PATHOGENIC MECHANISMS OF *Campylobacter jejuni*:

REGULATION OF *Campylobacter* INVASION ANTIGEN B

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of GARY ANDREW FLOM find it satisfactory and recommend that it be accepted.

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Chair

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PATHOGENIC MECHANISMS OF CAMPYLOBACTER JEJUNI: REGULATION OF *Campylobacter* INVASION ANTIGEN B

Abstract

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Campylobacter jejuni, a Gram-negative, spiral shaped bacterium is the leading cause of gastroenteritis in humans worldwide. However, little is known regarding the mechanisms *Campylobacter* employs to cause disease. Previous work has shown that *Campylobacter jejuni* secretes a set of proteins known as the *Campylobacter* invasion antigens (Cia proteins). Recent research has shown that the flagella apparatus acts as a type III secretion system allowing the delivery of the Cia proteins to intestinal epithelial cells. Furthermore, research has shown that the expression of the *cia* genes is upregulated in the presence of bile salts and fetal bovine serum. Given that an environmental signal was required to induce the expression of the *cia* genes, I sought to determine how the *cia* genes were regulated. I generated single crossover mutants of ten of the response regulators and one AraC-like transcriptional activator that are present in the *Campylobacter* NCTC 11168 genome. Upon subjecting the single crossover mutants to secretion assays, I determined that Cj0890c, Cj1024c (*flgR*), and Cj1042c (AraC-like) were secretion negative and may play a role in the regulation of the *cia* genes. Next, I generated double crossover mutants to validate this secretion negative phenotype. In addition I generated double crossover mutants of σ^{28} and σ^{54}, Cj0061c (*fliA*) and Cj0670 (*rpoN*). Secretion assays of

the double crossover mutants revealed that Cj0061c (*fliA*), Cj0890c, and Cj1042c (AraC-like) were secretion positive and do not play a role in the regulation of the *cia* genes. Cj0670 (*rpoN*) and Cj1024c (*flgR*) were found to be secretion negative, suggesting that Cj0670 (*rpoN*) and Cj1024c (*flgR*) may play a role in the regulation of the *cia* genes. Upon subjecting Cj0670 (*rpoN*) and Cj1024c (*flgR*) to RT-PCR and immunoblot analysis we observed that the *ciaB* was still transcribed and translated in the Cj0670 (*rpoN*) and Cj1024c (*flgR*) and therefore do not play a role in the regulation of *ciaB*. This finding strengthens the result that the flagella apparatus serves as the type III secretion system for the export of the Cia proteins. Collectively these data suggest that *ciaB* is under the control of σ^{70}.

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To my loving wife, Karen.

Chapter 1

INTRODUCTION

1.1 Background

The genus *Campylobacter*, a member of the family *Campylobacteraceae*, is comprised of 16 species and 6 subspecies (On, 2001). These species were identified primarily by performing partial 16S rRNA gene sequencing. *Campylobacter* was originally recognized as a species of *Vibrio*, but in 1963 it was proposed by Sebald and Véron that it be reclassified in 1963 (Sebald and Véron, 1963). The reasoning behind the reclassification was that *Campylobacter* organisms displayed genetic differences from *Vibrio* in mol% (G+C) DNA content and also lacked saccharolytic enzymes (Walker *et al.*, 1986). The first *Campylobacter* species that was identified was *Campylobacter fetus*, which causes abortion and infectious infertility in cattle, sheep, and humans (Sauerwein *et al.*, 1993; On, 2001). However, *Campylobacter* did not gain much recognition until the 1970's when it was shown to be highly prevalent in humans with diarrhea (Dekeyser *et al.*, 1972; Butzler *et al.*, 1973). Numerous species of *Campylobacter* (*C. concisus*, *C. curvus*, *C. gracilis*, *C. rectus*, *C. showae*, and *C. sputorum*) have been isolated from the oral cavities of humans (Macuch and Tanner, 2000). The most prevalent disease-causing species in humans are *Campylobacter jejuni* and *C. coli*, which are responsible for 95% of *Campylobacter* infections (Park, 2002).

Campylobacter sp. are Gram-negative, non-spore forming, spiral shaped rods, that are 1.5 to 6 μm long and 0.2 to 0.5 μm wide (Ketley, 1997). The organism is motile via unipolar or bipolar flagella. The growth *Campylobacter* is optimal under microaerobic conditions, requiring 5-10%

oxygen and 3-10% carbon dioxide, and at temperatures ranging from 30 to 42°C (Walker *et al.*, 1986).

Campylobacter jejuni, *C. coli*, and *C. lari* are recognized as the leading agents of *Campylobacter* infections in humans (Koenraad *et al.*, 1997). *C. jejuni* accounts for up to 95% of all *Campylobacter* infections (Lane and Baker, 1993), and is the leading cause of bacterial gastroenteritis in the United States, with an estimated 2.4 million cases per year (Allos, 2001). Recently, it has been suggested that other *Campylobacter* species, such as *C. upsaliensis*, *C. fetus*, and *C. concisus* may be significantly underdiagnosed as causes of gastrointestinal disorders due to inappropriate isolation and identification methods (Engberg *et al.*, 2000). Moreover, some species, such as *C. concisus*, *C. sputorum*, *C. curvus*, *C. rectus*, and *C. hyointestinalis*, require incubation in a hydrogen-enriched microaerobic atmosphere to allow for recovery (Engberg *et al.*, 2000). Due to the complexity of isolating *Campylobacter* sp., it is unclear as to the prevalence of these taxa in causing infection.

In developed countries, a majority of *Campylobacter* infections result from handling and consumption of undercooked poultry. Infections are also acquired from drinking unpasteurized milk and contaminated water. The majority of infections are sporadic in nature (Friedman *et al.*, 2000). While *Campylobacter* infections may occur throughout the year, there is a marked peak in summer and early fall (Blaser, 1997). It has been suggested that this seasonality of infection is due to an increase in picnics and barbeques (Berndtson, 1996). Infection of individuals with *Campylobacter* in developing countries does not appear to display a strong pattern of seasonality (Ketley, 1997). *Campylobacter* infection can occur in all age groups; however, the age of

individuals infected differs between developed and developing countries. In developed countries the highest level of reported cases occurs in infants and adults ranging from 15-30 years in age (Blaser, 1997). In contrast, *Campylobacter* infections in developing countries occur more often in children under the age of two (Blaser, 1997). The lack of symptomatic infection seen in the adult population may be from protective immunity acquired through repeated childhood exposure (Blaser, 1997).

Symptoms of *C. jejuni* infections depend upon numerous variables, including the virulence of the *Campylobacter* isolate, infectious dose, host immune status, age, and history of infection. Due to this wide variety of factors, the symptoms and disease progression associated with *Campylobacter* infections are highly variable ranging from asymptomatic to sepsis and death. For example, studies with human volunteers have shown that as few as 500 organisms are required for infection and that the number of infected individuals increases with dose (Black *et al.*, 1988). In a typical *Campylobacter* infection, the standard incubation period after ingestion ranges from 24 to 72 hours; however, incubation periods greater than one week have been observed (Skirrow and Blaser, 1995). Prodromal symptoms may include headache, myalgias, chills, fever, nausea, and acute abdominal pain so severe that they may be mistaken for appendicitis (Blaser, 1997). Symptoms progress to profuse watery diarrhea with more than eight bowel movements per day, often leading to inflammatory diarrhea containing both blood and leukocytes (Blaser *et al.*, 1982). Biopsies have revealed that diffuse inflammatory colitis and enteritis may occur (Blaser, 1997). The majority of *Campylobacter* infections are self-limiting and last less than one week; however, up to 20% of individuals infected with *C. jejuni* may experience a relapse or a prolonged illness (Blaser *et al.*, 1983). Individuals expressing mild

symptoms usually do not require treatment. Some patients may need fluid and electrolyte treatment in order counteract dehydration (Allos and Blaser, 1995). Studies have shown that antimicrobial therapy can speed the rate of recovery if administered at the onset of symptoms (Salazar-Lindo *et al.*, 1986; Allos and, 1995). The antibiotic of choice for most cases of *Campylobacter* enteritis is erythromycin (Allos and Blaser, 1995). However, erythromycin-resistant strains of *Campylobacter* are beginning to emerge (Reina *et al.*, 1992).

Campylobacter infections have been implicated in several sequelae aside from acute gastroenteritis, the most prominate being Guillain-Barré syndrome (GBS) and Miller Fisher syndrome (MFS). GBS is considered to be the common cause of acute flaccid paralysis in the post polio era (Ho *et al*., 1998). GBS is an acute post-infectious immune-mediated disorder associated with demylinating polyneuropathy that leads to flaccid paralysis (Nachamkin, 2002). Approximately one in every 1000 individuals infected with *C. jejuni* will develop GBS (Allos, 1997). Through culture and serological methods it has been estimated that 30-40% of patients with GBS display evidence of *C. jejuni* infection (Nachamkin, 2002). Common symptoms include symmetrical weakness in the limbs, weakness of the respiratory muscles and areflexia (loss of reflexes) (Nachamkin *et al*., 1998). Generally, the weakness begins in the legs spreading upward to the trunk of the body. In severe cases, up to 20% of patients may require mechanical ventilation (Hadden and Gregson, 2001). GBS is often self-limiting, with most individuals recovering muscle strength completely anywhere from a few weeks to months (Nachamkin *et al.*, 1998). However, 15-20% of individuals with GBS are left with severe neurologic deficits and 2-3% of these individuals die despite respiratory care (Nachamkin *et al.*, 1998). MFS is considered a rare variant of GBS, characterized by the acute onset of ophthalmoplegia (inability

to move the move eyes), ataxia (unsteady movements and staggering gait), and areflexia (Nachamkin *et al.*, 1998). Studies have shown that *C. jejuni* isolates resulting in post infection sequelae induce the production of cross-reactive autoimmune IgG antibodies directed against various human gangliosides. For example, individuals with GBS produce antibodies against GM_1, while those with MFS generate antibodies toward GQ_{1b} (Hadden and Gregson, 2001).

The genomes of *C. coli* and *C. jejuni* are approximately 1.6 to 1.7 Mbp (Chang and Taylor, 1990). In contrast, the *C. upsaliensis* genome is approximately 2 Mbp. This variance in size may have arisen due to a large duplication of chromosomal sequences (Bourke *et al.*, 1995). The small size of the *Campylobacter* genome helps to explain their requirement for a complex growth media, and their inability to ferment carbohydrates and to degrade complex substances (Griffiths and Park, 1990; Parkhill *et al.*, 2000). The complete genome of *Campylobacter jejuni* subspecies *jejuni* NCTC 11168 was sequenced at the Sanger Centre and published in 2000 (Parkhill *et al.*, 2000). Its genome is comprised of a circular chromosome of 1,641,481 base pairs [30.6 mol% (G+C) content] that encodes 1,654 proteins and 54 stable RNA species (Parkhill *et al.*, 2000). The genome of *C. jejuni* is unique in that it contains relatively few insertion sequences and phage-associated sequences and almost no repetitive sequences (Parkhill *et al.*, 2000). One interesting feature that was discovered was the presence of hypervariable sequences (Parkhill *et al.*, 2000). Evidence for extrachromosomal elements has been shown in *Campylobacter* in the form of bacteriophages and plasmids (Taylor, 1992). Bacon *et al.* (2000) reported that *C. jejuni* strain 81-176 contains two previously undescribed plasmids. One plasmid was termed pVir due to regions of DNA that encode a putative type IV secretion system similar to that in *Helicobacter pylori*. The second plasmid carries a *tetO* gene that confers tetracycline

resistance. Others have also reported the presence of small cryptic plasmids harbored by *C. jejuni* (Luo and Zhang, 2001; Alfredson and Korolik, 2003).

1.2 *C. jejuni* binding and internalization

The pathogenic mechanisms that *Campylobacter* utilizes are the subject of intense research; however, the understanding these factors is still in its infancy. There are numerous virulence factors that have been implicated, including motility and chemotaxis, adhesion and invasion, and toxin production. While no one model of infection can be agreed upon, it is believed that upon entering the host's intestine, *Campylobacter* display positive chemotaxic behavior toward mucin. Mucin lines the crypts of intestinal epithelial cells. The migration of the organism to the crypts allows adhesins, which are located on the surface of *Campylobacter* organisms, to bind to receptors situated on intestinal epithelial cells. Invasion of *Campylobacter* into intestinal epithelial cells can then occur by either a direct method through invasions (*Campylobacter* invasion antigens) or an indirect method through toxin (Cytolethal distending toxin) production.

Adhesins are known to play a major role in the pathogenesis of organisms such as *Salmonella* sp., *Shigella* sp., and enteropathogenic *Escherichia coli* (EPEC). The binding of *Campylobacter* organisms to non-professional phagocytic cells serves as primary method of evading peristalsis, and allows *Campylobacter* to colonize the host. Fauchère *et al.* (1986) reported that *C. jejuni* recovered from individuals displaying fever and diarrhea exhibited a greater binding affinity to epithelial cells than isolates recovered from asymptomatic individuals.

In vitro adherence assays have been used extensively to ascertain the identity of *Campylobacter* adhesins. Early assays demonstrated that heat-killed and sodium azide-killed *C. jejuni* bound to epithelial cells at levels equivalent to metabolically active organisms (Konkel and Cieplak, 1992). Additionally, *C. jejuni* treated with chloramphenicol (inhibitor of bacterial protein synthesis) had no effect on adherence (Konkel and Cieplak, 1992). Taken together these data indicated that the adhesins employed by *Campylobacter* are constitutively synthesized. Several molecules that have been proposed to play a role in adherence include the flagellum, lipooligosaccharides, the major outer membrane protein, and pili (Konkel *et al.*, 2001). The more widely recognized and studied adhesions include PEB1 (Pei and Blaser, 1990; Pei *et al.*, 1998), CadF (Konkel *et al.*, 1997; Konkel *et al.*, 1999; Ziprin *et al.*, 1999; Ziprin *et al.*, 2001; Monteville and Konkel, 2002; Monteville *et al.*, 2003), and JlpA (Jin *et al.*, 2001; Jin *et al.*, 2003).

Work by de Melo and Pechère (1990) laid the groundwork for the identification and characterization of *Campylobacter* adhesins. Using a ligand-binding assay de Melo and Pechère (1990) identified four outer membrane proteins from *C. jejuni*, with molecular masses of 28, 32, 36, and 42 kDa, that were able to bind to HEp-2 cells. Pei and Blaser (1990) went on to clone a gene, which codes for a protein with a molecular mass of 28,181 Da by screening a *C. jejuni* genomic λgt11 library with an antibody raised against the purified 28 kDa protein. The 28 kDa protein was termed PEB1, and is a product of the *peb1A* gene (Pei and Blaser, 1990). A null mutant of *peb1A* exhibited a 50- to 100-fold reduction in binding to HeLa cells when compared to wild-type *C. jejuni*, and a 15-fold reduction in invasion into INT 407 cells when compared to

the paternal isolate (Pei *et al.*, 1998). The *peb1A* mutant also displayed a reduction in the duration of mouse intestinal colonization compared to wild-type *C. jejuni* (Pei *et al.*, 1998).

Konkel *et al.* (1997) observed, via scanning electron microscopy, that *C. jejuni* appear to posses the ability to bind to the extracellular matrix of INT 407 cells. Studies also revealed that a 37 kDa outer membrane protein of *C. jejuni* was capable of binding fibronectin (Konkel *et al.*, 1997). The gene encoding the 37 kDa protein was termed *cadF* (*Campylobacter* adhesion to fibronectin). The *cadF* gene is highly conserved among *C. jejuni* and *C. coli* isolates (Konkel *et al.*, 1999; Bang *et al.*, 2003). Studies conducted on new hatched chickens revealed that wild-type isolates of *C. jejuni* can readily colonize the cecum of chickens, whereas a *cadF* null mutant failed to colonize the cecum, indicating that CadF is required to establish colonization in newly hatched leghorn chickens (Ziprin *et al.*, 1999). Additional work by Ziprin *et al.* (2001) has shown that chickens challenged with the *cadF* null mutant provided protection from a subsequent challenge of wild-type *C. jejuni*. Recent work involving CadF has shown that *C. jejuni* preferentially invaded the basolateral surface of intestinal epithelial cells (Monteville and Konkel, 2002) and that CadF is required for maximal adherence and invasion of intestinal epithelial cells (Monteville *et al.*, 2003). Additionally, the binding of CadF to fibronectin has been shown to stimulate a signal transduction pathway allowing for microfilament reorganization (Monteville *et al.*, 2003).

Jin *et al.* (2001) identified a 42.3 kDa lipoprotein, termed JlpA (*jejuni* lipoprotein A). Adherence to HEp-2 cells by a *jlpA* null mutant was reduced 18-19.4% when compared to wild-type *C. jejuni*; however, no difference in invasion was observed (Jin *et al.*, 2001). Recent work by Jin *et*

al. (2003) reported that JlpA interacts with heat shock protein (Hsp) 90□ on the surface on HEp-2 cells. The adherence of JlpA to Hsp90□ leads to the activation of NF-□B and p38 MAP kinase, suggesting that JlpA triggers inflammatory/immune responses in host cells following *C. jejuni* infection (Jin *et al.*, 2003).

The mechanism that *Campylobacter* utilizes to invade epithelial cells, though widely studied, is still poorly characterized. Konkel and Cieplak (1992) demonstrated that *C. jejuni* treated with chloramphenicol were significantly hindered in their ability to invade intestinal epithelial cells. In addition, metabolically inactive (heat-killed and sodium azide-killed) *C. jejuni* failed to internalize (Konkel and Cieplak, 1992). Taken together these data suggest that *C. jejuni* produce proteins required for entry. Metabolic-labeling assays revealed the presence of at least 14 *de novo* synthesized proteins when *C. jejuni* were incubated with INT 407 cells or in INT 407 cell conditioned media as judged by two dimensional gel electrophoresis (Konkel and Cieplak, 1992; Konkel *et al.*, 1993). Konkel *et al.* (1993) went on to develop two antiserums: one against *C. jejuni* cultured in the presence of INT 407 cells (Cj + INT) and one against *C. jejuni* cultured in the absence of INT 407 cells (Cj – INT). The former antiserum was capable of inhibiting internalization in a dose dependent manner, but did not hinder adherence of *C. jejuni* to INT 407 cells (Konkel *et al.*, 1993). This antiserum was used to clone a gene termed *ciaB* (*Campylobacter* invasion antigen B), which codes for a 73kDa protein (Konkel *et al.*, 1999). A *ciaB* null mutant maintained a similar adherence level as wild-type *C. jejuni* to INT 407 cells; however, internalization was significantly reduced (Konkel *et al.*, 1999). The CiaB protein displays a low level of similarity with *Salmonella* SipB, *Shigella* IpaB, and *Yersinia* YopB (Konkel *et al.*, 1999). These proteins lack a typical signal sequence and are translocated from

bacterial cells to eukaryotic cells via type III secretion systems (Hueck *et al.*, 1998). Konkel *et al.* (2004) have recently reported that the secretion of the Cia proteins is dependent upon a functional flagellar export apparatus.

An antiserum generated against a recombinant CiaB protein revealed through immunofluorescence microscopy that CiaB was translocated into the cytoplasm of host cells (Konkel *et al.*, 1999). Metabolic labeling experiments, coupled with one-dimensional gel electrophoresis, revealed 8 proteins in the supernatant fluids of *C. jejuni* co-cultured with INT 407 conditioned media (Konkel *et al.*, 1999). These proteins were designated CiaA-CiaH ranging in size from 108-12.8 kDa (Konkel *et al.*, 1999). Interestingly, a *ciaB* null mutant contained no secreted proteins in the supernatant fluid (Konkel *et al.*, 1999). To date, the remaining Cia proteins have not been identified.

Studies by Rivera-Amill *et al.* (2001) demonstrated that bile salts (deoxycholate, cholate, chenodeoxycholate) induce the expression of the *cia* genes. In addition, a component of fetal bovine serum allowed for the synthesis and secretion of the Cia proteins (Rivera-Amill *et al.*, 2001). Culturing *C. jejuni* on bile salt supplemented plates retarded the inhibitory effect of chloramphenicol on *C. jejuni* invasion (Rivera-Amill *et al.*, 2001).

In vivo studies have revealed that a *ciaB* null mutant failed to colonize the cecum of chickens (Ziprin *et al.*, 2001). Moreover, the initial exposure of chicks to the *ciaB* null mutant followed by challenge with a *C. jejuni* parental strain did not provide significant protection against colonization in chicks (Ziprin *et al.*, 2001). Whereas newborn piglets infected with the wild-type

C. jejuni developed diarrhea within 24 hours post-infection, the *ciaB* null mutant did not develop diarrhea until 72 hours post-inoculation (Konkel *et al.*, 2001).

1.3 Virulence gene regulation

Even with the availability of the *C. jejuni* NCTC 11168 genome (Parkhill *et al.*, 2000) and emerging new mutagenesis strategies (Bleumink-Pluym *et al.*, 1999; Golden *et al.*, 2000; Colegio *et al.*, 2001; Hendrixson *et al.*, 2001), there is still relatively little understood about gene regulation in *C. jejuni*. Based on the genome there are three sigma factors present in *C. jejuni*, σ^{70}, σ^{54}, and σ^{28}. σ^{70} is known as the house-keeping sigma factor and is encoded by *rpoD*. σ^{54} (*rpoN*) and σ^{28} (*fliA*) are involved in the transcription of flagellar genes.

One major obstacle in gene regulation in *Campylobacter* has been the determination of the promoter regions recognized by the three sigma factors. Wösten *et al.* (1998) attempted to characterize the σ^{70} promoter and concluded the promoter was unusual and poorly conserved. Recently, Peterson *et al.* (2003) used a hidden Markov model (HMM) to predict the consensus sequence of the σ^{70} promoter. Using the HMM a TATA box at the –10 promotor region, preceded by a conserved TG at the –16 promotor region was observed (Peterson *et al.*, 2003). This conserved –16 promotor region is similar the –16 promotor region found in Gram-positive bacteria (Peterson *et al.*, 2003). No conserved –35 promotor region was present in *C. jejuni* (Peterson *et al.*, 2003). Research has shown that *flaB*, which encodes a minor component of the flagellin filament, *flgE* (hook) and *flhB* (flagellar biosynthesis) are under the control of a promoter recognized by σ^{54} (Guerry *et al.*, 1990; Kinsella *et al.*, 1997; Matz *et al.*, 2002). FlaA, the major component of the flagellar filament encoded by *flaA*, is controlled by a

promoter recognized by σ^{28} (Guerry *et al.*, 1990). Hendrixson and DiRita (2003) have shown that FlaA transcription is also regulated by σ^{70}. Recently, Carrillo *et al.* (2004) performed genome-wide expression analyses of two variant *Campylobacter jejuni* NCTC 11168 strains. Microarray profiles of the two variants revealed differences in the expression of several flagellar genes as while as virulence factors (Carrillo *et al.*, 2004). Examination of these genes lead to the identification of putative σ^{54} and σ^{28} promoters for several of genes encoding the flagellar structural components and virulence factors including those involved in flagellar glycosylation and cytolethal distending toxin production (Carrillo *et al.*, 2004). Additionally work by Wösten *et al.* (2004) has identified promoter sequences to several of the *Campylobacter* flagellar genes.

The flagellar apparatus of *Campylobacter* has been implicated by Konkel *et al.* (2004) to serve as a type III secretion apparatus for the *Campylobacter* invasion antigens (Cia). Work by Young *et al.* (1999) has demonstrated that *Yersinia* secretes flagellar outer proteins (Fops) via the flagellar type III secretion apparatus. In addition, the expression of the Fops was coregulated with the flagella system; moreover, the expression of *yplA*, a member of the Fops, is dependent upon σ^{28} and the *flhDC* flagella master regulon (Young *et al.*, 1999). Studies in other bacteria, such as *Salmonella*, *Vibrio*, and *Shigella*, have shown that virulence gene regulation and type III secretion systems are coregulated (Prouty and Gunn, 2000; Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999; Konkel and Tilly, 2000).

The flagellum of *C. jejuni* is composed of a basal body, hook, and filament. The filament is comprised of two proteins, FlaA and FlaB, with FlaA being the major component. Until recently flagellar gene regulation was poorly understood in *C. jejuni* due to the lack of a recognizable master regulon (*flhDC*) and an anti-sigma factor (*flgM*) as seen in other organisms, such as

Salmonella. However, recent work by several labs has started to shed light on the regulation of flagellar apparatus in *Campylobacter*. Colland *et al.* (2001) have suggested that Cj1464 may severe as an anti-sigma factor in *C. jejuni*. Hendrixson and DiRita (2003) have shown that FlgM (Cj1464) only slightly reduces *flaA* expression and does not appear to play a significant role in the regulation of the σ^{28} dependent transcription of *flaA* as seen in other organisms. σ^{54}, σ^{28}, and FlgR have been implicated in the regulation of the flagella (Jagannathan *et al.*, 2001). FlaB (filament), FlgE (hook), and FlhB (flagellar biosynthesis) are regulated by a σ^{54} promoter (Guerry *et al.*, 1990; Kinsella *et al.*, 1997; Matz *et al.*, 2002), whereas FlaA (filament) is under the control of a σ^{28} promoter (Guerry *et al.*, 1990). FlgR, a member of the NtrC family of transcriptional regulators as well as a response regulator, serves as a σ^{54} enhancer element binding upstream of σ^{54} promoters. Deletion of *flgR* in *C. jejuni* abolished flagella production (Jagannathan *et al.*, 2001). Recently, Wösten *et al.* (2004) demonstrated that the two component regulatory system FlgS/FlgR is regulated by σ^{70}, and is at the top of the *Campylobacter flagellar* hierarchy. Along with FlgS/FlgR, the other σ^{70} regulated genes include *rpoN* (σ^{54}), the genes involved in production of the secretion apparatus (*flhA*, *B*; *fliH*, *I*, *O*, *P*, *Q*, *R*), the genes involved in the rotor (*fliF*, *G*, *M*, *N*, *Y*), and the motor genes (*motA*, *B*). Together these genes make up the Class I flagellar genes (Carrillo *et al.*, 2004; Wösten *et al.*, 2004). FlgR and RpoN work in conjunction to regulate the Class II flagellar genes, which make up the basal body (*flgF*, *G*, *H*, *I*), the hook (*flgE*, *D*, *K*, *L*), and the filament gene *flaB* (Carrillo *et al.*, 2004; Wösten *et al.*, 2004). Other Class II genes are under the regulation of σ^{70}. These include the sigma factor *fliA* (σ^{28}) and the basal body genes (*flgB*, *C*) (Carrillo *et al.*, 2004). The Class III genes encode for the anti-sigma factor *flgM*, the major filament *flaA*, and the filament cap *fliD*. The Class III genes are primarily under the control of σ^{28}; however, *fliD* and *flgM* are dually regulated by σ^{54} and σ^{28}

promoters. The assembly of the flagellar apparatus begins with the construction of the secretion apparatus and the motor. Next, the basal body and hook are constructed. Upon the completion of the hook it is believed that FlgM is exported allowing for increased production of FliA, which in turn allows the filament (*flaA*) and filament cap genes to be transcribed. Unlike other organisms, such as *Salmonella*, FlgM in *Campylobacter* does not completely repress the transcription of the σ^{28} dependent genes (Hendrixson and DiRita, 2003). A better understanding of the regulational control of the flagellar apparatus may help reveal how the Cia proteins are regulated and exported by the flagellar apparatus.

Bile salts play an important role in the disease progression of several enteric bacteria. Under normal conditions in the human intestine bile salt concentrations range from 0.2-2% (Gunn, 2000). Bile salts act as a detergent to disaggregate the lipid bilayer structure of the cellular membranes, thus acting as an effective antimicrobial (Gunn, 2000). Bile salts can enter directly through the outer membrane of bacteria or by passage through porins (OmpF in *Escherichia coli*) (Thanassi *et al.*, 1997). Enteric pathogens have developed mechanisms to resist the damaging effects of bile salts. For example, *C. jejuni* encodes a multidrug resistance efflux pump termed CmeABC, which confers the organism's resistance to bile salts and enables the organism to colonize the intestine (Lin *et al.*, 2003).

Bile salts are known to play an important role in the regulation of virulence genes in many enteric pathogens. In *Shigella,* bile salts increase Ipa (invasion plasmid antigens) protein secretion as while as increase invasion of epithelial cells due to enhanced adherence (Pope *et al.*, 1995). In addition, enhanced invasion was not observed when chloramphenical was used to stop

protein synthesis prior to growth in bile salts (Pope *et al.*, 1995). How bile salts are sensed in *Shigella* and how bile salts influence the regulation of virulence genes is unknown. What is known is that virulence genes in *Shigella* are controlled by transcriptional activator and repressor proteins and indirectly via the two component regulatory system EnvZ/OmpR, which detects changes in osmolarity (Maurelli *et al.*, 1992; Konkel and Tilly, 2000). At 30°C and low osmolarity, the invasion proteins and a type III secretion system are repressed by VirR, which binds to the operator region of *virF* (Konkel and Tilly, 2000). However, if the temperature is raised to 37°C and the osmolarity of the external environment is increased, VirR will dissociate from *virF* causing the derepression of *virF* (Konkel and Tilly, 2000). VirF acts as a transcriptional activator, which helps recruit RNA polymerase and allows the expression of *virB* (Konkel and Tilly, 2000). The transcriptional activator VirB allows for the expression of the *Shigella* invasion proteins (Ipa) and a type III secretion system (Konkel and Tilly, 2000).

As mentioned earlier, *Campylobacter* synthesizes the Cia proteins in response to growth in the presence of deoxycholate (Rivera-Amill *et al.*, 2001). Bile salts have also been shown to upregulate *flaA* promoter activity in *C. jejuni* (Allen and Griffiths, 2001). Recently, Konkel *et al.* (unpublished data) observed that *Campylobacter* grown on deoxycholate supplemented plates were able to invade epithelial cells immediately upon binding.

The methods that bacteria utilize to sense the presence of bile may include two component regulatory systems and AraC-like regulatory factors. Two component regulatory (TCR) systems allow for global gene regulation by bacteria, plants, and lower forms of eukaryotes such as *Saccharomyces cerevisiae*. Global gene regulation allows organisms to adapt to numerous environmental changes such as temperature, pH, nutrients, and osmolarity (Hoch and Silhavy,

1995). A typical two-component regulatory system is comprised of two subunits: a histidine/sensor kinase (HK) and a response regulator (RR) (Hoch and Silhavy, 1995). A standard HK consists of a transmembrane protein that contains two functional domains: an N-terminal signal or input domain and a C-terminal sensor kinase. The N-terminal portion of the signal domain of the HK usually resides within the periplasmic space and either directly interacts with an external signal or another protein that serves to relay the external signal to the input domain. Upon receiving the external stimulus the signal domain will activate the C-terminal sensor kinase domain, located in the cytoplasm. The activated sensor kinase hydrolyzes ATP, causing the phosphorylation of a conserved histidine residue in the sensor kinase domain. The second subunit of a typical TCR system is the RR. The RR is localized in the cytoplasm and contains an N-terminal response regulator domain and a C-terminal output domain. Once the HK is phosphorylated, a conformational change occurs allowing the docking of the N-terminus of the RR with the C-terminus of the HK. Docking allows the HK to transfer the phosphate, via a phosphorelay system, to a conserved aspartic acid residue located in the N-terminal response regulator domain. Phosphorylation of the RR will activate the C-terminal output domain. A typical phosphorylated/activated RR binds to DNA, via a DNA-binding motif, stimulating the induction or repression genes controlled by that RR. Some RRs lack the C-terminal output domain and are unable to bind to DNA; however, these RRs are capable of binding other proteins. Phosphorylated CheY (RR), a member of the TCR system that controls chemotaxis, will bind to FliM and reverse the direction of flagellar rotation (McEvoy *et al.*, 1999). In other TCR systems, a phosphorylated RR can transfer its phosphate to another HK.

Studies in pathogenic organisms (*Salmonella* and *Vibrio*) have shown that bile salts act as an external signal to regulate TCR systems that control the expression of virulence genes (Gunn, 2000). In *Salmonella,* the PhoQ/P TCR system is necessary for enhanced resistance to bile salts (Van Velkinburgh and Gunn, 1999). The presence of bile salts acts on the *Salmonella* TCR system, BarA/SirA, to repress the invasion regulatory proteins SirC, SirB, HilD, HilA, and InvF, thus preventing the transcription of *Salmonella* pathogenicity island 1 (Prouty and Gunn, 2000). *Salmonella* pathogenicity island 1 codes for a type III secretion system and *Salmonella* secreted proteins. Bile salts have also been shown to serve as an external signal to stimulate motility in *Vibrio cholerae* (Gupta and Chowdhury, 1997). Furthermore, bile salts negatively regulate ToxT (RR)-dependent transcription of genes encoding for cholera toxin and the toxin-coregulated pilus (Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999).

An alternative method that has been suggested for bacteria to sense bile salts is through the use of AraC-like transcriptional regulators. These transcriptional regulators respond to environmental signals such as oxidative stress, temperature, osmolarity, calcium concentration, and pH (Tobes and Ramos, 2002). Some contain a signal-receptor domain in addition to the DNA-binding domain (Tobes and Ramos, 2002). In a second group of AraC family transcriptional regulators, an unlinked signal receptor protein controls the synthesis of the AraC regulator protein (Tobes and Ramos, 2002). The proteins belonging to the AraC regulator family share three main regulatory functions: carbon metabolism, stress response and pathogenesis (Tobes and Ramos, 2002). Recently, Rosenberg *et al.* (2003) have shown that Rob, a member of the AraC family, is able to bind bile salts thus allowing Rob to mediate the transcription of an AcrAB efflux pump in *E. coli* and protect the bacteria from the harmful effects of bile salts.

Analysis of the *C. jejuni* genome has identified seven histidine kinases (HK) and eleven response regulators (RR), as well as an AraC family transcriptional regulator. Three genes were found that encode two component regulatory proteins involved with chemotaxis (*cheA, cheY*, and *cheV*). Work by Yao *et al.* (1997) demonstrated that a *C. jejuni cheY* null mutant was able to adhere to and invade INT 407 cells at a three-fold higher level than *C. jejuni* parental strain 81-176. In addition, a *C. jejuni cheY* null mutant was unable to colonize and cause disease in mice and ferrets (Yao *et al.*, 1997). A two component regulatory system involving *racS* (HK) and *racR* (RR) was identified as a temperature-dependent signaling pathway in *C. jejuni* (Brás *et al.*, 1999). A *racR* null mutant resulted in the reduction of colonization in the intestines of chickens (Brás *et al.*, 1999). The *racR* null mutant displayed temperature dependent changes in its protein profile and growth characteristics (Brás *et al.*, 1999). FlgR, a σ^{54} associated response regulator has been shown to be involved in the regulation of flagellin expression in *C. jejuni* (Jagannathan *et al.*, 2001; Hendrixson and DiRita, 2003; Wöston *et al.*, 2004). Recently, Wöston *et al.* (2004) have shown that the two component system FlgS/FlgR is involved in the gene regulation of the flagellar of apparatus. A better understanding of the functions of *C. jejuni* two component regulatory systems and AraC family transcriptional regulators will allow researchers to better understand how virulence factors in *C. jejuni* are regulated.

REFERENCES

Alfredson, D.A., Korolik, V. (2003) Sequence analysis of a cryptic plasmid pCJ419 from *Campylobacter jejuni* and construction of an *Escherichia coli-Campylobacter* shuttle vector. *Plasmid* 50, 152-60.

Allen, K.J., Griffiths, M.W. (2001) Effect of environmental and chemotactic stimuli on the activity of the *Campylobacter jejuni flaA* σ^{28}promoter. *FEMS Microbiol Lett* 205, 43-8.

Allos, B.M. (1997) Association between *Campylobacter* infection and Guillain-Barré syndrome. *J Infect Dis* 176, S125-8.

Allos, B.M. (2001) *Campylobacter jejuni* infections: update on emerging issues and trends. *Clin Infect Dis* 32, 1201-6.

Allos, B.M., Blaser, M.J. (1995) *Campylobacter jejuni* and the expanding spectrum of related infections. *Clin Infect Dis* 20, 1092-1101.

Bacon, D.J., Alm, R.A., Burr, D.H., Hu, L., Kopecko, D.J., Ewing, C.P., Trust, T.J., Guerry, P. (2000) Involvement of a plasmid in virulence of *Campylobacter jejuni* 81-176. *Infect Immun* 68, 4384-90.

Bang, D.D., Nielsen, E.M., Scheutz, F., Pedersen, K., Handberg, K., Madsen, M. (2003) PCR detection of seven virulence and toxin genes of *Campylobacter jejuni* and *Campylobacter coli* isolates from Danish pigs and cattle and cytolethal distending toxin production of the isolates. *J Appl Microbiol* 94, 1003-14.

Berndtson, E. (1996) *Campylobacter* in broiler chickens. Thesis. Swedish University of Agricultural Sciences, Uppsala, Sweden.

Black, R.E., Levine, M.M., Clements, M.L., Hughes, T.P., Blaser, M.J. (1988) Experimental *Campylobacter jejuni* infection in humans. *J Infect Dis* 157, 472-9.

Blaser, M.J. (1997) Epidemiologic and clinical features of *Campylobacter jejuni* infections. *J Infect Dis* 176, S103-5.

Blaser, M.J., Reller, L.B., Luechtefled, N.W., Wang, W.L.L. (1982) *Campylobacter* enteritis in Dever. *West J Med* 136, 287-90.

Blaser, M.J., Taylor, D.E., Feldman, R.A. (1983) Epidemiology of *Campylobacter jejuni* infections. *Epidemiol Rev* 5, 157-175.

Bleumink-Pluym, N.M.C., Verschoor, F., Gaastra, W., van der Zeijst, B.A.M., Fry, B.N. (1999) A novel approach for the construction of a *Campylobacter* mutant library. *Microbiol* 145, 2145-51.

Bourke, B., Sherman, P., Louie, H., Hani, E., Islur, P., Chan, V.L. (1995) Physical and genetic map of the genome of *Campylobacter upsaliensis*. *Microbiol* 141, 2417-24.

Brás, A.M., Chatterjee, S., Wren, B.W., Newell, D.G., Ketley, J.M. (1999) A novel *Campylobacter jejuni* two-component regulatory system important for temperature-dependent growth and colonization. *J Bacteriol* 181, 3298-302.

Butzler, J.P., Dekeyser, P., Detrain, M., Dehaen, F. (1973) Related *vibrio* in stools. *J Pediatr* 82, 493-5.

Carrillo, C.D., Taboada, E., Nash, J.H.E., Lanthier, P., Kelly, J., Lau, P., Verhulp, R., Mykytczuk, O., Sy, J., Findlay, W.A., Amoako, K., Gomis, S., Willson, P., Austin, J.W., Potter, A., Babiuk, L., Allan, B., Szymanski, C.M. (2004) Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. *J Biol Chem* Feb. 25 [Epub ahead of print].

Chang, N., Taylor, D.E. (1990) Use of pulsed-field agarose gel electrophoresis to size genomes of *Campylobacter* species and to construct a *Sal*I map of *Campylobacter jejuni* UA580. *J Bacteriol* 172, 5211-7.

Colland, F., Rain, J.C., Gounon, P., Labigne, A., Legrain, P., De Reuse H. (2001) Identification of the Helicobacter pylori anti-σ^{28} factor. *Mol Microbiol* 41, 477-87.

Colegio, O.R., Griffin, T.J. 4th., Grindley, N.D.F., Galán, J.E. 2001 In vitro transposition system for efficient generation of random mutants of *Campylobacter jejuni*. *J Bacteriol* 183, 2384-8.

de Melo, M.A., Pechère, J-C. (1990) Identification of *Campylobacter jejuni* surface proteins that bind to Eucaryotic cells *in vitro*. *Infect Immun* 58, 1749-56.

Dekeyser, P., Gossuin-Detrain, M., Butzler, J.P., Sternon, J. (1972) Acute enteritis due to related *vibrio*: first positive stool cultures. *J Infect Dis* 125, 390-2.

Engberg, J., On, S.L.W., Harrington, C.S., Gerner-Smidt, P. (2000) Prevalence of *Campylobacter*, *Arcobacter*, *Helicobacter*, and *Sutterella* spp. in human fecal samples as estimated by a reevaluation of isolation methods for Campylobacters. *J Clin Microbiol* 38, 286-91.

Fauchère, J.L., Rosenau, A., Véron, M., Moyen, E.N., Richard, S., Pfister, A. (1986) Association with HeLa cells of *Campylobacter jejuni* and *Campylobacter coli* isolated from human feces. *Infect Immun* 54, 283-7.

Friedman, C., Neimann, J., Wegener, H., Tauxe, R. (2000) Epidemiology of *Campylobacter jejuni* infections in the United States and other industrialized nations. *In Campylobacter 2nd Ed*. Nachamkin, I., Blaser, M.J. eds. American Society for Microbiology, Washington, D.C. pp. 121-138.

Golden, N.J., Camilli, A., Acheson, D.W.K. (2000) Random transposon mutagenesis of *Campylobacter jejuni*. *Infect Immun* 68, 5450-3.

Griffiths, P.L., Park, R.W. (1990) Campylobacters associated with human diarrhoeal disease. *J Appl Bacteriol* 69, 281-301.

Guerry, P., Logan, S.M., Thornton, S., Trust, T.J. (1990) Genomic organization and expression of *Campylobacter* flagellin genes. *J Bacteriol* 172, 1853-60.

Gunn, J.S. (2000) Mechanisms of bacterial resistance and response to bile. *Microbes Infect* 2, 907-13.

Gupta, S., Chowdhury, R. (1997) Bile affects production of virulence factors and motility of *Vibrio cholerae*. *Infect Immun* 65, 1131-4.

Hadden, R.D., Gregson, N.A. (2001) Guillain-Barré syndrome and *Campylobacter jejuni* infection. *Symp Ser Soc Appl Microbiol* 90, 145S-54S.

Hendrixson, D.R., Akerley, B.J., DiRita, V.J. (2001) Transposon mutagenesis of *Campylobacter jejuni* identifies a bipartite energy taxis system required for motility. *Mol Microbiol* 40, 214-24.

Hendrixson, D.R., DiRita, V.J. (2003) Transcription of σ^{54}-dependent but not σ^{28}-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. *Mol Microbiol* 50, 687-702.

Hueck, C.J. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* 62, 379-433.

Ho, T.W., McKhann, G.M., Griffin, J.W. (1998) Human autoimmune neuropathies. *Annu Rev Neurosci* 21, 187-226.

Hoch, J.A., Silhavy, T.J. (1995) *Two-component signal transduction*. ASM Press, Washington, D.C.

Jagannathan, A., Constantinidou, C., Penn, C.W. (2001) Roles of *rpoN*, *fliA*, and *flgR* in expression of flagella in *Campylobacter jejuni*. *J Bacteriol* 183, 2937-42.

Jin, S., Joe, A., Lynett, J., Hani, E.K., Sherman, P., Chan, V.L. (2001) JlpA, a novel surface-exposed lipoprotein specific to *Campylobacter jejuni*, mediates adherence to host epithelial cells. *Mol Microbiol* 39, 1225-36.

Jin, S., Song, Y.C., Emili, A., Sherman, P.M., Chan, V.L. (2003) JlpA of *Campylobacter jejuni* interacts with surface-exposed heat shock protein 90α and triggers signalling pathways leading to the activation of NF-κB and p38 MAP kinase in epithelial cells. *Cell Microbiol* 5, 165-74.

Ketley, J.M. (1997) Pathogenesis of enteric infection by *Campylobacter*. *Microbiol* 143, 5-21.

Kinsella, N., Guerry, P., Cooney, J., Trust, T.J. (1997) The *flgE* gene of *Campylobacter coli* is under the control of the alternative sigma factor σ^{54}. *J Bacteriol* 179, 4647-53.

Koenraad, P.M.F.J., Rombouts, F.M., Notermans, S.H.W. (1997) Epidemiological aspects of thermophilic *Campylobacter* in water-related environments: a review. *Water Environ Res* 69, 52-63.

Konkel, M.E., Cieplak, W., Jr. (1992) Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. *Infect Immun* 60, 4945-9.

Konkel, M.E., Garvis, S.G., Tipton, S.L., Anderson, D.E., Jr., Cieplak, W., Jr. (1997) Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol* 24, 953-63.

Konkel, M.E., Gray, S.A., Kim, B.J., Garvis, S.G., Yoon, J. (1999) Identification of the enteropathogens *Campylobacter jejuni* and *Campylobacter coli* based on the *cadF* virulence gene and its product. *J Clin Microbiol* 37, 510-7.

Konkel, M.E., Kim, B.J., Rivera-Amill, V., Garvis, S.G. (1999) Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Mol Microbiol* 32, 691-701.

Konkel, M.E., Klena, J.D., Rivera-Amill, V., Monteville, M.R., Biswas, D., Raphael, B., Mickelson, J. (2004) Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J Bacteriol* 186.

Konkel, M.E., Mead, D.J., Cieplak, W., Jr. (1993) Kinetic and antigenic characterization of altered protein synthesis by *Campylobacter jejuni* during cultivation with human epithelial cells. *J Infect Dis* 168, 948-54.

Konkel, M.E., Monteville, M.R., Rivera-Amill, V., Joens, L.A. (2001) The pathogenesis of *Campylobacter jejuni*-mediated enteritis. *Curr Issues Intest Microbiol* 2, 55-71.

Konkel, M.E., Tilly, K. (2000) Temperature-regulated expression of bacterial virulence genes. *Microbes Infect* 2, 157-66.

Lane, L., Baker, M. (1993) Are we experiencing an epidemic of *Campylobacter* infection? *Communicable Disease New Zealand* 93, 57-69.

Lin, J., Sahin, O., Michel, L.O., Zhang, Q. (2003) Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infect Immun* 71, 4250-9.

Luo, N., Zhang, Q. (2001) Molecular characterization of a cryptic plasmid from *Campylobacter jejuni*. *Plasmid* 45, 127-33.

Macuch, P.J., Tanner, A.C. (2000) *Campylobacter* species in health, gingivitis, and periodontitis. *J Dent Res* 79, 785-92.

Matz, C., van Vliet, A.H.M., Ketley, J.M., Penn, C.W. (2002) Mutational and transcriptional analysis of the *Campylobacter jejuni* flagellar biosynthesis gene *flhB*. *Microbiol* 148, 1679-85.

Maurelli, A.T., Hromockyj, A.E., Bernardini, M.L. (1992) Environmental regulation of *Shigella* virulence. *Curr Top Microbiol Immun* 180, 95-116.

McEvoy, M.M., Bren, A., Eisenbach, M., Dahlquist, F.W. (1999) Identification of the binding interfaces on CheY for its targets, the phosphatase CheZ and the flagellar switch protein FliM. J Mol Biol 289, 1423-33.

Monteville, M.R., Konkel, M.E. (2002) Fibronectin-facilitated invasion of T84 eukaryotic cells by *Campylobacter jejuni* occurs preferentially at the basolateral cell surface. *Infect Immun* 70, 6665-71.

Monteville, M.R., Yoon, J.E., Konkel, M.E. (2003) Maximal adherence and invasion of INT 407 cells by *Campylobacter jejuni* requires the CadF outer-membrane protein and microfilament reorganization. *Microbiol* 149, 153-65.

Nachamkin, I. (2002) Chronic effects of *Campylobacter* infection. *Microbes Infect* 4, 399-403.

Nachamkin, I, Allos, B.M., Ho, T. (1998) *Campylobacter* species and Guillain-Barré syndrome. *Clin Microbiol Rev* 11, 555-67.

On, S.L.W. (2001) Taxonomy of *Campylobacter*, *Arcobacter*, *Helicobacter*, and related bacteria: current status, future prospects, and immediate concerns. *Symp Ser Soc Appl Microbiol* 90**,** 1S-15S.

Park, S.F. (2002) The physiology of *Campylobacter* species and its relevance to their role as foodborne pathogens. *Int J Food Microbiol* 74, 177-88.

Parkhill, J., Wren, B.W., Mungall, K., Ketley, J.M., Churcher, C., Basham, D., Chillingworth, T., Davies, R.M., Feltwell, T., Holroyd, S., Jagels, K., Karlyshev, A.V., Moule, S., Pallen, M.J., Penn, C.W., Quail, M.A., Rajandream, M-A., Rutherford, K.M., van Vliet, A.H., Whitehead, S., Barrell, B.G. (2000) The genome sequence of the food-borne pathogen *Campylobacter jejuni* reveals hypervariable sequences. *Nature* 403, 665-8.

Pei, Z., Blaser, M.J. (1993) PEB1, the major cell-binding factor of *Campylobacter jejuni*, is a homolog of the binding component in gram-negative nutrient transport systems. *J Biol Chem* 268, 18717-25.

Pei, Z., Burucoa, C., Grignon, B., Baqar, S., Huang, X-Z., Kopecko, D.J., Bourgeois, A.L., Fauchere, J-L., Blaser, M.J. (1998) Mutation in the peb1A locus of *Campylobacter jejuni* reduces interactions with epithelial cells and intestinal colonization of mice. *Infect Immun* 66, 938-43.

Petersen, L., Larsen, T.S., Ussery, D.W., On, S.L.W., Krogh, A. (2003) *Rpo*D promoters in *Campylobacter jejuni* exhibit a strong periodic signal instead of a -35 box. *J Mol Biol* 326, 1361-72.

Pope, L.M., Reed, K.E., Payne, S.M. (1995) Increased protein secretion and adherence to HeLa cells by *Shigella* spp. following growth in the presence of bile salts. *Infect Immun* 63, 3642-8.

Prouty, A.M., Gunn, J.S. (2000) *Salmonella enterica* serovar typhimurium invasion is repressed in the presence of bile. *Infect Immun* 68, 6763-9.

Reina, J., Borrell, N., Serra, A. (1992) Emergence of resistance to erythromycin and fluoroquinolones in thermotolerant *Campylobacter* strains isolated from feces 1987-1991. *Eur J Clin Microbiol Infect Dis* 11, 1163-6.

Rivera-Amill, V., Kim, B.J., Seshu, J., Konkel, M.E. (2001) Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J Infect Dis* 183, 1607-16.

Rosenberg, E.Y., Bertenthal, D., Nilles, M.L., Bertrand, K.P., Nikaido, H. (2003) Bile salts and fatty acids induce the expression of *Escherichia coli* AcrAB multidrug efflux pump through their interaction with Rob regulatory protein. *Mol Microbiol* 48, 1609-19.

Salazar-Lindo, E., Sack, R.B., Chea-Woo, E., Kay, B.A., Piscoya, Z.A., Leon-Barua, R., Yi, A. (1986) Early treatment with erythromycin of *Campylobacter jejuni*-associated dysentery in children. *J Pediatr* 109, 355-60.

Sauerwein, R.W., Bisseling, J., Horrevorts, A.M. (1993) Septic abortion associated with *Campylobacter fetus* subspecies *fetus* infection: case report and review of the literature. *Infection* 21, 331-3.

Schuhmacher, D.A., Klose, K.E. (1999) Environmental signals modulate ToxT-dependent virulence factor expression in *Vibrio cholerae*. *J Bacteriol* 181, 1508-14.

Sebald, M., Véron, M. (1963) Teneur en bases da l'ADN et classification des vibrions. *Annales de L'institut Pasteur (Paris)* 105, 897-910.

Skirrow, M.B., Blaser, M.J. (1995) *Campylobacter jejuni*. *In Infections of the gastrointestinal tract*. Blaser, M.J., Smith, P.D., Ravdin, J.I., Greenberg, H.B., Guerrant, R.L. eds. Raven Press, New York City. pp. 285-48.

Taylor, D.E. (1992) Genetics of *Campylobacter* and *Helicobacter*. *Annu Rev Microbiol* 46, 35-64.

Thanassi, D.G., Cheng, L.W., Nikaido, H. (1997) Active efflux of bile salts by *Escherichia coli*. *J Bacteriol* 179, 2512-8.

Tobes, R., Ramos, J.L. (2002) AraC-XylS database: a family of positive transcriptional regulators in bacteria. *Nucleic Acids Res* 30, 318-21.

Van Velkinburgh, J.C., Gunn, J.S. (1999) PhoP-PhoQ-regulated loci are required for enhanced bile resistance in *Salmonella* spp. *Infect Immun* 67, 1614-22.

Walker, R. I., Caldwell, M.B., Lee, E.C., Guerry, P., Trust, T.J., Ruiz-Palacios, G.M. (1986) Pathophysiology of *Campylobacter* enteritis. *Microbiol Rev* 50, 81-94.

Wösten, M.M.S.M., Boeve, M., Koot, M.G.A., van Nuenen, A.C., van der Zeijst, B.A.M. (1998) Identification of *Campylobacter jejuni* promoter sequences. *J Bacteriol* 180, 594-9.

Wösten, M.M.S.M., Wagenaar, J.A., Van Putten, J.P.M. (2004) The FlgS/FlgR two-component signal transduction system regulates the *fla* regulon in *Campylobacter jejuni*. *J Biol Chem* Feb. 11 [Epub ahead of print].

Yao, R., Burr, D.H., Guerry, P. (1997) CheY-mediated modulation of *Campylobacter jejuni* virulence. *Mol Microbiol* 23, 1021-31.

Young, G.M., Schmiel, D.H., Miller, V.L. (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc Natl Acad Sci USA* 96, 6456-61.

Ziprin, R.L., Young, C.R., Byrd, J.A., Stanker, L.H., Hume, M.E., Gray, S.A., Kim, B.J., Konkel, M.E. (2001) Role of *Campylobacter jejuni* potential virulence genes in cecal colonization. *Avian Dis* 45, 549-57.

Ziprin, R.L., Young, C.R., Stanker, L.H., Hume, M.E., Konkel, M.E. (1999) The absence of cecal colonization of chicks by a mutant of *Campylobacter jejuni* not expressing bacterial fibronectin-binding protein. *Avian Dis* 43, 586-9.

Chapter 2

REGULATION OF *Campylobacter* INVASION ANTIGEN B

Running Title: Regulation of *ciaB*

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Manuscript in preparation

INTRODUCTION

Campylobacter jejuni, a Gram-negative bacterium motile via bipolar flagella is considered a major cause of gastroenteritis. Individuals infected with *C. jejuni* display symptoms such as fever, severe abdominal cramping, and diarrhea often containing blood and leukocytes. Studies by Rivera-Amill *et al.* (2001) demonstrated that bile salts induce the expression the genes encoding the *Campylobacter* invasion antigens (Cia). In addition, a component of fetal bovine serum allowed for the synthesis and secretion of the Cia proteins (Rivera-Amill *et al.*, 2001). Recently, work by Konkel *et al.* (2004) has demonstrated that the flagellum of *C. jejuni* serves as a type III export apparatus for the secretion of the Cia proteins. Furthermore, studies in other pathogenic organisms have revealed that type III secretion apparatus and virulence gene expression are coregulated (Young *et al.*, 1999; Konkel and Tilly, 2000; Prouty and Gunn, 2000).

The flagellum of *C. jejuni* is composed of a basal body, hook, and filament. The filament is comprised of two proteins, FlaA and FlaB, with FlaA being the major component. Flagellar gene regulation was poorly understood in *C. jejuni* due to the lack of a recognizable master regulon (*flhDC*) and an anti-sigma factor (*flgM*) as seen in other organisms, such as *Salmonella*. However, recent findings have begun to shed light on the regulation of flagellar genes. Colland *et al.* (2001) suggested that Cj1464 may serve as an anti-sigma factor in *C. jejuni*. Recently, Hendrixson and DiRita (2003) have shown that Cj1464 (*flgM*) only slightly reduces *flaA* expression and does not appear to play a significant role in the regulation of the σ^{28} dependent transcription of *flaA* as seen in other organisms. σ^{54}, σ^{28}, and FlgR have been implicated in the regulation of the flagella (Jagannathan *et al.*, 2001). *flaB* (filament), *flgE* (hook), and *flhB*

(flagellar biosynthesis) are regulated by a σ^{54} promoter (Guerry *et al.*, 1990; Kinsella *et al.*, 1997; Matz *et al.*, 2002), whereas *flaA* (filament) is under the control of a σ^{28} promoter (Guerry *et al.*, 1990). Recently, Carrillo *et al.* (2004) suggested census sequences for σ^{54} and σ^{28} by using the flagellar genes. FlgR, a member of the NtrC family of transcriptional regulators as well as a response regulator, serves as a σ^{54} enhancer element binding upstream of σ^{54} promoters. Deletion of *flgR* in *C. jejuni* abolished flagella production (Jagannathan *et al.*, 2001). Furthermore, research by Wösten *et al.* (2004) has shown that the FlgS/FlgR two component regulatory system under the control of σ^{70}, is at the top of the flagellar gene hierarchy. A model has been set forth dividing the regulation of the flagellar genes into three classes (Wösten *et al.*, 2004; Carrillo *et al.*, 2004). The Class I genes are regulated by σ^{70} and comprise the secretion apparatus (*flhA*, *B*; *fliH*, *I*, *O*, *P*, *Q*, *R*), the rotor (*fliF*, *G*, *M*, *N*, *Y*) and the motor (*motA*, *B*). Class II genes are primarily under the control of σ^{54} working in conjunction with FlgR. These genes encode the basal body (*flgF*, *G*, *H*, *I*), the hook (*flgE*, *D*, *K*, *L*), and the filament *flaB*. The *flgB* and *flgC* genes that encode part of the basal body are transcribed by σ^{70}. The Class III genes are under the regulation of σ^{28} and include the filament *flaA*, the filament cap *fliD*, and *flgM*. *fliD* and *flgM* transcription is also controlled by σ^{54}.

Virulence genes regulation in *C. jejuni* is poorly understood; however, in other enteric pathogens virulence genes are regulated via environmental signals such as temperature, pH, osmolarity, and bile salts (Hoch and Silhavy, 1995; Gunn, 2000). These environmental signals are sensed by two-component regulatory (TCR) systems and AraC-like transcriptional factors. Studies in pathogenic organisms (*Salmonella* and *Vibrio*) have shown that bile salts act as an external signal to regulate TCR systems that control the expression of virulence genes (Gunn, 2000). The

presence of bile salts acts on the *Salmonella* TCR system, BarA/SirA, to repress the invasion regulatory proteins SirC, SirB, HilD, HilA, and InvF, thus preventing the transcription of *Salmonella* pathogenicity island 1 (Prouty and Gunn, 2000). *Salmonella* pathogenicity island 1 encodes for a type III secretion system and *Salmonella* secreted proteins. Bile salts have also been shown to serve as an external signal to stimulate motility in *Vibrio cholerae* (Gupta and Chowdhury, 1997). Furthermore, bile salts negatively regulate ToxT (response regulator)-dependent transcription of genes encoding for cholera toxin and the toxin-coregulated pilus (Gupta and Chowdhury, 1997; Schuhmacher and Klose, 1999). Virulence gene expression and a type III secretion apparatus in *Shigella* are controlled by transcriptional activator and repressor proteins and indirectly via the two component regulatory system EnvZ/OmpR, which detects changes in osmolarity (Maurelli *et al.*, 1992; Konkel and Tilly, 2000). In *Shigella*, VirR will dissociate from *virF* (AraC-like regulatory factor) causing the derepression of *virF*, when the temperature is raised from 30°C to 37°C and the osmolarity of the external environment is increased. VirF acts as a transcriptional activator, which helps recruit RNA polymerase and allows the expression of *virB* and in turn allows for the expression of the *Shigella* invasion proteins (Ipa) and a type III secretion system. Young *et al.* (1999) demonstrated that *Yersinia* secretes flagellar outer proteins (Fops) via the flagellar type III secretion apparatus. In addition, the expression of the Fops was coregulated with the flagella system; moreover, the expression of *yplA*, a member of the Fops, is dependent upon σ^{28} and the *flhDC* flagella master regulon (Young *et al.*, 1999).

Due to the fact that virulence gene regulation and type III secretion systems are coregulated in other bacteria (*Salmonella*, *Vibrio*, *Shigella*, and *Yersinia*), a study was undertaken to determine

if the Cia proteins are coregulated with the flagella export apparatus. To accomplish this we sought to identify the sigma factor controlling the transcription of *ciaB*. In addition, we wanted to determine if a two component regulatory system or an AraC-like transcriptional factor regulates the expression of *ciaB*.

MATERIALS AND METHODS

Bacterial isolates and growth conditions. *C. jejuni* isolate F38011 was cultured on Mueller-Hinton (MH) agar plates containing 5% bovine citrated blood (MH-blood) under microaerobic conditions at 37°C. Where appropriate, MH-blood plates were supplemented with 12.5 μg/ml tetracycline, 15 μg/ml chloramphenicol, and 200 μg/ml kanamycin. *Campylobacter* isolates were routinely passed every 24-48 hours. For certain experiments, *C. jejuni* was harvested from MH agar plates supplemented with sodium deoxycholate (0.1% wt/vol). *Escherichia coli* XL-1 Blue MRF (Stratagene, La Jolla, CA) and Inv□F' (Invitrogen, Carlsbad, CA) were cultured in Luria-Bertani (LB) broth (10 g Bacto-tryptone, 5 g yeast extract, and 15 g Bacto-agar/L) in a 37°C incubator. LB plates were supplemented with 12.5 μg/ml tetracycline, 15 μg/ml chloramphenicol, and 50 μg/ml kanamycin as appropriate.

Construction of single crossover response regulator and AraC-like mutants. *C. jejuni* strain NCTC 11168 Cj0285c (*cheV*), Cj0355c, Cj0643, Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj1223c, Cj1227c, Cj1261 (*racR*), Cj1491c, Cj1608, Cj0670 (*rpoN*), and Cj0061c (*fliA*) gene sequences were obtained from the Sanger Centre website (http://www.sanger.ac.uk /Projects/C. jejuni). The Cj0285c (*cheV*), Cj0355c, Cj0643, Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj1223c, Cj1227c, Cj1261 (*racR*), Cj1491c, and Cj1608 genes in *C. jejuni* F38011 were disrupted by recombination via a single crossover event between the chromosomal gene and an internal fragment of the homologous gene in a suicide vector harboring the *aphA-3* gene encoding kanamycin resistance (Konkel *et al.*, 1997). The primers used to amplify the internal gene fragments are listed in Table 1.

Construction of Cj0890c chromosomal mutant. Chromosomal DNA isolated from the Cj0890 Kan single crossover mutant was digested with *Bgl*II and electroporated into *C. jejuni* parental strain F38011. Cj0890c chromosomal mutants were selected on kanamycin plates and Cj0890c disruption and *aphA-3* gene acquisition was confirmed by PCR.

Construction of double crossover response regulator, AraC-like, and sigma factor mutants. The Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj0670 (*rpoN*), and Cj0061c (*fliA*) genes in *C. jejuni* F38011 were disrupted by allelic replacement via a double crossover event between the chromosomal gene and a homologous gene containing an internal deletion and the *cat* gene (encodes chloramphenicol resistance) in a suicide vector harboring the *aphA-3* gene (Konkel *et al.*, 1997). The primers used to amplify the 5' and 3' ends of the genes [Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj0670 (*rpoN*), and Cj0061c (*fliA*)] as well as the *cat* gene are listed in Table 2. The forward primer for the 5' end of the genes contained a *Bam*HI restriction site and the reverse primer contained *Nhe*I and *Sac*II restriction sites. The forward primer for the 3' end of the genes contained a *Nhe*I restriction site; while the reverse primer contained a *Sac*II restriction site. Following the ligation of the DNA fragments harboring the 5' and 3' regions of the genes; the *cat* gene was ligated into the *Nhe*I restriction site. The recombinant vectors were introduced into *C. jejuni* F38011 by electroporation. *C. jejuni* F38011 mutants were identified by acquisition of kanamycin or chloramphenicol resistance/kanamycin sensitivity. Additionally, specific gene disruption was confirmed by PCR.

Complementation of the *fliA* double crossover mutant. A 2000 bp fragment containing the entire *fliA* gene and flanking DNA sequences was amplified from *C. jejuni* F38011 by PCR using the following primers: 5' -TTATTGAAAGATTTTAGC (forward primer) and 5' -GCATCAATTTCTTCTTGG (reverse primer). The forward primer is 348 bp upstream of the AUG methionine initiation codon and the reverse primer extends 34 bp beyond UAA stop codon. Following an intermediate cloning step into pCR2.1 (Invitrogen), the gel-purified insert was ligated into the *BamH*I site of pMEK80 (Rivera-Amill *et al.*, 2001). The resultant shuttle plasmid was introduced into *C. jejuni* strain F38011 *fliA*$^-$ mutant by electroporation. Transformants were identified by resistance to tetracycline and plasmid carriage was confirmed by PCR.

Motility assay. Motility assays were preformed using MH medium supplemented with 0.4% Bacto Agar. A 10 □l suspension of each bacterial isolate was spotted on the surface of the medium. Motility plates were incubated under microaerophilic conditions for 48 hr and then scored for motility on whether the isolate migrated from the center spot.

Preparation of secreted proteins. *C. jejuni* were harvested from MH-blood agar plates in Minimal Essential Medium (MEM), pelleted by centrifugation at 6000 x *g*, washed twice in MEM. The pellets were resuspended in MEM or MEM with 1% fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) to an optical density (OD_{540}) of 0.3 [approximately $5x10^8$ colony forming units (CFU)]. Metabolic labeling experiments were performed in 3 ml of MEM without methionine (ICN Biomedicals Inc., Aurora, OH) and [^{35}S]-methionine (Perkin Elmer Life Sciences Inc., Boston, MA) as described in Konkel and Cieplak (1992). For some

experiments, albumin was removed from the FBS using a Swell Gel Albumin removal kit (Pierce, Rockford, IL). Following a 3 hr labeling period, bacterial cells were pelleted by centrifugation at 6000 x *g* and supernatant fluids collected.

One dimensional gel electrophoresis. For some experiments, supernatant fluids were concentrated 4-fold by the addition of 5 volumes of ice-cold 1 mM HCl-acetone. For experiments where the albumin was removed from the FBS, the supernatant fluids were concentrated 20-fold. The pellets were air dried, resuspended in water, and mixed with an equal volume of double strength electrophoresis sample buffer (Konkel and Cieplak, 1992). Prior to electrophoresis, the samples were heated to 95°C for 5 min and cooled to room temperature. Proteins were resolved in sodium dodecylsulfate (SDS)-12.5% polyacrylamide gels using the discontinuous buffer system (Laemmli, 1970). Gels were treated with Entensify (Life Sciences Products, Boston, MA) according to the supplier's instructions and dried. Labeled bacterial proteins were visualized by autoradiography using Kodak BioMax MR film at -70°C.

Deoxycholate sensitivity assay. Equivalent amounts (OD_{540} = 0.18) of the single crossover mutants [Cj0285c (*cheV*), Cj0355c, Cj0643, Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj1223c, Cj1227c, Cj1261 (*racR*), Cj1491c, and Cj1608], the double crossover mutants [Cj0061c (*fliA*), Cj0670 (*rpoN*), Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like)], Cj0890c chromosomal mutant, and *C. jejuni* parental strain F38011 were plated on MH agar plates supplemented with sodium deoxycholate (0.1% wt/vol) and incubated under microaerobic conditions overnight. The plates were then scored for growth or no growth.

Transmission electron microscopy. *C. jejuni* bacterial suspensions were prepared from MH-blood agar plates using phosphate buffered saline and were added dropwise to formvar-coated copper grids. Bacteria were stained with 1% phosphotungstic acid. Samples were analyzed with a 1200 EX transmission electron microscope (JOEL).

RNA extractions and RT-PCR analysis. Cultures were grown overnight in a CO_2 incubator in MH broth supplemented with 0.1% deoxycholate, pelleted, and resuspended in 200 µl of TE (10 mM Tris Cl, 1 mM EDTA pH 7.4) buffer. The samples were then treated with the following: 100 µl of 50 mM glucose, 25 mM Tris Cl, 10 mM EDTA, 200 µl of 0.1M NaOH, 1% SDS and 150 µl of 5 M potassium acetate and centrifuged 5 min at 13,000 x *g*. All solutions were pretreated with 0.1% diethyl pyrocarbonate (Sigma, St. Louis, MO). The supernatant was treated with an equal volume of (1:1) phenol/chloroform. The resulting supernatant was added to an equal volume of 90% isopropanol and centrifuged for 5 min at 13,000 x *g*. The pellet was washed in 70% isopropanol, repelleted, dried by vacuum aspiration and treated with 50 U of RNase-free DNase (Roche, Indianapolis, IN) for 15 min at 37°C. After DNase treatment, an equal volume 7.5 M sodium acetate was added as well as two volumes of 90% isopropanol and centrifuged for 20 min at 4°C, and washed in 70% isopropanol and re-centrifuged. The pellet was resuspended in 50 µl of RNase-free water (Maniatis *et al.*, 1982).

Ten µg of the resulting RNA was used for cDNA preparation using 3 µl of the provided random primers from the ProSTAR First Strand RT-PCR kit (Stratagene) according to the manufacturer's instructions. Then, 2.5 µl of the cDNA was amplified by PCR using primers within the coding region of *ciaB* (Forward 5'- CTA TGC TAG CCA TAC TTA GGC; Reverse

5’-GCC CGC CTT AGA ACT TAC) and previously described primers for the *aspA* gene were used as a control (Dingle *et al.*, 2001). The cycling conditions were as follows: 1 cycle of 5 min at 94°C; 5 cycles of 30 sec at 94°C, 30 sec at 45°C, and 1 min at 72°C; 20 cycles at 94°C, 30 sec at 49°C, and 1 min at 72°C; with a final extension of 5 min at 72°C. The resulting products were resolved by electrophoresis through 1% agarose in Tris-borate-EDTA buffer, and bands visualized by UV light after ethidium bromide staining.

Immunoblot analysis. Following SDS-PAGE, proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore Corp., Bedford, MA). The membranes were washed three times in phosphate buffered saline (PBS)-Tween (0.1%) and incubated 18 hr at 4°C with a 1:250 dilution of rabbit anti-*C. jejuni* CiaB antibody (#407) in 20% FBS/PBS-Tween 0.1%. Bound antibodies were detected using peroxidase-conjugated rabbit anti-goat IgG (Sigma) and 4-chloro-1-napthol (Sigma) as the chromgenic substrate.

RESULTS

Generation of *C. jejuni* single crossover response regulator and AraC-like mutants.

Previous work in our laboratory has shown that the expression of the *ciaB* gene is induced by bile salts and a component of FBS (Rivera-Amill *et al.*, 2001). These data suggests that a two component regulatory (TCR) system or an AraC-like transcriptional factor may sense bile salts or a component of FBS and induce the transcription of the Cia proteins. Therefore, experiments were undertaken to determine if the genes encoding the Cia proteins are regulated by a TCR system or an AraC-like transcriptional factor. Upon searching the *C. jejuni* NCTC 11168 genome, 7 histidine kinases (HK), 11 response regulators (RR), and 1 AraC-like transcriptional factor were found. Mutants were generated for 10 of the RRs [Cj0285c (*cheV*), Cj0355c, Cj0643, Cj0890c, Cj1024c (*flgR*), Cj1223c, Cj1227c, Cj1261 (*racR*), Cj1491c, and Cj1608] and the one AraC-like transcriptional factor, Cj1042c. The eleventh RR, *cheY*, was not disrupted because a deletion in its HK partner, *cheA*, does not affect secretion of the Cia proteins (unpublished data). Mutants were generated using homologous recombination via a single crossover event between the chromosomal gene and an internal fragment of the homologous gene. After generation of the mutants, we assessed whether each mutant was motile and if the Cia proteins were secreted.

Motility assays were initially performed to determine if a RR or an AraC-like transcriptional factor played a role in the expression of the flagella. The expression of flagellar genes is critical, since a functional flagellar export apparatus is required for the secretion of the Cia proteins (Konkel *et al.*, 2004). Mutants in genes encoding the response regulators Cj0355c, Cj0643, Cj1261 (*racR*), Cj1491c, and Cj1608 were deficient in motility as judged by lack of migration on semi-solid agar (Table 3). Conversely, disruption of the following response regulators genes

displayed positive motility: Cj0285c (*cheV*), Cj0890c, Cj1024c (*flgR*), Cj1223c, and Cj1227c (Table 3). The Cj1042c (AraC-like) mutant was also motile (Table 3). Interestingly, the Cj1024c (*flgR*) mutant was motile. This result is in contrast with Jagannathan *et al.* (2001) and indicated that the *flgR* mutant could still aid in the transcription of the flagella σ^{54} genes, leading us to question the validity of our mutant.

The mutants were also subject to secretion assays to determine if a RR or an AraC-like transcriptional factor played a role in the expression of the *cia* genes. Konkel *et al.* (2004) has shown that the flagella apparatus is required for the secretion of the Cia proteins. Furthermore, a *ciaB* null mutant resulted in a secretion deficient phenotype (Konkel *et al.*, 1999). We therefore used a secretion negative profile as an initial marker to determine if a response regulator or an AraC-like transcriptional factor played a role in the regulation of the secreted proteins. Initially, the *C. jejuni* RR single crossover and AraC-like mutants were plated on MH agar supplemented with sodium deoxycholate (0.1% wt/vol) to upregulate the synthesis of the Cia proteins (Rivera-Amill *et al.*, 2001). We observed that on the plates containing Cj0890c, Cj1223c, and Cj1608 little or no growth had occurred; therefore, the bacteria used for this experiment were harvested from MH blood plates. Metabolic labeling experiments were preformed on the single crossover mutants with labeling medium in the presence and absence of FBS. FBS serves as an artificial signal to stimulate the synthesis and secretion of the Cia proteins (Rivera-Amill *et al.*, 2001). A negative secretion profile was observed in all single crossover mutants when incubated in labeling medium minus FBS (Table 3). A positive secretion profile was observed in 8 of the 10 response regulator mutants in the presence of 1% FBS: Cj0285c (*cheV*), Cj0355c, Cj0643, Cj1223c, Cj1227c, Cj1261 (*racR*), Cj1491c, and Cj1608 (Table 3). The Cj0890c and Cj1024c

(*flgR*) response regulator mutants were secretion negative in the presence of 1% FBS (Table 3). Additionally, the Cj1042c (AraC-like) mutant was secretion negative in the presence of 1% FBS (Table 3). The following results suggest that Cj0890c, Cj1024c (*flgR*), and Cj1042c (AraC-like) may play a role in the regulation of the *cia* genes.

Deoxycholate resistance and sensitivity. The deoxycholate sensitive phenotype observed in the Cj0890c, Cj1223c, and Cj1608 single crossover mutants was interesting, since previous work has shown that *C. jejuni* mutants sensitive to bile salts fail to colonize chickens (Lin *et al.*, 2003). To confirm the deoxycholate sensitive phenotype associated with the mutation in the Cj0890c, Cj1223c, and Cj1608 genes, additional isolates of the Cj0890c, Cj1223, and Cj1608 single crossover mutants were tested for sensitivity to deoxycholate. Upon plating equivalent amounts (OD_{540} = 0.180) onto MH agar supplemented with sodium deoxycholate (0.1% wt/vol), 2/6 isolates of Cj0890c displayed no growth; whereas, 4/6 isolates of Cj0890c a lawn of bacteria was present (Table 3). A deoxycholate sensitive phenotype was observed in 6/6 isolates of Cj1223c and Cj1608 (Table 3). To determine the true phenotype of the Cj0890c RR mutant, a chromosomal Cj0890c mutant was generated using chromosomal DNA from a deoxycholate sensitive Cj0890c single crossover mutant. The chromosomal DNA from the Cj0890c single crossover mutant was digested with *Bgl*II and elecroporated into *C. jejuni* F38011 wild-type isolate. The restriction enzyme *Bgl*II was chosen because this restriction enzyme site was not contained within the internal *Cj0890c* gene fragment or the *aphA-3* gene in the suicide vector used to generate the Cj0890c single crossover mutant. Upon confirming the disruption of the Cj0890c gene and the acquisition of *aphA-3* gene, the new Cj0890c chromosomal mutant was tested for deoxycholate sensitivity. Equivalent amounts (OD_{540} = 0.180) of the *C. jejuni* F38011

wild-type isolate, Cj0890c chromosomal mutant, and Cj0890c single crossover (deoxy^s) mutant were plated on MH agar supplemented with sodium deoxycholate (0.1% wt/vol). No growth was displayed by the Cj0890c single crossover (deoxy^s) mutant, but a lawn of bacteria was observed on the plates containing the *C. jejuni* F38011 wild-type isolate and the newly constructed Cj0890c chromosomal mutant. We concluded that the Cj0890c single crossover (deoxy^s) mutant contains a secondary mutation unrelated to Cj0890c.

Generation of *C. jejuni* double crossover response regulator, AraC-like, and sigma factor mutants. Given the motility positive phenotype in the Cj1024c (*flgR*) single crossover mutant and the deoxycholate phenotype in the Cj0890c single crossover mutant, and the fact that response regulators and AraC-like transcriptional factors are multi-domain proteins, we sought to confirm the secretion negative phenotype of Cj0890c, Cj1024c (*flgR*) and Cj1042c (AraC-like) single crossover mutants. Therefore, double crossover mutants were generated in Cj0890c, Cj1024c (*flgR*) and Cj1042c (AraC-like). While the deoxycholate sensitive phenotype observed in the Cj1223c and Cj1608 single crossover mutants was interesting, double crossover mutants were not generated due to their secretion positive phenotype. Furthermore, we generated double crossover mutants of Cj0670 (*rpoN*) and Cj0061c (*fliA*), which encode for σ^{54} and σ^{28}, to determine the sigma factor involved in *ciaB* transcription.

Motility assays were performed to validate the motility phenotypes of these mutants. The zones of migration were measured after 48 hours (Figure 1). Cj0890c and Cj1042c (AraC-like) mutants were motile as seen in the single crossover mutants
(Figure 1). The Cj0061c (*fliA*) mutant was partially motile, whereas the Cj1024c (*flgR*) and Cj0670 (*rpoN*) mutants were nonmotile (Figure 1), which is in agreement with Jagannathan *et al.*

(2001). TEM of the Cj0061c (*fliA*) mutant revealed a flagellum approximately one-third the length of *C. jejuni* F38011 wild-type flagella (Figure 2). This result is in agreement with Jagannathan *et al.* (2001). *C. jejuni* F38011 wild-type isolate was used as a positive control to determine motility.

A deoxycholate sensitivity assay was performed on the double crossover mutants to determine the validity of the deoxycholate phenotype observed in the single crossover mutants. Equivalent amounts (OD_{540} = 0.180) of the *C. jejuni* F38011 wild-type isolate, Cj0061c (*fliA*), Cj0670 (*rpoN*), Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like) were plated on MH agar supplemented with sodium deoxycholate (0.1% wt/vol). A lawn was observed with the *C. jejuni* F38011 wild-type isolate as well as with all of the double crossover mutants, indicating that Cj0061c (*fliA*), Cj0670 (*rpoN*), Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like) are resistant to deoxycholate. These results are in agreement with deoxycholate resistant phenotype seen in the Cj1024c (*flgR*) and Cj1042c (AraC-like) single crossover mutants. The deoxycholate resistant phenotype of the Cj0890c double crossover mutant further supports the conclusion that a secondary mutation is present in the Cj0890c single crossover (deoxy^{s}) mutant. Given that the motility phenotype and the deoxycholate resistance phenotypes had been validated, metabolic labeling experiments were performed to address whether these mutants were capable of Cia protein secretion.

Secretion of the Cia proteins requires σ^{54} and FlgR.

Metabolic labeling experiments were performed with labeling medium in the presence and absence of FBS. In this experiment, the FBS was passed through a Swell Gel Albumin removal kit (Pierce) to remove the albumin from the FBS and allow the sample to be concentrated 20-

fold. As expected, no secreted proteins were observed in the absence of FBS in the supernatants of *C. jejuni* F38011 wild-type and the double crossover mutants, Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj0670 (*rpoN*), and Cj0061c (*fliA*) (Figure 3, Panel A). The *Cj0890c* and *Cj1042c* (AraC-like) double crossover mutants were positive for secretion in the presence of FBS (Figure 3, Panel B). This result is in contrast to the secretion negative result observed in the Cj0890c and Cj1042c (AraC-like) single crossover mutants, indicating that the Cj0890c and Cj1042c (AraC-like) genes were not sufficiently disrupted in the single crossover mutants. Furthermore, these results suggest that the *cia* genes are not transcribed by the response regulator Cj0890c or the AraC-like transcriptional factor Cj1042c. The Cj0061c (*fliA*) double crossover mutant was positive for the secretion of the Cia proteins; however, it appeared that a single secreted protein was missing from the secreted protein profile (Figure 3, Panel B). Upon further investigation it was determined that the F38011 strain used to generate the Cj0061c (*fliA*) was different from the F38011 strain used to make the Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj0670 (*rpoN*) double mutants. When a secretion assay was performed using the F38011 wild-type isolate used to generate the *Cj0061c* (*fliA*) and the *Cj0061c* (*fliA*) double crossover mutant, no difference in the secretion profile was observed (Figure 4). This finding indicated that this strain of F38011 had lost the ability to produce one of the secreted proteins. Furthermore, this finding demonstrated that σ^{28} (encoded by *fliA*) is not required for the transcription of the *cia* genes. The Cj1024c (*flgR*) and Cj0670 (*rpoN*) double crossover mutants were secretion negative in the presence of 1% FBS, suggesting that Cj1024c (FlgR) and Cj0670 (RpoN) may play a role in the transcription of the *cia* genes. To determine if Cj1024c (FlgR) and Cj0670 (RpoN) were involved in the transcription of the *cia* genes, RT-PCR was performed using *ciaB*-specific primers.

***ciaB* is transcribed and translated in σ^{54} and FlgR knockouts.**

Previous results by Rivera-Amill *et al.* (2001) have shown using RT-PCR that *ciaB* is transcribed in the presence of deoxycholate and 1% FBS. We isolated RNA from Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj0670 (*rpoN*), and Cj0061c (*fliA*) double crossover mutants grown on MH supplemented with sodium deoxycholate (0.1% wt/vol) and used *ciaB*- and *aspA*- specific primers to determine if *ciaB* gene transcription was regulated by Cj0670 (RpoN) or Cj1024c (FlgR). The *aspA* gene was chosen as a control since it is constitutively expressed. Using *ciaB*-specific primers, a product was amplified in the Cj0890c, Cj1042c (AraC-like), Cj0061c (*fliA*), Cj1024c (*flgR*), and Cj0670 (*rpoN*) double mutants (Figure 5). This result indicates that Cj0670 (RpoN) and Cj1024c (FlgR) are not involved in the transcription of *ciaB*. Furthermore, these data suggest that *ciaB* transcription is under the control a σ^{70} promoter.

To validate the results above, whole cell lysates were prepared from the *C. jejuni* F38011 wild-type, *ciaB* null mutant, and the double crossover mutants [Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj0670 (*rpoN*), and Cj0061c (*fliA*)] grown on MH supplemented with sodium deoxycholate (0.1% wt/vol). Immunoblot analysis using an anti-*C. jejuni* CiaB antibody (# 407) revealed that the CiaB protein was made in the *C. jejuni* F38011 wild-type, Cj0890c, Cj1024c (*flgR*), Cj1042c (AraC-like), Cj0670 (*rpoN*), and Cj0061c (*fliA*) double crossover mutants (not shown). As expected, the *ciaB* null mutant displayed no reactive band at 73 kDa, the estimated size of CiaB.

DISCUSSION

This study was undertaken to gain a better understanding of how *cia* gene expression is regulated in *Campylobacter*. We sought to determine if a response regulator or an AraC-like transcriptional factor played a role in the regulation *cia* gene transcription. Studies by Konkel *et al.* (1999) identified eight secreted proteins, designated CiaA-CiaH, ranging in size from 108-12.8 kDa. To date only one of these proteins has been identified, CiaB (Konkel *et al.*, 1999). Interestingly, a *ciaB* null mutant contained no secreted proteins in the supernatant fluid (Konkel *et al.*, 1999). Studies by Rivera-Amill *et al.* (2001) demonstrated that bile salts (deoxycholate, cholate, chenodeoxycholate) induce the synthesis of Cia proteins. In addition, a component of fetal bovine serum was required for the synthesis and secretion of the Cia proteins (Rivera-Amill *et al.*, 2001). Konkel *et al.* (2004) has indicated that the secretion of the Cia proteins is dependent upon a functional flagellar export apparatus.

Upon completion of this work, it became clear that there was phenotypic variation between the response regulator and AraC-like transcriptional factor single crossover mutants compared to the double crossover mutants. For Cj1024c (*flgR*), the single crossover mutant displayed motility, whereas the double crossover mutant displayed a nonmotile phenotype. In the Cj0890c single crossover mutants, 2/6 were sensitive to deoxycholate. In contrast the Cj0890c chromosomal mutant and the Cj0890c double crossover mutant were deoxycholate resistant. The Cj0890c and Cj1042c (AraC-like) single crossover mutants exhibited a secretion deficient phenotype in the presence of 1% FBS, while in the Cj0890c and Cj1042c (AraC-like) double crossover mutants a positive secretion profile was observed in the presence of 1% FBS. Given that inconsistent phenotypes were observed between the single and double crossover mutants, it is imperative that

single crossover mutants, generated in genes coding for multiple domain proteins be mapped as to where the insertion occurred. In addition, the phenotypes of the mutants must be validated by a second independent method such as complementation of the knocked out gene, reinsertion of chromosomal DNA containing the suicide vector from the mutant into a wild-type strain, or the generation a of a double crossover mutant. A potential problem that can arise with single crossover mutants is the reversion of the disrupted gene to a functional wild-type copy of the gene by excision of the inserted suicide plasmid. Furthermore, the use of kanamycin as a selective marker in *Campylobacter* suicide vectors can lead to problems because *Campylobacter* can spontaneously mutate to confer kanamycin resistance, removing the selective pressure that retains the suicide vector. In this paper, the double crossover mutants were designed so that a large central portion of the gene being disrupted was deleted and replaced by the *cat* gene. This method reduces the possibility that functional domains are maintained in the disrupted genes.

This study has shown that the Cj0670 (*rpoN*) and Cj1024c (*flgR*) are required for the production of a functional secretion apparatus. Recent work confirms that Cj0670 (*rpoN*) and Cj1024c (*flgR*) are required to produce a functional flagella in *C. jejuni* (Wösten *et al.*, 2004; Carrillo *et al.*, 2004). These results strengthen the previous finding that the flagella are required for secretion of the Cia proteins (Konkel *et al.*, 2004). In addition, Konkel *et al.* (2004) has shown that mutants in the basal body, rod, or hook prevent a functional flagella apparatus from being assembled. Furthermore, either the FlaA or FlaB filament is required for the secretion of the Cia proteins (Konkel *et al.*, 2004).

Through this study we have shown that Cj0670 (*rpoN*) and Cj0061c (*fliA*) double crossover mutants transcribe and translate the *ciaB* gene. Thus, the *ciaB* gene must be expressed from a σ^{70} promoter.

Transcriptional regulatory systems under the control of σ^{70} have been observed in several bacteria including *Escherichia coli* and *Rhodobacter capsulatus*. In *E. coli*, TyrR, a protein that shows homology to NtrC (a σ^{54}-dependent transcriptional activator) is required for the regulation of σ^{70} genes encoding for aromatic amino acid biosynthesis and transport (Yang *et al.*, 2002). Expression of *ntrC* in *R. capsulatus* allows for the regulation of σ^{70} transcribed genes (Xu and Hoover, 2001). In *E. coli*, the response regulator, PhoB interacts with σ^{70} to transcribe over 40 genes in phosphate depletion conditions (Blanco et al., 2002). Given that transcriptional regulatory elements can allow for the expression of σ^{70} regulated genes it is possible that *ciaB* transcription may be under the control of a σ^{70} transcriptional element.

While this study did not reveal if the transcription of *cia* genes are regulated by a two component regulatory system, the mutants generated in this paper will allow us to identify genes transcribed by response regulators through the use of recently developed microarray technology. Furthermore, studies are under way to generate double crossover mutants in the remaining response regulators [Cj0285c (*cheV*), Cj0355c, Cj0643, Cj1223c, Cj1227c, Cj1261 (*racR*), Cj1491c, and Cj1068] to reexamine the phenotypes observed in the single crossover mutants. In addition, double crossover mutants of all the transcriptional regulatory genes identified in the *C. jejuni* NCTC 11168 genome are being generated.

ACKNOWLEGEMENTS

We thank Dr. Debabrata Biswas for performing the secretion assays on the single crossover response regulator mutants and the F38011 and Cj0061c (*fliA*) double crossover mutant. We thank Nicole Lindstrom for assisting with TEM. We thank Amy Keech for performing the RT-PCR analysis. We thank Dr. John D. Klena and Dr. Brian H. Raphael for critical review of this manuscript.

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REFERENCES

Blanco, A.G., Sola, M., Gomis-Ruth, F.X., Coll, M. (2002) Tandem DNA recognition by PhoB, a two-component signal transduction transcriptional activator. *Structure* (Camb) 10, 701-13.

Colland, F., Rain, J.C., Gounon, P., Labigne, A., Legrain, P., De Reuse H. (2001) Identification of the Helicobacter pylori anti-σ^{28} factor. *Mol Microbiol* 41, 477-87.

Carrillo, C.D., Taboada, E., Nash, J.H.E., Lanthier, P., Kelly, J., Lau, P., Verhulp, R., Mykytczuk, O., Sy, J., Findlay, W.A., Amoako, K., Gomis, S., Willson, P., Austin, J.W., Potter, A., Babiuk, L., Allan, B., Szymanski, C.M. (2004) Genome-wide expression analyses of *Campylobacter jejuni* NCTC11168 reveals coordinate regulation of motility and virulence by *flhA*. *J Biol Chem* Feb. 25 [Epub ahead of print].

Dingle, K.E., Colles, F.M., Wareing, D.R.A., Ure, R, Fox, A.J., Bolton, F.E., Bootsma, H.J., Willems, R.J.L., Urwin, R., Maiden, M.C.J. (2001) Multilocus sequence typing system for *Campylobacter jejuni*. *J Clin Microbiol* 39, 14-23.

Guerry, P., Logan, S.M., Thornton, S., Trust, T.J. (1990) Genomic organization and expression of *Campylobacter* flagellin genes. *J Bacteriol* 172, 1853-60.

Gunn, J.S. (2000) Mechanisms of bacterial resistance and response to bile. *Microbes Infect* 2, 907-13.

Gupta, S., Chowdhury, R. (1997) Bile affects production of virulence factors and motility of *Vibrio cholerae*. *Infect Immun* 65, 1131-4.

Hendrixson, D.R., DiRita, V.J. (2003) Transcription of σ^{54}-dependent but not σ^{28}-dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. *Mol Microbiol* 50, 687-702.

Hoch, J.A., Silhavy, T.J. (1995) *Two-component signal transduction*. ASM Press, Washington, D.C.

Jagannathan, A., Constantinidou, C., Penn, C.W. (2001) Roles of *rpoN*, *fliA*, and *flgR* in expression of flagella in *Campylobacter jejuni*. *J Bacteriol* 183, 2937-42.

Kinsella, N., Guerry, P., Cooney, J., Trust, T.J. (1997) The *flgE* gene of *Campylobacter coli* is under the control of the alternative sigma factor σ^{54}. *J Bacteriol* 179, 4647-53.

Konkel, M.E., Cieplak, W., Jr. (1992) Altered synthetic response of *Campylobacter jejuni* to cocultivation with human epithelial cells is associated with enhanced internalization. *Infect Immun* 60, 4945-9.

Konkel, M.E., Garvis, S.G., Tipton, S.L., Anderson, D.E., Jr., Cieplak, W., Jr. (1997) Identification and molecular cloning of a gene encoding a fibronectin-binding protein (CadF) from *Campylobacter jejuni*. *Mol Microbiol* 24, 953-63.

Konkel, M.E., Klena, J.D., Rivera-Amill, V., Monteville, M.R., Biswas, D., Raphael, B., Mickelson, J. (2004) Secretion of virulence proteins from *Campylobacter jejuni* is dependent on a functional flagellar export apparatus. *J Bacteriol* 186.

Konkel, M.E., Kim, B.J., Rivera-Amill, V., Garvis, S.G. (1999) Bacterial secreted proteins are required for the internalization of *Campylobacter jejuni* into cultured mammalian cells. *Mol Microbiol* 32, 691-701.

Konkel, M.E., Tilly, K. (2000) Temperature-regulated expression of bacterial virulence genes. *Microbes Infect* 2, 157-66.

Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680-5.

Lin, J., Sahin, O., Michel, L.O., Zhang, Q. (2003) Critical role of multidrug efflux pump CmeABC in bile resistance and in vivo colonization of *Campylobacter jejuni*. *Infect Immun* 71, 4250-9.

Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY. pp. 89-90.

Matz, C., van Vliet, A.H.M., Ketley, J.M., Penn, C.W. (2002) Mutational and transcriptional analysis of the *Campylobacter jejuni* flagellar biosynthesis gene *flhB*. *Microbiol* 148, 1679-85.

Maurelli, A.T., Hromockyj, A.E., Bernardini, M.L. (1992) Environmental regulation of *Shigella* virulence. *Curr Top Microbiol Immun* 180, 95-116.

Prouty, A.M., Gunn, J.S. (2000) *Salmonella enterica* serovar typhimurium invasion is repressed in the presence of bile. *Infect Immun* 68, 6763-9.

Rivera-Amill, V., Kim, B.J., Seshu, J., Konkel, M.E. (2001) Secretion of the virulence-associated *Campylobacter* invasion antigens from *Campylobacter jejuni* requires a stimulatory signal. *J Infect Dis* 183, 1607-16.

Schuhmacher, D.A., Klose, K.E. (1999) Environmental signals modulate ToxT-dependent virulence factor expression in *Vibrio cholerae*. *J Bacteriol* 181, 1508-14.

Wösten, M.M.S.M., Wagenaar, J.A., Van Putten, J.P.M. (2004) The FlgS/FlgR two-component signal transduction system regulates the *fla* regulon in *Campylobacter jejuni*. *J Biol Chem* Feb. 11 [Epub ahead of print].

Xu, H., Hoover, T.R. (2001) Transcriptional regulation at a distance in bacteria. *Curr Opin Microbiol* 4, 138-44.

Yang, J., Camakaris, H., Pittard, J. (2002) Molecular analysis of tyrosine-and phenylalanine-mediated repression of the *tyrB* promoter by the TyrR protein of *Escherichia coli*. *Mol Microbiol* 45, 1407-19.

Young, G.M., Schmiel, D.H., Miller, V.L. (1999) A new pathway for the secretion of virulence factors by bacteria: the flagellar export apparatus functions as a protein-secretion system. *Proc Natl Acad Sci USA* 96, 6456-61.

Table 1. Oligonucleotides used to generate the *C. jejuni* single crossover mutants

Primer Name	Sequence (5' to 3')
Cj0285c Forward 43-62	GAGCTTGTCGATTTCCGTAT
Cj0285c Reverse 551-571	TGCTGTCATCAAGTATAAGTG
Cj0355c Forward 11-33	TAGTTATAGAAGATGAGATTAGC
Cj0355c Reverse 535-556	CATTTGGAGTTACTAGCTCAGG
Cj0643 Forward 24-45	TGATGACAATAAAATGCTCGGC
Cj0643 Reverse 518-538	TTACTGGCATATTGACATCTG
Cj0890c Forward 28-49	GAAGATGATATAGATTTAAACG
Cj0890c Reverse 540-560	CTTGTAGTATCATCTTTACAA
Cj1024c Forward 19-38	GAAGATGATATTAATATGCG
Cj1024c Reverse 523-544	TAGAATGGGTGTGAATATAACG
Cj1042c Forward 21-37	ACCAGAAGATTTAAAGC
Cj1042c Reverse 575-592	AATACTCAAAAAGTTGGG
Cj1223c Forward 20-39	TTTTAGAAGATGATTTGAGC
Cj1223c Reverse 530-550	CCCAGAGTTCTTCAAAAATTC
Cj1227c Forward 23-43	AAGATGATTTAGAATTAGCAG
Cj1227c Reverse 533-552	CTCACTAATAGAAGAACAAT
Cj1261 Forward 38-55	GACTTTGCACAATTATTATCTG
Cj1261 Reverse 481-500	CCGTGTTGTTGGATAAGGTA
Cj1491c Forward 27-48	AATATTAGTTGAAGATGAG
Cj1491c Reverse 516-535	CATGCAAAACCAAAGCTCCA
Cj1608 Forward 25-44	GAAATTTATCTAGCGCAAAG
Cj1608 Reverse 718-739	CATTGCTATTGATATTTTTTTC

Table 2. Oligonucleotides used to generate the *C. jejuni* double crossover mutants

Primer	5' end Forward (5'to 3')	Enzyme
Cj0890c	TT(ggatcc)TTGCAAAAATGAAAGATGG	*Bam*HI
Cj1042c	AGATGTAAGTGAAAAACG	-
Cj1024c	AA(ggatcc)CAAAAAGAGCAGCGATTACAG	*Bam*HI
Cj0061c	TTGGAAGACATTTTAATAGAAG	-
Cj0670	GGGTTAAATTTCTTGGTCTTG	-
cat	TT(ggatcc)AGCTCTAGAGTCAACCGTGATATAG	*Nhe*I

Primer	5' end Reverse (5' to 3')	Enzyme
Cj0890c	AA(ccgcgg)AAA(gctagc)TAAAGATATAACTTCATAACC	*Sac*II, *Nhe*I
Cj1042c	AA(ccgcgg)TTT(gctagc)GTTTGTGTATATTTAGC	*Sac*II, *Nhe*I
Cj1024c	AA(ccgcgg)AAA(gctagc)CTTAAAGTAGCATTTCCTGTC	*Sac*II, *Nhe*I
Cj0061c	AA(ccgcgg)AAA(gctagc)CACAAGCTCATCTTGCTCTTTC	*Sac*II, *Nhe*I
Cj0670	AA(ccgcgg)AAAA(gctagc)GTGTTCAAAATATCCTTCCTCATTTAAG	*Sac*II, *Nhe*I

Primer	3' end Forward (5' to 3')	Enzyme
Cj0890c	TT(gctagc)GATAAAACAATCAATATAGC	*Nhe*I
Cj1042c	TT(gctagc)TTGGTTTATTGAAAGATTTAAAGAG	*Nhe*I
Cj1024c	TT(gctagc)TGGTAATATTAGAGAATTAATATCC	*Nhe*I
Cj0061c	TT(gctagc)CACGAAGTGCTAGATGATCTTAAAG	*Nhe*I
Cj0670	TT(gctagc)GCTACTCTTTATAAAATAGGGCTTATG	*Nhe*I

Primer	3' end Reverse (5' to 3')	
Cj0890c	AA(ccgcgg)GCTGAGTATTTTTTAAAGTG	*Sac*II
Cj1042c	AA(ccgcgg)CTACTTTGAATATTTTACC	*Sac*II
Cj1024c	AA(ccgcgg)AACAGGCACTCTAACACAAGTTGC	*Sac*II
Cj0061c	AA(ccgcgg)ATTTCTTTGATTTCATCTTTATC	*Sac*II
Cj0670	AA(ccgcgg)TAAAGCTACTAAAGCAATAGCTCCTAAG	*Sac*II
cat	AA(gctagc)GTATAGAAGTGCGCCCTTTAGTTCC	*Nhe*I

Table 3A. Phenotypes displayed by the *C. jejuni* response regulator and AraC-like transcriptional factor Single crossover mutants.

Isolate	Transcriptional Class	Motility	Secretion (No FBS)	Secretion (1% FBS)	Deoxcholate (resistant/sensitive)
Cj0285c (*cheV*)	Response Regulator	+	-	+	R
Cj0355c	Response Regulator	-	-	+	R
Cj0643	Response Regulator	-	-	+	R
Cj0890c	Response Regulator	+	-	-	S (2/6)
Cj1024c (*flgR*)	Response Regulator	+	-	-	R
Cj1223c	Response Regulator	+	-	+	S (6/6)
Cj1227c	Response Regulator	+	-	+	R
Cj1261 (*racR*)	Response Regulator	-	-	+	R
Cj1491c	Response Regulator	-	-	+	R
Cj1608	Response Regulator	-	-	+	S (6/6)
Cj0284c (*cheA*)	Histidine Kinase	+	-	+	R
Cj1042c	AraC-like	+	-	-	R

Table 3B. Phenotypes displayed by the *C. jejuni* double crossover mutants.

Isolate	Transcriptional Class	Motility	Secretion (No FBS)	Secretion (1% FBS)	Deoxcholate (resistant/sensitive)	*ciaB* mRNA	CiaB protein Synthesis
Cj0061c (*fliA*)	Sigma Factor	+	-	+	R	+	+
Cj0670 (*rpoN*)	Sigma Factor	-	-	-	R	+	+
Cj0890c	Response Regulator	+	-	+	R	+	+
Cj1024c (*flgR*)	Response Regulator	-	-	-	R	+	+
Cj1042c	AraC-like	+	-	+	R	+	+

Figure 1. Assessment of *C. jejuni* double crossover mutant motility on MH medium supplemented with 0.4% Bacto Agar after 48 hr.

FIG. 1

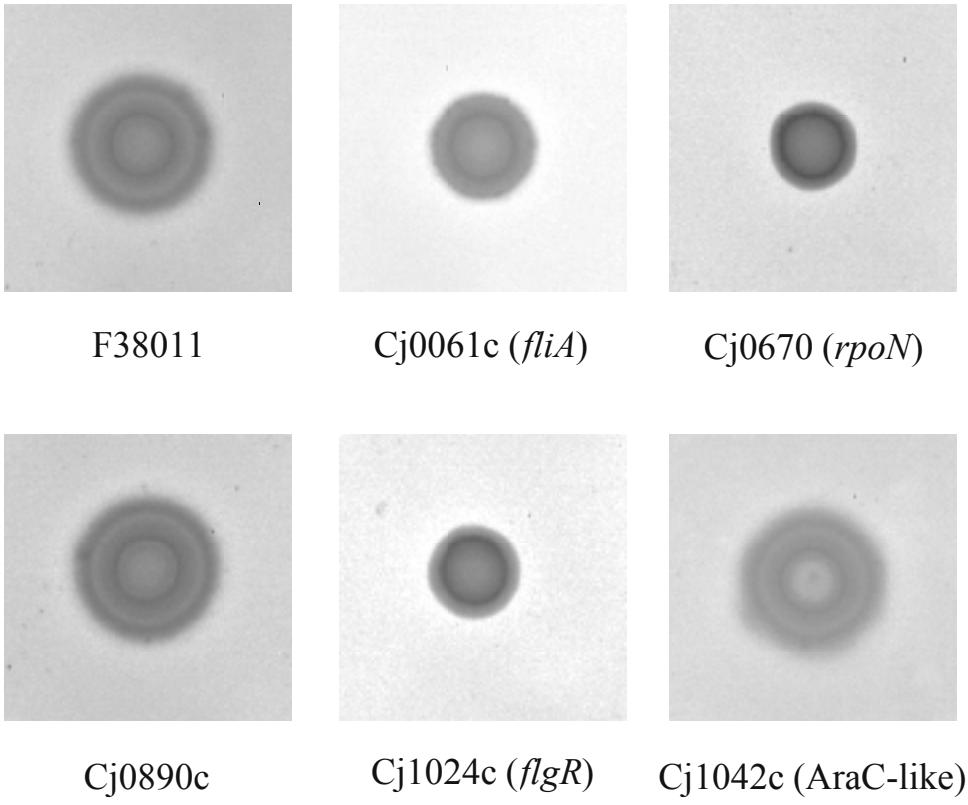
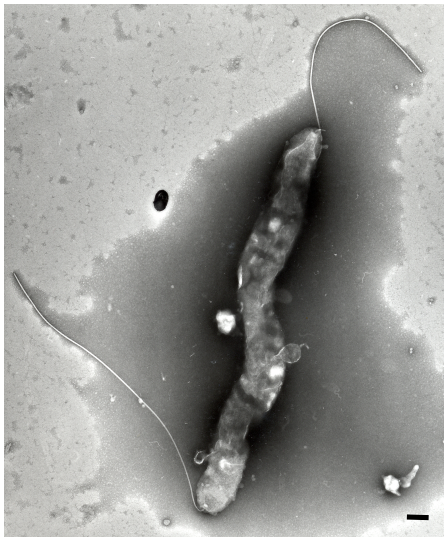

F38011
Cj0061c (*fliA*)
Cj0670 (*rpoN*)
Cj0890c
Cj1024c (*flgR*)
Cj1042c (AraC-like)

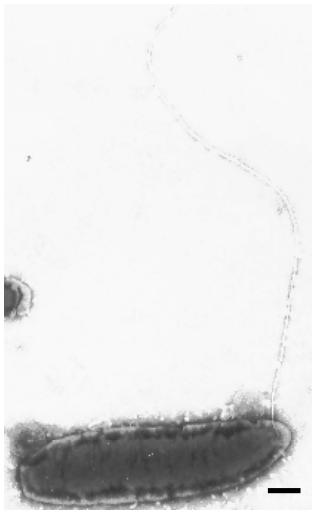
Figure 2. Transmission electron microscopy examination of *C. jejuni* wild-type F38011, *fliA* double crossover mutant, and *fliA* complement isolates. Bar = 0.2 □m.

FIG. 2



F38011

Cj0061c (*fliA*)



Cj0061c comp

Figure 3. *C. jejuni* Cia protein secretion requires Cj0670 (*rpoN*) and Cj1024c (*flgR*). *C. jejuni* cells were cultured on MH-blood plates and labeled in MEM in the absence (panel A) and presence (panel B) of FBS. Lanes: 1, *C. jejuni* F38011; 2, Cj0670 (*rpoN*) mutant; 3, Cj0061c (*fliA*) mutant; 4, Cj1024c (*flgR*) mutant; 5, Cj0890c mutant; and 6, Cj1042c (AraC-like) mutant. Molecular mass standards, in kDa, are indicated on the left.

FIG. 3

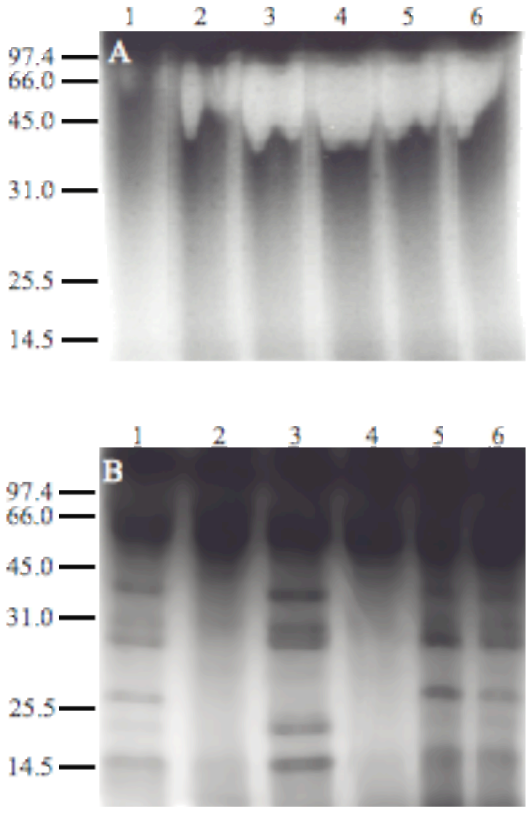

1
2
3
4
5
6
A
97.4
66.0
45.0
31.0
25.5
14.5
1
2
3
4
5
6
B
97.4
66.0
45.0
31.0
25.5
14.5

Figure 4. The Cj0061c (*fliA*) mutant secretion profile is identical to that of wild-type *C. jejuni* F38011. *C. jejuni* cells were cultured on MH-blood plates and labeled in MEM in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of FBS. Lanes: 1, *C. jejuni* F38011; 2, Cj0061c (*fliA*) mutant; 3, *C. jejuni* F38011; and 4, Cj0061c (*fliA*) mutant. Molecular mass standards, in kDa, are indicated on the left.

FIG. 4

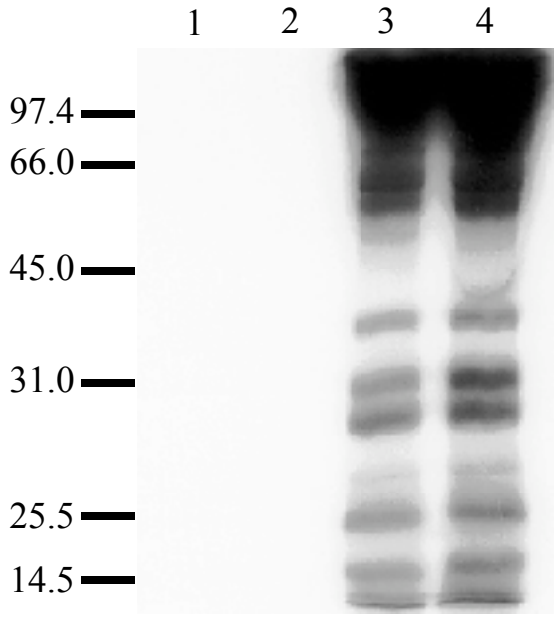
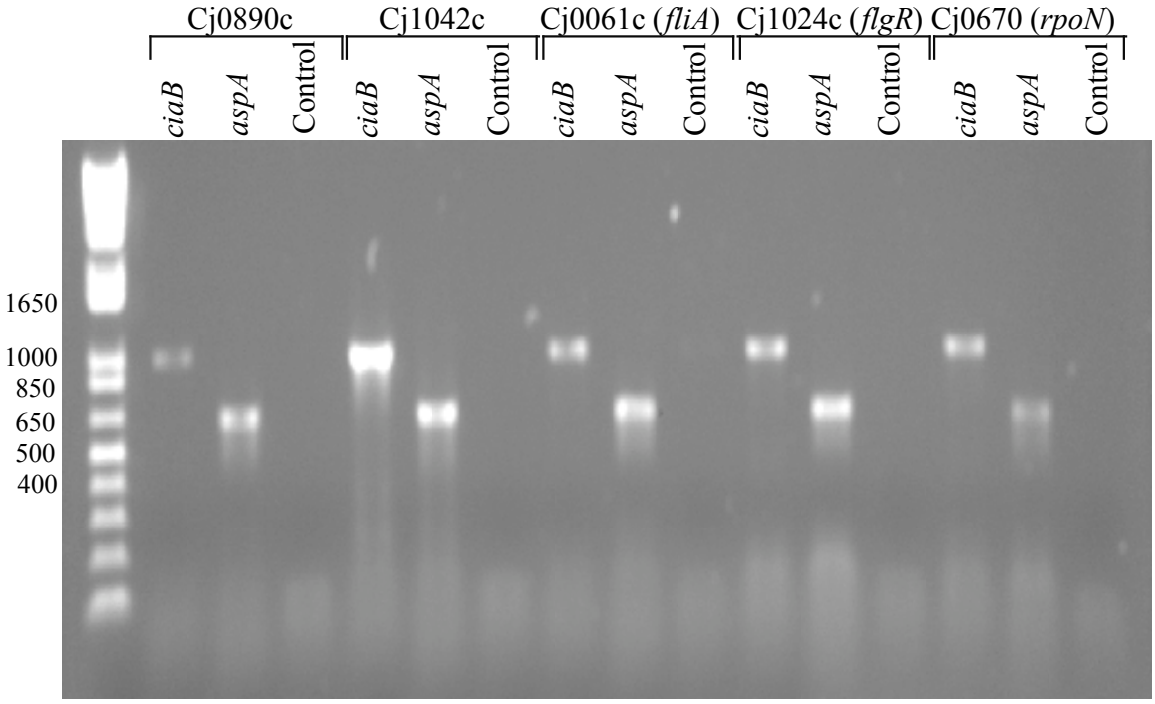


Figure 5. *ciaB* is transcribed in Cj0670 (*rpoN*) and Cj1024c (*flgR*) mutants. RT-PCR analysis was performed with *C. jejuni* cultured on MH agar supplemented with 0.1% deoxycholate. Bacterial RNA was extracted, and RT-PCR analysis was done, as described in Materials and Methods. RT-PCR analysis of each RNA sample was performed using *ciaB*-specific primers, *aspA*-specific primers and *ciaB* primers without the RT enzyme (control).

FIG. 5


Cj0890c
Cj1042c
Cj0061c (*fliA*)
Cj1024c (*flgR*)
Cj0670 (*rpoN*)
ciaB
aspA
Control
1650
1000
850
650
500
400

CONCLUSIONS AND FURTHER DIRECTIONS

Through this body of work we have shown that Cj0670 (*rpoN*) and Cj1024c (*flgR*) are required in the production of a functional secretion apparatus. Furthermore, one of the secreted proteins, CiaB, appears to be expressed by σ^{70}. However, the question still remains as to whether the expression of the other *cia* genes is regulated by a two component regulatory system. Therefore, double crossover mutants of the remaining response regulators [Cj0285c (*cheV*), Cj0355c, Cj0643, Cj1118c (*cheY*), Cj1223c, Cj1227c, Cj1261 (*racR*), Cj1491c, and Cj1608] need to be generated to validate their secretion phenotype.

Recently, the Konkel lab has sent RNA samples to Dr. Craig Parker (USDA, Albany, CA) for microarray analysis. *C. jejuni* F38011 RNA was isolated from MH broth and MH broth plus sodium deoxycholate (0.05% wt/vol). Upon microarray analysis four response regulators [Cj0355c, Cj1118c (*cheY*), Cj1227c, and Cj1491c] were shown to be upregulated in the presence of deoxycholate. Double crossover mutants of these four response regulators are currently being generated by Dr. Brian Raphael and will be subject to secretion assays and microarray analysis to determine possible genes under the control of these response regulators. Cj0285c (*cheV*), Cj0643, Cj1118c (*cheY*), Cj1223c, Cj1261 (*racR*), Cj1491c, and Cj1608 double crossover mutants have been generated and subjected to motility assays and deoxycholate sensitivity assays. Cj1118c (*cheY*) and Cj0285c (*cheV*), which are involved in chemotaxis, were found to be nonmotile. The remaining RRs [Cj0643, Cj1223c, Cj1261 (*racR*), Cj1491c, and Cj1608] displayed motility. Upon, testing for deoxycholate sensitivity, Cj0643 was determined to be extremely sensitive to growth in the presence of deoxycholate. Cj0285c (*cheV*), Cj1118c (*cheY*), Cj1223c, Cj1261 (*racR*), Cj1491c, and Cj1608 double crossover mutants showed no sensitivity

to deoxycholate. Immunoblot analysis using an anti-*C. jejuni* CiaB antibody (# 407) revealed that the CiaB protein was made in Cj0285c (*cheV*), Cj0643, Cj1118c (*cheY*), Cj1223c, Cj1261 (*racR*), Cj1491c, and Cj1608 double crossover mutants. Currently, Dr. Raphael is generating double crossover mutants in Cj0355c and Cj1227c. The Cj0643 double crossover mutant was sent to Dr. Zhang, to determine if its sensitivity to deoxycholate will hinder the colonization of the cecum in chickens.

With the use of microarray technology the identification of the genes under the control of response regulators may be ascertained by generating constitutively active response regulators. Response regulators can be made constitutively active by changing the conserved aspartic acid in the active site to a glutamic acid (Nohaile *et al.*, 1997). This substitution mimics the phosphorylation of the aspartic acid, thereby activating the response regulator without the need of the phosphate being transferred from the response regulator's histidine kinase partner (Nohaile *et al.*, 1997). By comparing the double crossover mutant response regulator to the constitutively expressed response regulator we will be able to identify the genes being regulated by that specific response regulator. By identifying these genes we may be able to determine potential signals that control the activation of the response regulators.

REFERENCE

Nohaile, M., Kern, D., Wemmer, D., Stedman, K., Kustu, S. (1997) Structural and functional analyses of activating amino acid substitutions in the receiver domain of NtrC: evidence for an activating surface. *J Mol Biol* 273, 299-316.

APPENDIX

This project is the result of the concerted efforts of many individuals. My contribution to this work includes: designing the oligonucleotides required to generate the single and double crossover mutants and the generation of all the *C. jejuni* mutants used in this study (Table 1 and 2). Furthermore, I performed the motility and deoxycholate sensitivity assays of the single and double crossover mutants (Table 3A and 3B; Fig. 1). I performed the secretion assay on the double crossover mutants (Fig. 3). Nicole Lindstrom performed the TEM (Fig. 2). D. Biswas performed the secretion assays on the single crossover mutants and the F38011 and Cj0061c (*fliA*) double crossover mutant (Table 3A; Fig. 4). A. Keech performed the RT-PCR analysis on the double crossover mutants (Fig. 5). B. Raphael performed the western blot analysis on the double crossover mutants using the anti-CiaB antibody (Table 3B). Future work towards the publication of this paper will be performed by M. Konkel and B. Raphael.