IDENTIFICATION OF CAMPYLOBACTER JEJUNI
SECRETED PROTEINS

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IDENTIFICATION OF CAMPYLOBACTER JEJUNI
SECRETED PROTEINS

Abstract

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*Campylobacter jejuni* is a leading cause of bacterial gastrointestinal illness worldwide. Several studies indicate the importance of the bacterium’s flagellum as an essential component for maximal and efficient invasion into host cells. To further understand the role of the flagellum in *C. jejuni* pathogenesis, studies were undertaken to characterize the flagellum as an apparatus that serves dual purposes for motility and secretion of virulence proteins. Specifically, we hypothesize that *C. jejuni* secretes virulence proteins termed, *Campylobacter secreted proteins* (CSP), in a Type III Secretion System (T3SS) dependent manner and that these proteins play a role in *C. jejuni*-mediated disease.

Previous work indicated that *C. jejuni* secretes 8-16 proteins *in vitro* via the flagellum. Since, the flagellum shares evolutionary homology to the T3SS injectisome, we surmised that the CSP must harbor characteristics that allow for recognition and secretion from the flagellum in a T3SS dependent manner. To test our hypothesis, we utilized the *Yersinia enterocolitica* flagellar T3SS to determine if homologous flagellar machinery would recognize and secrete the CSP, *Campylobacter* invasion antigen B (CiaB). Secretion of CiaB from the *Y. enterocolitica* flagellar apparatus provided proof
of concept for the generation of a genetic screen to identify the remaining Cia proteins. 321 amino termini of previously uncharacterized *C. jejuni* open reading frames (orfs) were tested for secretion by fusing putative secretion signals with the *Y. enterocolitica* YplA reporter. Recombinants were then tested for secretion under *Y. enterocolitica* flagellar induction conditions. One putative CSP that drove YplA secretion was encoded by the *C. jejuni* Cj1242 orf. A Cj1242 mutant was generated and subsequently characterized for the ability to invade host cells. A secretion profile from the Cj1242 mutant, designated *ciaC*, revealed an altered *C. jejuni* secretion profile whereby one band that corresponded to the predicted mass of CiaC was missing. The *ciaC* mutant also exhibited an invasion deficient phenotype, as judged by the gentamicin protection assay. In conclusion, our *C. jejuni* genome-wide screen resulted in the identification of at least one novel *C. jejuni* virulence determinant. Additional studies are underway to determine how CiaC contributes to *C. jejuni*-mediated disease.
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CHAPTER ONE
INTRODUCTION

Pathogenesis

The spiral shaped, strict microaerophile bacterium, *Campylobacter*, grows optimally between 37-42°C and harbors at least one polar flagellum. The Center for Disease Control and Prevention estimates that 0.8% of the general U.S. population is infected with campylobacteriosis annually (www.cdc.org). Campylobacteriosis may be caused by consuming as little as 500 microorganisms with consumption of improperly handled food, contaminated water sources, and unpasteurized milk as the most common routes of entry (Robinson, 1981; Allos, 2001). Only about 1% of campylobacteriosis is caused by *Campylobacter* species other than *C. jejuni*. Infected individuals often do not seek treatment due to the self-limiting nature of the disease. Thus, only about 13 cases per 100,000 individuals are actually diagnosed (www.cdc.org).

Campylobacteriosis is characterized by severe, abdominal cramping and bloody stools that often contain leukocytes. Symptoms usually arise 2-5 days post-ingestion and are followed by illness that may last from one to seven days. Complications from campylobacteriosis can result in the acute, demyelinating, polynueuropathy disease termed Guillain Barré Syndrome (GBS). In GBS patients, a peripheral nerve ganglioside, structurally resembling a *Campylobacter* lipooligosaccharide, is targeted by an individual’s immune system. Individuals then develop an auto-immune response, that destroys the myelin sheath surrounding peripheral nerves. Symptoms of GBS occur several weeks post-*C. jejuni* infection and recovery can often take several
months. In the U.S., 40% of GBS cases have resulted from a prior *Campylobacter* infection (Yuki et al., 2004).

**Motility**

Though *Campylobacter* exhibits a unique regulation scheme for expression of flagellar genes compared to motile Enterobacteriaceae, there remains significant homology amongst the flagellar apparatus proteins (Wosten et al., 2004; Hendrixon et al., 2003). Motility is essential for *C. jejuni* to maximally invade host cells as flagellar mutants exhibit a less invasive phenotype compared to wild-type strain (Grant et al., 1993; Nachamkin et al., 2003; Wassenaar et al., 1997). Colonization of the intestinal tract is also dependent on motility, as flagellar mutants fail to establish gut colonization (Chang and Miller 2006). The assembly of the apparatus itself requires the secretion of flagellar constituents in a T3SS dependent manner (MacNab, 2004; Minamino et al., 2004). The mechanism by which the flagellar basal body, hook, and filament proteins are secreted and assembled is similar to the mechanism by which the dedicated export pathways of the *Salmonella* SP1-1 and *Yersinia* Ysc apparati are constructed (Winstanley, 2001; Mota et al., 2005). In contrast to these virulence systems, the *Campylobacter* flagellum has evolved as the sole apparatus for the secretion of T3SS virulence proteins as well as to provide motility (Parkhill et al., 2000).

In addition to secretion of the basal body, hook, and filament proteins, the flagellar apparatus also recognizes and secretes regulatory proteins (*i.e.* anti-sigma factors) and virulence proteins (Hughes et al., 1998; Young et al., 1999; Nambu et al., 1999). Upon adherence to a host cell, the bacterium requires a functional flagellar apparatus to secrete a set of proteins, termed *Campylobacter* secreted proteins. The
subset of CSP required for invasion are termed *Campylobacter* invasion antigens (Cia) (Konkel *et al.*, 2004; Rivera-Amill *et al.*, 1999). *C. jejuni* mutants deficient in the basal body, hook, filament, and flagellar regulatory genes do not secrete Cia proteins and are not as invasive as wild-type strains (Konkel *et al.*, 1999; Rivera-Amill *et al.*, 2001).

**Adhesion/Intracellular Survival**

*C. jejuni* must adhere to a host cell prior to invasion. CadF, FlpA, CapA, JlpA, MOMP (major outer membrane protein), and PEB1 are known to contribute to *Campylobacter* adhesion to cells *in vitro* and to host colonization *in vivo* (Konkel *et al.*, 1997; Flanagan *et al.*, 2009; Ashgar *et al.*, 2007; Jin *et al.*, 2001; Moser *et al.*, 1997; Pei *et al.*, 1993). One adhesin, CadF (*Campylobacter* adhesin to Fibronectin), binds fibronectin to provide adhesion to the proximal host cell (Monteville *et al.*, 2003). A second adhesin, FlpA (Fibronectin-like protein A) has also been shown to be essential for maximal adhesion. FlpA harbors at least three fibronectin binding domains that may contribute to the interaction between the bacterium and the host cell extracellular matrix. As expected, the *C. jejuni* cadF, flpA, and cadF flpA mutants are significantly less adherent to host cells versus wild-type strain (Konkel *et al.*, 2010).

Once the microbe has adhered, the Cia proteins are secreted, and the bacterium invades by means of a trigger mechanism (Konkel *et al.*, 2004; Konkel *et al.*, 1999). INT407, HEp-2, and Caco-2 are the most commonly used mammalian cell lines in *Campylobacter* invasion studies (De Melo *et al.*, 1989; Konkel *et al.*, 1992; Russell *et al.*, 1994). Whether *C. jejuni* is taken up via an endocytic pathway or autophagic pathway is unclear. Different *Campylobacter* strains have showed varying invasive
capabilities after the addition of cytochalasin B, an inhibitor of actin polymerization (Hu et al., 1999; Monteville et al., 2003). Monteville et al. (2003) indicated that alteration of host cell microfilaments may play a role in *C. jejuni* translocation into host cells and that the host cell response is crucial for efficient invasion, whereas Hu et al. (1999) proposed a microtubule dependent invasive strategy. Consistent with known bacterial invasion strategies, Krause-Gruszczynska et al. (2007) further supported a microfilament dependent invasion mechanism by demonstrating the involvement of small Rho GTPases during *C. jejuni* invasion (Finlay, 2005).

Watson et al. (2008) demonstrated that post invasion, *Campylobacter* deviate from an endocytic pathway and reside in an intracellular compartment. Once established, the intracellular compartment does not fuse with lysosomes and provides an environment whereby survival and replication may occur. Additional evidence from ultrastructural studies revealed internalized bacteria within membrane bound vesicles post bacterial translocation (Konkel et al., 1992). Insight into *Campylobacter* intracellular survival would be greatly aided by mutants deficient in vacuole formation, which presently, are not available. Little is also understood about the mechanism of *C. jejuni* intracellular replication. Konkel et al. (1992) have observed a significant increase in bacterial numbers when *C. jejuni* is cocultured with an epithelial monolayer in vitro. Subsequent monolayer destruction indicates that *C. jejuni* replicates intracellularly to increase in sheer numbers and elicit a cytotoxic effect.

Debate on whether *C. jejuni* infection results in host cell apoptosis, or oncosis, is ongoing. Cytolethal distending toxin (Cdt) has been observed to contribute to prolonged survival in macrophages (Hickey et al., 2005). In endothelial cells, Cdt causes G₂/M
block and induces chemokine release (Whitehouse et al., 1998; Hickey et al., 2000; Zheng et al., 2008). In contrast to these studies, Kalischuck et al. (2007) have shown that cytotoxicity, ATP depletion, and DNA fragmentation occur independent of the cdt gene. Furthermore, post C. jejuni infection, enterocytes exhibit cytotoxicity and DNA fragmentation independent of a caspase mediated pathway. These findings argue in favor of an oncosis model whereby the cdt gene is dispensable for C. jejuni-mediated enteritis. Collectively, these studies suggest that the mechanism that C. jejuni employs to cause the host cell’s destruction may be strain specific.

**Type III Secretion System Characteristics**

Proteins secreted via a Type III Secretion Systems (T3SS) bypass the periplasmic space. Effector proteins translocated by a T3SS travel directly from the bacterial cytoplasm into the host cell cytosol via the translocon conduit. Chaperones (necessary for efficient synthesis and secretion of specific proteins) may also be associated with T3SS complexes to pilot secreted substrates to the secretory dock, prevent aggregation of multiple components, and determine an ordered secretion hierarchy (Cornelis, 2002).

The T3SS must be able to distinguish specific T3SS substrates designated for export from amongst hundreds of bacterial proteins (Cheng et al., 2000; Aldridge et al., 2001). T3SS have evolved cognate secreted proteins with a nonconsensus secretion signal that resides in the amino-terminal portion of the substrate. Debate on whether the actual signal resides within the mRNA sequence or that of the peptide sequence has been controversial. Ramamurthi et al. (2003) made frameshift mutations the first 10
codons of the amino-terminal secretion signal of *Yersinia* outer protein Q (YopQ). Mutations in this region resulted in non-YopQ secretion. However, frameshifted codons 11-15 were found to be tolerable to mutations, thus allowing the protein to be secreted. When the third amino acid, Ile, was substituted for a synonymous codon (*i.e.* another codon that also codes for Ile), secretion of the substrate was nearly abolished. This indicates that the mRNA sequence of the YopQ secretion signal has an important role in YopQ secretion.

The peptide signal hypothesis relies on precedent in biological protein export systems. This model is founded on the observation that a secreted protein from another T3SS is exported in a heterologous system. In contrast to YopQ observations, mutated secretion signals of YopE, favor support of the peptide signal hypothesis. Synonymous codon substitutions for the first 17 amino acids of YopE result in secretion of the protein. Frameshift mutations in the first 11 codons of the YopE secretion signal result in a nonsecretable substrate. Collectively, these observations support that the T3SS secretion signal does not wholly reside in the mRNA sequence (Lloyd *et al.*, 2001).

Despite their unique differences, YopQ and YopE share T3SS substrate commonalities. Both proteins do not contain a cleavable secretion signal and both proteins harbor the secretion signal within the amino-terminal portion of the protein (or 5' end of the mRNA). Last, both secretion signals are recognized by the Ysc needle-complex.

**Pathogens that utilize T3SS to export virulence proteins**

T3SS secreted proteins can be recognized universally amongst heterologous systems (Blocker *et al.*, 2003). Bacterial pathogens that harbor T3SS exhibit profound
homology amongst the individual components that comprise the secretion apparatus. Though the apparatus itself is highly conserved, the effectors vary from one T3SS to another T3SS. Each unique set of effector proteins provides a tailored niche for an individual species (Cornelis et al., 2000).

T3SS are often found encoded on pathogenicity islands (PI) within the bacterial chromosome (Winstanley et al., 2001). In the case of Y. enterocolitica, the Ysc needle complex is encoded on a 70 kb virulence plasmid, termed pYV (Cornelis et al., 1998). Y. enterocolitica also harbors two additional chromosome encoded T3SS. The Ysa and Flagellar T3SS are operational under low temperature and high salt concentration or no salt conditions, in vitro, respectively (Young et al., 2002; Foultier et al., 2002). Secreted proteins from Yersinia aid in retardation of macrophage phagocytosis. The effector proteins allow the bacterium to escape, migrate to lymph node tissue, and replicate (Bleves et al., 2000).

The genome of Salmonella typhimurium may also encode more than one T3SS as well (Gal-Mor et al., 2006). Recent evidence suggests that Salmonella harbors several T3SS pathogenicity islands, but the two pathogenicity islands, termed SP-I and SP-2 (Salmonella Pathogenicity Island -1,2 respectively) are the best characterized (Baker et al., 2007; Schlumberger et al., 2006). Salmonella species utilize their T3SS to mediate rearrangement of the host cell cytoskeleton (ruffling effect) leading to cell uptake. Once engulfed, the bacterium secretes proteins (encoded on the SP-2 locus) that return the host microfilament structure back to a non-ruffling state. Additional T3SS substrates aid in creating a Salmonella containing vacuole (SCV) for intracellular survival (Bakowski et al., 2008).
The enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) also harbor a needle complex T3SS. Like *Salmonella*, the EPEC and EHEC utilize a T3SS apparatus for the delivery of proteins to hijack cellular processes responsible for actin rearrangement. Export of the Translocated intimin receptor, or Tir, into a host cell is required for intimate contact and attachment, resulting in the formation of a unique pedestal formation (Dean *et al.*, 2009). This strategy is yet another clever example of how Gram-negative bacterial pathogens subvert cellular functions to create a niche by means of a T3SS.

In addition to enteric pathogens, plant pathogens also utilize T3SS (Hueck, 1998). The Gram-negative bacteria, *Xanthomonas campestris pv. vesicatoria*, *Erwinia* *spp.*, *Pseudomonas syringae*, and *Ralstonia solanacearum* cause a wide range of disease in plants. These include bacterial spot disease of pepper and tomato plants (*Xanthomonas*), soft rot and fireblight in rosaceous plants (*Erwinia*), bacterial speck (*Pseudomonas*), and bacterial wilt (*Ralstonia*).

**SIGNIFICANCE**

Since the early 1970’s, *C. jejuni* has become recognized as a leading cause of gastrointestinal illness. Little is known about the invasive mechanism(s) that the bacterium employs. The flagellum has been observed to have a greater role in pathogenicity than previously thought. The *C. jejuni* flagellum provides dual function in motility and secretion of virulence proteins. Identifying these proteins is crucial to elucidate the strategy of the bacterium’s invasive nature. The characterization of the
Cia proteins may provide insight into novel therapeutics that may be used in disease prevention and treatment for individuals infected with campylobacteriosis.

The sequence of the NCTC 11168 genome failed to show that *Campylobacter* harbors a T3SS pathogenicity island that may encode a needle apparatus (Parkhill et al., 2000). However, it is well accepted that *C. jejuni* harbors a flagellum and that the flagellar genes encoding the apparatus are dispersed throughout the genome. During growth in vitro, *C. jejuni* secretes a subset of proteins upon contact with host cells, or, when an additional stimulant, serum, is supplemented into the medium (without host cells) (Konkel et al., 1999; Rivera-Amill et al., 2001). The flagellum serves as the T3SS pathway for the export of (at least) eight proteins, termed *Campylobacter* invasion antigens (Cia). To date, only one Cia, CiaB, has been identified. CiaB is required for maximal invasion of host cells as *C. jejuni ciaB* mutants are less invasive than wild-type strain and are secretion minus for all Cia in vitro. Additionally, gnotobiotic (i.e. pathogen free) piglets infected with the *ciaB* mutant exhibit severe attenuated disease as compared to wild-type strain, in vivo (Konkel et al., 2001).

*C. jejuni* secretes the Cia proteins upon co-cultivation with epithelial cells. *In vitro* invasion assays of INT 407 cells infected with *C. jejuni* flagellar mutants revealed that maximal invasion of epithelial cells required secretion competent, motile bacteria. Complementation of the *C. jejuni flaA, flaB* double mutant with a shuttle vector harboring either the *flaA* or *flaB* gene restored Cia protein secretion, suggesting that Cia export requires at least one of the two filament proteins (Konkel et al., 2004). Collectively, these data indicate that the secretion of the Cia proteins requires a functional flagellar export apparatus.
Additional secretion studies in the heterologous T3SS of *Y. enterocolitica* will aid in characterizing the Cia proteins. The goal of this research is to identify one or more additional Cia proteins and to characterize these proteins as T3SS substrates. These studies will focus on observing CiaB export in *Y. enterocolitica* T3SS, characterizing the secretion signal, and generating a genomic library of putative *C. jejuni* cia genes.
References


CHAPTER 2
IDENTIFICATION OF A CAMPYLOBACTER JEJUNI FLAGELLAR SECRETED PROTEIN REQUIRED FOR MAXIMAL INVASION OF HOST CELLS

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CHAPTER 2 ATTRIBUTION PAGE

All manuscripts included in this thesis represent the culmination of work by a number of people, some of who are listed as authors in addition to myself. The following is a general summary of my contribution to each manuscript as listed by chapter.

Ideas for the project were conceived by Dr. Konkel, Dr. Jeffrey Christensen, Dr. Brian Raphael, and Dr. John Klena. I assisted with the preliminary work for demonstrating CiaB protein secretion via the flagellum (Figure 1), and constructed the ciaC mutant and the complemented ciaC mutant strain. I also assisted with the preliminary work for identifying the altered secretion profile (Figure 4) and demonstrating the invasion deficiency of the C. jejuni ciaC mutant (Table 3).
Summary

The food-borne pathogen *Campylobacter jejuni* is dependent on a functional flagellum for motility and the export of virulence proteins that promote maximal host cell invasion. Both the flagellar and non-flagellar proteins exported via the flagellar type III secretion system (T3SS) contain a sequence within the amino-terminus that directs their export from the bacterial cell. Accordingly, we developed a genetic screen to identify *C. jejuni* genes that encode a T3SS amino-terminal sequence that utilizes the flagellar T3SS of *Yersinia enterocolitica* and a phospholipase reporter (*yplA*). We screened a library of 321 *C. jejuni* genes and identified proteins with putative T3SS amino-terminal sequences. One gene identified by the screen was *Cj1242*. We generated a mutation in *Cj1242*, and performed growth rate, motility, secretion, and INT 407 cell binding and internalization assays. The *C. jejuni Cj1242* mutant was not altered in growth rate or motility when compared with the wild-type strain, but displayed an altered secretion profile and a reduction in host cell internalization. Based on the phenotype of the *C. jejuni Cj1242* mutant, we designated the protein *Campylobacter invasion antigen C* (CiaC). Collectively, our findings indicate that CiaC is a potentially important virulence factor.
Introduction

Campylobacter jejuni, a Gram-negative pathogen, is one of the leading bacterial causes of gastroenteritis worldwide (Allos, 2001; Westrell et al., 2009). The clinical presentation of C. jejuni-mediated disease varies from one individual to another, where some individuals have watery diarrhea and others experience diarrhea with blood (Blaser et al., 1983; Friedman et al., 2004). The reason for the variation in clinical presentation is not known. We speculate that both the unique virulence factors of the infecting strain as well as the host innate immune response influence the presentation and severity of disease (Larson et al., 2008). The most severe form of campylobacteriosis, which is characterized by fever, severe abdominal cramps, and diarrhea containing blood and leukocytes, likely results from C. jejuni invasion of the intestinal epithelium. Indeed, intracellular bacteria have been observed by electron microscopy examination of samples from C. jejuni-infected individuals with acute infectious colitis characterized by diarrhea with blood (van Spreeuwel et al., 1985).

C. jejuni must be metabolically active and secrete proteins from the flagellar type III secretion system (T3SS) for maximal invasion of host epithelial cells (Konkel & Cieplak, 1992; Konkel et al., 1993; Konkel et al., 2004). The proteins synthesized and secreted by C. jejuni upon co-cultivation with epithelial cells are termed Campylobacter invasion antigens (Cia) (Konkel et al., 1999). The importance of the Cia proteins in C. jejuni pathogenesis has been demonstrated with a ciaB mutant, which is deficient in Cia protein secretion. The severity and time of onset of disease in piglets inoculated with a C. jejuni ciaB null mutant is significantly attenuated when compared with a C. jejuni wild-


type isolate. The piglets inoculated with the *C. jejuni ciaB* null mutant did not develop diarrhea until 3 days post inoculation whereas all piglets inoculated with a *C. jejuni* wild-type isolate developed diarrhea within 24 hr (Raphael *et al.*, 2005).

Gram-negative bacteria have evolved distinct secretion systems to actively transport proteins across their membranes (Kostakioti *et al.*, 2005; Pallen *et al.*, 2003; Thanassi & Hultgren, 2000). The T3SS is characterized by the export of proteins across both membranes of the bacterium, which normally occurs upon bacteria-host cell contact (Cornelis, 2006, Galan & Wolf-Watz, 2006). In *C. jejuni*, the only T3SS is the flagellar apparatus (Parkhill *et al.*, 2000). Previous work has demonstrated that the secretion of *C. jejuni* Cia and other virulence proteins is dependent on a functional flagellar T3SS (Konkel *et al.*, 2004, Poly *et al.*, 2007). Precedence for the secretion of a virulence factor from the flagellum was first demonstrated with *Yersinia enterocolitica* (Schmiel *et al.*, 1998; Schmiel *et al.*, 2000; Young *et al.*, 1999), which utilizes the flagellar T3SS to export a phospholipase termed YplA.

The majority of the proteins secreted from *C. jejuni*, including the Cia virulence proteins, have not yet been identified due in part to low levels of protein secretion under *in vitro* conditions. The aim of this study was to identify a virulence protein that is secreted from the *C. jejuni* flagellar T3SS. As a first step in the identification of putative *Campylobacter* secreted proteins (Csp), we tested if CiaB would be recognized and secreted from the well-characterized flagellar T3SS of *Y. enterocolitica* (Warren & Young, 2005). Based on the finding that CiaB was secreted from *Y. enterocolitica*, we
developed a screen that utilized *Y. enterocolitica* and the YplA effector protein to identify *C. jejuni* genes that encode amino-terminal residues that facilitate protein secretion in a T3SS-dependant manner (i.e., T3S amino-terminal sequences) (Schmiel *et al.*, 2000; Berring *et al.*, 2004; Warren & Young, 2005). We demonstrated that the screen had the potential to identify putative Csp with T3S amino-terminal sequences using known *C. jejuni* flagellar secreted proteins. We report the identification of 42 *C. jejuni* proteins with amino-terminal sequences that promote secretion from the *Y. enterocolitica* flagellar T3SS. From this list, one gene (*Cj1242*) encoding a hypothetical protein was selected for additional study. We generated a mutation in *Cj1242*, and examined the growth rate, motility, secretion profile, and binding and invasion properties of the *C. jejuni Cj1242* mutant relative to the wild-type isolate. The *C. jejuni Cj1242* mutant displayed an altered secretion profile and reduced host cell-invasion, demonstrating that *Cj1242* is a virulence protein.
Results

The CiaB protein is secreted via the Y. enterocolitica flagellar T3SS

Based on the finding that CiaB is secreted via the flagellar T3SS of C. jejuni (Konkel et al., 1999), we reasoned that CiaB should be recognized and secreted in a T3SS-dependent manner in a heterologous system. To test this possibility, the full-length ciaB gene was cloned into the pMMB207 plasmid and conjugated into the Y. enterocolitica JB580v wild-type strain and Y. enterocolitica GY4492, a mutant lacking any functional T3SS (pYV8081_{flhDC ysaT}). These bacterial strains and plasmids are described in Table 1. Whole cell lysate and supernatants were collected from the Y. enterocolitica strains cultured under conditions to induce the secretion of the flagellar outer proteins (Fops) (i.e., 2 hr at 26°C in TYE broth medium). The Fops represent a set of at least 12 proteins secreted from the flagellar T3SS, including the flagellar filament proteins FleABC. As expected, the Y. enterocolitica JB580v wild-type strain secreted the Fops, whereas the Y. enterocolitica pYV8081_{flhDC ysaT} mutant did not secrete the Fops (Fig. 1A). The supernatants were also probed with the mouse monoclonal flagellin specific antibody 15D8 for the detection of the Y. enterocolitica FleABC flagellar filament proteins (38-40 kDa) (Kapatral & Minnich, 1995). The FleABC proteins were detected in the supernatants of Y. enterocolitica JB580v wild-type strain, demonstrating that the flagellar T3SS was functional, whereas the FleABC proteins were not detected from supernatants of the Y. enterocolitica pYV8081_{flhDC ysaT} T3SS mutant (Fig. 1B). Importantly, the CiaB protein (73 kDa) was detected in the supernatant of the Y. enterocolitica JB580v wild-type strain, but not the Y. enterocolitica flagellar mutant, as judged by immunoblot analysis with a rabbit polyclonal CiaB specific antibody (Fig. 1C).
The detection of CiaB protein in the supernatant was not due to bacterial cell lysis, as the cytoplasmic protein sigma 70 ($\sigma^{70}$) was not detected in the supernatants (Fig. 1D). As an additional control, we found that CiaB was synthesized and could be detected in the whole cell lysate of the Y. enterocolitica pYV8081 $\Delta$flhDC ysaT T3SS mutant (Fig. 1E). As expected, the Y. enterocolitica cytoplasmic protein $\sigma^{70}$ was detected in the whole cell lysates prepared from each of the bacterial strains (Fig. 1F). Collectively, these results indicate that CiaB is recognized as a flagellar T3SS protein by Y. enterocolitica.

C. jejuni T3S amino-terminal sequences promote secretion from the Y. enterocolitica flagellar T3SS

All proteins exported via a T3SS contain an amino-terminal sequence to direct their export from the bacterial cell. Moreover, previous work has shown that a T3SS protein can be: a) recognized and secreted by more than one T3SS in the same bacterium, and b) recognized and secreted from bacteria that belong to other genera (Badea et al., 2009; Lee & Galan, 2004; Young & Young, 2002). Since CiaB was secreted via the C. jejuni and Y. enterocolitica flagellar T3SS, we hypothesized that the CiaB amino-terminus would direct the export of a fusion protein from Y. enterocolitica in a T3SS-dependent manner. In addition, we hypothesized that the amino-termini of two other C. jejuni flagellar secreted proteins, FlaA and FlaC, would also promote secretion of a fusion protein. To test this hypothesis, we generated the pCSP50 shuttle vector encoding the Y. enterocolitica yplA phospholipase gene as a reporter (Fig. 2). The Y. enterocolitica YplA enzyme is an A2 phospholipase and is secreted under flagellar
T3SS inducing conditions *in vitro* (Schmiel *et al*., 1998; Berring *et al*., 2004). Warren and Young (Warren & Young, 2005) determined that the YplA enzyme’s T3S amino-terminal sequence is localized within the first 20 residues. The pCSP50 shuttle vector incorporates a constitutive promoter (*cat*) upstream of Ndel and BgIII cloning sites, a 5’-truncated *yplA* gene (eliminating the first 50 amino acids including the T3S amino-terminal sequence), and the *yplB* chaperone gene. The amino-terminal deletion of the *Y. enterocolitica* YplA protein abolished its secretion, but not its enzymatic (phospholipase) activity (not shown) (Hatic *et al*., 2002).

The *Y. enterocolitica* JB580v wild-type strain secretes YplA under flagellar T3SS inducing conditions and the enzymatic activity can be detected on phospholipase agar (PLA) plates (not shown). The hydrolysis of Tween 80 in PLA plates results in a fatty acid precipitate that forms a halo surrounding the YplA secretion competent colonies. In contrast, the *Y. enterocolitica yplAB* strain GY4757, generated for use in conjunction with a YplA reporter, showed no detectable phospholipase activity. Similarly, the *Y. enterocolitica yplAB* strain harboring the native pCSP50 vector was secretion negative (Fig. 3A). However, when the first 108 nucleotides of *yplA* (1-36 AA encoding sequence) was fused to the truncated *yplA* gene, the YplA fusion protein was secreted and detected on PLA plates.

To provide proof of concept for the screen for *C. jejuni* genes harboring T3S amino-terminal sequences, we generated *yplA* fusions with the first 108 nucleotides of three genes encoding proteins known to be secreted via the *C. jejuni* flagellar T3SS (FlaA,
As predicted, all three fusions with YplA were secreted and detected on PLA plates. In contrast, a fusion of the first 108 nucleotides of the *C. jejuni* gene for CysM was generated to serve as a T3SS negative control, and no secretion was observed. CysM is a 32.4 kDa cytoplasmic protein (O-acetylserine sulfhydrylase B) involved in cysteine biosynthesis (Garvis *et al.*, 1997).
Identification of C. jejuni genes harboring putative T3S amino-terminal sequences

The results from the native CiaB secretion assay and the YplA reporter assay demonstrated that the Y. enterocolitica flagellar system could be utilized to identify a C. jejuni protein with a T3S amino-terminal sequence. Thus, a total of 359 genes from the 1654 identified ORFs from the C. jejuni NCTC 11168 sequence were selected to test via the YplA reporter assay (Parkhill et al., 2000). These genes/ORFs were chosen for analysis as the deduced amino acid sequences lack predicted membrane-spanning domains, periplasmic domains, Sec-dependent signals, or Tat-dependent signals. No genes were found to encode Type I Sec-independent motifs. Primers were designed to amplify the first 108 encoding bases of all 359 ORFs and facilitate directional cloning into the shuttle vector pCSP50 to generate translational fusions with the truncated YplA reporter. The first 108 bp for 341 of the 359 ORFs were successfully cloned and sequence confirmed in the E. coli S17-1 λ-pir donor strain. From this fusion library, 321 vectors were successfully conjugated into the Y. enterocolitica yplAB host strain and characterized for YplA secretion on PLA plates (Table S1). Table 2 lists the 42 C. jejuni genes that harbor amino-terminal sequences that resulted in YplA secretion zone widths greater than or equal to that obtained with the CiaB amino-terminus from the Y. enterocolitica yplAB strain after 12 hrs incubation on PLA plates.

C. jejuni-YplA fusion proteins are secreted to the culture supernatant by the Y. enterocolitica flagellar T3SS

To confirm that the YplA fusion enzyme activity measured by the PLA plate assay was the result of secretion through the Y. enterocolitica flagellar T3SS, we tested several
strains by immunoblot analysis. *Y. enterocolitica* strains harboring the pCSP50 vector with *C. jejuni* amino-terminal sequences were grown in broth culture under conditions that induced or repressed synthesis of the flagellar system. Importantly, the *C. jejuni* amino-terminal sequences fused to YplA were only detected in the supernatants of strains cultured under flagellar inducing conditions (Fig. 3B). The amount of protein secreted into the supernatant, as judged by immunoblot analysis, was roughly proportional to that measured by the PLA plate assay and varied according to the *C. jejuni* amino-terminal sequence. As predicted from the PLA plate assays, there were no reactive bands detected from the supernatants for the strains harboring the CysM:YplA fusion or the pCSP50 truncated YplA. To evaluate the supernatants for bacterial lysis, which would result in the release of cytoplasmic proteins, the blots were probed using a mouse monoclonal antibody to the cytoplasmic protein σ70. No reactive band was detected for σ70 in any supernatants (Fig. 3C). Immunoblot analysis of the whole cell lysates with a rabbit polyclonal YplA specific antibody confirmed that the YplA fusion proteins (33.0-33.4 kDa, depending on amino-terminal sequence) were being synthesized under both flagellar and non-flagellar conditions (Fig. 3D). In addition, a band of consistent intensity was detected for both growth conditions corresponding to σ70 in the whole cell lysate samples (Fig. 3E). Cumulatively, these data indicate that the YplA fusion proteins were secreted from the flagellar T3SS.

*Functional classification of C. jejuni proteins harboring putative T3S amino-terminal sequences*
The functional classifications of the 42 proteins harboring putative T3S amino-terminal sequences were obtained from the Sanger Institute web site (http://www.sanger.ac.uk/) (Gundogdu et al., 2007). The majority of the C. jejuni NCTC 11168 proteins were classified as either conserved hypothetical proteins (16 proteins) or proteins of unknown function (14 proteins) Noteworthy is that two flagellar related proteins (FlgM, FlgJ) and a pathogenicity related protein (FspA) were identified among the proteins harboring a T3S amino-terminal sequence, which had not been characterized when this study commenced.

*Cj1242 is secreted from C. jejuni*

To confirm that one of the proteins identified using the phospholipase indicator agar assay was secreted from the flagellar T3SS of C. jejuni, we generated a *Cj1242* deletion mutant. *Cj1242* was chosen because the *Cj1242*-YplA fusion protein was highly secreted from *Y. enterocolitica* (Table 2), the gene is predicted to be monocistronic, and is upregulated when *C. jejuni* is cultured under conditions that induce virulence genes (Malik-Kale et al., 2008). The *Cj1242* gene is capable of encoding a protein with a *M*$_r$ 12,164. The growth rate and motility of the *Cj1242* deletion mutant was indistinguishable from that of the *C. jejuni* wild-type strain (not shown). We then performed secretion assays to determine if the *C. jejuni Cj1242* mutant was capable of Cia protein export.

The profile of Cia proteins detected from the *C. jejuni* F38011 wild-type strain was similar to that observed in previous work (Konkel et al., 2004). In contrast with the wild-
type strain, the secretion profile of the *C. jejuni* Cj1242 mutant lacked one band of 12.2 kDa (Fig. 4A). The 12.2 kDa band was restored in the *C. jejuni* Cj1242 complemented strain, which was transformed with a plasmid harboring a wild-type copy of *Cj1242 in trans*. Secreted proteins were not detected for the *C. jejuni* wild-type strain when FBS was omitted from the labeling medium, which is consistent with previous work indicating that components within serum are sufficient to induce Cia protein secretion (Konkel *et al.*, 1999; Rivera-Amill *et al.*, 2001). In addition, secreted proteins were not detected for the *C. jejuni* flgB mutant incubated with FBS, which is consistent with previous work indicating that Cia protein secretion is dependent on a functional flagellar secretion apparatus (Konkel *et al.*, 2004). The presence of the Cia proteins in the supernatants from the wild-type strain, Cj1242 mutant, and Cj1242 complemented strain was not due to bacterial lysis, because a 32.4 kDa band was not detected in supernatants probed with the CysM antibody (Fig. 4B). Coomassie brilliant blue (CBB R-250) staining of cell lysates from the secretion assay confirmed that equivalent quantities of protein were loaded (not shown), and an autoradiograph of the dried gel demonstrated equivalent labeling of cellular proteins with $[^{35}\text{S}]$-methionine (Fig. 4C). A 32.4 kDa band was detected in the whole cell lysates with the CysM antibody (Fig. 4D). Cumulatively, these data indicate that Cj1242 (CiaC) was secreted from the flagellar T3S system.

*Cj1242* (CiaC) is required for maximal *C. jejuni* invasion of host cells

Possible differences in bacterial adhesion and invasion between the *C. jejuni* wild-type strain and Cj1242 mutant were explored by the inoculation of human INT 407 epithelial cells. Quantification of adherent (i.e., cell-associated) and intracellular bacteria by the
gentamicin-protection assay revealed that the binding of the *C. jejuni* wild-type and the *Cj1242* mutant to the INT 407 cells was indistinguishable from one another, but that the *C. jejuni Cj1242* mutant was reduced in host cell-invasion when compared to the wild-type isolate (*p* < 0.01) (Table 3). Based on the deficiency in host cell internalization, we designated the protein encoded by *Cj1242* as *Campylobacter* invasion antigen C (CiaC).
Discussion

The goal of this study was to identify a *C. jejuni* secreted protein. To accomplish this goal, we developed a screen using *Y. enterocolitica* to identify genes from *C. jejuni* that contained a T3SS amino-terminal sequence. As a first step, we showed that the full-length CiaB protein from *C. jejuni* was synthesized by *Y. enterocolitica* and exported via the flagellar T3SS. We then demonstrated that the amino-terminal sequences of the *C. jejuni* CiaB, FlaA, and FlaC proteins were sufficient to drive secretion of a YplA fusion protein from *Y. enterocolitica*. FlaA, FlaC, and CiaB proteins are known to be secreted from the *C. jejuni* flagellum (Konkel *et al.*, 2004; Song *et al.*, 2004). Collectively, these data demonstrate proof of concept for screening *C. jejuni* proteins for T3SS amino-terminal sequences using the *Y. enterocolitica* PLA plate assay. We then utilized the assay to test for the presence of T3SS amino-terminal sequences in 321 genes from *C. jejuni*. Using the criteria outlined, a total of 42 *C. jejuni* genes were identified that encode amino-terminal sequences that promoted YplA fusion secretion from *Y. enterocolitica* at levels equal to or higher than the CiaB:YplA fusion protein. One of the 42 genes identified was *Cj1242*, which we demonstrate is a potentially important virulence determinant.

While the study was in progress, information on three of the 42 *C. jejuni* proteins identified in the YplA screen was published by other research groups. These studies identified two flagellar-related proteins (FlgM, FlgJ) and a pathogenicity-related protein (FspA1). FlgM (*Cj1464*) is an anti-sigma factor involved in blocking the promoter binding activity of *σ*\(^{28}\) and the cytoplasmic levels can be controlled by secretion through
the flagellar T3SS (Hendrixson & DiRita, 2003, Wosten et al., 2004). Although the precise role of FlgJ (Cj1463) in C. jejuni is unknown, FlgJ of Salmonella enterica is a two domain protein consisting of an N-terminal domain (including the T3SS amino-terminal sequence) involved in flagellar rod formation and a C-terminal region involved in flagellar L ring and hook formation (Hirano et al., 2001; Nambu et al., 1999). Interestingly, the C. jejuni FlgJ protein contains the corresponding N-terminal region as found in other ε-proteobacteria (including H. pylori) but it lacks the C-terminal acetylmuramidase region found in most β- and γ-proteobacteria (Nambu et al., 2006; Pallen et al., 2005). FspA is a 15.5 kDa protein that is secreted from C. jejuni via the flagellum (Poly et al., 2007). Two variant forms of FspA (A1 and A2) have been identified among C. jejuni strains. FspA2 was found to associate with the host cell monolayer and induce apoptosis when added to cell culture in purified form. Validation of the YplA screen described herein lies in the finding that the amino-termini of FlaA, FlaC, CiaB, FlgM, FlgJ, and FspA all drive YplA export from Yersinia via the flagellar T3SS, whereas fusion of the amino-terminus of a known cytoplasmic protein (CysM) to YplA did not. Importantly, FlaA, FlaC, CiaB, FlgM, and FspA all contribute to C. jejuni pathogenesis.

Previous work in our laboratory has demonstrated that culturing C. jejuni with physiological concentrations of the bile acid deoxycholate (DOC) results in the upregulation of 150 genes (Malik-Kale et al., 2008). Deoxycholate is also known to induce the synthesis of the Campylobacter invasion antigens (Cia) that are secreted via the flagellar T3SS (Konkel & Cieplak, 1992; Konkel et al., 1993; Konkel et al., 2004;
Konkel et al., 1999; Rivera-Amill et al., 2001). We found that eight of the genes induced by DOC also harbor T3S amino-terminal sequences as judged by PLA plate assay. These genes are of interest because C. jejuni cultured in the presence of DOC stimulates this bacterium’s pathogenic activity, which is evidenced by an increase in the kinetics of C. jejuni-host cell invasion (Malik-Kale et al., 2008).

The ultimate goal of this study was to identify a C. jejuni Cia virulence protein. The first Cia protein (CiaB) was identified in 1999 (Konkel et al., 1999), but the remaining Cia proteins have proven difficult to identify using traditional proteomic approaches, due in part to low levels of protein secretion under in vitro conditions. We selected Cj1242 for further characterization because the Cj1242-YplA fusion protein resulted in a high level of secretion and the gene is upregulated in C. jejuni cultured with deoxycholate. We generated a Cj1242 mutant and then performed growth rate, motility, protein secretion and cell binding/internalization assays. The C. jejuni Cj1242 mutant growth rate in Mueller-Hinton (MH) broth and its motility on 0.4% agar were indistinguishable from the C. jejuni wild-type strain (not shown). The profile of secreted proteins from the C. jejuni Cj1242 mutant lacked one band of the mass predicted for the Cj1242 protein (12.2 kDa). We performed binding and internalization assays with the Cj1242 mutant and INT407 cells, and found that there was no significant difference in the adherence of this mutant to INT 407 cells relative to the C. jejuni wild-type strain. However, the gentamicin-protection assay revealed the internalization of the C. jejuni Cj1242 mutant was significantly reduced when compared to the wild-type strain (p < 0.01). Based on the deficiency in host cell internalization, we designated the protein encoded by Cj1242
as *Campylobacter* invasion antigen C (CiaC).

We consider a *C. jejuni* strain yielding a percent I/A of greater than 1 as both invasive and pathogenic, as inoculation of piglets with these strains results in clinical symptoms that resemble those of human campylobacteriosis, including diarrhea with blood in the stool (Raphael *et al.*, 2005). Inoculation of newborn piglets with *C. jejuni* wild-type strain (secretion-positive isolates) results in more severe disease when compared with a *C. jejuni ciaB* isogenic mutant (*i.e.*, deficient in secretion of all Cia proteins). Noteworthy is that the I/A ratio (*i.e.*, the percent of adherent bacteria that invade epithelial cells) for the *C. jejuni ciaC* mutant is less than 1 (I/A = 0.82%), which is similar with the *C. jejuni ciaB* mutant (I/A = 0.56%). Based on this invasion ratio, we hypothesize that the *C. jejuni ciaC* mutant (*i.e.*, deficient in secretion of one Cia protein) would also cause less severe disease than a wild-type strain. Our findings indicate that CiaC is required for *C. jejuni* to efficiently invade epithelial cells, and invasion is a virulence attribute of strains known to cause severe campylobacteriosis.

Analysis of the deduced amino acid sequences of the *C. jejuni* proteins found to harbor a putative T3S amino-terminal sequence revealed some additional information. We utilized two recently developed programs for prediction of T3S proteins (Arnold *et al.*, 2009; Löwer & Schneider, 2009) to analyze the *C. jejuni* proteins for T3 amino-terminal sequences and compare the results with our YplA fusion data. While the results were slightly different for each program, at most only 10.6 % of the 321 *C. jejuni* proteins tested via the YplA reporter assay are predicted to be secreted. In contrast, 14 of the
42 (33.3 %) C. jejuni proteins listed in Table 2 are predicted to be secreted by one or both of the prediction programs. Of interest, both algorithms predicted CiaC to be secreted, but neither predicted FlaA and CiaB to be secreted. The failure of these programs to identify known C. jejuni flagellar secreted proteins, including FlaA and CiaB, highlight the need for experimental validation of prediction algorithms.

The deduced amino sequences of two of the 42 proteins contain domains that suggest they could be localized to the cytoplasm. Cj0012c is annotated as ruberythrin, a protein that protects against oxidative stress (Mydel et al., 2006; Sztukowska et al., 2002). The amino-terminus (i.e., 36 amino acids) of Cj0012c contains a small non-heme iron domain found in the desulfoferredoxin and desulfoferrodoxin proteins of some methanogens and sulfate/sulfur reducers (Marchler-Bauer et al., 2007). Cj0363c is annotated as a putative oxidoreductase by inclusion in the cluster of an orthologous group (COG0635) for oxygen-independent coproporphyrinogen III oxidase (hemN). Noteworthy is that Cj0363c is distantly related to other proteins in COG0635C (Cj0363c, Cj0580c, and Cj0992c) and it does not reside in the vicinity of other hem cluster genes on the C. jejuni chromosome. Moreover, the predicted products of Cj0363c, Cj0580c, and Cj0992c contain a radical S-adenosylmethionine (SAM) domain. Radical SAM proteins catalyze diverse reactions, including methylation, isomerization, sulphur insertion, ring formation, anaerobic oxidation and protein radical formation. Evidence exists that these proteins generate radical species by reductive cleavage of S-adenosylmethionine (SAM) through an unusual iron-sulfur center (Sofia et al., 2001). Although there is no experimental evidence indicating the cellular localization of either
Cj0012c or Cj0363c in *C. jejuni*, these two examples highlight the need to analyze each putative T3S protein identified in our screen. It is possible that some of the genes identified using the PLA plate assay may not possess functional T3S amino-terminal sequences recognized in *C. jejuni*, or the amino-terminal region may be folded and/or inaccessible in the native protein. However, recent work also indicates that some bacteria secrete virulence proteins that were previously believed to be located solely in the cytosol (Boel *et al.*, 2005).

*C. jejuni* harbors only one T3SS, the flagellum. As a first step in the identification of a *C. jejuni* virulence protein, we sought to identify genes from *C. jejuni* that harbor T3S amino-terminal sequences that direct their export from the flagellum. We report 42 *C. jejuni* proteins with putative T3S amino-terminal sequences. Moreover, we demonstrated that a mutation in one previously uncharacterized *C. jejuni* gene, *Cj1242*, resulted in an isolate with an altered secretion profile and reduced host cell invasion.

We have also demonstrated that the secretion of CiaC is dependent upon a functional flagellar apparatus, which serves to further highlight the importance of the flagellar secretion system in the export of *C. jejuni* virulence proteins. We are currently investigating whether the other proteins identified in this study are secreted from *C. jejuni* and contribute to pathogenesis. The phospholipase reporter assay described herein demonstrates that there is a remarkable level of conservation in protein recognition among the proteobacteria; *C. jejuni* is a member of the delta-epsilon subdivision of proteobacteria and *Y. enterocolitica* is a member of the gamma subdivision. Based on this finding, we submit that the phospholipase reporter system
can be used to identify genes harboring T3SS amino-terminal sequences from a variety of bacteria that possess less-well characterized T3SS.
Experimental procedures

Bacterial strains, plasmids, and media

The bacterial strains and plasmids are described in Table 1. All *Y. enterocolitica* strains used in this study were derived from strain JB580v (Kinder *et al*., 1993). *C. jejuni* strains were cultured with Mueller-Hinton (MH) broth or agar supplemented with 5% citrated bovine blood and incubated at 37°C under microaerobic conditions (5% O$_2$, 10% CO$_2$, 85% N$_2$) with chloramphenicol (Cm, 8 µg ml$^{-1}$), kanamycin (Kan, 50 µg ml$^{-1}$), or tetracycline (Tet, 2 µg ml$^{-1}$). *E. coli* strains were cultured at 37°C with Luria-Bertani (LB) broth or agar with Cm (15 µg ml$^{-1}$), Kan (50 µg ml$^{-1}$), or Tet (15 µg ml$^{-1}$). *Y. enterocolitica* strains were incubated at 26°C in LB broth or agar supplemented with Cm (10 µg ml$^{-1}$), nalidixic acid (Nal, 20 µg ml$^{-1}$), or Tet (10 µg ml$^{-1}$).

*C. jejuni* gene selection for T3S amino-terminal sequence screen

We selected genes to screen for T3S amino-terminal sequences from the original annotation of the *C. jejuni* NCTC 11168 sequence (Parkhill *et al*., 2000). 359 of 1654 ORFs were chosen for analysis following the elimination of genes encoding proteins with known functions or containing membrane-spanning domains, periplasmic domains, Sec-dependent signals, or Tat-dependent signals. No genes were identified with known Type I Sec-independent motifs.

Recombinant DNA procedures with the *pMMB207* and *pCSP* vectors

Vector pMMB207, harboring a 1.9 kb fragment encompassing the full length *ciaB* gene, was PCR amplified from *C. jejuni* NCTC11168 chromosomal DNA using primers CiaB-
F1 (5’ GGA TCC AAA GTT AAA AAG GAG AAT AAA AGT ATG) and CiaB-R1 (5’ TTA TTT TTT CTT ATA TCT TTC AAA TTC TC). Correct orientation of the ciaB gene was determined by inducing expression from the Ptac promoter with 5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Constructs were confirmed by DNA sequencing and conjugated into the Y. enterocolitica wild-type and mutant strains.

To facilitate the identification of C. jejuni genes that harbor T3S amino-terminal sequences, the pCSP50 shuttle vector was generated. The pCSP50 vector includes a tet cassette, a constitutive promoter (cat), a 5’ truncated yplA gene (lacking 150 nucleotides encoding the native T3S amino-terminal sequence), and the yplB gene (cognate chaperone). The Ndel and BglII sites facilitated directional cloning of C. jejuni sequences as fusions with the truncated yplA. The first 108 bp of the amino-terminal regions of 328 C. jejuni genes were PCR amplified with primers containing restriction sites for directional cloning into pCSP50. The amplicons and pCSP50 vector were digested with Ndel and BglII, DNA fragments ligated, and E. coli S17-1 λ-pir was transformed with Tet selection. Cloned C. jejuni gene fragments were confirmed by PCR fragment size and sequence analysis. Vectors were conjugated into Y. enterocolitica strains and confirmed by agarose gel electrophoresis of restriction digested plasmid preparations.

Phospholipase indicator agar (PLA) assay and analysis

Medium for detecting secretion of the YplA phospholipase and YplA fusion proteins from Y. enterocolitica was prepared as described previously (Young & Young, 2002). Y.
*Enterococcal* strains were incubated overnight in LB broth with shaking at 26°C. Fop secretion was induced by spotting 1.5 µl of culture on TYE PLA medium (1% tryptone, 0.5% yeast extract, 1.5% agar, 1% Tween 80, and 1 mM CaCl$_2$), and incubation at 26°C. Each isolate was tested for secretion at least three times from at least two independent PLA plate assays to ensure reproducible results. The conjugates were tested on PLA plates in groups of 16 in addition to a *Y. enterocolitica* strain expressing wild-type YplA as a positive control. All plates were scanned at 300 dpi resolution (12, 24, and 48 hr) to create a digital archive of the secretion results. The secretion zone widths were measured manually from digital images using select tools in Adobe Photoshop CS2 version 9.0.2 (Adobe Systems Incorporated, USA). The 24 hr secretion zone widths for the positive controls were consistent for all PLA plates ($n = 22$, avg = 3.3 mm, Std. Dev. = 0.12 mm).

**Rabbit antibodies to YplA and CysM**

Polyclonal antibodies against recombinant YplA and recombinant CysM were produced in female New Zealand White rabbits by subcutaneous injection of 100 µg of the immunogens in TiterMax Gold (Sigma). Subsequent booster injections of 50 µg of the immunogens in Freund's incomplete adjuvant were administered two and four weeks after the primary immunizations. Blood was collected from the rabbits by terminal bleeds. The sera were processed and stored at −80°C. Antibody generation in the New Zealand White rabbits was performed using a protocol approved by the Institutional Animal Care and Use Committee (IACUC protocol #2433) at Washington State University.
**Determination of Fop and YplA fusion protein secretion by immunoblot**

*Y. enterocolitica* strains were incubated overnight in LB broth with shaking at 26°C. Fop secretion was induced by inoculation of TYE broth (1% tryptone, 0.5% yeast extract) with 1x TYE broth washed *Y. enterocolitica* cultures and incubation with shaking at 26°C for 4-6 hr. The OD$_{540}$ of all cultures was determined, the cells washed 1x with TYE to remove secreted proteins, and suspended in fresh TYE at an OD$_{540}$ of 0.5 for the 0 hr time-point of the secretion assay. After 2 hr of shaking at 26°C, OD$_{540}$ were determined for normalization of whole cell lysate samples, and 1 ml of each supernatant harvested by filtration through 0.22 µM sterile filters. Secreted proteins were precipitated by addition of 111 µl of 6.1 N trichloroacetic acid (10% vol vol$^{-1}$ TCA final), minimum of 1 hr incubation at -20°C, and centrifugation with two acetone washes. Precipitated proteins were dissolved in 50 µl of single-strength electrophoresis sample buffer and heated to 95°C for 5 min. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (12% polyacrylamide) SDS-PAGE with the discontinuous buffer system described by Laemmli (Laemmli, 1970). The proteins were electrophoretically transferred to polyvinylidene fluoride membranes (PVDF) (Immobilon P; Millipore Corp., Bedford, MA) for immunoblot analysis. Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G or peroxidase-conjugated goat anti-mouse immunoglobulin G. Immunoblot development was done by chemiluminescence (Western Lightning, PerkinElmer Life Sciences, Inc.) and film exposure (Biomax MR film, Kodak).
**Generation of the C. jejuni Cj1242 deletion mutant and complement strain**

The Cj1242 gene was disrupted by homologous recombination between the disrupted Cj1242 gene on a suicide vector and the Cj1242 gene in the chromosome. The Cj1242 gene on the suicide vector had been disrupted by insertion of a TetO cassette as outlined below. A 900 base pair fragment upstream of the C. jejuni F38011 Cj1242 gene was amplified using the primers Cj1242F1Sstl (5’ TTG AGC TCG CTC TAG CTA TAA TGG TCA CAG) and Cj1242R1SstII (5’ AAC CGC GGC ATT TGA TGT TTT TTG AGT ATT ATC) and cloned into the pCR2.1 cloning vector (TA cloning system; Invitrogen) as outline by the supplier. An 806 base pair fragment downstream of the Cj1242 gene was amplified using the primers Cj1242F2SstII (5’ TTC CGC GGA CTT CGG CAG ATG AAT TTC AAG) and Cj1242R2XhoI (5’ AAC TCG AGG TAA GCT TTA AGG CAT CAT AGA C) and cloned into a separate pCR2.1 cloning vector. The upstream fragment was then restriction digested with SstI and SstII, gel purified, and ligated into the pCR2.1 cloning vector harboring the downstream fragment. A 2.4 kb TetO cassette was amplified from pUOA3 (Taylor et al., 1987) with primers containing SstII sites and cloned into the SstII site of the pCR2.1 Cj1242 construct. The resultant 4.1 kb insert was then excised by SstI and XhoI restriction digest, gel purified, and ligated into pBSK-Kan2. The resultant suicide vector was sequence confirmed and electroporated into the C. jejuni F38011 isolate. Transformants were selected on MH blood agar containing Tet 2 µg ml⁻¹. Tet resistant isolates were screened for Kan sensitivity, indicating a double-crossover homologous recombination event and loss of the suicide vector. Tet cassette integration into the C. jejuni Cj1242 gene was confirmed by PCR.
Construction of a complementation vector for the Cj1242 gene was accomplished by cloning a PCR product obtained with primers Cj1242F1SstI and Cj1242R2XhoI. The 1.7 kb amplicon encompassing Cj1242 was digested with SstI and XhoI, gel purified, and ligated into shuttle vector pRY111. The resultant pRY111:Cj1242 complementation vector was sequence confirmed and electroporated into the C. jejuni F38011 wild-type strain. Transformants were selected on MH blood agar containing Cm 8 μg ml\(^{-1}\) and presence of the vector encoded copy of Cj1242 was confirmed by PCR.

**INT 407 cell-adherence and internalization assays**

A stock culture of INT 407 cells (human embryonic intestine, ATCC CCL 6) was obtained from the American Type Culture Collection. The cells were cultured in MEM supplemented with 10% FBS at 37°C in a humidified, 5% CO\(_2\) incubator. The day prior to an assay, each well of a 24-well tissue culture tray was seeded with 1.5 X 10\(^5\) cells and incubated for 18 hr at 37°C in a humidified, 5% CO\(_2\) incubator. The following day, the cells were rinsed with MEM-1% FBS and inoculated with approximately 5 X 10\(^7\) CFU of a bacterial suspension. The tissue culture trays were centrifuged at 600 x g for 5 min to promote bacteria-host cell contact, and incubated at 37°C in a humidified, 5% CO\(_2\) incubator. For the adherence assays, the plates were incubated for 30 min. The cells monolayers were then rinsed 3 times with PBS, epithelial cells lysed with a solution of 0.1% (vol vol\(^{-1}\)) Triton X-100 (Calbiochem, La Jollo, CA), and bacterial suspensions were serially diluted and spread onto MH blood plates. The number of viable, adherent bacteria was determined by counting the resultant colonies. To assess bacterial
internalization, the inoculated cell monolayers were incubated for 3 hr, rinsed 3 times with MEM-1% FBS, and incubated for an additional 3 hr in MEM-1% FBS containing a bactericidal concentration of gentamicin (250 µg ml\(^{-1}\)). The number of internalized bacteria was then determined as outlined above for the adherence assays. The reported values represent the mean counts ± standard deviations derived from triplicate wells. All assays in this study were repeated a minimum of 3 times to ensure reproducibility and performed at a multiplicity of infection (MOI) between 50 and 500. Regardless of the MOI, the phenotype of the \textit{C. jejuni Cj1242} mutant relative to the wild-type strain was always the same.

\textit{C. jejuni secretion assay}

The \textit{C. jejuni} F38011 strain and isogenic \textit{Cj1242} mutant was metabolically labeled with \(^{35}\text{S}\)-methionine as described elsewhere (Konkel & Cieplak, 1992). Briefly, isolates were harvested from biphasic culture on MH agar supplemented with 0.1% deoxycholate and resuspended in MEM lacking methionine supplemented with or without dialyzed albumin depleted fetal bovine serum (FBS) to an \(\text{OD}_{540} = 0.3\). \(^{35}\text{S}\)-methionine was then added and inocula were incubated at 37°C for 3 hrs under microaerophilic conditions. After incubation, supernatant fluids were concentrated ten-fold by precipitation with four volumes of ice-cold 1 mM HCl-acetone. The pellets were air dried and dissolved in an equal amount of water and double strength sample buffer. Equal volumes of the concentrated samples were subjected to 12% SDS-PAGE. The gel was dried, exposed to film for five days, and developed to acquire the auroradiograph.
*Bioinformatics*

Operon and regulon prediction was performed by query of the MicrobesOnline site (Alm *et al.*, 2005). In silico Type III secretion protein prediction was performed using “EffectiveT3” ([http://www.chlamydiadb.org](http://www.chlamydiadb.org); Arnold *et al.*, 2009) and “Modlab” software ([http://gecco.org.chemie.uni-frankfurt.de/index.html](http://gecco.org.chemie.uni-frankfurt.de/index.html); Löwer & Schneider, 2009).
Acknowledgements

We thank Dr. Glenn M. Young (University of California-Davis) for providing the Y. enterocolitica strains, Dr. Scott A. Minnich (University of Idaho-Moscow) for providing the mouse monoclonal flagellin specific antibody 15D8, Dr. William G. Miller (US Department of Agriculture, Albany, CA) for sequence analysis of the C. jejuni NCTC 11168 genome, Rebecca C. Flanagan for performing INT 407 cell binding and internalization assays, and Dr. Daelynn Buelow for performing C. jejuni secretion assays. We also thank Dr. Buelow, Charles L. Larson, Jason M. Neal-McKinney for critical review of the manuscript.

This work was supported from funds awarded to MEK from the National Institute of Health, Department of Health and Human Services under contract number NO1-AI-30055.
References


Figure Legends

Fig. 1. The C. jejuni CiaB protein is secreted via the Y. enterocolitica flagellar T3SS. Supernatants (Panels A-D) and whole cell lysates (Panels E and F) were analyzed by SDS-PAGE coupled with silver staining or immunoblot analysis. Panels: A) Silver stain showing the flagellar outer proteins (Fops) and FleABC; B) Immunoblot probed with the flagellin antibody (FleABC, 38-40 kDa); C) Immunoblot probed with the CiaB antibody (CiaB, 73 kDa); D) Immunoblot probed with the RNA polymerase $\sigma^{70}$ antibody; E) Immunoblot probed with the CiaB antibody; and F) Immunoblot probed with the $\sigma^{70}$ antibody. Lanes: 1, Y. enterocolitica wild-type harboring the empty pMMB207 vector (WT); 2, Y. enterocolitica pYV8081-ΔflhDC ysaT flagellar mutant harboring the empty pMMB207 vector (ΔT3SS); 3, Y. enterocolitica wild-type harboring the pMMB207 vector containing the C. jejuni ciaB gene (WT + ciaB); and 4, Y. enterocolitica pYV8081-ΔflhDC ysaT flagellar mutant harboring the pMMB207 vector containing the C. jejuni ciaB gene (ΔT3SS + ciaB).
**Fig. 2.** The pCSP50 shuttle vector. The Ndel and BgIII sites flank the 5’ end of a truncated *yplA* gene and facilitate directional cloning to generate fusions with 108 bp amino-terminal sequences from *C. jejuni* genes.
Fig. 3. Secretion of YplA fusion proteins under flagellar T3SS inducing conditions. The first 36 amino acids of each indicated protein was fused to YplA encoded on vector pCSP50. The YplA secretion zone widths (mm) were measured from the edge of the bacterial growth to the outer edge of the fatty acid precipitate. Detection of the YplA fusion protein by immunoblot analysis was done with cultures grown under flagellar inducing conditions (lanes marked “+”) and non-inducing conditions (lanes marked “-”). Panels: A) A representative PLA assay is shown indicating YplA fusion proteins and secretion zone widths; B) Immunoblot analysis of supernatants probed with the YplA antibody; C) Immunoblot analysis of supernatants probed with the RNA polymerase $\sigma^{70}$ antibody; D) Immunoblot analysis of whole cell lysates probed with the YplA antibody; and E) Immunoblot analysis of whole cell lysates probed with a $\sigma^{70}$ antibody.
A) Secretion (mm) for various proteins:

<table>
<thead>
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<th>Protein</th>
<th>Secretion (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>YplA</td>
<td>2.4</td>
</tr>
<tr>
<td>FiaA</td>
<td>1.7</td>
</tr>
<tr>
<td>FiaC</td>
<td>2.8</td>
</tr>
<tr>
<td>CiaB</td>
<td>2.4</td>
</tr>
<tr>
<td>FspA1</td>
<td>3.6</td>
</tr>
<tr>
<td>FlgJ</td>
<td>3.1</td>
</tr>
<tr>
<td>FlgM</td>
<td>3.2</td>
</tr>
<tr>
<td>CysM</td>
<td>0.0</td>
</tr>
<tr>
<td>pCSP50</td>
<td>0.0</td>
</tr>
</tbody>
</table>

B) Flagellar induction for YplA:

- (+) indicates induction
- (-) indicates no induction

C) Western blot analysis of YplA and σ70:

- YplA: 40-15 kDa
- σ70: 50 kDa

D) Western blot analysis of YplA and σ70 truncated:

- Δ2-50 AA: 40-15 kDa

E) Western blot analysis of YplA and σ70:

- Δ2-50 AA: 40-15 kDa
**Fig. 4.** Secretion of Cj1242 (CiaC) from the *C. jejuni* flagellar T3SS. Isolates were incubated in medium containing $[^{35}\text{S}]-\text{methionine}$ and supplemented with 1% FBS or without FBS as described in 'Materials and Methods.' Supernatants (Panels A and B) and whole cell lysates (Panels C and D) were analyzed by SDS-PAGE coupled with autoradiography and immunoblot analysis. Panels: A) Autoradiograph of supernatant samples; CiaC (12.2 kDa) protein is indicated by an arrowhead; B) Immunoblot of supernatant samples probed with the CysM antibody; C) Autoradiograph of whole cell lysates; D) Immunoblot of whole cell lysates probed with the CysM antibody (32.4 kDa). Lanes: 1) *C. jejuni* F38011 wild-type with 1% FBS; 2) *C. jejuni* F38011 Cj1242 mutant with 1% FBS; 3) *C. jejuni* F38011 Cj1242 mutant complemented with pRY111:Cj1242 with 1% FBS; 4) *C. jejuni* F38011 flgB mutant with 1% FBS; 5) *C. jejuni* F38011 wild-type without FBS.
<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype</th>
<th>Source or Reference</th>
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</thead>
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<tr>
<td><strong>C. jejuni</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCTC 11168</td>
<td>Wild-type genome sequenced strain</td>
<td>(Parkhill <em>et al.</em>, 2000)</td>
</tr>
<tr>
<td>F38011</td>
<td>Clinical isolate</td>
<td>(Rivera-Amill &amp; Konkel, 1999)</td>
</tr>
<tr>
<td>F38011 <em>flgB</em></td>
<td>Insertion disruption of <em>flgB</em>, non-motile and Cia protein secretion deficient</td>
<td>(Konkel <em>et al.</em>, 2004)</td>
</tr>
<tr>
<td><strong>Y. enterocolitica</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JB580v</td>
<td>Serogroup O:8, Nal yenR (r- m+)</td>
<td>(Kinder <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>GY4478</td>
<td>JB580v, pYV8081-</td>
<td>(Young &amp; Young, 2002)</td>
</tr>
<tr>
<td>GY4757</td>
<td>JB580v ∆yplAB, pYV8081-</td>
<td>(Warren &amp; Young, 2005)</td>
</tr>
<tr>
<td>GY4492</td>
<td>JB580v ∆<em>flhDC</em> ysaT::TnMod-RKm, pYV8081-</td>
<td>(Young &amp; Young, 2002)</td>
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<tr>
<td><strong>Escherichia coli</strong></td>
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<td></td>
</tr>
<tr>
<td>S17-1 lambda <em>pir</em></td>
<td><em>recA</em> <em>thi pro hsdR</em>- M+ RP4::2-Tc::Mu::Km Tn7 <em>pir</em></td>
<td>(Simon <em>et al.</em>, 1983)</td>
</tr>
<tr>
<td>Inv-alpha <em>F'</em></td>
<td>F' <em>endA1</em> <em>recA1</em> <em>hsdR17</em> (r-, m+) <em>supE44</em> <em>thi-1</em> <em>gyrA96</em> <em>relA1</em> <em>f80lacZDM15</em> D(lacZYA-argF) U169I-</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>XL1-Blue</td>
<td><em>recA1</em> <em>endA1</em> <em>gyrA96</em> <em>thi-1</em> <em>hsdR17</em> <em>supE44</em> <em>relA1</em> <em>lac</em> (F' <em>proAB</em> <em>lacZΔM15</em> Tn10)</td>
<td>Stratagene</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pMMB207</td>
<td><em>mob</em>+, low copy vector containing an inducible <em>tac</em> promoter (Ptac), Cm</td>
<td>(Morales <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td>pMEK250</td>
<td>pMMB207 harboring the full-length 1.9 kb <em>ciaB</em> gene driven by <em>Ptac</em></td>
<td>This study</td>
</tr>
<tr>
<td>pTM100</td>
<td><em>mob</em>+, derivative of pACYC184, Cm Tet</td>
<td>(Michiels &amp; Cornelis, 1991)</td>
</tr>
<tr>
<td>pCSP50</td>
<td><em>P</em>cat upstream of <em>NdeI</em> and <em>BglII</em> sites for directional cloning of fusions with 5’ truncated <em>yplA</em> (lacking nucleotides 4-150) and complete <em>yplB</em> locus cloned into pTM100 <em>EcoRI</em> site, Tet</td>
<td>This study</td>
</tr>
<tr>
<td>pCSP50-<em>yplA</em> 1-108</td>
<td>Nucleotides 1-108 of <em>yplA</em> fused to truncated <em>yplA</em> in pCSP50</td>
<td>This study</td>
</tr>
<tr>
<td>pCSP50-<em>flaA</em> 1-108</td>
<td>Nucleotides 1-108 of <em>flaA</em> (Cj1339c) fused to</td>
<td>This study</td>
</tr>
<tr>
<td>Vector Name</td>
<td>Description</td>
<td>Source</td>
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<td>------------</td>
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<tr>
<td>pCSP50-flaC 1-108</td>
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</tr>
<tr>
<td>pCSP50-ciaB 1-108</td>
<td>Nucleotides 1-108 of ciaB fused to truncated yplA in pCSP50</td>
<td>This study</td>
</tr>
<tr>
<td>pCSP50-cysM 1-108</td>
<td>Nucleotides 1-108 of cysM fused to truncated yplA in pCSP50</td>
<td>This study</td>
</tr>
<tr>
<td>pBluescript II SK+</td>
<td>Phagemid cloning vector</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pMW10</td>
<td>C. jejuni–E. coli shuttle vector, Kan</td>
<td>(Wosten et al., 1998)</td>
</tr>
<tr>
<td>pBSK-Kan2</td>
<td>pBluescript II SK+ with original ampicillin cassette replaced by the native promoter and apha3 gene from pMW10, Kan</td>
<td>This study</td>
</tr>
<tr>
<td>pBSK-Kan2:delCj1242</td>
<td>pBSK-Kan2 with Cj1242 internal deletion and disrupted with tetO from pUOA3, Kan Tet</td>
<td>This study</td>
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<td>pRY111</td>
<td>C. jejuni–E. coli shuttle vector, pWKS29 MCS Cm</td>
<td>(Yao et al., 1993)</td>
</tr>
<tr>
<td>pRY111:Cj1242</td>
<td>pRY111 with a 1.7 kb fragment encompassing Cj1242, Cm</td>
<td>This study</td>
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### Table 2. *C. jejuni* genes encoding a putative T3S amino-terminal sequence

<table>
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<tr>
<th>Gene</th>
<th>Locus</th>
<th>Product description</th>
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<tbody>
<tr>
<td><em>flgM</em> B, D</td>
<td>Cj1464</td>
<td>anti-sigma 28 factor</td>
</tr>
<tr>
<td><em>fspA1</em> B, E</td>
<td>Cj0859c</td>
<td>Flagellar secreted protein, virulence factor</td>
</tr>
<tr>
<td><em>rrc</em> B</td>
<td>Cj0012c</td>
<td>non-haem iron protein, rubrerythrin</td>
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<tr>
<td>Cj0036</td>
<td>Cj0036</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>Cj1242 C</td>
<td>Cj1242</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td><em>flgJ</em> B, C, F</td>
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<td>flagellar rod protein</td>
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<td>conserved hypothetical protein</td>
</tr>
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<td>Cj0122</td>
<td>hypothetical protein</td>
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<td>hypothetical protein</td>
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<td>Cj0140</td>
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<td>Cj0239c</td>
<td>NifU protein homolog</td>
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<td>Cj0251c</td>
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<td>Cj0787 C</td>
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<tr>
<td>Cj0788 C</td>
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<td>hypothetical protein</td>
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<td>Cj0021c</td>
<td>putative fumarylacetoacetate (FAA) hydrolase family protein</td>
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<td>Cj0030</td>
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<tr>
<td><em>hemN</em></td>
<td>Cj0363c</td>
<td>putative oxygen-independent coproporphyrinogen III oxidase</td>
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<td>Cj0416</td>
<td>Cj0416</td>
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<td>Cj0849c</td>
<td>Cj0849c</td>
<td>conserved hypothetical protein</td>
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<td>Cj1300 B</td>
<td>Cj1300</td>
<td>putative SAM domain containing methyltransferase</td>
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<td>Cj1543 B</td>
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<td>putative allophanate hydrolase subunit 2</td>
</tr>
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<td>Cj0188c C</td>
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<td>putative kinase</td>
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<td>putative ArsC family protein</td>
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<td>Cj1310c</td>
<td>Cj1310c</td>
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<tr>
<td>Cj0916c</td>
<td>Cj0916c</td>
<td>conserved hypothetical protein</td>
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</table>
Cj1162c B  Cj1162c  putative heavy-metal-associated domain protein
Cj1232  Cj1232  hypothetical protein
Cj1505c B  Cj1505c  putative two-component response regulator (SirA-like protein)

A:  C. jejuni gene yplA fusions with a secretion zone width greater than or equal to the zone obtained for theciaB:yplA fusion strain. Listed in descending order of secretion zone width; ascending locus number for equivalent zones.
B:  Not annotated in original NCTC 11168 sequence analysis (Parkhill et al., 2000)
C:  Upregulated when grown in the presence of deoxycholate (Malik-Kale et al., 2008)
D:  (Hendrixson & DiRita, 2003; Wosten et al., 2004)
E:  (Poly et al., 2007)
F:  (Pallen et al., 2005)
Table 3. Binding and internalization of the *C. jejuni* wild-type strain and isogenic mutants.

<table>
<thead>
<tr>
<th>Bacterial Strain</th>
<th>Numbers of viable bacteria</th>
<th>I/A&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adherent</td>
<td>Internalized</td>
</tr>
<tr>
<td><em>C. jejuni</em> wild-type</td>
<td>$(7.1 \pm 0.6) \times 10^5$</td>
<td>$(3.3 \pm 0.5) \times 10^4$</td>
</tr>
<tr>
<td><em>C. jejuni</em> Cj1242</td>
<td>$(7.6 \pm 1.2) \times 10^5$</td>
<td>$(6.2 \pm 1.9) \times 10^3$</td>
</tr>
<tr>
<td><em>C. jejuni</em> ciaB</td>
<td>$(7.2 \pm 1.2) \times 10^5$</td>
<td>$(4.0 \pm 0.6) \times 10^3$</td>
</tr>
<tr>
<td><em>E. coli</em> XL1-Blue</td>
<td>$(1.8 \pm 0.4) \times 10^5$</td>
<td>$(1.7 \pm 1.2) \times 10^2$</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percent of internalized bacteria relative to adherent bacteria.

<sup>b</sup>Internalization of the *C. jejuni* Cj1242 and *C. jejuni* ciaB mutants was significantly different from the wild-type strain ($p < 0.01$) as judged by analysis using unpaired Student's *t*-tests.
**Table S1.**  *C. jejuni* genes screened for secretion as *yplA* fusions

<table>
<thead>
<tr>
<th>Gene</th>
<th>Locus</th>
<th>Product (Gundogdu <em>et al.</em>, 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>rrc</em></td>
<td>Cj0012c</td>
<td>non-haem iron protein</td>
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<td>Cj0015c</td>
<td>Cj0015c</td>
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<td><em>dbα</em></td>
<td>Cj0018c</td>
<td>disulphide bond formation protein</td>
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<td>Cj0021c</td>
<td>Cj0021c</td>
<td>putative fumarylacetoacetate (FAA) hydrolase family protein</td>
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<tr>
<td><em>thYX</em></td>
<td>Cj0026c</td>
<td>thymidylate synthase</td>
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<td>Cj0030</td>
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<td>hypothetical protein</td>
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<tr>
<td>Cj0038c</td>
<td>Cj0038c</td>
<td>putative poly(A) polymerase family protein</td>
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<td>Cj0040</td>
<td>hypothetical protein</td>
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<tr>
<td><em>fliK</em></td>
<td>Cj0041</td>
<td>putative flagellar hook-length control protein</td>
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<td>Cj0044c</td>
<td>hypothetical protein</td>
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<td>Cj0045c</td>
<td>putative iron-binding protein</td>
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<td>Cj0054c</td>
<td>Cj0054c</td>
<td>putative lysine decarboxylase family protein</td>
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<td>Cj0062c</td>
<td>Cj0062c</td>
<td>putative integral membrane protein</td>
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Cj0463  zinc protease-like protein
ctb  group III truncated haemoglobin
dapA  putative dihydrodipicolinate synthase
Cj0485  putative oxidoreductase
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Cj0496  hypothetical protein
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Cj0500  putative rhodanese-like domain protein
Cj0504c  putative oxidoreductase
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Cj0519  putative rhodanese-like domain protein
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ubiD  putative 3-octaprenyl-4-hydroxybenzoate carboxy-lyase
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Cj0556  putative amidohydrolase family protein
Cj0559  putative pyridine nucleotide-disulphide oxidoreductase
Cj0563  hypothetical protein
Cj0571  hypothetical protein
Cj0573  putative GatB/Yqey family protein
nudH  putative NUDIX hydrolase family protein
Cj0583  hypothetical protein
Cj0600  hypothetical protein
Cj0602c  MOSC-domain containing protein
Cj0604  putative polyphosphate kinase
Cj0611c  putative acyltransferase family protein
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Cj0621  hypothetical protein
holA  putative DNA polymerase III, delta subunit
Cj0635  putative Holliday junction resolvase
Cj0636  NOL1\NOP2\sun family protein
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Cj0644  putative TatD-related deoxyribonuclease protein
Cj0647  putative HAD-superfamily hydrolase
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Cj0954c Cj0954c  putative dnaJ-like protein
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Cj0962 Cj0962  putative acetyltransferase
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Cj1309c Cj1309c  hypothetical protein
Cj1310c Cj1310c  hypothetical protein Cj1310c (617 family)
maf3    Cj1334  (Motility accessory factor, function unknown)
maf4    Cj1335  hypothetical protein Cj1335 (1318 family)
pseE/maf5 Cj1337  hypothetical protein
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Cj1349c Cj1349c  possible fibronectin/fibrinogen-binding protein
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Cj1367c Cj1367c  possible nucleotidyltransferase
Cj1370  Cj1370  putative nucleotide phosphoribosyltransferase
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Cj1383c Cj1383c  hypothetical protein
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Cj1397  Cj1397  putative ferrous iron transport protein
nadD    Cj1404  putative nicotinate-nucleotide adenylyltransferase
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tilS    Cj1453c  putative tRNA(Ile)-lysidine synthase
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Cj1543  Cj1543  putative allophanate hydrolase subunit 2
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Cj1679  Cj1679  hypothetical protein
Cj1710c Cj1710c putative metallo-beta-lactamase family protein
Cj1712  Cj1712  hypothetical protein
Cj1713  Cj1713  putative radical SAM domain protein
Cj1714  Cj1714  small hydrophobic protein
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<th>Cj1715</th>
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<td>Cj1724c</td>
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<td>putative GTP cyclohydrolase I</td>
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CHAPTER 3

CAMPYLOBACTER JEJUNI SECRETES PROTEINS VIA THE FLAGELLAR
TYPE III SECRETION SYSTEM THAT CONTRIBUTE TO HOST CELL INVASION
AND GASTROENTERITIS

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CHAPTER 3 ATTRIBUTION PAGE

All manuscripts included in this thesis represent the culmination of work by a number of people, some of who are listed as authors in addition to myself. The following is a general summary of my contribution to each manuscript as listed by chapter.

Ideas for the book chapter were conceived by Dr. Konkel, Dr. Jeffrey Christensen, and Charles Larson. I assisted in editing and formatting. My contribution to the article was generating the *C. jejuni* Cia secretion profile (Figure 3). I also provided information and draft revisions for the cartoon image of the *C. jejuni* flagellum (Figure 4). The actual image was generated by Mark Mikelsen (Technical Services, WSU).
ABSTRACT

_Campylobacter jejuni_, a flagellated, spiral-shaped, Gram-negative bacterium, is a member of the delta-epsilon group of proteobacteria and a frequent cause of gastrointestinal disease in humans. Gram-negative bacteria have evolved at least six distinct pathways to actively transport proteins across their membranes. The secretion systems are defined by the characteristics of the secreted substrates. Type III secretion system (T3SS) substrates share the following characteristics: 1) the absence of a Sec-dependent signal sequence; 2) an amino terminal signal that facilitates secretion; and 3) export through a specialized conduit spanning the bacterial inner and outer membranes. Based on the properties of the secreted proteins (substrates), the flagellum represents a T3SS. In this chapter we present a model of _C. jejuni_-mediated enteritis, review the literature indicating that _C. jejuni_ utilizes its flagellum as an export apparatus for T3SS virulence substrates, and discuss the potential contribution of secreted proteins in the development of _C. jejuni_-mediated enteritis. Published work indicates that _C. jejuni_ secrete proteins via the flagellar T3SS that contribute to host cell invasion and apoptosis. Moreover, inoculation of newborn piglets with _C. jejuni_ secretion-positive isolates results in more severe disease when compared with secretion-deficient isolates. The identification and functional characterization of the secreted proteins will shed new light on the virulence mechanisms employed by _C. jejuni_ to survive and multiply within a human host, and refine the model of _C. jejuni_ pathogenesis.
I. INTRODUCTION

*Campylobacter jejuni* is a leading bacterial cause of gastroenteritis worldwide. The clinical presentation of *C. jejuni*-mediated disease varies in symptoms, severity, and duration. The spectrum of disease observed in infected individuals likely results from differences in *C. jejuni* strain virulence and host immunity. While research indicates that *C. jejuni* strains have differences in gene content and expression, *C. jejuni* virulence requires motility, host (target) cell adherence, host cell invasion, alteration of host cell signaling pathways, induction of host cell death, evasion of host immune defenses, iron acquisition, and drug/detergent resistance. This list is not comprehensive, but rather illustrates our belief that *C. jejuni* disease occurs in a susceptible host from a combination of virulence attributes working in concert. We propose that the most severe form of disease, which is characterized by fever, severe abdominal cramps, and diarrhea containing blood and leukocytes, involves *C. jejuni* invasion of the intestinal epithelium. In the context of bacteria-host cell invasion, we have found that *C. jejuni* secrete proteins, and that the secreted proteins contribute to the organism’s ability to maximally invade epithelial cells.

The body of this chapter is divided into three major sections. In the first section we present a model of *C. jejuni*-mediated enteritis. The second section presents a general overview of the organism’s pathogenic mechanisms and virulence determinants. Finally, in the third section, we discuss various aspects of *C. jejuni*-host cell invasion and protein secretion. Specifically, in this section we discuss *C. jejuni* protein export via the flagellar type III secretion system (T3SS), the development of an assay to identify *C.
C. jejuni secreted proteins, the evolutionary relatedness of the flagellum and virulence T3SS, and the putative roles of C. jejuni secreted proteins in disease. While much remains unknown regarding the identity and functional characteristics of the proteins exported via the flagellar apparatus, we will highlight evidence supporting the proposal that these proteins contribute to C. jejuni-mediated enteritis.

II. MODEL OF Campylobacter jejuni-MEDIATED ENTERITIS

Figure 1 illustrates our model of C. jejuni pathogenesis. An accumulation of events during the C. jejuni–host interaction produce the clinical manifestation of C. jejuni infection. Although the exact sequence of events that occur between colonization of the host intestine and C. jejuni-mediated enteritis are unclear, we have incorporated knowledge from different sources to produce a disease model.

The model presented herein describes severe cases of C. jejuni infection, in which individuals exhibit fever, abdominal cramps and diarrhea containing blood and leukocytes (Figure 1). Clearly, this is not the only disease course resulting from C. jejuni infections. Individuals infected with C. jejuni may experience mild diarrhea symptoms, or they may develop post-infection sequelae such as Guillain-Barré Syndrome (GBS) or irritable bowel syndrome (Schwerer, 2002, Thornley et al., 2001). Clinical manifestations of C. jejuni infection, other than the acute disease state described herein, require separate models. In essence, our model is a platform to analyze the specific C. jejuni virulence factors involved during various stages of acute infection.
Early infection: Key roles for virulence factors

Ingestion of viable *C. jejuni* is the first step of the infectious process. Individuals most often acquire *C. jejuni* infection from the consumption of foods cross-contaminated with raw or undercooked poultry products; however milk, eggs, untreated water, and contact with animals colonized with *C. jejuni* have also been implicated as sources of infection (Friedman *et al.*, 2004, Gillespie *et al.*, 2006). The number of *C. jejuni* required to infect an individual varies significantly. One study revealed that 800 organisms were sufficient to cause disease in 50% of the individuals inoculated, while approximately $10^8$ bacteria were required to infect 100% of a population. Moreover, doses greater than 800 organisms did not produce a more severe disease or increase duration (Black *et al.*, 1988). The factors that determine the infective dose of *C. jejuni* are not well defined, however, the pathogenicity of the *C. jejuni* strain and the host immune response are both important determinants.

*C. jejuni* undergoes an adaptive response in which global changes in gene expression occur that facilitate survival in and infection of the host (Andersen *et al.*, 2005, Hendrixson, 2006, Lin *et al.*, 2005, Malik-Kale *et al.*, 2007). Passage of the organism through the gastrointestinal tract triggers the expression of genes encoding virulence factors that help establish infection, including an efflux pump that confers resistance to bile salts and antibiotics (Lin *et al.*, 2005, Lin *et al.*, 2002, Raphael *et al.*, 2005). Once through the stomach and duodenum, *C. jejuni* localizes to the jejunum/ileum of the small intestine and occupies specific niches within the host intestinal epithelium in order to
resist the peristaltic forces and flushing of the small intestine. *C. jejuni* responds to the environment, and in a directed manner, the flagellum drives the bacterium through the viscous mucosal layer covering the epithelium where it adheres to the host cell surface. Debate remains regarding the specific cell types within the ileum/jejunum that *C. jejuni* is localized, but studies have indicated targeting of the glandular crypts and M cells (Everest *et al.*, 1993a, Everest *et al.*, 1993b, Walker *et al.*, 1988). Regardless, *C. jejuni* is able to survive, replicate, and interact with host epithelial cells once it traverses the mucosal layer.

Motility is pivotal throughout *C. jejuni* infection. Flagellar locomotion is controlled by chemotaxis and quorum sensing mechanisms (Jeon *et al.*, 2003, Yao *et al.*, 1997). During the initial stages of infection, bacterial motility promotes access to the apical and basolateral surfaces of host cells. Upon bacteria-host cell contact, flagellar activity may be modulated to maintain adherence to host cell surfaces and secrete proteins that contribute to infection. The binding of *C. jejuni* adhesins to specific host cell ligands, which are concentrated on the basolateral surface of the epithelium, facilitates maximal bacteria-host cell invasion (Chen *et al.*, 2006, Monteville and Konkel, 2002). Establishment of adherence facilitates the invasive and cytotoxic activities of *C. jejuni* responsible for the manifestation of enteric disease. The initiation and establishment of *C. jejuni* infection involves adherence, protein secretion, and invasion, which can stimulate the host cell inflammatory response and, in turn, promote additional bacteria-host cell interactions (Figure 2).
C. jejuni appears to have properties that diminish its recognition by immune system and aid in establishing a niche in the host. While the C. jejuni FlaA and FlaB filament proteins are highly immunogenic, the monomers comprising the filament lack the recognized TLR-5 consensus domain of other enteric pathogens. As such, the C. jejuni flagellin proteins do not trigger IL-8 secretion (Ramos et al., 2004). In addition, the C. jejuni flagellar filament is heavily glycosylated with pseudaminic acid, containing more glycan modifications than any other known bacterial proteins (see chapter by Logan and colleagues) (Guerry et al., 2006). Glycosylation of outer surface proteins by mucosal pathogens contributes to protection against proteolytic cleavage, provides antigenic variation, and aids in immune evasion (Szymanski and Wren, 2005). It is also interesting that the genes involved in flagellar biosynthesis and glycosylation contain numerous polymorphisms amongst C. jejuni strains, resulting in antigenic diversity (Szymanski et al., 2003).

Late infection

During late infection the disruption of tight junctions, alteration of net water flow, and induction of premature apoptosis (or necrosis) contribute to the severe disease state. These consequences are likely due to a combination of C. jejuni-host cell interactions that include adherence, invasion, and secretion of bacterial effector proteins. The disruption of tight junctions during bacterial infections may also result from host immune processes (Chen et al., 2006, MacCallum et al., 2005, Perdomo et al., 1994b, Sansonetti et al., 1999). Although the precise virulence attributes that contribute to this stage of disease remain to be defined, the net result is severe gastroenteritis.
As mentioned previously, *C. jejuni* binds and invades more efficiently from the basolateral surface of cells, suggesting localization to the lamina propria may be an important step in infection. M-cell adsorption, demonstrated with the rabbit ileal loop model, is one route that *C. jejuni* can pass through the epithelium into the lamina propria (Walker *et al.*, 1988). *In vitro* observations indicate transepithelial migration is accompanied by tight junction alteration (Chen *et al.*, 2006, MacCallum *et al.*, 2005, Monteville and Konkel, 2002, Walker *et al.*, 1988). Tight junctions connect adjacent epithelial cells separating the apical and basolateral surfaces, thus creating a barrier that impedes bacteria in the lumen from entering the lamina propria. In addition, tight junctions provide a fencing function, which serve to limit receptors (integrins, focal adhesions, etc.) to the basolateral surface of the epithelium (Blikslager *et al.*, 2007). Tight junctions are an important facet of the gut innate immune defense, and their disruption would result in two processes that could potentiate *C. jejuni* infection. First, their disruption would allow a bacterium increased access to the lamina propria and to bind the basolateral surfaces of host cells, which might further increase host cell invasion. Second, the loss of fencing function provided by tight junctions would allow host membrane receptors to migrate to the apical surfaces of cells, thereby facilitating additional bacterial adherence/invasion directly from the lumen.

The influx of fluid and professional phagocytes during *C. jejuni* infection could result in the disruption of tight junctions (Black *et al.*, 1988, Blaser *et al.*, 1983, Gillespie *et al.*, 2006). During the inflammatory response, neutrophils recruited by the release of
cytokines migrate across the intestinal epithelium and disrupt tight junctions. In this process, host ligands like fibronectin and integrin receptors become available at the apical surface of the cell, thereby facilitating adherence and invasion. In addition to loss of fencing function, temporary lesions created by neutrophil transepithelial migration may provide a path for *C. jejuni* to enter the lamina propria. Evidence of this phenomenon is observed during *Shigella* infection (Perdomo et al., 1994a, Perdomo et al., 1994b).

Prior to the manifestation of diarrhea, individuals infected with *C. jejuni* are often febrile. The increased interaction of *C. jejuni* with cells in the lamina propria likely coincides with the intestinal inflammation characteristic of later stages of infection. Specialized cells located within the lamina propria have an abundance of pathogen recognition receptors (PRRs) that recognize specific pathogen associated molecular patterns (PAMPs). Recognition of *C. jejuni* PAMPs by host PRRs stimulates the release of proinflammatory cytokines causing an influx of fluid and professional phagocytes (Magalhaes et al., 2007, O'Hara and Shanahan, 2006). In addition, resident macrophages and dendritic cells phagocytize bacteria and present antigens to cells involved in acquired humoral immunity. *C. jejuni* are rapidly killed by complement mediated lysis by both classical and alternative pathways (Blaser et al., 1985). *In vitro* observations indicate *C. jejuni* can persist and replicate in both epithelial cells and macrophages, which possibly represents an adaptation by *C. jejuni* to avoid complement-mediated destruction (De Melo et al., 1989, Kiehlbauch et al., 1985, Konkel et al., 1992, Myszewski and Stern, 1991, Naikare et al., 2006, Wassenaar et al., 1997). While the breakdown of some
innate defenses during the inflammatory response facilitates infection, the self-limiting nature of *C. jejuni*-mediated disease within several days of the onset of symptoms indicates the pathogen can be effectively cleared by innate processes prior to full activation of humoral responses (Blaser *et al.*, 1983, Perez-Perez *et al.*, 1989).

III. **CAMPYLOBACTER JEJUNI FACTORS THAT CONTRIBUTE TO DISEASE**

*C. jejuni* strains exhibit diverse virulence phenotypes

A simple, cost effective animal model that accurately mimics *Campylobacter* infection of humans is not yet widely available to researchers. Therefore, virulence determinants and their phenotypes have been studied primarily by performing *in vitro* assays to assess bacterial adherence, invasion, protein secretion, intracellular survival, and toxin production. Based on these assays, it is clear that *C. jejuni* strains exhibit significant genotypic and phenotypic variations (Dorrell *et al.*, 2001, Konkel and Joens, 1989, Newell *et al.*, 1985b). Comparisons of *C. jejuni* genomes by sequencing and subtractive hybridization analysis have both revealed variation in genomic content amongst strains, providing a basis for some of the phenotypic variation observed. *C. jejuni* are naturally competent for DNA transformation, and horizontal gene transfer between strains within a host has been documented (Hepworth *et al.*, 2007). The exchange of DNA between strains within a host organism is widely supported as the mechanism responsible for *C. jejuni* genome diversity (Manning *et al.*, 2003, McCarthy *et al.*, 2007). However, differences in genomic content do not account for all the phenotypic diversity observed. Different adherence, invasion, and colonization phenotypes have been recorded with genetically matched strains of *C. jejuni* and
subsequently attributed to a point mutation in a sensor kinase gene (Hendrixson, 2006, Malik-Kale et al., 2007). These findings suggest the ability of \textit{C. jejuni} to sense the environment and regulate expression of the genome varies between strains and contributes to their pathogenicity. Thus, it is a combination of genomic content and gene regulation that ultimately determines the pathogenicity of \textit{C. jejuni}.

\textbf{\textit{C. jejuni} pathogenic mechanisms}

Motility, adherence, invasion, protein secretion, intracellular survival, and toxin production may contribute to the pathogenicity of a given \textit{C. jejuni} strain. As these topics and others dealing with \textit{C. jejuni} virulence factors are covered in detail elsewhere in this book, we present a general overview of motility, adherence, and invasion to set a foundation for an in-depth discussion of \textit{C. jejuni} protein secretion and the possible roles of secreted proteins in the development of gastrointestinal disease.

\textit{C. jejuni} motility is provided by either monotrichous or amphitrichous flagella, and \textit{C. jejuni} must be flagellated to colonize chickens or cause disease in humans (Black \textit{et al.}, 1988, Hendrixson, 2006, Newell \textit{et al.}, 1985a). Flagellar locomotion facilitates penetration of the mucosal boundary lining the host intestine. Motility also allows \textit{C. jejuni} to colonize specific niches within the host where the bacterium can avoid the peristaltic motion and flushing of the intestine. Disrupting flagellar function impairs motility and reduces \textit{C. jejuni} binding and invasion of host cells (Carrillo \textit{et al.}, 2004, Konkel and Joens, 1989, Konkel \textit{et al.}, 2004, Malik-Kale \textit{et al.}, 2007, Wassenaar \textit{et al.}, 1991).
Adherence to intestinal epithelial cells is proposed to be fundamental to \textit{C. jejuni} colonization and pathogenesis. Proteins proposed to act as \textit{C. jejuni} adhesins include CadF, CapA, PorA (MOMP), PEB1, and JlpA. CadF, \textit{Campylobacter} adhesion to fibronectin, is a 37 kDa outer membrane protein (Konkel \textit{et al.}, 1997). A \textit{C. jejuni} cadF mutant shows a reduction in binding and invasion of INT 407 cells when compared with a wild-type isolate (Monteville and Konkel, 2002, Monteville \textit{et al.}, 2003), and is incapable of colonizing chickens (Ziprin \textit{et al.}, 1999). CapA is a putative member of the autotransporter family of exported proteins. Similar to a mutation in cadF, a capA mutant shows reduced adherence to Caco-2 cells and does not colonize chickens (Ashgar \textit{et al.}, 2007). The \textit{porA} gene of \textit{C. jejuni} encodes a 43 kDa \textit{major outer membrane protein} (MOMP), which facilitates transport of hydrophilic molecules across the bacterium’s outer membrane barrier and provides structural stability to the outer membrane (Bolla \textit{et al.}, 1995, De \textit{et al.}, 2000). MOMP purified from outer membrane preparations was shown to bind to INT 407 cells via ligand immunoblot assays and microadhesion ELISAs (Moser \textit{et al.}, 1997, Schroder and Moser, 1997). \textit{C. jejuni} porA mutants have yet to be characterized, as mutations in \textit{porA} are lethal to \textit{C. jejuni} due to the protein’s critical structural and transport functions. PEB1 is a 28 kDa protein and disruption of peb1A reduces \textit{C. jejuni} adherence to HeLa cells by 50- to 100-fold (Pei and Blaser, 1993, Pei \textit{et al.}, 1998). In addition, a \textit{C. jejuni} peb1A null mutant exhibits a reduction in the duration of mouse intestinal colonization when compared to the \textit{C. jejuni} wild-type isolate (Pei \textit{et al.}, 1998). JlpA, \textit{jejuni lipoprotein A}, is a \textit{C. jejuni} 43.2 kDa protein. Disruption of jlpA reduces \textit{C. jejuni} adherence to HEp-2 cells by 18 to 19.4%
relative to the wild-type strain (Jin et al., 2001). These studies support the hypothesis that adherence mediated by specific adhesins is necessary for colonization of a host.

The most extensively characterized adhesin is the outer membrane protein CadF, which mediates binding to the fibronectin component of the host cell extracellular matrix via four amino acid residues – phenylalanine, arginine, leucine, and serine (FRLS, residues 133 to 137 of the full length CadF protein) (Konkel et al., 2005). Fibronectin is a ligand for the α5β1 integrin receptor. Binding of fibronectin to the extracellular domain triggers lateral migration of multiple integrins across the plasma membrane. This integrin clustering results in increased signaling activity and subsequent formation of focal adhesion complexes, which provide a physical link and transmit signals between the extracellular matrix and the actin cytoskeleton. Numerous scaffolding and signaling molecules are associated with focal adhesions that regulate actin polymerization in response to external stimuli (Gilcrease, 2007). CadF binding to fibronectin has been shown to specifically induce phosphorylation of the focal adhesion component paxillin, indicating C. jejuni attachment to fibronectin triggers host cell signal transduction from the extracellular matrix through the α5β1 integrin receptors to focal adhesion complexes (Hu et al., 2006, Monteville et al., 2003). The ability of pathogens to influence focal adhesion signaling cascades has several putative implications for invasion (Bruce-Staskal et al., 2002, Eto et al., 2007, Kierbel et al., 2007, McCormick et al., 1997, Shi and Casanova, 2006). The binding of CadF may trigger receptor clustering that recruits focal adhesion complexes to the site of bacterial adherence. During this process, host cell signaling molecules would be brought into close proximity to bacteria, increasing the
probability that other *C. jejuni* virulence proteins would bind to host cell factors and promote bacterial internalization. Noteworthy is that GTPase dependent cell-signaling events, which are necessary for *C. jejuni* invasion of human cells, were decreased with a cadF mutant relative to the wild-type strain (Krause-Gruszczynska *et al.*, 2007). Although the binding of *C. jejuni* to a host cell is sufficient to trigger focal adhesion signaling, host cell invasion is not merely a consequence of the interaction between bacterial adhesins and host cell receptors alone; bacteria must be viable and able to secrete proteins for maximal invasion (Konkel *et al.*, 1992, Konkel *et al.*, 1999).

Many putative pathogenic mechanisms of *C. jejuni* are derived from processes known to occur during infections of other enteric pathogens. *C. jejuni* utilizes a T3SS, the flagellum, to secrete proteins that contribute to host cell invasion (Konkel *et al.*, 2004, Rivera-Amill and Konkel, 1999). We speculate that these secreted proteins modulate host cell signaling pathways. Microfilament reorganization is observed during internalization of *C. jejuni* (De Melo *et al.*, 1989). Inhibition of either host cell actin dynamics, or *C. jejuni* protein secretion, reduces the invasive potential of *C. jejuni* (Konkel *et al.*, 2004, Krause-Gruszczynska *et al.*, 2007, Rivera-Amill and Konkel, 1999). Studies have shown that microtubules also play a role during the internalization of *C. jejuni* (Biswas *et al.*, 2003, Kopecko *et al.*, 2001). In addition to facilitating invasion, other *C. jejuni* proteins disrupt host cell processes and trigger apoptosis (Poly *et al.*, 2007, Siegesmund *et al.*, 2004), potentially contributing to the degradation of the host intestinal epithelium (Blaser *et al.*, 1983, Everest *et al.*, 1993b).
IV. INVASION AND PROTEIN SECRETION

*C. jejuni* secretes a set of proteins required for host cell invasion upon target cell contact

Our knowledge of *C. jejuni* host cell interactions indicates that *C. jejuni* synthesizes and secretes a set of proteins upon host cell contact using the flagellar type III secretion system. It has been demonstrated that at least two of these proteins, Cj0914c (CiaB) and Cj0859c (FspA), are delivered to epithelial cells, indicating that the flagellum of *C. jejuni* serves the dual function of cell motility and virulence protein secretion. In this section, the observations leading to this conclusion will be reviewed.

**Invasion studies**

To gain a better understanding of the ability of *C. jejuni* to enter, survive, and replicate in eukaryotic cells, researchers have used the gentamicin-protection assay. This *in vitro* assay involves inoculation of a monolayer of eukaryotic cells with a known number of bacteria, followed by an incubation period to allow the bacteria to bind to and internalize within the eukaryotic cells. After this incubation period, the cell monolayer is rinsed and medium containing gentamicin, which does not penetrate eukaryotic cell membranes (Hale and Bonventre, 1979), is added to kill the extracellular bacteria. The number of intracellular *C. jejuni* is determined by dilution plating after lysis of the host cells with a detergent. *In vitro* tissue culture assays have provided a method to study bacteria-host cell binding and invasion, and to characterize specific *C. jejuni* mutants. As noted in the previous section, a number of genes have been identified, including *cadF, capA, porA, peb1*, and *jlpA*, that encode putative adhesins. As an alternative to the percent of the
inoculum internalized as a measure of a strain’s invasive potential, we report the percent of adherent bacteria that are internalized [(number of internalized bacteria divided by the number of adherent bacteria) x 100]. The reason for this distinction is to normalize the effect of variable adherence on a strain’s invasive capacity, because the ability of a *C. jejuni* strain to bind to a eukaryotic cell is a prerequisite for host cell invasion. Moreover, ample evidence exists demonstrating that generating a knockout in a *C. jejuni* gene encoding an adhesin (i.e., CadF, CapA, and PEB1a) results in a reduction in host cell adherence, with a corresponding decrease in host cell invasion (Ashgar *et al.*, 2007, Monteville *et al.*, 2003, Pei *et al.*, 1998). Similarly, if a *C. jejuni* strain is non-motile, it shows a reduction in binding to host cells relative to its isogenic, motile counterpart. We consider a *C. jejuni* strain yielding a percent I/A of greater than 1 as both invasive and pathogenic. The reason for using the percent I/A is because strains yielding a value of greater than 1 cause piglets to develop clinical symptoms that resemble those of human campylobacteriosis, including diarrhea with blood in the stool.

It is well documented that some bacteria must be metabolically active for maximal cell invasion, as shown for *Haemophilus influenzae* (St Gme and Falkow, 1990), *Neisseria gonorrhoeae* (Richardson and Sadoff, 1988), *Rickettsia prowazekii* (Walker and Winkler, 1978), *Salmonella typhimurium* (Finlay *et al.*, 1989, Lee and Falkow, 1990), and *Shigella flexneri* (Hale and Bonventre, 1979, Headley and Payne, 1990). Early studies on *C. jejuni* invasion were consistent with this theme (Konkel and Cieplak, 1992). Internalization of *C. jejuni* is significantly reduced when protein synthesis is inhibited by exposure to chloramphenicol prior to co-culture with host cells (Konkel and
Cieplak, 1992, Konkel et al., 1993, Oelschlaeger et al., 1993). Similar results (loss of invasion capacity) are obtained if *C. jejuni* is heat- or sodium azide-killed. Together, these results strongly inferred that *de novo* protein synthesis was required upon target cell contact. Indeed, examination of this parameter in more detail demonstrated that *C. jejuni* respond to culture with epithelial cells by synthesizing a novel set of proteins. One and two-dimensional electrophoretic analyses of metabolically labeled *C. jejuni* cultured with and without epithelial cells revealed that proteins were synthesized either exclusively or preferentially in the presence of epithelial cells, while others were selectively repressed (Konkel and Cieplak, 1992, Konkel et al., 1993). Panigrahi et al. (Panigrahi et al., 1992) also reported that in rabbit ileal loops, *C. jejuni* synthesized a number of proteins that were not synthesized under standard laboratory conditions. Two of the newly synthesized proteins, with apparent molecular masses of 84 and 47 kDa, were detectable using convalescent sera from *C. jejuni*-infected individuals. Additional work revealed that the *de novo* synthesized proteins by *C. jejuni* upon co-cultivation with INT 407 cells were unique from those proteins induced by thermal stress of *C. jejuni* (Konkel et al., 1998). These findings suggest *C. jejuni* responds in a coordinated fashion to the host epithelial cell microenvironment. As noted below, this response includes induction of genes encoding invasion-promoting proteins (Konkel et al., 1992).

The link between *C. jejuni* cell invasion and protein secretion
In an attempt to identify *C. jejuni* proteins induced by epithelial cell contact, we screened a *C. jejuni* genomic DNA-phage expression library with two antisera (Konkel et al.,
1999). One antiserum was collected from a rabbit injected with a whole cell lysate of *C. jejuni* cultured with INT 407 epithelial cells (Cj + INT antiserum) and the other antiserum collected from a rabbit injected with a whole cell lysate of *C. jejuni* cultured in the absence of epithelial cells (i.e., tissue culture medium alone) (Cj - INT antiserum). Phage clones that reacted positively with the Cj + INT antiserum were then screened with the Cj - INT antiserum. From this differential screen, one recombinant phage was identified that reacted with the Cj + INT antiserum but did not react with the Cj - INT antiserum. The *C. jejuni* genomic DNA fragment within this phage clone contained an ORF predicted to encode a 73 kDa protein. A *C. jejuni* mutant strain was constructed and demonstrated an invasion-deficient phenotype compared with the isogenic *C. jejuni* wild-type strain. This gene was designated *ciaB* for *Campylobacter* invasion antigen B.

Confocal microscopy examination of *C. jejuni* infected INT 407 cells with an anti-CiaB antibody revealed staining of the host cell cytoplasm, suggesting that the CiaB protein was secreted from the bacterial cell (Konkel *et al.*, 1999). The induction of specific genes upon host cell contact further suggested that *C. jejuni* may secrete protein as demonstrated for other enteric pathogens. Therefore, screening of cell-free culture supernatants for candidate secreted proteins was conducted. Cells were grown in medium supplemented with $[^{35}S]$-methionine under invasion-conducive conditions with subsequent removal of cells by filtration and protein concentration of the culture supernatants. The resultant concentrate was screened for radiolabeled proteins by autoradiography. By this method, at least 8 proteins, ranging in size from 12.8 to 108 kDa, were originally identified in culture supernatants from *C. jejuni* cells in contact with
INT 407 epithelial cells (Konkel et al., 1999). Protein secretion was not detected when
*C. jejuni* is incubated in the absence of epithelial cells. Modification of the original
secretion assay has allowed for greater sensitivity and improved resolution, revealing
additional secreted proteins. A profile of *C. jejuni* proteins is shown in Figure 3. The *M*,
of the secreted proteins is indicated in Table 1.

Further experiments showed that the *ciaB* mutant was non-invasive and defective in
protein secretion, demonstrating a phenotypic link between invasion and protein
secretion (Konkel et al., 1999). Both invasion- and protein secretion-deficiencies of the
*ciaB* mutant are restored by *in trans*-complementation. Finally, using a polyclonal rabbit
anti-CiaB antibody, it was determined that CiaB is one of the eight proteins originally
detected in culture supernatants under invasion-conducive growth conditions. The ‘B’
designation reflects that CiaB was the second protein in descending molecular weight
order detected in the secretion protein gel profile (Konkel et al., 1999).

The correlation between the differential protein synthetic response, protein secretion,
and host cell invasion is further supported by the finding that there is a temporal
association shared amongst the three responses. *C. jejuni* demonstrate an increased
rate of radioactive methionine incorporation, an altered synthetic response, and secrete
the Cia proteins immediately prior to a rapid increase in *C. jejuni*-host cell internalization
(Konkel et al., 1993, Rivera-Amill and Konkel, 1999). We have also noted that inclusion
of serum in the growth medium induces Cia secretion and thus could substitute the
requirement of *C. jejuni*/eukaryotic cell co-cultivation. It was also determined that
inclusion of bile salts promoted Cia expression even though this stimulus did not promote Cia secretion; hence expression and secretion signals could be uncoupled by varying media constituents (Rivera-Amill and Konkel, 1999).

In summary, Cia protein-secretion is dependent upon C. jejuni-host cell contact or another biological stimulus (serum). Furthermore, CiaB lacks a cleavable signal peptide leader suggesting export is accomplished in lieu of the Sec-dependent general secretory pathway. While these characteristics are reminiscent of either a virulence-associated type III secretion system (T3SS) or a type IV secretion system (T4SS), the genomic sequence of C. jejuni NCTC 11168 did not reveal candidate genes predicted to encode a non-flagellar T3SS or T4SS. The only T3SS encoded by the C. jejuni genome is the flagellar apparatus (Parkhill et al., 2000) (http://www.sanger.ac.uk/Projects/C.jejuni). Based on these findings, we hypothesized that the CiaB protein was a T3SS substrate and was exported by the C. jejuni flagellar T3SS. In part, this prediction was based on the precedence that Y. enterocolitica was reported to secrete at least one virulence factor, a phospholipase (YplA), from the flagellum. To test this hypothesis we have followed two basic strategies. First, we reasoned that if the flagellum is required for secretion of Cia proteins, mutations in both flagellar regulatory and structural genes should be non-motile and should block both Cia export and C. jejuni host cell invasion. Second, because T3SS are generally indiscriminant in recognition and secretion of substrates (Galan and Wolf-Watz, 2006, Lloyd et al., 2002, Schlumberger and Hardt, 2006, Sorg et al., 2005), we reasoned that
if CiaB is a T3SS substrate, it should be recognized and exported by heterologous T3SS such as those harbored by *Y. enterocolitica*.

**C. jejuni flagellar mutants do not secrete Cia proteins**

To test the prediction that the flagellar system was used as the export apparatus for virulence proteins, a number of flagellar gene mutations were constructed directly to test if the Cia proteins require a functional flagellum for secretion (Konkel et al., 2004). From our genetic studies we determined that the secretion of the Cia proteins required a functional basal body, hook, and at least one of the filament proteins. Mutations that affect either the export of flagellar components (*flhB*), or the non-filament structural components (*flgB, flgC, and flgE2*), likewise result in a Cia secretion-negative phenotype. At least a partial filament is also required for Cia secretion. CiaB is secreted in a *flaA* mutant, but CiaB secretion is not detected when both filament genes are deleted (*flaAB* mutation). Finally, we recovered *C. jejuni* strains from poultry that were non-motile, as they did not synthesize the flagellum, and found that they were Cia secretion-negative and poorly invasive for INT 407 cells (Malik-Kale et al., 2007).

Together, the genetic evidence obtained to date is consistent with Cia protein secretion through the flagellar export system (Figure 4).

Two observations suggest that the roles of the Cia proteins are distinct from flagellar proteins. First, a *C. jejuni ciaB* mutant is motile (Konkel et al., 2004). Second, the expression of the *cia* genes is regulated in a manner distinct from flagellar genes. More specifically, $\sigma^{54}$ is responsible for directing the expression of the flagellar class II
components that comprise the basal body, hook proteins, and the FlaB filament protein, while $\sigma^{28}$ is responsible for the expression of the C. jejuni flagellar class III genes, which includes the gene encoding the FlaA filament protein. The expression of the C. jejuni ciaB gene appears to be independent of both the $\sigma^{54}$ and $\sigma^{28}$ sigma factors as judged by real-time RT-PCR (Konkel et al., unpublished observations). Studies are currently in progress to dissect the regulation of the C. jejuni Cia-encoding genes in relation to flagellar gene regulation. Based on these data, it is evident that the regulation of the genes encoding the flagellar and Cia proteins is separate, but that both sets of proteins require a flagellar T3SS for export.

We have designated the proteins secreted from the flagellum as the *Campylobacter* secreted proteins (Csp). As discussed above, the subset of the Csp proteins that contribute to host cell invasion have been termed the *Campylobacter* invasion antigens (Cia). To date, the Csp and Cia mostly remain unidentified. The low level of cia gene expression and Cia protein export *in vitro* has made it difficult to identify invasion associated proteins. Detection of these proteins requires addition of serum to the culture medium and radiolabeling of cells. The presence of serum proteins in these preparations, combined with low concentration of secreted proteins, has negated traditional proteomic approaches to identify additional Cia proteins. However, the recognition that the flagellar T3SS is required for Cia secretion and host cell invasion has provided the insight for the development of an assay to screen for and identify the Csp.
Recognition and export of CiaB by a heterologous T3SS

Gram-negative bacteria possess at least six different mechanisms to actively transport proteins across the bacterial membranes, one of which is the T3SS [reviewed in (Kostakioti et al., 2005)]. While the flagellum is a T3SS, other T3SS have evolved solely to transport bacterial effector proteins from the bacterial cytoplasm into the host cell cytosol via a specialized conduit comprised of a basal body and a translocon. Secretion of effector proteins is triggered by bacterial contact with the host cell. Though the general structure of the T3SS apparatus is similar amongst the Gram-negative pathogens that harbor these systems, the biological function of the secreted proteins (effector molecules) varies. Requirements of T3SS protein substrates include: 1) the absence of a Sec-dependent signal sequence; 2) an amino terminal signal that facilitates secretion; and 3) export through a specialized conduit spanning the inner and outer membranes of Gram-negative bacteria (Cornelis, 2006). Several animal and plant pathogens have evolved unique strategies that alter recipient host cell signaling pathways with effector proteins. Ultimately, the effector proteins modulate the host to facilitate intracellular survival, bacterial multiplication, and/or immune evasion (Journet et al., 2005). Throughout the remainder of the text, when necessary for clarity, we refer to the flagellar T3SS as “flagellar T3SS” and the classical T3SS that is dedicated to the secretion of proteins as “T3SS”.

*Yersinia enterocolitica* is an intensely studied gastrointestinal pathogen that harbors three T3SS. The three *Y. enterocolitica* protein export pathways are termed the flagellar, Ysa, and Ysc T3SS, which respond to known environmental stimuli of
temperature and salt concentration (Young and Young, 2002). The flagellar outer proteins (or Fops) are secreted by the flagellar T3SS, the *Yersinia* secreted proteins (or Ysps) are secreted by the Ysa T3SS, and the *Yersinia* outer proteins (or Yops) are secreted by the Ysc T3SS. The Ysc system is encoded on the pYV plasmid, whereas the former two systems are chromosomally encoded. The Ysc system is induced at 37°C in LB base medium supplemented with 0.2 M sodium oxalate (high salt/high temperature), the Ysa system is induced at 26°C in LB base medium supplemented with 0.29 M NaCl (high salt/low temperature), and the flagellar system is induced at 26°C in base medium without supplemented NaCl (low salt/low temperature). Both the Ysa and Ysc T3SS are capable of secreting the YopE, YopN, and YopP effector proteins, indicating the promiscuous nature of the export pathways (Lee and Galan, 2004, Matsumoto and Young, 2006, Young and Young, 2002).

In *C. jejuni*, the flagellum is required for motility and secretion of the Csp. Based on the hypothesis that CiaB is a T3SS protein, we reasoned that it would be recognized in a heterologous system and secreted in a T3SS-dependent manner. To test this hypothesis, the full-length ciaB gene was cloned into the inducible expression vector pMMB207 and transformed into *Y. enterocolitica* wild-type and flagellar mutant isolates. After induction of the *Y. enterocolitica* flagellar T3SS, supernatant proteins were harvested and probed with polyclonal rabbit anti-CiaB serum. The 73 kDa CiaB protein was detected in the supernatant fluids of the *Y. enterocolitica* wild-type isolate, whereas the supernatant fluids of the *Y. enterocolitica* flhDC flagellar mutant lacked a band of equivalent mass (Christensen *et al.*, 2009). When the whole cell lysates of both the
wild-type and flagellar mutant harboring a copy of the \textit{ciaB} gene were probed with the polyclonal rabbit anti-CiaB serum, a band at 73 kDa was observed in both isolates. This demonstrated that the CiaB protein was synthesized in the \textit{Y. enterocolitica} flagellar mutant cytosol, but was not secreted. Silver stained Fop profiles revealed that only the \textit{Y. enterocolitica} wild-type isolate secretes the Fop proteins. The \textit{Y. enterocolitica} flagellar mutant cannot secrete the Fop proteins. This result indicated that the CiaB protein was secreted along with the Fops in a T3SS-dependent manner. It also suggested that the CiaB protein harbors a T3SS signal that is recognized for export via the flagellar pathway. This latter point was verified using a new assay (described below), whereby the amino terminus of \textit{ciaB} was found to harbor a T3SS signal.

In summary, two lines of evidence support the hypothesis that the \textit{C. jejuni} flagellar T3SS is used to secrete virulence proteins in the host environment. First, a functional flagellum is a prerequisite for Cia protein export. This assertion is based on extensive genetic studies showing that flagellar regulatory and structural gene mutations abolish both motility and Cia export but not \textit{cia} expression. Second, the N-terminal coding sequence of CiaB contains a T3SS export signal that is recognized by the \textit{Y. enterocolitica} flagellar T3SS.

**Identification of \textit{C. jejuni} secreted proteins**

To facilitate the identification of \textit{C. jejuni} genes that harbor a T3SS signal, the pCSP50 shuttle vector was generated (Figure 5). The pCSP50 shuttle vector incorporates a
constitutive promoter (cat) upstream of cloning sites for the encoded signal sequences, the 150 nucleotide amino-terminus truncated yplA gene (eliminating the first 50 amino acids including the T3SS signal), and the yplB chaperone gene. An amino-terminal deletion of the Y. enterocolitica YplA enzyme abolishes its secretion. However, if a T3SS signal is fused to the truncated yplA gene, YplA is secreted and detected on phospholipase indicator plates. More specifically, a fatty acid precipitate, which results from cleavage of Tween 80, can be visualized as a halo surrounding the YplA secretion competent colonies. The pCSP50 vector with C. jejuni gene fragments were used to transform Y. enterocolitica yplAB (phospholipase A and the YplB chaperone) mutants also lacking the pYV plasmid. Using this approach, we detected secretion and extracellular phospholipase activity by the ciaB-yplA fusion construct expressed in a yplA chromosomal deletion background. This finding further indicated that the ciaB 5’ end (N-terminus) encodes a T3SS signal. In contrast, fusing the N-terminal coding region of C. jejuni genes encoding cytosolic proteins with yplA, did not promote YplA export.

We then selected genes to screen for T3SS signal sequences from the C. jejuni NCTC 11168 sequence database (Parkhill et al., 2000). 359 of 1625 ORFs were chosen for analysis following the elimination of genes encoding proteins with known functions or containing membrane-spanning domains, periplasmic domains, Sec-dependent signal sequences, or Tat-dependent signal sequences. No genes were identified with known Type I Sec-independent motifs. Not surprisingly, most of the 359 ORFs encoded proteins of unknown function and conserved hypothetical proteins. Primers were
designed to amplify the first 108 encoding bases of all the ORFs and facilitate directional cloning into the shuttle vector pCSP50 to generate translational fusions with the truncated YplA. Thus far, 329 of the 359 ORFs have been analyzed. Each vector was sequence confirmed and used to transform the *Y. enterocolitica yplAB* (phospholipase A and the YpIB chaperone) mutant also lacking the pYV plasmid. The transformants were then screened for both flagellar and Ysa T3SS secretion by spot inoculation onto phospholipase indicator plates (low and high salt, respectively).

A total of 42 of 329 *C. jejuni* ORFs were identified that strongly drive the export of the *C. jejuni*-YplA fusion proteins from via the *Y. enterocolitica* flagellar and Ysa T3SS (Christensen and Konkel, *manuscript in preparation*). Included within this list are signal sequences from two known *C. jejuni* secreted virulence factors Cj0914c (CiaB) and Cj0859c (FspA). In addition, the signal sequences from two putative flagellar related proteins, Cj1463 (FlgJ) and Cj1464 (FlgM), resulted in strong secretion from both T3SS. The functional categories (Gundogdu *et al.*, 2007) of 26 of the strongly secreted proteins are either conserved hypothetical proteins or proteins of unknown function (Figure 6). From the remaining list of 16 proteins, all but 4 are annotated as putative functions.

**C. jejuni secreted proteins (CiaB, FlaA, FlaB, FlaC, and FspA)**

We have found that CiaB is a T3SS protein and hypothesize that the remaining Cia proteins are also T3SS substrates. Proteins secreted via this pathway will fall into two general categories: 1) Proteins that comprise the flagellum, and 2) Proteins that are not
part of the flagellar structural apparatus, some of which serve as effector proteins.

Evidence indicates that the Cia proteins enhance *C. jejuni* invasion of host cells, and we speculate that this occurs through their modification of host cell behavior.

FlaA, FlaB, FlaC, and FspA were also found to harbor a T3SS signal as evidenced by our *Y. enterocolitica* phospholipase screen (not shown). Song *et al.* (Song *et al.*, 2004) reported that FlaC (Cj0720c), a 26 kDa protein, shares N- and C-terminus homology to the flagellar FlaA and FlaB filament proteins in the *C. jejuni* TGH9011 strain. This group also detected the FlaC protein in the supernatant milieu of isolates harboring an intact flagellum. Studies revealed that the *flaC* mutant was less invasive for INT 407 cells as compared with the wild-type isolate, suggesting that the FlaC protein may play a role in *C. jejuni* pathogenesis. Poly *et al.* (Poly *et al.*, 2007) determined that the *C. jejuni* FspA gene encoded a 15.5 kDa protein designated FspA, for Flagellar secreted protein A. Similar to FlaC, FspA was only detected in supernatant fluids harvested from isolates harboring a functional flagellum. Additionally, Poly *et al.* (Poly *et al.*, 2007) found two variant forms of the FspA protein (A1 and A2) within a variety of *C. jejuni* strains including *C. jejuni* 8486 and *C. jejuni* 81-176. Only FspA2 was associated with the host cell monolayer and induced apoptosis. The FspA protein does not appear to play a role in cell adherence and invasion, as differences were not observed between the wild-type strain and a *fspA1* mutant. Interestingly, the FlaC protein and two FspA protein variants did not require an external stimulus for protein secretion (i.e., host contact, or co-culture with conditioned medium) and were not required for motility (Poly *et al.*, 2007).
Collectively, the data further support our hypothesis that the *C. jejuni* flagellum is essential for motility and also facilitates the secretion of non-flagellar proteins.

**Evolutionary relatedness of the flagellum and virulence T3SS pathways**

The bacterial flagellum and T3SS injectisome share an evolutionary and functional relationship (Gophna *et al.*, 2003). The first indication of this relationship was the predicted protein similarities between the *Caulobacter crescentus* flagellar gene *flbB* (now *flhA*) and the *Y. enterocolitica* virulence plasmid-encoded *lcrD* (Ramakrishnan *et al.*, 1991, Sanders *et al.*, 1992). Following these two reports, additional flagellar protein and virulence protein similarities were found. Because *Y. enterocolitica* phase varied flagellin and virulence protein secretion, and because both systems were Sec-independent processes, Minnich and Rohde (Minnich and Rohde, 2007) postulated the simplest explanation was that the *Yersinia* basal body might be reorganized for virulence protein secretion in the host environment [see review by (Harshey and Toguchi, 1996)]. As such, this broadened the traditional view of the flagellum as a motility organelle to one that could also be viewed as a standalone highly efficient protein secretory device (i.e., depending upon the circumstances, the basal body could serve a dual purpose). Studies performed in the late 1990’s demonstrated for *Yersinia* and other enteric pathogens that the flagellar T3SS and the classical T3SS, which is dedicated to the secretion of virulence proteins, were separate but parallel systems. As described in the preceding section, *C. jejuni* is a unique example in which the flagellum is the sole channel to export flagellar components and virulence proteins.
Despite the varying roles T3SS serve for pathogens, the mechanism of assembly, protein secretion and the apparatus itself are remarkably similar to the flagellar basal body. About 25 proteins are required to build the non-flagellar T3SS. Eight of these proteins are conserved with proteins that comprise the flagellum [reviewed by (Cornelis, 2006, Galan and Wolf-Watz, 2006)]. Non-flagellar T3SS harbor basal body structures that serve to anchor the apparatus into the bacterial inner and outer membranes and peptidoglycan layer. Basal body assembly must occur before the integration of the inner rod, needle, and regulatory proteins, and involves the incorporation of an ATPase that is involved in the recognition and unfolding of T3SS apparatus substrates. Once these structures have been inserted, conformational changes on the cytoplasmic side of the bacterial membrane allow for protein selection and secretion. Flagellar T3SS are also comprised of a similar basal body structure that serves to anchor the flagellar engine to the bacterial membrane [reviewed by (Macnab, 2004)].

There has been speculation about the evolutionary phylogeny of the flagellar and T3SS systems. Saier (2004) proposed three possibilities, which include: 1) both the T3SS and flagellum are derived from a common ancestral protein secretory system; 2) the T3SS is the precursor for the more complex flagellum; or, 3) the flagellum served as the precursor for the T3SS. Pallen and Matzke (Pallen and Matzke, 2006) have argued for the first possibility above. Gophna et al. (Gophna et al., 2003) argued for the second possibility, in part, because the Chlamydia, deeply rooted in the phylogenetic tree, contains only a virulence T3SS. Finally, Saier (Saier, 2004) favors the third possibility because all groups of eubacteria contain flagella, but the T3SS are thus far limited to a
small subset of Gram-negative bacteria. Further, Saier points out that bacterial motility more than likely predated the appearance of eukayotes and the resulting opportunity for symbiosis or parasitism. Finally, mutation densities in flagellar proteins are much greater than those associated with T3SS, suggesting the latter are evolutionarily more recent. Our findings demonstrate that the *C. jejuni* flagellum can provide motility and secretes virulence proteins. Because of this observation, postulating a common ancestral system giving rise to both flagellar T3SS and classical T3SS may not be necessary. In fact, the evidence from *C. jejuni* argumentatively favors the flagellum being the ancestral organelle from which the classical T3SS developed. The fact that the CiaB flagellar-dependent T3SS signal of *C. jejuni* (Epsilon proteobacteria) is recognized by the *Yersinia* (Gamma proteobacterium) flagellar T3SS and classical T3SS highlights a remarkable conservation. The evidence that the T3SS is the ancestral apparatus inferred from observations with *Chlamydia* is not disputed. However, given that *Chlamydia* spp. are obligate intracellular pathogens and that the transition to such a specialized lifestyle often involves a significant loss of genetic information, one could speculate these organisms may have lost the locomotion functionality of a flagellum while retaining the ability to secrete proteins to facilitate their intracellular survival. Such examples of system losses have been observed for non-motile *Y. pestis*, *Shigella* spp., and *Bordetella pertussis*, and recent isolates of nonmotile *E. coli* O157, all of which require a T3SS injectisome for virulence and demonstrate clear evidence of mutationalty-inactivated flagellar systems. In fact, expressing flagella in the host may be a liability for pathogens since flagellin is a potent inducer of innate immunity via toll-like receptors specifically positioned to recognize
these PAMPS. In summary, our findings on the dual nature of the *C. jejuni* flagellar T3SS add an interesting twist to the possible origin of these two systems.

**Motility, Cia protein secretion, host cell invasion, and *C. jejuni*-mediated enteritis**

The role of motility in *C. jejuni* colonization and subsequent disease production has been intensely studied. Predictably, motility was found to be important in promoting the colonization of animals by *C. jejuni* (Nachamkin *et al.*, 1993, Pavlovskis *et al.*, 1991). Also expected was the finding that motility, and the expression of the flaA gene, is necessary for the maximal invasion of *C. jejuni* into host mammalian cells and for the translocation of polarized cell monolayers (Grant *et al.*, 1993, Wassenaar *et al.*, 1991). However, when these early studies were performed, it was observed that the *C. jejuni flaA* (flaB<sup>+</sup>) strain is more invasive than a *C. jejuni flaA flaB* strain (Konkel *et al.*, 2004). Also interesting was that the invasiveness of a *C. jejuni flaA* (flaB<sup>+</sup>) strain was enhanced 10-fold by promoting bacteria-host cell contact via centrifugation. In contrast, the centrifugation step did not change the invasive potential of the *C. jejuni* wild-type strain (Wassenaar *et al.*, 1994). Based on these findings, Grant *et al.* (Grant *et al.*, 1993) concluded that the flagellar structure played a role in internalization that was independent of motility. Subsequently, we found that the Cia proteins were secreted from a *C. jejuni* 81116 flaA (flaB<sup>+</sup>) mutant but not from a *C. jejuni* 81116 flaA flaB mutant (Grant *et al.*, 1993). Thus, it was clear that a *C. jejuni* non-motile strain can be either secretion-positive (i.e., the flaA flaB<sup>+</sup> mutant) or secretion-negative (i.e., the flaA flaB mutant), and the ability of the bacterium to secrete proteins resulted in an increase in its invasive potential. While these data helped clarify the relationship between *C. jejuni*
motility, secretion, and host cell-invasion, the significance of protein secretion and host cell invasion in *C. jejuni*-mediated gastroenteritis was not known. This question was addressed directly with a *C. jejuni ciaB* mutant, which is motile but secretion-negative. Importantly, *in vitro* assays revealed that the *C. jejuni* F38011 wild-type strain (motile and secretion-positive) was 50-fold more invasive than the *C. jejuni ciaB* mutant (motile and secretion-negative). Moreover, inoculation of piglets with the *C. jejuni* wild-type and complemented *ciaB* strains resulted in diarrhea 24 hours post-infection, whereas diarrhea was not observed in piglets infected with the *C. jejuni ciaB* mutant until 3 days post-infection (Konkel *et al.*, 2001). More severe histological lesions (i.e., shortening of the villi and the production of an exudate in the lumen of the intestine) were also observed in piglets inoculated with the *C. jejuni* complemented *ciaB* isolate when compared to the *C. jejuni ciaB* mutant. While the undifferentiated cells in the crypt remained normal in appearance, *C. jejuni* destroyed the fully differentiated epithelial cells located at the tips of the villi. Collectively, the infection studies performed with the newborn piglets and the *C. jejuni* Cia secretion-competent and secretion-deficient isolates revealed that host cell invasion and the secreted Cia proteins are major contributors to the pathology of *C. jejuni*-mediated enteritis.

**V. SUMMARY**

*C. jejuni* is an interesting pathogen that has evolved a unique set of virulence mechanisms to cause disease. Based on the work described herein, we submit that *C. jejuni* utilizes its flagellar T3SS for the secretion of virulence proteins that contribute to *C. jejuni*-mediated enteritis. To our knowledge, this is first example where the flagellum
functions in both motility and as the primary conduit for virulence protein secretion. Our current focus is to identify the secreted proteins and to gain an understanding of how these proteins modulate host cell functions. Further, *C. jejuni* strains show significant genetic and phenotypic diversity. How this diversity contributes to the spectrum of disease profiles in susceptible hosts is of keen interest. The question of why *C. jejuni* elicits disease in humans but establishes a commensal relationship with other animals is critical in understanding *C. jejuni* pathogenesis. The dynamic interplay of *C. jejuni* virulence determinants and host responses is necessary to understand why disease develops in susceptible hosts. A more accurate and comprehensive understanding of *C. jejuni*-mediated enteritis will emerge as researchers unravel the virulence attributes unique to particular *C. jejuni* strains.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 1. Model of *C. jejuni* pathogenesis. The clinical manifestation of *C. jejuni* infection is a result of bacterial activities (white box) and the host immune response (gray box). Dotted lines indicate processes that can potentiate infection.
C. jejuni

Inflammatory response
- Stimulate innate immune functions
  - Influx of fluid
  - Complement activation
  - Recruitment of phagocytes
  - Phagocytosis
  - Bacterial lysis/death
  - Clearing the infection

Adherence
- Protein secretion
  - Invasion
- Bacterial effects on cellular events
  - Cytoskeletal rearrangement
  - Host cell death
  - Tight junction disruption
  - Cytokine induction

Tissue destruction
- Loss of epithelial cell function
  - Compromised barrier and absorptive functions

Disease manifestation
- Humoral response
  - Antigen presentation

Early responses

Late responses
Fig. 2. Adherence, protein secretion, and invasion are a few of the *C. jejuni* virulence attributes that contribute to acute infection. As depicted, bacterial colonization of the intestinal tract can occur via different routes. Several virulence attributes may stimulate the host inflammatory response and, in turn, promote additional bacteria-host cell interactions. Other factors (not listed) are also capable of triggering the host inflammatory response. The dotted line represents the possibility that secreted proteins may enhance the cytokine response.
Fig. 3. Secretion of the Cia proteins is dependent on an intact flagellar T3SS. Culture medium supplemented with fetal bovine serum (FBS) triggers secretion of the Cia proteins. *C. jejuni* were suspended in MEM, either with or without FBS, and radiolabeled with [³⁵S]-methionine for 3 h. Supernatant fluids were harvested, concentrated four-fold, and solubilized in double-strength sample buffer. Equal volumes of samples were separated in a SDS-12.5% polyacrylamide gel. The gel was dried and analyzed by phosphorimaging (Molecular Dynamics, Inc., ImageQuant®, Sunnyvale, CA). Lanes: 1) *C. jejuni* 81-176 (+) FBS; 2) *C. jejuni* 81-176 (-) FBS.
C. jejuni secretion profile
Fig. 4. The *C. jejuni* type III secretion system (T3SS) is the flagellum, which secretes the *Campylobacter* secreted proteins (Csp). A subset of the Csps, termed the *Campylobacter* invasion antigens (Cia), are required for maximal invasion. Both the Csp and Cia proteins harbor non-cleavable, non-consensus secretion signals, required for export.
**Fig. 5.** Depiction of the pCSP50 shuttle vector and phospholipase indicator plate results for controls. The pCSP50 vector includes a *tet* cassette, a constitutive promoter (*cat*), a 5’ truncated *yplA* (lacking 150 nucleotides encoding the native T3SS signal), and the *yplB* gene (cognate chaperone). The NdeI and BglII sites facilitate directional cloning of *C. jejuni* sequences as fusions with the truncated *yplA*. Panels A-E are scans of phospholipase indicator plates under flagellar T3SS induction: A, *Y. enterocolitica* JB580v (wild-type); B, *Y. enterocolitica yplAB* mutant; C, *Y. enterocolitica yplAB* mutant with pCSP50; D, *Y. enterocolitica yplAB* mutant with pCSP50:ciaB<sub>1-108</sub>; and E, *Y. enterocolitica yplAB* mutant with pCSP50:cySM<sub>1-108</sub>. All strains show strong growth; panels A and D show strong secretion (of YplA and CiaB<sub>1-36</sub>:YplA fusion protein, respectively) resulting in a zone of precipitate due to hydrolysis of fatty acids by the phospholipase.
Fig. 6. Functional categories of *C. jejuni* secreted proteins. The numbers of *C. jejuni* proteins, categorized according to function, encoding signal sequences that elicited strong secretion from both the *Y. enterocolitica* flagellar and Ysa T3SS.
Table 1. Relative molecular mass of *C. jejuni* secreted proteins

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Despite being a leading cause of bacterial gastroenteritis worldwide, the mechanism by which C. jejuni mediates disease is ill-defined. Disease prevention includes proper food handling techniques and minimizing water and milk reservoirs from contamination. Alternatively, reduction of Campylobacter numbers in poultry is an emerging field of interest, as the microbe is a commensal organism of the avian cecum. Poultry farms have attempted to address reduction of avian environmental exposures to Campylobacter (via biosafety measures), increase host resistance to reduce Campylobacter carriage in the gut (e.g., vaccinate and use competitively exclusion strains), and use antimicrobial alternatives to reduce and eliminate the organism from colonized chickens (via bacteriophage therapy and bacteriocin treatment) (Lin, 2009).

Once ingested, the bacterium utilizes the flagellar apparatus for motility to reach the small intestinal tract. Initial C. jejuni infection occurs in the jejunum and ileum. Once in the intestinal tract, bile salts in the lumen upregulate the cia genes, thus priming the bacterium for invasion (Malik-Kale et al., 2008). Adhesion to host cells is a critical step that ensures the bacterium will not be flushed from the intestinal tract by peristaltic force. The adhesins CadF and FlpA are essential for C. jejuni binding to host cells. Mutants in either adhesin result in 50% reduction in Campylobacter adherence. Bacteria-host cell contact triggers delivery of the Cia into the host cell cytosol. Delivery of the Cia are crucial for maximal invasion efficiency, as nonmetabolically active bacteria cannot induce uptake. Furthermore, Konkel et al. (1999) have demonstrated
that CiaB is translocated into the host cell cytosol and that translocation of the CiaB protein requires an intact flagellum.

The mechanism of Cia effector translocation (i.e. delivery to the host cell) via the flagellum will no doubt be an exciting field of exploration. Canonical T3SS injectisomes harbor multimeric complexes at the tips of the apparati that act in a concerted fashion to ultimately produce a pore whereby effectors from the bacterial cytoplasm are “injected” directly into the host cell cytosol (Cornelis, 2006). Electron microscopy (EM) studies have provided a helpful tool to yield further information on how T3SS apparati are assembled (Enninga et al., 2009). The iterative helical real space reconstruction (IHRSR) method was recently applied to cryo-EM images to determine that the C. jejuni flagellum flagellar subunits pack differently than that of the Salmonella flagellum (Galkin et al., 2008). The authors found that the C. jejuni flagellum is made up of 7 protofilaments, or 3 protofilments less than the Salmonella flagellum, and that overall the C. jejuni filament is about 180Å in diameter whereby the Salmonella filament is about 220Å. Regardless of this observation, the lumen of both apparati are similar in size. Future studies are required to determine how the unique features of the Campylobacter flagellum effect Cia secretion. It is also interesting to speculate if there is a T3SS substrate(s) located at the tip of the flagellum that harbors transmembrane domains to make up the pore by which the Cia proteins are translocated into the eukaryotic host cell cytosol.

Paxillin phosphorylation and subsequent microfilament reorganization are essential for Campylobacter invasion (Monteville et al., 2003). Host cell membrane ruffling at the site of C. jejuni invasion has been observed and has lead to speculation
that small Rho GTPases are "hijacked," resulting in the activation of actin rearrangement signaling cascades (Krause-Gruszczynska et al., 2007). Further evidence provided by Krause-Gruszczynska et al. (2007) indicate the involvement of small Rho GTPase activation during the entry of the organism. Collectively, these results are consistent with similar observations that occur with *Salmonella*-mediated invasion. *Salmonella* requires five SP-1 encoded effector proteins to maximize invasion (Schlumberger et al., 2006). SipA and SipC act directly and cooperatively on actin by mediating polymerization and bundling. Neither SipA or SipC can mediate ruffling or invasion alone as evidenced by a *sopE sopE2 sopB* triple mutant (Zhou et al., 2001). SopE and SopE2 have been extensively characterized as activators of Cdc42 (SopE and SopE2) and Rac1 (SopE) (Schlumberger et al., 2003 and Williams et al., 2004). These effectors mimic G-nucleotide exchange factors in a cooperative fashion with SopB to ultimately recruit and activate actin nucleation complexes and promote subsequent actin branching for bacterial invasion. Interestingly, mutants deficient in either *sopE* or *sopE2* severely impair *Salmonella* invasion whereas a *sopB* mutant exhibits a delayed invasion phenotype *in vitro* (Zhou et al., 2001). These results indicate that *Salmonella* requires SopE and SopE2 for efficient invasion and relies on the ability of these effectors to interact with small Rho GTPases.

To date, the *C. jejuni* CiaB, CiaC, and FspA proteins are the only *Campylobacter* nonmotility associated T3SS substrates to have been identified. Similar to the CiaB and CiaC proteins, FspA is secreted in a T3SS manner via the flagellum. Further analysis of FspA has revealed that host cells undergo apoptosis when purified FspA is coincubated with a cell monolayer (Poly et al., 2007). This finding is unexpected as T3SS effector
proteins have only been characterized to elicit an effect on host cell machinery after translocation into the host cell cytosol. Further studies are required to elucidate the mechanism by which FspA causes apoptosis to a cell monolayer. The CiaB and CiaC proteins are T3SS virulence proteins that require a functional flagellum for maximal invasion in vitro and in vivo (CiaC results – unpublished data). As such, one would predict that these effectors could harbor intrinsic properties that mimic eukaryotic proteins. Though of bacterial origin, both the CiaB protein and the CiaC protein harbor multiple predicted PDZ peptide binding motifs as determined by the Eukaryotic Linear Motif tool for functional sites in proteins (http://elm.eu.org/). The PDZ acronym refers to the proteins for which the domains were first described (Jelen et al., 2003). PDZ domains are composed of 80-100 amino acids and can occur in multiple copies within a protein. PDZ domains specifically recognize short (e.g., 3-6 residue) peptide motifs often located within the carboxy terminus of cognate ligand partners. Interestingly, PDZ-containing proteins are overwhelmingly involved in localizing cellular components to the plasma membrane where an opportunity to bind many different types of target proteins and subsequently “glue” targets into networks of supramolecular signaling complexes can scaffold (Jelen et al., 2003). Though the function of the C. jejuni CiaB protein is unknown, it is interesting to speculate if any of the 26 predicted PDZ peptide binding motifs within CiaB contribute to a molecular “glue stick” for the recruitment and subsequent scaffolding of focal adhesion proteins (e.g., paxillin, vinculin, c-Src, Talin) (Table 1). By mimicking PDZ motifs for cellular components harboring PDZ domains, CiaB may be stabilizing interactions amongst focal adhesion proteins that may
otherwise be transient. This in turn could also benefit other Cia effector proteins by situating cognate ligands near the bacterial invasion site.

Ectopic expression of the *C. jejuni* CiaC protein in HeLa cells results in spiked projections emanating from the cell membrane, suggesting massive microfilament rearrangement (unpublished results). The underlying mechanism for this phenotype is unknown, but may be explained by the two predicted PDZ recognition motifs harbored within the CiaC protein. Studies indicate that PDZ domain containing proteins and interacting ligand partners can activate biochemical pathways that trigger Rho GTPase activity (Chikumi *et al*., 2002; Garrard *et al*., 2003; Van Leeuwen *et al*., 2003; Yamada *et al*., 2005; Lin *et al*., 2008). One example is focal adhesion kinase (FAK), a key signaling protein-tyrosine kinase involved in recruiting and stabilizing focal adhesion complexes (Tomar *et al*., 2009). FAK has been characterized to interact with PDZ domain containing proteins such as lysophosphatidic acid (LPA) and PDZ-RhoGEF, which can lead to Rho GTPase activation (Iwanicki *et al*., 2007). Future studies are required to determine if the two predicted CiaC PDZ peptide binding motif sites can influence activation of Rho GTPase signaling cascades (Table 2).

Once within the cell, *Campylobacter* may continue to secrete T3SS substrates to create a *Campylobacter* containing vacuole (CCV). Watson *et al*. (2008) observed CCV localization to the golgi apparatus post invasion. The mechanism required for migration and replication thereafter in the vacuole remains unclear. Clearly, identification of a virulence factor involved in *Campylobacter* intracellular survival will aid in understanding the aforementioned observations by Watson *et al*. 

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The characterization of the CiaB protein as a bona fide T3SS substrate provided the proof of concept to generate a genome-wide genetic screen study for the identification of putative cia genes. The genetic screen led to the identification of at least one Cia that is involved in invasion. This finding in itself is a milestone in the identification of a virulence determinant involved in *C. jejuni*-mediated pathogenesis. Future directions of particular interest are identifying the remaining secreted proteins, characterizing the function of the secreted proteins, and determining how these proteins cooperatively interact within the host. To further strengthen our model, it will also be critical to demonstrate that the flagellar T3SS apparatus can function as a virulence protein injectisome. In conclusion, while our model of *C. jejuni* pathogenesis is far from complete, it will be very exciting to see how putative csp mutants provide insight into the *C. jejuni* mechanism of invasion, intracellular survival, and evasion of immune detection.
References


Table 1. Results of Eukaryotic Linear Motif (ELM) search after globular domain filtering, structural filtering and context filtering for the CiaB protein. Results were computed from entering the entire CiaB protein sequence at the ELM website (http://elm.eu.org).

<table>
<thead>
<tr>
<th>Elm Name</th>
<th>Instances (Matched Sequence)</th>
<th>Positions</th>
<th>Elm Description</th>
<th>Cell Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLV_NDR_NDR_1</td>
<td>VRK</td>
<td>1-13 55-57 162-164</td>
<td>N-Arg dibasic convertase (nardilysine) cleavage site (Xaa[-]Arg-Lys or Arg[-]Arg-Xaa)</td>
<td>extracellular, Golgi apparatus, cell surface</td>
</tr>
<tr>
<td></td>
<td>RRL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>LRK</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLV_PCSK_PC1ET2_1</td>
<td>KRV</td>
<td>418-420 484-486</td>
<td>NEC1/NEC2 cleavage site (Lys-Arg-[-Xaa)</td>
<td>Golgi membrane, extracellular, Golgi apparatus</td>
</tr>
<tr>
<td></td>
<td>KRA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLV_PCSK_SKI1_1</td>
<td>KLAFC</td>
<td>238-242 248-252 248-331 352-356 372-376 400-404 407-411 538-542 559-563</td>
<td>Subtilisin/kexin isozyme-1 (SKI1) cleavage site ([RK]-X-[hydrophobic]-[LTKF]-[-X])</td>
<td>endoplasmic reticulum, endoplasmic reticulum lumen, Golgi apparatus</td>
</tr>
<tr>
<td></td>
<td>RVIKA</td>
<td></td>
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<td>KEVLS</td>
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<td>KGLFS</td>
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<tr>
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<td>KKIFA</td>
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<td>RNILF</td>
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<tr>
<td></td>
<td>KELTL</td>
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</tr>
<tr>
<td></td>
<td>KEFLS</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>LIG_14-3-3_2</td>
<td>RSLISLS</td>
<td>35-41</td>
<td>Longer mode 2 interacting phospho-motif for 14-3-3 proteins with key conservation RxxxS#p.</td>
<td>nucleus, mitochondrion, cytosol, internal side of plasma membrane</td>
</tr>
<tr>
<td>LIG_BRCT_BRCA1_1</td>
<td>LSTQF</td>
<td>148-152 394-398 446-450 509-513 600-604</td>
<td>Phosphopeptide motif which directly interacts with the BRCT (carboxy-terminal)</td>
<td>BRCA1-BARD1 complex, nucleus</td>
</tr>
<tr>
<td></td>
<td>SSEVF</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>QSGFF</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>LSLLF</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>NSGEF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LIG_BRCT_BRCA1_2</strong></td>
<td><strong>SSEVFDK</strong></td>
<td><strong>394-400</strong></td>
<td>Phosphopeptide motif which directly interacts with the BRCT (carboxy-terminal) domain of the Breast Cancer Gene BRCA1 with high affinity.</td>
<td>nucleus, BRCA1-BARD1 complex</td>
</tr>
<tr>
<td>----------------------</td>
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<td>------------------------------------------------------------------------------------------------------------------</td>
<td>-----------------------------</td>
</tr>
<tr>
<td><strong>LIG_CYCLIN_1</strong></td>
<td><strong>RSLI</strong></td>
<td><strong>35-38</strong></td>
<td>Substrate recognition site that interacts with cyclin and thereby increases phosphorylation by cyclin/cdk complexes. Predicted protein should have the MOD_CDK site. Also used by cyclin inhibitors.</td>
<td>nucleus, cytosol</td>
</tr>
<tr>
<td></td>
<td><strong>RRLI</strong></td>
<td><strong>55-58</strong></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td><strong>KGLF</strong></td>
<td><strong>352-355</strong></td>
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<tr>
<td></td>
<td><strong>KELTL</strong></td>
<td><strong>538-542</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LIG_EH1_1</strong></td>
<td><strong>VHNIGLMN</strong></td>
<td><strong>122-130</strong></td>
<td>The engrailed homology domain 1 motif is found in homeodomain containing active repressors and other transcription families, and allows for the recruitment of Groucho/TLE corepressors.</td>
<td>nucleus</td>
</tr>
<tr>
<td><strong>LIG_FHA_1</strong></td>
<td><strong>DKTTMLA</strong></td>
<td><strong>46-52</strong></td>
<td>Phosphothreoni</td>
<td>nucleus</td>
</tr>
<tr>
<td>SWTKEII NYTHAVA ICTPMIF ATTGGLI YYTEGLI</td>
<td>135-141 279-285 340-346 458-464 501-507</td>
<td>ne motif binding a subset of FHA domains that show a preference for a large aliphatic amino acid at the pT+3 position.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LIG_MAPK_1</td>
<td>KQDKTTMLAI KVKSPLQVKKIFAFINKTKPFMKIKCKEFVKF</td>
<td>44-53 262-269 372-380 386-393 579-586</td>
<td>MAPK interacting molecules (e.g. MAPKKs, substrates, phosphatases) carry docking motif that help to regulate specific interaction in the MAPK cascade. The classic motif approximates (R/K)xxxx#x# where # is a hydrophobic residue.</td>
<td></td>
</tr>
<tr>
<td>Ligand</td>
<td>Sequence</td>
<td>Position</td>
<td>Function</td>
<td></td>
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</tr>
<tr>
<td><strong>LIG_PP1</strong></td>
<td>FGRNILFY NLKINFD</td>
<td>405-412</td>
<td>Protein phosphatase 1 catalytic subunit (PP1c) interacting motif binds targeting proteins that dock to the substrate for dephosphorylation. The motif defined is ([RK]{0,1}[VI][^P][FW]).</td>
<td></td>
</tr>
<tr>
<td><strong>LIG_SH2_GRB2</strong></td>
<td>YKNI YENA</td>
<td>315-318</td>
<td>GRB2-like Src Homology 2 (SH2) domains binding motif.</td>
<td></td>
</tr>
<tr>
<td><strong>LIG_SH2_SRC</strong></td>
<td>YENA</td>
<td>382-385</td>
<td>Src-family Src Homology 2 (SH2) domains binding motif.</td>
<td></td>
</tr>
<tr>
<td><strong>LIG_SH2_STAT3</strong></td>
<td>YHEQ</td>
<td>468-471</td>
<td>YXXQ motif found in the cytoplasmic region of cytokine receptors that bind STAT3 SH2 domain.</td>
<td></td>
</tr>
<tr>
<td>LIG_SH3_3</td>
<td>WMKVKSPLQVGHPIFLPIMP</td>
<td>260-266 266-272 572-578</td>
<td>This is the motif recognized by those SH3 domains with a non-canonical class I recognition specificity</td>
<td>cytosol, plasma membrane, focal adhesion</td>
</tr>
<tr>
<td>LIG_SH3_4</td>
<td>KPFMKS</td>
<td>388-395</td>
<td>This is the motif recognized by those SH3 domains with a non-canonical class II recognition specificity</td>
<td>cytosol, focal adhesion</td>
</tr>
<tr>
<td>LIG_TRAF2_1</td>
<td>SSEE SEESEEEAWQE</td>
<td>223-226 224-227 252-255</td>
<td>Major TRAF2-binding consensus motif. Members of the tumor necrosis factor receptor (TNFR) superfamily initiate intracellular signaling by recruiting the C-domain of the TNFR-associated factors (TRAFs) through their cytoplasmic tails.</td>
<td>cytosol</td>
</tr>
<tr>
<td>LIG_TRAF6</td>
<td>GHPLEYYE</td>
<td>270-278</td>
<td>TRAF6 binding site. Members of the tumor necrosis factor receptor (TNFR) superfamily initiate intracellular signaling by</td>
<td>cytosol</td>
</tr>
</tbody>
</table>
recruiting the C-domain of the TNFR-associated factors (TRAFs) through their cytoplasmatic tails.

<table>
<thead>
<tr>
<th>LIG_WW_4</th>
<th>YQKTPE KVKSPL YICTPM</th>
<th>168-173 262-267 339-344</th>
<th>Class IV WW domains interaction motif; phosphorylation-dependent interaction.</th>
<th>nucleus, cytosol</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOD_CK1_1</td>
<td>SLISLSE</td>
<td>36-42</td>
<td>CK1 phosphorylation site</td>
<td>nucleus, cytosol</td>
</tr>
<tr>
<td>MOD_CK2_1</td>
<td>SLISLSE IFASSEE FASSEE FASSEE EVLSNIE</td>
<td>36-42 220-226 221-227 328-334</td>
<td>CK2 phosphorylation site</td>
<td>nucleus, cytosol, protein kinase CK2 complex</td>
</tr>
<tr>
<td>MOD_Cter_Amidation</td>
<td>AGKK</td>
<td>370-373</td>
<td>Peptide C-terminal amidation</td>
<td>extracellular, secretory granule</td>
</tr>
<tr>
<td>MOD_GlcNHglycan</td>
<td>FSAQ QSGF DNSGE NSGE</td>
<td>355-358 446-449 599-603 600-603</td>
<td>Glycosaminoglycan attachment site</td>
<td>extracellular, Golgi apparatus</td>
</tr>
<tr>
<td>MOD_GSK3_1</td>
<td>TEKTMNQS</td>
<td>440-447</td>
<td>GSK3 phosphorylation recognition site</td>
<td>nucleus, cytosol</td>
</tr>
<tr>
<td>MOD_N-GLC_1</td>
<td>DNYTHA ANDTEK MNQSGF</td>
<td>278-283 437-442 444-449</td>
<td>Generic motif for N-glycosylation. Shakin-Eshleman et al. showed that Trp, Asp, and Glu are uncommon before the Ser/Thr position. Efficient glycosylation</td>
<td>extracellular, endoplasmic reticulum, Golgi apparatus</td>
</tr>
</tbody>
</table>
usually occurs when ~60 residues or more separate the glycosylation acceptor site from the C-terminus

<table>
<thead>
<tr>
<th>MOD_PIKK_1</th>
<th>ILSTQFP IECTQLY</th>
<th>147-153 333-339</th>
<th>(ST)Q motif which is phosphorylated by PIKK family members</th>
<th>nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOD_PLK</td>
<td>FEISWTK TEKTMNQ KEKTLKN</td>
<td>132-138 440-446 538-544</td>
<td>Site phosphorylated by the Polo-like-kinase</td>
<td>nucleus, cytosol</td>
</tr>
<tr>
<td>MOD_ProDKin_1</td>
<td>YQKTEPG KVKSPLQ YICTPMI</td>
<td>168-174 262-268 339-345</td>
<td>Proline-Directed Kinase (e.g. MAPK) phosphorylation site in higher eukaryotes.</td>
<td>nucleus, cytosol</td>
</tr>
<tr>
<td>MOD_SUMO</td>
<td>LKEE LKLE</td>
<td>60-63 205-208</td>
<td>Motif recognised for modification by SUMO-1</td>
<td>nucleus, PML body</td>
</tr>
<tr>
<td>TRG_ENDOCYTIC_2</td>
<td>YEFL YGVL YARF YKNI YEKF YHEL YYDL YEKI</td>
<td>22-25 181-184 196-199 315-318 534-537 545-548 587-590 591-594</td>
<td>Tyrosine-based sorting signal responsible for the interaction with mu subunit of AP (Adaptor Protein) complex</td>
<td>cytosol, plasma membrane, clathrin-coated endocytic vesicle</td>
</tr>
<tr>
<td>TRG_LysEnd_APsa cLL_1</td>
<td>EIAKLV EQDDLI RAIGLI ESEVLI</td>
<td>6-11 470-475 485-490 514-519</td>
<td>Sorting and internalisation signal found in the cytoplasmic juxta-membrane region of type I transmembrane proteins. Targets them from the Trans</td>
<td>cytosol, Endocytic vesicle</td>
</tr>
</tbody>
</table>
Golgi Network to the lysosomal-endosomal-melanosomal compartments. Interacts with adaptor protein (AP) complexes.

| TRG_PEX | WKRVY | 417-421 | Specific ELM present in Pex5p and binding to Pex13p and Pex14p. Part of the peroxisomal matrix protein import system | peroxisome |
Table 2. Results of Eukaryotic Linear Motif (ELM) search after globular domain filtering, structural filtering and context filtering for the CiaC protein. Results were computed from entering the entire CiaC protein sequence at the ELM website (http://elm.eu.org).

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<th>Positions</th>
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<th>Cell Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLV_PCSK_SKI1_1</td>
<td>KLFTT</td>
<td>90-94</td>
<td>Subtilisin/kexin isozyme-1 (SKI1) cleavage site ([RK]-X-[hydrophobic]-[LTKF]-X)</td>
<td>endoplasmic reticulum, endoplasmic reticulum lumen, Golgi apparatus</td>
</tr>
<tr>
<td>LIG_14-3-3_3</td>
<td>KLFTTI</td>
<td>90-95</td>
<td>Consensus derived from reported natural interactors which do not match the Mode 1 and Mode 2 ligands.</td>
<td>nucleus, cytosol, internal side of plasma membrane</td>
</tr>
<tr>
<td>LIG_BRCT_BRCA1_1_1</td>
<td>NSSKF</td>
<td>49-53</td>
<td>Phosphopeptide motif which directly interacts with the BRCT (carboxy-terminal) domain of the Breast Cancer Gene BRCA1 with low affinity</td>
<td>nucleus, BRCA1-BARD1 complex</td>
</tr>
<tr>
<td>LIG_FHA_1</td>
<td>STTTKVK</td>
<td>15-21</td>
<td>Phosphothreonine motif binding a subset of FHA domains that show a preference for a large aliphatic amino acid at the pT+3 position.</td>
<td>nucleus</td>
</tr>
<tr>
<td>LIG_FHA_2</td>
<td>KNTSADE QATLNEV KFTNEDI LFTTIDA</td>
<td>23-29 31-37 52-58 91-97</td>
<td>Phosphothreonine motif binding a subset of FHA domains that have a preference for an acidic amino acid at the pT+3 position.</td>
<td>nucleus, Replication fork</td>
</tr>
<tr>
<td>LIG_PDZ_3</td>
<td>NEDI EETL</td>
<td>55-58 84-87</td>
<td>Class III PDZ domains binding motif</td>
<td>cytosol, plasma membrane, membrane</td>
</tr>
<tr>
<td>LIG_SH2_STAT3</td>
<td>YAWQ</td>
<td>70-73</td>
<td>YXXQ motif found in the cytoplasmic region of cytokine receptors that bind STAT3 SH2 domain.</td>
<td>cytosol</td>
</tr>
<tr>
<td>MOD_CK1_1</td>
<td>STTTKVK SKFTNED</td>
<td>15-21 51-57</td>
<td>CK1 phosphorylation site</td>
<td>nucleus, cytosol</td>
</tr>
<tr>
<td>MOD_CK2_1</td>
<td>KNTSADE FQATLNE</td>
<td>23-29 30-36</td>
<td>CK2 phosphorylation site</td>
<td>nucleus, cytosol, protein kinase CK2 complex</td>
</tr>
<tr>
<td>MOD_GlcNHglycan</td>
<td>TSAD</td>
<td>25-28</td>
<td>Glycosaminoglycan attachment site</td>
<td>extracellular, Golgi apparatus</td>
</tr>
<tr>
<td>MOD_GSK3_1</td>
<td>VDNNTQKTS KTNSSKFT</td>
<td>3-10 47-54</td>
<td>GSK3 phosphorylation recognition site</td>
<td>nucleus, cytosol</td>
</tr>
<tr>
<td>MOD_N-GLC_1</td>
<td>KNTSAD TNSSKF GNATNN TNNTKA</td>
<td>23-28 48-53 98-103 101-106</td>
<td>Generic motif for N-glycosylation. Shakin-Eshleman et al. showed that Trp, Asp, and Glu are uncommon before the Ser/Thr position. Efficient</td>
<td>extracellular, endoplasmic reticulum, Golgi apparatus</td>
</tr>
</tbody>
</table>
glycosylation usually occurs when ~60 residues or more separate the glycosylation acceptor site from the C-terminus

| MOD_PIKK_1 | VDNTQKT | 3-9 | (ST)Q motif which is phosphorylated by PIKK family members | nucleus |
| MOD_PLK   | NEETLLN | 83-89 | Site phosphorylated by the Polo-like-kinase | nucleus, cytosol |