IDENTIFICATION AND CHARACTERIZATION OF FLAGELLAR CO-EXPRESSED DETERMINANTS (FEDS) OF *CAMPYLOBACTER JEJUNI*

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DEDICATION

To my mother, Marleny Tobon.
For being my foundation and my strength.

To Victor, my favorite person in the world.
For being the best friend a girl can have.

IDENTIFICATION AND CHARACTERIZATION OF FLAGELLAR CO-EXPRESSED DETERMINANTS (FEDS) OF *CAMPYLOBACTER JEJUNI*

By

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ABSTRACT

IDENTIFICATION AND CHARACTERIZATION OF FLAGELLAR CO-EXPRESSED DETERMINANTS (FEDS) OF *CAMPYLOBACTER JEJUNI*

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The University of Texas Southwestern Medical Center, 2013

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Campylobacter jejuni is the leading cause of bacterial gastroenteritis in humans throughout the world. In contrast to infection of humans, *C. jejuni* is a commensal organism of the intestinal tracts of wild and agriculturally-significant animals and avian species. Flagellar motility is the only virulence and colonization factor proven to be required for infection of human volunteers to promote disease and infection of poultry for commensalism. Expression of many flagellar genes is dependent on two alternative sigma factors, σ^{54} and σ^{28}. Many rod and hook genes are dependent on σ^{54} for expression, whereas σ^{28} is involved in the expression of the major flagellin and other filament genes. We investigated the σ^{28} regulon and identified five genes that are dependent on σ^{28} and flagellar components for maximal expression, but are not required for motility. One gene, *ciaI*, has previously been shown to function in intracellular survival after invasion of human intestinal epithelial cells. The four remaining genes, which we annotated as *fedA-fedD* (for *f*lagellar co-*e*xpressed *d*eterminants), encode proteins that have not been characterized. Mutants lacking any one of these *fed*s or *ciaI* demonstrated a reduced commensal colonization capacity in a natural chick model of colonization. Similar to the σ^{28}-

dependent gene product FspA1, a subset of these Feds is secreted by the bacterium in a flagellar-dependent manner. To further investigate the secretion requirements of these σ^{28}-dependent proteins (FedB, CiaI and FspA1), we examined putative flagellar chaperones, flagellar components and other aspects of flagellar biosynthesis such as flagellar protein glycosylation for a role in secretion of Feds. We discovered that, like in other motile organisms, the FliJ chaperone is required for secretion of flagellar components in general, and that FliS is likely the chaperone for the major flagellin, FlaA. However, FliS and other putative flagellar chaperones are not required for secretion of the Feds. We also discovered that secretion of the Feds occurs during or just after hook biosynthesis, suggesting that construction of a hook is required for maximal secretion of these proteins via the flagellum. In addition, in the absence of the flagellar cap or flagellin glycosylation, we observed an increase in secretion of FedB, CiaI and FspA1, suggesting a possible inverse correlation between the amount of Fed proteins secreted via the flagella and length of the flagellar filament. Furthermore, we have identified N- and C-terminal intramolecular determinants within FedB and CiaI that are required for maximal secretion. Based on how other flagellar proteins are secreted, these findings indicate that a flagellar Type III secretion system (T3SS)-specific signal sequence is likely found at the N-terminus, and that an unidentified chaperone may bind to the C-terminus. Both of these factors appear to be required for maximal flagellar-dependent secretion of the Feds. We also examined the importance of secretion of Feds during commensal colonization and invasion of human colonic epithelial cells *in vitro*. Gentamicin-protection assays revealed that secretion of CiaI is not required for invasion of T84 cells. Furthermore, preliminary studies using a chick model of commensal colonization showed that secretion of FedB is important for colonization of the chick intestinal tract. However, whereas CiaI is required for colonization, secretion of CiaI was not

important for colonization of the chick cecum. In summary, our work provides evidence that the flagellar system is a global regulatory system that coordinates production of flagella with colonization and virulence determinants, some of which are secreted in a flagellar-dependent manner, to promote maximal fitness during colonization and virulence.

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PREVIOUS PUBLICATIONS

Barrero-Tobon, A. M and Hendrixson, D. R. (2012) Identification and analysis of flagellar coexpressed determinants (Feds) of *Campylobacter jejuni* involved in colonization. *Mol. Microbiol.* 84: 352-369.

Ayong, L., Pagnotti, G., **Tobon, A. B.**, and Chakrabarti, D. (2007) Identification of Plasmodium falciparum family of SNAREs. Mol Biochem Parasitol 152:113-122.

CHAPTER ONE

Literature Review

Pathogenesis of *Campylobacter jejuni*

C. jejuni is a spiral-shaped, Gram-negative bacterium. This microaerophilic organism is a member of the ε-proteobacteria family, which also includes *Helicobacter* and *Wolinella* species. The genome of *C. jejuni* is relatively small, which is characteristic of the epsilon class of proteobacteria, containing 1.6 Mb coding for an estimated 1700 proteins [1]. This bacterium naturally colonizes the gastrointestinal tract of chickens and other avian species where it establishes a long-term relationship with its host. However, it is also a common foodborne pathogen that causes disease in humans. Although this organism grows well at 37 °C, the optimal temperature for growth of the bacterium is 42 °C, which is the body temperature of many avian species.

In avian species, *C. jejuni* primarily colonizes the large intestine and cecum, but can also spread to the spleen and liver. More specifically, this organism colonizes the mucosal layer lining the intestinal tract and cecum, with very little invasion of cecal or intestinal epithelia. In the avian host, *C. jejuni* is a commensal organism and does not cause any symptoms of disease. The primary route of transmission to humans is by handling or consuming undercooked poultry, unpasteurized milk or contaminated water [2-4]. In humans, *C. jejuni* primarily colonizes the colon and the infection may progress to campylobacteriosis, which is the most common foodborne bacterial diarrheal disease in developed countries [5,6]. Infection in humans can be achieved with approximately 500-800 organisms. The symptoms of disease can range from

mild, watery diarrhea to bloody diarrhea likely caused by inflammation in the intestinal tract [7]. These symptoms are suggestive of invasion and disruption of the human intestinal epithelial layer upon colonization.

Campylobacteriosis can be diagnosed upon detection of the bacterium using PCR methods or microscopy analyses of stool samples obtained from patients suffering from diarrhea [7]. The incubation period of the bacterium before disease presentation is approximately 2-5 days, with diarrhea as the typical symptom [8,9]. In some cases, patients can also experience malaise, abdominal pain and fever even after the diarrhea subsides. During the period of intestinal colonization, fecal samples from infected patients show signs of leukocyte infiltration indicative of inflammation [8]. In most cases, disease caused by *C. jejuni* is self-limiting and treatment with an antibiotic, such as erythromycin, is only administered to patients with persistent symptoms or immunocompromised individuals [8,9]. One complication that can arise due to infection with *C. jejuni* is the development of Guillain-Barré syndrome (GBS), which is an acute paralysis of the peripheral nervous system. In most GBS cases, antibodies against *C. jejuni* can be detected in serum from patients. Neurological symptoms for GBS can be observed as early as 1-3 weeks after infection [9-11]. This sequela is likely caused by molecular mimicry of the LOS to the human gangliosides, leading to an autoimmune response that damages peripheral nerves to cause paralysis.

Adherence and Invasion of Human Intestinal Cells

Inflammation of the human colon and intestinal tract upon *C. jejuni* infection suggests invasion, disruption and damage of the host intestinal epithelial layer. To endure the clearing forces of peristalsis, microbial organisms adhere to host cells, which is an early step for host cell

invasion. In the case of *C. jejuni*, attachment is achieved through the binding of adhesins, such as CadF, to surface molecules found on human intestinal epithelial cells. CadF is an outer membrane protein that binds fibronectin found on the surface of the intestinal epithelial layer [12]. Adherence and invasion assays using polarized T84 cells showed that *C. jejuni* favors binding to fibronectin found on the basolateral surface [13]. Although the mechanism by which the bacterium can reach the basolateral surface is not fully understood, it was suggested that *C. jejuni* translocates across a cell monolayer to bind fibronectin. CadF has also been shown to be required during commensal colonization of the chick intestinal tract, suggesting that this adhesin has a major role for virulence and commensal colonization [14]. Other examples of *C. jejuni* adhesins include JlpA, which is a surface-bound lipoprotein [15]. Further characterization of JlpA revealed it binds Hsp90α on the host cell and this interaction is important for wild-type levels of adherence of the bacterium to HEp-2 cells. Binding to Hsp90α initiates a cascade of events that include the phosphorylation and degradation of IκBα, which is the inhibitor of NF-κB, and the phosphorylation of the p38 MAP. These signaling events result in NF-κB-dependent transcription of genes involved in pro-inflammatory and immune responses [16]. Interestingly, Peb1 is another adhesin of *C. jejuni* that is part of an ABC transporter system, but has been shown to be important for adherence of HeLa cells [17,18]. Peb1 localizes to the outer membrane where it presumably acts as an adhesin, but it is also found in the periplasm. The dual localization of Peb1 facilitates both of its functions in adherence and in amino acid transport [19,20]. Other proteins found at the surface of *C. jejuni* that have been associated with adherence to human epithelial cells are the autotransporter CapA and the major outer membrane protein (MOMP) [21]. MOMP was first described as a pore forming protein, however a role in adherence to INT 407 cells has been reported as well [22,23].

Like other enteric pathogens, *C. jejuni* is able to enter host epithelial cells, but the mechanisms by which the bacterium mediates entry into eukaryotic cells and ensures intracellular survival are not fully understood. An intact flagellum has been implicated in the invasion process, but is not clear whether the flagellum, motility, or both are required for this process. To address this question, invasion of human epithelial cells was assessed using *C. jejuni* mutants lacking PflA (paralyzed flagella protein) or MotA (motility protein A), both of which produce a flagellum but lack components necessary for motility. The internalization of these *C. jejuni* mutants was reduced, indicating that perhaps motility, and not the flagellar structure, is responsible for entry into host cells [24,25].

One characteristic of *C. jejuni* internalization into host cells is that this process is mediated by microfilaments as opposed to the actin cytoskeleton [26,27]. After internalization, the bacterium associates with microtubules and this interaction may be important for trafficking *C. jejuni* within the host cell [28]. Another aspect of the intracellular phase of *C. jejuni* is that it is found enclosed in a membrane-bound compartment known as the *Campylobacter*-containing vacuole (CCV) [29]. In human intestinal epithelial cells, the CCV is decorated by early endosomal markers such as EEA-1, Rab4 or Rab5, but segregates from the endocytic pathway and does not fuse to lysosomes. This activity is important to ensure the intracellular survival of the bacterium [30].

Once inside a host epithelial cell, *C. jejuni* is thought to alter its physiology to adapt to an intracellular environment with low oxygen. Evidence for this change is observed during prolonged *in vitro* cell invasion assays in which the bacteria must be first recovered in low oxygen conditions before resuming normal microaerophilic growth [30]. In addition, analyses of *C. jejuni* 20 hours post-invasion showed that after internalization, the bacterium undergoes a

modification of its proteome including a decrease in expression of proteins involved in metabolic pathways or aerobic respiration [31]. A transposon mutagenesis screen also identified other proteins involved in invasion of host epithelial cells. For instance, mutations in *aspA*, *aspB* and *sodB* resulted in a significant decrease in invasion of epithelial cells compared to wild-type [25]. AspA and AspB are enzymes involved in carbon metabolism and SodB is a superoxide dismutase that functions during oxidative stress. Further analysis revealed that the *sodB* mutant had a slight defect in adherence to epithelial cells and a decreased ability to survive intracellularly [25]. AspA and AspB on the other hand are indirectly necessary for invasion since the addition of fumarate to the culture medium relieved the defect [25].

Lastly, a homologue for VirK in *C. jejuni* was shown to be required for intracellular survival upon invasion of human intestinal epithelial cells [32]. VirK homologues were first described as a virulence factor in *Salmonella* and *Shigella flexneri* due to the involvement of VirK in dissemination and intracellular replication upon invasion of host cells [33,34]. Although the mechanism of action of VirK in *C. jejuni* is not completely understood, VirK was found to localize to the cytoplasmic side on the inner membrane and found to be required for resistance to antimicrobial agents such as polymyxin B [32].

Animal Models Used to Study *C. jejuni* Infection

Chick model of commensal colonization. As mentioned above, *C. jejuni* naturally colonizes the intestinal tract of poultry. Because avian species are the natural reservoir of *C. jejuni*, birds that become colonized do not show any symptoms of disease such as reduction of weight gain or diarrhea [6]. Chickens are a common model for analyzing *C. jejuni* commensal colonization. Newly hatched chicks are inoculated by oral gavage with 10^2-10^8 CFU of *C.*

jejuni. In this model, chicks are sacrificed days to weeks after infection to determine the bacterial burden of the intestinal tract. The principal region for colonization is the lower intestinal tract, which includes the cecum and the large intestine. The proximal and distal small intestines (which form the upper intestinal tract) are also colonized at lower levels [35]. The bacterium can also be recovered from the bursa and the spleen early after infection. Histology analysis of tissues collected from infected chicks revealed lack of invasion or tissue damage, which is consistent with the absence of symptoms indicative of disease. Colonization may be prolonged, lasting many weeks to months.

Ferret diarrhea model. Ferrets have been used to study virulence factors of *C. jejuni* that play a role in disease. Ferrets can be orally inoculated to mimic the normal route of infection in humans. Symptoms of infection can be observed as early as 24 hours and are characterized by a soft stool with green mucus and in some cases bacteremia [36]. Some of the limitations of this model are the need for opiates to suppress peristalsis, extremely high inocula (10^8-10^{10} CFU), and a narrow window of time for symptoms [37]. The relevance of this model of infection in human disease is questionable due to differences in immunological responses and other factors that are unique to the different species. However, this model is one tool that may be used for identification and assessment of virulence factors.

Newborn piglet model. The colostrum-deprived newborn piglet is another animal model to study *C. jejuni* virulence. The use of piglets at an early age is essential to this model since older piglets do not develop symptoms of infection [37]. The piglets are inoculated orally with approximately 10^8 CFU and are observed daily for symptoms of infection. Some of the advantages of this model include the development of diarrhea that can last up to 12 days and damage to the intestinal epithelia as shown by histological analyses [38]. These symptoms are

also observed during human infection. In addition, the newborn piglets lack two important factors that help establish infection: the absence of intestinal flora and lack of maternal antibodies against *C. jejuni*, which are present in the colostrum.

Galleria mellonella. *G. mellonella* larvae, commonly known as the wax worm, have recently been proposed as a model for infection with *C. jejuni* [39]. The larvae mortality rate has been tested with different *C. jejuni* strains that were isolated from human infections and are currently used for research [39]. This model has been used before to identify virulence factors from other bacterial species such as *Francisella tularensis*, *Burkholderia mallei* or from the fungi *Candida albicans* [40-42]. The larvae are generally injected with 10^4-10^6 CFU and this results in *C. jejuni* colonization of the gut and hemocoel (the body cavity). Upon infection, an immunological response against the bacterium can be visualized by changes in pigmentation of the larvae [43]. Histopathological analyses of *C. jejuni*-infected larvae showed damage to the cells lining the gut wall as well as the presence of nodules. One of the caveats of this model is that *C. jejuni* cells loose their spiral shape when they colonize the larvae gut, which suggest a gross alteration of the biology of *C. jejuni* [43]. However, this model could be useful to identify potential virulence factors based on the survival of the larvae and changes in pigmentation upon colonization with *C. jejuni*.

MyD88-deficient mouse model. *C. jejuni* is unable to naturally colonize adult immunocompetent mice. However, it was shown that mice deficient in MyD88 are readily colonized by wild-type *C. jejuni* [44]. MyD88 is required for signal transduction to stimulate an innate immune response upon activation of Toll-like receptor (TLR) signaling. The absence of innate immune response provides *C. jejuni* with the opportunity to colonize the intestinal tract of mice that have been inoculated orally or intraperitoneally. *C. jejuni* can be recovered from mice

feces up to three weeks post-infection. In addition to MyD88$^{-/-}$, Nramp1$^{-/-}$ is another mutation in mice that enhances colonization by intracellular pathogens such as *C. jejuni* [44]. The dosage for infection tends to be high, with 10^9 CFU required for oral inoculation and 10^6 CFU required for intraperitoneal inoculation. Even though the inoculum requires a high number of bacteria, *C. jejuni* mutants lacking known colonization or virulence factors do not colonize the MyD88$^{-/-}$ Nramp1$^{-/-}$ mice. Infection of these animals does not result in any symptoms of infection, but it is a good model to assess the virulence potential of many *C. jejuni* components.

Virulence Factors of *C. jejuni* Involved in Human Infection and Pathogenesis

Capsule. The potential for capsular polysaccharide production in *C. jejuni* was first identified by genome sequence analysis, which recognized the presence of several genes with homology to characterized genes encoding components involved in capsule biosynthesis from other bacteria [1]. The capsule of *C. jejuni* varies in composition and linkage, which results in high diversity with a total of 47 different serotypes based on the Penner serotyping scheme used to classify *C. jejuni* strains [45]. The diversity of *C. jejuni* capsular polysaccharides arises from the ability of the different capsular genes to undergo phase variation. This variation can occur within the conserved capsule locus that encodes proteins functioning in capsular assembly and transport, or within genes that encode capsular sugar biosynthesis and modification enzymes [46]. Furthermore, *in vitro* analyses have shown that capsule biosynthesis can also be influenced by the presence of iron. This type of regulation occurs at the transcription level and targets the production of specific sugars incorporated in the capsule [45,47,48].

The importance of capsule for virulence is not well understood. However, the ability to switch capsular polysaccharide production between “ON” and “OFF” states could suggest that

the capsule plays an important role in *C. jejuni* biology in different niches that the bacterium encounters. Disruption of *kpsM*, (encoding an ABC transporter required for capsule transport) or a mutant lacking O-methyl phosphoramidate (MeOPN, a modification prevalent in *C. jejuni* capsule) demonstrated a reduction in adherence to and invasion of human intestinal cells *in vitro*, resistance to complement, colonization of ferrets, and prolonged colonization of mice [49-51]. Another piece of evidence that adds to the importance of capsule in the biology of *C. jejuni* is that disruption of different factors involved in capsule production resulted in reduced ability to colonize the chick intestinal tract [52]. This suggested that capsule is necessary for commensal colonization and for colonization of hosts in which *C. jejuni* causes disease. Lastly, recent developments of capsule conjugate vaccine suggest that capsule has potential as a vaccine target to prevent infection of *C. jejuni* in humans [53].

O- and N-linked glycosylation. *C. jejuni* has two systems for protein glycosylation. The *O*-linked protein modification is found on the flagellins, and is important for efficient polymerization of the flagellin subunits to form the filament structure [54]. Therefore, this post-translational modification of flagellins is indirectly necessary for motility of *C. jejuni*, virulence and colonization [55]. *O*-linked protein glycosylation consists of the addition of a pseudaminic acid glycan to a serine or threonine on flagellins in areas that are eventually surface exposed when a flagellin is incorporated into the flagellum [56,57]. The addition of the post-translational modification of flagellins requires the precursor molecule UDP-GlcNAc, which is modified by several enzymes or transferases (PseB-PseI) in a cascade of events to produce pseudaminic acid [58]. Specific genes of this glycosylation pathway are partially regulated by the flagellar transcriptional regulatory cascade [54]. However, glycosylation of flagellins likely takes place in the cytoplasm and is not dependent on the formation of other flagellar sub-structures [57].

N-linked protein glycosylation occurs on some periplasmic and outer membrane proteins. This modification consists of the addition of a heptasaccharide on specific asparagine residues of target proteins. *N*-linked protein modification has only been observed in *Haemophilus influenzae* and *C. jejuni* [59]. This modification is carried out by the Pgl enzyme family, which mediates sugar biosynthesis and transport. Disruption of different members of this family results in defects in commensal colonization and *in vitro* adherence to and invasion of human intestinal epithelial cells [60]. To further investigate the importance of *N*-linked glycosylation, a series of *C. jejuni* mutants were analyzed for invasion of human intestinal epithelial cells. These mutants lacked individual proteins that contained the sequence for glycosylation (NXS/T) and are found in the periplasm, suggesting that they could be glycosylated. One protein in particular, Cj1496, was shown to play a role in adherence and invasion of INT-407 cells. However, its activity did not require the post-translational modification as shown by a mutant protein containing point mutations that could not be modified. In other words, the absence of *N*-linked glycosylation had no effect on the invasion ability of the mutant [61]. Recently, the production of free oligosaccharides (fOS) by the *N*-linked glycosylation system has been observed, and is the result of activity of the transferase PglB [62]. fOSs have been found to have a protective function in the periplasm during osmotic stress [62]. Based on these findings, the role of *N*-linked glycosylation system during virulence and commensal colonization has so far been shown to be independent of the modification of proteins, but rather for the production of free sugars during osmotic stress.

Cytolethal distending toxin. Genome analysis of *C. jejuni* revealed the presence of genes (*cdtABC*) coding for a cytolethal distending toxin [1]. This type of toxin is also found in several other bacteria such as *E. coli* and *Haemophilus ducreyi* [63,64]. The CDT of *C. jejuni* causes

host cell cycle-arrest and induces a morphological change in host cells. When HeLa cells were cultured with *C. jejuni* extracts that contained the toxin, the cells appeared to swell and eventually died [65]. Further analysis revealed that CDT halts cell-cycle progression at the G_2 phase and induces nuclear breakdown. For the most part, activity of the toxin requires the presence of the three subunits (CdtA, CdtB and CdtC). Although the role of CDT in *C. jejuni* pathogenesis is not fully understood, studies have shown that the most conserved subunit, CdtB, is responsible for nuclear localization [66]. CdtB also possesses DNase activity, which is important because the mechanism of action of this toxin is based on DNA damage, perhaps due to double-strand breaks that cause host cell cycle arrest [66,67]. In contrast, an isogenic mutant lacking the toxin colonized chicks to similar levels as a wild-type strain [68]. Interestingly, neutralizing α-CDT antibodies were not detected in the chick sera upon colonization with *C. jejuni*. However, sera obtained from human patients suffering from campylobacteriosis did contain neutralizing α-CDT antibodies. These findings suggest that the toxin is not essential for commensal colonization, but may play an important role during infection of humans [68].

Role of flagella during colonization and invasion. Experimental evidence supports the concept that *C. jejuni* requires flagella for successful colonization of humans [69]. This observation was obtained by analyzing stools collected from human volunteers who were challenged with a *C. jejuni* solution. The inoculum administered was a mixed inoculum of flagellated and aflagellated *C. jejuni* strains. Stool cultures collected from infected volunteers showed that only flagellated *C. jejuni* were able to colonize. *In vitro*, *C. jejuni* invades human intestinal epithelial cells and translocates across polarized epithelial layers of Caco-2 cells [70]. Adherence of *C. jejuni* to epithelial cells does not involve the flagella or motility since aflagellated or nonmotile *C. jejuni* cells can still bind to INT407 cells *in vitro* if the bacterium-

host cell interaction is enhanced by centrifugation [70]. However, non-motile or aflagellated mutants are defective in internalization into human epithelial cells [70]. Furthermore, flagella of *C. jejuni* are also required for commensal colonization of the gastrointestinal tract of chicks [35,71]. Therefore, flagella are another virulence factor that plays a major role during human infection, but are also important for commensal colonization.

The role of flagella during infection expands beyond its function in motility. For instance, flagella of *C. jejuni* also serve as an specialized secretion system that is required for secretion of different virulence and colonization factors that play a role during commensal colonization and/or invasion of human intestinal epithelial cells [72,73]. In addition, transcription of some of these factors is co-regulated with flagellar components [74].

Determinants for Commensal Colonization of Chickens

A number of laboratories have identified factors of *C. jejuni* required for commensal colonization of chickens. One successful approach involved a signature-tagged transposon mutagenesis strategy to identify colonization factors of *C. jejuni* required for colonization of the cecum [35]. This analysis revealed 22 genes required for wild-type levels of commensal colonization. Interestingly, this study revealed several genes that are required for flagellar gene expression, biosynthesis or motility. These results were in agreement with previous observations indicating that flagella are necessary for commensal colonization of chickens and human infection. Not all genes identified in the signature-tagged transposon screen were genes involved in flagellar motility. For example, one transposon mutant that retained motility, but was unable to colonize the chick ceca, was a cytochrome *c* peroxidase (CCP) mutant. This gene was annotated *docA* (determinant of chick colonization). DocA is a periplasmic protein that binds

heme, but does not perform the known functions of a CCP such as reduction of hydrogen peroxide to water to reduce toxicity during oxidative stress [75].

Several other factors that are recognized as *C. jejuni* virulence factors have also been shown to play a role in commensal colonization of the chick intestinal tract, such as capsular polysaccharide production [52]. Also, *C. jejuni* mutants defective in *O*- or *N*-linked protein glycosylation show reduction in the ability to colonize chicks [60,76]. In addition, a recent study of all known adhesins of *C. jejuni* revealed that CapA, CadF, Peb1 and a newly identified adhesin, FlpA, are required for wild-type levels of *in vitro* adherence to chick hepatocellular carcinoma epithelial cells [77]. Some of these adhesins have additional roles in prolonged commensal colonization of the chick cecum. For instance, a *capA* mutant was not detected at 6-weeks post-infection, but wild-type *C. jejuni* was capable of prolonged colonization [21]. CadF, Peb1 and FlpA are also important for chick colonization as determined by the reduced number of bacteria recovered 7-days post-infection [14,77].

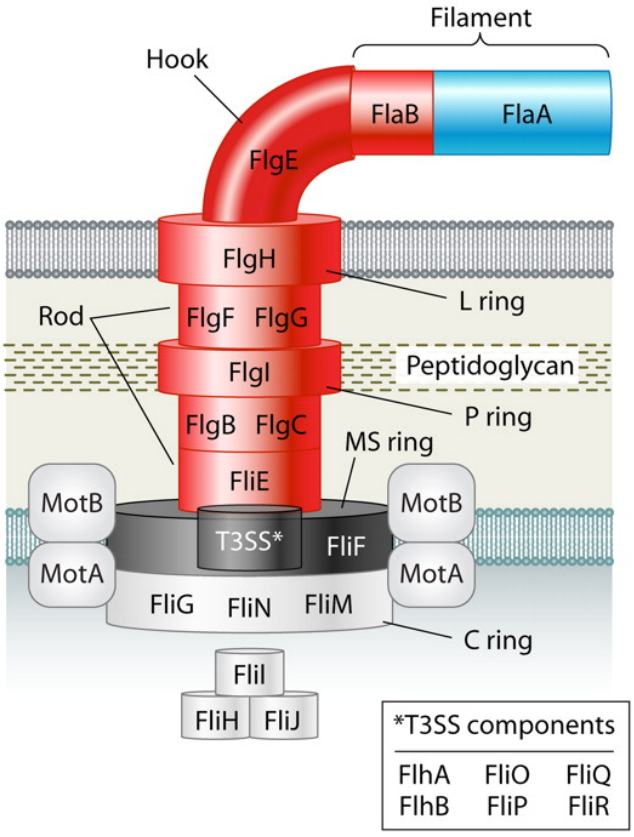
Flagellar Biosynthesis

Flagellar biosynthesis is a complex process that requires the coordinated expression, interaction and secretion of over 40 different proteins. A flagellum can be divided into substructures that include a flagellar basal body, hook and the filament. The basal body is composed of a rod that spans from the inner membrane to the outer membrane, and ring structures in the cytoplasm, periplasm, inner membrane and outer membrane. The expression, secretion and assembly of the many components associated with the flagellar apparatus occur in a highly organized manner with the proximal structures constructed first, followed by more distal structures [78]. The early steps of flagellar biosynthesis include the formation of the flagellar

T3SS at the inner membrane, followed by the formation of the MS ring and the cytoplasmic ring [79,80]. Formation of the periplasmic rod is next, followed by the hook structure and lastly the filament. In *C. jejuni*, flagellar biosynthesis occurs at the poles and only one flagellum per pole is synthetized making the bacterium an amphitrichous organism. The spatial arrangement of flagella is currently being studied in our laboratory. FlhF and FlhG are important for the proper placement and number of flagella at the poles [81]. In *C. jejuni,* FlhF is a GTPase whose activity is important for the polar localization of flagella [82]. FlhF also influences flagellar gene expression to some extent at an early stage of the flagellar regulatory cascade [83]. On the other hand, FlhG has been shown to have two activities important for *C. jejuni* biology: to limit the number of flagella at the pole to only one; and to inhibit cell division at the poles [84].

To add complexity to this process, there are other factors such as phase variation that can affect the expression of flagellar components [85,86]. The FlgSR two-component system is required for activation of σ^{54} and expression of many genes that form the rod and hook (as explained in detail below; [83,87]). The production of the FlgS sensor kinase or the FlgR response regulator undergoes phase variation to switch between the ON and OFF states. This process occurs by modifying the number of bases within homopolymeric tracts or the number of repeating units in heteropolymeric tracts found in the genes encoding both proteins [85,86]. The OFF (aflagellated) position can revert to the ON position (flagellated) in the chick intestinal tract where motility promotes maximal fitness [85,86].

The flagellar T3SS and motor. The flagellar (T3SS) is located in the inner membrane and is surrounded by the MS and C rings (Figure 1). The flagellar T3SS is formed by the inner membrane proteins FlhA, FlhB, FliP, FliQ and FliR. The cytoplasmic components FliI and its regulator FliH, are also important for activity of the flagellar T3SS. FliI is an ATPase involved



Adapted from Gilbreath J J et al. (2011) [88]

Figure 1. Flagellar Structure of *C. jejuni*. Shown here is a diagram depicting the different components that make up a functional flagellum in *C. jejuni*. The flagellar T3SS, which localizes to the inner membrane, is shown in black and gray alongside the stators. The basal body rod, which connects the inner membrane to the outer membrane, is shown in red. The hook structure is also shown in red and it connects the basal body to the filament. The major flagellin FlaA (blue) and the minor flagellin FlaB (red) polymerize to form the filament.

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in the dissociation of secretion substrates from their respective chaperones [89]. All of these components are necessary for protein export of rod, hook and filament proteins [90]. The C-termini of FlhA and FlhB are located in the cytoplasm, whereas the remainder proteins are found embedded within the inner membrane with small periplasmic and cytoplasmic loops. FlhB, as studied in *Salmonella*, exists in a native and a processed form with each form possessing different specificity for flagellar components. The native form is found at the early stages of

flagellar biosynthesis in which the C-terminus of FlhB interacts with rod and hook components (FliE, FlgB, FlgE and FlgD; [91]), which may allow for specific recognition and secretion. Processing of FlhB takes place after hook biosynthesis and results in a substrate specificity switch. This processed form of FlhB interacts with filament and other proteins including the anti-σ-factor FlgM [91].

In addition to the flagellar T3SS, the MS ring, the C ring and the stator form the base of the flagellar structure. The MS ring is formed by the polymerization of FliF proteins, which results in enclosure of the flagellar T3SS (Figure 1). The C ring is a cytoplasmic structure that is formed by the switch complex (FliM and FliN) and the rotor [92]. The rotor portion of the C ring is made of FliG proteins that polymerize into a structure that interacts with the MS ring. The rotor is responsible for the movement of the flagellum in a clockwise or counterclockwise motion [93,94]. The direction of flagellar rotation is determined by interaction of the FliM and FliN switch proteins with the chemotaxis response regulator CheY [95]. The C ring also functions as an "inverted cup" to collect proteins and promote efficient delivery of secretion substrates to the flagellar T3SS [96].

The stator is embedded into the inner membrane and is made of two proteins, MotA and MotB. These two proteins interact to form a channel that allows proton flow across the inner membrane. The proton flux generates torque, which powers rotation of the flagella [97]. MotB contains at its C-terminus a peptidoglycan-binding region and MotA interacts with FliG, both of which allow the stator to integrate into the flagellar motor for function [98,99].

Basal body and hook. The basal body is formed by a periplasmic rod structure that spans from the inner membrane to the outer membrane. The rod is surrounded by the P ring, which is located within the peptidoglycan layer, and the L ring, which is located in the outer membrane

(Figure 1). The P and the L ring subunits are thought to be secreted prior to the formation of the rod since their secretion is dependent on the Sec pathway rather than the flagellar T3SS [100]. The P and L ring are thought to allow the rod structure to cross the peptidoglycan and outer membrane layers. Proteins that compose the rod are the first ones secreted after formation of the MS ring, C ring and flagellar T3SS [101].

The hook is an extracellular curved cylinder made up of FlgE monomers that connect the basal body to the filament (Figure 1). Length of the extracellular hook structure is determined by FliK, which has given this protein the designation of a "molecular ruler" for hook biosynthesis [102,103]. A *fliK* mutant produces an abnormally long hook (referred to as a "polyhook" phenotype) and is unable to form a filament [103]. There is evidence suggesting that FliK facilitates the transition of FlhB from a native form to a processed form. This modification of FlhB causes the flagellum to switch from secreting hook proteins to secreting filament proteins. Once the hook reaches the proper length, FliK interacts with the C-terminus of FlhB, which stimulates autocleavage of FlhB [104-106]. Cleavage of FlhB is essential for proper secretion of filament proteins, but not rod or hook proteins [105]. These findings suggest that the interaction between FlhB and FliK is important for the latter stages of flagellar biosynthesis [103,106].

The mechanism by which FliK functions as a molecular ruler is not fully understood. It has been suggested that hook biosynthesis in wild-type cells continues until a certain hook length is reached. FliK is thought to be secreted periodically during hook biosynthesis. FliK appears to inhibit further hook elongation when the N-terminus of FliK interacts with the hook cap (FlgD) at the same time that its C-terminus interacts with FlhB. The FliK-FlhB interaction induces the cleavage event that results in substrate specificity switch [96,107].

As mentioned before, a properly formed hook serves as a signal to switch substrate specificity of secretion by the flagellar T3SS so that it can initiate secretion of late flagellar proteins. In *Salmonella* species, the rod and hook proteins belong to an early export class of flagellar proteins. There appears to be no differentiation between these two types of flagellar components for secretion [90]. The only switch occurs after formation of the hook structure and this change results in higher secretion specificity for filament proteins and decreased secretion specificity for rod and hook class proteins. The importance of this secretion specificity switch lies in the fact that a high number of flagellin monomers are needed to be secreted to polymerize into the filament structure.

Flagellar filament. In *C. jejuni*, the filament is a long whip-like and cylindrical structure composed of several hundred subunits of the major flagellin, FlaA, and many fewer subunits of the minor flagellin, FlaB (Figure 1; [108]). The first proteins to be secreted after the specificity switch that stops secretion of hook proteins are the hook-filament junction proteins FlgK and FlgL. Next the flagellar cap, FliD, is secreted and forms a cap of five monomers that binds to the end of the completed hook structure. As flagellins are secreted, the FliD cap traps flagellins so that they polymerize on the distal end of the growing filament [109-111]. While both FlaA and FlaB are capable of polymerizing into a filament, only FlaA is essential for motility since a mutant lacking *flaB* retains wild-type levels of motility [108]. Although FlaA and FlaB have 90% identity, they differ in secretion efficiency as shown in studies where the N-termini of both proteins, which are predicted to contain the secretion signal, were swapped and filament formation and length was monitored. This study suggested that secretion efficiency, rather than flagellin expression is what drives the preference of FlaA over FlaB and this has an impact on filament length and motility [112].

In *Salmonella,* the flagellin is encoded by *fliC*. The structure of a folded FliC subunit can be divided into four connected domains (D_0-D_3). The N-terminus (which contains the secretion signal) and C-terminus together are denoted as the D_0 domain [113]. The disordered D_0 domain is the region where adjacent flagellins interact to polymerize and form the filament [114].

Flagellar Chaperones

Flagellar chaperones are proteins that play an essential role in secretion of flagellar substrates. Functional characteristics of flagellar chaperones include the ability to target their substrate to the flagellar T3SS for secretion and to promote efficient secretion by inhibiting multimerization of the substrates in the cytoplasm. Another important characteristic of flagellar chaperones is their specificity for their cognate substrate. The known flagellar chaperones include: FliJ, FlgN, FliT, FliS and FliW. Their functions in flagellar biosynthesis are described below.

FliJ. Initially, the FliJ chaperone was attributed a role in aiding with secretion of rod and hook proteins [90]. However, recombinant FliJ was shown to interact not only with rod and hook proteins, but also hook-filament junction proteins such as FlgL. In addition, FliJ in excess stimulated the secretion of FliC (the flagellin in *Salmonella* species), suggesting that this flagellar chaperone has the ability to interact with different classes of substrates [91,115]. Further characterization of this chaperone showed that FliJ prevented the aggregation of overexpressed rod substrates, which is another function of flagellar chaperones [115].

Recent studies have shown however, that the role of FliJ may be to recycle used chaperones and facilitate their interaction with new cognate secretion substrates [116]. FliJ was found to interact with free chaperones and bind the same region of a flagellar chaperone that

interacts with the substrate. FliJ delivers the chaperone to a free cognate substrate subunit. Due to the chaperone having a higher binding affinity to its substrate compared to FliJ, the chaperone binds the substrate causing its release from FliJ. The importance of this mechanism is that chaperones that are recycled more often than others by FliJ are those for the minor flagellar substrates such as filament cap or hook-filament junction proteins. These proteins are secreted in much lesser amounts compared to flagellins, and must compete with flagellins for secretion by the flagellar T3SS. FliJ is positioned in close proximity to the flagellar T3SS as it interacts with the cytoplasmic region of FlhA, FliI (the ATPase that mediates the release of flagellar chaperones from their substrates) and FliH (the regulator of FliI) [117]. It seems that the role of FliJ during flagellar substrate secretion is to bind chaperones to enhance the secretion of minor flagellar proteins close to the flagellar T3SS. These proteins are found amid an excess pool of flagellin proteins, yet they need to be secreted for proper formation of the flagellum.

FlgN, FliT and FliS. In *Salmonella*, FlgN, FliT and FliS are chaperones required for secretion of filament proteins. FlgN is the flagellar chaperone for the hook-filament junction proteins FlgK and FlgL, whereas FliT is the chaperone for the flagellar cap FliD [118]. FlgN and FliT are found naturally as a homodimer within the bacterium [119]. These two chaperones perform the known activities of flagellar chaperones: they bind their substrates to promote efficient secretion by inhibiting intracellular aggregation and they interact with components of the flagellar T3SS to deliver their substrates for secretion [120,121]. However, studies have also shown that in *Salmonella,* FlgN plays a role in regulating translation of the anti-σ-factor FlgM [122]. A *flgN* mutant had lower intracellular FlgM levels compared to wild-type bacteria. This reduction in protein levels is not due changes in stability of FlgM, but rather changes in translation of the *flgM* transcript.

FliS is the cognate flagellar chaperone for flagellins. The polymerization of flagellins into the filament structure requires more than 10,000 subunits. This highlights the necessity for a flagellar chaperone to bind its substrate and act as a "bodyguard" to prevent cytoplasmic association between subunits. In *Salmonella*, the absence of FliS results in the production of a short flagellum or no flagellum at all [123]. FliS has been shown to bind FliC with high specificity and functions as a chaperone since it is required for the efficient export of the flagellin subunits [124]. FliS specifically binds the disordered C-terminal portion of the D_0 domain of FliC, thus inhibiting association with other flagellin subunits [125].

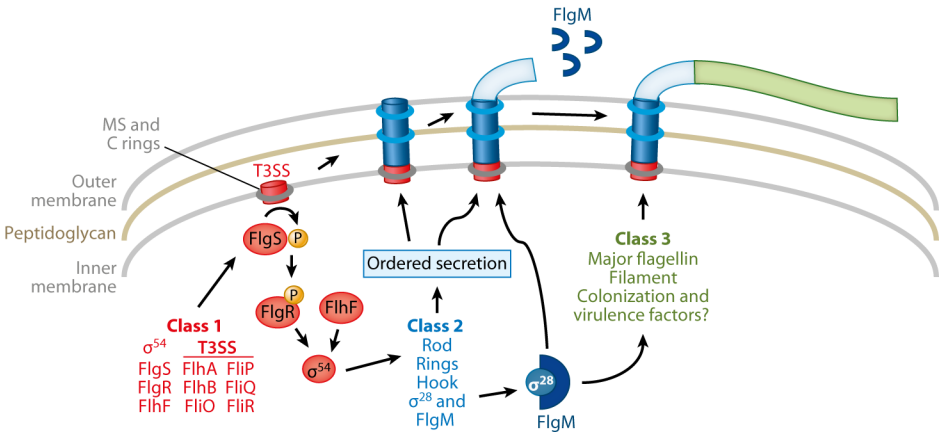
FliW. FliW was first identified in a yeast two-hybrid screen as a protein required for assembly of the flagellar apparatus in *Treponema pallidum* [126]. In a similar way to FliS, FliW interacts with the C-terminus of the flagellin FlaB1 in *T. pallidum*. The role of FliW in flagellin secretion is not well understood. Initially, it was suggested that FliW had a role in stabilizing flagellins since co-expression of recombinant *T. pallidum* flagellin and FliW increased the stability of the flagellin [126]. However, studies performed in *Bacillus subtilis* indicated that FliW plays a role in Hag (flagellin) production. A *B. subtilis fliW* mutant produces short flagella, which is likely due to reduced levels of flagellins present in the cell. The mechanism by which FliW affects Hag production involves the protein CsrA, which is an RNA-binding protein that regulates mRNA stability or translation of certain transcripts. During the early stages of flagellar biosynthesis, CsrA blocks binding of ribosomes to the *hag* transcript and down-regulates *hag* translation at the same time that residual flagellins are bound to FliW before the flagellar basal body is formed [127]. Once the flagellar basal body is formed, Hag is released from FliW to be secreted. FliW is then free to physically interact with CsrA, which releases CsrA-mediated repression on *hag* translation [128]. Increased Hag production and secretion result in the

formation of the filament. This mechanism involving FliW and CsrA suggests that FliW does not behave like a canonical chaperone. Instead, its main role is the homeostatic regulation of flagellin production in the cell. This idea is supported by the fact that FliS and FliW can bind Hag simultaneously and FliW does not displace FliS, suggesting that they do not share a binding site within Hag [129].

Flagellar Gene Regulation

Flagellar gene transcription is a tightly regulated process so that flagellar genes are transcribed in the correct order to promote proper flagellar biosynthesis. The flagellar regulatory cascade was first analyzed in *Salmonella* species. Since then, similarities and differences have been elucidated in *C. jejuni* and other motile bacteria. There is a common theme governing flagellar formation, which involves the organized expression of specific flagellar genes followed by sequential secretion and assembly of the different substructures. Once a specific structure is formed, a genetic switch is flipped to then promote expression of the next class of flagellar genes until the organelle is completed.

Flagellar genes are divided into classes based on their temporal order of expression and function of the encoded proteins. Usually a master transcriptional regulator is required for expression of class I genes that make up the early flagellar components [93]. In contrast, the flagellar regulatory cascade in *C. jejuni* begins with expression of class I genes, which form the T3SS and the MS and C rings (Figure 2). The proper polymerization of FliF and FliG in a flagellar T3SS-dependent manner activates the FlgSR two-component system, which in turn activates σ^{54} to induce expression of several flagellar genes [130,131]. In addition to σ^{54} activity, expression of class II genes requires FlhF, although its mechanism of action is not well



Adapted from Lertsethtakarn, P. et al. (2011) [93].

Figure 2. Diagram of the flagellar regulatory cascade of *C. jejuni*. The proper formation of the MS ring serves as a signal to activate the FlgSR two-component system in a flagellar T3SS-dependent manner. FlgSR in turn activates σ^{54}, which is required for the expression of genes encoding hook and rod proteins, *fliA* (encoding σ^{28}) and *flgM* (encoding the anti-σ factor for σ^{28}). The GTPase FlhF is also required for full levels of expression of σ^{54}-dependent genes and functions in a pathway separate from the T3SS-FlgSR pathway. Activity of σ^{28} is inhibited by FlgM before the flagellar rod and hook are formed. Once FlgM is secreted from the cytoplasm, σ^{28} is free to interact with the RNA polymerase to transcribe genes that encode minor filament proteins and FlaA, as well as genes encoding virulence and commensalism determinants.

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understood [82]. The class II genes code for the components of the rod, rings, hook, as well as σ^{28} and its anti-σ-factor FlgM. FlgM represses σ^{28} activity through direct binding until FlgM is secreted out of the cell through the flagellar T3SS, rod and hook (Figure 2, [132,133]). Once σ^{28} is free from repression, it initiates the expression of the class III genes, which include the major flagellin (FlaA) and minor filament proteins [74]. Studies have shown that the interaction of FlgM with σ^{28} is temperature-dependent in *C. jejuni*, where tighter interactions between FlgM and σ^{28} are observed at 37 °C compared to 42 °C. The biological significance of this temperature-regulated interaction is that the avian body temperature is 42 °C, suggesting that FlgM is secreted at higher levels and the inhibition of σ^{28} activity is less within the chick gut. Thus, there is likely an increase in class III gene expression to result in a longer filament when

the bacterium is found in the avian gut, which may be beneficial to commensal colonization [134].

A microarray study was performed comparing genome expression of a *C. jejuni* strain lacking *fliA*, the gene that encodes σ^{28}, to wild-type *C. jejuni*. Transcriptome and bioinformatic analyses to identify a consensus sequence for σ^{28} binding in *C. jejuni* promoters revealed putative members of the σ^{28} regulon. Among some of the genes identified as part the σ^{28} regulon are FlaA and other minor flagellar filament genes. In addition, this study suggested that σ^{28} is also required for the expression of six genes that were not predicted to encode components of the flagella [74].

Two genes that were identified as members of the σ^{28} regulon from these transcriptome and bioinformatic analyses were further characterized. The gene *cj0977* (or *cjj0996* in the *C. jejuni* 81-176 genome, which is the strain examined and presented in this study) was shown to be dispensable for flagellar formation and motility in viscous media [135]. A mutant lacking *cj0977* exhibited reduced invasion of intestinal cells and was attenuated in a ferret diarrheal disease model. Recent studies showed that this mutant retained motility in viscous media, but was completely non-motile in liquid media [24]. This phenotype could perhaps explain the defect observed during *in vitro* invasion of epithelial cells and infection of ferrets. The mechanism by which this σ^{28}-dependent protein confers motility in media with low viscosity, but is not required in high viscosity medium, is unknown.

The other σ^{28}-dependent gene that has been studied is *fspA*. This gene product is observed as two alleles in different *C. jejuni* isolates, FspA1 and FspA2 [136]. The FspA1 protein produced by *C. jejuni* 81-176 is secreted into the supernatant fraction in a flagellar-dependent manner. Further characterization of the secretion of FspA1 revealed that intact

flagella are required. In terms of virulence, FspA1 is not required for *in vitro* adherence to or invasion of intestinal cells. However, FspA2 of other *C. jejuni* strains induces apoptosis of intestinal cells when recombinant protein is added to eukaryotic cells [136]. Other putative σ^{28}-dependent genes that are not predicted to be involved in motility were characterized and are the subject of this thesis.

Protein Secretion in *C. jejuni*

As in many other bacteria, *C. jejuni* likely utilizes the Sec pathway to transport specific proteins across the inner membrane. Although the Sec system has not been studied in *C. jejuni*, many proteins predicted to localize in the periplasm or outer membrane contain an N-terminal signal peptide to differentiate them from cytoplasmic proteins [137]. In addition, putative components involved in type II protein export were identified within the *C. jejuni* genome [1].

The only T3SS present in *C. jejuni* is the flagellar T3SS. The flagellar proteins and the T3SS injectisome found in many bacterial pathogens share morphological and sequence similarities [138]. The secretion signal of the flagellar T3SS substrates is usually found at the N-terminus and the substrates must be in a semi-folded state to be secreted. Another important component for secretion by the flagellar T3SS are chaperones, which bind the C-terminus of their substrate [138,139]. Once a secretion substrate is expressed, it interacts with its cognate chaperone and is then delivered to the T3SS where the N-terminal secretion signal is recognized followed by translocation of the substrate.

The T3SS is specific for secretion substrates such as effector proteins or flagellar components. However, a few cases have been noted where a T3SS can secrete non-effector or non-flagellar proteins [140]. The first evidence that the flagellar T3SS could secrete substrates

with different function was shown by Young et. al. [140]. In this study, *Y. enterocolitica* was used as a model to identify a set of proteins termed flagellar outer proteins (Fops), which required a fully functional flagellar apparatus for secretion [140]. They also concluded that formation of flagella (and therefore motility) was coupled with the secretion of the Fops. More importantly, the genes encoding the Fops could be found within the flagellar operons. YplA, a σ^{28}-dependent phospholipase involved in virulence, was identified as a Fop that required secretion for activity, but was not required for motility [140]. Further characterization of YplA showed that this protein was also secreted by the other two T3SS in *Y. enterocolitica* (Ysa and Ysc) when they are active under the right environmental conditions [141]. In addition, YplA can be secreted from *Escherichia coli* K-12, which only has the flagellar T3SS [142]. The secretion signal for YplA was located within the first 10 amino acids, which is consistent with other flagellar T3SS substrates that contain their secretion signal at the N-terminus [142].

Proteins that utilize the flagellar T3SS for their secretion were also identified in *C. jejuni*. The *Campylobacter* invasion antigens (Cia) were identified as a family of proteins secreted by *C. jejuni* strain F38011 and shown to be important for internalization, but not for adherence to epithelial cells [143]. Synthesis of Cia proteins is stimulated by the presence of bile acid components (i.e. deoxycholate, cholate, and chenodeoxycholate), or upon co-culture of the bacterium with human epithelial cells (INT407) or with INT407-conditioned medium that contains serum [143,144]. Secretion of Cia proteins was shown to depend on stimulants such as FBS, and is also a requirement for invasion of human epithelial cells *in vitro* [145,146].

An important observation presented by the Konkel laboratory indicated that the Cia proteins are likely to be secreted via the flagellum. Secretion was not detected in different flagellar mutants lacking components of the flagellar T3SS, basal body, rod or hook

[73,146,147]. Furthermore, the Cia proteins are expressed, but not secreted in the absence of the major flagellin (FlaA) or the minor flagellin (FlaB; [146]). However, when at least one flagellin is present either endogenously or expressed from a plasmid, secretion of Cia proteins is restored but with lower levels compared to wild-type. Although the Cia proteins require an intact flagellar apparatus for secretion, the flagellar regulatory cascade does not control their expression (with exception of CiaI, which will be discussed in this work).

CiaB was the first member of the Cia family to be identified [143]. This protein shares approximately 40% similarity to effectors such as SipB. *C. jejuni* F38011 strains lacking *ciaB* are defective for internalization of INT407 cells. However, this phenotype seems to be specific to the F38011 strain since analysis of *ciaB* mutants of other *C. jejuni* strains are not defective for invasion of human intestinal epithelial cells [24,135]. Furthermore, analysis of radiolabeled supernatants of the F38011 *ciaB* mutant strain suggested that this protein is required for secretion of other Cia proteins (CiaA-CiaH, [143]). Secretion of CiaB was further characterized using the *Yersenia enterocolitica* T3SS. In this system, CiaB expressed in *Y. enterocolitica* was secreted into the supernatant fraction, and its secretion was dependent on an intact T3SS in the same way as the Fops [147]. This finding was possible due to unique characteristics of T3SS substrates: they contain a secretion signal at their N-terminus, and this secretion signal can be recognized by T3SSs from other species [147]. CiaC is another member of the Cia protein family. This protein was first identified in a screen aimed to identify secreted proteins that contained a T3SS signal at their N-terminus and could be secreted via the *Y. enterocolitica* flagellar T3SS [147]. CiaC is another example of a secreted protein that requires an intact flagellar apparatus for secretion, and is also involved in *C. jejuni* internalization of host epithelial cells.

As mentioned before, one of the early observations about Cia proteins is that the bile salt deoxycholate (DOC) serves as a signal to induce expression in a dose-dependent manner [144]. This finding would suggest that when *C. jejuni* is exposed to physiologically-relevant concentrations of DOC, its pathogenicity is enhanced, which is possibly due to an increase in expression of virulence factors such as the Cia proteins [145]. To identify more genes whose expression is affected by DOC, a microarray analysis was performed using *C. jejuni* grown in the presence DOC. One gene in particular, *ciaI,* displayed a 1.7-fold change in expression when comparing strains grown in the absence and presence of DOC [145]. However, *ciaI* was also identified in another microarray screen performed to identify members of the σ^{28} regulon. The discrepancies in regulation of gene expression for *ciaI* will be addressed in a later chapter [74]. Further characterization of CiaI revealed that this protein is secreted via the flagellar T3SS and that it plays a role in long-term survival of *C. jejuni* after invasion of intestinal epithelial cells [72]. In these analyses, HeLa cells were infected with wild-type *C. jejuni* and an isogenic mutant lacking *ciaI*. Analysis of markers located on the campylobacter containing vacuole (CCV) surface showed that after a prolonged infection, the CCVs containing the *ciaI* mutant were associated with cathepsin D, while CCVs containing wild-type *C. jejuni* were not. This finding suggested that CiaI may play a role in blocking fusion of the CCV with lysosomes. To further explore this idea, a CiaI-GFP fusion was transfected into HeLa cells to determine the localization of this protein once it enters the host cell. The CiaI-GFP fusion had a punctate pattern as observed by fluorescence microscopy and it co-localized with late-endosome markers like LAMP-1 [72]. The authors from this study concluded that CiaI may localize to vesicles to inhibit fusion of the CCV with lysosomes. A predicted double-leucine motif in CiaI may be important for this localization and for intracellular survival.

Another protein from *C. jejuni* that is secreted via the flagellar T3SS is FlaC. This protein is characterized by high similarity to the N- and C-terminus of FlaA and FlaB, but is not required for flagellar filament formation or motility [148]. FlaC requires a functional rod structure for its secretion into the supernatant fraction [148]. Although its mechanism of action is not fully understood, GST-FlaC can bind to HEp-2 cells. In addition, a *flaC* mutant exhibited reduced invasion of HEp-2 cells.

Recent studies identified a 13-gene operon, denoted *tssA-M*, that encodes a functional T6SS in *C. jejuni* strain 108 [149]. This study noted the presence of gene products with similarities to two well-conserved T6SS components, Hcp and VgrG. The hemolysin co-regulated protein (Hcp) polymerizes to form the needle structure while the valine-glycine repeat protein G (VgrG) polymerizes to form the spike that localizes to the tip of the needle [150]. Secretion of Hcp was monitored to determine the functionality of the T6SS in *C. jejuni*. The newly identified T6SS seems to have a role in contact-dependent red blood cell lysis as shown by *in vitro* experiments [149]. The role of this secretion system during pathogenesis and colonization is yet to be determined. Furthermore, analysis of 80 different *C. jejuni* isolates revealed that only ~10% of strains analyzed contained the T6SS locus. Therefore this system is not well conserved among *Campylobacter* species.

CHAPTER TWO

Materials and Methods[§]

Bacterial strains and plasmids

C. jejuni strain 81-176 was originally isolated from a patient with gastroenteritis [151]. Subsequent studies verified the capacity of this strain to infect human volunteers and promote commensal colonization of chicks [35,69]. *C. jejuni* was typically grown in microaerobic conditions (85% N_2, 10% CO_2, 5% O_2) on Mueller-Hinton (MH) agar or in MH broth at 37 ºC. As required, antibiotics were added to MH media at the following concentrations: 10 μg/ml trimethoprim (TMP), 20 μg/ml chloramphenicol, 100 μg/ml kanamycin, 30 μg/ml cefoperazone or 0.5, 1, 2, or 5 mg/ml streptomycin. All *C. jejuni* strains were stored at -80 ºC in a 85% MH broth and 15% glycerol solution. For routine growth to perform most experiments, *C. jejuni* strains were grown from frozen stocks for 48 h in microaerobic conditions at 37 °C, then streaked on MH agar and grown for additional 16 h in identical conditions. *E. coli* DH5α, XL1-Blue and BL21 strains were grown on Luria-Bertani (LB) agar or in LB broth containing 100 μg/ml ampicillin, 50 μg/ml kanamycin or 15 μg/ml chloramphenicol as appropriate. All *E. coli* strains were stored at -80 ºC in a 80% LB broth and 20% glycerol solution.

Construction of mutants

C. jejuni mutants were constructed by electroporation following previously described methods [87]. Genes to be deleted from the *C. jejuni* 81-176 Sm^R chromosome were first

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amplified by PCR using primers containing 5' BamHI or EcoRV restriction sites. Each fragment contained the gene of interest with 750 bases of flanking sequence. Cloning of the fragments into the BamHI site of pUC19 resulted in the creation of the following plasmids: pDRH3022 (pUC19::*fspA1*), pABT253 (pUC19::*Cjj81176_0996*), pABT115 (pUC19::*ciaI*), pABT249 (pUC19::*fedA*), pABT113 (pUC19::*fedB*), pABT328 (pUC19::*fedC*), pABT111 (pUC19::*fedD*), pDRH259 (pUC19::*flgK*), pDRH2428 (pUC19::*fliDS*), pDRH3235 (pUC19::*pseBC*), pLKB648 (pUC19::*rdxA*), pABT1044 (pUC19::*fliJ*), pABT945 (pUC19:: *fliW*) and pABT824 (pUC19::*ciaB*). For some genes it was necessary to create restriction sites within the coding sequence by PCR-mediated mutagenesis or by SOEing mutagenesis [152,153]. These reactions created an EcoRV site in *fspA1* (pDRH3025), *pseB* (pDRH3237), *rdxA* (pLKB653), *fliS* (pABT823), *fliD* (pABT822) and *ciaB* (pABT828); a PmeI site in *fedA* (pABT322); a StuI site in *Cjj81176_0996* (pABT329), *fedC* (pABT355), *fedD* (pABT152), *Cjj81176_1458* (pABT953) and *fliW* (pABT954); and a SwaI site in *fliJ* (pABT1211). Also, to create a *flaAB* double mutant, plasmid pDRH519 (pUC19::*flaAB*) was digested with EcoRV, which removed a large amount of the coding sequence of *flaA* and *flaB*. A SmaI-digested *cat-rpsL* cassette was obtained from pDRH265 and ligated into plasmids in the appropriate restriction sites to interrupt each gene [87].

Each plasmid generated above was electroporated into *C. jejuni* 81-176 Sm^R (DRH212) or *C. jejuni* 81-176 Sm^R Δ*astA* (DRH461) to interrupt each respective gene on the chromosome with the *cat-rpsL* cassette. Transformants were recovered on MH agar containing chloramphenicol. Mutations were verified by colony PCR and the following isogenic mutants of 81-176 Sm^R were obtained: ABT103 (*fspA1*::*cat-rpsL*), ABT366 (*Cjj81176_0996*::*cat-rpsL*), ABT214 (*ciaI*::*cat-rpsL*), ABT353 (*fedA*::*cat-rpsL*), ABT261 (*fedB*::*cat-rpsL*), ABT370

(*fedC*::*cat-rpsL*), ABT233 (*fedD*::*cat-rpsL*), DRH1249 (*flgK*::*cat-rpsL*), DRH2623 (Δ*astA* *flgG*::*cat-rpsL*), DRH3240 (*pseB*::*cat-rpsL*), ABT1246 (*fliJ*::*cat-rpsL*), ABT1021 (*Cjj81176_1458*::*cat-rpsL*), ABT861 (*fliS*::*cat-rpsL*), ABT1019 (*fliW*::*cat-rpsL*), ABT919 (*ciaB*::*cat-rpsL*), ABT1176 (*flaAB*::*cat-rpsL*), and ABT857 (*fliD*::*cat-rpsL*). In addition, creation of 81-176 Sm^R Δ*astA* Δ*flgM* was accomplished by electroporation of 81-176 Sm^R Δ*astA* (DRH461) with a previously constructed plasmid, pDRH552, to result in strain DRH557 (Δ*astA* *flgM*::*cat-rpsL*; [83]).

Primers were designed to construct in-frame deletions of genes originally cloned in pUC19 using PCR-mediated mutagenesis. Alternatively, some deletions were created by SOEing mutagenesis to result in fragments that were then cloned into the BamHI site of pUC19 (see above and; [153]). After sequencing to verify correct construction of in-frame deletions, the following plasmids were obtained: pDRH565 (pUC19::Δ*flgM*), pABT205 (pUC19::Δ*fspA1*), pABT325 (pUC19::Δ*Cjj81176_0996*), pABT173 (pUC19::Δ*ciaI*), pABT280 (pUC19::Δ*fedA*), pABT357 (pUC19::Δ*fedB*), pABT356 (pUC19::Δ*fedC*), pABT164 (pUC19::Δ*fedD*), pDRH2425 (pUC19::Δ*flgG*), pABT1057 (pUC19::Δ*fliJ*), pABT1031 (pUC19::Δ*Cjj81176_1458*) and pABT931 (pUC19::Δ*fliS*). These plasmids were electroporated into strains containing *cat-rpsL* interruptions of the respective genes on the chromosome. Transformants were recovered on MH agar with 0.5, 1, 2 or 5 mg/ml of streptomycin and then screened for chloramphenicol sensitivity. Deletion of each gene was verified by colony PCR, which resulted in creation of the following 81-176 Sm^R mutant strains: DRH604 (Δ*astA* Δ*flgM*), ABT361 (Δ*fspA1)*, ABT501 (Δ*Cjj81176_0996*), ABT279 (Δ*ciaI*), ABT477 (Δ*fedA*), ABT473 (Δ*fedB*), ABT472 (Δ*fedC*), ABT278 (Δ*fedD*), SNJ925 (Δ*astA* Δ*flgG)*, ABT1266 (Δ*fliJ)*, ABT1055 (Δ*Cjj81176_1458*) and ABT952 (Δ*fliS*).

To create plasmids containing *astA* transcriptional fusions to genes of interest, a SmaI-digested *astA-kan* cassette from pDRH580 was ligated into the EcoRV site of *fspA1* pDRH3025, the EcoRV site of *ciaI* in pABT115, the HpaI site of *fliA* in pDRH263, and the StuI site of *fedD* in pABT152 [83]. As a result, *astA* transcriptional fusions were created in the following plasmids: pABT405 (*fliA*::*astA-kan*), pDRH3027 (*fspA1*::*astA-kan*), pABT119 (*ciaI*::*astA-kan*), and pABT236 (*fedD*::*astA-kan*). These plasmids were then electroporated into 81-176 Sm^R Δ*astA* (DRH461) and isogenic strains lacking σ^{28}, *flgM*, or components of the σ^{54} regulatory pathway. Transformants were recovered on MH agar containing kanamycin and acquisition of the *astA* transcriptional reporter at the native locus on the chromosome of each gene was verified by colony PCR.

PCR-mediated mutagenesis was used to introduce point mutations in the coding sequence of *ciaI* in pABT115 to result in pABT673 (pUC19::$ciaI_{K42A}$) and pABT674 ($ciaI_{LL153\text{-}154AA}$) [153]. These plasmids were electroporated into ABT214 to replace *ciaI*::*cat-rpsL* on the chromosome with genes encoding the mutant proteins. Transformants were recovered on MH agar containing 0.5, 1, 2 or 5 mg/ml of streptomycin and screened for chloramphenicol sensitivity. Putative transformants were verified by colony PCR and sequencing to result in 81-176 Sm^R $ciaI_{K42A}$ (ABT704) and 81-176 Sm^R $ciaI_{LL153\text{-}154AA}$ (ABT706).

Plasmids carrying wild-type *ciaI* or *fedB* were constructed by amplifying DNA fragments containing 258 bases upstream of the start codon and 23 bases downstream of the stop codon of *ciaI* or 252 bases upstream of the start codon and 25 bases downstream of the stop codon of *fedB* by PCR using primers containing 5' BamHI restriction sites. These fragments were then cloned into the BamHI site of pRY108 to result in the creation of pABT720 (pRY108::*ciaI*) and pABT951 (pRY108::*fedB*). Primers were designed to generate truncated mutants lacking 6 to 25

codons at the 5' or 3' end of the coding sequence of *ciaI* or *fedB*. The plasmids were confirmed by sequencing. As a result, the following plasmids were created pABT956 (pRY108::*ciaI*Δ1), pABT1128 (pRY108::*ciaI*Δ1CD), pABT1354 (pRY108::*ciaI*Δ1ABC), pABT1032 (pRY108::*ciaI*Δ4), pABT1151 (pRY108::*ciaI*Δ4EF), pABT1152 (pRY108::*ciaI*Δ4GH), pABT1024 (pRY108::*fedB*Δ1), pABT1129 (pRY108::*fedB*Δ1AB), pABT1070 (pRY108::*fedB*Δ1A), pABT1056 (pRY108::*fedB*Δ1B), pABT1130 (pRY108::*fedB*Δ1CD), pABT1356 (pRY108::*fedB*Δ1ABC), pABT961 (pRY108::*fedB*Δ2), pABT1025 (pRY108::*fedB*Δ4), pABT1135 (pRY108::*fedB*Δ4EF) and pABT1370 (pRY108::*fedB*Δ4GH). These plasmids were transformed into the *E. coli* conjugation strain DH5α/RK212.1. The donor strains were used to conjugate the plasmids into the appropriate 81-176 mutant strains (ABT279 or ABT473).

To create Δ*ciaB* or Δ*fedB* strains expressing wild-type and mutant FedB or CiaI *in cis*, the wild-type and *ciaI* or *fedB* mutants were cloned with native promoters into the *rdxA* locus of *C. jejuni*. pLKB653 (pUC19::*rdxA*), which contains an EcoRV site, was further manipulated to create a PmeI restriction site using PCR-mediated mutagenesis to result in plasmid pABT1265 [153]. A SmaI-digested *kan* cassette obtained from pILL600 was ligated into the PmeI site of *rdxA* to create pABT1307 (pUC19::*rdxA-kan*). BamHI-digested fragments of wild-type *ciaI* and *fedB* or mutant alleles were ligated into pABT1307 digested with BglII. All inserts were confirmed by DNA sequencing. This resulted in the creation of pABT1342 (pUC19::*rdxA-ciaI-kan*), pABT1322 (pUC19::*rdxA-ciaI*Δ1-*kan*), ABT1403 (pUC19::*rdxA-ciaI*Δ4GH-*kan*), ABT1340 (pUC19::*rdxA-fedB-kan*) and pABT1321 (pUC19::*rdxA-fedB*Δ1-*kan*). These plasmids were then electroporated into *C. jejuni* Δ*ciaI* (ABT279) or *C. jejuni* Δ*fedB* (ABT473). Transformants were recovered on MH agar containing kanamycin and confirmed by colony

PCR. This resulted in the isolation of ABT1347 (Δ*ciaI rdxA*::*ciaI-kan*), ABT1331 (Δ*ciaI rdxA*::*ciaI*Δ1-*kan*), ABT1413 (Δ*ciaI rdxA*::*ciaI*Δ4GH-*kan*), ABT1344 (Δ*fedB rdxA*::*fedB-kan*), ABT1328 (Δ*fedB rdxA*::*fedB*Δ1-*kan*).

SOEing mutagenesis was employed to construct chimeric proteins in which the N-terminus of FedB or CiaI was fused to $FlaA_{\Delta N'36}$. First, a DNA fragment containing 203 bases upstream of the start codon thru the stop codon of *flaA* was amplified by PCR using primers containing 5' BamHI restriction sites. Additionally, primers were designed to construct an in-frame deletion of codons 1 to 36 of *flaA* using SOEing mutagenesis. BamHI-digested *flaA* and $flaA_{\Delta N'36}$ DNA fragments were then ligated into BglII-digested pABT1307, resulting in plasmids ABT1465 (pUC19::*rdxA-flaA-kan*) and ABT1412 (pUC19::*rdxA-*$flaA_{\Delta N'36}$*-kan*). To construct the chimeric proteins, a PCR was initially performed to create a DNA fragment that fused the promoter region of *flaA* to a fragment of *ciaI* or *fedB* encoding the first 11 residues. A second DNA fragment was generated using a long primer that fused the region of *ciaI* or *fedB* encoding the first 26 residues to *flaA*, at codon 37. Both DNA fragments were fused by PCR to create a final fragment containing 5' BamHI restriction sites. The final fragment consisted of the promoter of *flaA* followed by the first 26 codons of *ciaI* or *fedB* fused to $flaA_{\Delta N'36}$, which encoded $CiaI_{N'26}$-$FlaA_{\Delta N'36}$ or $FedB_{N'26}$-$FlaA_{\Delta N'36}$. The BamHI-digested PCR fragments were ligated into BglII-digested pABT1307. The resulting plasmids were sequenced for confirmation yielding plasmids pABT1466 (pUC19::*rdxA-*$ciaI_{N'26}$*-*$flaA_{\Delta N'36}$*-kan*) and pABT1468 (pUC19::*rdxA-*$fedB_{N'26}$*-*$flaA_{\Delta N'36}$*-kan*). Finally, pABT1412, pABT1465, pABT1466 and pABT1468 were

electroporated into *C. jejuni flaAB::cat-rpsL* (ABT1176). Transformants were recovered on MH agar containing kanamycin and chloramphenicol and insertion was confirmed by colony PCR. This resulted in the isolation of ABT1470 (Δ*flaAB rdxA*::*flaA-kan*), ABT1420 (Δ*flaAB rdxA*::*flaA*$_{\Delta N'36}$-*kan*), ABT1473 (Δ*flaAB rdxA*::*ciaI*$_{N'26}$-*flaA*$_{\Delta N'36}$-*kan*) and ABT1477 (Δ*flaAB rdxA*::*fedB*$_{N'26}$-*flaA*$_{\Delta N'36}$-*kan*).

Gene expression analyses

Arylsulfatase assays were employed to measure the level of expression of *astA* transcriptional fusions located on the chromosome of *C. jejuni* strains as previously described [83,154,155]. Each strain was analyzed in triplicate and each assay was performed three times. The level of expression of each transcriptional fusion in mutant strains was calculated relative to the expression in wild-type *C. jejuni* 81-176 Sm^R Δ*astA*, which was set to 100 units.

Semi-quantitative real time RT-PCR (qRT-PCR) was performed by extracting total RNA from wild-type and mutant *C. jejuni* 81-176 Sm^R strains with Trizol (Invitrogen) according to manufacturer's instructions. RNA was treated with DNaseI (GenHunter) and diluted to a final concentration of 30 - 50 ng/μl. For qRT-PCR analysis, 2.5 μg RNA was mixed with 0.2 μM forward and reverse primers and 0.1 μl Multiscribe reverse transcriptase along with Sybr green PCR mix (Applied Biosystems). A control sample was prepared by omitting the reverse transcriptase. A 7500 real-time PCR system (Applied Biosystems) was used to perform the reactions with the following conditions: 48 °C for 30 min and 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Detection of *gyrA* or *secD* served as endogenous controls for normalization of results. Relative expression of each gene was calculated using the ΔΔ*CT*

method and reported as the level of expression compared to wild-type *C. jejuni* 81-176 Sm^R, which was set to 1. Each assay was performed in triplicate.

Primer extension analyses

RNA was isolated from wild-type *C. jejuni* 81-176 Sm^R and isogenic mutants. To identify transcriptional start sites for *fliA*, two primers were used: the primers (5'-TGTAAGAAAGCACAAGCTCA-3') and (5'-AAAGGCTTCATCTATACTAA-3') bound 94 bases upstream and 223 bases downstream of the start codon, respectively. For identification of the *ciaI* transcriptional start site, a primer (5'-CATCAAGATCATTTTGTGTG-3') that bound 119 bases downstream from the start codon was used. The primer (5'-AAGTCTTTAAAATACTGAAA-3') was used for analysis of the transcriptional start site of *fedC*, which bound 74 nucleotides downstream of the start codon. The primers were end-labeled with γ^{32}[P]-ATP using polynucleotide kinase from the Excel Cycle-Sequencing kit (Epicentre Tech). The end-labeled primer was then mixed with RNA and Superscript II reverse transcriptase (Invitrogen) to generate labeled cDNA. The cDNA was analyzed on a 6% acrylamide gel by running alongside a sequencing ladder generated using the same end-labeled primer and a plasmid containing each gene. The gel was dried and analyzed using a Storm 820 Phosphorimager according to manufacturer's instructions (Amersham Biosciences).

Motility Assays

C. jejuni strains were suspended from plates in MH broth and diluted to an OD_{600} 0.8. Each bacterial strain was stabbed into semisolid MH motility media containing 0.4% agar using an inoculation needle. The motility of each strain was tested six times. The plates were

incubated for 30 h at 37 °C in microaerobic conditions and the area of the motility zone for each strain was calculated and averaged. For darkfield microscopy, the cultures were further diluted 1:10 in MH broth. Strains were immediately analyzed for motility by applying 3 μl of culture between a glass slide and glass coverslip.

Analysis of *in vitro* growth

Wild-type and mutant *C. jejuni* 81-176 Sm^R strains were suspended from plates in MH broth and diluted to an OD_{600} 0.7 to 1.0. Fifty milliliters of MH broth with TMP were inoculated with 3 ml of each diluted bacterial culture and placed in 500 mL flasks. Flasks were then incubated at 37 °C in microaerobic conditions without shaking for 48 h. OD_{600} readings were taken at time 0, 4, 8, 24, and 48 h. The experiment was repeated three times and the OD_{600} readings at each time point were averaged.

Chick colonization assays

The ability of wild-type or mutant *C. jejuni* 81-176 Sm^R strains to colonize the ceca of chicks after oral inoculation was determined as previously described [35]. Briefly, fertilized chicken eggs (SPAFAS) were incubated for 21 d at 37.8 °C with appropriate humidity and rotation in a Sportsman II model 1502 incubator (Georgia Quail Farms Manufacturing Company). Approximately 12 to 36 h after hatching, chicks were orally infected with 100 μl of PBS or MH broth containing approximately 10^2, 10^4 or 10^6 cfu of a single wild-type *C. jejuni* or mutant strain. To prepare strains for infection, *C. jejuni* strains were suspended from plates after growth at 37 °C in microaerobic conditions and diluted in PBS or MH broth to obtain the appropriate inoculum for oral gavage of chicks. Dilutions of the inocula were spread on MH

agar to determine the number of bacteria in each inoculum. Seven days post-infection, chicks were sacrificed and the cecal contents were recovered and suspended in PBS or MH broth. Serial dilutions were spread on MH agar containing TMP and cefoperazone. Bacteria were grown for 72 h at 37 °C in microaerobic conditions and then counted to determine the cfu per gram of cecal contents.

***In vitro* invasion assays**

Internalization of *C. jejuni* into T84 colonic epithelial cells was assessed using a gentamicin-protection assay. Semi-confluent monolayers of T84 cells (2.5 x 10^5 cells/ml) were seeded in 24-well tissue culture plates in DME/F12 (HyClone) with 5% FBS 24 h before infection. Wild-type and mutant *C. jejuni* 81-176 Sm^R strains were suspended from plates in MH broth to an OD_{600} 0.4 and then diluted 1:10 in MH broth. Prior to infection, media was removed from the T84 cells and 300 μl of tissue culture media were added back to the cells. Monolayers were then infected with 15 μl of each diluted bacterial culture (~3 x 10^6 cfu per monolayer). Each inoculum was diluted and plated on MH agar to verify the actual number of bacteria used to infect each monolayer. Tissue culture plates were then centrifuged for 5 min at 960 rpm at room temperature to enhance contact between *C. jejuni* and colonic epithelial cells. The plates were then incubated for 4 h at 37 °C in a 5% CO_2 incubator. T84 cells were washed three times with PBS and fresh tissue culture media containing 250 μg/ml of gentamicin was added to the monolayer. After a 2 h incubation at 37 °C in 5% CO_2, cells were rinsed three times with PBS. Monolayers were released from the plates with 0.25% trypsin and the cells were disrupted by repeated pipetting. Serial dilutions were then spread on MH agar. After incubation for 72 h at 37 °C in microaerobic conditions, the number of internalized bacteria were determined. Percent

invasion was determined by dividing the number of internalized bacteria by the number of bacteria in the inoculum.

Generation of antisera

Specific antiserum to *C. jejuni* proteins was generated from purified recombinant proteins with N- or C-terminal 6XHis-, maltose-binding protein (MBP)- or glutathione-S-transferase (GST)-fusion. For generation of a N-terminal 6XHis-tag to FspA1, primers containing 5' in-frame BamHI restriction sites to codon 2 and the stop codon were used to amplify *fspA1* from the *C. jejuni* 81-176 genome. The BamHI-digested PCR product was then ligated into BamHI-digested pQE30, generating pABT363. This plasmid was then transformed into *E. coli* XL-1 Blue for protein induction and purification from the soluble fraction with Ni-NTA agarose according to manufacturer's instructions (QIAGEN).

Cjj81176_0996 was amplified from the *C. jejuni* 81-176 genome by PCR with a primer containing a 5' in-frame NdeI restriction site fused to codon 2. The other primer contained a 5' BamHI restriction site and an in-frame 6XHis-tag fused to the last codon of the gene. This PCR fragment was then digested with NdeI and BamHI and ligated into NdeI- and BamHI-digested pT7-7 to create pABT522. This plasmid was then transformed into *E. coli* BL21 (DE3) for protein production and purification from the soluble fraction with Ni-NTA agarose according to manufacturer's instructions (QIAGEN).

For purification of recombinant CiaI, primers containing 5' in-frame BamHI restriction sites were used. In addition, one of the primers contained an in-frame 6XHis-tag to the last codon of *ciaI*. The BamHI-digested PCR product was cloned into pMal-C2x to create pABT613, which encoded a MBP-CiaI-6XHis tag protein. The plasmid was transformed into *E. coli* BL21

(DE3) cells for protein production and purification from the soluble fraction with amylose resin according to manufacturer's instructions (New England Biolabs).

Specific antiserum to *C. jejuni* FlgD was generated from purified recombinant GST-fusion. Primers containing 5' in-frame BamHI restriction sites to codon 58 and codon 225 were used to amplify *flgD* from the *C. jejuni* 81-176 genome. The BamHI-digested PCR product was then ligated into BamHI-digested pGEX-4T-2 (GE Healthcare), generating pDRH2933. The resulting plasmid was then transformed into *E. coli* BL21(DE3) for protein induction and purification from the soluble fraction with glutathione Sepharose 4B according to manufacturer's instructions (GE Healthcare). Purified recombinant proteins were then used to immunize five mice for production of polyclonal antisera (Cocalico Biologicals, Inc).

Analysis of production and secretion of σ^{28}-dependent proteins

C. jejuni strains were suspended from MH agar plates into MH broth to an OD_{600} of 0.6. For each strain, 20 ml of diluted culture were incubated at 37 °C in microaerobic conditions without shaking for 4 h. At the end of the incubation period, final OD_{600} measurements were obtained. For preparation of proteins from whole-cell lysates (WCL), 1 ml of culture for each strain was pelleted in a microcentrifuge at full speed for 3 min, washed once with PBS and resuspended in 25 µl of PBS and 25 µl of 2X SDS-PAGE loading buffer. For recovery of supernatant proteins, the remaining 18 ml of culture were centrifuged for 30 min at 13,000 rpm. The supernatants were recovered and the centrifugation step was repeated twice to ensure removal of all bacteria. Proteins present in the supernatant were precipitated by combining 18 ml of supernatant with 2 ml of trichloroacetic acid (TCA; 10% final concentration) followed by a 30 min incubation on ice. Precipitated proteins were recovered by centrifugation for 15 min at

10,000 rpm. The protein pellets were rinsed with 0.5 ml of cold acetone and dried. Precipitated proteins were resuspended in 20 μl of 1M Tris, pH 8.0 and 20 μl of 2X SDS-PAGE loading buffer.

All protein samples were boiled for 5 min prior to loading on 12.5% SDS-PAGE gels. For wild-type *C. jejuni* and mutant strains WCL, 10 μl of WCL (representing 200 μl of culture) were analyzed for detection of CiaI and RpoA, 15 μl were used for analysis of FspA1 and FlgD, 2μl were used for analysis of FedB and 2 μl of a 1:5 dilution of WCL were used for detection of FlaAB. The volumes of WCL of mutant strains loaded onto gels were normalized based on the final OD_{600} readings of wild-type and mutant strains to ensure analysis of equal amounts of proteins between strains. For TCA precipitated supernatants, 4 μl (for analysis of FlaAB and FedB), 10 μl (for analysis of CiaI and RpoA) or 15 μl (for analysis of FspA1 and FlgD) of precipitated proteins were separated by 12.5% SDS-PAGE. For immunoblot analysis, primary murine antisera was used at the following concentrations to detect proteins: α-FspA1 M140, 1:2000; α-Cjj81176_0996 M151, 1:2000; α-CiaI M154, 1:2000; α-FlgD M92; and α-RpoA M60, 1:2500 [156]. For detection of FedB or FlaAB, polyclonal rabbit antiserum (Dr. Patricia Guerry, Naval Medical Research Center) was used at a dilution of 1:10,000 or 1:22,000 respectively [157]. A 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit antiserum (Bio-Rad) was used as the secondary antibody. For FlaAB analysis, a 1:20,000 dilution of anti-rabbit secondary antibody was used. Immunoblots were developed using the Western Lightning Plus ECL kit (Perkin-Elmer).

Electron microscopy analyses

C. jejuni strains were grown for 16 h then suspended from MH agar plates with PBS and diluted to an OD_{600} of 0.8. One milliliter of the diluted culture was then pelleted for 3 min at full speed in a benchtop microcentrifuge and the pellet was washed twice with PBS. The pellet was resuspended in 2% gluteraldehyde in 0.1 M cacodylate and incubated on ice for 1 h to allow the cells to be fixed. Cells were then stained with 2% uranyl acetate and visualized using a FEI Technai G2 Spirit BioTWIN transmission electron microscope.

Statistical analyses

Tests for statistical significance in gene expression, motility and invasion assays were conducted by using the Student's *t* test (two-tailed distribution with two-sample, equal variance calculations). As indicated in figures or figure legends, statistically-significant differences between relevant strains possessed *P*-values < 0.05. For chick colonization assays, statistical analyses were performed by the Mann-Whitney U test, with statistically-significant differences between wild-type and mutant strains indicated with *P*-values < 0.01 or 0.05.

CHAPTER THREE

Identification and Analysis of Flagellar Co-expressed Determinants (Feds) of *Campylobacter Jejuni* Involved in Colonization[**]

Introduction

To understand factors required by *C. jejuni* to colonize hosts, we previously used a 1-day old chick model of commensalism to identify 29 *C. jejuni* genes necessary for wild-type levels of colonization of the chick ceca [35]. Other studies revealed that *C. jejuni* requires specific transport systems, metabolic pathways, cytochrome *c* peroxidases, protein glycosylation systems, capsular polysaccharide production and fibronectin-binding proteins to promote commensalism [60,75,77,158-161]. An ideal *in vivo* virulence model that mimics *C. jejuni* diarrheal disease in humans remains elusive. Instead, cell culture models of infection are available to assess the ability of *C. jejuni* to invade and survive within human small intestinal or colonic epithelial cells. One transposon mutant screen revealed the importance of oxidative stress resistance and fumarate metabolism in promoting *C. jejuni* entry into or survival within colonic epithelial cells [24].

Flagellar motility is one factor of *C. jejuni* required for both commensal colonization of poultry and infection of human volunteers [35,69,71,131,162]. Furthermore, flagella and flagellar motility are required for interactions with and invasion of human intestinal epithelial cells [25,70,163]. In addition to secreting proteins for flagellar biosynthesis, the *C. jejuni* flagellar type III secretion system (T3SS) has been implicated in secretion of proteins

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required for interactions with eukaryotic cells [136,143,146,148]. Some of these proteins have been annotated as the *Campylobacter* invasion antigens (Cias) [143]. Production of Cia proteins has been reported to be induced by bile salts, with flagellum-dependent secretion of these proteins requiring either a factor produced by intestinal cells or components of serum [144-146]. *C. jejuni* mutants lacking different Cia proteins (CiaB, CiaC, and CiaI) are reduced in their ability to invade or survive within eukaryotic cells [72,143,147]. However, conflicting reports exist on the universal requirement of Cia proteins for cell invasion among different *C. jejuni* strains [24,135]. Other proteins of *C. jejuni*, such as FlaC and the FspA proteins, are secreted by the flagellum but are not dependent on bile salts for expression or serum for secretion. FlaC, a flagellin-like protein involved in motility, is required for full level of invasion of eukaryotic cells [148]. Two related FspA proteins are produced by different *C. jejuni* strains [136]. Of these proteins, FspA2, but not FspA1 promotes apoptosis of eukaryotic cells.

As in many motile bacteria, regulation of flagellar gene expression and biosynthesis is a complex process in *C. jejuni* [93]. Two alternative σ factors in *C. jejuni* control expression of flagellar genes encoding extracytoplasmic components of the flagellar organelle [87]. σ^{54} is required for expression of most flagellar rod and hook genes, whereas σ^{28} is required for expression of *flaA*, encoding the major flagellin, and other filament genes [74,83,131,134]. The flagellar T3SS components (including FlhA, FlhB, FliP, and FliR), the FlgSR two-component regulatory system, and the FlhF GTPase form a regulatory system required to initiate transcription by σ^{54}-RNA polymerase holoenzyme [82,83,130,164,165]. A current model proposes that the FlgS histidine kinase may sense the formation of the MS ring, which encloses the flagellar T3SS, resulting in activation of the FlgR response regulator for σ^{54}-dependent expression of flagellar rod and hook genes [130,164]. The FlhF GTPase is also essential for σ^{54}-

dependent flagellar gene expression, but specific details regarding its requirement are not yet known [82]. Like in other motile bacteria, activity of σ^{28} in *C. jejuni* is repressed by the FlgM anti-σ factor until the flagellar rod and hook are synthesized [131,134]. After rod and hook formation, FlgM is secreted from the cytoplasm to relieve σ^{28} from repression, which results in *flaA* expression necessary for filament synthesis. Additional mechanisms involving FlhF and the putative FlhG ATPase spatially and numerically regulate flagellar biosynthesis so that only one flagellum is produced per bacterial pole [82,84]. These two proteins, along with the flagellar MS ring and switch complex also influence a mechanism to spatially control cell division [84].

A previously reported microarray analysis provided information regarding potential members of the σ^{28} regulon [74]. This analysis not only identified genes encoding proteins known to be involved in motility, but also genes with functions other than in flagellar motility that potentially may be dependent on σ^{28} for expression [74,135]. Genes encoding the FspA proteins described above are dependent on σ^{28} for expression [136]. *Cj0977* (encoded by *Cjj81176_0996* in the *C. jejuni* 81-176 genome; for the remainder of this work, this gene will be referred to as *0996* to be consistent with the annotation of the 81-176 genome) is another member of the σ^{28} regulon initially not thought to be required for motility [135]. However, further analysis revealed that 0996 is required for flagellar motility in liquid media, but not in semi-solid motility agar [24]. This motility defect may explain the reduced invasion and pathogenicity observed with a *C. jejuni 0996* mutant [135].

In this work, we provide an extensive analysis that establishes the σ^{28} regulon of *C. jejuni* and identifies a new class of *C. jejuni* virulence and colonization factors. We identified five σ^{28}-dependent genes (including *ciaI*) that are not required for motility, but are required for wild-type levels of commensal colonization of poultry. In addition to CiaI, we found another σ^{28}-

dependent factor involved in invasion of epithelial cells. Further exploration of CiaI analyzed potential domains for a role in commensal colonization or invasion and revealed an influence of bile salts on translation of a specific *ciaI* mRNA. Due to their co-expression with flagellar genes and their requirement for wild-type levels of colonization, and in some cases virulence, we propose annotating four of these previously uncharacterized proteins as Feds (for flagellar co-expressed determinants). Furthermore, this work establishes the flagellar system of *C. jejuni* as a regulatory system required for expression of genes not only required for motility, but also genes with broader functions than previously realized that include commensalism and virulence.

Results

fliA *expression is partially dependent on the* σ^{54} *regulatory pathway*

A previous analysis of flagellated and aflagellated *C. jejuni* NCTC11168 strains revealed that expression of *fliA*, encoding σ^{28}, was reduced in the aflagellated mutant [74]. In addition, a potential σ^{54}-binding site was identified within *flhG*, which is two genes upstream of *fliA*. Therefore, we hypothesized that *fliA* may be a member of the σ^{54} regulon and examined if factors belonging to the σ^{54} regulatory pathway influence *fliA* expression.

For all analyses in this work, we examined *C. jejuni* 81-176, a strain capable of infecting human volunteers and promoting commensal colonization of the chick cecum [35,69]. We compared expression of the *fliA*::*astA* transcriptional reporter in wild-type *C. jejuni* 81-176 Sm^R Δ*astA* and isogenic mutants lacking σ^{54} (Δ*rpoN*), the FlgSR two-component system (Δ*flgS* or Δ*flgR*), flagellar T3SS components (Δ*flhA* or Δ*flhB*), or the FlhF GTPase (Δ*flhF*). Expression of *fliA*::*astA* was reduced approximately ~25-80% in these mutants relative to wild-type *C. jejuni* (Figure 3), suggesting that *fliA* is partially dependent on the σ^{54} regulatory pathway for

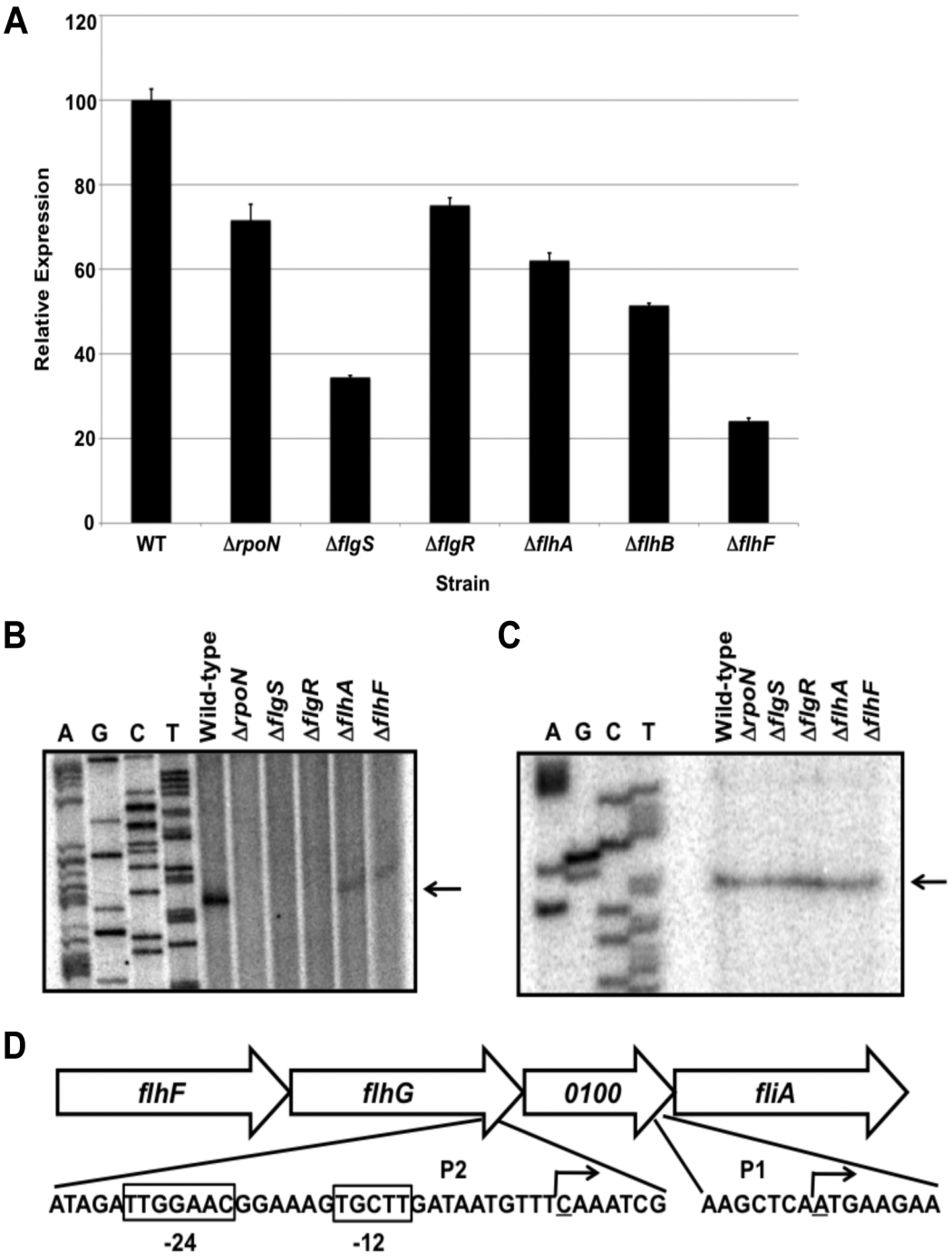
A
120
100
80
60
40
20
0
Relative Expression
WT
ΔrpoN
ΔflgS
ΔflgR
ΔflhA
ΔflhB
ΔflhF
Strain
B
A G C T
Wild-type
ΔrpoN
ΔflgS
ΔflgR
ΔflhA
ΔflhF
C
A G C T
Wild-type
ΔrpoN
ΔflgS
ΔflgR
ΔflhA
ΔflhF
D
flhF
flhG
0100
fliA
P2
P1
ATAGATTGGAACGGAAAGTGCTTGATAATGTTTCAAATCG
AAGCTCAATGAAGAA
-24
-12

Figure 3. Analysis of *fliA* expression and transcriptional start sites in wild-type and mutant *C. jejuni* strains. (A) Arylsulfatase assays measuring expression of a *fliA::astA* transcriptional fusion in wild-type *C. jejuni* 81-176 Sm^R and isogenic mutant strains lacking a component of the σ^{54} regulatory pathway. The amount of *fliA*::*astA* expression in each strain is relative to wild-type *C. jejuni,* which was set to 100 units. Error bars indicate standard error of the average arylsulfatase activity analyzed from three samples. The reporter activity in each mutant was significantly different from the activity in the wild-type strain (*P*-value < 0.05). (B and C) Primer extension analyses to identify transcriptional start sites for *fliA*. Two different primers were used to identify transcriptional start sites dependent (B) and independent (C) of the σ^{54} regulatory pathway. Primer extension reactions were performed with RNA from *C. jejuni* 81-176 Sm^R or isogenic mutant strains lacking a component of the σ^{54} regulatory pathway. Reactions were run alongside and to the right of a sequencing ladder generated with the same primer used in primer extension reactions. Arrows indicate transcriptional start sites. (D) Location of the transcriptional start sites for *fliA*. The transcriptional start site generated from the σ^{54}-dependent promoter (P2) is located within the 3' end of *flhG*. Boxed nucleotides indicate conserved -24 and -12 binding sites for σ^{54}. The transcriptional start site from the σ^{54}-independent promoter (P1) is located immediately upstream of *fliA* and within *Cjj81176_0100*. The underlined nucleotides and bent arrows indicate transcriptional start sites.

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expression. We identified two transcriptional start sites for *fliA* by primer extension analysis (Figures 3B and 3C). The transcriptional start site for the σ^{54}-independent promoter (P1) was located 27 nucleotides upstream of the *fliA* start codon (Figure 3C and 3D). The transcriptional start site for a σ^{54}-dependent promoter (P2) was located 382 nucleotides upstream of *fliA* and within the 3' end of *flhG* (Figure 3B and 3D). Consistent with this observation, a highly conserved σ^{54}-binding site was found directly upstream of the P2 transcriptional start site (Figure 3D).

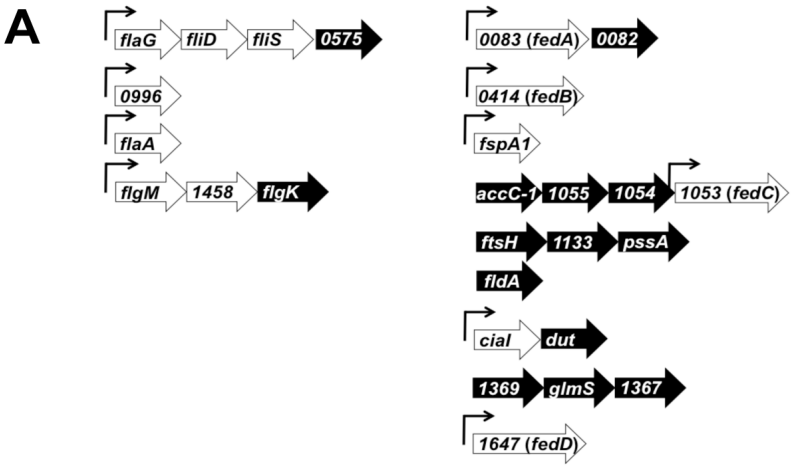
Establishment of the σ^{28} regulon and identification of flagellar co-expressed determinants (Feds)

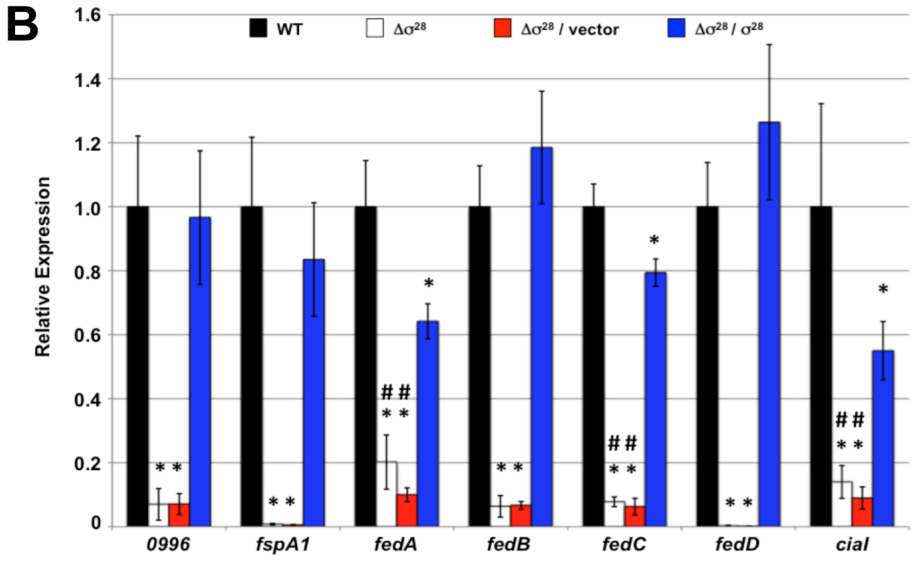
Initial data from a previous microarray analysis suggested involvement of σ^{28} and FlhA, a flagellar T3SS protein and component of the σ^{54} regulatory pathway, in the expression of approximately 30 genes, with some genes encoding proteins not previously associated with a role

in flagellar motility in other motile bacteria [74]. Therefore, we analyzed expression of these genes to determine if they indeed compose part of the *C. jejuni* σ^{28} regulon.

Expression of potential σ^{28}-dependent genes in wild-type *C. jejuni* 81-176 Sm^R and an isogenic Δ*fliA* mutant (for the remainder of this report, *fliA* will be referred to as σ^{28} for clarity and to avoid confusion with *flaA* encoding the major flagellin) was compared by semi-quantitative real-time RT-PCR (qRT-PCR) (Figure 4A). Of 27 genes examined, expression of 12 genes was reduced 4- to over 100-fold in the $\Delta\sigma^{28}$ mutant (Figure 4A and Table 1). Transcription of these genes increased upon complementation with a plasmid expressing σ^{28} *in trans*. In addition, expression of *fliS*, encoding a putative flagellin chaperone, was reduced 25% in the $\Delta\sigma^{28}$ mutant, and complementation with σ^{28} *in trans* resulted in two-fold overexpression of the gene (Table 1). Transcription of the remaining 14 genes was not affected by deletion of σ^{28}, and these genes were not further analyzed.

Of the 13 σ^{28}-dependent genes, seven encode proteins that either have been verified for a role in flagellar motility or are predicted to be involved in motility (Figure 4A). These genes include *flaA* (encoding the major flagellin; [70,71,163]), *flaG* (encoding a filament length control protein; [166]), *fliD* (encoding the putative filament cap; [146,167]), *fliS* (encoding the putative flagellin chaperone; [167]), *flgM* (encoding the anti-σ factor that regulates σ^{28} activity; [83,131,134]), *Cjj81176_1458* (encoding a possible chaperone for flagellar hook-associated proteins), and *0996* (encoding a protein required for motility in low viscosity media; [24]). However, six genes encode proteins with no homology to any known flagellar proteins. These genes and our proposed annotation based on additional findings described below include: *fspA1* [136], *ciaI* [72], *Cjj81176_0083* (*fedA*), *Cjj81176_0414* (*fedB*), *Cjj81176_1053* (*fedC*), and *Cjj81176_1647* (*fedD*) (Figure 4A). Similar to expression of *fspA1*, expression of the *fed* genes


A
flaG
fliD
fliS
0575
0996
flaA
flgM
1458
flgK
0083 (fedA)
0082
0414 (fedB)
fspA1
accC-1
1055
1054
1053 (fedC)
ftsH
1133
pssA
fldA
cial
dut
1369
glmS
1367
1647 (fedD)


B
WT
Δσ28
Δσ28 / vector
Δσ28 / σ28
Relative Expression
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0996
fspA1
fedA
fedB
fedC
fedD
cial

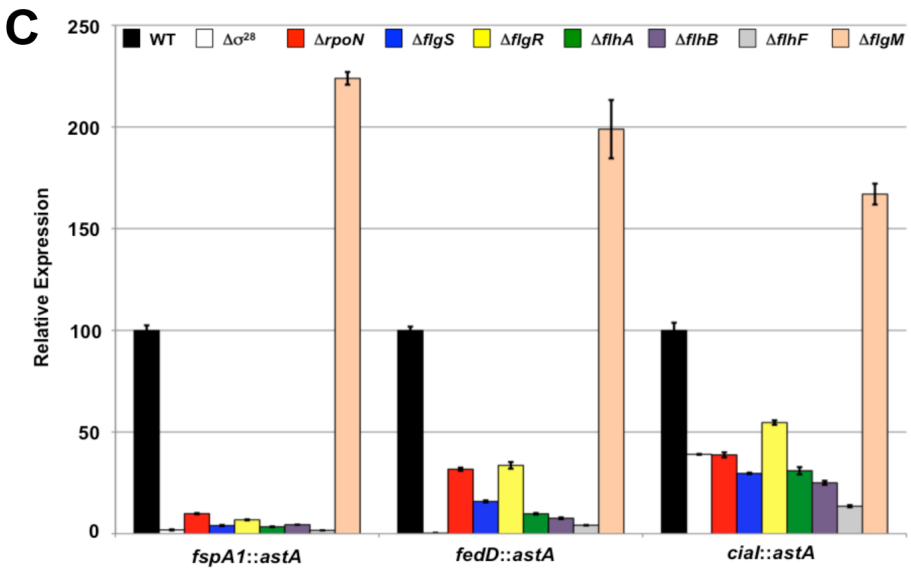

C
WT
Δσ28
ΔrpoN
ΔflgS
ΔflgR
ΔflhA
ΔflhB
ΔflhF
ΔflgM
Relative Expression
250
200
150
100
50
0
fspA1::astA
fedD::astA
cial::astA

Figure 4. Identification and analysis of expression of the *C. jejuni* σ^{28} regulon. (A) Individual genes and operons examined for potential dependence on σ^{28} for expression. Genes and operons were selected for investigation based on a previous microarray analysis that suggested at least partial dependence on σ^{28} and FlhA, a flagellar T3SS protein and component of the σ^{54} regulatory pathway, for expression [74]. Numerical annotation of each gene and location of genes in potential operons are based on the *C. jejuni* 81-176 genome [168]. Genes in the left column are homologous to known flagellar genes or have been shown to be involved in flagellar motility of *C. jejuni*. Genes to the right are not homologous to any known flagellar genes. Genes in white were verified in this work to be at least partially dependent on σ^{28} for expression in *C. jejuni* 81-176 Sm^R. Genes in black were not found to depend on σ^{28} for wild-type levels of expression. Bent arrows indicate location of consensus σ^{28}-binding sites in the promoter for each gene or operon. (B) Semi-quantitative real time RT-PCR (qRT-PCR) analysis of expression of *0996*, *fspA1*, the *fed* genes, and *ciaI* in wild-type *C. jejuni* 81-176 Sm^R (black) and an isogenic $\Delta\sigma^{28}$ mutant (white). Also included for analysis were the $\Delta\sigma^{28}$ mutant strains complemented with vector alone (pECO102; red) or pECO102 expressing σ^{28} *in trans* (blue). Expression of each gene in each mutant is relative to the expression in wild-type *C. jejuni*, which was set to 1. All strains were examined in triplicate and the error bars indicate the standard error. Statistically-significant differences in gene expression between wild-type *C. jejuni* and $\Delta\sigma^{28}$ mutant strains with or without σ^{28} *in trans* are indicated (* *P*-value < 0.05). Statistically-significant differences in gene expression between *C. jejuni* $\Delta\sigma^{28}$ with σ^{28} expressed *in trans* and the $\Delta\sigma^{28}$ mutants alone or with empty vector are indicated (# *P*-value < 0.05). (C) Arylsulfatase assays measuring expression of *fspA1*::*astA*, *fedD*::*astA*, and *ciaI*::*astA* transcriptional fusions in wild-type *C. jejuni* or mutant strains lacking σ^{28}, FlgM, or a component of the σ^{54} regulatory pathway. Strains used in analysis include: wild-type *C. jejuni* 81-176 Sm^R (black) and isogenic mutants lacking σ^{28} (white), *rpoN* (encoding σ^{54}; red), *flgS* (blue), *flgR* (yellow), *flhA* (green), *flhB* (purple), *flhF* (grey), and *flgM* (peach). The amount of expression of the transcriptional reporter in each mutant is relative to wild-type *C. jejuni*, which was set to 100 units. Error bars indicate the standard error of the average arylsulfatase activity analyzed from three samples. The reporter activities in each mutant were significantly different from the activity in the wild-type strain (*P*-value < 0.05).

	Strain			
Gene[a]	Wild-type	$\Delta\sigma^{28}$	$\Delta\sigma^{28}$/ vector	$\Delta\sigma^{28}$/ σ^{28}
0083 (*fedA*)	1.00 ± 0.14[b]	0.20 ± 0.08	0.10 ± 0.02	0.64 ± 0.05
0082	1.00 ± 0.45	0.64 ± 0.14	0.68 ± 0.15	0.78 ± 0.26
0414 (*fedB*)	1.00 ± 0.13	0.06 ± 0.03	0.07 ± 0.01	1.19 ± 0.18
flaG	1.00 ± 0.13	0.28 ± 0.19	0.38 ± 0.16	1.17 ± 0.17
fliD	1.00 ± 0.12	0.27 ± 0.20	0.46 ± 0.18	1.11 ± 0.14
fliS	1.00 ± 0.07	0.77 ± 0.28	1.10 ± 0.70	2.00 ± 0.28
0575	1.00 ± 0.41	1.23 ± 0.13	0.93 ± 0.29	1.33 ± 0.49
0875 (*fspA1*)	1.00 ± 0.22	0.01 ± 0.00	0.01 ± 0.00	0.84 ± 0.18
0996	1.00 ± 0.22	0.07 ± 0.05	0.07 ± 0.04	0.97 ± 0.21
accC-1	1.00 ± 0.23	0.91 ± 0.17	0.78 ± 0.15	0.70 ± 0.15
1055	1.00 ± 0.38	1.68 ± 0.43	1.14 ± 0.91	1.03 ± 0.31
1054	1.00 ± 0.26	0.65 ± 0.47	0.69 ± 0.09	0.61 ± 0.10
1053 (*fedC*)	1.00 ± 0.07	0.08 ± 0.02	0.06 ± 0.03	0.79 ± 0.04
ftsH	1.00 ± 0.08	0.62 ± 0.30	0.68 ± 0.09	1.04 ± 0.09
1133	1.00 ± 0.03	1.26 ± 0.31	1.32 ± 0.36	1.22 ± 0.05
pssA	1.00 ± 0.17	0.80 ± 0.36	0.67 ± 0.03	0.67 ± 0.08
flaA	1.00 ± 0.24	0.01 ± 0.03	0.01 ± 0.01	0.72 ± 0.11
fldA	1.00 ± 0.13	1.14 ± 0.31	0.85 ± 0.17	0.91 ± 0.11
1369	1.00 ± 0.46	1.03 ± 0.30	0.93 ± 0.28	1.04 ± 0.50
glmS	1.00 ± 0.13	0.63 ± 0.15	0.79 ± 0.10	1.29 ± 0.19
1367	1.00 ± 0.21	1.20 ± 0.28	0.75 ± 0.08	1.33 ± 0.26
1443 (*ciaI*)	1.00 ± 0.32	0.14 ± 0.05	0.09 ± 0.03	0.55 ± 0.09
dut	1.00 ± 0.49	0.52 ± 0.16	0.47 ± 0.17	0.67 ± 0.19
flgM	1.00 ± 0.31	0.17 ± 0.06	0.21 ± 0.02	0.73 ± 0.21
1458	1.00 ± 0.46	0.06 ± 0.05	0.22 ± 0.07	0.52 ± 0.11
flgK	1.00 ± 0.55	1.18 ± 0.46	1.80 ± 0.50	0.47 ± 0.12
1647 (*fedD*)	1.00 ± 0.14	<0.01 ± 0.00	<0.01 ± 0.00	1.26 ± 0.24

Table 1. Semi-quantitative reverse transcriptase PCR analysis of expression of potential σ^{28}-dependent genes in wild-type *C. jejuni* 81-176 Sm^R and mutant strains. [a] Genes are listed in order as annotated for the *C. jejuni* 81-176 genome [168]. Genes encoding FspA1, CiaI, or each Fed protein are indicated. [b] The levels of expression of each gene in each strain is relative to wild-type *C. jejuni*, which was set to 1.0. All analyses were performed in triplicate and the standard errors were calculated.

and *ciaI* was reduced 5- to 100-fold in the $\Delta\sigma^{28}$ mutant compared to wild-type *C. jejuni* (Figure 4B and Table 1; [136]). Transcription of these genes was largely restored by complementation with σ^{28} *in trans*, with no statistically-significant differences in transcription of *0996, fspA1*, *fedB*, or *fedD* between wild-type and complemented strains. Although expression of *fedA*, *fedC*, and *ciaI* did not reach wild-type levels upon complementation with σ^{28}, transcription of these genes in the complemented strain was significantly higher than in the $\Delta\sigma^{28}$ mutant with or without the empty vector (Figure 4B).

Considering that the *fed*s, including *fspA1* and *ciaI*, were dependent on σ^{28} for expression, we predicted that expression of these genes would decrease in σ^{54}-regulatory pathway mutants. These mutants have reduced expression of σ^{28} and lack rod and hook biogenesis, which can inhibit residual σ^{28} activity due to cytoplasmic retention of the FlgM anti-σ factor (Figure 3A; [82,83,130,131,134,164,165]). In addition, we predicted that transcription of these genes would increase in a Δ*flgM* mutant, due to derepression of σ^{28} activity [131,134]. As expected, expression of *fspA1*::*astA* and *fedD*::*astA* transcriptional fusions was reduced approximately 3- to 62-fold (Figure 4C). Expression of *ciaI*::*astA* was more modestly reduced about 2- to 4-fold in σ^{54}-regulatory pathway mutants and the $\Delta\sigma^{28}$ mutant (Figure 4C). In addition, expression of these transcriptional fusions increased 67 to 123% in a Δ*flgM* mutant, which possesses augmented σ^{28}-dependent activity due to lack of FlgM-mediated repression (Figure 4C).

We characterized the promoter of *fedC* since a previous bioinformatic analysis did not identify a potential σ^{28}-binding site upstream of this gene [74]. In addition, we characterized the *ciaI* promoter because expression of this gene was not entirely dependent upon σ^{28}. By primer

extension analysis, we identified a single transcriptional start site 61 nucleotides upstream of the *fedC* start codon that was absent in the $\Delta\sigma^{28}$ mutant and restored upon complementation with σ^{28} *in trans* (Figure 5A). Immediately upstream of the σ^{28}-dependent *fedC* transcriptional start site and within the 3' end of the upstream gene *Cjj81176_1054*, a potential σ^{28} consensus-binding site was identified (Figure 5A). Similarly, we identified a σ^{28}-dependent transcriptional start site for *ciaI* 28 nucleotides upstream of the start codon of the gene (Figure 5B). As with the *fedC* promoter, a potential σ^{28}-binding site upstream of this transcriptional start site was identified (Figure 5B; [74]). We also identified one or two other potential transcriptional start sites for *ciaI* further upstream and into the coding sequence of *Cjj81176_1442* that were not dependent on σ^{28} (Figure 5B). These alternative transcriptional start sites may contribute to residual expression of *ciaI* in the $\Delta\sigma^{28}$ mutant (Figure 4B and 4C and Table 1). These data are strong evidence that the *fed* genes and *ciaI* are members of the σ^{28} regulon and dependent on the flagellar regulatory system for expression.

The Fed proteins and CiaI are required for commensal colonization but not for flagellar motility

Although dependent on σ^{28} for expression, the *fed* genes and *ciaI* do not encode proteins with homology to any known motility proteins. FedA is most homologous to single-domain hemerythrins, which are diiron- and oxygen-binding proteins primarily found in anaerobic and microaerobic bacteria and some invertebrates [169]. FedC contains a putative DnaJ-domain, suggesting the protein may be part of the Hsp70 chaperone machinery and involved in protein folding or degradation [170]. FedB and FedD are not homologous to any proteins with known functions.

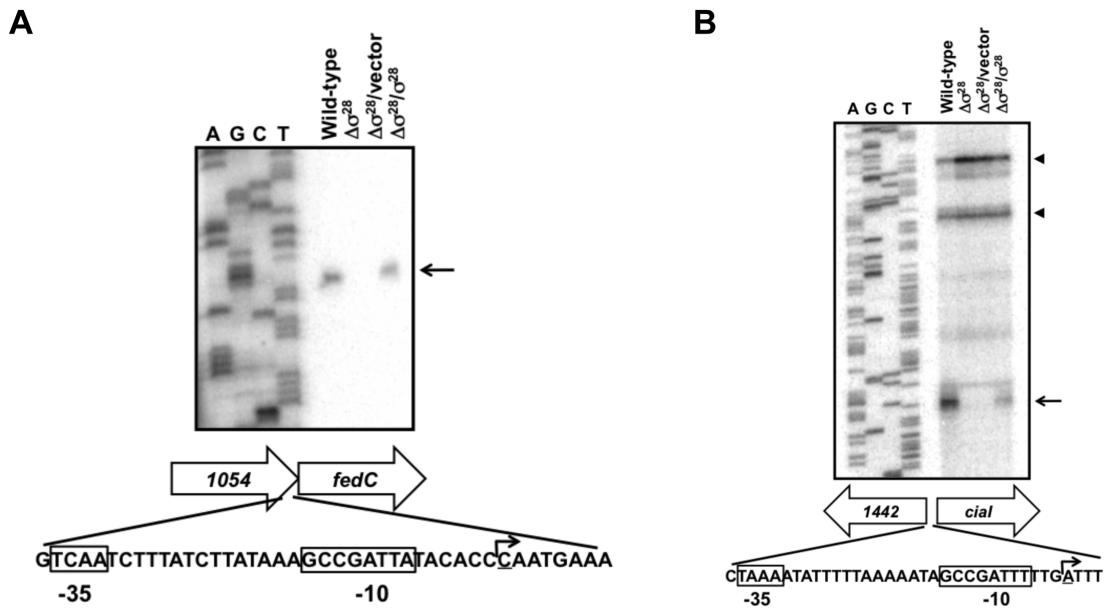


Figure 5. Identification of transcriptional start sites for *fedC* and *ciaI*. Primer extension analyses identified transcriptional start sites for *fedC* (A) and *ciaI* (B). Strains analyzed include wild-type *C. jejuni* 81-176 Sm^R and an isogenic $\Delta\sigma^{28}$ mutant. Additional strains included in analysis were the *C. jejuni* 81-176 $\Delta\sigma^{28}$ mutant complemented with vector alone (pECO102) or pECO102 expressing σ^{28} *in trans*. Primer extension reactions were performed using RNA from wild-type *C. jejuni* and mutant strains. Reactions were run alongside and to the right of a sequencing ladder generated with the same primer used in primer extension reactions. Arrows indicate transcriptional start sites generated from σ^{28}-dependent promoters. Arrowheads include one or two possible transcriptional start sites generated from σ^{28}-independent promoters for *ciaI*. Shown below each gel are the locations of σ^{28}-dependent transcriptional start sites for *fedC* and *ciaI*. Boxed nucleotides indicate conserved -35 and -10 binding sites for σ^{28}. The underlined nucleotides and bent arrows indicate transcriptional start sites.

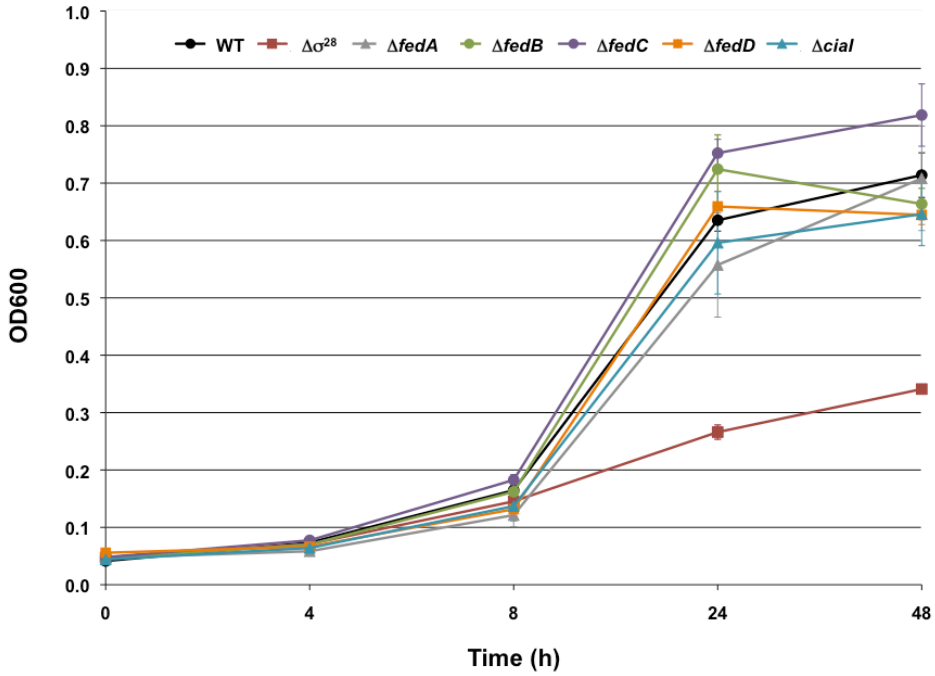


Figure 6. *In vitro* growth analysis of wild-type *C. jejuni* 81-176 Sm^R and mutant strains. Wild-type *C. jejuni* 81-176 Sm^R and isogenic mutant strains were inoculated in MH broth at an OD_{600} of approximately 0.05 and incubated at 37 °C in microaerobic conditions without shaking. OD_{600} readings were obtained at 4, 8, 24, and 48 h post-inoculation. Assays were performed three times and averaged. The error bars indicate standard error.

To characterize a role for the Fed proteins, CiaI, and other σ^{28}-dependent proteins in the biology of *C. jejuni*, we constructed in-frame chromosomal deletions of each gene in *C. jejuni* strain 81-176 Sm^R. All strains grew similarly to wild-type *C. jejuni* in Mueller-Hinton (MH) broth at 37 ºC in microaerobic conditions (Figure 6). We also examined the *C. jejuni* $\Delta\sigma^{28}$ mutant as a control. We noted that the $\Delta\sigma^{28}$ mutant demonstrated a growth defect in standing broth cultures, which may be due to the reduced motility of the mutant (Figure 6 and Figure 7A and 7B). As observed in semi-solid motility agar and in liquid broth by dark-field microscopy, no significant motility defects were observed in any of the Δ*fed* mutants or the Δ*ciaI* mutant (Figure 7A and 7B; data not shown). In fact, deletion of *fedD* and *ciaI* appeared to cause a

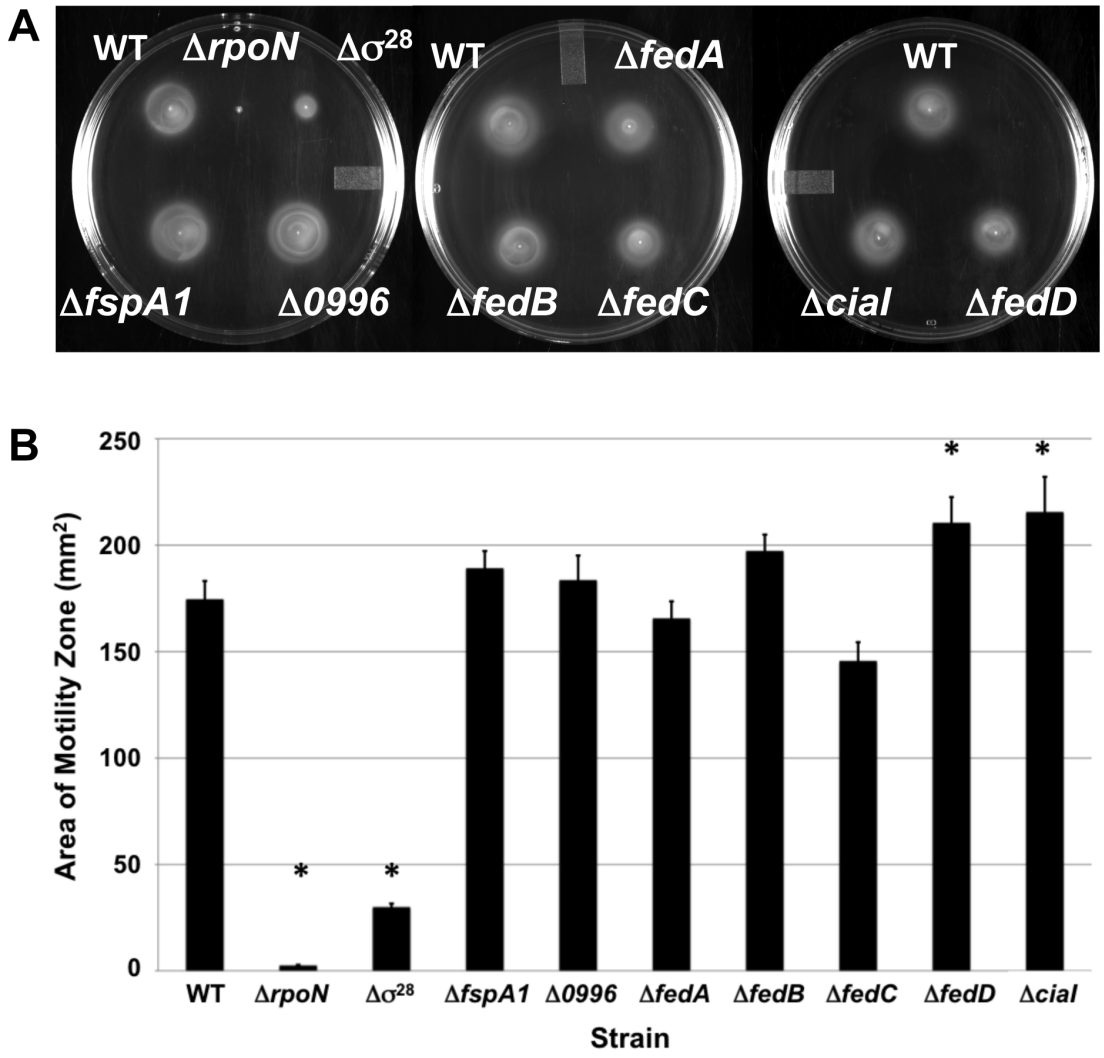


Figure 7. Motility phenotype of mutants lacking σ^{28}-dependent genes. Assays were performed by stabbing cultures of wild-type *C. jejuni* 81-176 Sm^R and isogenic mutants at similar optical densities in MH motility medium containing 0.4% agar. Plates were incubated in microaerobic conditions at 37 °C for 30 h. (A) Motility of wild-type *C. jejuni* and each mutant strain in motility agar. (B) Area of motility zone for each wild-type and mutant *C. jejuni* strains as determined by averaging six assays. Error bars indicate the standard error of the average area. Statistically-significant differences in motility between wild-type *C. jejuni* and mutant strains are indicated (* *P*-value < 0.05).

slight, but statistically significant, increase in motility (Figure 7B). As controls, we examined motility in the Δ*rpoN* mutant, which lacks σ^{54} and expression of many flagellar rod and hook genes, and the $\Delta\sigma^{28}$ mutant, which expresses reduced levels of *flaA* (Table 1). Both mutants were defective for motility in motility agar (Figure 7A and 7B). Similar to previous observations [24], the *C. jejuni* Δ*0996* mutant was non-motile in liquid broth but motile in semi-solid media (Figure 7A and 7B; data not shown).

We previously found that a *C. jejuni* 81-176 Sm^R $\Delta\sigma^{28}$ mutant was defective for commensal colonization of chicks [35]. The colonization defect of this mutant was thought to be due solely to its greatly reduced motility phenotype. However, we considered if the *fed* genes or *ciaI*, which require σ^{28} for expression, may be necessary for colonization and contribute to the reduced colonization capacity of the $\Delta\sigma^{28}$ mutant. For these experiments, we infected 1-day old chicks orally with either 10^4 or 10^2 cfu of wild-type *C. jejuni* or mutants lacking one of the *fed*s or *ciaI*. At seven days post-infection, the levels of wild-type *C. jejuni* in the ceca of chicks given an oral inoculum of 10^4 cfu averaged 2.8 x 10^8 cfu per gram of cecal content (Figure 8A). In contrast, the Δ*fedA*, Δ*fedC*, Δ*fedD*, and Δ*ciaI* mutants colonized at 4- to 16-fold lower levels than the wild-type strain in the ceca of chicks. These were statistically-significant differences (Figure 8A). However, the Δ*fspA1* and the Δ*fedB* mutants did not show a colonization defect when administered at this inoculum. As expected, the Δ*0996* mutant was reduced 42-fold for colonization compared to wild-type *C. jejuni*, which is likely due to its non-motile phenotype under certain conditions. When the inoculum was lowered to 10^2 cfu, all mutants except for the Δ*fspA1* mutant showed statistically-significant colonization defects relative to wild-type *C. jejuni*. These colonization defects ranged from 14-fold lower for the *C. jejuni* Δ*fedB* mutant to over 1,000-fold lower for the Δ*fedD* mutant (Figure 8B). These data indicate that many of the

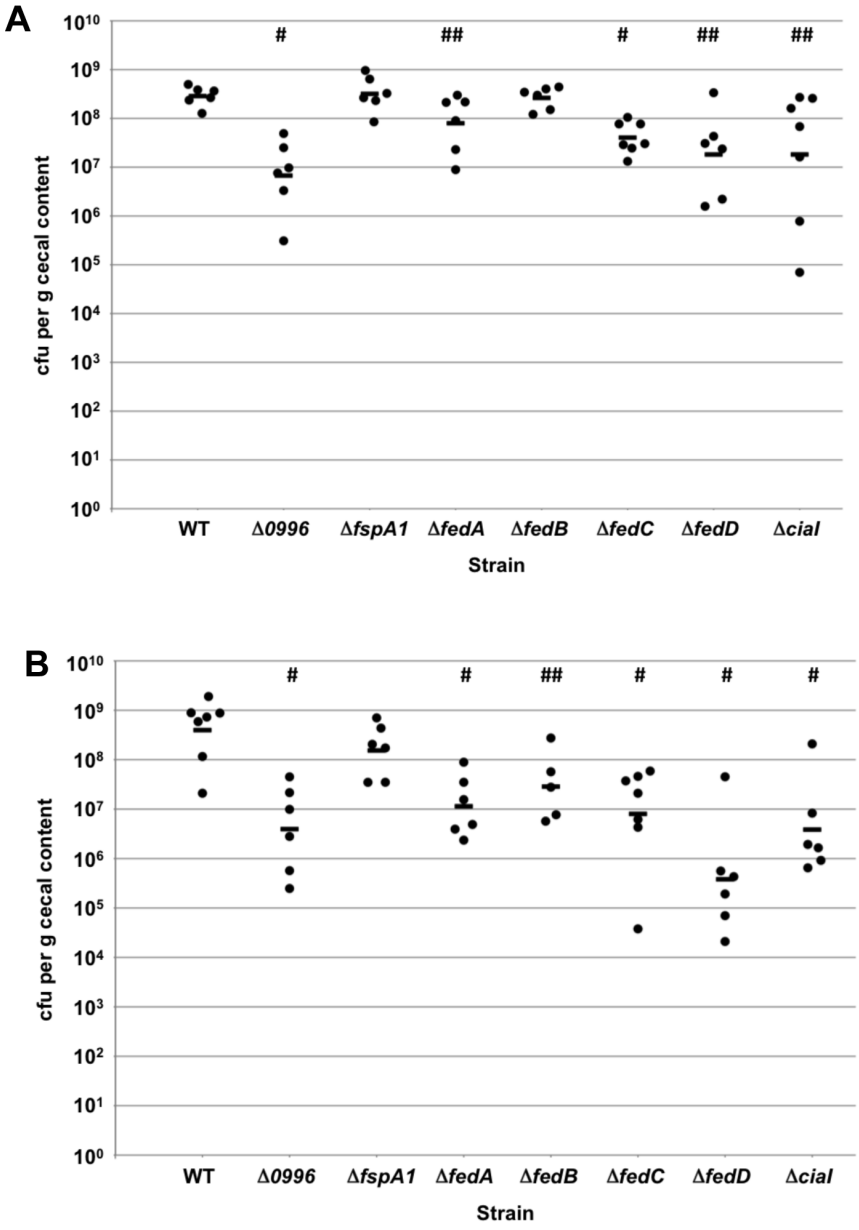


Figure 8. Commensal colonization capacity of wild-type *C. jejuni* and mutant strains. One-day old chicks were orally inoculated with approximately 10^4 (A) or 10^2 (B) cfu of wild-type *C. jejuni* 81-176 Sm^R or isogenic mutant strains. Each dot represents the amount of *C. jejuni* recovered from the ceca of each chick seven days post-infection. The geometrical mean for each group is depicted by the horizontal bar. Statistical analysis was performed using the Mann-Whitney U test (## $P < 0.05$; # $P < 0.01$).

σ^{28}-dependent genes not required for motility are required for optimal commensal colonization of chicks.

FedA is involved in invasion of epithelial cells

Because a *ciaI* mutation in another *C. jejuni* strain was previously shown to possess a two-fold invasion defect for human intestinal epithelial cells [72], we examined the *fed* mutants for defects in invasion of T84 colonic epithelial cells at six hours post-infection by using a standard gentamicin-protection assay. In these assays, approximately 2.2% of the wild-type *C. jejuni* inoculum was found intracellularly at the end of the assay (Table 2). Consistent with previous reports, the Δ*ciaI* and Δ*0996* mutants demonstrated 2- and 10-fold reductions in invasion, respectively [72,135]. Upon examination of each *fed* mutant, only the Δ*fedA* mutant showed a significantly reduced invasion capacity, which was approximately 10-fold lower than the wild-type strain. This invasion defect was similar to the levels of the $\Delta\sigma^{28}$ mutant, which is minimally motile and expresses reduced levels of *fedA* (Figure 4B and Figure 7A and 7B). Thus, in addition to being a determinant for commensal colonization of chicks, FedA is also a virulence determinant required for invasion of human colonic epithelial cells.

A ciaI *transcript is dependent on DOC for translation*

Previous analysis in *C. jejuni* strain F38011 suggested that transcription of *ciaI* and production of Cia proteins are augmented in the presence of the bile salt sodium deoxycholate (DOC) [145]. However, our results presented above indicated that approximately 60 - 85% of *ciaI* transcription originates from the σ^{28}-dependent promoter in *C. jejuni* 81-176 (Figure 4B and Table 1). Therefore, we analyzed if expression of *ciaI* from σ^{28}-independent and -dependent

Strain	Invasion of T84 cells (% inoculum)[a]
Wild-type	2.23 ± 0.28
Δσ^{28}	0.16 ± 0.06*
Δ*0996*	0.20 ± 0.05*
Δ*fspA1*	2.59 ± 0.15
Δ*fedA*	0.22 ± 0.05*
Δ*fedB*	1.80 ± 0.34
Δ*fedC*	2.17 ± 0.38
Δ*fedD*	1.61 ± 0.30
Δ*ciaI*	1.11 ± 0.35*

Table 2. Invasion capacity of wild-type *C. jejuni* 81-176 Sm^R and mutant strains for T84 colonic cells. [a] Percent invasion was determined by comparing the number of intracellular bacteria surviving a 2 h gentamicin treatment of infected T84 cells compared to the number of bacteria in the infecting inoculum (approximately 3.0 x 10^6 cfu). Each assay was performed in triplicate, and at least three biological replicates were performed. The average percent invasion +/- standard error for each strain is presented. Statistically-significant differences in invasion between wild-type *C. jejuni* and mutant strains are indicated (* *P*-value < 0.05).

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promoters in *C. jejuni* 81-176 strains was increased in the presence of 0.1% DOC, a concentration previously shown to be required for CiaI production [72]. When wild-type and Δσ^{28} mutant strains were grown in the presence of DOC, we actually observed a 13 to 30% decrease in *ciaI*::*astA* expression, respectively (Figure 9). Growth in higher concentrations of DOC resulted in similar decreased levels of *ciaI*::*astA* expression (data not shown). Therefore, contrary to a previous report analyzing CiaI production in a different strain of *C. jejuni*, we were unable to link DOC to a mechanism regulating transcription of *ciaI* in *C. jejuni* 81-176 [144].

To determine if production of CiaI was influenced by DOC, proteins from whole-cell lysates of wild-type and Δσ^{28} mutant strains grown on media with and without 0.1% DOC were examined by immunoblot analysis. We observed an increase in CiaI levels in wild-type *C. jejuni* 81-176 Sm^R grown in the presence of DOC (Figure 10A). Furthermore, we discovered almost complete dependence on DOC for CiaI production in the Δσ^{28} mutant. Levels of RpoA, the α

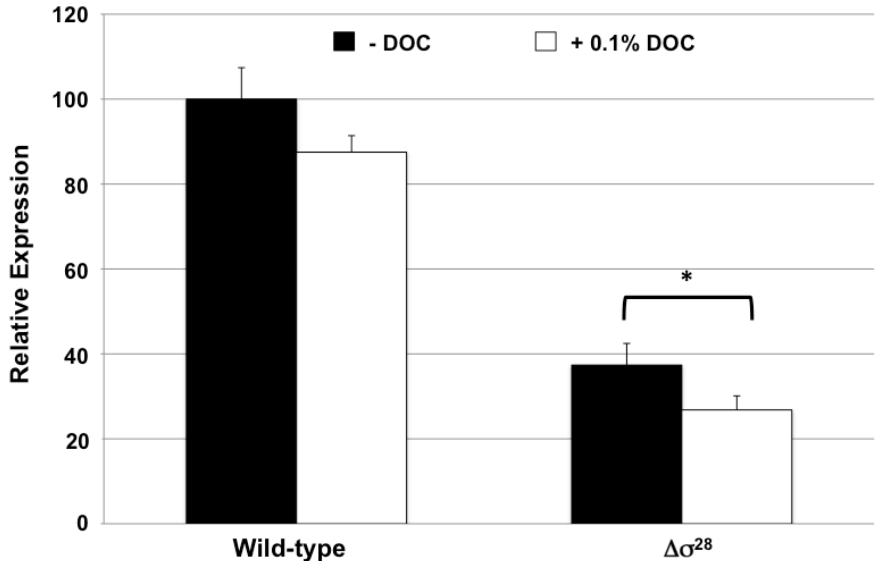

Figure 9. Analysis of expression of *ciaI*::*astA* in wild-type *C. jejuni* 81-176 Sm^R and an isogenic Δσ^{28} mutant grown with or without sodium deoxycholate. The amount of *ciaI*::*astA* expression was determined by performing arylsulfatase assays after growing *C. jejuni* strains in the absence or presence of 0.1% sodium deoxycholate (DOC). The level of expression in each strain is relative to wild-type *C. jejuni* grown in the absence of DOC, which was set to 100 units. Error bars indicate standard error of the average arylsulfatase activity analyzed from three samples. Asterisk indicates a statistically-significant difference (*P*-value < 0.05) between samples.

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component of RNA polymerase, or other σ^{28}-dependent proteins such as FspA1, FedB, and 0996 did not increase with growth in the presence of DOC (Figure 10A and data not shown), suggesting that the effect of DOC was specific for translation of CiaI. These findings suggest a DOC-dependent post-transcriptional mechanism that influences production of CiaI specifically from a σ^{28}-independent transcript.

FedB and CiaI are secreted by the flagellum in the absence of serum

Secretion of CiaI and other Cias from *C. jejuni* strain F38011 was previously found to be dependent on both flagella and serum [72,144]. In contrast, a previous investigation found that

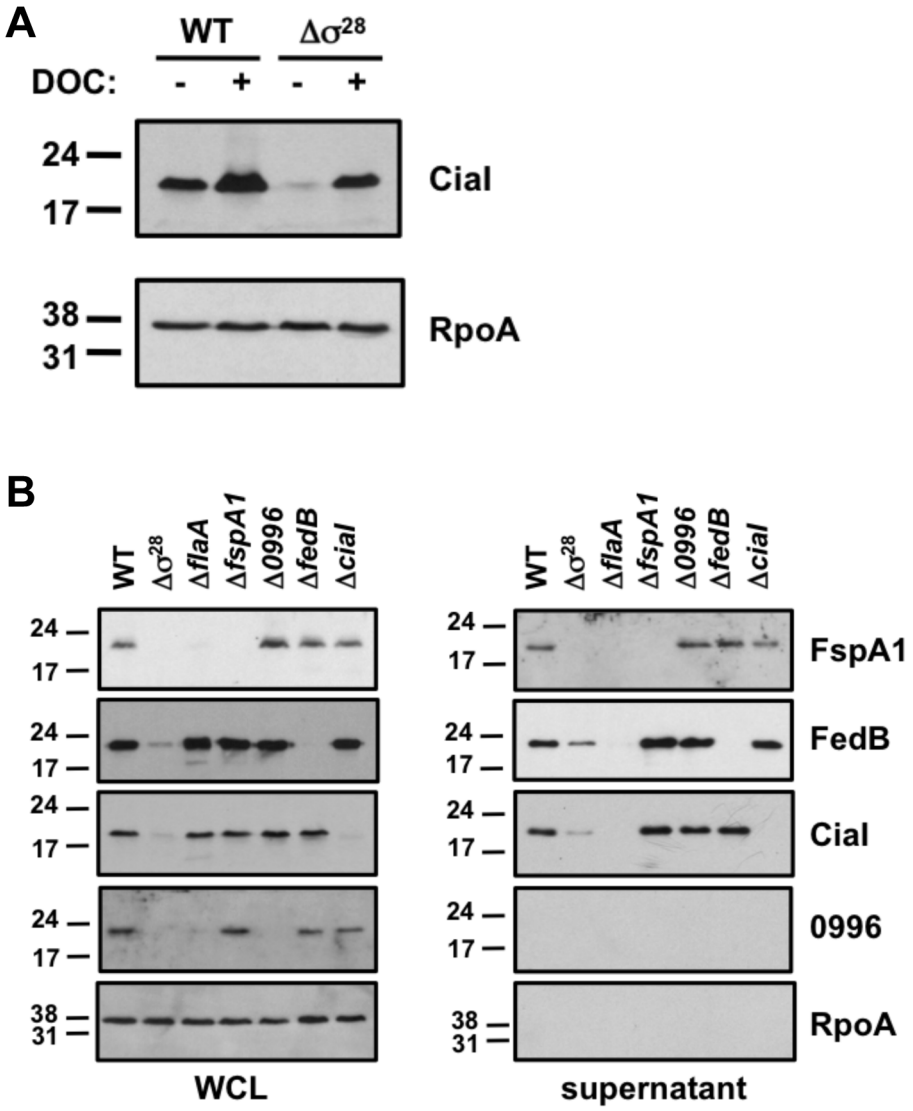


Figure 10. Analysis of production and secretion of CiaI and other σ^{28}-dependent proteins. (A) Production of CiaI in wild-type *C. jejuni* 81-176 Sm^R and an isogenic $\Delta\sigma^{28}$ mutant. Strains were grown on MH agar in the absence (-) or presence (+) of 0.1% sodium deoxycholate (DOC). Equal amount of proteins in whole-cell lysates (WCL) were examined by immunoblot analysis using antiserum against CiaI or RpoA, the α subunit of RNA polymerase. Molecular weight markers are indicated in kDa. (B) Production of σ^{28}-dependent proteins and CiaI in bacterial strains and supernatants after growth in MH broth. Wild-type *C. jejuni* 81-176 Sm^R and isogenic mutants lacking σ^{28}, *flaA* (encoding the major flagellin), *fspA1*, *0996*, *fedB*, and *ciaI* were grown in MH broth for 4 h at 37 °C in microaerobic conditions. WCL and supernatant proteins were recovered and analyzed by immunoblots using antiserum specific for each protein or RpoA. Molecular weight markers are indicated in kDa.

the FspA proteins, which are other σ^{28}-dependent proteins, are secreted by the flagellum of *C. jejuni* 81-176 in the absence of serum [136]. To analyze secretion of FspA1, FedB, CiaI and 0996, we generated or obtained antisera specific for these proteins. We were unable to generate antisera to FedA, FedC, and FedD as these proteins were refractory to purification or antisera generation. In addition, these proteins could not be detected with antiserum specific for a 6XHis tag as addition of this epitope to the N- or C-terminus of these proteins made the proteins unstable in *C. jejuni* (data not shown).

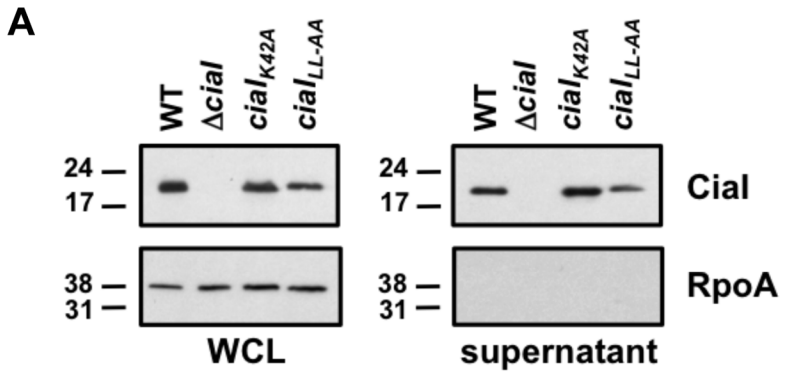
After growth of wild-type and mutant *C. jejuni* strains in MH broth alone, proteins from whole bacteria and supernatants were analyzed. We also analyzed an 81-176 Sm^R Δ*flaA* mutant, which lacks the major flagellin. Secretion of Cia proteins has been shown to be reduced in a *C. jejuni* F38011 *flaA* mutant [146]. As a negative control, we analyzed a $\Delta\sigma^{28}$ mutant, which lacks these proteins or produces the proteins at greatly reduced levels (Figure 10B). As previously reported, FspA1 was secreted from wild-type *C. jejuni* 81-176 Sm^R in MH broth without addition of serum, but not from the Δ*flaA* mutant (Figure 10B; [136]). RpoA was only observed in the whole-cell lysates of these strains, suggesting that our procedures were adequate for recovering secreted proteins. In addition, the 0996 protein remained associated with bacteria as previously reported [135]. We also noted that FspA1 and 0996 were absent in whole-cell lysate of the Δ*flaA* mutant (Figure 10B).

Contrary to a previous report, we observed that CiaI was secreted abundantly in MH broth alone without serum addition (Figure 10B; [72]). Secretion of CiaI was dependent on flagella as this protein was absent from supernatants of the Δ*flaA* mutant. In addition, we found FedB to be secreted in MH broth alone in a flagellum-dependent manner (Figure 10B). In the absence of FlaA and secretion, both CiaI and FedB were stable in *C. jejuni* (Figure 10B). In

addition, deletion of any σ^{28}-dependent gene analyzed with the exception of *flaA* did not impair secretion or stability of other σ^{28}-dependent proteins. These results identified FedB as a new flagellum-dependent secreted protein and suggest that serum-dependent secretion of CiaI is not a universal feature among *C. jejuni* strains. Furthermore, our results suggest that FlaA directly or indirectly plays a role in the production**,** stability, or secretion of σ^{28}-dependent proteins.

The potential nucleotide-binding domain of CiaI, but not the dileucine motif, mildly influences colonization and invasion

A previous study indicated that CiaI contains a dileucine motif that may be important for *C. jejuni* to promote invasion of eukaryotic cells [72]. This domain has been suggested to function as an endosomal-targeting motif. Analysis of CiaI-GFP ectopically produced in HeLa cells revealed a punctate distribution, but a GFP fusion to CiaI with a mutated dileucine motif appeared diffuse [72]. Together, these data suggested that the dileucine motif of CiaI may be important in localizing the protein to endosomal vesicles and influencing the biology or survival of intracellular *C. jejuni*. However, the effect of CiaI with a mutation of the dileucine motif on invasion when produced from *C. jejuni* was not examined [72]. In addition, bioinformatic analysis indicates that CiaI may also contain a putative ATP- or GTP-binding motif. We tested if either of these domains are required for CiaI to function as a commensal colonization factor or a virulence determinant for *C. jejuni*. Therefore, we replaced wild-type *ciaI* on the chromosome of *C. jejuni* 81-176 Sm^R with $ciaI_{K42A}$, which is predicted to disrupt the nucleotide-binding motif, or $ciaI_{LL153\text{-}154AA}$, which disrupts the dileucine motif. We attempted to analyze potential ATP- or GTP-binding activity or hydrolysis with purified wild-type CiaI, but the recombinant protein did not show either activity *in vitro* (data not shown).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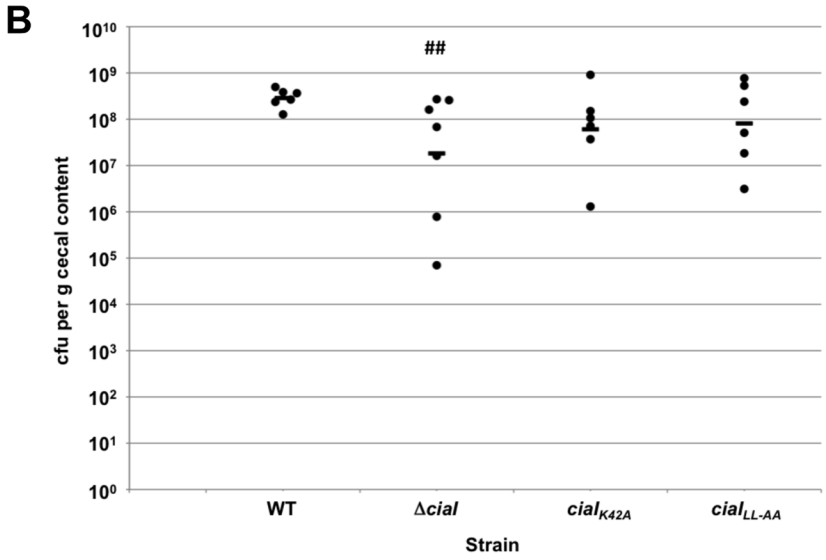
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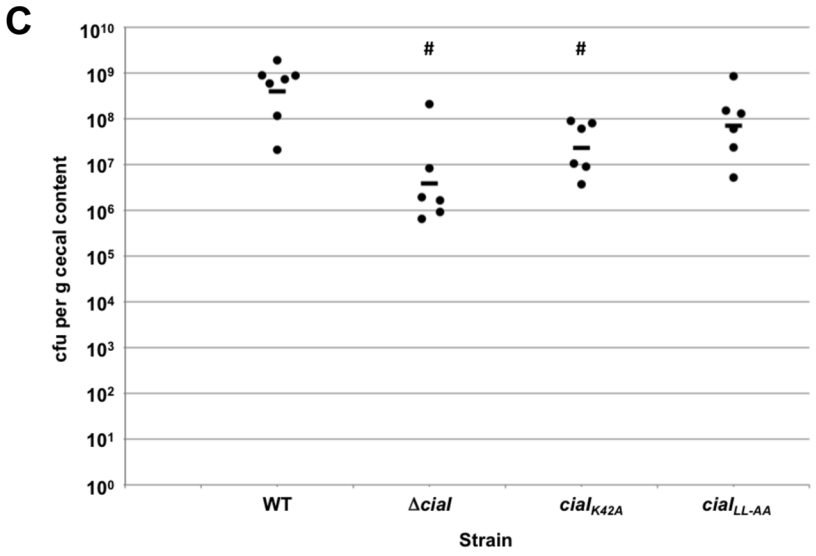
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Figure 11. Analysis of the commensal colonization capacity of *C. jejuni ciaI* mutants. (A) Production of CiaI proteins in bacteria and supernatants after growth in MH broth. Wild-type *C. jejuni* 81-176 Sm^R and isogenic mutants lacking *ciaI*, or containing $ciaI_{K42A}$ or $ciaI_{LL153\text{-}153AA}$ ($ciaI_{LL\text{-}AA}$) were inoculated in MH broth and grown for 4 h at 37 °C in microaerobic conditions. Proteins from whole-cell lysates (WCL) and supernatants were recovered and examined by immunoblot analysis using CiaI or RpoA specific antiserum. For both WCL and supernatants, equal amount of proteins were loaded across strains. Molecular weight markers are indicated in kDa. (B and C) Commensal colonization capacity of wild-type *C. jejuni* 81-176 Sm^R and *ciaI* mutants. One-day old chicks were orally inoculated with 10^4 cfu (B) or 10^2 cfu (C) of *C. jejuni* strains. Each dot represents the amount of *C. jejuni* recovered from the ceca of each chick seven days post-infection. The geometrical mean for each group is depicted by the horizontal bar. Statistical analysis was performed using the Mann-Whitney U test (## $P < 0.05$; # $P < 0.01$). For wild-type *C. jejuni* and the Δ*ciaI* mutant, the same data is shown as in Figure 8A and 8B.

Immunoblot analysis revealed that the mutant proteins were produced and secreted from *C. jejuni*, with perhaps a slight decrease in production or stability of the $CiaI_{LL153\text{-}154AA}$ protein (Figure 11A). Both mutants demonstrated a 4- to 5-fold decreased colonization capacity for chicks relative to wild-type *C. jejuni* 81-176 Sm^R when administered at an inoculum of 10^4 cfu (Figure 11B). However, these decreases were not significant compared to the level of colonization promoted by the wild-type strain. When administered at an inoculum of 10^2 cfu, the $ciaI_{K42A}$ mutant demonstrated a 17-fold decrease in commensal colonization, which was a statistically significant difference from wild-type *C. jejuni* (Figure 11C). The $ciaI_{LL153\text{-}154AA}$ mutant showed only a 6-fold decrease in colonization when administered at an inoculum of 10^2 cfu, which was not statistically significant. Neither mutant was as defective for commensal colonization as the Δ*ciaI* mutant at either inoculum.

We also tested the *C. jejuni ciaI* mutants for invasion of T84 cells. Whereas the Δ*ciaI* mutant was reduced approximately two-fold for invasion, the invasion capacity of the $ciaI_{K42A}$ mutant was reduced only about 30%, which was not statistically significant (Table 3). Despite a previous report attributing the CiaI dileucine motif as being important for vesicular localization and possibly invasion, we did not detect a significant invasion defect of the *C. jejuni* $ciaI_{LL153\text{-}}$

Strain	Invasion of T84 cells (% inoculum)[a]
Wild-type	2.08 ± 0.22
Δ*ciaI*	1.28 ± 0.13*
ciaI K42A	1.44 ± 0.27
ciaI LL153-154AA	1.74 ± 0.18

Table 3. Invasion capacity of wild-type *C. jejuni* 81-176 Sm^R and *ciaI* mutant strains for T84 colonic cells. [a] Percent invasion was determined by comparing the number of intracellular bacteria surviving a 2 h gentamicin treatment of infected T84 cells compared to the number of bacteria in the infecting inoculum (approximately 3.0 x 10^6 cfu). Each assay was performed in triplicate, and at least three biological replicates were performed. The average percent invasion +/- standard error for each strain is presented. Statistically-significant differences in invasion between wild-type *C. jejuni* and mutant strains are indicated (* *P*-value < 0.05).

154AA mutant *in vitro*. These results suggest that the dileucine motif is likely not important for CiaI to promote invasion of *C. jejuni*. Instead, our results suggest that the putative nucleotide-binding domain of CiaI may have a limited role in commensal colonization or invasion by *C. jejuni*.

Discussion

Flagella and flagellar motility are well-established virulence and colonization factors of *C. jejuni* required for infection of humans to promote diarrheal disease and natural commensal colonization of avian species [35,69,71,131,162]. Flagella not only provide chemotactic motility required for bacterial migration to proper replicative niches in hosts, but also likely facilitate initial contact of *C. jejuni* with human intestinal and colonic epithelial cells for subsequent invasion [25,70,163]. Furthermore, the flagellum and its secretory system have been implicated in secretion of some proteins that are not required for motility, including the Cia proteins, FspA

proteins, and FlaC, which may modulate either interactions with or the biology of eukaryotic cells [136,146,148].

The intricate regulatory pathway that governs σ^{54}-dependent gene expression in *C. jejuni* involves the flagellar T3SS, the FlgSR two-component system, and the FlhF GTPase [82,83,130,131,164]. Because the σ^{54} regulon largely includes flagellar rod and hook genes [165], disruption of the σ^{54} regulatory pathway results in an aflagellated and non-motile phenotype, which has consequences for the ability of *C. jejuni* to promote commensalism or disease in multiple hosts. In this work, we established a more direct role for the flagellar regulatory system in commensalism and pathogenesis of disease. We found that *fliA*, encoding σ^{28}, is a member of the σ^{54} regulon of *C. jejuni* 81-176. Furthermore, in addition to σ^{28} being required for expression of flagellin and other filament genes, we discovered that σ^{28} is directly involved in expression of *ciaI* and four genes we annotated as *fed*s. We found that CiaI and each Fed protein are required for wild-type levels of commensal colonization of poultry, and that CiaI and FedA are involved in invasion of colonic epithelial cells. Because expression of the *fed*s and *ciaI* is largely dependent on σ^{28} and the σ^{54} regulatory pathway, our results increase the number of genes whose expression is influenced by the flagellar regulatory system to include those involved in motility and also genes directly involved in commensalism and invasion.

Our findings indicate that a substantial proportion of genes within the *C. jejuni* σ^{28} regulon have functions other than in flagellar motility. Previous analysis revealed that *C. jejuni fspA1* is a σ^{28}-dependent gene, but a mutant lacking *fspA1* did not demonstrate a noticeable *in vitro* motility defect [136]. Similarly, we did not detect any *in vitro* motility defects in mutants lacking CiaI or any Fed proteins. Instead, we found these proteins are required for wild-type levels of commensal colonization of the chick ceca. Mutants lacking a single Fed protein or CiaI

demonstrated 4- to over a 1000-fold commensal colonization defects. Furthermore, we confirmed the previously noted modest invasion defect of the Δ*ciaI* mutant and found that the Δ*fedA* mutant possessed a 10-fold defect in invasion of T84 colonic cells [72]. The only σ^{28}-dependent factor not found to be involved in motility, commensalism, or invasion was FspA1.

Through our analysis, we propose that the Feds and CiaI are a new collection of colonization and virulence factors co-expressed with flagella. Currently, we do not know how CiaI or each Fed functions for *C. jejuni in vivo* to promote optimal levels of commensal colonization of chicks. FedA, which we found to be involved in both commensalism and invasion, shares most homology to hemerythrins. These proteins are found mainly in anaerobic and microaerobic bacteria and some invertebrates, but the biological function of many bacterial hemerythrins is unknown [169]. FedA contains a conserved domain that has been shown in a few characterized bacterial hemerythrins to bind iron and oxygen [171,172]. As such, hemerythrins of anaerobes and microaerobes are predicted to function in biological processes involving iron or oxygen. Thus, we hypothesize that FedA may be involved in one or more iron- or oxygen-dependent activities for *C. jejuni* during commensalism or invasion. FedC contains a C-terminal region with homology to a DnaJ domain. Proteins with DnaJ domains often interact with DnaK or similar proteins to serve as a co-chaperone in the Hsp70 chaperone machine, which assists in protein folding or degradation in bacteria [170]. Mutants lacking the DnaJ-like co-chaperone component of an Hsp70 chaperone system are often sensitive to thermal or oxidative stress. Due to the increased body temperature of avian species compared to humans (42 °C versus 37 °C), one possibility is that FedC may be required for folding or maintaining stability of one or more specific proteins essential for colonization of poultry. FedB and FedD do not share homology with any proteins of known function. Although we have ruled out *in*

vitro motility defects of mutants lacking the Fed proteins, it is possible that the mutants possess motility defects in certain *in vivo* settings.

Our work uncovered some significant findings for CiaI that differed from prior investigations regarding its expression, production, and importance in *C. jejuni* biology. Previous work suggested that transcription of *ciaI* and production of the encoded protein are induced by DOC in *C. jejuni* strain F38011 [72,145]. Furthermore, secretion of CiaI was previously determined to be both flagellum- and serum-dependent [72]. A defect in invasion of human intestinal epithelial cells of a *ciaI* mutant was hypothesized to be due to CiaI localizing to and influencing development of *C. jejuni*-containing vacuoles for intracellular survival [72]. Ectopic production of CiaI in HeLa cells suggested that a dileucine motif in CiaI is essential for the protein to localize to vesicles and perhaps *C. jejuni*-containing vacuoles.

In this study, we discovered specific details regarding the regulated transcription and production of CiaI. First, we found that σ^{28} and the flagellar regulatory system are responsible for approximately 60 - 85% of the transcription of *ciaI* in *C. jejuni* 81-176. Consistent with this finding, we identified a σ^{28}-dependent transcriptional start site and at least one possible σ^{28}-independent transcriptional start site. Expression from neither promoter was induced upon growth in the presence of DOC, which contradicts a previous study that indicated *ciaI* transcription was increased when *C. jejuni* strain F38011 was grown with DOC [144]. Instead, we discovered that translation of CiaI was induced by DOC specifically from the σ^{28}-independent transcript. These results suggest that a majority of CiaI is dependent on σ^{28} and the flagellar regulatory system for production, but not DOC. In addition, residual CiaI is produced by a DOC-dependent mechanism that influences translation of a *ciaI* mRNA that originates independently of σ^{28} and the flagellar regulatory system. It is interesting to speculate that DOC-

dependent production of CiaI may be important for the bacterium *in vivo* when the flagellar regulatory cascade may be inactive and not promote expression of the σ^{28} regulon. Whether translation, rather than transcription of other Cia proteins, such as CiaB and CiaC, is induced by DOC remains to be determined. In additional analysis, we did not observe DOC to be required for production of any other σ^{28}-dependent protein. Consistent with previous analysis of secretion of FspA1, we found that CiaI and FedB also did not require serum to be secreted in a flagellum-dependent manner in *C. jejuni* 81-176 [136]. These results suggest that serum is not universally required for flagellum-dependent secretion among *C. jejuni* strains.

Our work also provides new insights into the role of CiaI in the biology of *C. jejuni*. We found that CiaI is a commensal colonization determinant as the Δ*ciaI* mutant displayed 16- to over a 100-fold defect in cecal colonization of chicks. We were able to verify a modest, two-fold defect at an early step in invasion previously reported for the Δ*ciaI* mutant in a different strain of *C. jejuni* [72]. However, the $ciaI_{LL153\text{-}154AA}$ mutant did not demonstrate an invasion defect at 6 h post-infection, which suggests that the dileucine motif of CiaI previously determined to be required for vesicular-localization of the protein may not influence invasion. In addition, alteration of the dileucine motif did not influence the ability of *C. jejuni* to promote commensal colonization of chicks. We did note a modest invasion and colonization defect when the putative nucleotide-binding motif of CiaI was altered, but these defects of the $ciaI_{K42A}$ mutant were not as severe as the Δ*ciaI* mutant. As a note, we were unable to verify an *in vitro* nucleotide-binding or -hydrolysis activity of recombinant CiaI. Even though CiaI appears to be involved in invasion of human intestinal cells, we believe that CiaI is likely required for a different function in commensalism. During colonization of chicks, *C. jejuni* primarily localizes to the mucosa layer atop the cecal and intestinal epithelium, with little invasion of epithelial cells evident [6]. As a

commensal in the intestinal tract of the natural avian host, we do not expect CiaI to be influencing an invasion mechanism for *C. jejuni*. The role of CiaI in colonization of poultry remains to be fully elucidated.

We also noticed a curious requirement for FlaA, the major flagellin of the flagellar filament of *C. jejuni*, in the production, stability or secretion of multiple proteins encoded by σ^{28}-dependent genes. We found that both CiaI and FedB are dependent on FlaA for secretion. In the absence of FlaA, both proteins remain stably associated with the bacterium. We also found that FspA1 requires FlaA for either production or stability. As a result, FspA1 is not found in the whole-cell lysate or in the secreted protein fraction in the absence of FlaA. Lastly, the 0996 protein, which we and others did not find to be secreted [135], requires FlaA for either production or stability in *C. jejuni*. These results suggest an additional function of FlaA outside of its role as a flagellin composing the filament for flagellar motility. Currently, it is unknown if the effect of FlaA on these proteins is direct or indirect. For instance, it is possible that FlaA may need to be secreted first to alter the flagellar secretory system for secretion of other σ^{28}-dependent proteins. Alternatively, these proteins may directly complex with FlaA for increased stability or secretion or FlaA may be involved in a mechanism influencing translation of the proteins. It is intriguing to speculate that in addition to being a secreted flagellin, FlaA may have some chaperone activity for other σ^{28}-dependent proteins. This latter possibility may provide a reason why these proteins are part of the σ^{28} regulon and co-expressed with FlaA.

Previous work from our group identified a requirement of flagellar components for proper spatial regulation of division [84]. In this work, we continued to expand our understanding of the requirements of flagella and the flagellar regulatory system in the biology of *C. jejuni*. This study demonstrates that the flagellar regulatory system of *C. jejuni* is directly

required for expression of genes essential for broad biological functions, such as motility, commensalism and virulence. Furthermore, we established the Fed proteins and CiaI as a new class of colonization factors co-expressed with flagella. Due to the dependence of many of these proteins on FlaA for stability or secretion, our findings suggest a possible new function for the major flagellin in *C. jejuni*. Continued exploration will undoubtedly further contribute to our understanding of the global requirement of flagella for many diverse aspects of *C. jejuni* biology.

CHAPTER FOUR

Characterization of Intra- and Intermolecular Requirements for Fed Secretion and the Role of Feds Upon Secretion

Introduction

In humans, infection with *C. jejuni* results in a mild, watery to bloody diarrheal disease. These symptoms are thought to be the result of the ability of this bacterium to adhere to and invade epithelial cells lining the intestinal tract of its host [173]. Invasion of the intestinal epithelium also elicits host responses that contribute to the inflammatory enteritis that is characteristic of disease [173]. Many studies have identified requirements of *C. jejuni* for infection of human and avian hosts and adherence to and invasion of human epithelial cells. One determinant of *C. jejuni* required for infection of human volunteers and avian hosts is flagellar motility [35,69,71,131,162]. *C. jejuni* produces a single flagellum at each pole to promote motility. The motility imparted by the flagellum is essential for wild-type levels of commensal colonization of the chick intestinal tract and invasion of human colonic and intestinal epithelial cells [25,35,70,163].

In addition, the *C. jejuni* flagellum also serves as a secretion system for two groups of proteins that have been implicated in commensal colonization of chickens or invasion of human intestinal epithelial cells. Some members of the Fed and Cia family of proteins are secreted in a flagellar-dependent manner [72,73,143,146,147,174]. The Feds are six proteins whose expression is controlled by the flagellar regulatory cascade and σ^{28} [174]. As such, Fed proteins are co-expressed with σ^{28}-dependent flagellar proteins such as the FlaA major flagellin

and other filament proteins, but the Feds are not required for motility. The Feds include FedA (a putative bacterial hemerythrin), FedB, FedC (a DnaJ-domain containing protein), FedD, FspA1, and CiaI. FedA, FedB, FedC, FedD, and CiaI are required for wild-type levels of commensal colonization of chicks with respective mutants attenuated 4- to 1000-fold for colonization [174]. The remaining Fed, protein FspaA1, is not required for infection of chicks, but the related protein FspA2 produced by some *C. jejuni* strains promotes apoptosis of eukaryotic cells [136,174]. In addition to CiaI and FspA1, we discovered that FedB is secreted in a flagellar-dependent manner [72,136,174].

The Cia proteins have been implicated in *C. jejuni* invasion of human intestinal epithelial cells and survival within these cells [72,143,146,147]. Although the mechanisms are unclear, expression of the Cia proteins is induced in the presence of bile salts and their secretion via the flagellum requires serum or contact with eukaryotic cells [144-146]. It has been suggested that at least eight Cia proteins may exist, but only three Cia proteins have been identified and characterized so far, CiaB, CiaC, and CiaI [72,73,143,147]. These proteins are required for wild-type levels of invasion of intestinal epithelial cells or intracellular survival within these cells. CiaB itself is secreted by the flagellum, but it is also required for the flagellar-dependent secretion of other Cia proteins [143]. Although the role that CiaC performs during invasion is unknown, it is believed that CiaI is required to prevent fusion of the *Campylobacter*-containing vacuole to a lysosome to assist in intracellular survival [72]. Consistent with this observation, transfection of GFP-CiaI into host epithelial cells resulted in localization with late endosomal vesicles. A dileucine motif in the chimeric protein was thought to mediate this vesicular localization. However, a *C. jejuni ciaI* mutant with an altered dileucine motif was not impaired for invasion, which calls into question the mechanism by which CiaI assists in intracellular

survival after invasion [174]. Another conflicting issue with CiaI is that the production of the protein was originally reported to be induced by deoxycholate similar to other Cia proteins [72]. However, we found that CiaI is also a member of the σ^{28} regulon and a Fed that is co-expressed with flagella [174].

A recent study using chimeric proteins composed of CiaC and CiaI fused to an adenylate cyclase domain showed delivery of the chimeras to the cytoplasm of intestinal epithelial cells via flagella [73]. Secretion of Cia proteins requires an N-terminal domain of 36 residues within the proteins and a flagellar structure consisting of at least a flagellar hook and hook-filament junction complex [73,147]. Furthermore, whereas the Cia proteins require serum or epithelial cell contact to be secreted, the Feds do not [143,145]. Although a flagellum is known to be required for secretion of the Feds, the minimal flagellar structure required for secretion is not known. In addition, intermolecular requirements for secretion of the Feds such as possible chaperones or other factors and any intramolecular domains within the Feds that facilitate secretion via the flagellum are unknown. Because the Fed proteins are co-expressed with the FlaA major flagellin, which is one of the most abundant proteins in *C. jejuni*, secretion of FedB, CiaI, and FspA1 must have a means to be secreted efficiently by the flagellum in the presence of an abundance of FlaA (estimated at 10,000 – 20,000 proteins per flagellum). These factors may include a secretion signal within the Feds and/or a specific chaperone. Many minor flagellar proteins require chaperones to be secreted in a T3SS-dependent manner [119,123]. These chaperones bind their secretion substrates to prevent their premature polymerization which would hinder secretion, or to facilitate delivery of the substrates to the T3SS so that these minor proteins can compete with other proteins for efficient secretion [118,119,124,175,176].

While mechanisms by which flagellar proteins are secreted via the flagellar T3SS have been characterized, it is unclear how a flagellum secretes proteins not involved in motility. Furthermore, it is unclear whether mechanisms to secrete these proteins differ from those to secrete native flagellar substrates. In this work, we characterized the secretion of Fed proteins to: 1) understand requirements of the flagellum for secretion of a non-flagellar protein; 2) identify inter- and intramolecular determinants used by non-flagellar proteins to promote specific secretion via the flagellar T3SS; 3) determine whether the Fed proteins compete with flagellar proteins (specifically the FlaA and FlaB flagellins) for secretion; 4) identify any regulatory determinants for secretion of the Feds; and 5) determine if secretion of FedB and CiaI is required for commensal colonization of chicks (and invasion for CiaI). This information will provide a better understanding of how these proteins promote infection of hosts. By pursuing these goals, we found that FedB and CiaI both possessed N-terminal and C-terminal domains that influenced secretion. However, mutation of all known flagellar chaperones of *C. jejuni* did not directly impair secretion of FedB, CiaI, or FspA1. We found that FedB and CiaI are secreted quite efficiently by the flagellum and the presence of flagellins did not influence secretion, indicating that FedB and CiaI do not appear to be in competition with flagellins for secretion. Furthermore, we found that FedB, CiaI, and FspA1 are secreted during hook biosynthesis and a major factor controlling the level of Fed secretion is flagellar filament polymerization upon hook completion. Thus, we found that filament biosynthesis regulates secretion of the Fed proteins, establishing a temporal control of Fed secretion that is dependent on the length of the extracellular flagellar structure that is constructed. Lastly, we found that secretion of CiaI was not required for invasion, which contradicts a previous study suggesting that CiaI localizes to vesicles to inhibit lysosomal fusion with the CCV. Furthermore, secretion of FedB, but not CiaI, was required for

optimal colonization of chicks. Our work provides new insights into the flagellum as a secretion apparatus for virulence and colonization determinants and provides new information regarding requirements of these proteins for *C. jejuni* infection of hosts.

Results

Intramolecular requirements for secretion of FedB and CiaI

Many secreted flagellar proteins contain an intramolecular determinant that is recognized by the flagellar T3SS for translocation into the central channel within the flagellum for eventual transit to the growing tip of the flagellar organelle [112,177-179]. For example, flagellins possess an N-terminal domain that is recognized by the T3SS and a C-terminal domain that is bound by the FliS chaperone to prevent premature polymerization of flagellins in the cytoplasm prior to secretion [112,113,124,175,177]. Proteins that are minor components of the flagellum, such as hook-filament junction proteins and the filament cap, rely on a cognate chaperone to bind to the C-terminal regions of these proteins and target them to the flagellar T3SS to increase their efficiency of secretion [118,119,180].

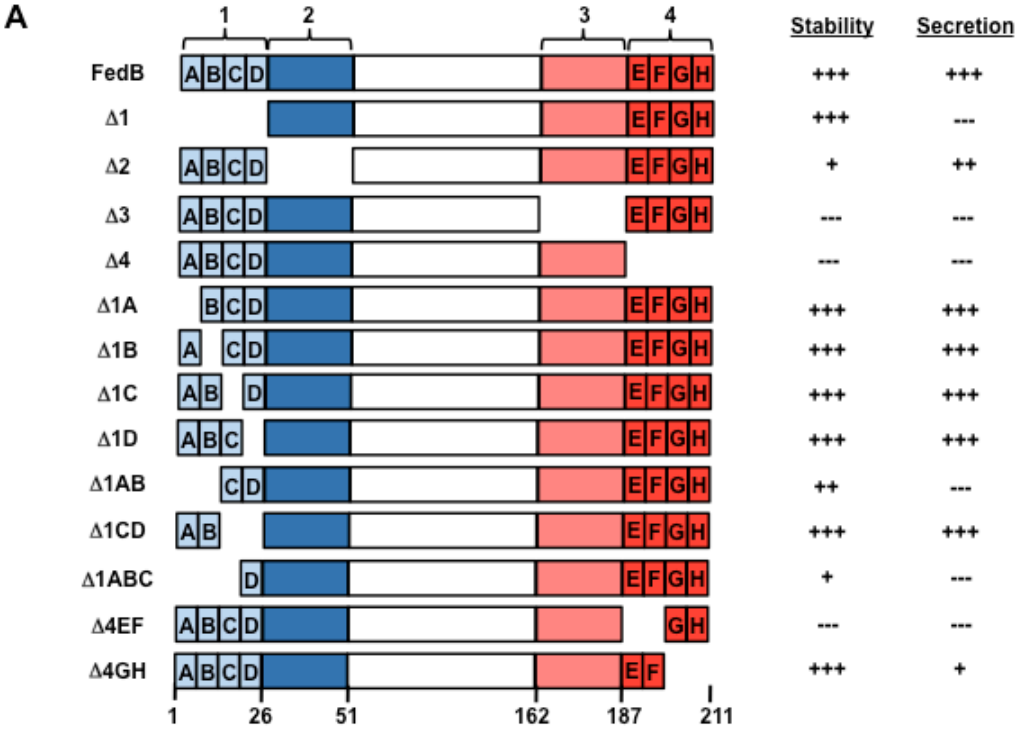
Based on this knowledge, we deleted N- and C-terminal domains of FedB and CiaI to determine whether these regions are required for flagellum-dependent secretion. For these assays, we divided the N- and C-terminal regions into 25-amino acid domains (e.g., domain 1 extends from residues 2-26; domain 2 extends from residues 27-51; domain 3 contains the C-terminal 26-51 residues; and domain 4 contains the last 25 residues of the proteins; Figure 12A and 13A). Domains 1 and 4 of FedB and CiaI were further divided into subdomains (e.g, subdomain 1A-D and 4E-H) each containing six or seven amino acids. Mutant genes encoding

the various domain deletions of FedB and CiaI were constructed and expressed *in trans* from native promoters from *C. jejuni* 81-176 Δ*fedB* or Δ*ciaI* (Figure 12A and 13A).

In analyzing FedB, we discovered that deletion of domain 1 (FedBΔ1) resulted in a stable protein that was not secreted by *C. jejuni* (Figure 12A and 12B). In contrast, FedB lacking domain 2 (FedBΔ2) was produced less abundantly than wild-type FedB, but the protein was still secreted, indicating that domain 2 was not required for secretion. Smaller deletions within domain 1 of FedB revealed that removal of residues 2-14 (FedBΔ1AB) or residues 2-20 (FedBΔ1ABC) abolished secretion (Figure 12A and 12B). However, deletion of only domain 1A or 1B from FedB did not affect secretion. These results indicate that an intramolecular determinant required for secretion is largely contained within the N-terminal 14 amino acids of FedB. In these assays, we did not detect secretion of the cytoplasmic RNA polymerase subunit A (RpoA) protein in the supernatant, which verified that our procedures were adequate for analyzing secreted proteins rather than proteins from lysed cells.

Many FedB mutant proteins lacking various regions of the C-terminus were unstable (e.g., FedBΔ3, FedBΔ4, FedBΔ4EF; Figure 12A, 12B, and data not shown), which hindered our analysis and interpretation whether these domains were required for secretion. We were able to create a stable FedB mutant by deleting the last 12 residues of FedB (FedBΔ4GH). Secretion of this protein was reduced compared to wild-type FedB (Figure 12A and 12B), suggesting that this C-terminal region of FedB influences secretion of the protein to a certain extent.

Intramolecular secretion determinants required for secretion of CiaI were located in similar regions of the protein as in FedB. Deletion of domain 1 from CiaI (CiaIΔ1) abolished secretion of the protein (Figure 13A and 13B). However, deletion of each subdomain A-D individually within domain 1 did not hinder secretion of CiaI. We were unable to create a stable


A
1
2
3
4
Stability
Secretion
FedB
Δ1
Δ2
Δ3
Δ4
Δ1A
Δ1B
Δ1C
Δ1D
Δ1AB
Δ1CD
Δ1ABC
Δ4EF
Δ4GH
+++
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1
26
51
162
187
211

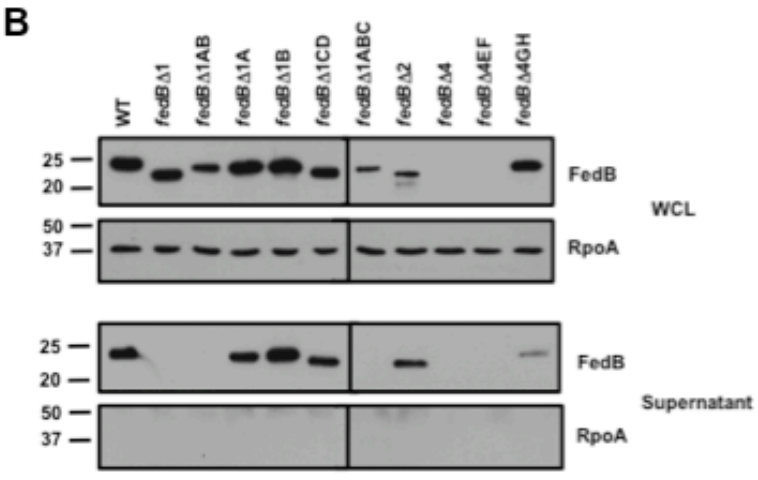
B
WT
fedBΔ1
fedBΔ1AB
fedBΔ1A
fedBΔ1B
fedBΔ1CD
fedBΔ1ABC
fedBΔ2
fedBΔ4
fedBΔ4EF
fedBΔ4GH
25
20
50
37
FedB
WCL
RpoA
25
20
50
37
FedB
Supernatant
RpoA

Figure 12. Identification of intramolecular domains of FedB required for secretion. (A) Domains and subdomains of *C. jejuni* FedB and analysis of their involvement in stability and secretion of the protein. The N- and C-terminal domains of FedB were divided into regions of 25 amino acids in length. FedB N-terminal domains are indicated in light blue (domain 1) or dark blue (domain 2). FedB C-terminal domains are indicated in pink (domain 3) or red (domain 4). Domains 1 and 4 were further divided into subdomains A-D or E-H, respectively, that were each six or seven amino acids in length. Each mutant protein that was constructed and analyzed is shown. A summary of the level of stability and production of wild-type and mutant FedB proteins as assessed qualitatively by immunoblots is shown on the right. The level of each protein produced and secreted was assessed on a scale relative to wild-type FedB, which was set to "+++". (B) Immunoblot analysis of wild-type and FedB mutant proteins in *C. jejuni* whole-cell lysates (WCL) and supernatants after growth in MH broth. Wild-type and FedB mutant proteins were expressed from the native promoter *in trans* in *C. jejuni* 81-176 Δ*fedB*. All strains were grown in MH broth for 4 h at 37 °C in microaerobic conditions. WCL and supernatant proteins were recovered and analyzed by immunoblotting with antiserum specific for FedB or RpoA, which served as a control for a cytoplasmic protein. Molecular weight markers are indicated in kDa.

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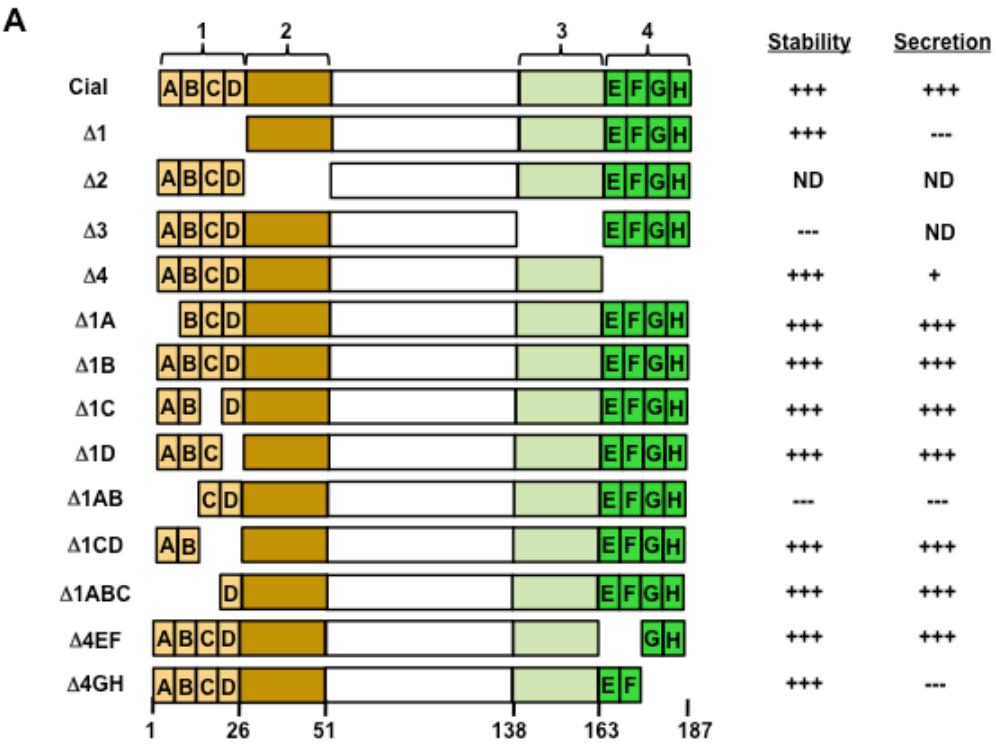
protein by deleting subdomains 1A and 1B that we could confidently analyze for secretion, but deletion of subdomains 1C and 1D together did not affect secretion of CiaI (Figure 13A and 13B; data not shown). Curiously, CiaIΔCD migrated at slightly higher mobility than wild-type CiaI, which counters what would be predicted for a truncated protein. This observation suggests a possible gross disruption of the protein structure by deleting these domains to cause the aberrant migration. Taken together, we discovered that a region within residues 2-26 of CiaI was required for secretion, perhaps excluding residues 15-26 that are encompassed by domains 1C and 1D. When we examined the role of C-terminal CiaI domains for secretion, we were only able to create a stable protein by deleting domain 4 (CiaIΔ4), which removes the C-terminal 25 residues (Figure 13A and data not shown). CiaIΔ4 was secreted at much lesser quantities than wild-type CiaI (Figure 13B). Deletion of smaller subdomains within domain 4 revealed that CiaIΔ4EF was secreted, but CiaIΔ4GH was not (Figure 13B), indicating that the C-terminal 12 residues of CiaI are required for secretion. As expected RpoA was not secreted in any of the *C. jejuni* strains expressing *ciaI* mutants (Figure 13A and 13B).


A
1
2
3
4
Stability
Secretion
CialABCDEFGH +++ +++
Δ1EFGH +++ ---
Δ2ABCDEFGH ND ND
Δ3ABCD--- ND
Δ4ABCDEFGH +++ +
Δ1ABCDEFGH +++ +++
Δ1BABCDEFGH +++ +++
Δ1CABDEFGH +++ +++
Δ1DABCEFGH +++ +++
Δ1ABCDEFGH --- ---
Δ1CDABEFGH +++ +++
Δ1ABCDEFGH +++ +++
Δ4EFABCDGH +++ +++
Δ4GHABCDEF +++ ---
1
26
51
138
163
187

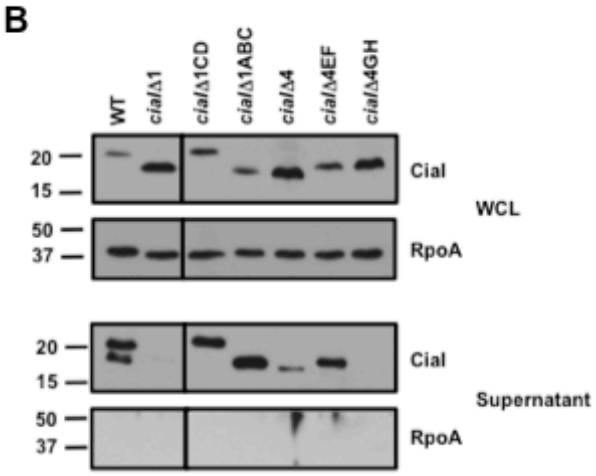

B
WT
cial/Δ1
cial/Δ1CD
cial/Δ1ABC
cial/Δ4
cial/Δ4EF
cial/Δ4GH
20
15
50
37
Cial
WCL
RpoA
Cial
Supernatant
RpoA

Figure 13. Identification of intramolecular domains of CiaI required for secretion. (A) Domains and subdomains of *C. jejuni* CiaI and analysis of their involvement in stability and secretion of the protein. The N- and C-terminal domains of CiaI were divided into regions of 25 amino acids in length. CiaI N-terminal domains are indicated in light yellow (domain 1) or dark yellow (domain 2). CiaI C-terminal domains are indicated in light green (domain 3) or dark green (domain 4). Domains 1 and 4 were further divided into subdomains A-D or E-H, respectively, that were each six to seven amino acids in length. Each mutant protein that was constructed and analyzed is shown, except where indicated by "ND" (not determined). The level of each protein produced and secreted was assessed on a scale relative to wild-type CiaI, which was set to "+++". (B) Immunoblot analysis of wild-type and CiaI mutant proteins in *C. jejuni* WCL and supernatants after growth in MH broth. Wild-type and CiaI mutant proteins were expressed from the native promoter *in trans* in *C. jejuni* 81-176 Δ*ciaI*. All strains were grown in MH broth for 4 h at 37 °C in microaerobic conditions. WCL and supernatant proteins were recovered and analyzed by immunoblotting with antiserum specific for CiaI or RpoA, which served as a control for a cytoplasmic protein. Molecular weight markers are indicated in kDa.

N-terminal regions of FedB and CiaI are sufficient to promote secretion of a C. jejuni *flagellin mutant*

We next determined if N-terminal regions of FedB and CiaI were sufficient to mediate the secretion of a protein via the flagellum. We first attempted to create chimeric proteins by fusing the N-terminal 26 residues of FedB ($FedB_{N'26}$) or CiaI ($CiaI_{N'26}$) to Cjj81176_0996 (0996). ORF *0996* requires σ^{28} for expression and the encoded protein is required for motility in liquid medium, but is not secreted and remains associated with *C. jejuni* [24,135,174]. However, $FedB_{N'26}$-0996 or $CiaI_{N'26}$-0996 chimeras expressed in a *Δ0996* background were not secreted and remained associated with *C. jejuni* (data not shown). We suspect that the native folding of 0996 likely hindered the flagellum-dependent secretion of the chimeras since flagellar proteins that are secreted by the flagellar T3SS are predicted to remain in a semi-unfolded state during transit through the flagellar structure.

To circumvent this problem, we fused $FedB_{N'26}$ and $CiaI_{N'26}$ to the *C. jejuni* FlaA flagellin, which is normally secreted through the flagellum. We expressed wild-type FlaA and FlaA lacking the initial 36 amino acids ($FlaA_{\Delta N'36}$) from the native *flaA* promoter *in cis* by

inserting these constructs in *rdxA* in the chromosome of a *C. jejuni* 81-176 *flaA flaB* double mutant that lacks flagellins. A previous study of *C. jejuni* FlaA flagellin identified an intramolecular secretion determinant within the initial 36 residues of FlaA [112]. Wild-type FlaA expressed *in cis* in the Δ*flaA* Δ*flaB* mutant was secreted at similar levels as FlaA in wild-type *C. jejuni*, but $FlaA_{\Delta N'36}$ was not (Figure 14). When we examined strains producing $FedB_{N'26}$-$FlaA_{\Delta N'36}$ or $CiaI_{N'26}$-$FlaA_{\Delta N'36}$, we noticed that the cellular levels of these chimeras were lower than wild-type FlaA (Figure 14). However, these proteins were clearly secreted from *C. jejuni*, demonstrating that the N-terminal regions of FedB and CiaI were sufficient to restore secretion to $FlaA_{\Delta N'36}$ lacking its native secretion signal (Figure 14). These results verify that the N-terminal domains of FedB and CiaI contain a determinant to mediate secretion via the *C. jejuni* flagellum.

Analysis of putative C. jejuni *flagellar chaperones in secretion of the Fed proteins*

As discussed above, minor flagellar proteins that compose the hook-filament junction and the filament cap possess a C-terminal domain that is bound by a specific chaperone to enhance secretion of these proteins via the flagellar T3SS [118,119]. Because we observed some involvement of the C-terminal regions of FedB and CiaI in the secretion of these proteins, we assessed if any putative flagellar chaperones of *C. jejuni* are directly required for FedB, CiaI or FspA1 secretion. Although much information is available regarding how flagellins and other flagellar proteins are secreted during *E. coli* and *Salmonella* flagellar biosynthesis, little is known regarding the requirements for secretion of *C. jejuni* flagellar proteins. Therefore, we took this

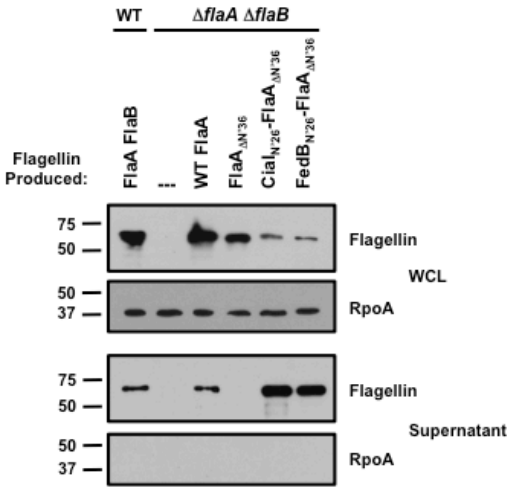


Figure 14. Secretion of chimeric proteins containing N-terminal regions of FedB and CiaI fused to a secretion-deficient FlaA mutant. Immunoblot analysis of wild-type and chimeric FlaA proteins in whole-cell lysates or supernatant fractions. Wild-type and *C. jejuni* Δ*flaA* Δ*flaB* mutant strains expressing $FedB_{N'26}$- and $CiaI_{N'26}$-$FlaA_{\Delta N'36}$ chimeric proteins from the *rdxA* locus were grown in MH broth for 4 h at 37 °C in microaerobic conditions. The wild-type *C. jejuni* 81-176 Sm^R strain produces both the FlaA and FlaB flagellins, which both react with the flagellin antiserum (lane 1). Wild-type and chimeric FlaA proteins were expressed from the native *flaA* promoter. Whole-cell lysates (WCL) and supernatant proteins were recovered and analyzed by immunoblotting with antisera specific for flagellin or RpoA, which served as a control for a cytoplasmic protein. Molecular weight markers are indicated in kDa.

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opportunity to also learn more regarding intermolecular requirements for secretion of *C. jejuni* flagellar proteins.

The flagellar chaperones and substrates of well characterized bacterial flagellar systems include FlgN (a chaperone for the hook-filament junction proteins FlgK and FlgL), FliT (a chaperone for the FliD filament cap protein), and FliS (a chaperone for flagellins; Table 4) [118,119,124,175]. The FliJ protein was once thought to be a chaperone for rod and hook proteins. Early studies showed that FliJ could interact with both rod and hook proteins to prevent their intracellular aggregation [90,115]. However, recent investigations revealed that FliJ docks to the flagellar T3SS and is involved in the recycling of free FlgN and FliT

Flagellar Chaperones[a]	Function	Reference[b]
FliJ	Recycle unbound flagellar chaperones	[116]
Cjj81176_1458	Putative chaperone for FlgK and FlgL	[118,119]
FliS	Chaperone for flagellins	[124,125]
FliW	Putative chaperone for flagellins	[126]

Flagellar Structural Protein[a]	Function	Reference[b]
FlgG	Distal rod	[181,182]
FlgE	Hook subunits	[181,182]
FlgK	Distal hook-filament junction	[181,183,184]
FliD	Filament cap	[123,185]
FlaAB	Major and minor flagellins	[108,186]

Table 4. List of *C. jejuni* flagellar chaperones and flagellar structural components analyzed for a role in secretion of Feds. [a] Subset of flagellar structural proteins and known flagellar chaperones with homologues in *C. jejuni*. [b] References describe identification, localization or function of genes encoding flagellar chaperones or flagellar proteins.

chaperones to assist them in binding free cognate substrates for secretion [116]. We constructed *C. jejuni* 81-176 mutants lacking *fliS*, *fliJ*, and ORF *Cjj81176_1458 (Cjj1458;* a putative *flgN* homologue). Moreover, we were unable to identify an obvious *fliT* homologue encoded within the *C. jejuni* genome. We also analyzed a *C. jejuni fliW* mutant, which has been proposed to encode a chaperone that stabilizes flagellins in *Treponema* [126]. However, in *B. subtilis,* FliW was shown to be involved in the translational control of the Hag flagellin [128,129]. The role of FliW in *C. jejuni* biology has not been characterized. It has only been shown that a transposon insertion in *fliW* results in a reduction of *C. jejuni* motility [167]. In addition, because CiaB has been shown to be required for the flagellum-dependent secretion of some Cia proteins [143], we analyzed a *C. jejuni ciaB* mutant to determine if the respective protein was required for secretion of Fed proteins.

For analysis of secretion of flagellar and Fed proteins in *C. jejuni* 81-176 chaperone mutants, we examined the presence of three secreted Fed proteins (FedB, CiaI, and FspA1) and three flagellar proteins (the FlaA major flagellin, FlaB minor flagellin, and the FlgD hook cap) in culture supernatants of wild-type *C. jejuni* and isogenic chaperone mutants. In the whole-cell lysates of wild-type *C. jejuni* and all mutants, FedB, CiaI, and FspA1 were expressed at comparable levels. The only exceptions were modest reductions in FedB and FspA1 levels in *C. jejuni* Δ*fliS* and Δ*fliW* (Figure 15). We also observed that the flagellins (FlaA and FlaB) and FlgD were produced near wild-type levels in all mutants. In a culture supernatant of the Δ*fliJ* mutant, no flagellar or Fed proteins were present (Figure 15B), which is similar to a gross defect in flagellar protein secretion seen in *fliJ* mutants of other motile bacteria [115]. Examination of *C. jejuni* Δ*fliJ* by electron microscopy revealed a complete lack of a flagellar hook on the bacterial surface, which is consistent with the lack of secretion of the FlgD hook cap necessary for hook biosynthesis (Figure 15B and 15C). Thus, FliJ is likely indirectly required for secretion of the Fed proteins, by being necessary for at least flagellar hook biosynthesis.

Cjj1458 is a predicted FlgN homologue involved in transport the FlgL and FlgK hook-junction proteins that structurally transition the nascent flagellum from hook to filament biosynthesis. Consistent with this prediction, electron microscopy revealed that *C. jejuni* Δ*Cjj1458* only produced hook structures that lack filaments (Figure 15C). In this mutant, all Feds and flagellar proteins were detected in the supernatant fraction at higher levels compared to a wild-type strain (Figure 15B). Only flagellar hooks without filaments were also produced by the Δ*fliS* and Δ*fliW* mutants (Figure 15C), which is reflected in a possible role of the respective proteins as flagellin chaperones. Consistent with this observation, very little to no flagellin was present in the supernatants (Figure 15B). However, FliS and FliW were not required for

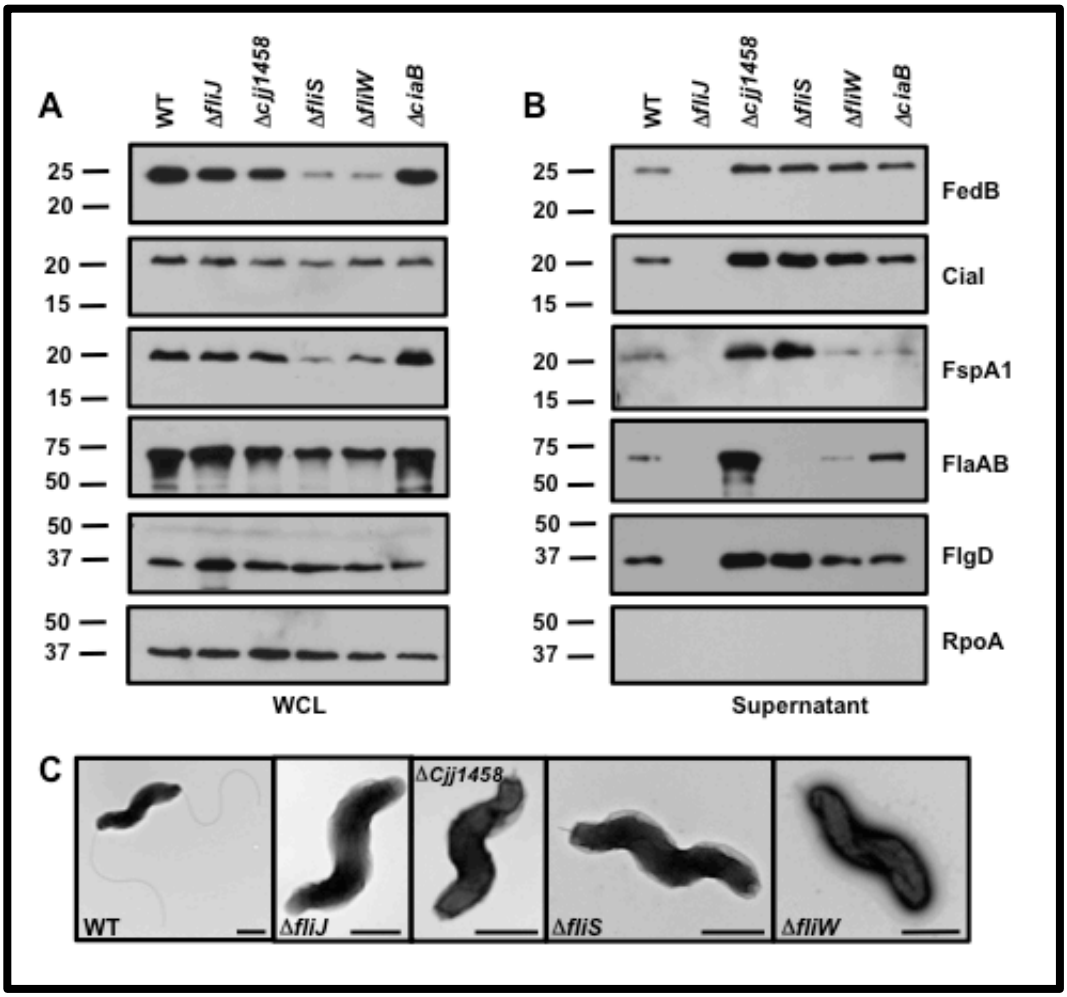

Figure 15. Analysis of flagellar-dependent secretion of flagellar and Fed proteins in *C. jejuni* mutants lacking putative flagellar chaperones. Immunoblot analysis of Fed or flagellar proteins in (A) whole-cell lysates or (B) supernatants of cultures. Wild-type and *C. jejuni* isogenic mutants lacking putative flagellar chaperones or CiaB, which is required for secretion of Cia proteins, were grown in MH broth for 4 h at 37 °C in microaerobic conditions. Whole-cell lysates (WCL) and supernatant proteins were recovered and analyzed by immunoblotting with antisera specific for each protein or RpoA, which served as a control for a cytoplasmic protein. The secreted Fed proteins include FedB, CiaI, and FspA1. The secreted flagellar proteins include the FlgD flagellar hook cap and the FlaA and FlaB flagellins. The antisera for flagellins recognize both the FlaA major flagellin and FlaB minor flagellin. Molecular weight markers are indicated in kDa. (C) Electron micrographs of wild-type and isogenic *C. jejuni* 81-176 Sm^R lacking genes encoding putative flagellar chaperones. The scale bar represents 0.5 μm.

secretion of the Feds (Figure 15B). In fact, we noted slightly higher levels of FedB and CiaI in the supernatants of both the Δ*fliS* and Δ*fliW* mutants compared to the wild-type strain. We also observed increased levels of FspA1 in the supernatants of the Δ*fliS* mutant, but the level of the protein in the supernatants of the Δ*fliW* mutant was similar to the wild-type strain (Figure 15B). Curiously, we noticed lower levels of FedB and FspA1 produced in the lysates of the Δ*fliS* and Δ*fliW* mutants, but this reduction did not correlate with a reduction in secretion of these proteins relative to the wild-type strain (Figure 15A and 15B). Analysis of the *C. jejuni* Δ*ciaB* mutant revealed no reductions in secretion of any flagellar or Fed protein, which was surprising at least for CiaI, considering that CiaB had been previously implicated in the secretion of Cia proteins [143]. These results suggest that no predicted flagellar chaperone proteins are directly required for secretion of the Fed proteins.

Fed secretion in C. jejuni *mutants impaired for hook or filament biosynthesis*

As described above, *C. jejuni* Δ*fliJ* lacked hook biosynthesis and did not secrete the Fed proteins, suggesting that at least a hook structure may be required for Fed secretion (Figure 15B and 15C). For the Cia proteins, the FlgE hook protein and the FlgK and FlgL hook-filament junction proteins were required for secretion [73]. Therefore, we constructed *C. jejuni* mutants that lacked FlgG (the distal rod protein), FlgE, and FlgK (Table 4). We also attempted to create a *flgD* mutant which lacked the hook cap and should be impeded in flagellar biosynthesis after completion of the flagellar rod, but this mutation caused the *C. jejuni* FlgSR two-component system that regulates flagellar gene expression to switch to the phase 'OFF' state [85,86]. In all of these mutants, production of the Fed proteins was not affected (Figure 16A). Deletion of *flgG*, which would result in formation of an incomplete flagellar rod structure and impaired hook

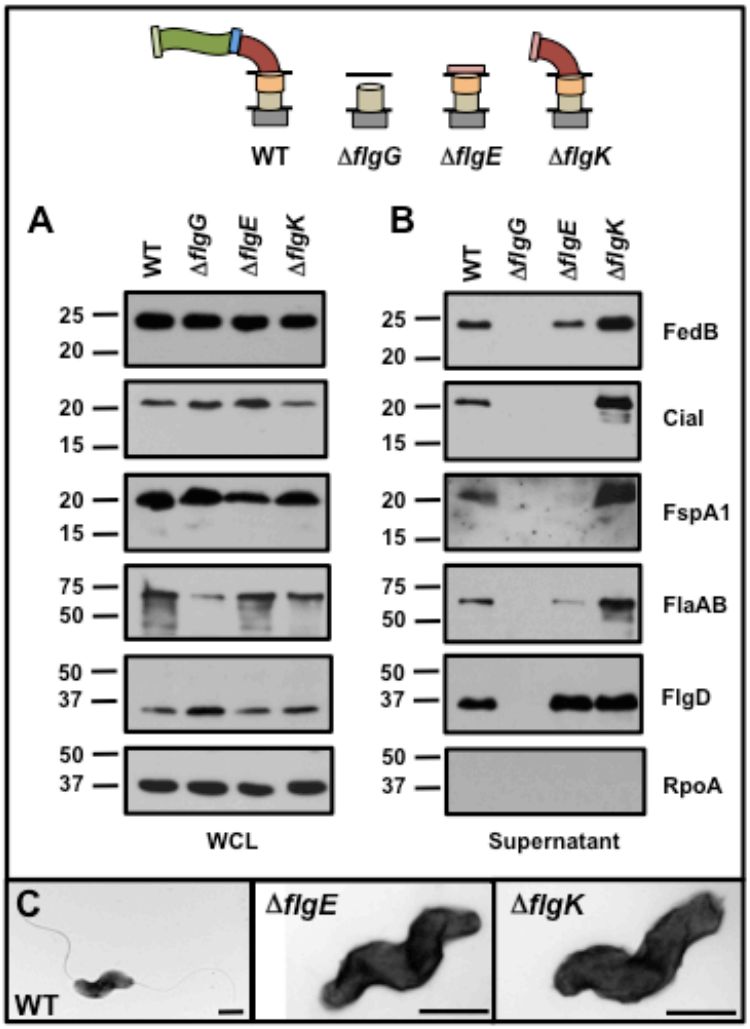

Figure 16. Secretion of Fed and flagellar proteins in *C. jejuni* rod and hook mutants. Immunoblot analysis of Fed or flagellar proteins in (A) whole-cell lysates (WCL) or (B) supernatants of cultures. Wild-type and *C. jejuni* isogenic mutants lacking flagellar rod and hook proteins were grown in MH broth for 4 h at 37 °C in microaerobic conditions. Whole-cell lysates and supernatant proteins were recovered and analyzed by immunoblotting with antisera specific for each protein or RpoA, which served as a control for a cytoplasmic protein. The secreted Fed proteins include FedB, CiaI, and FspA1. The secreted flagellar proteins include the FlgD flagellar hook cap and the FlaA and FlaB flagellins. The antisera for flagellins recognized both the FlaA major flagellin and FlaB minor flagellin. Molecular weight markers are indicated in kDa. A diagram of the flagellar structure produced by each mutant is shown at the top. The flagellar components shown include: the flagellar T3SS (grey rectangle); proximal rod (light grey cylinder); distal rod (orange cylinder); hook cap (pink rectangle); hook (red curved cylinder); hook-filament junction (blue rectangle); filament (dark green wavy bar); and filament cap (light green rectangle). (C) Electron micrographs of wild-type and isogenic *C. jejuni* 81-176 Sm^R lacking *flgE*, encoding the flagellar hook, and *flgK*, encoding a hook-filament junction protein. The scale bar represents 0.5 μm.

biosynthesis, resulted in the absence of any flagellar or Fed protein in the culture supernatant (Figure 16B). In *C. jejuni* Δ*flgE*, which lacks the major component of the flagellar hook, CiaI and FspA1 were not secreted and secretion of FedB and the flagellins was slightly reduced compared to the wild-type strain. As expected, secretion of the FlgD hook cap, which occurs before FlgE secretion, was not affected in the Δ*flgE* mutant (Figure 16B). In contrast, higher levels of all Feds and flagellins were found in the culture supernatants from *C. jejuni* Δ*flgK*, which lacks the distal hook-filament junction protein and is locked into producing hooks without filaments (Figure 16C). These results suggest that secretion of FedB occurs to a certain extent before initiation of hook biosynthesis, and that secretion of the other Feds begins during hook biogenesis. Furthermore, the hook-filament junction formed by FlgK and FlgL is not required for secretion of the Feds, which is counter to their involvement in secretion of Cia proteins.

Filament biosynthesis decreases extracellular secretion of the Feds

Considering our results described above, we thought it was curious that we observed enhanced levels of the Fed proteins in the supernatants of mutants that do not synthesize the flagellar filament (e.g, the *C. jejuni* Δ*Cjj1458*, Δ*fliS*, and Δ*flgK* mutants; Figure 15B and 16B). Therefore, we considered whether the length of the flagellar organelle during biosynthesis controls the levels of Fed secretion. Furthermore, since the FlaA and FlaB flagellins are secreted at the same time during flagellar biogenesis as the Feds, we tested whether the Fed proteins are in competition with flagellins for secretion via the flagellar T3SS. Therefore, we constructed specific mutants in *C. jejuni* 81-176 that would affect level of flagellin secretion or directly impact the ability of the flagellins to form a filament (Table 4).

We first compared the level of Feds in the culture supernatants of wild-type *C. jejuni,* a Δ*fliS* mutant, and a Δ*flaA* Δ*flaB* double mutant (which lacks all flagellins). As described above, the *C. jejuni* Δ*fliS* mutant lacks the chaperone to promote flagellin secretion and filament biosynthesis. Both mutants resulted in increased levels of secretion of FedB, CiaI, and FspA1 in the absence of flagellin expression altogether or secretion of the flagellins (Figure 17 and 17B). We then analyzed the role of *C. jejuni* FliD, which forms the filament cap after hook-filament junction biosynthesis to trap flagellins at the growing end of the flagellar tip to promote polymerization of flagellins into the filament. Similar to *fliD* mutants in other motile bacteria, the *C. jejuni* flagellins were present in increased levels in the culture supernatant of *C. jejuni* Δ*fliD* relative to wild-type *C. jejuni* (Figure 17B). Even in the presence of increased flagellin secretion in the Δ*fliD* mutant, all Feds were secreted at higher levels than in the wild-type strain (Figure 17B). We also observed similar high levels of the Feds in the culture supernatant of *C. jejuni* Δ*pseB* (Figure 17B). PseB is one of many enzymes that form the *O*-linked protein glycosylation system that modifies *C. jejuni* flagellins with pseudaminic acid. This modification is required by the *C. jejuni* flagellins to polymerize into the filament. In this mutant, we observed increased levels of unmodified flagellins (which are of smaller size in immunoblots) and continued high levels of the Fed proteins in the supernatant relative to wild-type *C. jejuni* (Figure 17A and 17B). These results suggest that the Feds are not in competition with flagellins for secretion via the flagellar T3SS. Instead, these proteins inherently are secreted efficiently amid a vast excess of flagellins to be secreted. In addition, our data suggest a temporal regulation of Fed secretion that is controlled by flagellar biosynthesis. The level of Fed secretion and Fed proteins in the extracellular milieu is inversely proportional to the length of the flagellar filament.

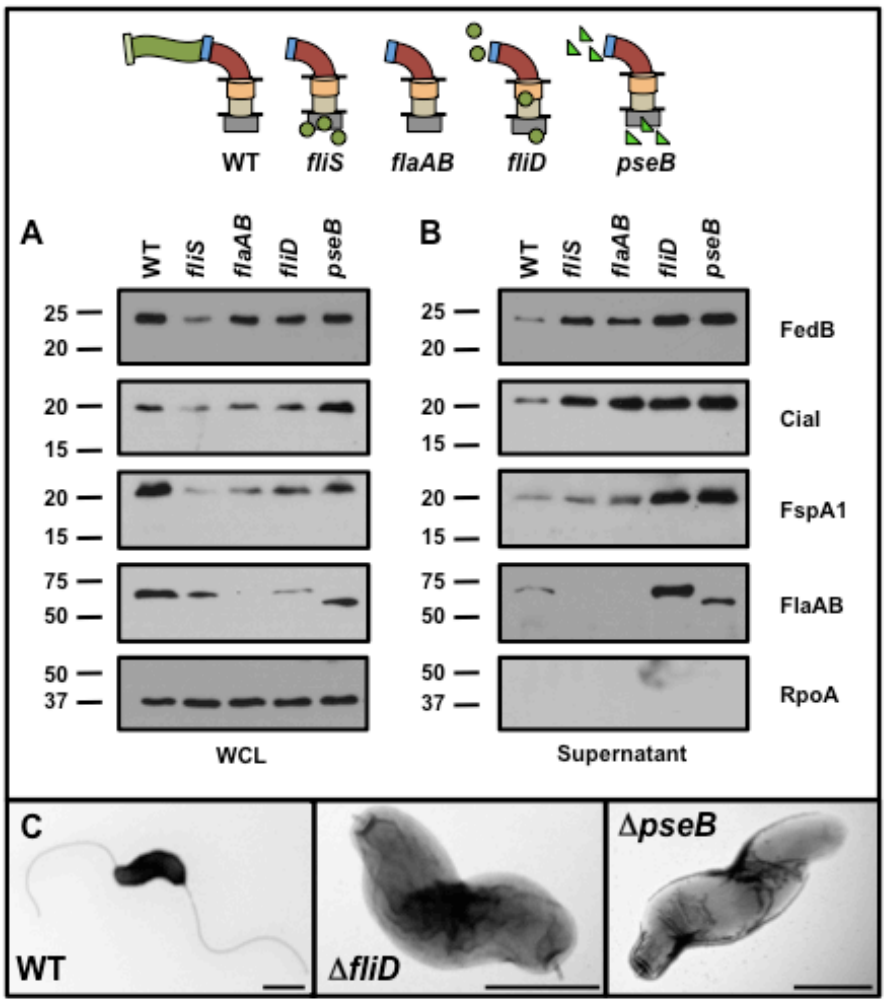

Figure 17. Secretion of Fed and flagellar proteins in *C. jejuni* mutants defective in filament biosynthesis. Immunoblot analysis of Fed or flagellar proteins in (A) whole-cell lysates (WCL) or (B) supernatants of cultures. Wild-type and *C. jejuni* isogenic mutants lacking proteins required for filament biosynthesis were grown in MH broth for 4 h at 37 °C in microaerobic conditions. Whole-cell lysates and supernatant proteins were recovered and analyzed by immunoblotting with antisera specific for each protein or RpoA, which served as a control for a cytoplasmic protein. The secreted Fed proteins include FedB, CiaI, and FspA1 and the secreted flagellar proteins include the FlaA and FlaB flagellins. The antisera for flagellins recognized both the FlaA major flagellin and FlaB minor flagellin. Molecular weight markers are indicated in kDa. A diagram of the flagellar structure produced by each mutant is shown at top. The flagellar components shown include: the flagellar T3SS (grey rectangle); proximal rod (light grey cylinder); distal rod (orange cylinder); hook (red curved cylinder); hook-filament junction (blue rectangle); filament (dark green wavy bar); filament cap (light green rectangle); glycosylated flagellins (green circles); unglycosylated flagellins (green triangles). (C) Electron micrographs of wild-type and isogenic *C. jejuni* 81-176 Sm^R lacking *fliD*, encoding the filament cap, and *pseB*, encoding a enzyme required for *O*-linked glycosylation of flagellin. The scale bar represents 0.5 μm

Analysis of the requirements of secretion of FedB and CiaI for commensal colonization of chicks and invasion of human colonic cells

Although FedB and CiaI are secreted into culture supernatants *in vitro*, it is unknown if these proteins must be secreted for interactions with hosts. To test the requirement of FedB and CiaI secretion for commensal colonization of chicks, we took advantage of the FedBΔ1 and CiaIΔ4GH domain deletion mutants that are stably produced but are not secreted from *C. jejuni* and remain associated with the bacterium (Figure 12B and 13B). We expressed these mutant proteins or the corresponding wild-type proteins with native promoters *in cis* from the *rdxA* locus in respective *C. jejuni* Δ*fedB* and Δ*ciaI* mutants (Figure 18A and 18B). These mutants were then assessed for the ability to colonize 1-day old chicks.

As a note, the commensal colonization data presented below is preliminary and requires further testing due to new and unexpected changes we have noticed in the *C. jejuni* chick colonization model. In Figure 19, we present the results of the bacterial loads in chicks seven days post-infection with an inoculum of 10^6. Wild-type *C. jejuni* colonized chicks at levels mainly between $10^8 - 10^9$ cfu per gram of cecal content. Although Δ*ciaI* producing wild-type CiaI did not fully reach the levels of colonization of wild-type *C. jejuni*, the Δ*ciaI* mutant producing CiaIΔ4GH did (Figure 19). These results suggest that CiaI likely does not need to be secreted to function in a mechanism to promote colonization of chick.

In analyzing the requirement of secretion of FedB for colonization, we observed that the Δ*fedB* mutant producing wild-type FedB was not consistently at the levels of wild-type *C. jejuni* (Figure 19). However Δ*fedB* producing FedBΔ1A showed a statistically significant decrease in colonization that averaged at 10,000-fold lower that wild-type *C. jejuni* (Figure 19). Keeping in mind that this N-terminal mutation may have affected the biological activity of FedB, these data

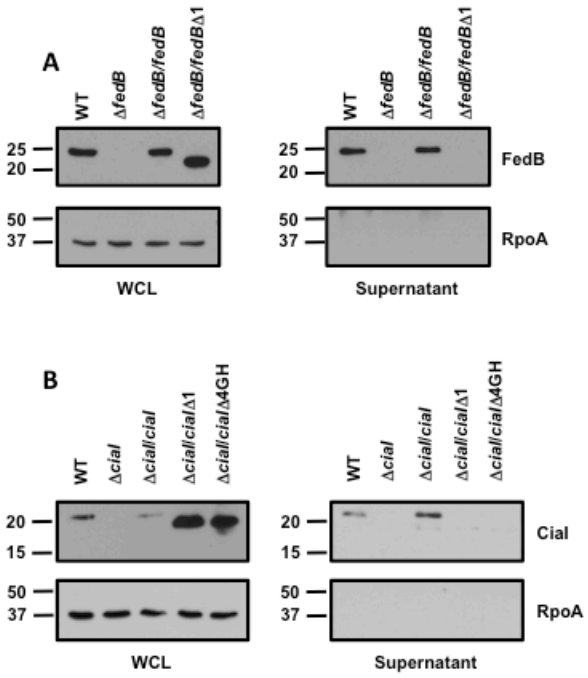

Figure 18. Analysis of wild-type FedB and CiaI or mutant proteins expressed *in cis* from the *rdxA* locus. Immunoblot analysis of whole-cell lysates or supernatant fractions of wild-type *C.* jejuni and mutant strains expressing wild-type FedB (A) or CiaI (B) mutant proteins from the *rdxA* locus. Strains were grown in MH broth for 4 h at 37 °C in microaerobic conditions. Wild-type and secretion-deficient proteins were expressed from their respective native promoters. Whole-cell lysates (WCL) and supernatant proteins were recovered and analyzed by immunoblotting with antisera specific for FedB, CiaI or RpoA, which served as a control for a cytoplasmic protein. Molecular weight markers are indicated in kDa.

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may suggest that FedB must be secreted to function in a mechanism to promote *C. jejuni* colonization of chicks.

CiaI is also required for wild-type level of invasion of human colonic cells [72,174]. CiaI has previously been proposed to contain a dileucine motif that is required for the protein to localize to vesicles within eukaryotic cells; and this activity might be important for preventing fusion of the CCV with lysosomes [72]. However, we have shown that a CiaI mutant protein produced by *C. jejuni* that lacked the dileucine motif was not impaired for invasion [174], which calls into question if CiaI must be secreted at all or into host cells to mediate a role in invasion.

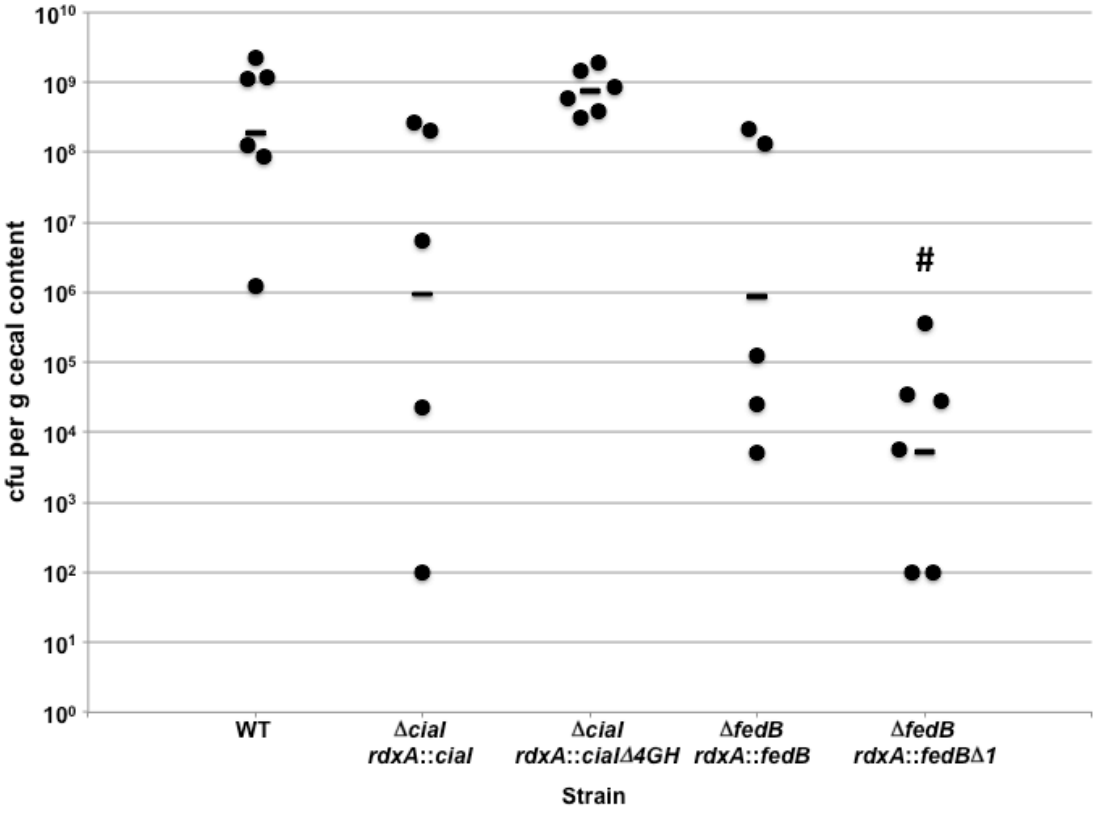

Figure 19. Commensal colonization capacity of wild-type *C. jejuni* and *C. jejuni* mutants producing wild-type and secretion-deficient CiaI and FedB proteins. One-day old chicks were orally inoculated with 10^6 cfu of *C. jejuni* strains. Each dot represents the amount of *C. jejuni* recovered from the ceca of chicks at day 7 post-infection. The geometric mean for each group is depicted by the horizontal bar. Statistical analysis was performed using the Mann-Whitney *U* test (#$P < 0.01$).

Strain	Invasion of T84 cells (% inoculum)[a]
Wild-type	14.51 ± 1.43
Δ*ciaI*	7.51 ± 0.74*
Δ*ciaI rdxA*::*ciaI*	12.89 ± 1.56
Δ*ciaI rdxA*::*ciaI* Δ1	7.12 ± 1.07*
Δ*ciaI rdxA*::*ciaI* Δ4GH	11.14 ± 1.01

Table 5. Invasion capacity of wild-type *C. jejuni* and isogenic *ciaI* mutants lacking domains for secretion. [a] Percent invasion was determined by comparing the number of intracellular bacteria surviving a 2 h gentamicin treatment of infected T84 cells compared to the number of bacteria in the infecting inoculum (approximately 3.0 x 10^6 cfu). Each assay was performed in triplicate, and at least three biological replicates were analyzed. The average percent invasion +/- standard error for each strain is presented. Statistically-significant differences in invasion between wild-type *C. jejuni* and mutant strains are indicated (* *P*-value < 0.05).

To assess this, we looked at invasion of T84 colonic epithelial cells. We analyzed *C. jejuni* Δ*ciaI* expressing wild-type and CiaI mutant proteins that could not be secreted *in cis* from the *rdxA* locus. For this analysis, we studied the relevance during invasion of the mutant proteins CiaIΔ1 and CiaIΔ4GH, both of which are not secreted from *C. jejuni* (Figure 18B). In the invasion assay, approximately 14.5% of the wild-type *C. jejuni* inoculum was found intracellularly at the end of the 6-h long assay (Table 5). The *C. jejuni* Δ*ciaI* showed an approximately two-fold reduction in colonization that could be restored by expression of wild-type CiaI. When we analyzed the Δ*ciaI* mutant producing the non-secreted CiaIΔ1 or CiaIΔ4GH mutant proteins, we found that CiaIΔ1 could not restore invasion, whereas CiaIΔ4GH did restore invasion levels close to those obtained with wild-type infection (Table 5). Since CiaIΔ4GH, which cannot be secreted *in vitro* from *C. jejuni*, appeared to be functional for invasion, we interpret these data as suggesting that CiaI likely does not need to be secreted to facilitate *in vitro* invasion of *C. jejuni*. We suspect that the lack of invasion upon expression of CiaIΔ1 in the Δ*ciaI* mutant may indicate

that the domain deletion within this mutant protein may interfere with its biological activity resulting in decreased ability to invade host cells.

Discussion

The *C. jejuni* flagellum is a versatile organelle that functions in multiple aspects of biology for the bacterium. As with other motile bacteria, the *C. jejuni* flagellum confers swimming motility [83]. We recently discovered that the *C. jejuni* flagellum also impacts cellular division by functioning in a mechanism to inhibit septation at polar regions so that division occurs at the cellular midpoint [84]. The *C. jejuni* flagellum has also been implicated as a secretory organelle for both flagellar proteins and for the Cia and Fed proteins that contribute to invasion of human epithelial cells and commensal colonization of chicks [72,73,136,146,147,174]. Although secretion of flagellar proteins has been studied in other motile bacteria, mechanisms used by the flagellum to secrete proteins not involved in motility remain fairly uncharacterized. Therefore, it was unclear whether the requirements and mechanisms for secretion of non-flagellar proteins via the flagellum are different than those to secrete *bona fide* flagellar proteins.

Many flagellar proteins possess an N-terminal region that is required for secretion. Depending on the flagellar protein, these regions usually are located within the initial 36 residues of the N-terminus [112,147]. These regions are thought to form a type of secretion signal that is recognized by the flagellar T3SS, although it has not been determined whether these domains interact with the T3SS or how these regions are required for secretion of the proteins. For FedB and CiaI, we identified a region within the first 13 and 26 residues, respectively, that were required for secretion of the proteins. We attempted to identify any conserved residues within

these sequences and within the N-terminus of FspA1 that may form a consensus motif possibly representing a secretion signal for recognition by the flagellar T3SS. However, we could not identify a clear consensus motif. Although deletion of the first 13 residues of FedB or 26 residues of CiaI inhibited secretion, deletion of 6-7 consecutive residues within these minimal domains did not diminish secretion of these proteins. Considering this information, we suspect that a specific primary sequence within the N-termini of the secreted Fed proteins is not recognized by the *C. jejuni* flagellar T3SS. Instead, we suspect that a structural motif such as a partially unfolded or disordered domain of at least a minimal length within the initial 26 residues is required for mediating secretion via the flagellar T3SS. This type of specific but disordered motif recognized by the flagellar T3SS has been postulated to exist at the N-termini of secreted flagellar proteins [113,187].

Some flagellar proteins are bound at their C-termini by specific chaperones that can assist in targeting the proteins to the flagellar T3SS for secretion [118,119]. We observed that deletion of C-terminal regions of FedB or CiaI severely reduced or even abolished flagellar-dependent secretion of these proteins. This requirement for a C-terminal domain for secretion was reminiscent of a chaperone-binding domain at the end of some flagellar proteins. However, we were unable to demonstrate that any predicted *C. jejuni* flagellar chaperones were required for secretion of FedB, CiaI, or FspA1. Therefore, if a chaperone is required, it remains elusive. It is possible that the secreted Fed proteins are organized similarly as secreted type III effector proteins, which bind cognate chaperones via an N-terminal domain and a C-terminal domain is recognized by the respective type III injectisome machinery [138]. Further investigation will require both broad and specific analyses for possible interacting proteins and flagellar T3SS

components to understand intermolecular determinants of *C. jejuni* required for secretion of the Fed proteins.

Previous studies have identified some intramolecular and intermolecular determinants required for flagellar-dependent secretion of the *C. jejuni* Cia proteins [72,73,144,146,147]. Drawing from these works and our present study, clear differences exist for requirements of flagellar-dependent secretion of the Cia and Fed proteins. The Cia proteins require the presence of serum or host cell contact for secretion [144]. One protein, CiaB, is not only secreted via the flagellum, but is also required for the secretion of other Cia proteins [146]. Furthermore, a flagellum structure consisting of at least a T3SS, rod, hook, and hook-filament junction are required for secretion of the Cia proteins [73]. In addition, a N-terminal region of no more than 36 residues is required for secretion via a heterologous T3SS [147]. However, requirement of a C-terminal domain in the Cia proteins for secretion has not been analyzed. In contrast, the Fed proteins, FedB, CiaI, and FspA1, are secreted via the flagellum *in vitro* in MH broth with no requirement for serum, host cell contact, or CiaB [174]. In addition, a shorter N-terminal domain of no more than 13 or 26 residues (for FedB and CiaI, respectively) and a C-terminal domain are required for wild-type levels of secretion. We also observed that FedB can be secreted without a hook structure (as demonstrated by a *C. jejuni* Δ*flgE* mutant), but a hook is required for maximal secretion of FedB, CiaI, and FspA1. Unlike the Cia proteins, a hook-filament junction within the flagellum was not required for secretion of the Feds. Our data suggest that Fed secretion mirrors many aspects of how the flagellins and the FliD filament cap, which compose the filament, are secreted. However, Cia proteins appear to be different with additional requirements for secretion. Currently, it is unknown how serum, host cell contact, or CiaB impact the flagellum or other aspects of *C. jejuni* biology for secretion of the Cia proteins.

As a note, we previously reported that mutation of *C. jejuni flaA* resulted in the absence of FspA1 in lysates and the lack of all secreted Fed proteins in culture supernatants. We initially concluded from this work that the FlaA major flagellin was required for production, stability, and secretion of some Fed proteins. For complete analysis in this work, we constructed a new *C. jejuni* mutant that lacked both FlaA and the FlaB minor flagellin. Our data in the current work clearly show that production or secretion of FedB, FspA1, or CiaI was not reduced in a *C. jejuni* flagellin mutant. We are confident in the results reported in this work and we suspect that our original *C. jejuni flaA* mutant had a secondary mutation affecting aspects of Fed production or secretion.

As our work shows, FedB, CiaI, FspA1 and flagellins require the flagellar hook for wild-type levels of secretion, suggesting that the Feds and flagellins are likely simultaneously secreted. Considering that during the late stages of flagellar biosynthesis after hook completion, ~20,000 flagellin subunits must be secreted to polymerize into the filament, we postulated that the Fed proteins may be in competition with flagellins for secretion via the flagellar organelle. Indeed, we observed that the Feds were present at higher levels in culture supernatants in the flagellin mutant compared to the wild-type strain, demonstrating that the lack of secretion of flagellins did result in increased Fed secretion. However, when we analyzed *C. jejuni* Δ*fliD* or Δ*pseB* mutants, in which flagellins are unable to polymerize into the filament and are present in increased amounts in culture supernatants, the Feds were also found at higher levels in the supernatants relative to the wild-type strain. These results suggest that FspA1, FedB, and CiaI are not in competition with the flagellins for secretion via the flagellum. Instead, the Fed proteins are secreted quite well when flagellins are also found in increased amounts in the culture

supernatants. Thus, the Fed proteins have evolved a mechanism to be secreted efficiently and simultaneously through the flagellum with native flagellar proteins.

Considering the data that Fed proteins were secreted at higher levels in *C. jejuni* mutants that do not construct a filament, we propose that one major factor that governs Fed secretion is the length of the growing filament during flagellar biosynthesis. We propose that once hook biosynthesis is completed, a bolus of Fed protein secretion occurs that gradually decreases as the flagellins are secreted and initiate filament polymerization. Decreased secretion of the Fed proteins into the extracellular environment may be due to increasing steric hindrance during transit through the central channel of the flagellar filament. Alternatively, there may be a change in the efficiency of Fed secretion once the flagellins polymerize into the filament. It is known that during different stages of flagellation, the substrate specificity of the flagellar T3SS changes from secreting rod proteins, then hook proteins, and then filament substrates. It is conceivable that filament polymerization triggers a change in the *C. jejuni* flagellar T3SS that alters its substrate specificity to reduce secretion of the Feds and then primarily secrete flagellins. Regardless of the mechanism, we propose a temporal regulation of secretion of Fed proteins that is controlled by the length of the flagellar filament. To our knowledge, our work is the first to report that the length of the flagellar organelle controls the levels of secretion of virulence and colonization factors of a bacterium.

Another intriguing finding from our work is the apparent absence of the requirement for secretion of CiaI for commensal colonization of chicks or invasion of human colonic epithelial cells. We assumed that since CiaI is secreted from *C. jejuni in vitro*, secretion of the protein would be required to mediate a function for host interactions. However, when we analyzed a *C. jejuni* strain producing a CiaI mutant protein that could not be secreted, the strain colonized

chicks and invaded human colonic cells as well as wild-type *C. jejuni*. These results strongly suggest that secretion of CiaI is not required for host interactions. Instead, CiaI likely performs a function inside the bacterium that is required for host interactions. It is possible that during prolonged colonization of chicks or during infection of humans, secretion of CiaI may be required for optimal interactions with these hosts. We did observe that a N-terminal 13-residue deletion of FedB abolished secretion of the protein and caused a reduction in colonization of chicks. It is tempting to conclude that secretion of FedB is required for commensal colonization of chicks. However, we cannot fully exclude the possibility that that this small domain deletion within FedB altered the structure or function of the protein to cause the colonization defect.

From a historical perspective, the *Yersinia enterocolitica* flagellum was the first flagellar organelle observed to secrete a non-flagellar protein [140]. Subsequently, the *C. jejuni* flagellum was found to secrete the Cia proteins. However, how the flagellum secretes non-flagellar proteins is not fully understood. In this work, we have revealed new knowledge regarding how the *C. jejuni* flagellum accomplishes transport of cognate flagellar proteins and non-flagellar proteins that are not required for motility but are required for host interactions. Furthermore, we have uncovered new biological information about secreted Fed proteins and factors that influence secretion of these proteins. An interesting question to be addressed in future endeavors would be to explore whether other motile bacteria employ their flagella to secrete non-flagellar proteins for various activities.

CHAPTER FIVE

Discussion

Overview

C. jejuni is the leading cause of gastroenteritis in developed countries. This food-borne pathogen has the ability to colonize the intestinal tract of different hosts, such as humans and agriculturally significant animals and birds. The interaction of *C. jejuni* with both hosts requires a functional whip-like organelle called the flagellum. The flagellum facilitates motility for the cell, which is essential to establish colonization. The work herein expands the importance of the flagellum in the biology of *C. jejuni*. We have shown that flagellar genes are co-regulated with a group of factors that are required for commensal colonization, with some factors also promoting invasion of human intestinal epithelial cells. We annotated this group of determinants as Feds (flagellar co-expressed determinants). In addition, we showed that a subset of the Feds is secreted in a flagellar-dependent manner. The purpose of this work was to expand our knowledge of factors controlled by the flagellar regulatory cascade and to identify new genes required for host interactions. In addition, this work allowed us to explore how non-flagellar proteins are secreted via the flagellar T3SS and to analyze the role of secretion of these proteins during commensal colonization and *in vitro* invasion of human intestinal epithelial cells.

The *C. jejuni* Flagellar Regulatory Cascade is a Global Regulatory System.

The flagellar regulatory cascade is a highly organized transcriptional and biosynthesis pathway required for motility. A fundamental step in the initiation of this regulatory cascade is

the formation of the MS ring and the flagellar T3SS. Both of these structures are essential for the activation of the FlgSR two-component system [80]. Activation of FlgSR results in the expression of genes that belong to the σ^{54} regulon [130,131]. The GTPase FlhF also plays a role in the activation of σ^{54} independently of FlgSR [82]. Analyses of the promoter of *fliA* (encoding σ^{28}) along with gene expression studies revealed that *C. jejuni fliA* belongs to the class II flagellar genes whose expression is dependent on σ^{54}. Furthermore, we analyzed 28 putative members of the σ^{28} regulon and found that transcription of 13 of these genes was decreased in a σ^{28} mutant. Seven of these genes encode flagellar proteins or proteins predicted to have a role in motility. However, a significant finding was that the remaining six genes included two previously annotated genes coding for the FspA1 and CiaI virulence factors and the other four genes were annotated as *fed*s. Analysis of the promoter region of the *fed*s and *ciaI* revealed a consensus sequence for σ^{28} binding upstream of the transcriptional start site for these genes. This observation led us to propose that the flagellar regulatory cascade of *C. jejuni* is also involved in the regulation of virulence and colonization factors. For this reason, we consider the flagellar regulatory cascade a global regulatory system that governs expression of factors involved in motility, commensalism and virulence.

DOC is Required for Translation of a σ^{28}-independent *ciaI* Transcript.

A previous study performed with *C. jejuni* strain F38011 found that expression of *ciaI* required physiological levels of DOC in bacterial media [145]. However, all our gene expression analyses demonstrating the σ^{28}-dependent transcription of *ciaI* were performed in the absence of DOC. To understand the discrepancies between our observations and the studies previously reported, we analyzed the effects of DOC on transcription and translation of *ciaI*. Transcription

studies revealed that DOC did not affect σ^{28}-dependent and σ^{28}-independent expression of *ciaI*. We then analyzed the levels of CiaI in whole-cell lysates of wild-type and σ^{28} mutant strains in the absence and presence of DOC. We observed that σ^{28}-independent production of CiaI relied on the presence of DOC. Analyses of the promoter region of *ciaI* showed a putative σ^{28}-independent promoter upstream of the σ^{28} binding sequence. The *ciaI* transcript that is σ^{28}-independent and requires DOC for production possesses a longer untranslated region (UTR). For now, we can only speculate that DOC influences the UTR of the σ^{28}-independent *ciaI* transcript to promote translation. This mechanism needs to be further investigated since it suggests a translational regulation of CiaI production under specific conditions in which the bacterium encounters bile salts, such as during *in vivo* colonization of a host intestinal tract.

Characterization of the Role of Feds in *C. jejuni* Biology

Feds are determinants for commensalism and virulence. We showed that a *C. jejuni* mutant lacking σ^{28} was reduced for expression of specific flagellar and *fed* genes (which include *fedA-D*, *ciaI* and *fspA1*). Previous findings showed that a *C. jejuni* σ^{28} mutant was defective for colonization of the cecum of chicks [35]. We investigated if this decrease in commensal colonization was due solely to the lack of motility, since σ^{28} regulates the expression of flagellar filament proteins, or also due to reduction in expression of one or more *fed*s. To answer this question, we analyzed the colonization ability of *C. jejuni* 81-176 mutants lacking individual Feds in a chick model of commensalism. We observed a decrease in the bacterial levels recovered from the ceca of chicks infected with mutants lacking any one of five Feds. The only mutant that did not have a significant reduction in commensal colonization compared to wild-type was the Δ*fspA1* mutant. These findings suggested that, in addition to the non-motile

phenotype of a σ^{28} mutant strain, the lack of expression of five *fed*s resulted in a reduction in commensal colonization.

Another aspect of the Feds that we explored was a possible role in virulence. CiaI has been shown to be required for intracellular survival after internalization of human intestinal epithelial cells. We analyzed different *C. jejuni* mutants lacking individual Feds for the ability to invade unpolarized T84 cells *in vitro*. These studies revealed that one Fed, FedA, is important for *in vitro* invasion of human colonic epithelial cells.

Possible functions of Feds in C. jejuni biology. Through bioinformatic analyses, we found that FedA contains a well–conserved hemerythrin (Hr) domain. The Hr domain consists of a series of five conserved histidines, one glutamate and one aspartate that together coordinate the binding of a non-heme diiron molecule and one oxygen molecule [188]. Hemerythrins were first identified in marine invertebrates where they function as oxygen transporters, scavengers of heavy metals or as components of innate immune responses [169]. Recently, the conserved Hr domain has been identified in some proteins of prokaryotes, mainly in marine bacteria, anaerobes or microaerophilic organisms [171,189]. A methyl-accepting chemotaxis protein from *Desulfovibrio vulgaris* (DcrH) was the first Hr identified in prokaryotes [189]. DcrH contains an Hr domain at its C-terminus cytoplasmic portion. Although the function of DcrH has not been fully elucidated, the authors of this study postulated that the Hr domain could play a role in oxygen sensing.

Analysis of the *C. jejuni* genome revealed the presence of two other genes, *Cjj81176_0266* and *Cjj81176_1237*, in which the encoded proteins contain the well-conserved Hr domain. FedA, Cjj0266 and Cjj1237 are small proteins that only contain the Hr domain, which classifies them as single-domain Hrs (SDHrs). To further elucidate the role of SDHr in

the biology of *C. jejuni*, we collaborated with the Kelly laboratory at the University of Sheffield. *Cjj81176_0266* is located upstream of genes involved in the biosynthesis of iron-sulfur (Fe-S) clusters, which are sensitive to oxygen. Due to the microaerobic nature of *C. jejuni*, the bacterium requires mechanisms to protect or repair enzymes that have been exposed to oxidative damage. One characteristic of Hr-containing proteins is that they can bind oxygen molecules. Therefore, there is the possibility that in *C. jejuni* these proteins can have a protective role during environmental insults such as exposure to excess oxygen.

To characterize the role of Cjj0266, Cjj1237 and FedA in the biology of *C. jejuni*, the Kelly laboratory devised a procedure in which the activity of oxygen-sensitive enzymes that contain Fe-S clusters can be monitored after exposure to oxygen. Two candidates for this analysis are the pyruvate and 2-oxoglutarate: acceptor oxidoreductases (Por and Oor), which are essential metabolic enzymes in *C. jejuni*. Comparison of mutants lacking each SDHr to wild-type *C. jejuni* revealed that Cjj0266 and Cjj1237 confer protection of Por and Oor from oxidative damage during microaerobic growth. However, this was not the case for FedA (Kendall et al., manuscript in revision). Whereas Cjj0266 and Cjj1237 are important to prevent oxidation of their target proteins, they are not involved in repairing enzymes that have been damaged. Our studies with a Δ*fedA* mutant showed for the first time that a SDHr can play an important role for colonization and virulence. However, the other *C. jejuni* SDHrs, Cjj0266 and Cjj1237, are dispensable for commensal colonization (Kendall et al., manuscript in revision). Although FedA, Cjj0266 and Cjj1237 contain a well-conserved Hr domain, these proteins appear to perform different functions for *C. jejuni*. The mechanism of action of FedA during commensal colonization and *in vitro* invasion is something that could be explored further. First, it would be necessary to establish whether FedA behaves like a canonical SDHr that binds iron and oxygen.

Also, we could determine if the conserved Hr domain is important for host interactions by disrupting each conserved residue that composes the Hr domain.

The C-terminus of FedC contains a region common to the DnaJ (Hsp40) cochaperone protein [170]. DnaJ and GrpE, a nucleotide-exchange factor, function as co-chaperones that interact with DnaK (Hsp70) to either help refold damaged proteins or target these proteins for degradation [170]. DnaJ binds DnaK through its J-domain and stimulates ATP hydrolysis of DnaK, which allows the delivery of an unfolded or damaged substrate to the DnaK chaperone machinery [190,191]. In *E. coli*, the DnaK-DnaJ-GrpE machinery is induced during stress conditions such as heat shock [170,192]. *C. jejuni* appears to produce a complete DnaK-DnaJ-GrpE chaperone machinery, although it has not been fully characterized. During colonization of the intestinal tract of avian species, *C. jejuni* must grow at 42°C, which is the natural body temperature of chicks and a temperature that usually induces heat stress in most bacteria. It is tempting to speculate that FedC could have a DnaJ-like cochaperone function for DnaK to assist during commensal colonization and *in vivo* growth at 42°C. Future work will need to be performed to determine if the J-domain is essential for FedC function and whether FedC functions as a cochaperone in the DnaK chaperone machinery.

FedB and FedD do not share homology to any characterized proteins. However, FedD has a few characteristics that could be helpful in determining a function for this protein. FedD is a small protein with a predicted size of only 6 kDa. Due to its small size, we speculate that FedD is likely secreted via the flagellum similar to FedB, CiaI and FspA1. Furthermore, secondary structure prediction analyses propose that FedD folds into an alpha-helical structure. The size and predicted structure of FedD are characteristics shared by some bacterial antimicrobial peptides referred to as bacteriocins [193]. Microcins, a subcategory of bacteriocins, are small

peptides usually less than 10 kDa in size produced by *E. coli* and other enterobacteria [194]. One important feature of microcins is that they have a narrow spectrum of targets and are mostly active against species that are closely related to the producer cell [195]. In our studies, we showed that a mutant strain lacking *fedD* had the highest reduction of chick colonization of all *fed* mutants compared to wild-type (over 1,000-fold). If FedD is in fact a microcin, it is possible that this protein plays a crucial part in establishing *C. jejuni* colonization amidst other bacteria colonizing the host intestinal tract.

A Subset of Feds is Secreted in a Flagellar-Dependent Manner

Previous work by others has shown that two σ^{28}-dependent gene products, FspA1 and CiaI, are secreted and required an intact flagellar apparatus for secretion [72,136]. Immunoblot analyses of whole-cell lysate and supernatant fractions of wild-type *C. jejuni* and flagellar mutants showed that FedB is also secreted in a flagellar-dependent manner. This observation led us to inquire about the mechanisms and requirements for secretion of some Feds. In addition, we took advantage of this opportunity to use *C. jejuni* as a model to understand how a flagellum is able to secrete flagellar and non-flagellar proteins.

Intramolecular requirements for Fed secretion. For analysis of secretion of a subset of Feds, one of the first issues we addressed was the possibility that secretion of Feds is mediated by a secretion signal encoded within the protein. Characterization of FliC, the flagellin in *Salmonella* species, showed that a secretion signal is located within the N-terminus of the protein [113]. In addition, the C-terminus of FliC is the region where the flagellin chaperone, FliS, interacts to prevent cytoplasmic polymerization of FliC [125]. With this in mind, we concentrated our studies on the N- and C-termini of FedB and CiaI. We choose to further

explore the secretion of these proteins since both are required for interactions with the avian host and because CiaI is involved in invasion of human intestinal epithelial cells. Analysis of a series of truncated proteins indicated that portions of the N- and C-termini of these proteins are important for secretion. These regions include the first 14 or 26 amino acids of FedB and CiaI respectively and the last 12 amino acids of both proteins.

To further explore the sufficiency of the putative secretion signals, we created chimeric proteins in which the first 26 amino acids of FedB or CiaI were fused to a truncated FlaA that is defective for secretion due to the absence of its native secretion signal. Analysis of the supernatant fraction of *C. jejuni* expressing these chimeras showed that the N-terminus of FedB and CiaI is sufficient to promote secretion via the flagellum. In these chimeras, the C-terminus of FlaA is intact and can be readily bound by the chaperone FliS to inhibit intracellular polymerization. For future studies, we will analyze expression and secretion of the chimeras in a *C. jejuni* strain lacking *fliS* to determine if the N-terminus of FedB or CiaI is sufficient to promote secretion of the FlaA chimeras.

In addition, we noticed a lack of sequence similarity among the N-terminal regions of FedB, CiaI, FspA1 and the flagellins, suggesting that their secretion signals are not composed of a specific sequence of amino acids. Instead, we propose that, similar to flagellins in *Salmonella*, the secretion signal of the secreted Feds is an N-terminal motif of a minimal length and disordered structure that is recognized by the flagellar T3SS to then initiate translocation [125,187]. Another possibility is that the N-terminal region of the secreted Feds functions as a binding site for putative chaperones, as is the case of T3SS effector proteins [138]. Effector proteins have a different arrangement than flagellins where their cognate chaperones bind the N-terminus instead of the C-terminus.

Known flagellar chaperones are not required for Fed secretion. As discussed above, the C-terminus of CiaI and FedB also influence flagellar-dependent secretion. These regions in flagellar proteins are usually bound by chaperones, which assist in secretion. Thus, we postulate that a chaperone might be necessary for secretion of FedB and CiaI. We analyzed secretion of FedB, CiaI, FspA1, and flagellins in mutants lacking proteins with homology to characterized flagellar chaperones such as FlgN and FliS, or FliJ, which is involved in chaperone-substrate recycling. We observed that secretion of these Feds was not affected by the absence of known flagellar chaperones. Secretion of FedB, CiaI and FspA1 was reduced in a Δ*fliJ* mutant, but this defect can be attributed to the lack of proper formation of a flagellar rod and hook structure in this mutant. These findings indicate that, even though the Feds are secreted through the flagellar apparatus, no predicted flagellar chaperones were required for secretion. It is possible that uncharacterized chaperones are produced by *C. jejuni* to mediate Fed secretion, or that the C-terminus of the Feds are required for secretion by a chaperone-independent mechanism.

As mentioned before, FedC contains a DnaJ-like domain conserved in a family of proteins with cochaperone activity. Even though FedC could have a cochaperone role and interact with other proteins in *C. jejuni*, preliminary analyses of secreted Feds in a Δ*fedC* mutant revealed that this putative cochaperone is not required for secretion of these Feds. If secretion of FedB, CiaI and FspA1 is dependent on the activity of chaperones, we could attempt to identify possible chaperones by determining what proteins physically interact with the secreted Feds. Identification of FedB, CiaI or FspA1 binding partners could be beneficial to learn more about the mechanism for Fed secretion via the flagellar T3SS or the role of secreted Feds in *C. jejuni*. More importantly, we could use this new class of putative chaperones to find conserved

homologues in other bacteria and perhaps identify other non-flagellar secreted proteins in other species.

Structural requirements of the flagellum for Fed secretion. Another important aspect of FedB, CiaI and FspA1 that we addressed was to determine the minimal flagellar structure required for their secretion. This knowledge would help us determine at what stage of flagellar biosynthesis these Feds are secreted. For this study we analyzed secretion of Feds from different *C. jejuni* mutant strains lacking specific components of the flagellar structure. We found that FedB and the flagellins were secreted simultaneously and their secretion likely begins at the very early stages of hook biosynthesis. However, CiaI and FspA1 secretion required a constructed hook conduit.

Another observation in this study was the hypersecretion of Feds and flagellins in the absence of the hook-filament junction protein FlgK. This has been observed before in *Salmonella,* where flagellins are expelled into the extracellular medium, likely due to the absence of a transitional protein between the hook and filament to facilitate filament polymerization [101]. Since FedB, CiaI, FspA1 and flagellins are likely secreted at the same time, we wondered whether the Feds competed with flagellins for secretion and if the excess secretion of flagellins affected secretion of these Feds. The supernatant fraction of mutants such as Δ*Cjj81176_1458* or Δ*flgK*, which lacked the putative chaperone for the hook-filament junction proteins and a hook filament-junction protein respectively, revealed hypersecretion of both flagellins and Feds. These findings indicate that Feds and flagellins do not compete for secretion by the flagellar T3SS. Thus, the Feds are inherently very competitive with flagellins for secretion via the filament.

Secretion of Feds is temporally controlled by filament biosynthesis. An initial observation of a *C. jejuni* Δ*fliS* mutant, which does not secrete FlaAB or make a filament, indicated an increase in Fed secretion. We questioned if the length of the filament could regulate secretion of FedB, CiaI or FspA1. Since we cannot create mutations to control the length of the filament, we looked at secretion of these Feds in other flagellar mutants that are unable to construct a filament. Immunoblot analyses of the supernatant fraction of a Δ*pseB* mutant, which fails to glycosylate flagellins to promote filament polymerization, or a Δ*flaAB* mutant, which lacks flagellins altogether, showed an increase in Fed secretion. Considered together, we postulate that secretion of Feds in a wild-type strain occurs in a burst at the early stages of filament biosynthesis, and that the efficiency of secretion is diminished as the filament elongates. Thus, secretion of FedB, CiaI and FspA1 appears to be temporally regulated relative to flagellar filament biosynthesis. To our knowledge, this type of regulation for secretion of virulence and colonization determinants via a flagellar organelle has not been reported before.

We are considering two models to explain how filament biogenesis affects the levels of secretion of FedB, CiaI and FspA1. The first model states that elongation of the filament results in a type of steric hindrance that disrupts the mobility of the secreted Feds traveling through the narrow conduit within the growing flagellum. However, a problem with this model is that it suggests that at some point during filament biosynthesis, the filament channel becomes blocked with the secreted Feds that cannot reach the extracellular environment. Another model proposes that a second secretion specificity switch for the *C. jejuni* flagellar T3SS could exist to transition from hook biosynthesis to filament biosynthesis. In this model, maximal secretion of FedB, CiaI and FspA1 could take place after completion of a hook structure and before flagellins begin to polymerize into the filament. Once the filament begins to polymerize, the specificity of the

flagellar T3SS may shift towards a preference for flagellins only with reduced secretion of Feds. Future work will require to determine whether such specificity switch exists and to determine an activation signal for this process.

Role of secretion of FedB and CiaI in colonization and pathogenesis. To enhance our understanding of the importance of secretion of FedB and CiaI for the biology of *C. jejuni*, we performed commensal colonization analyses and *in vitro* invasion experiments with *C. jejuni* expressing FedB or CiaI mutants that are not secreted. Preliminary colonization studies revealed that secretion of FedB might be important for wild-type levels of colonization of the chick cecum. Secretion of CiaI, on the other hand, was dispensable for commensal colonization. CiaI is the only Fed that we know of that is secreted and plays a role in invasion of human intestinal epithelial cells *in vitro* [174]. For this reason, we performed gentamicin-protection assays using *C. jejuni* mutant strains defective for secretion of CiaI. Our studies showed that secretion of CiaI is also not important for its role during invasion of unpolarized T84 colonic cells. Thus, it appears that CiaI functions intracellularly within *C. jejuni* for colonization and invasion. Additional evidence that supports this idea are the chick colonization analyses and *in vitro* invasion experiments using a CiaI mutant with a defective dileucine motif. This motif was reported to be important in localizing CiaI to vacuoles, an activity that allegedly is necessary to inhibit fusion of the vacuole with lysosomes [72]. However, we showed that secretion of CiaI or a functional dileucine motif is not important for interactions between *C. jejuni* and its hosts [174]. On the other hand, we also showed that a putative nucleotide-binding domain is relevant for invasion of T84 cells *in vitro* and colonization of the avian host [174]. This information led us to believe that the primary role of CiaI is executed intracellularly in the bacterium. Although

the mechanism of action of CiaI is not fully understood, there is likely a reason for this protein to be secreted by the bacterium. It is possible that the protein may be secreted for a function during prolonged colonization of chicks or during infection of humans for pathogenesis.

Proposed Model for Fed Secretion

The continuous production of all secreted and non-secreted Feds depends on the activation of flagellar gene expression. It is worth noting that FedB and CiaI are also produced from σ^{28}-independent transcripts in the absence of flagellar gene expression, but cannot be secreted unless flagellar biosynthesis is taking place. Secretion of FedB, CiaI and FspA1 likely reaches its peak at the point between hook and filament biosynthesis, and their secretion gradually declines as the filament elongates. We postulate that after the bacterium undergoes cell division, the flagellar regulatory cascade is turned on to build a flagellum at the new pole. At this point, maximal expression of Feds occurs, possibly beginning at the stage of hook biosynthesis of the nascent flagellum. If this model applies during commensal colonization or human infection, it would indicate that the subpopulation of *C. jejuni* cells that have undergone division and are in the process of building a new flagellum are producing Feds for colonization. Although the role of Fed secretion in colonization is not entirely clear, it is likely that newly dividing cells that are building a flagellum are also producing the secreted Feds for the entire *C. jejuni* population.

Closing Remarks

Previous analyses of *C. jejuni* motility mutants showed that flagellar motility is essential to establish colonization at the mucosal layer of the host intestinal tract. However, in this study

we showed that flagella are also important for the regulation of expression of genes that encode commensal colonization determinants and virulence factors. More importantly, we showed that flagella in *C. jejuni* are also required for secretion of some of these factors. Through this work, we assigned a direct role to the flagellar regulatory cascade for commensalism and pathogenesis.

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6xHis	hexahistidine tag
A	alanine
B. subtilis	*Bacillus subtilis*
C. jejuni	*Campylobacter jejuni*
Caco-2	human epithelial colorectal adenocarcinoma cells
CCV	*Campylobacter* containing vacuole
CDT	cytolethal distending toxin
cfu	colony forming units
Cia	*Campylobacter* invasion antigens
d	day(s)
DME/F12	Dulbecco's modified eagle's medium: nutrient mixture F12
DOC	deoxycholate
DocA	determinant of chick colonization A
E. coli	*Escherichia coli*
ECL	enhanced chemilumescent substrates
FBS	fetal bovine serum
Fe-S	Iron-Sulfur clusters
Feds	Flagellar co-expressed determinants
Fops	Flagellar outer proteins
fOS	free oligosaccharides
FspA1	Flagellar secreted protein A1

g	gram(s)
G. mellonella	*Galleria mellonella*
GBS	Guillain-Barré syndrome
GFP	Green fluorescent protein
GST	glutathione-S-transferase
h	hour(s)
Hcp	Hemolysin coregulated protein
HeLa	Henrietta Lacks immortalized cell line
HEp-2	HeLa derived epithelial cells
Hr	hemerythrin
HRP	horseradish peroxidase
Hsp40	Heat shock protein 40
Hsp70	Heat shock protein 70
Hsp90α	Heat shock protein 90α
INT407	human intestinal epithelial cells
K	lysine
kDa	kilodaltons
L	leucine
LB	Luria-Bertani
LOS	Lipooligosaccharide
M	Molar
Mb	megabases
MBP	maltose-binding protein

MeOPN	O-methyl phosphoramidate
MH	Mueller-Hinton
min	minutes(s)
ml	milliliters
MOMP	Major outer membrane protein
MotA	Motility protein A
ND	not determined
Ni-NTA	nickel nitrilotriacetic acid
OD	optical density
Oor	2-oxoglutarate: acceptor oxidoreductase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PflA	Paralyzed flagella protein
Por	Pyruvate oxidoreductase
qRT-PCR	semiquantitative real-time reverse-transcription PCR
rpm	revolutions per minute
s	second(s)
SDHr	single-domain hemerythrin
SDS	sodium dodecyl sulfate
Sm^R	Streptomycin resistant
SodB	Superoxide dismutase B
SOEing	Splicing by overlap extensions

T. pallidum	*Treponema pallidum*
T3SS	Type III secretion system
T6SS	Type VI secretion system
T84	human colonic epithelial cells
TCA	trichloroacetic acid
TLR	Toll-like receptor
TMP	trimethoprim
UTR	untranslated region
VgrG	Valine-glycine repeat protein G
WCL	whole-cell
WT	Wild-type
Y. enterocolitica	*Yersinia enterocolitica*
μl	microliter
μM	micromolar

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