IDENTIFICATION OF CAMPYLOBACTER JEJUNI SECRETED PROTEINS

By

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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 predicated 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, polyneuropathy 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 *C*ampylobacter 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 G_2/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 dispensible 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 rearrangment 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.

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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$^-$ Δ*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 26ºC 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$^-$ Δ*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$^-$ Δ*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 (σ^{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^{-} Δ*flhDC ysaT* T3SS mutant (Fig. 1E). As expected, the *Y. enterocolitica* cytoplasmic protein σ^{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 NdeI and BglII 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,

FlaC, CiaB) (Fig. 3A). 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 σ^{70}. No reactive band was detected for σ^{70} 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 σ^{70} 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}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 desulforedoxin 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 37ºC 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 37ºC 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 26ºC 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 P*tac* 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 NdeI 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 NdeI 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.*

enterocolitica strains were incubated overnight in LB broth with shaking at 26ºC. 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 26ºC. 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 26ºC 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 26ºC, 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 -20ºC, 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 Cj1242F1SstI (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 *C. jejuni Cj1242* mutant relative to the wild-type strain was always the same.

C. jejuni *secretion assay*

The *C. jejuni* F38011 strain and isogenic *Cj1242* mutant was metabolically labeled with [^{35}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 OD_{540} = 0.3. [^{35}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://wwwchlamydiadb.org; Arnold *et al*., 2009) and "Modlab" software (http://gecco.org.chemie.uni-frankfurt.de/index.html; Löwer & Schneider, 2009).

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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 σ^{70} antibody; E) Immunoblot probed with the CiaB antibody; and F) Immunoblot probed with the σ^{70} antibody. Lanes: 1, *Y. enterocolitica* wild-type harboring the empty pMMB207 vector (WT); 2, *Y. enterocolitica* pYV8081^{-} Δ*flhDC 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^{-} Δ*flhDC ysaT* flagellar mutant harboring the pMMB207 vector containing the *C. jejuni ciaB* gene (ΔT3SS + *ciaB*).

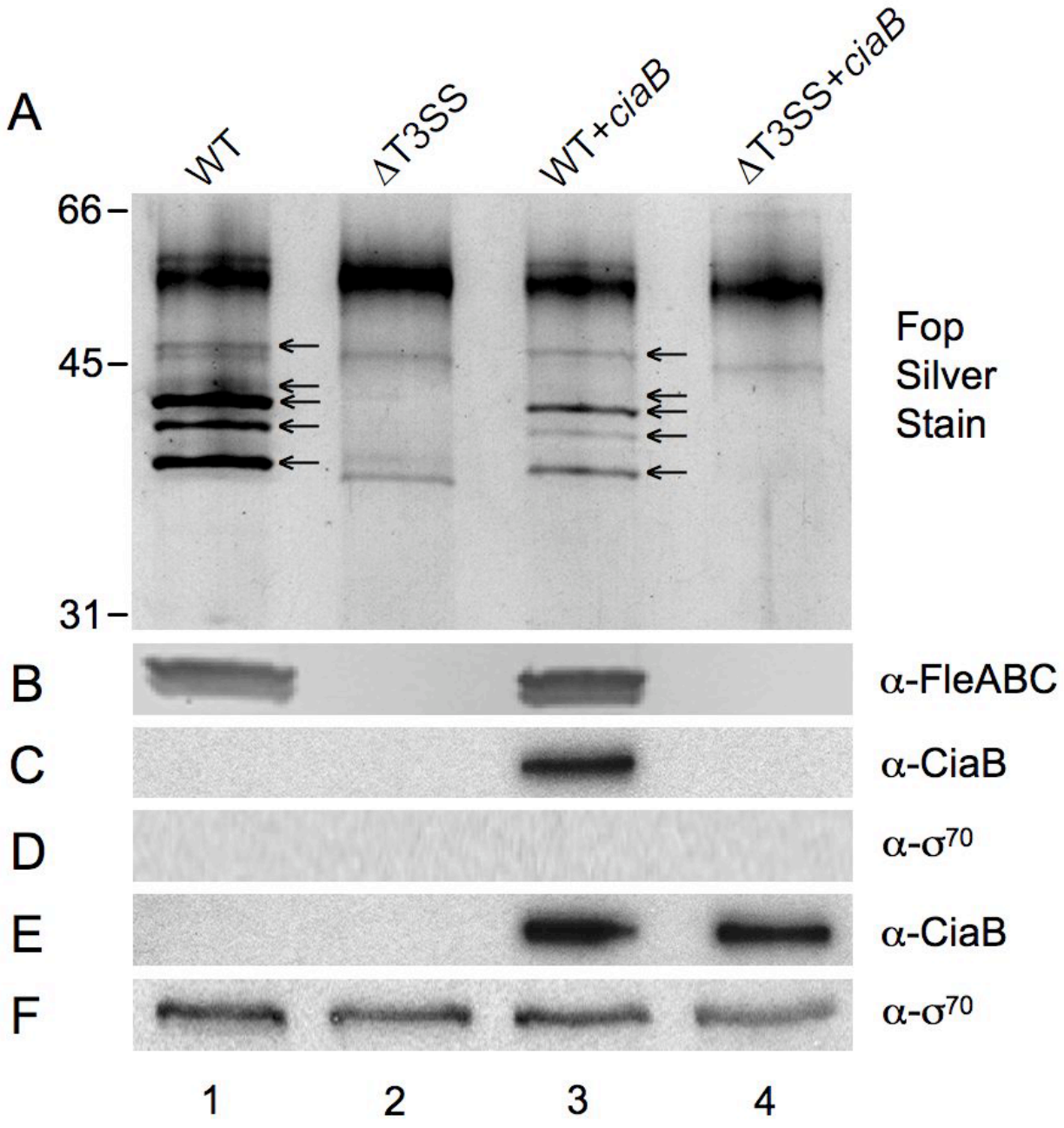
A
WT
ΔT3SS
WT+ciaB
ΔT3SS+ciaB
66
45
31
Fop Silver Stain
B
α-FleABC
C
α-CiaB
D
α-σ70
E
α-CiaB
F
α-σ70
1
2
3
4

Fig. 2. The pCSP50 shuttle vector. The NdeI and BglII 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.

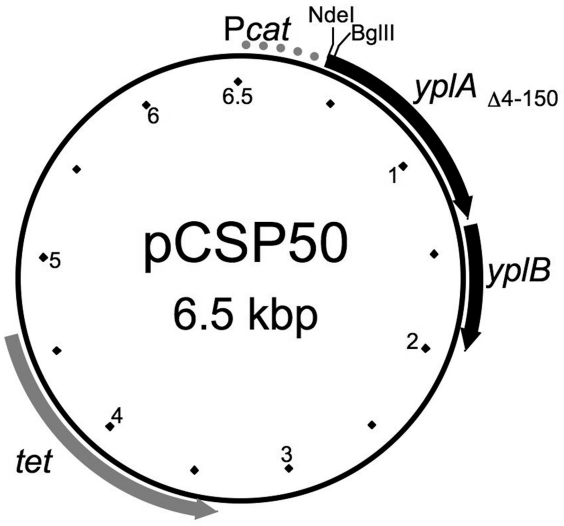
Pcat
NdeI
BglII
yplA Δ4-150
yplB
tet
pCSP50
6.5 kbp
6.5
1
2
3
4
5
6

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 σ^{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 σ^{70} antibody.

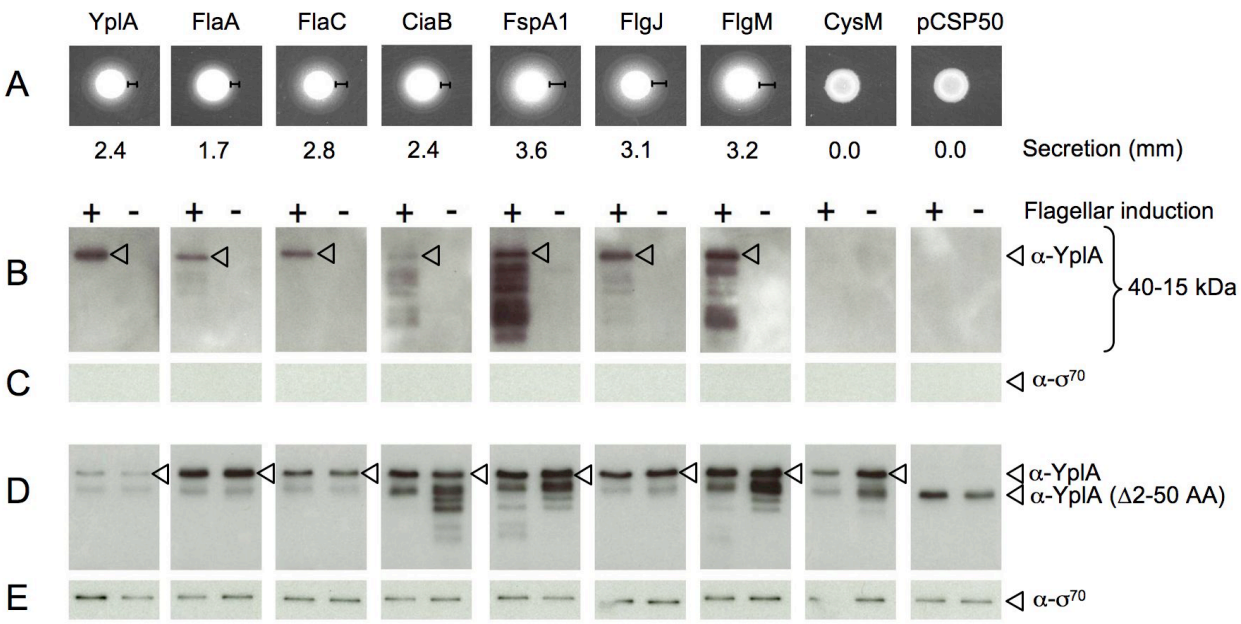
YplA
FlaA
FlaC
CiaB
FspA1
FlgJ
FlgM
CysM
pCSP50
A
2.4
1.7
2.8
2.4
3.6
3.1
3.2
0.0
0.0
Secretion (mm)
+ - + - + - + - + - + - + - + - + -
Flagellar induction
B
α-YplA
40-15 kDa
C
α-σ70
D
α-YplA
α-YplA (Δ2-50 AA)
E
α-σ70

Fig. 4. Secretion of Cj1242 (CiaC) from the *C. jejuni* flagellar T3SS. Isolates were incubated in medium containing [^{35}S]-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.

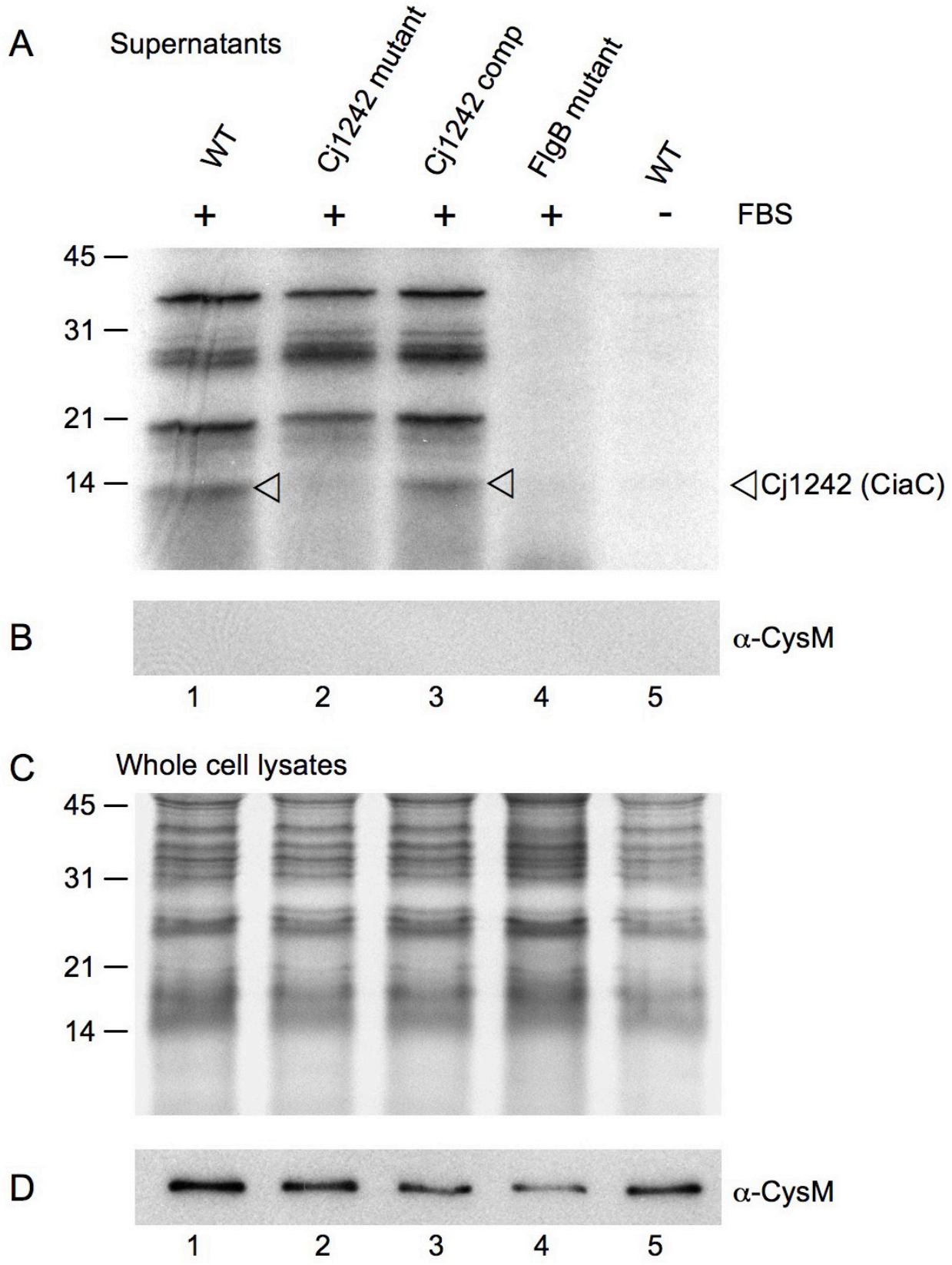
A
Supernatants
WT
Cj1242 mutant
Cj1242 comp
FlgB mutant
WT
+
+
+
+
-
FBS
45
31
21
14
Cj1242 (CiaC)
B
α-CysM
1
2
3
4
5
C
Whole cell lysates
45
31
21
14
D
α-CysM
1
2
3
4
5

Table 1. Strains and plasmids

Strains	Genotype	Source or Reference
C. jejuni		
NCTC 11168	Wild-type genome sequenced strain	(Parkhill *et al*., 2000)
F38011	Clinical isolate	(Rivera-Amill & Konkel, 1999)
F38011 *flgB*	Insertion disruption of *flgB*, non-motile and Cia protein secretion deficient	(Konkel *et al*., 2004)
Y. enterocolitica		
JB580v	Serogroup O:8, Nal *yenR* (r- m+)	(Kinder *et al*., 1993)
GY4478	JB580v, pYV8081-	(Young & Young, 2002)
GY4757	JB580v Δ*yplAB*, pYV8081-	(Warren & Young, 2005)
GY4492	JB580v Δ*flhDC ysaT*::Tn*Mod*-RKm, pYV8081-	(Young & Young, 2002)
Escherichia coli		
S17-1 lambda *pir*	*recA thi pro hsd*R- M+ RP4::2-Tc::Mu::Km Tn7 *pir*	(Simon *et al.*, 1983)
Inv-alpha F'	F' *end*A1 *rec*A1 *hsd*R17 (r-, m+) *sup*E44 *thi*-1 *gyr*A96 *rel*A1 f80*lac*ZDM15 D(*lac*ZYA-*arg*F) U169l-	Invitrogen
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* (F´ *proAB lacIZ*Δ*M15* Tn*10*)	Stratagene
Plasmids		
pMMB207	*mob*+, low copy vector containing an inducible *tac* promoter (P*tac*), Cm	(Morales *et al.*, 1991)
pMEK250	pMMB207 harboring the full-length 1.9 kb *ciaB* gene driven by P*tac*	This study
pTM100	*mob*+, derivative of pACYC184, Cm Tet	(Michiels & Cornelis, 1991)
pCSP50	P*cat* upstream of NdeI and BglII sites for directional cloning of fusions with 5' truncated *yplA* (lacking nucleotides 4-150) and complete *yplB* locus cloned into pTM100 *Eco*RI site, Tet	This study
pCSP50-*yplA* 1-108	Nucleotides 1-108 of *yplA* fused to truncated *yplA* in pCSP50	This study
pCSP50-*flaA* 1-108	Nucleotides 1-108 of *flaA* (Cj1339c) fused to	This study

	truncated *yplA* in pCSP50	
pCSP50-*flaC* 1-108	Nucleotides 1-108 of *flaC* (Cj0720c) fused to truncated *yplA* in pCSP50	This study
pCSP50-*ciaB* 1-108	Nucleotides 1-108 of *ciaB* fused to truncated *yplA* in pCSP50	This study
pCSP50-*cysM* 1-108	Nucleotides 1-108 of *cysM* fused to truncated *yplA* in pCSP50	This study
pBluescript II SK+	Phagemid cloning vector	Stratagene
pMW10	*C. jejuni–E. coli* shuttle vector, Kan	(Wosten *et al.*, 1998)
pBSK-Kan2	pBluescript II SK+ with original ampicillin cassette replaced by the native promoter and *apha3* gene from pMW10, Kan	This study
pBSK-Kan2:del*Cj1242*	pBSK-Kan2 with *Cj1242* internal deletion and disrupted with *tetO* from pUOA3, Kan Tet	This study
pRY111	*C. jejuni–E. coli* shuttle vector, pWKS29 MCS Cm	(Yao *et al.*, 1993)
pRY111:*Cj1242*	pRY111 with a 1.7 kb fragment encompassing *Cj1242*, Cm	This study

Table 2. *C. jejuni* genes encoding a putative T3S amino-terminal sequence

Gene [A]	Locus	Product description
flgM [B, D]	Cj1464	anti-sigma 28 factor
fspA1 [B, E]	Cj0859c	Flagellar secreted protein, virulence factor
rrc [B]	Cj0012c	non-haem iron protein, rubrerythrin
Cj0036	Cj0036	hypothetical protein
Cj1242 [C]	Cj1242	hypothetical protein
flgJ [B, C, F]	Cj1463	flagellar rod protein
Cj0073c [C]	Cj0073c	conserved hypothetical protein
Cj0122	Cj0122	hypothetical protein
Cj0125c	Cj0125c	hypothetical protein
Cj0140	Cj0140	hypothetical protein
Cj0239c	Cj0239c	NifU protein homolog
Cj0251c	Cj0251c	conserved hypothetical protein
Cj0787 [C]	Cj0787	conserved hypothetical protein
Cj0788 [C]	Cj0788	hypothetical protein
Cj0015c	Cj0015c	hypothetical protein
Cj0021c [B]	Cj0021c	putative fumarylacetoacetate (FAA) hydrolase family protein
Cj0030	Cj0030	hypothetical protein
hemN	Cj0363c	putative oxygen-independent coproporphyrinogen III oxidase
Cj0416	Cj0416	hypothetical protein
Cj0449c	Cj0449c	conserved hypothetical protein
Cj0849c	Cj0849c	conserved hypothetical protein
Cj1300 [B]	Cj1300	putative SAM domain containing methyltransferase
Cj1543 [B]	Cj1543	putative allophanate hydrolase subunit 2
Cj0188c [C]	Cj0188c	putative kinase
Cj0254	Cj0254	hypothetical protein
Cj0391c	Cj0391c	hypothetical protein
Cj0717 [B]	Cj0717	putative ArsC family protein
Cj0973	Cj0973	hypothetical protein
Cj1006c [B, C]	Cj1006c	putative MiaB-like tRNA modifying enzyme
Cj1057c [B]	Cj1057c	putative coiled-coil protein
Cj1089c	Cj1089c	hypothetical protein
Cj1310c	Cj1310c	hypothetical protein (617 family)
Cj1348c [B]	Cj1348c	putative coiled-coil protein
Cj1497c	Cj1497c	hypothetical protein
Cj0069	Cj0069	hypothetical protein
Cj0668 [B]	Cj0668	putative ATP/GTP-binding protein
Cj0681	Cj0681	hypothetical protein
Cj0706 [C]	Cj0706	conserved hypothetical protein
Cj0916c	Cj0916c	conserved hypothetical protein

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 the *ciaB: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.

Bacterial Strain	Numbers of viable bacteria		I/A[a]
	Adherent	Internalized	
C. jejuni wild-type	$(7.1 \pm 0.6) \times 10^5$	$(3.3 \pm 0.5) \times 10^4$	4.6%
C. jejuni Cj1242	$(7.6 \pm 1.2) \times 10^5$	$(6.2 \pm 1.9) \times 10^3$	0.82%[b]
C. jejuni ciaB	$(7.2 \pm 1.2) \times 10^5$	$(4.0 \pm 0.6) \times 10^3$	0.56%[b]
E. coli XL1-Blue	$(1.8 \pm 0.4) \times 10^5$	$(1.7 \pm 1.2) \times 10^2$	0.09%

[a]Percent of internalized bacteria relative to adherent bacteria.

[b]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

Gene	Locus	Product (Gundogdu *et al*., 2007)
rrc	Cj0012c	non-haem iron protein
Cj0015c	Cj0015c	hypothetical protein
dba	Cj0018c	disulphide bond formation protein
Cj0021c	Cj0021c	putative fumarylacetoacetate (FAA) hydrolase family protein
thyX	Cj0026c	thymidylate synthase
Cj0030	Cj0030	hypothetical protein
Cj0036	Cj0036	hypothetical protein
Cj0038c	Cj0038c	putative poly(A) polymerase family protein
Cj0040	Cj0040	hypothetical protein
fliK	Cj0041	putative flagellar hook-length control protein
Cj0044c	Cj0044c	hypothetical protein
Cj0045c	Cj0045c	putative iron-binding protein
Cj0054c	Cj0054c	putative lysine decarboxylase family protein
Cj0062c	Cj0062c	putative integral membrane protein
Cj0067	Cj0067	putative amidohydrolase family protein
Cj0069	Cj0069	hypothetical protein
Cj0073c	Cj0073c	hypothetical protein
Cj0074c	Cj0074c	putative iron-sulfur protein
Cj0075c	Cj0075c	putative oxidoreductase iron-sulfur subunit
Cj0085c	Cj0085c	putative amino acid recemase
Cj0118	Cj0118	hypothetical protein
Cj0120	Cj0120	hypothetical protein
Cj0121	Cj0121	hypothetical protein
Cj0122	Cj0122	hypothetical protein
Cj0125c	Cj0125c	hypothetical protein
Cj0126c	Cj0126c	hypothetical protein
Cj0128c	Cj0128c	putative inositol monophosphatase family protein
Cj0133	Cj0133	putative glycoprotease family protein
Cj0135	Cj0135	hypothetical protein
Cj0138	Cj0138	hypothetical protein
Cj0140	Cj0140	hypothetical protein
Cj0148c	Cj0148c	hypothetical protein
Cj0152c	Cj0152c	putative membrane protein
Cj0156c	Cj0156c	hypothetical protein
Cj0157c	Cj0157c	putative integral membrane protein
Cj0159c	Cj0159c	putative 6-pyruvoyl tetrahydropterin synthase
Cj0160c	Cj0160c	putative radical SAM domain protein
Cj0162c	Cj0162c	putative periplasmic protein
Cj0163c	Cj0163c	hypothetical protein
Cj0172c	Cj0172c	putative saccharopine dehydrogenase
Cj0184c	Cj0184c	possible serine\threonine protein phosphatase
Cj0188c	Cj0188c	putative kinase
Cj0189c	Cj0189c	hypothetical protein

Cj0229	Cj0229	putative acetyltransferase
Cj0230c	Cj0230c	putative transferase protein
Cj0232c	Cj0232c	putative integral membrane protein
Cj0239c	Cj0239c	nifU protein homolog
Cj0241c	Cj0241c	putative iron-binding protein
Cj0249	Cj0249	hypothetical protein
Cj0251c	Cj0251c	highly acidic protein
Cj0253	Cj0253	hypothetical protein
Cj0254	Cj0254	hypothetical protein
Cj0258	Cj0258	putative bacterial regulatory protein, ArsR family
Cj0270	Cj0270	putative tautomerase family protein
Cj0272	Cj0272	hypothetical protein
Cj0286c	Cj0286c	hypothetical protein
Cj0323	Cj0323	hypothetical protein
Cj0327	Cj0327	putative endoribonuclease L-PSP family protein
Cj0331c	Cj0331c	hypothetical protein
Cj0340	Cj0340	putative nucleoside hydrolase
Cj0344	Cj0344	hypothetical protein
Cj0353c	Cj0353c	phosphatase
glmM	Cj0360	phosphoglucosamine mutase
hemN	Cj0363c	putative oxygen-independent coproporphyrinogen III oxidase
Cj0364	Cj0364	hypothetical protein
Cj0372	Cj0372	putative glutathionylspermidine synthase
Cj0373	Cj0373	putative D-2-hydroxyacid dehydrogenase
Cj0374	Cj0374	hypothetical protein
Cj0377	Cj0377	probable AAA family ATPase
Cj0380c	Cj0380c	hypothetical protein
Cj0386	Cj0386	putative GTP-binding protein
Cj0391c	Cj0391c	hypothetical protein
mqo	Cj0393c	putative malate:quinone oxidoreductase
Cj0394c	Cj0394c	putative transcriptional activator
Cj0395c	Cj0395c	hypothetical protein
Cj0397c	Cj0397c	hypothetical protein
Cj0403	Cj0403	hypothetical protein
Cj0404	Cj0404	putative transmembrane protein
Cj0411	Cj0411	putative ATP/GTP binding protein
Cj0412	Cj0412	putative ATP/GTP binding protein
Cj0415	Cj0415	putative GMC oxidoreductase subunit
Cj0416	Cj0416	hypothetical protein
Cj0421c	Cj0421c	putative integral membrane protein
Cj0427	Cj0427	hypothetical protein
Cj0429c	Cj0429c	hypothetical protein
Cj0436	Cj0436	putative pyridoxamine 5'-phosphate oxidase
Cj0447	Cj0447	putative NUDIX hydrolase family protein
Cj0449c	Cj0449c	hypothetical protein
Cj0456c	Cj0456c	hypothetical protein

Cj0457c	Cj0457c	putative lipoprotein
Cj0462	Cj0462	putative radical SAM domain protein
Cj0463	Cj0463	zinc protease-like protein
ctb	Cj0465c	group III truncated haemoglobin
dapA	Cj0481	putative dihydrodipicolinate synthase
Cj0485	Cj0485	putative oxidoreductase
Cj0487	Cj0487	putative amidohydrolase
Cj0488	Cj0488	hypothetical protein
Cj0496	Cj0496	hypothetical protein
Cj0499	Cj0499	putative histidine triad (HIT) family protein
Cj0500	Cj0500	putative rhodanese-like domain protein
Cj0504c	Cj0504c	putative oxidoreductase
Cj0505c	Cj0505c	putative aminotransferase (degT family)
Cj0510c	Cj0510c	hypothetical protein
Cj0519	Cj0519	putative rhodanese-like domain protein
Cj0539	Cj0539	hypothetical protein
ubiD	Cj0546	putative 3-octaprenyl-4-hydroxybenzoate carboxy-lyase
Cj0550	Cj0550	hypothetical protein
Cj0556	Cj0556	putative amidohydrolase family protein
Cj0559	Cj0559	putative pyridine nucleotide-disulphide oxidoreductase
Cj0563	Cj0563	hypothetical protein
Cj0571	Cj0571	putative transcriptional regulator
Cj0573	Cj0573	putative GatB/Yqey family protein
nudH	Cj0581	putative NUDIX hydrolase family protein
Cj0583	Cj0583	hypothetical protein
Cj0600	Cj0600	hypothetical protein
Cj0602c	Cj0602c	MOSC-domain containing protein
Cj0604	Cj0604	putative polyphosphate kinase
Cj0611c	Cj0611c	putative acyltransferase family protein
Cj0620	Cj0620	hypothetical protein
Cj0621	Cj0621	hypothetical protein
holA	Cj0630c	putative DNA polymerase III, delta subunit
Cj0635	Cj0635	putative holliday junction resolvase
Cj0636	Cj0636	NOL1\NOP2\sun family protein
Cj0641	Cj0641	putative inorganic polyphosphate/ATP-NAD kinase
Cj0644	Cj0644	putative TatD-related deoxyribonuclease protein
Cj0647	Cj0647	putative HAD-superfamily hydrolase
Cj0650	Cj0650	putative ATP/GTP binding protein
Cj0667	Cj0667	putative S4 domain protein
Cj0668	Cj0668	putative ATP/GTP-binding protein
Cj0681	Cj0681	hypothetical protein
Cj0682	Cj0682	hypothetical protein
Cj0700	Cj0700	hypothetical protein
Cj0701	Cj0701	putative protease
Cj0703	Cj0703	hypothetical protein
Cj0706	Cj0706	hypothetical protein

Cj0708	Cj0708	putative ribosomal pseudouridine synthase
Cj0711	Cj0711	hypothetical protein
Cj0716	Cj0716	putative phospho-2-dehydro-3-deoxyheptonate aldolase
Cj0717	Cj0717	putative ArsC family protein
Cj0719c	Cj0719c	hypothetical protein
Cj0724	Cj0724	hypothetical protein
Cj0729	Cj0729	putative type I phosphodiesterase/nucleotide pyrophosphatase
Cj0733	Cj0733	putative HAD-superfamily hydrolase
Cj0760	Cj0760	hypothetical protein
Cj0761	Cj0761	hypothetical protein
Cj0786	Cj0786	small hydrophobic protein
Cj0787	Cj0787	hypothetical protein
Cj0788	Cj0788	hypothetical protein
Cj0791c	Cj0791c	putative aminotransferase
Cj0792	Cj0792	hypothetical protein
Cj0794	Cj0794	hypothetical protein
Cj0797c	Cj0797c	hypothetical protein
Cj0800c	Cj0800c	putative ATPase
Cj0805	Cj0805	putative zinc protease
Cj0808c	Cj0808c	small hydrophobic protein
Cj0809c	Cj0809c	putative hydrolase
Cj0823	Cj0823	hypothetical protein
Cj0829c	Cj0829c	putative CoA binding domain containing protein
Cj0833c	Cj0833c	oxidoreductase
Cj0837c	Cj0837c	hypothetical protein
Cj0839c	Cj0839c	hypothetical protein
mobB	Cj0841c	putative molybdopterin-guanine dinucleotide biosynthesis protein
Cj0844c	Cj0844c	putative integral membrane protein
Cj0849c	Cj0849c	hypothetical protein
Cj0852c	Cj0852c	putative integral membrane protein
Cj0859c	Cj0859c	hypothetical protein
Cj0878	Cj0878	hypothetical protein
Cj0880c	Cj0880c	hypothetical protein
Cj0881c	Cj0881c	hypothetical protein
Cj0883c	Cj0883c	putative transcriptional regulator
Cj0898	Cj0898	HIT-family protein
Cj0915	Cj0915	putative hydrolase
Cj0916c	Cj0916c	hypothetical protein
Cj0930	Cj0930	putative GTP-binding protein
Cj0939c	Cj0939c	hypothetical protein
Cj0947c	Cj0947c	putative carbon-nitrogen hydrolase
Cj0949c	Cj0949c	putative peptidyl-arginine deiminase family protein
Cj0954c	Cj0954c	putative dnaJ-like protein
Cj0957c	Cj0957c	hypothetical protein
Cj0959c	Cj0959c	hypothetical protein
Cj0962	Cj0962	putative acetyltransferase

Cj0963	Cj0963	hypothetical protein
Cj0965c	Cj0965c	putative acyl-CoA thioester hydrolase
Cj0971	Cj0971	hypothetical protein
Cj0972	Cj0972	hypothetical protein
Cj0973	Cj0973	hypothetical protein
Cj0976	Cj0976	putative methyltransferase
Cj0977	Cj0977	hypothetical protein
Cj0984	Cj0984	hypothetical protein
Cj0989	Cj0989	putative membrane protein
Cj0990c	Cj0990c	hypothetical protein
Cj0993c	Cj0993c	hypothetical protein
Cj1002c	Cj1002c	putative phosphoglycerate/bisphosphoglycerate mutase
Cj1006c	Cj1006c	putative MiaB-like tRNA modifying enzyme
Cj1009c	Cj1009c	hypothetical protein
Cj1011	Cj1011	putative CorA-like Mg2+ transporter protein
Cj1028c	Cj1028c	possible purine/pyrimidine phosphoribosyltransferase
Cj1034c	Cj1034c	possible dnaJ-like protein
ate	Cj1035c	putative arginyl-tRNA-protein transferase
Cj1036c	Cj1036c	hypothetical protein
Cj1056c	Cj1056c	putative carbon-nitrogen hydrolase family protein
Cj1057c	Cj1057c	putative coiled-coil protein
Cj1063	Cj1063	possible acetyltransferase
Cj1075	Cj1075	hypothetical protein
Cj1084c	Cj1084c	putative ATP/GTP-binding protein
Cj1086c	Cj1086c	hypothetical protein
Cj1089c	Cj1089c	hypothetical protein
lnt	Cj1095	putative apolipoprotein N-acyltransferase
Cj1100	Cj1100	hypothetical protein
clpS	Cj1107	ATP-dependent Clp protease adaptor protein
Cj1112c	Cj1112c	putative SelR domain containing protein
Cj1113	Cj1113	hypothetical protein
Cj1115c	Cj1115c	putative phosphatidylserine decarboxylase-related protein
Cj1144c	Cj1144c	hypothetical protein
gmhB	Cj1152c	D,D-heptose 1,7-bisphosphate phosphatase
Cj1162c	Cj1162c	putative heavy-metal-associated domain protein
Cj1164c	Cj1164c	hypothetical protein
Cj1172c	Cj1172c	hypothetical protein
tatA	Cj1176c	Sec-independent protein translocase (TatA/E homolog)
Cj1178c	Cj1178c	highly acidic protein
Cj1199	Cj1199	putative iron/ascorbate-dependent oxidoreductase
Cj1203c	Cj1203c	putative integral membrane protein
Cj1208	Cj1208	putative 5-formyltetrahydrofolate cyclo-ligase family protein
Cj1211	Cj1211	putative competence family protein
Cj1216c	Cj1216c	hypothetical protein
Cj1217c	Cj1217c	hypothetical protein
Cj1224	Cj1224	putative iron-binding protein

Cj1225	Cj1225	hypothetical protein
Cj1232	Cj1232	hypothetical protein
Cj1233	Cj1233	putative HAD-superfamily hydrolase
Cj1236	Cj1236	hypothetical protein
Cj1237c	Cj1237c	possible phosphatase
Cj1242	Cj1242	hypothetical protein
Cj1245c	Cj1245c	putative membrane protein
Cj1247c	Cj1247c	hypothetical protein
Cj1249	Cj1249	hypothetical protein
Cj1251	Cj1251	hypothetical protein
Cj1254	Cj1254	hypothetical protein
Cj1256c	Cj1256c	putative membrane protein
Cj1268c	Cj1268c	putative FAD dependent oxidoreductase
Cj1270c	Cj1270c	putative 2-nitropropane dioxygenase, oxidoreductase protein
Cj1285c	Cj1285c	hypothetical protein
pseC	Cj1294	C4 aminotransferase specific for PseB product
Cj1295	Cj1295	hypothetical protein
Cj1300	Cj1300	putative SAM domain containing methyltransferase
Cj1305c	Cj1305c	hypothetical protein Cj1305c (617 family)
Cj1306c	Cj1306c	hypothetical protein Cj1306c (617 family)
Cj1307	Cj1307	putative amino acid activating enzyme
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/maf 5	Cj1337	hypothetical protein
Cj1342c	Cj1342c	(Motility accessory factor, function unknown)
Cj1348c	Cj1348c	putative coiled-coil protein
Cj1349c	Cj1349c	possible fibronectin/fibrinogen-binding protein
Cj1360c	Cj1360c	putative proteolysis tag for 10Sa_RNA
Cj1361c	Cj1361c	hypothetical protein
Cj1367c	Cj1367c	possible nucleotidyltransferase
Cj1370	Cj1370	putative nucleotide phosphoribosyltransferase
Cj1374c	Cj1374c	hypothetical protein
Cj1377c	Cj1377c	putative ferredoxin
Cj1383c	Cj1383c	hypothetical protein
Cj1384c	Cj1384c	hypothetical protein
Cj1386	Cj1386	ankyrin-repeat containing protein
Cj1397	Cj1397	putative ferrous iron transport protein
nadD	Cj1404	putative nicotinate-nucleotide adenylyltransferase
Cj1405	Cj1405	hypothetical protein
Cj1449c	Cj1449c	hypothetical protein
Cj1450	Cj1450	putative ATP/GTP-binding protein
tilS	Cj1453c	putative tRNA(Ile)-lysidine synthase
truD	Cj1457c	tRNA pseudouridine synthase D

Cj1459	Cj1459	hypothetical protein
Cj1460	Cj1460	hypothetical protein
flgJ	Cj1463	hypothetical protein
flgM	Cj1464	hypothetical protein
flgN	Cj1465	hypothetical protein
Cj1467	Cj1467	hypothetical protein
Cj1476c	Cj1476c	pyruvate-flavodoxin oxidoreductase
Cj1477c	Cj1477c	putative hydrolase
Cj1482c	Cj1482c	hypothetical protein
Cj1493c	Cj1493c	putative integral membrane protein
Cj1495c	Cj1495c	hypothetical protein
Cj1496c	Cj1496c	putative periplasmic protein
Cj1497c	Cj1497c	hypothetical protein
Cj1501	Cj1501	hypothetical protein
Cj1505c	Cj1505c	putative two-component response regulator (SirA-like protein)
Cj1507c	Cj1507c	putative regulatory protein
Cj1514c	Cj1514c	hypothetical protein
Cj1521c	Cj1521c	putative CRISPR-associated protein
Cj1522c	Cj1522c	putative CRISPR-associated protein
coaE	Cj1530	putative dephospho-CoA kinase
Cj1533c	Cj1533c	putative helix-turn-helix containsing protein
Cj1534c	Cj1534c	possible bacterioferritin
Cj1541	Cj1541	hypothetical protein
Cj1542	Cj1542	putative allophanate hydrolase subunit 1
Cj1543	Cj1543	putative allophanate hydrolase subunit 2
Cj1548c	Cj1548c	putative NADP-dependent alcohol dehydrogenase
Cj1558	Cj1558	putative membrane protein
Cj1585c	Cj1585c	putative oxidoreductase
Cj1602	Cj1602	hypothetical protein
Cj1613c	Cj1613c	putative pyridoxamine 5'-phosphate oxidase
Cj1627c	Cj1627c	hypothetical protein
Cj1637c	Cj1637c	putative periplasmic protein
Cj1639	Cj1639	nifU protein homolog
Cj1640	Cj1640	hypothetical protein
Cj1642	Cj1642	hypothetical protein
Cj1649	Cj1649	putative lipoprotein
Cj1650	Cj1650	hypothetical protein
Cj1656c	Cj1656c	hypothetical protein
Cj1667c	Cj1667c	repA protein homolog
Cj1671c	Cj1671c	hypothetical protein
Cj1674	Cj1674	hypothetical protein
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

Cj1715	Cj1715	putative acetyltransferase
Cj1720	Cj1720	hypothetical protein
Cj1724c	Cj1724c	putative GTP cyclohydrolase I

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.*

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 *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 *C. jejuni*.

C. jejuni pathogenic mechanisms

Motility, adherence, invasion, protein secretion, intracellular survival, and toxin production may contribute to the pathogenicity of a given *C. jejuni* strain. As these topics and others dealing with *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 *C. jejuni* protein secretion and the possible roles of secreted proteins in the development of gastrointestinal disease.

C. jejuni motility is provided by either monotrichous or amphitrichous flagella, and *C. jejuni* must be flagellated to colonize chickens or cause disease in humans (Black *et al.*, 1988, Hendrixson, 2006, Newell *et al.*, 1985a). Flagellar locomotion facilitates penetration of the mucosal boundary lining the host intestine. Motility also allows *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 *C. jejuni* binding and invasion of host cells (Carrillo *et al.*, 2004, Konkel and Joens, 1989, Konkel *et al.*, 2004, Malik-Kale *et al.*, 2007, Wassenaar *et al.*, 1991).

Adherence to intestinal epithelial cells is proposed to be fundamental to *C. jejuni* colonization and pathogenesis. Proteins proposed to act as *C. jejuni* adhesins include CadF, CapA, PorA (MOMP), PEB1, and JlpA. CadF, *Campylobacter* adhesion to fibronectin, is a 37 kDa outer membrane protein (Konkel *et al.*, 1997). A *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 *et al.*, 2003), and is incapable of colonizing chickens (Ziprin *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 *et al.*, 2007). The *porA* gene of *C. jejuni* encodes a 43 kDa 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 *et al.*, 1995, De *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 *et al.*, 1997, Schroder and Moser, 1997). *C. jejuni porA* mutants have yet to be characterized, as mutations in *porA* are lethal to *C. jejuni* due to the protein's critical structural and transport functions. PEB1 is a 28 kDa protein and disruption of *peb1A* reduces *C. jejuni* adherence to HeLa cells by 50- to 100-fold (Pei and Blaser, 1993, Pei *et al.*, 1998). In addition, a *C. jejuni peb1A* null mutant exhibits a reduction in the duration of mouse intestinal colonization when compared to the *C. jejuni* wild-type isolate (Pei *et al.*, 1998). JlpA, jejuni lipoprotein A, is a *C. jejuni* 43.2 kDa protein. Disruption of *jlpA* reduces *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 $\alpha_5\beta_1$ 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 $\alpha_5\beta_1$ 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 Geme 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_r 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, σ^{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 σ^{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 σ^{54} and σ^{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 *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 *Y. enterocolitica* flagellar mutant cytosol, but was not secreted. Silver stained Fop profiles revealed that only the *Y. enterocolitica* wild-type isolate secretes the Fop proteins. The *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 *ciaB* was found to harbor a T3SS signal.

In summary, two lines of evidence support the hypothesis that the *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 *cia* expression. Second, the N-terminal coding sequence of CiaB contains a T3SS export signal that is recognized by the *Y. enterocolitica* flagellar T3SS.

Identification of *C. jejuni* secreted proteins

To facilitate the identification of *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 YplB 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 cresecentus* 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 mutationally-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$^+$)* 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$^+$)* 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$^+$)* 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$^+$* 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.

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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.

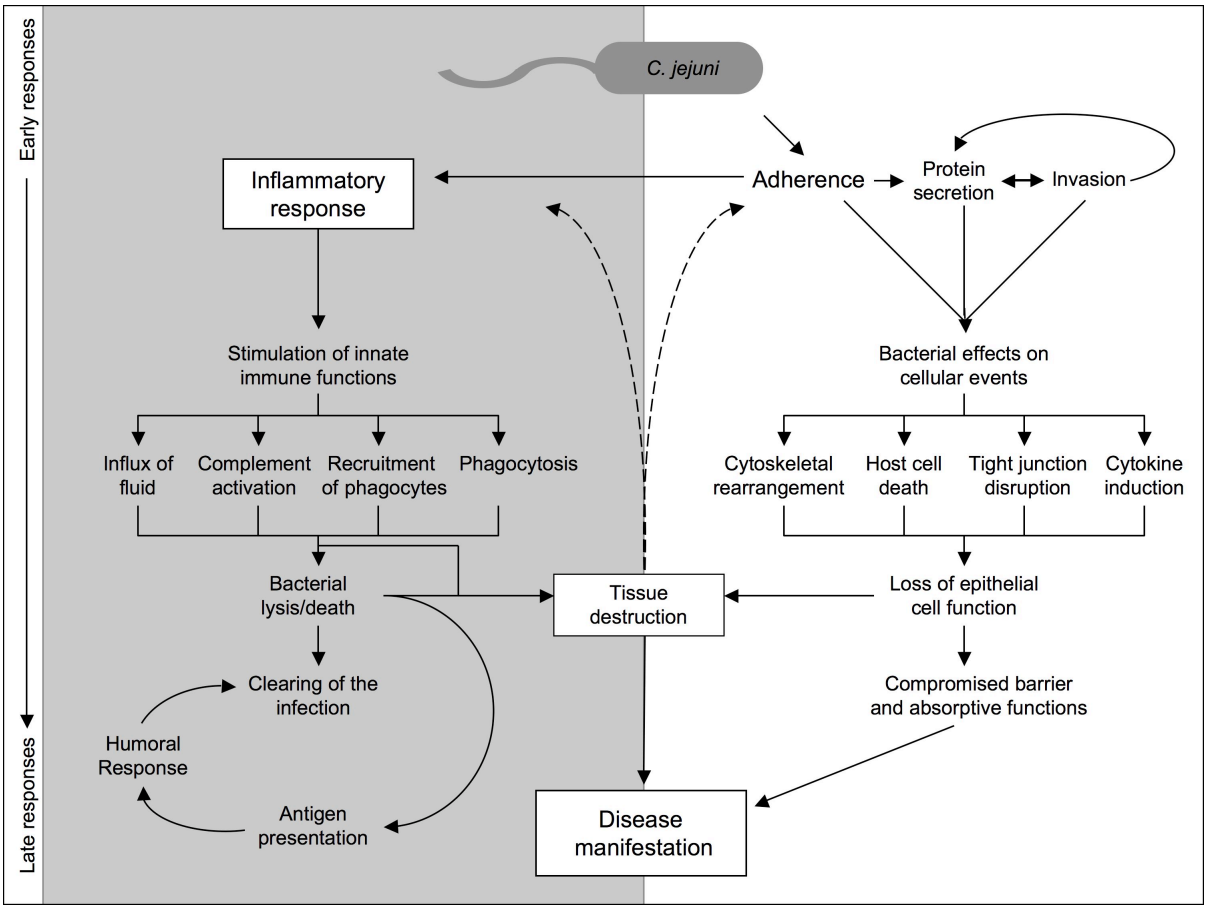

Early responses
Late responses
C. jejuni
Inflammatory response
Adherence
Protein secretion
Invasion
Stimulation of innate immune functions
Bacterial effects on cellular events
Influx of fluid
Complement activation
Recruitment of phagocytes
Phagocytosis
Cytoskeletal rearrangement
Host cell death
Tight junction disruption
Cytokine induction
Bacterial lysis/death
Tissue destruction
Loss of epithelial cell function
Clearing of the infection
Compromised barrier and absorptive functions
Humoral Response
Antigen presentation
Disease manifestation

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.

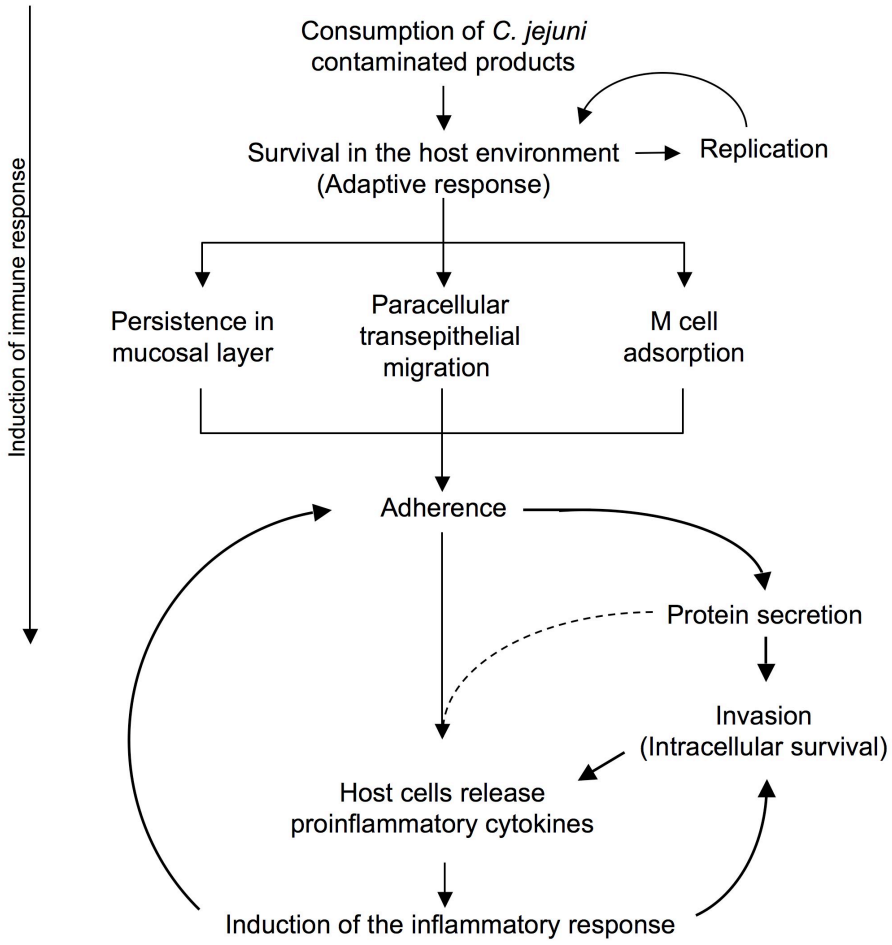

Induction of immune response
Consumption of C. jejuni contaminated products
Survival in the host environment (Adaptive response)
Replication
Persistence in mucosal layer
Paracellular transepithelial migration
M cell adsorption
Adherence
Protein secretion
Invasion (Intracellular survival)
Host cells release proinflammatory cytokines
Induction of the inflammatory 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 [^{35}S]-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

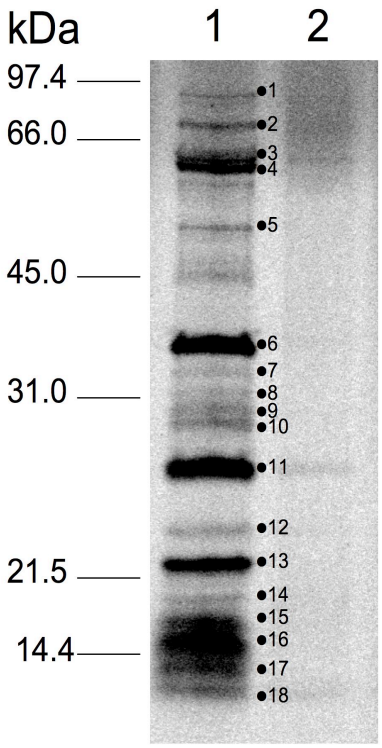


Fig. 4. The *C. jejuni* type III secretion system (T3SS) is the flagellum, which secretes the *Campylobacter* secreted proteins (Csp). A subset of the Csps, termed the *Campylobacter* invasion antigens (Cia), are required for maximal invasion. Both the Csp and Cia proteins harbor non-cleavable, non-consensus secretion signals, required for export.

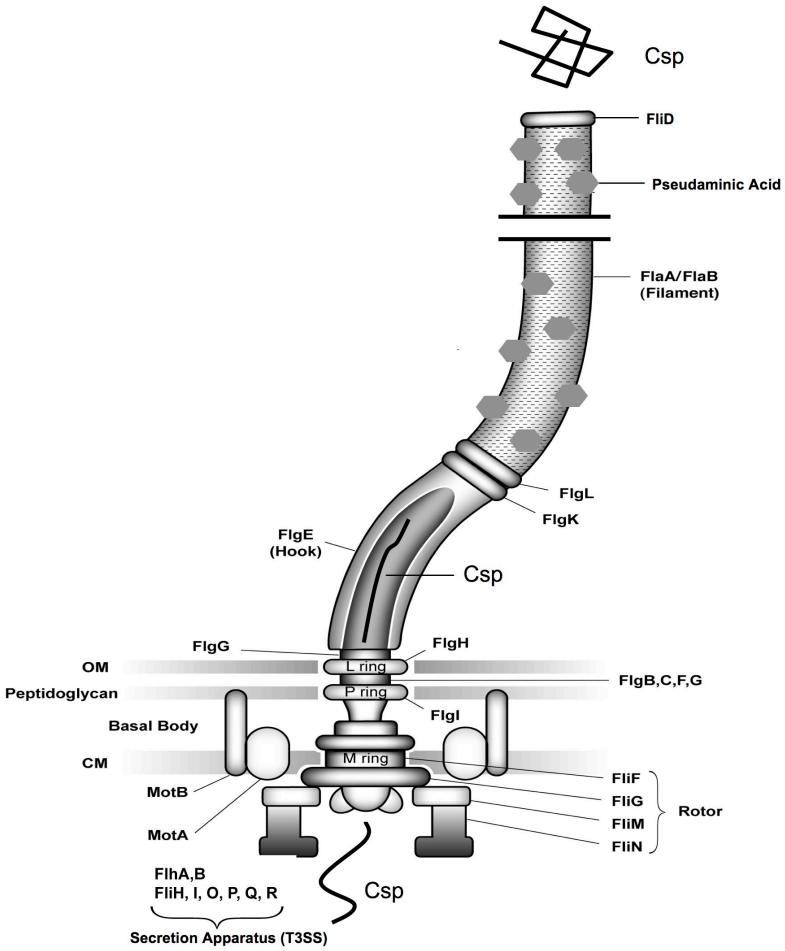
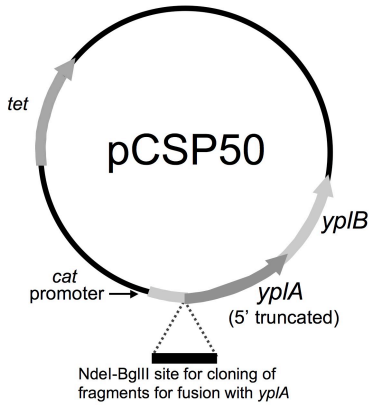

Csp
FliD
Pseudaminic Acid
FlaA/FlaB
(Filament)
FlgL
FlgK
FlgE
(Hook)
Csp
FlgG
FlgH
OM
L ring
FlgB,C,F,G
Peptidoglycan
P ring
FlgI
Basal Body
CM
M ring
FliF
FliG
FliM
FliN
Rotor
MotB
MotA
FlhA,B
FliH, I, O, P, Q, R
Csp
Secretion Apparatus (T3SS)

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*$_{1\text{-}108}$; and E, *Y. enterocolitica yplAB* mutant with pCSP50:*cysM*$_{1\text{-}108}$. All strains show strong growth; panels A and D show strong secretion (of YplA and CiaB$_{1\text{-}36}$:YplA fusion protein, respectively) resulting in a zone of precipitate due to hydrolysis of fatty acids by the phospholipase.


tet
pCSP50
yplB
cat
promoter
yplA
(5' truncated)
NdeI-BglII site for cloning of
fragments for fusion with yplA

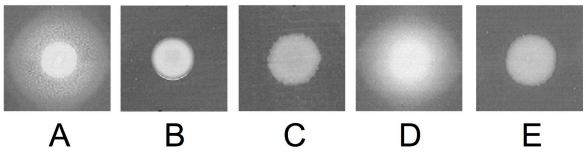

A
B
C
D
E

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.

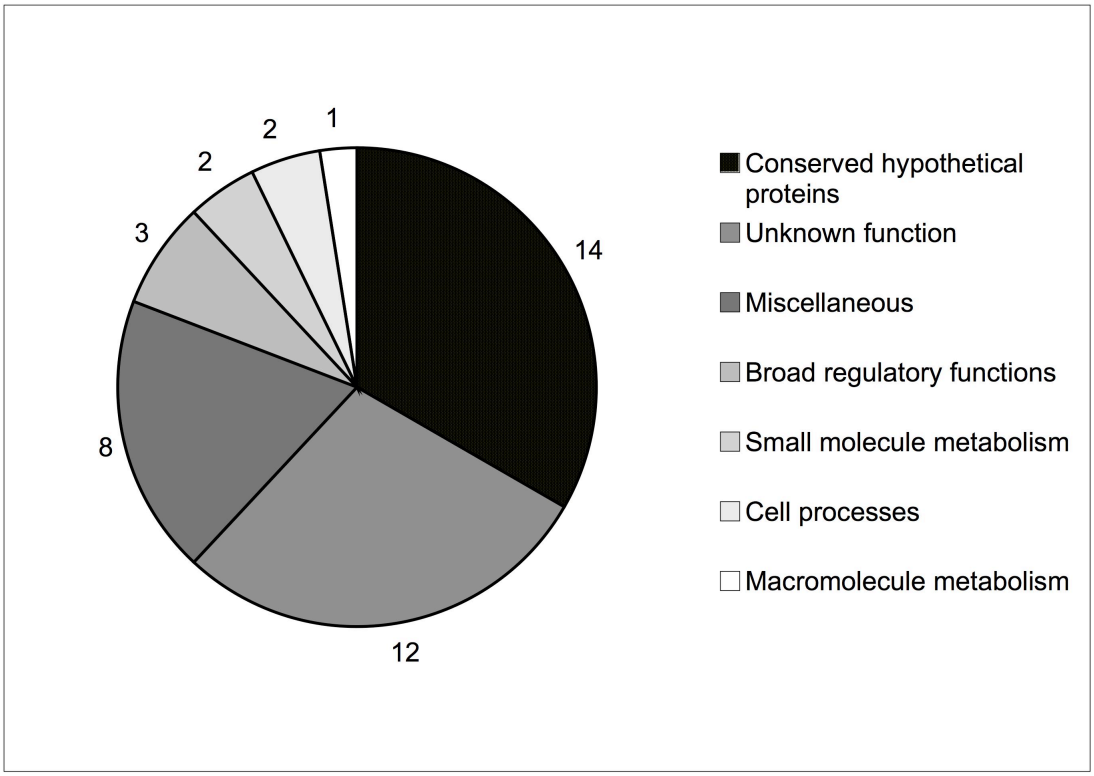

1
2
2
3
8
14
12
Conserved hypothetical proteins
Unknown function
Miscellaneous
Broad regulatory functions
Small molecule metabolism
Cell processes
Macromolecule metabolism

Table 1. Relative molecular mass of *C. jejuni* secreted proteins

Protein #	% of total secreted protein	M_r
1	0.2	86.0
2	0.7	72.0
3	2.0	63.0
4	5.4	60.5
5	1.2	51.5
6	19.0	38.5
7	1.5	36.0
8	1.6	32.0
9	1.8	30.0
10	2.4	29.5
11	17.2	27.0
12	1.2	24.0
13	12.0	22.0
14	1.5	18.5
15	6.4	17.0
16	14.6	15.5
17	7.0	13.0
18	4.3	11.5

CHAPTER 4

SUMMARY

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.*

The characterization of the CiaB protein as a bonified 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.

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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).

Elm Name	Instances (Matched Sequence)	Positions	Elm Description	Cell Compartment
CLV_NDR_NDR_1	VRK RRL LRK	1-13 55-57 162-164	N-Arg dibasic convertase (nardilysine) cleavage site (Xaa-|-Arg-Lys or Arg-|-Arg-Xaa)	extracellular, Golgi apparatus, cell surface
CLV_PCSK_PC1ET2_1	KRV KRA	418-420 484-486	NEC1/NEC2 cleavage site (Lys-Arg-|-Xaa)	Golgi membrane, extracellular, Golgi apparatus
CLV_PCSK_SKI1_1	KLAFC RVIKA KEVLS KGLFS KKIFA KEFLD RNILF KELTL KEFLS	238-242 248-252 327-331 352-356 372-376 400-404 407-411 538-542 559-563	Subtilisin/kexin isozyme-1 (SKI1) cleavage site ([RK]-X-[hydrophobic]-[LTKF]-|-X)	endoplasmic reticulum, endoplasmic reticulum lumen, Golgi apparatus
LIG_14-3-3_2	RSLISLS	35-41	Longer mode 2 interacting phospho-motif for 14-3-3 proteins with key conservation RxxxS#p.	nucleus, mitochondrion, cytosol, internal side of plasma membrane
LIG_BRCT_BRCA1_1	LSTQF SSEVF QSGFF LSLLF NSGEF	148-152 394-398 446-450 509-513 600-604	Phosphopeptide motif which directly interacts with the BRCT (carboxy-terminal)	BRCA1-BARD1 complex, nucleus

			domain of the Breast Cancer Gene BRCA1 with low affinity.	
LIG_BRCT_BRCA1_2	SSEVFDK	394-400	Phosphopeptide motif which directly interacts with the BRCT (carboxy-terminal) domain of the Breast Cancer Gene BRCA1 with high affinity.	nucleus, BRCA1-BARD1 complex
LIG_CYCLIN_1	RSLI RRLI KGLF KELTL	35-38 55-58 352-355 538-542	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.	nucleus, cytosol
LIG_EH1_1	VHNIGLIMN	122-130	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.	nucleus
LIG_FHA_1	DKTTMLA	46-52	Phosphothreoni	nucleus

	SWTKEII NYTHAVA ICTPMIF ATTGGLI YYTEGLI	135-141 279-285 340-346 458-464 501-507	ne motif binding a subset of FHA domains that show a preference for a large aliphatic amino acid at the pT+3 position.	
LIG_MAPK_1	KQDKTTML AI KVKSPLQV KKIFAFINF KTKPFMKI KCKEFVKF	44-53 262-269 372-380 386-393 579-586	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.	nucleus, cytosol
LIG_PDZ_3	YEFL KEDV EENL EDKI KEII MEFL NEIL IDQL FEKL EDRV FDVL FEHV KEVL DEFV KEFL QEKI QDDL DDLI	22-25 27-30 62-65 76-79 138-141 159-162 202-205 215-218 234-237 246-249 298-301 311-314 327-330 363-366 400-403 413-416 471-474 472-475	Class III PDZ domains binding motif	cytosol, plasma membrane, membrane

	HELI VDEV TEGL SEVL KEFL EDNI KEFV YEKI	480-483 495-498 503-506 515-518 559-562 569-572 581-584 591-594		
LIG_PP1	FGRNILFY NLKINFD	405-412 524-530	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].	protein phosphatase type 1 complex, nucleus, cytosol
LIG_SH2_GRB2	YKNI YENA	315-318 382-385	GRB2-like Src Homology 2 (SH2) domains binding motif.	cytosol
LIG_SH2_SRC	YENA	382-385	Src-family Src Homology 2 (SH2) domains binding motif.	cytosol
LIG_SH2_STAT3	YHEQ	468-471	YXXQ motif found in the cytoplasmic region of cytokine receptors that bind STAT3 SH2 domain.	cytosol
LIG_SH2_STAT5	YFRS YIEY YFEK YTHA YICT	33-36 230-233 233-236 280-283 339-342	STAT5 Src Homology 2 (SH2) domain binding motif.	cytosol

	YTEG	502-505		
LIG_SH3_3	WMKVKSP PLQVGHP IFLPIMP	260-266 266-272 572-578	This is the motif recognized by those SH3 domains with a non-canonical class I recognition specificity	cytosol, plasma membrane, focal adhesion
LIG_SH3_4	KPFMKISS	388-395	This is the motif recognized by those SH3 domains with a non-canonical class II recognition specificity	cytosol, focal adhesion
LIG_TRAF2_1	SSEE SEEE AWQE	223-226 224-227 252-255	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.	cytosol
LIG_TRAF6	GHPLEYYE D	270-278	TRAF6 binding site. Members of the tumor necrosis factor receptor (TNFR) superfamily initiate intracellular signaling by	cytosol

			recruiting the C-domain of the TNFR-associated factors (TRAFs) through their cytoplasmatic tails.	
LIG_WW_4	YQKTPE KVKSPL YICTPM	168-173 262-267 339-344	Class IV WW domains interaction motif; phosphorylation-dependent interaction.	nucleus, cytosol
MOD_CK1_1	SLISLSE	36-42	CK1 phosphorylation site	nucleus, cytosol
MOD_CK2_1	SLISLSE IFASSEE FASSEEE EVLSNIE	36-42 220-226 221-227 328-334	CK2 phosphorylation site	nucleus, cytosol, protein kinase CK2 complex
MOD_Cter_Amidation	AGKK	370-373	Peptide C-terminal amidation	extracellular, secretory granule
MOD_GlcNHglycan	FSAQ QSGF DNSGE NSGE	355-358 446-449 599-603 600-603	Glycosaminoglycan attachment site	extracellular, Golgi apparatus
MOD_GSK3_1	TEKTMNQS	440-447	GSK3 phosphorylation recognition site	nucleus, cytosol
MOD_N-GLC_1	DNYTHA ANDTEK MNQSGF	278-283 437-442 444-449	Generic motif for N-glycosylation. Shakin-Eshleman et al. showed that Trp, Asp, and Glu are uncommon before the Ser/Thr position. Efficient glycosylation	extracellular, endoplasmic reticulum, Golgi apparatus

			usually occurs when ~60 residues or more separate the glycosylation acceptor site from the C-terminus	
MOD_PIKK_1	ILSTQFP IEKTQLY	147-153 333-339	(ST)Q motif which is phosphorylated by PIKK family members	nucleus
MOD_PLK	FEISWTK TEKTMNQ KELTLKN	132-138 440-446 538-544	Site phosphorylated by the Polo-like-kinase	nucleus, cytosol
MOD_ProDKin_1	YQKTPEG KVKSPLQ YICTPMI	168-174 262-268 339-345	Proline-Directed Kinase (e.g. MAPK) phosphorylation site in higher eukaryotes.	nucleus, cytosol
MOD_SUMO	LKEE LKLE	60-63 205-208	Motif recognised for modification by SUMO-1	nucleus, PML body
TRG_ENDOCYTIC_2	YEFL YGVL YARF YKNI YEKF YHEL YYDL YEKI	22-25 181-184 196-199 315-318 534-537 545-548 587-590 591-594	Tyrosine-based sorting signal responsible for the interaction with mu subunit of AP (Adaptor Protein) complex	cytosol, plasma membrane, clathrin-coated endocytic vesicle
TRG_LysEnd_APsAcLL_1	EIAKLV EQDDLI RAIGLI ESEVLI	6-11 470-475 485-490 514-519	Sorting and internalisation signal found in the cytoplasmic juxta-membrane region of type I transmembrane proteins. Targets them from the Trans	cytosol, Endocytic vesicle

			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).

Elm Name	Instances (Matched Sequence)	Positions	Elm Description	Cell Compartment
CLV_PCSK_SKI1_1	KLFTT	90-94	Subtilisin/kexin isozyme-1 (SKI1) cleavage site ([RK]-X-[hydrophobic]-[LTKF]-|-X)	endoplasmic reticulum, endoplasmic reticulum lumen, Golgi apparatus
LIG_14-3-3_3	KLFTTI	90-95	Consensus derived from reported natural interactors which do not match the Mode 1 and Mode 2 ligands.	nucleus, cytosol, internal side of plasma membrane
LIG_BRCT_BRCA1_1	NSSKF	49-53	Phosphopeptide motif which directly interacts with the BRCT (carboxy-terminal) domain of the Breast Cancer Gene BRCA1 with low affinity	nucleus, BRCA1-BARD1 complex
LIG_FHA_1	STTTKVK	15-21	Phosphothreonine motif binding a subset of FHA domains that show a preference for a large aliphatic amino acid at the pT+3 position.	nucleus

LIG_FHA_2	KNTSADE QATLNEV KFTNEDI LFTTIDA	23-29 31-37 52-58 91-97	Phosphothreonine motif binding a subset of FHA domains that have a preference for an acidic amino acid at the pT+3 position.	nucleus, Replication fork
LIG_PDZ_3	NEDI EETL	55-58 84-87	Class III PDZ domains binding motif	cytosol, plasma membrane, membrane
LIG_SH2_STAT3	YAWQ	70-73	YXXQ motif found in the cytoplasmic region of cytokine receptors that bind STAT3 SH2 domain.	cytosol
MOD_CK1_1	STTTKVK SKFTNED	15-21 51-57	CK1 phosphorylation site	nucleus, cytosol
MOD_CK2_1	KNTSADE FQATLNE	23-29 30-36	CK2 phosphorylation site	nucleus, cytosol, protein kinase CK2 complex
MOD_GlcNHglycan	TSAD	25-28	Glycosaminoglycan attachment site	extracellular, Golgi apparatus
MOD_GSK3_1	VDNTQKTS KTNSSKFT	3-10 47-54	GSK3 phosphorylation recognition site	nucleus, cytosol
MOD_N-GLC_1	KNTSAD TNSSKF GNATNN TNNTKA	23-28 48-53 98-103 101-106	Generic motif for N-glycosylation. Shakin-Eshleman et al. showed that Trp, Asp, and Glu are uncommon before the Ser/Thr position. Efficient	extracellular, endoplasmic reticulum, Golgi apparatus

			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