disruption of *cj1427c, cj1428c* and *cj1430c* do not affect CmeDEF, it does not come as a surprise that the susceptibility of both the wild type and the mutants to SDS are similar. It is known that the CPS is hydrophilic (13); the fact that our strains of *C. jejuni* displayed similar levels of susceptibility to SDS likely indicates that the charged surface of the bacteria has not changed due to the absence of a heptose in the CPS. To assay this, it would be interesting to carry out a salt agglutination test (37, 107, 147, 151). This method measures cell surface hydrophobicity and net negative charge by agglutination in varying concentrations of ammonium sulfate. Agglutination in salt concentrations less than or

equal to 1 M indicates a hydrophobic surface, whereas agglutination in high concentrations of salt (greater than or equal to 2 M) indicates a hydrophilic surface.

Once *C. jejuni* gains entry into the host intestine, it has the opportunity to gain access to the vascular space and cause systemic infection (18). Amongst the multiple innate immune defense mechanisms that are present in blood, complement is a key component that can be found in serum. We thus sought to investigate whether alterations in the CPS played a role in serum resistance. In contrast to the findings for detergents, we found that, intriguingly, the *cj1427*::CAT mutant was killed nearly completely in the presence of low concentrations of serum (20%), while the wild type strain showed only 60% death. In addition, timecourse experiments at this concentration of serum showed that the *cj1427*::CAT mutant was killed very quickly, within the first 15 minutes of exposure to serum. This would seem to indicate that serum resistance is largely conveyed by the presence of heptose in the CPS and is critical for resistance; however, this is not the case, as our other heptoseless mutants, *cj1428*::CAT and *cj1430*::CAT do not show the same pattern of susceptibility to serum. While they are slightly more sensitive than wild type, the same level of extreme sensitivity as the *cj1427*::CAT mutant is not observed, indicating that there is another factor at play.

It was previously thought that LOS does not play a role in serum resistance (13), however, work by Guerry *et al* (56, 59) showed that a *C. jejuni* LOS mutant lacking distal sialic acid residues is serum-sensitive, despite the presence of CPS. Contrasting this, Keo *et al* (89) found that in a systemic strain of *C. jejuni*, LOS plays no role in serum resistance. This may indicate that the composition and physical presentation of unique sugars on the surface of *C. jejuni,* and perhaps more importantly, strain specificity, may

play a role in conveying differing resistance against complement mediated killing. Based on silver staining of the capsule (**Figure 6A, B**), the *cj1430*::CAT mutant was shown to have a different CPS staining pattern than the *cj1428*::CAT and *cj1427*::CAT mutants. While CPS was being produced, the amount of material was much less than the mutants, indicating a potential defect in export of the complete capsular polysaccharide. In addition, there are fewer bands compared to the wild type, indicating that fewer species of CPS with multiple sugar repeats are being produced. As changes in capsular composition can alter the ability of sugars to be silver stained (81, 158), it is possible that mutations in *cj1427c, cj1428c* and *cj1430c* affect the CPS in different ways that results in the variation seen for serum resistance. Alternatively, it may be that mutations in *cj1427c*, *cj1428c* or *cj1430c* affect expression of phosphoramidate related genes, leading to varying levels of phosphoramidate within the CPS of each mutant, as previously elucidated via NMR. The phosphoramidate moiety appears to be essential for serum resistance, as the *cj1427*::CATΔ mutant (lacking both the phosphoramidate and heptose moieties) that was previously investigated displayed extreme serum sensitivity. The addition of the phosphoramidate side-branch in the CPS may be essential to prevent C3 complement deposition, and subsequent complement-mediated killing of the bacteria.

It has been shown that CPS of other bacterial species plays a role in modulating the deposition of the C3 complement component (166). It would be interesting to determine whether C3 deposition on the surface of our mutants is affected by the lack of heptose.

Previous work done in our laboratory has shown that a capsuleless mutant of *C. jejuni* readily autoagglutinates over a period of three hours (100). This was hypothesized

that a lack of CPS in *C. jejuni* exposes underlying adhesins, which are normally hidden by the CPS. Our results indicated that the lack of a heptose in the CPS did not play a role in the autoagglutination of bacteria, and that no underlying surface adhesins were exposed.

As flagella and CPS are both anchored to the outer membrane of *C. jejuni*, we also sought to investigate whether changes in the CPS would lead to changes in motility. Interestingly, the *cj1427*::CAT mutant showed a significant decrease in motility compared to the wild type, indicating that subtle changes in the CPS may lead to decreased motility. When comparing our other heptoseless mutants, it was found that all mutants also showed significantly less motility than the wild type. As it has been shown that the mutants all lack heptose in the CPS (see section 3.2.3), this suggests there may be a link between the surface composition of *C. jejuni* and its motility. While there have been no studies that link a heptoseless CPS and motility, the results in this thesis may show that there are complex interactions between the genes involved in modified heptose synthesis and the motility of the organism.

4.8 The interactions of *C. jejuni* with Caco-2 intestinal cells and RAW 264.7 macrophages

Once *C. jejuni* has gained access to the host intestine, adhesion and subsequent invasion of intestinal epithelial cells is important for virulence. Based on our results, *C. jejuni* ATCC 700819 was found to adhere to, and invade Caco-2 cells at a very low rate, consistent with what has been previously reported in the literature (39, 47, 157). As no differences were found between the wild type and the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT mutant strains, this suggests that the absence of a heptose on the CPS is

unimportant for adhesion to Caco-2 cells. The *cj1427*::CATΔ mutant showed an increase in adhesion (100); this may be due to the lack of the phosphoramidate moiety. Its absence from the CPS of this mutant may allow increased adhesion due to the lack of steric hindrance between adhesins and the host cells.

As the CFU counts for *C. jejuni* in the previous Caco-2 experiments were low, it was hypothesized the CFUs were simply cell surface adherent bacteria that were able to avoid killing by gentamicin. This could affect interpretation of the results particularly when investigating phagocytosis of the bacteria into host cells. Via confocal microscopy, verification that the bacteria were present intracellularly was conducted by Xuan Thanh Bui. Preliminary results showed that the bacteria were indeed internalized, validating our gentamicin treatment to kill external bacteria in invasion assays.

Results for the adhesion, phagocytosis and survival of *C. jejuni* within macrophages showed that CPS structure, specifically the absence of a heptose, does not play a direct role in anti-phagocytosis, as the wild type and mutant strains were phagocytosed by macrophages effectively.

In both infection and survival assays, the mutants showed slightly lower uptake by and survival within macrophages, respectively. Our results for each strain are taken as a CFU count in the presence of macrophages over the CFU count in the absence of macrophages. While the CFU counts for both the wild type and the mutants in the presence of macrophages were similar, in the absence of macrophages, the CFU counts for the mutants were significantly higher (approximately 10 fold) compared to the wild type strain, despite having the same initial multiplicity of infection. Due to the higher CFU counts, this may explain why infection and survival rates appear lower. This

increase in growth is supported by the data in 3.3.1 and work previously done by our laboratory where the mutants were found to grow more rapidly than the wild type (100). It is possible that the low apparent rates of the mutants are artifacts due to this increased growth rate.

When investigating the phagocytosis of the mutants compared to wild type, it was observed that the mutants, showed more or less a steady state within macrophages. For infection, it was expected that an increase in phagocytosis would be observed with an increase in duration of infection, as observed for wild type. As differences were observed, this suggests that there may be either an inability of the macrophages to phagocytose the bacteria or a problem of survival of the mutants within macrophages. However, the latter hypothesis was refuted when data for the survival within macrophages was analyzed; the mutants were killed at approximately the same rate as the wild type strain, indicating there was no significant difference in the ability to survive within macrophages in all strains.

As differences in phagocytosis, but not in survival were observed between the wild type and mutant strains, we hypothesized that the differences observed were due to changes in *C. jejuni*-macrophage interactions that occurred at earlier timepoints. Consequently, we dissected the adhesion of *C. jejuni* to macrophages. The adhesion of the mutants to macrophages showed no significant differences compared to wild type. The slightly higher adhesion of the *KpsM* mutant was previously hypothesized by our laboratory to be due to the loss of a capsule and the subsequent exposure of underlying adhesins (100). The slightly lower adhesion rate of the *cj1427*::CATΔ is puzzling; as it lacks both heptose and phosphoramidate, there should be less steric hindrance for the

underlying adhesins. As there were no defects for survival nor adhesion in the other mutants, the reason for the differences observed in phagocytic rates is unclear at this time. It is possible that the lack of a heptose indirectly confers a fitness advantage. One possibility for the decreased phagocytosis is due to a failure of the macrophages to activate. Macrophages are known to upregulate the production of inflammatory cytokines such as TNFα and IL-8 in the presence of other *Campylobacter* species (110). However, the caveat is that the production of these proinflammatory cytokines is both *Campylobacter* strain and species dependent. It is not unreasonable to consider that changes in the surface properties of *C. jejuni* will cause a defect in macrophage activation, and subsequently, lead to less phagocytosis of the bacteria. To test this possibility, enzyme-linked immunosorbent assays (ELISA) can be used to determine whether the macrophages are failing to activate (i.e. lack of TNFα or IL-8 production) in the presence of the heptoseless mutants.

Rather than a failure of the macrophages to activate, there is also the possibility that the decreased phagocytosis of the mutants is due to a pattern of bacterial gene expression that blocks further uptake of the bacteria. If this is indeed the case, it will be necessary to identify the genes that are differentially expressed between the mutants and the wild type strains both inside and outside macrophages. To do this, a method known as selective capture of transcribed sequences (SCOTS) can be employed (33). In this method, bacterial RNA can be isolated from infected macrophages, alleviating problems due to abundant eukaryotic RNA in downstream analyses. The pool of amplified expressed bacterial genes can then be analyzed using DNA microarrays to determine

whether differential gene expression plays in a role in conferring resistance against uptake by macrophages.

4.9 Complementation of the *C. jejuni* mutants

Creation of the complementation constructs was accomplished using the gene splicing by overlap extension (gene SOE-ing) method. In this method, multiple fragments are spliced together to generate the final complementation construct. The *porA* promoter is a constitutively expressed promoter that drives the expression of the complementation construct at high levels, as previously shown (100). The kanamycin resistance cassette was chosen so that complemented mutants could be selected. Once the complementation constructs were successfully constructed and sequenced, they were inserted into a pBluescript vector containing the 16S – 23S rRNA regions, and then transformed into *E. coli*. Great difficulty was encountered during attempts to transform *E. coli* with the constructed vectors containing the gene SOEd products. Controls at each step of the process (i.e. restriction digest of plasmid, dephosphorylation of the plasmid, T4 DNA ligation, transformation efficiency, etc) were conducted in parallel to verify that each step was occurring as envisioned. Upon acquiring data from the transformation efficiency control, it was determined that even for the pBluescript plasmid (positive control), the efficiency was very low per nanogram of DNA; the repeated inability to acquire transformants containing the complementation constructs was likely due to this extremely low transformation efficiency. However, despite these difficulties, the *cj1427*::CAT, *cj1428*::CAT and *cj1430*::CAT complementation constructs were transformed into *E. coli* by another student in our laboratory, Xuan Thanh Bui. Once the complementation constructs were transformed into *E. coli*, the plasmid was extracted in large quantities,

and then verified via DNA sequencing to determine if mistakes in the sequence were present.

Due to the nature of gene SOE-ing, multiple PCRs must be conducted to make the final complementation construct. As a consequence of PCR, single nucleotide polymorphisms (SNPs) may be inadvertently introduced into the complementation construct. This is likely due to the relatively low fidelity of the DNA polymerase mix that was chosen (Expand DNA Polymerase mix; a mix of Taq DNA polymerase and Tgo DNA polymerase). The *cj1427*::CAT complementation construct was found to contain three SNPs, resulting in a glycine to aspartic acid change (G9D), a leucine to proline change (L42P), and a serine to proline change (S142P). The G9D mutation results in the disruption of a Rossmann fold, a protein structural motif that binds cofactors such as nicotinamide adenine dinucleotide (NAD) and is vital for folding and activity of the enzyme (138). As this disruption would result in a non-functional protein, this SNP was fixed via QuikChange mutagenesis PCR. For the L42P mutation, comparisons to ATCC 81-176 were done and it was found that the leucine was conserved, indicating that it was likely important. Similar comparisons for the S142P also showed that it was conserved. In addition, proline is known to be a disruptor in the middle of regular secondary structural elements, such as alpha helices and beta sheets (146). As of final submission of this thesis, all SNPs have been fixed, and the complementation construct has been mobilized into the *cj1427*::CAT mutant. The *cj1428*::CAT complementation construct was found to contain several SNPs. The first was a valine to alanine (V21A) mutation; comparison to *C. jejuni* 81-176 revealed that a leucine was present, and thus, it was decided that this amino acid was not very conserved. The second mutation resulted in a

lysine to arginine (K100R) mutation. Comparison to *C. jejuni* 81-176 revealed that it also possessed a leucine in this region; however, further comparisons to protein homologs revealed that there was only a requirement for an amino acid with a polar NH_2 group for protein function. As a result, it was deemed that the SNPs were unlikely to affect the function of the Cj1428 protein. This complementation construct was used as is for our phenotypic studies. Finally, the *cj1430*::CAT complementation construct was found to contain no SNPs upon DNA sequencing.

With the successful completion of the QuikChange mutagenesis PCRs required to remove the SNPs in the complementation construct, it, along with the *cj1428*::CAT and *cj1430*::CAT complementation constructs were mobilized into their respective *C. jejuni* mutants using the method outlined by Donahue *et al* (36). Mobilization of the *cj1428*::CAT plasmid into the *cj1428*::CAT mutant was successful using both electroporation and natural transformation. It was noted that natural transformation was much more efficient than electroporation; this may be due to several factors. Firstly, it is known that *C. jejuni* is naturally competent (178); this in combination with the fact that the complementation construct has been selectively methylated to have the restriction endonuclease signature of *C. jejuni* ATCC 700819 results in highly efficient transformation. Electroporation was also thought to be less efficient than natural transformation due to the stress that is placed onto the cells during the procedure. Regardless of the method that was used, transformants for the *cj1428*::CAT complement were obtained. These potential clones were screened via PCR to ensure that the complementation construct was indeed present within *C. jejuni*. While PCR showed that the complementation construct was within *C. jejuni*, homologous recombination into the

16S – 23S rRNA region of the genome did not occur (**Figure 19A** and **B**, respectively). This is puzzling, as there are three copies of the 16S – 23S rRNA region within the *C. jejuni* genome (87); by design, the complementation construct should have integrated into one of these three regions. Due to this failure to integrate into the 16S – 23S rRNA region, we attempted to investigate the precise location of the complementation construct. The other two elements in the complementation construct were the *cj1428c* gene and the *porA* promoter. Each gene was investigated using flanking primers to determine whether the complementation construct inadvertently recombined into these areas as homologous recombination can occur with as few as 200-300 base pairs (3, 80). PCR revealed no increase in size corresponding to the insertion of the complementation construct in either gene (**Figure 19C** and **D**, respectively). It was also verified that the pBluescript vector was not replicating within *C. jejuni* by attempting to extract the plasmid. The failure to do so agrees with the literature that indicates the pBluescript vector is a suicide vector. While the complementation construct did not integrate into the 16S – 23S rRNA region as initially desired, the construct has been verified to be within *C. jejuni*, and thus, phenotypic studies involving this complemented mutant were conducted.

Interestingly, for the *cj1430*::CAT mutant, both electroporation and natural transformation were not successful at transforming the complementation plasmid into *C. jejuni*, despite it being methylated. At first, it was thought that the surface properties of the *cj1430*::CAT mutant were refractive to transformation. This would result in an inability of the plasmid to enter the cells. However, this theory was quickly disproved when it was found that the *cj1430*::CAT complementation plasmid was unable to enter the easily transformable *cj1428*::CAT mutant as well. In addition, the *cj1428*::CAT

complementation construct as well as other plasmids in our collection were easily transformed into the *cj1430*::CAT mutant, indicating that the problem was not due to differences in the surface properties of the *cj1430*::CAT mutant. The reason for the inability of the *cj1430*::CAT complementation construct to be mobilized into the *cj1430*::CAT mutant is unknown at this time. One possible explanation for this failure is that Cj1430c may require another interacting protein within the capsular cluster to be expressed at wild type levels. However, preliminary data in our laboratory by M. McCallum indicates Cj1430c does not interact with any of the other enzymes within the capsular cluster. In the future, to complement the *cj1430*::CAT mutant, a *cj1426c – cj1430c* complementation construct may be attempted. While the construction and subsequent transformation of this construct will be difficult due to the large size (> 8 Kb), it may alleviate the difficulties encountered when attempting to complement the *cj1430*::CAT mutant. The region from *cj1426c – cj1430c* will be used so that the expression of each gene product is at equimolar level; this will also ensure that if (an) interacting partner(s) is (are) required by any of the enzymes within the capsular cluster, this requirement will be met. A shuttle plasmid based complementation approach will be used if this method is attempted, for several reasons. Firstly, homologous recombination into the 16S – 23S rRNA region was shown not to work for the complemented *cj1428*::CAT mutant. Secondly, with a large region of the *C. jejuni* genome in the plasmid, there is a high chance that inadvertent homologous recombination will occur, causing additional problems.

Despite the failure of the *cj1428*::CAT complementation construct to integrate into the desired area of interest, the phenotypes that were previously investigated by our

laboratory (100) and showed striking differences compared to the wild type were analyzed in the complemented strain to determine whether the phenotypes would be restored to wild type levels. Specifically, complementation should restore the susceptibility of the mutant to be phagocytosed by macrophages, restore the survival kinetics of the mutant within macrophages to wild type susceptible levels and return motility to wild type levels.

When the survival within macrophages of the complemented *cj1428*::CAT mutant was investigated, it was found that complementation occurred (**Figure 22**). Interestingly, the complemented *cj1428*::CAT mutant is able to survive better within macrophages; this may be due to the overexpression of Cj1428c, as production of this enzyme is driven by a highly active *porA* promoter.

The phagocytosis of the complemented *cj1428*::CAT mutant was partially complemented to wild type levels (**Figure 23**), particularly at timepoint one. However, at later timepoints, complementation does not seem to be occurring. This indicates that partial complementation for the initial steps of infection (i.e. adhesion and initial internalization) is likely occurring. As the data from (**Figure 22**) shows that complementation is occurring, this indicates that there may be differences on the surface of the complemented *cj1428*::CAT mutant that results in the blockage of further internalization. While the *cj1428*::CAT complementation construct did not integrate into the desired 16S – 23S rRNA area, complementation is occurring due to the expression of Cj1428c (**Figure 20**).

It was found that motility of the *cj1428*::CAT mutant was significantly reduced compared to wild type (**Table 3**). Contrary to expectations, results showed that the

complemented mutant was less motile than both the wild type and the *cj1428*::CAT mutant. The decrease in motility was deemed significant ($p < 0.05$) when comparing the diameter of the halos to the wild type and also to the *cj1428*::CAT mutant.

The failure of the complementation construct to restore motility, while complementing the macrophage phenotypes that were studied cannot be explained at this time. It is possible that the complementation construct is somehow affecting genes involved in motility of *C. jejuni*. Further elucidation will need to be conducted to determine where the construct has integrated. This can be accomplished using inverse PCR after digestion of the *C. jejuni* genome with a low – medium frequency restriction endonuclease to create multiple fragments. As the construct must not be cut by the restriction endonuclease for this strategy to work, this necessitates a relatively uncommon restriction endonuclease. The fragments can then be ligated together to create circularized DNA, and inverse PCR carried out to generate PCR products that can then be sequenced to determine the precise integration site of the *cj1428*::CAT complementation construct.

4.10 Overall summary

C. jejuni is a pathogen that is able to cause disease in humans, and it has evolved to successfully achieve this goal. The work that has been carried out as part of this thesis has furthered our knowledge of the involvement of modified heptoses in the *C. jejuni* capsule in pathogenesis. Once ingested, *C. jejuni* must avoid the bile that is secreted by the gall bladder, and then adhere to and invade the target intestinal epithelial cells. Following this, the bacterium must evade avoid host defense such as complement and macrophages. With the disruption of putative genes involved in modified heptose production, we had in our possession the exclusively heptoseless *cj1428*::CAT, and

cj1430::CAT mutants, the capsuleless *KpsM* mutant and the side-branchless *cj1427*::CATΔ mutant. Since then, we have also completed the heptoseless *cj1427*::CAT mutant.

Nuclear magnetic resonance (NMR) has elucidated the composition of the capsule in each of these mutants, and revealed that the *cj1427*::CAT, *cj1428*::CAT, *cj1430*::CAT, and *cj1427*::CATΔ mutants all lack heptose. With exception of the *cj1427*::CATΔ mutant, the *cj1427*::CAT, *cj1428*::CAT, *cj1430*::CAT also showed the confirmed presence of phosphoramidate, while the wild type strain did not. This likely indicates that capsular phase variability is occurring. This phase variability may assist *C. jejuni* in the evasion of host defenses.

Real-time PCR was initially conducted to verify that the insertion of the chloramphenicol antibiotic resistance cassette into the mutants was non-polar. However, it was also found that the levels of some of the genes investigated within the capsular cluster showed very low levels of expression. This could either be due to the differential degradation of the mRNA or could be attributed to the actual low expression levels of the genes. Therefore, complementation was still necessary to assess gene specificity of the phenotypes.

Interestingly, the newly constructed *cj1427*::CAT mutant showed different phenotypes compared to the *cj1428*::CAT and *cj1430*::CAT mutants, despite all of these mutants lacking a heptose and having similar capsular compositions. The *cj1427*::CAT mutant was found to be significantly susceptible to serum, while comparatively resistant to bile salts. In addition, the *cj1427*::CAT mutant was found to be phagocytosed by macrophages as much as wild type, and showed similar survival kinetics within

macrophages, again differing from the observed trends for the *cj1428*::CAT and *cj1430*::CAT mutants. This indicates that the modified heptose is not directly responsible for changes in the pathogenesis of *C. jejuni,* and that another factor is at play.

While complementation of the *cj1427*::CAT and *cj1430*::CAT mutants has yet to be completed, thus far, the *cj1428*::CAT mutant has been complemented, despite the complementation construct not recombining into the desired 16S – 23S rRNA region. While restoration of motility of the mutant was not observed, restoration of the phagocytosis rate as well as survival kinetics to wild type susceptible levels was observed, indicating that the phenotypes observed for the *cj1428*::CAT mutant were gene specific.

4.11 Future Directions

The real-time PCR data that have been gathered as part of this thesis has raised several interesting questions. Further work is warranted to determine whether the low levels of transcript found by real-time PCR represent actual changes in gene expression. This can be accomplished using a reporter system to determine whether regulatory effects are causing the lowered expression levels observed. In addition, investigation into phosphoramidate synthetic genes will allow us to determine why the wild type lacks a phosphoramidate, while the *cj1427*::CAT, *cj1428*::CAT, and *cj1430*::CAT mutants possess a phosphoramidate. Upregulation of genes involved in phosphoramidate as a compensatory mechanism for the lack of heptose may be responsible, and thus determination if these genes are being more highly expressed is needed.

The different trends for the *cj1427*::CAT mutant that were observed in the phagocytic uptake as well as survival within macrophages experiments have also raised

several interesting questions. As the *cj1427*::CAT, *cj1428*::CAT, and *cj1430*::CAT mutants all have the same capsular composition as elucidated by NMR, it was expected that they would behave similarly. However, this was not the case, as the *cj1427*::CAT mutant behaved like the wild type, while the *cj1428*::CAT and *cj1430*::CAT mutants did not. Enzyme-linked immunosorbent assays (ELISA) will determine if the macrophages are failing to activate in the presence of these different mutants. Alternatively, using selective capture of transcribed sequences (SCOTS), it will be possible to determine genes that may be differentially expressed within each mutant and the wild type both inside and outside the macrophage. This will also aid in the understanding of bacterial factors necessary for prolonged survival of *C. jejuni* within macrophages.

Lastly, the work that has been presented as part of this thesis has shown that the role of the modified heptose in the *C. jejuni* CPS is not as straightforward as initially imagined. While the work presented here has shed light on some important aspects of *C. jejuni* pathogenesis, more importantly, it has laid the groundwork for future studies in the field. Elucidation of the various factors involved in *C. jejuni* pathogenesis and identification of novel targets for therapeutic agents against *C. jejuni* will help alleviate the symptoms caused by this organism and possibly eliminate this pathogen from the world.

APPENDICES

Vectors		
pHel 3	*H. pylori* shuttle vector containing a kanamycin resistance cassette	Heuermann and Haas, 1998
pRY111	*C. jejuni* shuttle vector containing a chloramphenicol resistance cassette	Yao *et al*, 1993
pET23*cj1427*::CAT	The CAT cassette (743 bp) inserted into *cj1427*	This study
pBluescriptKS(+)::16SrRNA-28SrRNA	The 16SrRNA-28SrRNA intragenic region (1400 bp) inserted into pBluescriptKS(+)	This study
pBluescriptKS(+)::16SrRNA-28SrRNA (-XbaI)	The 16SrRNA-28SrRNA intragenic region (1400 bp) inserted into pBluescriptKS(+) with the additional XbaI site in pBluescriptKS being removed	This study

Primers	**Sequence (5' – 3')**
Aph3 P1	ga**agatct**gataaacccagcgaacca
Aph3 P2	ag**ggtcca**tggagacatctaaatctaggtac
CatColi P2	gtc**ggtacc**ttatttattcagcaagtcttg
CatColi P3	gtcatc**gggccc**ttcctttccaagttaattgc
cj1418 P1	cactcttactgcttccatctg
cj1421 P1	ggtcaaactgtgatagaacatag
cj1421 P2	ccccattttctaccataagc
cj1421 P3	gctactatatctggacgatg
cj1421 P4	gattgcggcctttgtccttc
cj1422 P2	ggctgaatttgggttgagcatgg
cj1422 P3	ggatatagttggagacaagac

cj1422 P4	gaattggtataaaggaggtgg
cj1423 P1	gatattggtgtgcctgagg
cj1427 P3	gcgtc**ggatcc**ttaattaaaatttgcaaagcga
cj1427 P4	gtg**gggtacc**tagaatgagacttga
cj1427 P5	ctgcta**gggcccc**atattctgagatagg
cj1428 P2	aggta**ccatgg**gcatgcaaacaaattcaaaaatataa
cj1428 P3	gct**ggatcc**tcaattttgtgttttatacca
cj1428 P4	gtg**ggtacc**tgtagctatctatacgatgc
cj1428 P5	ctgcta**gggcccc**tcaggatacatataccca
cj1430 P2	agggt**ccatgg**caatagaatttgatata
cj1430 P3	gcgtc**ggatcc**ttatcctttatttttagttgcaa
cj1430 P4	gtg**ggtacc**aaatatgggaaattct
cj1430 P5	ctgcta**gggcccc**attttaaataggttgg
kpsM P2	a**ggtacc**atggtgagttatgattatagtttatg
kpsM P3	gct**ggatcc**tagattaattaactttatcattc
kpsM P4	ga**agatct**tgtatttcctgttcatttgc
kpsM P6	aggta**ccatgg**agttctagcaataaatacatg

Complementation primers	
KAN 1427 Rev	caaatggttcgctgggtttcttaattaaaatttgcaaagcgatta
KAN For 1427	taatcgctttgcaaattttaattaagaaacccagcgaaccatttg
KAN 1428 Rev	tcaaatggttcgctgggtttctcaattttgtgttttataccattc
KAN For 1428	gaatggtataaaacacaaaattgagaaacccagcgaaccatttga

KAN 1430 Rev	caaatggttcgctgggtttcttatcctttatttttagttgcaag
KAN For 1430	cttgcaactaaaaataaaggataagaaacccagcgaaccatttg
kpsM Rev CAT	gcaattaacttggaaaggaactcattctttttttaccgccgtt
CAT For kpsM	aacggcggtaaaaaaagaatgagttcctttccaagttaattgcg
16S rRNA IGS Top	ctggaactcaactgacgctaa
28S rRNA IGS Bottom	ctcttgcacattgcagtccta
OmpE prom Fwd IGS	ggttggatcacctcctttctctttagatgtttttatccttcgg
OmpE-for-XbaI	gc**tctaga**ctttagatgtttttatccttc
IGS Rev ompE Prom	ccgaaggataaaaacatctaaagagaaaggaggtgatccaacc
28S rRNA Fwd KAN	ttagtacctagatttagatgtcaaaagtaataagtctcacaactatt
Aph3P3	gc**tctaga**gacatctaaatctaggtac
28S rRNA Fwd CAT	gaggaaggaaataataaatggctaaagtaataagtctcacaactatt
CAT-reverse-XbaI	gc**tctag**agccatttattatttccttcctc
KAN rev 28S rRNA	aatagttgtgagacttattacttttgacatctaaatctaggtactaa
CAT rev 28SrRNA	aatagttgtgagacttattactttagccatttattatttccttcctc
1427 For ompE	taatttttgacaaggagaattctcatgtcaaaaaaagttttaattacag
1428 Fwd ompE	taatttttgacaaggagaattctcatgcaaacaaattcaaaaatatatata
1430 Fwd ompE	taatttttgacaaggagaattctcatggcaatagaatttgatatacaa
KpsM Fwd ompE	taatttttgacaaggagaattctcatgttaaatgtaatttatgctttattt
ompE pro Rev 1427	ctgtaattaaaacttttttttgacatgagaattctccttgtcaaaaatta
ompE Pro Rev 1428	tatatatatttttgaatttgtttgcatgagaattctccttgtcaaaaatta
ompE pro Rev 1430	ttgtatatcaaattctattgccatgagaattctccttgtcaaaaatta
ompE prom Rev kpsM	aaataaagcataaattacatttaacatgagaattctccttgtcaaaaatta

16S rRNA Top Primer – KpnI	ggggtac**cctgga**actcaactgacgctaa
28S rRNA Bottom Primer – BamHI	cg**ggatcc**ctcttgcacattgcagtccta
23S rRNA Bottom NotI	ataagaat**gcggccgc**tcttgcacattgcagtccta

Real-time PCR primers	
1425 RT Btm	accacttggtacatccgaataggt
1425 RT Top	tgcgactatatctttatacatacattg
1426 RT Btm	ttatcgacataaagcatcttgtaaaaaa
1426 RT Top	attgataatcatcagcaagctaggaa
1427 RT Btm	ccgcatgcactttatcgatccca
1427 RT Top	ctgatattattattcctctagctgct
1428 RT Top	caagcggttgctaaatttttaaagaa
1428 RT Btm	aaatcaccttgaaataaatattcctctt
1429 RT Btm	gcttgtaccaaagtaattgtattctc
1429 RT Top	gaataggtggtaatggagatggtg
1430 RT Top	cagatggaattaaatttaagcatgaca
1430 RT Btm	tggtggtaataaaataagttgctgatt
Acetyl RT Btm	tagccccaatccttgcacaagct
Acetyl RT Top	aatgtcttgatcgtcatatgaaaacaa
Cj1444 RT Top	agtggtggattctcaaggaaatattt
Cj1444 RT Btm	tgaatccgaactaagtccttgataaa
Cj1445 RT Btm	aaggtaaatctatatgttgctcttgat
Cj1445 RT top	atacattgattgcagcaccaagatat

RTCj1447c Fwd	cctttatttagtggtggaagacatta
RTCj1447c Rev	ttatctctagctgttaaagaaccttg
KpsM RT Btm	attacaaaataaatacaaaactcaagtaa
KpsM RT Top	gttagagaatatcatcatcaagttatg

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