ALTERATION OF GENETIC CONTENT AND GENE EXPRESSION MODULATE THE PATHOGENIC POTENTIAL OF *CAMPYLOBACTER JEJUNI*

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of PREETI MALIK-KALE find it satisfactory and recommend that it be accepted.

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Chair

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ALTERATION OF GENETIC CONTENT AND GENE EXPRESSION MODULATE THE PATHOGENIC POTENTIAL OF *CAMPYLOBACTER JEJUNI*

ABSTRACT

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A leading cause of bacterial gastroenteritis worldwide, *Campylobacter jejuni* is responsible for as many as 2.5 million reported cases per year in the United States of America alone. *Campylobacter* is a flagellated, spiral shaped, Gram-negative bacterium. The development of new genetic tools and availability of the *C. jejuni* genome sequence has accelerated the progress made in the field of *Campylobacter* pathogenesis in the past decade. Although a number of virulence determinants have been identified, to date their role in the ability of *Campylobacter* to cause disease remains unknown. The research presented in this dissertation highlights two distinct features by which *C. jejuni* is able to "toggle" between a virulent and an avirulent state. First, *C. jejuni* is able to alter its genetic content to exhibit variable motility phenotype. It is proposed that alternating between a motile and a non-motile state helps *Campylobacter* switch from its commensal lifestyle in one host to an invasive lifestyle in the other. The data presented in Chapter 2 indicate that the differences observed in the virulence phenotype of two poultry isolates CS and S2B were a result of a point mutation in the *flgR* and *rpoN* genes, respectively. Proteins encoded by both these genes are essential for flagellar biosynthesis, thus mutations in these genes rendered the organism non-motile and hence avirulent. Second, *Campylobacter* alters its virulence potential by modulating its gene expression in

response to an environmental stimulus. To adapt and survive within the intestinal tract *Campylobacter* must alter its genes expression in response to the varied *in vivo* conditions encountered including exposure to bile. The data presented in chapter three demonstrate that culture in the presence of bile salts enhances the virulence potential of *Campylobacter*. When cultured in the presence of the bile salt sodium deoxycholate, *C. jejuni* undergoes a synthetic response characterized by the upregulation of genes known to play a role in *Campylobacter* pathogenesis. Taken together, the data presented show that *Campylobacter* is able to vary its virulence potential by altering its genetic content and gene expression.

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This dissertation/thesis is dedicated to

My husband Ritesh R. Kale

CHAPTER ONE

INTRODUCTION

1.1. The Genus Campylobacter

The genus *Campylobacter* was recognized in 1963 when Sebald and Véron (138) applied the Hugh and Leifson's test for fermentative metabolism and the guanine and cytosine ratio to differentiate *Campylobacter fetus* and *Campylobacter bubulus* from other *Vibrio* species. In 1973 a more comprehensive taxonomic study by Véron and Chatelain (154) reclassified the other microaerobic and/or anaerobic 'Vibrio' taxa as *Campylobacter* spp. At present, the genus *Campylobacter* contains 16 species and six subspecies. A number of species formerly referred to as *Campylobacter* spp. or *Campylobacter*-like organisms have been assigned to other phylogenetically related genera, such as *Arcobacter*, *Helicobacter*, and *Sulfurospirillum*. However *Campylobacter*, *Arcobacter* and *Sulfurospirillum* share a number of characteristics and are considered members of the same family, Campylobacteraceae (152). These taxa form a distinct, but diverse phylogenetic group often referred to as rRNA superfamily IV (151), the epsilon division of the proteobacteria (142) or the epsilobacteria (19).

The genus *Campylobacter* consists of Gram-negative, slender, spiral rods, however some species are predominantly curved or straight rods. Most species are motile with a characteristic corkscrew-like motion driven by an unsheathed flagellum at one or both ends of the bacterium. *C. gracilis* is non-motile, while *C. showae* has multiple flagella. Campylobacters are microaerophilic, i.e., they require an atmosphere with an oxygen concentration between 3-15% and grow best at temperatures ranging from 37 to 42˚C.

Fastidious in nature, these organisms are generally unreactive in standard biochemical tests and their sugar catabolism cannot be detected by conventional methods.

Of the 16 species of the genus *Campylobacter*, *C. jejuni*, *C. coli* and *C. fetus* are frequently isolated from humans (76). *C. jejuni* comprises two subspecies that differ substantially in their ubiquity and to some extent ecology. *C. jejuni* subsp. *jejuni*, often referred to as *C. jejuni*, represents the taxon first described by Jones *et al.* (54) as '*Vibrio jejuni*' isolated from bovine intestinal contents. *C. jejuni* subsp. *doylei* fails to reduce nitrate or grow at 42˚C, properties that help differentiate it from *C. jejuni* subsp. *jejuni* (143). To date, no animal host has been identified for *C. doylei* while *C. jejuni* shares a commensal relationship with a wide range of animal hosts, including chickens, cattle, pigs sheep, dogs and ostriches (109).

1.2. Epidemiology

Campylobacter is the primary cause of bacterial diarrheal illness in the developed world, with an estimated 2-3 million *Campylobacter*-related illnesses occurring in the U. S. per year. Although thermophilic *Campylobacter* species are considered food-borne pathogens, most illnesses caused by *Campylobacter* occur sporadically, which makes it difficult to trace the cause of infection. The most common clinical symptom seen in humans infected with *C. jejuni* is gastroenteritis. The specific signs and symptoms vary from individual to individual presumably due to variations in the host and infecting organism. Following an incubation period of 24 to 72 h, an acute diarrheal illness develops. A nonspecific syndrome of fever, chills, myalgia and headache may follow.

Abdominal cramping and fever often accompany the diarrhea, which may range from loose to watery to bloody.

Although *C. jejuni*-associated illnesses are self-limiting and uncomplicated, in rare cases serious manifestations may occur following the diarrhea. Approximately 0.1% of the *C. jejuni* caused gastrointestinal illnesses are followed by Guillain-Barré syndrome (GBS), a life threatening polyneuropathy that can result in paralysis and other serious complications. *C. jejuni* has also been associated with other neuropathies such as Miller Fisher syndrome, and inflammatory diseases including reactive arthritis and myocarditis. Certain serotypes of *Campylobacter* are linked with GBS. While most cases of GBS in the U.S. are linked with serotype O:19, other serotypes including O:1, O:2, O:2/44, O:4 complex, O:5, O:10, O16, O23, O37, O:41, O:44 and O:64 have also been associated with GBS (100, 127, 139).

The prevalence of *Campylobacter* species in the environment, including wild and domesticated animals, animal waste, soil and water is ultimately related to the contamination of food with *Campylobacter*. While domesticated animals and avian species are considered major reservoirs of *C. jejuni*, these organisms have also been isolated from primates, ungulates, wildcats, canines, bears, pandas, ferrets, hedgehogs and badgers. In addition, *Campylobacter* has been found in rodents, including hamsters, hares, rats, voles, muskrats and squirrels. However, horizontal transmission between avian and non-avian species may be a zoonotic or water-borne event instead of food-borne (92).

Campylobacter prevalence in poultry is very high. *Campylobacter* colonizes the cecum and survives as a commensal at levels as high as 10^{10} CFU per gram of cecal content. Horizontal transfer is common due to contamination of food and water with fecal material and due to the coprophagous nature of chickens (140). Although vertical transmission is not very well documented, a study by Pearson *et al.* (116) suggests that within a shed of broiler chickens, low level of vertical transfer followed by horizontal transmission may occur. Although *C. jejuni* and *C. coli* are common contaminants of non-frozen poultry meat and meat products, the incidences of *Campylobacter* on frozen products are substantially lower (92).

C. jejuni is commonly isolated from cattle and sheep, while *C. coli* is isolated primarily from swine and pork (92). The high incidence of *Campylobacter* in livestock is contrasted by its low occurrence in beef, pork and lamb meat products (52). A reduction in incidence is attributed to the dehydration step during carcass chilling (38), as the same effect is not observed in the spray chilling process that results in a lower level of carcass dehydration (153).

Campylobacter outbreaks have been correlated with eight different sources, namely: 1) meat and meat related products; 2) milk and dairy products; 3) miscellaneous or mixed food sources; 4) poultry or poultry products; 5) produce or produce related products; 6) seafood/shellfish; 7) water; and 8) unknown source. Contamination of water and milk with *C. jejuni* continue to be the major sources responsible for outbreaks since 1978.

Consumption of raw milk and inadequately pasteurized milk has been associated with a number of *Campylobacter* outbreaks (92). Water is an important reservoir of *Campylobacter* as it acts as a potential source of infection for humans, poultry and livestock (115). The occurrence of *Campylobacter* in aqueous environments may be attributed to its ability to survive in a viable but non-cultivable (VBNC) state (21, 144). The number of bacteria isolated from water varies seasonally with the higher numbers recovered during autumn and winter while the lowest present during spring and summer (13, 51, 106, 107). The seasonality observed may be attributed to the ability of *Campylobacter* to survive in extreme conditions. For example, *C. jejuni* survive in cold water (107) and under UV radiation (13, 107). Additionally they are able to compete against aquatic micro-flora during summer months (14) and form biofilms in the water (15).

1.3. Strain Variation

Phenotypic and genotypic diversity among *Campylobacter* strains is well established (24, 25, 157). The sporadic nature of campylobacteriosis and the variety of different sources of infection may be responsible for the large diversity observed among human *Campylobacter* isolates. Although sequencing of the *C. jejuni* NCTC 11168 genome revealed few mechanisms by which the organism can generate genetic diversity, several lines of evidence indicate intraspecific genotypic and phenotypic diversity compared to other enteropathogens (25). Genetic exchange (90, 144), and more recently, gene rearrangement (22) have been implicated in the wide diversity observed among *Campylobacter* strains. The mechanism by which this occurs is not well understood.

The detection of strain variation within *C. jejuni* population has improved significantly since the introduction of serotyping methods in the early 1980s. These include the heat-stable (HS) (121) and the heat-labile (HL) (88) serotyping schemes. Limitations from cost, production, and quality control of antiserum reagents have led to the development of molecular techniques that targets genetic variations within the DNA sequence of the organism. Some of the molecular typing methods include pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST) and genomotyping. These molecular methods are often more universally applicable, reduce problems encountered with phenotypically "untypable" strains and provide a much higher level of discriminatory power (28).

Pulsed-field gel electrophoresis (PFGE) is a molecular typing method widely used in the epidemiological studies of *Campylobacter*. This technique employs relatively rare cutting restriction enzymes to digest whole-cell DNA into fragments, which are then separated by pulsed-field gel electrophoresis. PFGE is the method of choice used by the national molecular sub-typing network for food-borne disease surveillance or PulseNet established in the U.S. (28).

Multilocus sequence typing (MLST) uses the sequence of internal fragments from multiple housekeeping genes. Approximately 450-500 bp internal fragments from each gene are used, as these can be accurately sequenced on both strands using an automated DNA sequencer. Seven housekeeping genes are typically used to type *Campylobacter*

isolates. These are aspartase A (*aspA*), glutamine synthetase (*glnA*), citrate synthase (*gltA*), serine hydroxymethyltransferase (*glyA*), phosphoglucomutase (*pgm*), transketolase (*tkt*) and ATP synthase α subunit (*uncA*). For each gene, the different sequences present within a species are assigned as distinct alleles and, for each isolate, the alleles at each of the seven loci define the allelic profile or sequence type. Each isolate of a species is characterized by a series of seven integers, which corresponds to the alleles at the seven housekeeping loci (133, 134).

Genomotyping is a relatively new technique, which uses DNA microarrays to compare inter-strain variations in bacteria at the genomic level. Publication of the complete genome sequence of the *C. jejuni* strain NCTC 11168 has enabled the application of this technique to *Campylobacter* genomics. Dorell and colleagues (25) constructed the first *Campylobacter* whole genome DNA microarray containing all 1,654 annotated genes from the NCTC 11168 genome sequence.

Whole genome comparison of 11 *C. jejuni* strains of varying Penner serotypes identified a set of 1,300 "core" genes common to all strains, also referred to as species-specific genes (25). Species-specific or conserved genes in the strains studied included those involved in metabolic, biosynthetic, cellular and regulatory processes, as well as several putative virulence factors including cytolethal distending toxin (124), flagellar structural proteins (159), phospholipase A (37), PEB1 (120), *Campylobacter* invasion antigen B (CiaB) (72), *Campylobacter* adhesion to fibronectin (CadF) (67) and CheY (162). A number of studies (25, 80, 81, 113, 117, 147) have indexed the complete genomic

contents of several *C. jejuni* strains in relation to strain NCTC 11168 and identified regions of variability between strain NCTC 11168 and other *C. jejuni* strains such as the lipooligosaccharide biosynthesis, capsular biosynthesis, flagellar modification, iron acquisition and DNA restriction/modification loci.

The complete genomic sequence of the *C. jejuni* strain RM1221 shows that it is syntenic with the genome of NCTC 11168 except for insertions by four islands and smaller gene clusters (29). The four genomic islands in strain RM1221 are referred to as *C. jejuni*-integrated elements (CJIEs). CJIE1 is a *Campylobacter* Mu-like phage encoding several proteins with similarity to bacteriophage Mu (98) while CJIE2 and CJIE4 encode genes predicted to encode phage-related endonucleases, methylases, or repressors. However, CJIE3 may be an integrated plasmid as it encodes proteins similar to those encoded on the *C. coli* RM2228 megaplasmid and other *Campylobacter* plasmids (9). In a recent study by Parker *et al.* (113), the authors demonstrated the presence of these CJIEs among the 67 *C. jejuni* and 12 *C. coli* strains tested. Thus integrated elements including those observed in strain RM1221 contribute to the diversity among *C. jejuni* strains.

1.4. Virulence Determinants

Study of the molecular basis of *Campylobacter* pathogenesis has led to a plethora of new findings in the past decade. The development of new genetic tools and the availability of the genome sequence have helped in the identification of a number of virulence determinants. At present the factors known to contribute to *Campylobacter* pathogenesis

include motility, chemotaxis, translocation, adhesion, secretion, invasion, toxins, iron acquisition, lipopolysaccharides and capsular polysaccharides.

Chemotaxis is the movement of the bacterium towards or away from a chemical stimulus and has been shown to play an important role in *C. jejuni* pathogenesis (20, 33, 46, 148, 162). Chemotaxis mutants, *cheY* (162) and *cheA* (20) were found to be incapable of colonization in a murine model.

Translocation is the ability of the bacterium to move across a cellular barrier (in the host's intestinal tract) by disrupting the tight junctions, which allows them to infect the underlying tissues and disseminate throughout the host. *Campylobacter* has been shown to translocate via transcellular and/or paracellular route (75), although a study has demonstrated that the bacteria may access the submucosa via uptake by M-cells (156). However, the ability of *C. jejuni* to translocate is not related to its ability to invade host cells (45).

Adhesion is the ability of the bacterium to bind to a host cell receptor with the help of surface exposed ligands called adhesins. A number of adhesins including CadF (67), JlpA (53), PEB1 (61, 118, 119), and CapA (3), have been identified in *Campylobacter*, but their individual contribution to this process remains unknown. CadF is one of the most well characterized adhesin and is known to bind to the cell matrix protein fibronectin present extensively on the basolateral surface of intestinal epithelial cells (65,

95, 96). Recently it has been shown to play a role in the activation of host cell signaling molecules such as Rac1 and Cdc42 during infection of the host cell (77).

C. jejuni is known to encode a variety of toxins, including hemolysins (49, 94, 123), secreted protease (158), and a cytolethal distending toxin (CDT) (124, 125). CDT is a well-characterized tripartite AB toxin in which CdtA and CdtC comprise the binding component. CdtB is the active subunit, when transported into the nucleus causes double strand DNA breaks and arrests the host cell in the G2 phase (78, 79).

Campylobacter is not known to synthesize siderophores to help them chelate iron from biological systems (8, 27, 123). However, they are capable of using siderophores produced by other indigenous bacteria in the gut with the help of outer membrane receptors such as CfrA (41). In addition, *Campylobacter* is able to acquire iron through heme compounds present within host macromolecules such as hemoglobin via the enterochelin (encoded by the *chuABCD* operon) (129) and the ferrichrome (encoded by the *fhuABD* operon) (31) systems. FeoB protein is known to help in the process of ferrous iron uptake and is important for gut colonization (102). Thus, *Campylobacter* possess mechanisms whereby both ferric and ferrous iron may be acquired from the iron limited intestinal environment.

Lipopolysaccharide (LPS) is a major component of the outer membrane of Gram-negative bacteria that consists of three major components; lipid A- anchored to the inner membrane, a core composed of glycoses, and the O antigen. However, the

polysaccharide of *Campylobacter* lacks the O-antigen and is referred to as lipooligosaccharide (LOS). The LOS core in *C. jejuni* strains is hypervariable due to differences in composition and monosaccharide linkage. Eight different LOS loci have been identified containing 10 to 19 genes (112). In addition, sequencing of the LOS locus has demonstrated four mechanisms by which this variability is generated in *C. jejuni*. These include phase variation because of homopolymeric tracts (42, 87), gene inactivation due to deletion or insertion of a single base (32), mutation leading to the inactivation of glycosyltransferase (32) and multiple mutations in the glycosyltransferase leading to altered receptor specificities. More importantly, various *C. jejuni* LOS structures mimic human neuronal gangliosides, which are thought to be involved in the induction of autoimmune disorders including GBS (63, 101, 122).

Until recently a number of *C. jejuni* strains were thought to synthesize a high molecular weight LOS, which is now known to be a highly variable capsular polysaccharide (59). This capsule acts as the major determinant in the Penner serotyping method (59), indicating that the capsular polysaccharide is not only accessible to the host immune system, but also that the variability observed in the capsule may be responsible for immune evasiveness. The variation in the capsular structure is attributed to phase variation and *O*-methyl phosphoramidate modification (58, 59, 146). Mutations in genes responsible for capsular biosynthesis affect serum resistance, adherence and invasion of epithelial cells, chick colonization and virulence in a ferret model (5, 7, 40, 55).

The three virulence determinants studied as a part of this dissertation (motility, invasion and secretion) are addressed in detail below.

1.4.1. Motility

Campylobacter is characterized by rapid darting motility mediated by polar flagella at one or both ends of the bacterium. The *C. jejuni* flagellum consists of three main components: the basal body, hook, and filament. The filament itself is composed of two proteins, a major flagellin FlaA encoded by *flaA* and a minor flagellin FlaB encoded by *flaB*. The *flaA* and *flaB* genes in *C. jejuni* NCTC 11168 strain are each 1,731 base pairs in length (separated by a 173 bp region) and have nucleotide sequences that are 95% identical. A unique feature of the *Campylobacter* flagellum is that two different promoters control the two flagellar filament genes (39). The *flaA* gene is expressed from a σ^{28} promoter, while a σ^{54} promoter controls the expression of *flaB* (47).

The *C. jejuni* flagellum is assembled in a sequential and highly regulated fashion. First, the Class I flagellar genes, controlled by the housekeeping sigma factor σ^{70}, are expressed. These include genes encoding some of the initial proteins required to assemble the basal body. Expression of the *flhA* gene appears to be essential for activating expression of both Class II and Class III flagellar genes (18). The Class II genes are controlled by a σ^{54} promoter, and encode the majority of the hook and basal body genes in addition to *flaB*. In *C. jejuni*, expression of the σ^{54} -regulated genes is also dependent on the FlgS/FlgR two-component regulatory system. After phosphorylation by the FlgS sensor kinase, the FlgR response regulator interacts with RpoN, increasing its

synthesis and transcriptional activity (47, 161). Thus, the interaction between FlgR and RpoN leads to the completion of the hook and basal body structure. Until this stage the expression of the σ^{28}-controlled Class III genes is weakly repressed by the presence of the anti-sigma factor FlgM (47). When the product of the Class I and II genes have been assembled into a secretion apparatus, FlgM escapes the bacterium, derepressing FliA (σ^{28}), allowing for expression of the Class III genes (including *flaA* and *fliD*), and completion of a full-length flagellum.

The *Campylobacter* flagellum not only plays a role in motility and chemotaxis but it is also an important virulence determinant. It is involved in colonization, secretion, autoagglutination, biofilm formation and avoidance of the innate immune response. The importance of a functional flagellum in these processes is explained below.

The importance of motility in colonization was first demonstrated by Marooka *et al*. (99) who showed that non-motile isolates were unable to colonize suckling mice and were cleared from the intestinal tract within 2 days post challenge. Wassenaar *et al.* (159) later corroborated this finding, using genetically defined *flaA* and *flaB* mutants. He showed that the *C. jejuni flaA*$^{-}$*flaB*$^{+}$ mutant colonized the chicks 100 to 1000-fold less efficiently than the wild-type isolate. Studies elaborating the role of motility in cultured epithelial cells also showed that *flaA* expression was required for translocation. However, the expression of *flaB* alone in a *flaA*$^{-}$*flaB*$^{+}$ mutant was sufficient to enhance the invasive potential of the bacterium in comparison to a *flaA*$^{-}$*flaB*$^{-}$ mutant. Thus the flagellar

structure itself and not motility was important for invasion of epithelial cells by *C. jejuni* (36, 159).

The *C. jejuni* flagellum is also known to act as a secretory apparatus for the Cia proteins when cultured in the presence of epithelial cells (also see section 1.4.2). Secretion of the Cia proteins requires a minimum flagellar structure consisting of the hook and the basal body. Thus, *C. jejuni* mutants lacking a functional *flhB, flgB, flgC* and *flgE* gene were secretion deficient. Although secretion occurred in a $flaA^-$ and $flaB^-$ single mutant, it is not clear why secretion was not observed in the $flaA^-flaB^-$ double mutant as an intact secretory apparatus consisting of the basal body and the hook was present in all of these mutants (73).

Autoagglutination (AAG) is a marker for host cell interaction and virulence in a number of Gram-negative bacteria. In *C. jejuni,* AAG is mediated by the flagella and glycans on the flagella (93). A *C. jejuni* mutant lacking the acetamidino form of pseudaminic acid (a 9-carbon sugar) on the flagellin was AAG deficient. This mutant also showed reduced adhesion to epithelial cells and microcolony formation. Although the role of microcolony formation in bacterial virulence is not defined, it may help increase concentration of effector molecules while on the surface of target cells. Interestingly the *pseAM* mutant was also found to be attenuated in comparison with the wild-type in the ferret model of bacterial disease (33).

Proteomic analyses of planktonic and biofilm-grown cells demonstrated that proteins involved in the motility complex, including the filament protein FlaA, FlaB, the filament cap (FliD), the basal body (FlgG, FlgG2), and the chemotactic protein (CheA), all were expressed in biofilms at a higher level than in stationary-phase planktonic cells (57). An aflagellate *flhA* mutant not only lost the ability to attach to a solid matrix and form a biofilm, but also was unable to form a pellicle at the air-liquid interface; indicating that the flagellar motility complex plays a crucial role in the initial attachment of *C. jejuni* to solid surfaces during biofilm formation as well as in cell-to-cell interaction (56, 57, 135).

The *Campylobacter* flagella differ from that produced by other flagellated pathogens in two structural features. First the *Campylobacter* flagellins lack the residues responsible for stimulating Toll-like receptor (TLR) 5. The TLR 5 recognition motif maps to amino acids 89-96 in *Salmonella* but these motifs are modified in *Campylobacter* such that the bacteria is able to evade the host immune system (2). The other important feature of *Campylobacter* flagellins is that the proteins are heavily glycosylated (137, 149). The glycosylation is extremely important for filament assembly, as a *C. jejuni* 81-176 mutant deficient in glycosylation was found to be non-motile and accumulated unglycosylated flagellins intracellularly (35).

Flagella associated motility is also influenced by phase variation (105), and reversible non-motile variants have been reported after extended incubation (17). Phase variation is a mechanism that results in alteration of gene transcription or protein translation by random reversible changes in the length of short DNA sequence repeats. These repeats

may be homopolymeric nucleotide tracts or repetitive homopolymeric unit. Slipped-strand mismatch repair during DNA replication may then result in gain or loss of repeats within the gene that may affect positively or negatively the expression of a functional gene product. For motility, *maf1* (60), *flhA* (111) and *flgR* (46) genes are known to undergo phase variation. The phase variability in the *flgR* is hypothesized to play an important role in establishing a commensal relationship in chickens. The variable phenotype observed during colonization maybe due to selection pressures imposed by the altered host environment (60) or due to an apparent lack of mismatch repair enzymes together with an adenine-thymine rich genome (43, 114).

1.4.2. Invasion

Invasion of the intestinal mucosa is considered an important virulence mechanism for intestinal pathogens. Results of intestinal biopsies of human patients (150), infected primates (132) and infected model animals (4, 6, 27, 34, 44, 104, 119, 136, 155, 162, 163), in addition to experiments with cultured human intestinal epithelial cells (16, 23, 62, 69, 70, 97, 145) have established *Campylobacter* as an invasive pathogen. Although early work suggested that *Campylobacter* spp. were only able to invade HEp-2 and A549 cells when co-infected with *Salmonella* or *Shigella,* De Melo *et al.* (23) clearly showed that *C. jejuni* alone was able to invade HEp-2 cells. Later *C. jejuni* was found to invade other cell lines including the human colonic Caco-2 (75) and T84 cell lines (95), and the INT 407 (68, 108) cell line.

The invasion efficiency of *C. jejuni* for cultured cells is strain specific. Newell *et al.* (103) found clinical isolates to be much more invasive than environmental isolates, while Everest *et al.* (26) found strains from individuals with colitis more invasive than those from individuals with non-inflammatory disease. *In vitro* passage over a period of time is also thought to negatively effect invasion efficiency (64). Additionally, the invasion efficiency of *Campylobacter* is host cell line specific and, in general, bacteria appear to be most effectively internalized in cell lines of human origin. Semi-confluent monolayers of INT 407 cells and Caco-2 cells appear more favorable for invasion than fully confluent monolayers, indicating that the host cell adhesion and/or invasion receptors are located basolaterally (50). Biswas *et al*. (12) showed that a maximal number of internalized bacteria were present within epithelial cells when infected with a high multiplicity of infection (MOI). However, Hu and Kopecko (50) showed that the efficiency of invasion is highest at low MOI of 0.02 bacteria per host cell. Other factors of bacterial origin known to play an important role in cellular invasion include motility (See section 1.4.1), adhesion (See section 1.4), and the secretion of Cia proteins (See section 1.4.3).

A number of host related factors are also involved in the process of invasion. Several studies have demonstrated that the host cytoskeleton, comprising microtubules and microfilaments, is involved in the uptake of *Campylobacter*. Depending upon the cell line, the strain of *Campylobacter* and the experimental conditions used, the internalization of *C. jejuni* is reported to be dependent upon microfilaments and/or microtubules or neither of the two (11, 12, 16, 50, 96, 97, 108). While Hu and Kopecko

(50) demonstrated that the *C. jejuni* strain 81-176 exclusively required the host microtubules for internalization, Monteville *et al*. (96) found that both microtubules and microfilament were require for this process. The alteration of the host cytoskeleton by *C. jejuni* is mediated by Rac1 and Cdc42 proteins, which are members of the small RhoGTPase family. These proteins act as molecular switches and alter host signal transduction pathways (77). The interaction of *C. jejuni* with epithelial, macrophage and dendritic cells also activates nuclear transcription factor (NF-kappa B) (91) and mitogen-activated protein kinase (MAPK) signaling pathways (89), resulting in the release of cytokines including IL-8, which contributes to the pro-inflammatory response (160).

1.4.3. Secretion

It is well documented that *Campylobacter* must be metabolically active for maximal cell invasion, as its ability to invade epithelial cells is significantly reduced when pre-treated with a protein synthesis inhibitor (66, 74, 108). One and two-dimensional electrophoresis analyses of metabolically labeled *C. jejuni* cultured with and without epithelial cells indicated that *de novo* protein synthesis occurred upon target cell contact (66, 74). Further, Panigrahi *et al*. (110) reported that *C. jejuni* synthesized a number of proteins *in vivo* in rabbit ileal loops that were not synthesized under standard laboratory conditions. Further investigation of the proteins synthesized *de novo* by *C. jejuni* when cultured in the presence of epithelial cells led to identification of Cias (71, 130, 131). The secretion of the Cias is dependent on a minimal flagellar structure comprising the secretory apparatus and one of the flagellins as described previously (See section 1.4.1).

The Cias are a set of at least eight proteins that are secreted into the culture supernatant when incubated in the presence of epithelial cells or fetal bovine serum as an external stimulus mimicking an *in vivo* environment (130, 131). The identity of one of the Cias is known and it is designated CiaB, which is encoded by Cj0914c (*ciaB*). It is a 73.1 kDa protein. CiaB shows 40.6% to 45.4% similarity with the *Salmonella* SipB, *Shigella* IpaB and *Yersinia* YopB proteins (71). Further, its homologs are found in *C. coli*, *C. upsaliensis*, *C. lari*, *Wolinella succinogenes* and *Helicobacter hepaticus* (71). A *ciaB* mutant in the *C. jejuni* F38011 strain is a fully motile strain that fails to secrete any of the Cia proteins. The invasive potential of this mutant is significantly reduced in comparison to the wild type strain, highlighting the importance of the Cias in invasion. However, *ciaB* mutation in the *C. jejuni* 81-176 strain shows no significant effect on its invasive potential (34). Although its cellular partner(s) remains unidentified CiaB has been shown to be localized in INT 407 cells (71).

FlaC is another protein secreted by *C. jejuni*. It is recognized by the flagellar export machinery as another flagellin substrate owing to its identity to the N-terminus of FlaA and FlaB (141). FlaC is a 26.6 kDa protein that is highly conserved among *C. jejuni* strains. Mutation in *flaC* results in a fully motile strain, indicating that it is not incorporated into the flagellar filament. FlaC secretion is independent of any external stimulus but is dependent on a minimum flagellar structure comprising the hook and the basal body. Although *flaC* mutation in the *C. jejuni* TGH9011 strain resulted in 86% reduction in the invasion potential when compared with the wild-type, only a 58%

reduction was observed in the *flaC* mutant of the *C. jejuni* 81-176 strain as compared with the wild-type strain (34).

Similar to FlaC, a third secreted protein termed FspA has been identified (126). This protein is secreted into the culture supernatant of a biphasic culture of the *C. jejuni* 81-176 strain. FspA secretion is not observed in *flgI*, *flgK* or a *flgE* mutant strains. Sequencing of *fspA* from 40 *C. jejuni* strains helped in the identification of two different alleles, *fspA1* and *fspA2*. The proteins encoded by the two alleles are 41% identical to each other. Mutation in *C. jejuni* strain 81-176 harboring the *fspA1* allele did not show any difference in its invasive potential when compared to the wild-type isolate. The recombinant FspA2 bound to epithelial cells and induced apoptosis, whereas recombinant FspA1 did not, indicating that only one allelic form was toxic to the eukaryotic cell.

1.5. *Campylobacter* and bile salts

Whether acting as a pathogen or as a commensal, *C. jejuni* must encounter bile in the gut of its host. Bile is a yellow/green aqueous solution of organic and inorganic compounds whose major constituents include cholesterol, phospholipids, bile salts and the pigment biliverdin (10). Bile is produced as a sterile compound, but interacts with intestinal bacteria following secretion into the duodenum. The concentration of bile salts ranges from ~8% in the gall bladder to ~0.2 to 2% in the intestine (48). Being a surface-active agent, bile possesses potent antimicrobial activity. In addition to causing membrane damage, bile induces secondary structure formation in RNA, DNA damage, protein denaturation and misfolding (10).

Campylobacter is able to resist the deleterious effects of bile with the help of a RND-type multidrug efflux pump encoded by the *cmeABC* operon (85). CmeA is homologous to the membrane fusion protein of MDR pumps while CmeB forms the inner membrane transporter characterized by 12 transmembrane segments and two large hydrophilic extracytoplasmic loops. CmeC is an outer membrane protein similar to TolC of *E. coli*. The CmeABC MDR efflux pump is essential for growth on bile salt containing media. Lin *et al*. (83) showed that inactivation of the pump confers sensitivity to various bile salts. The minimum inhibitory concentrations (MICs) of selected bile salts were decreased 4000-fold (chenodeoxycholate) and 64-fold (cholic acid and taurocholic acid) in the mutant strains of *cmeABC* when compared to wild-type strain. Further the CmeABC multidrug efflux pump is essential for the colonization of chickens by *C. jejuni* (84, 86). The transcriptional regulator CmeR binds to inverted repeats in the promoter region of *cmeABC* and represses the transcription of the operon. Bile salts also interact directly with CmeR and bring about a conformational change in the repressor, resulting in overexpression of the *cmeABC* operon (82, 83). Further the response regulator CbrR was found to modulate bile resistance in *C. jejuni* and to play an important role in chicken colonization (128).

Bile salts, including sodium deoxycholate, cholate and chenodeoxycholate affect invasiveness by stimulating the synthesis of the Cia proteins. This phenomenon was found to be specific to bile, as alteration of pH, calcium concentration, osmolarity or temperature did not induce their synthesis (130). Bile was also found to upregulate the *C.*

jejuni flaA σ^{28} promoter (1). The increase in FlaA synthesis together with chemotaxis may play a role in colonization and secretion. Finally, proteomic analysis of *C. jejuni* NCTC 11168 cultured with ox-bile for 18 h showed that genes encoding elongation factor, ferritin, chaperones, and ATP synthase were upregulated (30).

1.6. Current Model of *Campylobacter jejuni* mediated enteritis

The first step of campylobacteriosis involves the ingestion of viable bacteria, which is most often acquired by consumption of food cross-contaminated by raw or undercooked poultry products. Humans, when challenged with low dose (500-800) of *C. jejuni,* showed symptoms of enteritis. Higher infection doses showed an increase in the number of infected individuals. The infective dose of *C. jejuni* is dependent on the pathogenicity of the infecting strain and the host immune response.

To survive in the human gastrointestinal tract, *C. jejuni* must endure numerous environmental extremes including variations in pH, low oxygen levels, bile salts, nutrient limitation and elevated osmolarity. Although the exact mechanism by which *C. jejuni* combats each of these environmental challenges is not known, it has been proposed that an adaptive response accompanied by global changes in gene expression facilitates bacterial survival and infection in the host.

C. jejuni is proposed to initially colonize the jejunum and ileum and then the colon of humans. Upon sensing its environment, *C. jejuni* uses its flagella to drive itself through the mucosal layer covering the epithelium in a directed manner where it adheres the host

cell surface. Thus, motility and chemotaxis help the bacteria resist the peristaltic forces and flushing of the small intestine. This also helps the bacteria access the apical and basolateral surfaces of the host cell. Although the cell types that comprise the microenvironment of *C. jejuni* are debatable, the bacteria are known to survive, replicate and interact with the host cells.

C. jejuni are known to traverse the intestinal epithelium via a paracellular and transcellular route, however, the significance of either route verses the role of M cells in this process is not known. It is known that ligands of bacterial adhesins are concentrated on the basolateral surface of the epithelium that may act as a chemoattractant. Once *C. jejuni* reaches its specific niche, it probably synthesizes proteins that help promote subsequent interactions with the host target cells. Thus, adherence facilitates the invasive and cytotoxic activities of *C. jejuni*, although the contribution of invasion verses protein secretion to the severity of *Campylobacter*-mediated enteritis is not defined.

During later stages of infection by *Campylobacter*, disruption of tight junction, influx of fluids and immune cells, and induction of premature apoptosis or necrosis contributes to the severe disease symptoms observed. The disruption of the tight junctions during infection may be attributed to either bacterial or host immune processes; irrespective, it allows the bacteria increased access to the lamina propria and that allows invasion of the sequestered host cells. The response of intestinal epithelial cells to *C. jejuni* infection is characterized by induction of cytokines (i.e., IL-8) that help recruit inflammatory cells to the site of infection.

1.7. Goals of this dissertation

The research outlined in this dissertation addresses two important aspects of *Campylobacter* pathogenesis:

- The work described in Chapter 2 is based on the hypothesis that differences in the genetic content or gene expression alters the degree of pathogenesis of *C. jejuni* strains. In this study *Campylobacter* poultry isolates were categorized as virulent and avirulent strains based on their motility, secretion and invasion phenotypes. The molecular mechanism behind the avirulent phenotype of two *C. jejuni* CS and S2B strains was then investigated.
- The work described in Chapter 3 is based on the hypothesis that the virulence potential of *Campylobacter* may be triggered by bile salt sodium deoxycholate. In this study *C. jejuni* was cultured in the presence of bile salts to enhance its virulence potential, and this was monitored by its ability to invade epithelial cells and synthesize CiaB, a known virulence factor.

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CHAPTER TWO

Characterization of genetically-matched isolates of *Campylobacter jejuni* reveals mutations in genes involved in flagellar biosynthesis alter the organism's virulence potential

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ABSTRACT

Phenotypic and genotypic evidence suggests that not all *Campylobacter jejuni* isolates are pathogenic for humans. We hypothesized that differences in the gene content or gene expression alter the degree of pathogenicity of *C. jejuni* isolates. A *C. jejuni* isolate (Turkey) recovered from a turkey and a second *C. jejuni* isolate (CS) recovered from a chicken differed in their degree of *in vitro* and *in vivo* virulence. The *C. jejuni* Turkey isolate invaded INT 407 human epithelial cells and secreted the *Campylobacter* invasion antigens (Cia proteins) while the *C. jejuni* CS isolate was non-invasive for human epithelial cells and did not secrete the Cia proteins. Newborn piglets inoculated with the *C. jejuni* Turkey isolate developed more severe clinical signs of campylobacteriosis than piglets inoculated with the *C. jejuni* CS isolate. Additional work revealed that flagellin was not expressed in the *C. jejuni* CS isolate. Microarray and real time RT-PCR analyses revealed that all flagellar class II genes were significantly downregulated in the *C. jejuni* CS isolate when compared to the *C. jejuni* Turkey isolate. Finally, nucleotide sequencing of the *flgR* gene revealed the presence of a single residue that was different in the FlgR protein of the *C. jejuni* Turkey and CS isolates. Complementation of *C. jejuni* CS isolate with a wild-type copy of *flgR* gene restored the isolate's motility. Collectively, these findings support the hypothesis that critical differences in gene content or gene expression can alter the pathogenic potential of *C. jejuni* isolates.

INTRODUCTION

Campylobacter jejuni strains are genetically diverse. The diversity of this organism has been observed by a number of techniques including pulsed-field gel electrophoresis (PFGE) (82), subtractive hybridization (2), microarray analysis (16, 42, 61, 64, 65), and multilocus sequence typing (MLST) (71). Some differences in the genetic content between strains can be explained by the presence of polynucleotide tracts in hypervariable loci including those encoding the lipo-oligosaccharide (LOS), flagellar biosynthesis, and the polysaccharide capsule (60). Such genetic diversity has been problematic in studies attempting to distinguish pathogenic from non-pathogenic isolates. Aeschbacher and Piffaretti (1) reported that there was no clustering of animal and human isolates based on multilocus enzyme electrophoresis (MLEE) using nine polymorphic loci. Based on this result, the authors concluded that every animal isolate is a potential human pathogen. Manning *et al.* (47) performed MLST on 266 *C. jejuni* isolates, and found that the populations of *C. jejuni* veterinary and human isolates overlapped among the 19 clonal complexes identified.

The ability of *C. jejuni* to cause disease is a complex, multifactorial process. Potential virulence determinants include toxins (7, 11, 26, 34, 40, 46, 48, 63, 69, 79, 85), adherence factors (adhesins) (13, 19, 20, 35, 38, 49, 53, 62), and entry-promoting molecules (invasins) (5, 18, 36, 55, 57, 70, 75). Also of interest are strain-variable genes, which encode for factors involved in iron acquisition, DNA restriction/modification, sialylation, flagellar biosynthesis, LOS biosynthesis, and capsular biosynthesis (60, 81). Investigators have proposed that strain-variable genes encode factors that contribute to

different disease presentations and enable the organism to survive in unique ecological niches. Regardless, an established set of *C. jejuni* virulence genes, and their contribution in disease production, has yet to emerge.

To better understand the metabolic capacity and virulence properties of *C. jejuni*, the genome sequence of strain NCTC 11168 was determined (60). Shortly thereafter a comparison of *C. jejuni* isolates by whole genome-microarray analysis revealed extensive genetic diversity (16). Based on the comparison of eleven *C. jejuni* isolates, 21% of the genes in the *C. jejuni* NCTC 11168 sequence strain were proposed to be dispensable as they were either absent or highly divergent among the other isolates included in the study. Dorrell *et al.* (16) also noted that many of the virulence genes identified to date are conserved among *C. jejuni* strains.

Published reports suggest that not all *C. jejuni* strains have the same virulence potential. Everest *et al.* (18) noted that 86% of *Campylobacter* isolates from individuals with colitis were able to translocate across Caco-2 polarized cells versus 48% of strains isolated from individuals with noninflammatory disease. Fauchere *et al.* (20) found that *C. jejuni* recovered from individuals with fever and diarrhea adhered to cultured cells at a greater efficiency than those isolated from asymptomatic individuals. The relative ability of *C. jejuni* to invade cultured cells also appears to be strain-dependent (18, 38, 54). Newell *et al.* (54) found that environmental isolates were much less invasive for HeLa cells than clinical isolates as determined by immunofluorescence and electron microscopy examination of *C. jejuni*-infected cells. Moreover, a statistically significant difference

has been observed in the level of invasion between *C. jejuni* isolated from individuals with colitis versus those isolated from individuals with noninflammatory diarrhea (18).

Poly *et al.* (65) compared two unrelated *C. jejuni* strains (ATCC 43431 and NCTC 11168) by a shotgun genomic DNA microarray. Not surprisingly, a large number of genes unique to ATCC 43431 encoded proteins in LOS and capsular synthesis. Interestingly, 36 of 130 DNA fragments unique to ATCC 43431 showed similarity to proteins encoding the *Helicobacter hepaticus* Imp locus located on a putative pathogenicity island. In other organisms, the Imp locus has been proposed to modulate host responses (9). More recently, Poly *et al*. (64) compared the genomic content of *C. jejuni* strain 81-176 with that of *C. jejuni* strain NCTC 11168 and found divergence at LOS, capsular biosynthesis, and restriction/modification system loci.

In this study, we assessed the putative virulence properties of *C. jejuni* isolates recovered from poultry. More specifically, we analyzed the virulence attributes of two *C. jejuni* isolates, designated Turkey and CS, because they were indistinguishable by PFGE, MLST, and genomotyping. The *C. jejuni* Turkey isolate showed greater pathogenic potential than the *C. jejuni* CS isolate based on both *in vitro* and *in vivo* assays. The lack in pathogenic potential of the *C. jejuni* CS isolate, relative to the *C. jejuni* Turkey isolate, was attributed to a loss in the synthesis of a functional flagellum. The flagellum, and associated motility, is a known *C. jejuni* virulence determinant (8, 51, 53, 77, 78, 84). In addition, host cell invasion and the secretion of the Cia (*Campylobacter* invasion antigen) proteins requires a functional flagellar export apparatus (39). We identified a point

mutation in the *flgR* gene of the *C. jejuni* CS isolate. The *flgR* gene in *C. jejuni* encodes for the response regulator of the FlgS/FlgR two-component signal transduction system that participates in flagellar biosynthesis. Phosphorylation of the C-terminal output domain of the FlgR response regulator protein presumably results in its binding to DNA, whereby it interacts with σ^{54} to initiate the expression of flagellar class II genes. The class II flagellar genes encode proteins that are required for the assembly of a functional flagellum (30, 33).

While other investigators have examined differences in *C. jejuni* isogenic strains or passage variants, we are unaware of another study undertaken to examine the virulence attributes of naturally occurring clonally-matched isolates. We identified a difference in the nucleotide sequences of the *flgR* gene in *C. jejuni* Turkey and CS isolates, which resulted in the non-motile and avirulent phenotype of the *C. jejuni* CS isolate. This mutation appears to provide the isolate a fitness advantage in the environment. More specifically, the *C. jejuni* CS isolate exhibited greater resistance against the antimicrobial activity of the bile salt deoxycholate, and is thus able to reach a greater cell density in deoxycholate supplemented medium when compared to a genotypically matched isolate. Further, the *C. jejuni* Turkey isolate harboring a mutation in *flgR* mirrored the phenotype of the *C. jejuni* CS isolate.

MATERIALS AND METHODS

Culture of bacterial isolates

Two *C. jejuni* human clinical isolates (*C. jejuni* F38011 and 81-176) and six *C. jejuni* poultry isolates were used throughout this study. Five of the six *C. jejuni* poultry isolates were recovered from chicken rinses (CS, S1, S2B, S3, SC) and one isolate was recovered from the liver of a turkey (Turkey) exhibiting clinical signs of poultry enteritis. All *C. jejuni* were cultured at 37°C under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) on Mueller-Hinton agar plates supplemented with 5% citrated bovine blood (MH-blood agar). Cultures were subcultured to a fresh plate every 24 to 48 h. In addition to the six *C. jejuni* isolates used throughout this study, we also screened an additional 99 *C. jejuni* poultry isolates for motility. These isolates used were recovered from either poultry processing plants located in Kansas (A1a, A2a, A3a, A4a, A5a, A7a, A8a, A9a, A11a, A12a, A13a, A14a, A15a, A16a, A18a, A20a, A22a, A23a, A24a, A25a, A37a), Iowa (D28a, D30a, D31a, D32a, D33a, D34a, D37a, D38a, D41a, D42a, D44a, D45a, D48a, D49a) and Washington state (B13a, B19a, C11a, C18a, C20a, C24a, C25a, G9a, G11a, G12a, G13a, G15a, G17a, G21a, G22a, G26a, H1a, H2a, H3a, H4a, H5a, H8a, H29a, I6a, I7a, I16a, I22a), or from cecal droppings collected at different broiler houses (2, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13, 14, 15, 16, 19, 21, 22, 23, 24, 25, 26, 33, 34, 35, 36, 39, 41, 42, 43a, 43c, 44, 77, 78, 79, 80, 81, 83). All the *C. jejuni* poultry isolates used in this study were passed less than five times in the lab. The primary stock of each of *C. jejuni* isolate was frozen in citrated bovine blood and stored at -80°C. *E. coli* INVαF' (Invitrogen,

Carlsbad, CA), S17-1 λ *pir*, and XL1-Blue MRF' (Stratagene, La Jolla, CA) were cultured on Luria-Bertani (LB) agar plates at 37°C.

Culture of INT 407 cells

A culture of INT 407 human epithelial cells (ATCC CCL6) was obtained from the American Type Culture Collection (Manassas, VA). Stock cultures of INT 407 cells were grown in Minimal Essential Medium (MEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT). Cultures were maintained at 37°C in a humidified, 5% CO_2 incubator.

C. jejuni-*INT 407 cell binding and invasion assay*

For experimental assays, each well of a 24-well tissue culture tray was seeded with 1.4 x 10^5 INT 407 cells per well and incubated for 18 h at 37°C in a humidified, 5% CO_2 incubator. The cells were rinsed with MEM-1% FBS and inoculated with a suspension of approximately 5 x 10^7 colony forming units (CFUs) of bacteria. Tissue culture trays were centrifuged at 600 x *g* for 5 min, and incubated at 37°C in a humidified, 5% CO_2 incubator. For binding, the infected monolayers were incubated for 30 min, rinsed 3 times with phosphate buffered saline (PBS), and the epithelial cells were lysed with 0.1% (vol/vol) Triton X-100 (Calbiochem, La Jolla, CA). The suspensions were 10-fold serially diluted and the number of viable, adherent bacteria was determined by counting the resultant colonies on MH-blood plates. To measure bacterial internalization, the infected monolayers were incubated for 3 h, rinsed 3 times with MEM-1% FBS, and incubated for an additional 3 h in MEM-1% FBS containing a bactericidal concentration

(250 μg/ml) of gentamicin. The number of internalized bacteria was determined as outlined above. Unless otherwise stated, the reported values represent the mean counts ± standard deviations derived from triplicate wells. All assays were performed a minimum of 3 times to ensure reproducibility. Significance between samples was determined using Student's *t* test following $\log_{10}$ transformation of the data. Two-tailed *P* values were determined for each sample, and a *P* value < 0.01 was considered significant.

Secretion assay

C. jejuni were harvested from MH-blood agar plates in PBS, pelleted by centrifugation at 6,000 x g, washed twice in MEM, and suspended in MEM lacking methionine (labeling medium; ICN Biomedicals, Inc., Aurora, OH) to an optical density (OD_{540}) of 0.3 [approximately 5 x 10^8 CFU]. Metabolic labeling experiments were performed in 3 ml of labeling medium with the addition of [^{35}S]-methionine (PerkinElmer Life Sciences, Inc., Boston, MA) as described elsewhere (37). Following a 3 h labeling period, bacterial cells were pelleted by centrifugation at 6,000 x g and supernatant fluids collected. The supernatant fluids were concentrated four-fold by the addition of five volumes of ice-cold 1 mM HCl-acetone followed by centrifugation. The pellets were air dried and resuspended in water. The secreted proteins were resolved in sodium dodecyl sulfate-12.5% polyacrylamide gels using the discontinuous buffer system described by Laemmli (41). Gels were treated with Amplify (Amersham, Piscataway, NJ) according to the manufacturer's instructions. Autoradiography was performed with Kodak BioMax MR film at -70°C.

Motility assay

Motility assays were performed using MH medium supplemented with 0.4% Select Agar (motility agar plates, Life Technologies, UK). A 10 µl suspension of each bacterial isolate was spotted on the surface of the semi-solid medium. Motility plates were incubated at 37°C under microaerobic conditions for 48 h.

Macrorestriction enzyme profile pulsed-field gel electrophoresis

C. jejuni were harvested from MH-blood agar plates in 3 ml PETT IV buffer (1 M NaCl, 10 mM Tris, 10 mM EDTA, pH 8.0) and cell densities adjusted to 0.75 using a Microscan Turbidity Meter (Dade Behring, West Sacramento, CA) in 12 X 75 mm Falcon round bottom tubes (Becton Dickinson, Franklin Lakes, NJ). Four hundred µl of 1.4% (wt/vol) molten (50°C) pulsed-field grade agarose (Bio-Rad, Hercules, CA) was added to an equivalent volume of each bacterial suspension, mixed gently and a 100 µl aliquot was pipetted into agarose plug molds. The agarose plugs were removed from the molds and incubated in 1 ml of ESP buffer (50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% (wt/vol) N-lauroyl sarcosine, 0.5 mg/ml proteinase K) at 50˚C for 1 h. Following cell wall lysis, the agarose blocks were washed 3 times in sterile water and 3 times in TE (10 mM Tris, pH 8.0, 1 mM EDTA). Each wash was performed at ambient temperature for 30 min. Individual agarose plugs were incubated with 100 µl of 1X restriction endonuclease buffer containing 20 U of SmaI. The reactions were incubated at 25˚C for a minimum of 4 h. Following incubation, the agarose plugs were loaded into an agarose gel. Restricted genomic DNA was separated in 1% (wt/vol) pulsed-field grade agarose prepared with 0.5X TBE [0.089 M Tris base, 0.089 M boric acid, 0.002 M EDTA (pH 8.0)]. Run

parameters consisted of a reorientation angle of 120 degrees with a constant voltage of 120 V and a constant temperature of 14˚C. An electrophoresis run time of 19 h and a ramped pulse time of 6.8-35.4 sec were used. For plugs digested with SalI, a run time of 18 h and a ramped pulse time of 5.2 – 42.3 sec was used. Gels were stained for 20 min in 3 μg/ml ethidium bromide and destained for 20 min in water. Images were captured using a Bio-Rad FluorS system and processed using Adobe Photoshop.

Multilocus sequence typing

Multilocus sequence typing was performed as described by Dingle *et al.* (15) in which a portion of the housekeeping genes [aspartase A (*aspA*), glutamine synthetase (*glnA*), citrate synthase (*gltA*), serine hydroxymethyl transferase (*glyA*), phosphoglucomutase (*pgm*), transketolase (*tkt*) and the ATP synthase alpha subunit (*uncA*)] were amplified and sequenced. Genomic DNA was recovered from *C. jejuni* isolates harvested from MH-blood agar plates. Amplification of each gene fragment was performed using approximately 10 ng of genomic DNA and 1.25 U of *Taq* DNA polymerase (Invitrogen) in a 50 μl reaction volume containing 1 μM forward and reverse primer, 1 X PCR buffer, 1.5 mM $MgCl_2$ and 0.8 mM dNTPs. Amplification reactions consisted of the following cycling conditions: an initial denaturation step (94ºC, 2 min) followed by an annealing (50ºC, 1 min) and extension step (72ºC, 1 min), which were repeated for 35 cycles. PCR amplicons were assessed for quantity and quality by agarose gel electrophoresis. Primers and unincorporated dNTPs were removed by passing amplicons through a commercially available batch column purification system (Qiaquick PCR kit, Qiagen, Valencia, CA). The nucleotide sequence of PCR amplicons were determined using a modification of

Sanger dideoxynucleotide sequencing, incorporating fluorescence dye technology at the DNA sequencing facility of Washington State University. Sequencing was performed on both strands for a segment of each allele from each isolate. Nucleotide sequences for each allele were aligned using the program Clustal X and the nucleotide sequences were assigned alleles from the MLST website (http://mlst.zoo.ox.ac.uk). Sequence types (STs), based on the combination of assigned alleles to the seven housekeeping genes, were determined using the MLST website (http://mlst.zoo.ox.ac.uk).

Inoculation of piglets

Newborn piglets were obtained from sows at the time of farrowing. Feces from each sow were plated on selective Abeyta Hunt Bark agar plates containing sodium cefoperazone (32 mg/L), rifampicin (10 mg/L), amphotericin B (2 mg/L), and 4 ml/L of FBP [62.5 g/L sodium pyruvate, 62.5 g/L ferrous sulfate, and 62.5 g/L sodium metabisulfite]. Suspect colonies were tested for *C. jejuni* as described by Nogva *et al.* (56). Only piglets obtained from *C. jejuni* free sows were used for *in vivo* studies.

Each piglet was fed 5 times daily with 50 ml of Similac formula (Ross Laboratories). Overnight grown cultures of *C. jejuni* were resuspended in Similac at a concentration of approximately 5 x 10^9 CFU/ml. Piglets were orally inoculated with approximately 20 ml of either the *C. jejuni* Turkey or CS isolate. Actual numbers of viable bacteria inoculated were determined by serial dilution and plating of the bacterial suspension.

Piglets were observed for diarrhea and rectal swabs were plated on Abeyta Hunt Bark medium prior to inoculation and daily thereafter to determine the presence of *C. jejuni* shedding. Piglets were euthanized when morbidity was observed or 6 days post-inoculation with 2 ml of Beuthanasia-D (Schering Corp.). Gross examination of the small intestine, colon, and cecum were recorded and tissue samples were fixed in 10% buffered formalin and examined for histological lesions at the University of Arizona Veterinary Diagnostic Laboratory (Tucson, AZ).

Preparation of whole cell lysates, outer membrane proteins and flagellin

C. jejuni whole cell lysates were generated by harvesting bacteria grown overnight on MH-blood plates in PBS and subjecting the bacterial suspension to sonication on ice (five 30 sec pulses).

Outer membrane proteins (OMP) were prepared as described by deMelo and Pechere (13) with minor modifications. Briefly, bacterial sonicates were centrifuged at 8,000 x *g* to remove whole bacterial cells. Supernatant fluids were centrifuged at 100,000 x *g* for 2 h. The resulting pellets were resuspended in a solution of 1% sarkosyl (Sigma, St. Louis, MO) in 10 mM Tris-HCl (pH 7.5) and allowed to incubate at ambient temperature for 30 min on a platform rocker. The solutions were centrifuged at 100,000 x g for 2 h and the supernatants were removed. The pellets were washed in 10 mM Tris-HCl (pH 7.5) and centrifuged at 100,000 x g for 2 h. Finally, the pellets were resuspended in 50 mM Tris-HCl (pH 7.5) and the protein concentration was determined by the BCA™ Protein Assay Kit according to the manufacturer's instructions (Pierce, Rockford, IL).

Flagella were purified as described by Alm *et al*. (3). Bacteria were resuspended in PBS and homogenized twice for 3 min. The bacterial suspensions were centrifuged at 8,000 x *g* to remove whole cells. The supernatant fluids were centrifuged at 100,000 x *g* for 1 h. The resulting pellets were resuspended in 1% sodium dodecyl sulfate (SDS) and allowed to incubate at ambient temperature. The solutions were centrifuged at 100,000 x *g* for 1 h and the resulting pellets were washed twice in distilled water and centrifuged as above. The final pellets were resuspended in 10 mM Tris-HCl (pH 7) and the protein concentration was determined as described above. All protein extracts were stored at -20°C.

Gel electrophoresis

Two-dimensional gel electrophoresis was performed using the parameters described elsewhere (37). One-dimensional gel electrophoresis was performed by mixing an equal volume of a bacterial suspension (an equivalent of 0.1 OD_{600} units) with double strength electrophoresis sample buffer (37). The samples were placed in boiling water for 5 min and allowed to cool to ambient temperature. Proteins were resolved by 12.5% SDS-PAGE using the discontinuous buffer system described by Laemmli (41).

Immunoblot analysis

Proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore Corp., Bedford, MA). The membranes were washed 3 times in PBS and incubated for 18 h at 4°C with a 1:500 dilution of a rabbit α-*C. jejuni* flagellin antibody in PBS pH 7.4/0.01% Tween-20 containing 9% dried milk.

Bound antibodies were detected using peroxidase-conjugated goat anti-rabbit IgG (Sigma) at a 1:1,000 dilution and 4-chloro-1-napthol (Sigma) as the chromogenic substrate.

RNA Isolation

Total cellular RNA was isolated from the *C. jejuni* Turkey and CS isolates grown to mid-log phase using the hot phenol method (74). Briefly, 5 ml of RNA degradation stop solution was added (10% phenol solution, buffered saturated with 0.1 M citrate buffer, pH 4.3 + 0.2, in 100% ethanol) to 50 ml of each bacterial culture. The bacterial suspension was then pelleted and resuspended in 3 ml of 1% buffer-saturated phenol solution. The culture was pipetted into 1.5 ml of boiling lysis buffer in a water bath until cell lysis occurred. This was followed by two extractions with phenol at 60ºC and one extraction with phenol-chloroform, the aqueous phase was precipitated with ethanol and resuspended in diethylpyrocarbonate-treated water. Contaminating genomic DNA was removed by two consecutive treatments with RQ1-DNase (Promega, Rockford, IL). The absence of genomic DNA was confirmed via PCR using *C. jejuni ciaB* gene-sequence specific primers (CiaB-FOR 5'-CTATGCTAGCCATACTTAGGC and CiaB-REV 5'-GCCCGCCTTAGAACTTAC). The PCR conditions used were 94ºC for 1 min, 50ºC for 1 min and 72ºC for 1 min with a final extension time of 5 min (35 cycles in total).

Construction of the C. jejuni *DNA microarray*

DNA fragments of individual open reading frames (ORFs) were amplified using ORF specific primers for those present in strain NCTC 11168 (Sigma Genosys, The

Woodlands, TX). Each PCR reaction (total reaction volume, 100 µl) consisted of 1X MasterAmp Taq PCR buffer, 1X MasterAmp Taq Enhancer, 2.5 mM $MgCl_2$, 200 µM each deoxynucleoside triphosphate, forward and reverse primers at 0.2 µM each, 0.5 U of MasterAmp Taq DNA polymerase (Epicentre Madison, WI), and approximately 50 ng of genomic DNA from strain NCTC 11168. Thermal cycling was performed using a Tetrad thermal cycler (MJ Research, Waltham, MA) with the following amplification parameters: 30 cycles of 25 sec at 94°C, 25 sec at 52°C, and 2 min at 72°C and a final extension at 72°C for 5 min. PCR products were analyzed by gel electrophoresis in a 1% (wt/vol) agarose gel (containing 0.5 µg of ethidium bromide/ml) in 1X Tris-acetate-EDTA buffer. DNA bands were examined under UV illumination. A total of 1530 PCR products were successfully amplified and then purified on a Qiagen 8000 robot using a Qiaquick 96-well Biorobot kit (Qiagen), dried and resuspended to an average concentration of 0.1-0.2 µg/µl in 20 µl of 50% dimethyl sulfoxide (DMSO) containing 0.3X saline sodium citrate (SSC). All of the PCR probes were then spotted in duplicate on GAPSII slides (Corning, Acton, MA) using an OmniGrid Accent (GeneMachines, Ann Arbor, MI) producing a final array that contained a total of 3060 features.

Fluorescent labeling of genomic DNA and cDNA

For each comparative genomic microarray hybridization reaction, genomic DNAs from the reference strain NCTC 11168 and a test strain were fluorescently labeled with Cy5 and Cy3, respectively. Approximately 2 µg of DNA was mixed with 5 µl 10x NEBlot labeling buffer containing random sequence octamer oligonucleotides (New England Biolabs, Beverly, MA) and water to a final volume of 41 µl. This mixture was heated to

95°C for 10 min and then stored for 5 min at 4°C. After this incubation, the remainder of the labeling reaction components were added: 5 µl of 10X dNTP labeling mix (1.2 mM each dATP, dGTP, dCTP; 0.5 mM dTTP in 10 mM Tris pH 8.0; 1 mM EDTA), 3 µl of Cy3 dUTP or Cy5 dUTP (Amersham Biosciences, Piscataway, NJ) and 1 µl of Klenow fragment. The labeling reactions were incubated overnight at 37°C. Fluorescently labeled DNA was purified using Qiaquick PCR purification columns (Qiagen, Valencia, CA) according to manufacturer's directions.

For the expression profiling arrays, an indirect comparison of gene expression was performed (83), where the expression from the various *C. jejuni* isolates was measured separately on different slides. In this microarray experimental design, each labeled cDNA is combined with a fixed reference source (83), allowing the comparison of different experiments in which a common reference has been used, as in previous studies (17, 45). Sixteen µg of total RNA from each *C. jejuni* isolate was labeled during reverse transcription to cDNA with Cy5-dUTP using Stratascript (Stratagene, Palo Alto, CA). Following 16 h labeling, RNA was degraded by the addition of NaOH to 0.3 M and incubation at 70°C for 10 min, followed by neutralization with an equimolar amount of HCl. The labeled cDNA was purified using Qiaquick PCR purification columns (Qiagen) according to manufacturer's directions. In all expression microarray hybridizations, genomic DNA from *C. jejuni* strain NCTC 11168 was labeled with Cy3-dUTP as described above. The labeled genomic DNA also served as a quality control for all the spots in the array. In this particular indirect comparison for gene expression, an

analysis of repeatable residual color bias from Cy-Dye-swap experiments was removed since the between-slide differences were taken into account (see below) (83).

Microarray hybridization

For each genomic indexing hybridization, Cy5 labeled reference DNA from the *C. jejuni* strain NCTC 11168 was mixed with Cy3 labeled test DNA or cDNA in 45 µl of Corning hybridization buffer (Corning, Acton, MA) and heated to 95°C for 5 min. Then 15 µl of the hybridization mixture was put onto the microarray slide and sealed with a coverslip in a GeneMachine hybridization chamber (Genomic Solutions, Ann Arbor, MA) and incubated at 42°C for 18 h. This method, known as differential labeling, allows the hybridization of fluorescently labeled control and test DNA or cDNA to be measured for each probe on the microarray. As the genetic composition of the sequenced strain *C. jejuni* NCTC 11168 is known, it served as a control fluorescence signal for each probe and was used for comparison with the test DNA signal during the statistical analysis (see below). Following hybridization, microarray slides were washed for 2 min in 2× SSC, 0.1% SDS at 42°C to remove the coverslip and then washed twice for 5 min in each of the following buffers: (a) 2X SSC, 0.1% SDS at 42°C, (b) 0.2X SSC, and finally (c) 0.01X SSC. Microarray slides were dried by centrifugation at 300 × *g* for 15 min before scanning.

Microarray data analysis

DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA) and the data for spot and background intensities

were processed using the GenePix 4.0 software. Poor features were excluded from analysis if they contained abnormalities or a reference signal lower than background plus three standard deviations. As described by Eriksson *et al.* (17), fluorescence ratios were calculated after local background was subtracted from spot signals. To compensate for any effect of the amount of template and uneven Cy-dye incorporation, data normalization was performed as previously described (4, 17, 45) by bringing the median natural logarithm of the ratios for each group of spots printed by the same pin to zero using the following equation: $ln(T_i) = ln(R_i/G_i) - c$, where T is the centered ratio, i is the gene index, R and G are the red and green intensities, respectively, and c is the 50th percentile of all red/green ratios. Normalized data that passed the quality controls were analyzed using GENESPRING 6.2 software (Silicon Genetics, Palo Alto, CA). For comparison of the *C. jejuni* Turkey and CS gene expression, at least four hybridization measurements were generated per biological experiment (two technical replicate arrays and two replicate features per array) and the experiment was repeated two times (biological replicate). Significance of the centered data at $P \geq 0.05$ was determined using a parametric-based statistical t-test adjusting the individual P value with the Benjamini and Hochberg false discovery rate multiple test correction within the GeneSpring analysis package.

Sequencing genes involved in flagellar biosynthesis

The genes of interests (*flhA, flhB, flhF, fliA, fliF, fliG, fliH, fliI, fliM, fliN, fliP, fliQ, fliR, fliY flgR, flgS, rpoN,* Cj0667, Cj0668, and Cj0669) were PCR amplified using KOD DNA polymerase (Novagen, San Diego, CA) and sequenced using gene specific primers. Both

strands, of every gene above, were sequenced from both the *C. jejuni* Turkey and CS isolates. All sequencing primers were designed based on *C. jejuni* NCTC 11168 genomic sequences (60). Sequencing was performed using the Big Dye terminator kit (Applied Biosystems, Foster City, CA) and the DNA sequencer ABI373 (Applied Biosystems, Foster City, CA). The sequences were analyzed using the MultAlin multiple sequence alignment program.

Real time RT-PCR

The relative expression of twenty-six genes (*clpB, cmeA, flaA, flaB, flaC, flgB, flgC, flgD, flgE, flgG, flgH, flgK, fliF, fliG, fliH, fliI, fliM, fliY, flhA, flhB, metA, rpoN,* Cj1514, Cj0667, Cj0668, and Cj0669) was determined by real time quantitative reverse transcription-PCR (real time RT-PCR). The cDNA was synthesized using the ThermoScript™ RT-PCR system (Invitrogen) according to the manufacturer's directions. Real time RT-PCR amplification of 0.5 µl of cDNA was performed in a reaction mix containing Power SYBR® Green PCR master mix (Applied Biosystems, Foster city, CA), 300 nM concentration of forward and reverse primers and diethylpyrocarbonate-treated water. Real time RT-PCR analysis was performed using Gene Amp 7000 thermocycler (Applied Biosystems, Foster city, CA). PCR conditions included one cycle of 2 min at 50°C, followed by 40 cycles of denaturation at 95°C for 15 sec and an annealing at 55°C for 1 min. Cycle threshold (Ct) values were determined using Prism SDS software version 1.0 (Applied Biosystems). The comparative threshold cycle ($\Delta\Delta C_T$) method was used to calculate fold change where samples were normalized to *glnA*, since it is a housekeeping gene and was not found to be differentially expressed by microarray

analysis. Reactions were performed in duplicate, and two biological replicates were performed for each sample.

Construction of reporter vectors

The promoter regions of the *flaB* and *metK* genes were PCR amplified using the following primer sets (P*metK*F: ATTTGGATCCCCTTGTGCTCCTGTTTGTGC, P*metK*R: ATATGGATCCAAAAAGTCCTTTCATTTAAAATGAACC, P*flaB*F: AAGGATCCACACTTAAAGGCGCTATGGCTGTGATG and P*flaB*R: AAGGATCCGATGTTGGTGTTTATCCTAAAACC) designed to include a BamHI site at the 5' end. The PCR products were cloned into pCR 2.1 using the TOPO® TA Cloning Kit (Invitrogen). The ligated vector was electroporated into *E. coli* INVαF' and the transformants were selected on LB agar supplemented with kanamycin (KAN, 50 µg/ml). The P*flaB*-pCR2.1 and P*metK*-pCR2.1 were digested with BamHI to give 0.8-kb and 0.78-kb fragments respectively. Each fragment was gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The pMW10 shuttle vector (80) was digested with BamHI, gel purified and ligated with the BamHI-P*flaB* and BamHI-P*metK* fragment for 18 h. The ligation mix was ethanol precipitated and electroporated into *E. coli* INVαF'. The transformants were selected on LB-KAN agar and tested for insert by blue white screening. The sequences of the constructs were verified using a primer that anneals to the *aphA3'* gene encoding kanamycin resistance (TACACTCAAATGGTTCGCTG) as described previously (80).

Electroporation of promoter-shuttle vectors in the C. jejuni *F38011 and CS isolates*

The vectors (pMW10, P*metK*-pMW10, P*flaB*-pMW10) were introduced into the *C. jejuni* F38011 and CS strains by electroporation. The resultant colonies were selected on MH blood agar plates supplemented with KAN (200 µg/ml).

β-galactosidase assay

β-galactosidase activity was determined as a measure of conversion of *o*-nitrophenyl-β-D-galactopyranoside (Sigma) to nitrophenol. *C. jejuni* transformants were grown for 16 h in MH broth. Harvested bacteria were diluted in PBS until the OD_{600} was between 0.4 and 0.5. The assay was carried out in triplicate as previously described (80).

Construction of the complementation vectors

To construct the *flgR* pRY107 (KAN resistant) complementation vector, the coding region of *flgR* gene along with its native promoter (350 bps upstream of the start codon) was PCR amplified using primers containing 5' BamHI restriction sites (*flgR*compF: TATATAGGATCCAATGGGTATGTGAAATTTCTTATAGTG and *flgR*compR: TATATAGGATCCCCCAACCGCACCAGTAGC). The PCR product was cloned into pCR2.1 using the TOPO® TA Cloning Kit (Invitrogen). The ligated vector was electroporated into *E. coli* INVαF' and the transformants were selected on LB-KAN plates (50 µg/ml). The pCR2.1 construct containing the BamHI *flgR* fragment was digested with BamHI, gel purified and cloned into the BamHI site of pRY107. The ligated vector was electroporated into *E. coli* INVαF' and transformants were selected on LB-KAN plates (50 µg/ml).

To construct the *rpoN* pRY111 (Cm resistant) complementation vector, the promoter region of Cj0667 was fused with the *rpoN* ORF. Noteworthy is that the *rpoN* gene is the fourth gene within the operon (5'- Cj0667, Cj0668, Cj0669, *rpoN*). The promoter region of Cj0667 was PCR amplified using primers containing 5' SstI and NdeI restriction sites (Cj0667-SstI-F:TATATAGAGCTCTCAAGGCGTCCTATACCGTG and Cj0667-NdeI-R2: ATATATACATATGTTCAGAAATAGCTCTTC). The *rpoN* ORF was PCR amplified using primers containing 5' NdeI and SstI restriction sites (*rpoN*-NdeI-F: TATAATCATATGTTAAAGCAAAAAATCACCCAAG and *rpoN*-SstI-R: TATAATCCGCGGAATATTTAAAAACAGTTATTATTGTATCAC). The PCR amplicons were cloned into pCR2.1 using the TOPO® TA Cloning Kit (Invitrogen). The ligated vector was electroporated into *E. coli* INVαF' and transformants were selected on LB-KAN plates (50 μg/ml). The *rpoN*-pCR2.1 construct was digested with NdeI and SstI and the gel purified fragment was ligated into the NdeI and SstI restricted PCj0667-pCR2.1 construct. The ligated vector was electroporated into *E. coli* INVαF' and transformants were selected on LB-KAN plates (50 μg/ml). The PCj0776-*rpoN*-pCR2.1 fusion vector was digested with SstI and the SstI-PCj0667-*rpoN* fragment was gel purified and cloned into the SstI site of pRY111. The resulting vector was electroporated into *E. coli* INVαF' and transformants were selected on LB agar plates supplemented with chloramphenicol (LB-CHL).

Conjugation

The complementation plasmids were electroporated into *E. coli* S17-1 λ *pir*. The *E. coli* S17-1 λ *pir* harboring the complementation plasmids *flgR*-pRY107 was then conjugated with *C. jejuni* CS isolate. The transconjugants were selected on MH-blood plates supplemented with KAN (200 μg/ml) and tetracycline (TET, 50 μg/ml). The *C. jejuni* CS isolate colonies resistant to both KAN and TET were screened for motility on motility agar plates (Life Technologies, UK).

The *E. coli* S17-1 λ *pir* harboring the PCj0667-*rpoN*-pRY111 complementation plasmid was conjugated with *C. jejuni* S2B isolate. The transconjugants were selected on MH-blood plates supplemented with CHL (20 μg/ml) and TET (50 μg/ml). The *C. jejuni* S2B isolate colonies resistant to both KAN and TET were screened for motility on motility agar plates.

Generation of the C. jejuni *Turkey* flgR *mutant*

A disrupted copy of the *flgR* gene was amplified from *C. jejuni* F38011 *flgR:cat* mutant using *flgR* gene specific primers (66), and the resultant product was cloned into pCR2.1. The *flgR:cat* fragment was digested using EcoRI, gel purified and cloned into the EcoRI site of the pBluescriptII SK+ vector. The ligated vector was electroporated into *E. coli* INVαF' and selected on LB-CHL. The mutagenesis vector was introduced into the *C. jejuni* Turkey isolate by electroporation and selected on MH-blood agar plates supplemented with CHL. The putative mutants were confirmed by PCR amplification of *flgR*, followed by digestion of the PCR product by NheI to release the *cat* cassette.

Growth curves

Overnight cultures of *C. jejuni* Turkey and CS isolates were used to inoculate 250 ml of MH broth at an OD_{540} of 0.01. The flasks were incubated at 37°C under microaerobic conditions, and shaken at 150 rpm. At different time points, 1 ml of bacterial culture was used to determine the OD_{540} until both cultures reached stationary phase.

Sensitivity to deoxycholate

Overnight cultures of *C. jejuni* Turkey, *C. jejuni* Turkey *flgR* mutant and CS isolates were used to inoculate tubes at an OD_{540} of 0.05. Different concentrations of sodium deoxycholate were added to each tube in triplicate and incubated for 48 h at 37°C under microaerobic conditions and shaken at 150 rpm. At the end of the incubation period, the OD_{540} for each tube was measured.

Other analytical procedures

Protein concentrations were determined by the bicinchoninic acid (BCA) method, with bovine serum albumin (BSA) as the standard, as outlined by the supplier (Pierce, Rockford, IL). To determine the identity of the protein present in the flagellar extract prepared from the *C. jejuni* Turkey isolate, the extract was mixed with an equal volume of double strength electrophoresis sample buffer sample and separated by SDS-PAGE. The proteins were transferred to PVDF membranes and stained with Coomassie brilliant blue G-250. The amino-terminal sequence of the 62 kDa protein was determined at the

University of British Columbia Biotechnology Laboratory (Protein Sequencing and Peptide Mapping, NAPS Unit, Vancouver, B.C., Canada).

RESULTS

C. jejuni *isolates display marked differences in virulence phenotypes*

While there are several reports documenting the variation in the efficiency of *C. jejuni* isolates to invade cultured cells (18, 38, 54), the molecular mechanisms for these variations have seldom been elucidated. To identify *C. jejuni* isolates with apparent differences in virulence potential, we performed INT 407 cell invasion assays and secretion assays with six *C. jejuni* poultry isolates. *C. jejuni* invasion of INT 407 cells was assessed using the gentamicin-protection assay. *C. jejuni*-INT 407 host cell contact was promoted by a low-speed centrifugation step immediately following inoculation. The invasiveness of the six *C. jejuni* poultry isolates was markedly different (TABLE 1). Relative to the *C. jejuni* 81-176 clinical strain, the *C. jejuni* Turkey strain was the most invasive environmental isolate tested and the *C. jejuni* S2B strain was the least invasive. Because *C. jejuni* binding is a prerequisite for host cell invasion, the data were transformed and presented as the percent I/A (intracellular/cell-associated bacteria) to more directly assess the invasive potential of each isolate. Three of the six isolates (CS, S2B, and S3) were readily discernable from the other isolates tested in that they displayed lower invasive potential.

C. jejuni synthesize and secrete a set of proteins upon co-culturing with epithelial cells, which aid in the invasion of the host cells. The secreted proteins are referred to, collectively, as *Campylobacter* invasion antigens (Cia proteins). Since three of the six *C. jejuni* poultry isolates showed low invasive potential, secretion assays were performed to

determine the ability of the isolates to secrete Cia proteins. The assays were performed in the presence of fetal bovine serum (FBS), which serves as an artificial signal to stimulate the synthesis and secretion of the Cia proteins (68). The Cia proteins were readily identifiable in the supernatant fluids of *C. jejuni* 81-176, S1, SC, and Turkey isolates, but not detectable in the supernatant fluids of *C. jejuni* CS, S2B, and S3 isolates (FIG. 1). The M_r of the Cia proteins exported from the *C. jejuni* poultry isolates was consistent with that of the *C. jejuni* 81-176 human clinical isolate (FIG. 1), and with previous work with other *C. jejuni* clinical isolates (39, 68). Noteworthy is that the *C. jejuni* CS, S2B, and S3 isolates displayed a lower invasive potential than the *C. jejuni* poultry isolates (S1, SC, and Turkey) that secreted the Cia proteins.

Previous work indicates that the *C. jejuni* Cia proteins are secreted from the flagellar apparatus (39). As a screen to determine whether each isolate possessed a functional flagellar export apparatus, motility assays were performed with each of the six *C. jejuni* poultry isolates (not shown). In contrast to *C. jejuni* SC, S1, and Turkey, the *C. jejuni* CS, S2B, and S3 isolates were non-motile. These findings raised the possibility that the *C. jejuni* CS, S2B, and S3 isolates were weakly invasive and did not secrete the Cia proteins because they did not possess a functional secretion apparatus/flagellum.

C. jejuni *isolates Turkey and CS are genotypically indistinguishable*

To unravel the molecular basis of the differences in pathogenesis exhibited by the isolates we wanted to compare their genetic relatedness. Since macrorestriction enzyme pulsed-field gel electrophoresis (MRP-PFGE) profiling is the currently accepted method to

compare the relatedness of *Campylobacter* isolates (12, 14, 21, 22, 25, 31, 32, 67, 72, 73, 76), we used it to assess the genomic diversity of the *C. jejuni* isolates used in this study. Initially, *C. jejuni* isolates were analyzed by PFGE following digestion of chromosomal DNA with the restriction enzyme SmaI. A representative gel is presented in FIG. 2A. PFGE of SmaI restricted *C. jejuni* chromosomal DNA yielded four to ten fragments ranging in size from < 97-kb to approximately 485-kb. The *C. jejuni* CS, SC, and Turkey isolates yielded indistinguishable macrorestriction profiles (MRP) using the SmaI restriction enzyme. We chose to narrow the remainder of this study to the Turkey and CS isolates because the Turkey isolate was found to be highly invasive for INT 407 cells and was Cia secretion-positive whereas the CS isolate displayed a non-invasive phenotype for INT 407 cells and was Cia secretion-negative. Noteworthy is that MRP of the *C. jejuni* Turkey and CS isolates were also indistinguishable using a second restriction enzyme, SalI (FIG. 2B).

Multilocus sequence typing (MLST) is a technique that characterizes isolates based upon combinations of alleles at seven "unlinked" housekeeping loci (15). Of interest was that the *C. jejuni* Turkey and CS isolates were found to belong to the same sequence type (ST-48).

We also examined the gene content of the *C. jejuni* Turkey and CS isolates by a comparative genomic hybridization analysis (genomotyping) using a *C. jejuni* NCTC 11168 DNA microarray. It should be noted that only genes that have a feature represented on the *C. jejuni* NCTC 11168 DNA microarray can be detected using this

method. Moreover, some mutations, including frameshift and point mutations that might be present in one isolate and not the other, would not be identified by this method. With these caveats in mind, we found no observable differences between the signal intensities for the *C. jejuni* Turkey and CS isolates, suggesting that among those genes conserved with *C. jejuni* strain NCTC 11168, that all three isolates have an identical gene content (not shown). However, both the *C. jejuni* Turkey and CS isolates exhibited divergence from NCTC 11168 strain for genes that are contained within the recognized hypervariable regions, including the capsular biosynthesis locus, LOS biosynthesis locus, and the restriction modification locus. To confirm divergence in the LOS locus among the isolates, we used a recently described PCR method (59). Both *C. jejuni* isolates CS and Turkey possess the class B LOS biosynthetic locus (not shown) while strain NCTC 11168 possesses the class C LOS locus.

The C. jejuni *Turkey isolate is more pathogenic for piglets*

Because the *C. jejuni* Turkey and CS isolates were genetically indistinguishable as judged by MRP-PFGE, MLST, and comparative genomic hybridization analysis, but differed significantly in their *in vitro* virulence capabilities, we focused our efforts on these two isolates to decipher the molecular basis for *C. jejuni* pathogenicity. However, we decided to perform *in vivo* experiments to assess the pathogenicity of the *C. jejuni* Turkey and CS isolates prior to comparing the protein and gene expression profiles. Noteworthy is that the infection of piglets with *C. jejuni* has been used previously as a model for *Campylobacter*-mediated enteritis as infected piglets exhibit overt symptoms and histopathology that are characteristic of human infections (4). The *C. jejuni* Turkey

isolate was more pathogenic than the CS isolate for piglets (TABLE 2). Diarrhea was noted in two of the three piglets inoculated with the *C. jejuni* Turkey isolate and none of the piglets inoculated with *C. jejuni* CS isolate. A single Turkey inoculated piglet developed hemorrhage in the colon which was noted upon gross examination. Histological examination of intestinal samples was performed on piglets inoculated with the *C. jejuni* Turkey and CS isolates. Two of the three piglets inoculated with the *C. jejuni* Turkey isolate developed significant degenerative lesions in the small intestine. Also apparent in the small intestines of these two piglets were areas of congestion and cell necrosis. In the colon, the crypts were filled with eosinophilic debris and mucosal erosion was apparent accompanied with a fibrinous exudate. One of the three *C. jejuni* CS inoculated piglets exhibited minor pathological changes. This *C. jejuni* CS-inoculated piglet had congested vessels and slight villus degeneration.

The C. jejuni *Turkey and CS isolates display differences in protein profiles*

To determine if there were differences in the proteins synthesized by the *C. jejuni* Turkey and CS isolates *in vitro*, bacterial whole-cell lysates were analyzed by one and two-dimensional gel electrophoresis and the proteins stained with Coomassie brilliant blue R-250 (CBB-R250) and silver stain (not shown). A minimum of 21 proteins varied in their relative amounts in the whole cell lysate of the *C. jejuni* Turkey isolate versus the whole cell lysate of the CS isolate as judged by two-dimensional electrophoresis coupled with silver staining (not shown). Further, CBB-R250 staining of the two-dimensional gels revealed a predominant band of 62 kDa in the whole cell lysate of the *C. jejuni* Turkey isolate that was not observed in the CS isolate. As *C. jejuni* FlaA monomers are known

to have a M_r of 62 kDa (28), this difference was further investigated. To confirm whether FlaA was indeed synthesized by the *C. jejuni* Turkey isolate and not by the *C. jejuni* CS isolate, bacterial extracts (e.g., whole-cell lysate, outer membrane protein, and flagellar protein) were prepared from both isolates using SDS-PAGE coupled with immunoblot analysis with the flagellin polyclonal antiserum. Again, a 62 kDa immunoreactive band was clearly visible in the various bacterial extracts prepared from the *C. jejuni* Turkey isolate and absent in the extracts prepared using the *C. jejuni* CS isolate (FIG. 3). The identity of the 62 kDa protein was confirmed to be FlaA filament protein by N-terminal sequence analysis. More specifically, the amino-terminal amino acid sequence of the mature protein was determined after SDS-PAGE and electrophoretic transfer to PVDF membranes to be: G F R I N T N V A A L. This sequence corresponds to residues two through 12 of the deduced amino acid of the FlaA protein. Collectively, these data indicate that the FlaA protein is synthesized in the *C. jejuni* Turkey isolate but not in the *C. jejuni* CS isolate.

Decreased expression of flagellar-related transcripts from the C. jejuni *CS isolate*

Based on the differences in the phenotypic properties and protein profiles of the *C. jejuni* Turkey and CS and isolates, we anticipated that there would be differences in the gene expression profiles of the two isolates. From MH broth cultures, total RNA was isolated from the *C. jejuni* Turkey and CS isolates and fluorescently labeled, as it was reverse transcribed to cDNA. The labeled cDNA was used to probe the *C. jejuni* NCTC 11168 DNA microarrays. The expression profiles for the isolates were distinct, showing that 47 genes had significantly reduced transcript levels of two-fold or more in *C. jejuni* CS

isolate versus the Turkey isolate (TABLE 3). Most strikingly, 20 of the 47 genes are related to flagellar biosynthesis and motility and this correlates with the decreased motility of the *C. jejuni* CS isolate. There were also 24 genes for which the transcript levels were increased two-fold or more in the *C. jejuni* CS isolate versus the Turkey isolate (TABLE 4). Eight of these genes have annotated functions involving purine or amino acid biosynthesis. However, the role that the upregulated genes play in the phenotype displayed by the *C. jejuni* CS isolate is not known. To assess the accuracy of the microarray data, real time RT-PCR used performed on four genes that were upregulated (*flaB, flgD, flgE, and flgK*) and three genes that were downregulated (*clpB, metA,* and Cj 0414) in the array experiments. The expression profile of *C. jejuni* Turkey and CS isolates using real time RT-PCR mirrored the microarray results.

Flagellar class II genes are downregulated in C. jejuni CS isolate

Microarray analysis revealed that 20 of the 47 genes expressed at a higher level in the *C. jejuni* Turkey isolate when compared to the CS isolate were associated with flagellar biosynthesis. To elucidate the molecular mechanism associated with disruption of flagellar biosynthesis in the *C. jejuni* CS isolate, we performed real time RT-PCR on eight class I genes (*flhA, flhB, fliF, fliG, fliH, fliI, fliM*, and *fliY*) and eight class II genes (*flaB, flaD, flgB, flgD, flgE, flgG, flgH,* and *flgK*) previously shown to be regulated by σ^{70} and σ^{54}, respectively (10). All class I genes were expressed at a higher level in the *C. jejuni* CS isolate when compared to the *C. jejuni* Turkey isolate. In contrast, the σ^{54} regulated flagellar genes were expressed at a lower level in *C. jejuni* CS isolate when compared to *C. jejuni* Turkey isolate (FIG. 4A). Moreover, *flgG* and *flgH* were also

found to be expressed at a lower level in the *C. jejuni* CS isolate when compared to *C. jejuni* Turkey isolate, even though differential expression of these genes were not detected by microarray analysis.

The σ^{54} regulated flaB *gene is poorly expressed in the* C. jejuni *CS isolate*

Real time RT-PCR indicated that σ^{54} regulated flagellar genes were downregulated in the *C. jejuni* CS isolate. To determine if σ^{54} is active in the *C. jejuni* CS isolate, we performed reporter assays with the pMW10 *lacZ* promoterless shuttle vector (80). The promoter region of the σ^{54} regulated *flaB* and the σ^{70} regulated *metK* genes were cloned upstream of the *lacZ* gene. *C. jejuni* F38011 was chosen as a positive control because we were unable to transform the *C. jejuni* Turkey isolate with a shuttle plasmid. Comparable levels of β-galactosidase activity were noted in the *C. jejuni* F38011 and CS isolates harboring the P*metK*-pMW10 construct. In contrast, the *C. jejuni* CS isolates harboring the P*flaB*-pMW10 construct showed almost no β-galactosidase activity (FIG. 4B) as compared to *C. jejuni* F38011 isolate. This finding further suggests that σ^{54} (RpoN) regulated flagellar genes are not expressed in the *C. jejuni* CS isolate.

To determine why the *flaB* flagellar gene is not expressed in the *C. jejuni* CS isolate, the entire *rpoN* operon (*rpoN,* Cj0667, Cj0668, and Cj0669) was sequenced in the *C. jejuni* Turkey and CS isolates to identify a frame-shift or point mutation. No differences were found in the sequences of the genes contained within the *rpoN* operon of the *C. jejuni* Turkey and CS isolates. Furthermore, real time RT-PCR indicated that all four genes in

the *rpoN* operon of the *C. jejuni* Turkey and CS isolates were expressed at comparable levels.

Identification of point mutations in the flgR *gene of* C. jejuni *CS isolate*

We sequenced the entire ORFs of genes whose product is involved in either flagellar gene regulation or biosynthesis to attempt to identify the molecular mechanism associated with disruption of flagellar biosynthesis in the *C. jejuni* CS isolate. The class I genes sequenced in the *C. jejuni* Turkey and CS isolates were *flhA, flhB, flhF, fliF, fliG, fliH, fliI fliM, fliN, fliP, fliQ*, and *fliR*. We also sequenced *fliA* (σ^{28}) and the genes known to interact or activate σ^{54} including *flgR and flgS.* No nucleotide alterations were noted in the *flhA, flhB, flhF, fliA, fliF, fliG, fliH, fliI, fliM, fliN, fliP, fliQ, fliR*, and *flgS* genes of the *C. jejuni* Turkey and CS isolates. However, a difference was noted in a single nucleotide in the *flgR* gene that resulted in an amino acid change in the *C. jejuni* CS when compared to the Turkey isolate (FIG. 5A). The *flgR* gene encodes the response regulator of the FlgS/FlgR two-component regulatory system associated with the flagellar regulon. The amino acid change resides within in the receiver domain of the FlgR protein.

Complementation of the C. jejuni *CS isolate with a copy of the* flgR *gene from* C. jejuni *NCTC 11168 restores motility*

To determine if FlgR is responsible for the phenotype of the *C. jejuni* CS isolate, the *flgR* gene from *C. jejuni* NCTC 11168 was cloned into the pRY107 shuttle vector and introduced into the *C. jejuni* CS isolate. The *C. jejuni* CS isolate transformed with the *flgR* pRY107 shuttle vector was motile (FIG. 5B). Collectively, these data indicate that a

single nucleotide in the *C. jejuni* CS *flgR* gene, which resulted in an amino acid change, is responsible for the non-motile phenotype displayed by the isolate. Subsequently, we generated a mutation in the *flgR* gene of the *C. jejuni* Turkey isolate. Analysis of this mutant revealed a phenotype that was indistinguishable from the *C. jejuni* CS isolate; the *C. jejuni* Turkey isolate is non-motile and incapable of invading INT 407 epithelial cells (data not shown).

C. jejuni *CS isolate displays greater resistance to sodium deoxycholate than the* C. jejuni *Turkey isolate*

C. jejuni flagellar mutants (i.e., *flgS, flgR*, and *rpoN*) have been shown previously to multiply at a rate three times greater than an isogenic wild-type isolate (81). While we did not find any differences in growth rates of the *C. jejuni* Turkey and CS isolates, the *C. jejuni* Turkey isolate displayed a longer lag phase as compared to the *C. jejuni* CS isolate. Also, the *C. jejuni* CS isolate achieved a greater maximum cell density in MH medium as compared to the *C. jejuni* Turkey isolate (FIG. 6).

Microarray analysis revealed that some genes encoding membrane-associated proteins were expressed at different levels in the *C. jejuni* CS isolate when compared to the *C. jejuni* Turkey isolate. Therefore, we studied the ability of the *C. jejuni* Turkey and CS isolates to resist the antimicrobial activity of the bile salt sodium deoxycholate. Both isolates were exposed to different concentrations of sodium deoxycholate and the cell density achieved at 48 h was measured. The growth of the *C. jejuni* Turkey isolate was significantly inhibited by the presence of 0.05 % and 0.1 % of sodium deoxycholate when

compared to the *C. jejuni* CS isolate (FIG. 7). This finding was reproduced with the *C. jejuni* Turkey isolate when compared to a *C. jejuni* Turkey *flgR* mutant, where the *C. jejuni* Turkey *flgR* mutant was found to be more resistant to deoxycholate. Not known is why the *C. jejuni* Turkey and CS isolates differ in their sensitivity to deoxycholate.

Screening C. jejuni *poultry isolates for non-motile phenotype*

To assess how common non-motile isolates occurs within a population of poultry isolates, we screened a total of 99 isolates to determine their motility phenotype. We identified one additional non-motile *C. jejuni* isolate, designated H8a. Counting the initial six *C. jejuni* isolates used in this study, a total of four non-motile isolates were identified (CS, S2B, S3, and H8a).

To identify the molecular mechanism for the motility defects, we attempted to transform the *C. jejuni* S2B, S3, and H8a isolates with the pMW10 *flaB* promoter construct. Although we were unable to transform the *C. jejuni* H8a isolate with the pMW10 *flaB* construct, negligible beta-galactosidase activity was detected in the S2B and S3 isolates. Upon sequencing the *rpoN* genes from each of these isolates, a thymine base was inserted between bp 1,004 and 1,005 in the *C. jejuni* S2B isolate. This insertion resulted in an early termination codon at bp 1,011. To determine if this frameshift mutation was responsible for the loss in σ^{54} activity, a wild-type copy of the *rpoN* from *C. jejuni* NCTC 11168 was introduced into the *C. jejuni* S2B isolate using the pRY111 shuttle vector. The *C. jejuni* S2B isolate harboring the *rpoN*-pRY111 shuttle vector was motile. The reason why the *C. jejuni* S3 and H8a isolates are non-motile is not known currently.

DISCUSSION

The clinical presentation of campylobacteriosis varies extensively among individuals. Symptoms range from mild diarrhea to severe abdominal pain associated with diarrhea containing blood and leukocytes. In addition, infections in developing countries are more often reported in children with symptoms limited to watery diarrhea while infections in developed countries are associated with more dysentery-like symptoms. Explanations for the variation in the severity of campylobacteriosis include differences in the virulence of *C. jejuni* strains, repeated exposure to the organism, and host susceptibility.

Genetic variation among strains of *Campylobacter* spp. is well documented (2, 16, 42, 61, 64, 65, 71, 79). Dingle *et al.* (15) reported that *C. jejuni* form a weakly clonal population based on the distribution of strains among MLST sequence type complexes. In addition, various groups have shown that strains differ in their genomic content using microarrays. Dorrell *et al.* (16) compared 11 *C. jejuni* strains with NCTC 11168 and found that at least 21% of the NCTC 11168 genome is divergent in one or more of the tested strains. Using a shotgun genomic microarray, Poly *et al.* (65) showed that there was significant variation between *C. jejuni* strains ATCC 43431 and NCTC 11168 strains especially at LOS, capsular biosynthesis and restriction-modification enzyme loci. Comparison of *C. jejuni* strains 81-176 and ATCC 43431 by the same method showed differences at similar loci (64). Finally, comparison of the whole genome sequence of NCTC 11168 and RM1221 identified the presence of phage insertion elements and polymorphisms at the LOS and capsular biosynthesis loci (23). Noteworthy is that both LOS and capsule biosynthesis have been implicated in the pathogenesis of *C. jejuni* (6, 27). Changes in the

poly-G tract of *cgtA* of *C. jejuni* strain 81-176 results in changes to the LOS core structure (27). A *cgtA* mutant in which the LOS mimics that of the GM_3 ganglioside rather than the GM_2 ganglioside demonstrates an increase in invasiveness in cultured epithelial cells. The *kpsM* gene is involved in the expression of the capsular high-molecular-weight glycan and a *C. jejuni* 81-176 *kpsM* isogenic mutant is reduced in invasiveness and ability to cause diarrhea in the ferret model (6).

In addition to strain-to-strain genetic variation, intra-strain variation has been observed in *Campylobacter* spp. For example, MRP-PFGE profile changes were found in five out of six *Campylobacter coli* isolates that had been passed up to 50 times in the laboratory after initial isolation from separate pig herds (58). With respect to *C. jejuni*, Mixter *et al.* (50) observed that genomic changes occurred in *C. jejuni* strain F38011, as determined by SmaI-MRP-PFGE, after intraperitoneal inoculation of mice. Carrillo *et al.* (10) reported that a *C. jejuni* NCTC 11168 strain that had undergone long term passage in the laboratory colonized chickens poorly and was less motile than the original stock strain (10). Although a specific mutation was not identified in a *C. jejuni* NCTC 11168 non-motile variant, microarray analysis revealed an expression profile that was comparable to a *flhA* flagellar biosynthetic mutant. Gaynor *et al.* (24) also found gene expression differences between the genome sequenced variant of *C. jejuni* NCTC 11168 and the original freezer stock; the two strains also showed differences in their ability to colonize chickens.

Virulence-related phenotypic differences between *C. jejuni* strains have been described. Multiple investigators have found differences in the invasion of *C. jejuni* strains into cultured epithelial cells (38, 54). Konkel and Joens (38) showed that the invasion of cultured epithelial cells by *C. jejuni* clinical isolates varied considerably. Overall, clinical isolates were more invasive than those obtained from culture collections (e.g., ATCC, American Type Culture Collection, Manassas, VA). Everest *et al.* (18) found that *C. jejuni* isolates recovered from individuals with colitis were more invasive than those isolated from individuals with non-inflammatory diarrhea. Newell *et al.* (54) observed that *C. jejuni* and *C. coli* isolates recovered from water sources were less invasive than clinical isolates. Variation in the invasion efficiency among *C. jejuni* strains supports the notion that strain-to-strain genetic differences may play a role in the range of clinical symptoms of campylobacteriosis.

In this study, we examined the genotypic and phenotypic characteristics of *C. jejuni* poultry isolates. These isolates differed in both their ability to invade INT 407 cells as well as secretion of virulence proteins. All the isolates found to be non-motile also showed lack of invasion and secretion potential. Conversely, all isolates that were able to secrete virulence proteins were also able to migrate through semi-solid media, which is widely used as an indication of swarming motility. These data are consistent with previously published work demonstrating that the flagellar apparatus is required for the secretion of virulence proteins by *C. jejuni* (39). In addition, motility has been associated with *C. jejuni* pathogenesis both in terms of invasion of epithelial cells (77) and colonization of chickens (78).

We found that the *C. jejuni* Turkey and CS isolates used in this study were indistinguishable by MRP-PFGE, and therefore constituted a clonally-matched pair. MLST confirmed this finding, with both strains belonging to ST-48. Genomotyping using a microarray consisting of ORFs from NCTC 11168 revealed no differences in the genomic content between the *C. jejuni* CS and Turkey isolates. Although clonally indistinguishable, the Turkey and CS isolates differed in their putative pathogenic potential as judged by *in vitro* assays. The *C. jejuni* Turkey isolate secreted virulence proteins, invaded INT 407 epithelial cells at a high level, and was motile, while the *C. jejuni* CS isolate failed to secrete, invaded INT 407 epithelial cells at a low level, and was non-motile. Also, the pathogenic potential of *C. jejuni* isolates CS and Turkey were tested in the neonatal piglet model as described by Babakhani *et al.* (5). In agreement with our *in vitro* assays, piglets inoculated with the *C. jejuni* CS isolate did not develop diarrhea. Taken together, a good correlation exits between the *in vitro* and *in vivo* models with respect to determining an isolate's pathogenic potential. Moreover, the *C. jejuni* Turkey isolate was assessed to be more pathogenic than the CS isolate in all assays.

No detectable differences were noted in the genomic content of the *C. jejuni* Turkey and CS isolates by genomotyping or genomic subtractive hybridization (not shown), indicating that a difference in gene expression might result in the observed phenotypes. The protein profiles of the *C. jejuni* Turkey and CS isolates were examined. Based on the fact that the *C. jejuni* CS isolate was non-motile, it is not surprising that the FlaA flagellin protein was not synthesized in the CS isolate as judged by the absence of a 62 kDa

protein in whole cell extracts, outer membrane protein preparations, and a flagellar extract. The flagellin protein in the *C. jejuni* Turkey isolate, but not the *C. jejuni* CS isolate, was confirmed to be FlaA by N-terminal sequence analysis. Total RNA was also extracted from the *C. jejuni* Turkey and CS isolates and used in an expression microarray to identify genes that differed in their expression. The *C. jejuni* Turkey isolate had significantly higher expression of genes involved in flagellar biosynthesis when compared to the *C. jejuni* CS isolate.

Flagellar biosynthesis in *C. jejuni* is divided into three stages. The expression of class I genes is under the control of σ^{70} and is critical for the expression of class II genes. Class II genes are under the control of σ^{54}. Class III genes are under the control of σ^{28}. In addition to the participation of different sigma factors, the FlgS/R two-component signal transduction system participates in the regulation of the flagellar regulon. Real time RT-PCR analysis of the isolates in this study indicated that all class I genes were upregulated in the *C. jejuni* CS isolate, whereas all the class II genes were downregulated. These findings suggested that a mutation might exist in either a class I flagellar biosynthesis gene (e.g., *flhA*) or a regulator of flagellar biosynthesis (*rpoN, flgR, flgS*). To test this possibility, the ORFs of genes involved in flagellar gene regulation from both isolates were sequenced including *flhA, flhB, flhF, fliA, fliF, fliG, fliH, fliI, fliM, fliN, fliP, fliQ, fliR, rpoN,* Cj0667, Cj0668, and Cj0669. Although we were unable to identify a point mutation in any gene contained within the *rpoN* operon, it was evident from the beta-galactosidase reporter assay that the σ^{54} regulated flagellar genes were not expressed in the *C. jejuni* CS isolate. Since FlgS and FlgR are required for σ^{54} activity, we sequence

the *flgS* and *flgR* open reading frames. Comparison of the open reading frame of the *flgR* gene of *C. jejuni* Turkey and CS revealed a difference in one nucleotide that altered the amino acid sequence. This change in the deduced amino acid sequence proved to be responsible for the non-motile phenotype of the *C. jejuni* CS isolate as indicated by the restoration of motility via complementation of the isolate with a wild-type copy of *flgR* gene on the pRY107 shuttle vector. Hendrixson (29) reported a phase variable mechanism responsible for modulating motility, which in turn helps the bacteria establish a commensal state. FlgR phase variation was achieved by the gain or loss of a nucleotide in homopolymeric adenine or thymine tract. Noteworthy is that the regions that the mutation occurred in the *flgR* gene of the *C. jejuni* CS isolate was not identified previously as a phase-variable region (29).

To assess the frequency of non-motile strains in a population of poultry isolates, we screened a total of 105 isolates and found four non-motile strains (CS, S2B, S3, and H8a). Of the four non-motile isolates recovered from poultry, we identified mutations in two distinct genes involved in flagellar biosynthesis. We were able to restore the motility of both the *C. jejuni* CS and S2B isolates and found a motile revertant of the S3 isolate (not shown). The latter finding indicates that the S3 isolate likely has a single point mutation that alters its motility phenotype. We have sequenced the *rpoN* and *flgR* genes from *C. jejuni* S3 isolate, and did not identify a nucleotide that altered the deduced amino acid sequence from that of the *C. jejuni* NCTC 11168 isolate. Thus, the S3 isolate possesses a mutation in a gene distinct from *rpoN* and *flgR*, indicating that a mutation of a third gene can also lead to a non-motile phenotype. Based on these findings, it appears

that a non-motile phenotype likely offers a selective advantage to *C. jejuni* within the host or environment.

One of the differences noted in the phenotypes of *C. jejuni* Turkey and CS isolates was in their resistance to the bile salt deoxycholate; the *C. jejuni* CS isolates is more resistant to deoxycholate. The difference in sensitivity appears to be specifically associated with the *flgR* mutation as the same observation was noted for the *C. jejuni* Turkey when compared to a *C. jejuni* Turkey *flgR* mutant. It is presently not known why a *flgR* mutant is more resistant to deoxycholate than a wild-type isolate. In *C. jejuni*, the CmeABC multi-drug efflux pump plays a major role in the organism's resistance to detergents, antimicrobials including different classes of antibiotics, and heavy metal-containing salts (43, 44). A *C. jejuni cmeB* mutant is 64-fold more sensitive to sodium deoxycholate when compared to a wild-type strain (44). In contrast to what one might predict, microarray analysis revealed that the *cmeABC* genes were downregulated in the *C. jejuni* CS isolates when compared to the *C. jejuni* Turkey isolate. The reduction in expression of the *cmeABC* genes in the CS isolate was confirmed by real-time RT-PCR. Clearly additional work is needed to understand this phenomenon.

Microarray analysis of the *C. jejuni* CS isolate (*flgR* mutant) and *rpoN* mutant revealed that many more genes were not expressed in a *rpoN* mutant when compared to its isogenic wild-type isolate versus the *C. jejuni* CS isolate when compared to the Turkey isolate (data not shown). Of the nine flagellar-related genes whose expression was lower in the *C. jejuni* F38011 *rpoN* mutant when compared to the F38011 wild-type isolate,

eight of the same nine genes were lower in the *C. jejuni* CS isolate when compared to the *C. jejuni* Turkey isolate. However, a total of 80 genes were downregulated in the defined *rpoN* mutant versus of total of 47 genes in the *C. jejuni* CS isolate. Based on this finding, we propose that FlgR is necessary for the expression of σ^{54} regulated flagellar genes, but that σ^{54} is responsible for the expression of other genes independent of the FlgR response regulator. Additional work is required to determine the genes of the σ^{54} regulon.

The role of motility has been well documented in the pathogenesis of *C. jejuni*. Newell (52) reported that a nonflagellated *C. jejuni* isolate colonized a murine intestinal colonization model poorly and was cleared within seven days post-infection. Nachamkin *et al*. (51) found that only *C. jejuni* isolates with intact and functional flagella colonized the three-day old chick model while Wassenaar *et al*. (78) found that expression of the FlaA flagellin subunit maximized colonization of one-day-old chicks by *C. jejuni.* Consistent with the hypothesis that motility is an important virulence attribute for *C. jejuni*, only motile variants were recovered from human volunteers challenged with a mixture of motile and non-motile variants of *C. jejuni* (8). The results of this study also support the hypothesis that motility is an important *C. jejuni* virulence determinant as it affects an isolate's pathogenicity in the neonatal piglet model of campylobacteriosis. Our findings further underscore the need to assess whether a *C. jejuni* isolate is motile prior to assessing its *in vitro* and *in vivo* virulence potential.

In summary, we have compared the genetically-matched *C. jejuni* Turkey and CS isolates, and found that the differences in the pathogenicity of these two isolates is due to

a point mutation in the *flgR* gene of the *C. jejuni* CS isolate. The *C. jejuni* CS isolate, when compared to the *C. jejuni* Turkey isolate, exhibited a shorter lag phase in MH broth and achieved a greater maximum cell density in MH medium (FIG. 6). Inspection of the data published in Wösten *et al*. (81) also revealed differences in lag phase and maximal cell density of a *flgR* mutant when compared to a wild-type isolate, but the basis of these differences were not analyzed. It is unclear if the increased expression of certain nucleotide and amino acid biosynthesis genes by the *C. jejuni* CS isolate contributes to its higher maximal cell density. Nevertheless, it seems possible that a mutation in *flgR* may provide an organism with a selective advantage in a host (e.g., chicken intestinal tract) because it can reach a higher cell density. The resistance of a *C. jejuni* isolate to the bile salt deoxycholate might provide a selective advantage to the organism in the intestinal tract of both humans and poultry. Finally, this study provides an approach to identify the molecular basis for *C. jejuni* pathogenicity. Moreover, we believe that it will be possible to identify novel *C. jejuni* virulence factors by comparing *C. jejuni* genetically-matched isolates that display differences in virulence potential.

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Table 1. Binding and internalization of *C. jejuni* poultry

Isolates	Origin	No. cell-associated bacteria[a]	No. internalized bacteria[b]	(I/A) X 100[c]
81-176	human	$(3.0 \pm 0.8) \times 10^5$	$(2.2 \pm 0.3) \times 10^4$	7.3
CS	chicken	$(5.8 \pm 1.0) \times 10^5$	$(5.0 \pm 1.8) \times 10^2$	0.09
S1	chicken	$(3.1 \pm 0.7) \times 10^4$	$(9.3 \pm 1.5) \times 10^2$	3.0
S2B	chicken	$(3.5 \pm 0.4) \times 10^5$	$(1.3 \pm 0.5) \times 10^2$	0.04
S3	chicken	$(3.9 \pm 0.4) \times 10^5$	$(9.0 \pm 2.0) \times 10^1$	0.02
SC	chicken	$(1.6 \pm 0.2) \times 10^5$	$(1.5 \pm 0.4) \times 10^3$	0.9
Turkey	turkey	$(1.2 \pm 0.2) \times 10^5$	$(4.3 \pm 0.5) \times 10^3$	3.6
E. coli XL1-Blue MRF'	Stratagene	$(3.3 \pm 1.0) \times 10^5$	$(2.1 \pm 0.4) \times 10^2$	0.07

[a] Number of bacteria per well of *C. jejuni* inoculated monolayers.

[b] Number of bacteria per well of *C. jejuni* inoculated monolayers treated with gentamicin for 3 h.

[c] (I/A) X 100 indicates the percentage of cell-associated bacteria that are internalized.

Table 2. The *C. jejuni* Turkey isolate is more virulent than the CS isolate in the neonatal piglet model

	C. jejuni strain	
Clinical Symptom	Turkey	CS
No. piglets with diarrhea	2/3	0/3
Onset of diarrhea	24 h	not detected
Death (pre-necropsy)	0/3	0/3
Hemorrhage	1/3	0/3

Table 3. Transcripts detected in lower levels in the *C. jejuni* CS isolate when compared to the *C. jejuni* Turkey isolate

Gene	Fold Change	Description
Cj0040[a,b]	4.30	Conserved hypothetical protein
Cj0041[a,b]	3.01	Conserved hypothetical protein
flgD[a,b]	6.55	Flagellar hook assembly protein
flgE[a,b]	6.52	Flagellar hook protein
Cj0044c[a]	2.11	Conserved hypothetical protein
Cj0045c[a]	2.60	Conserved hypothetical protein
aspA	2.52	Aspartate ammonia-lyase
dcuA	2.57	C4-dicarboxylate transporter, anaerobic
Cj0168c	3.50	Hypothetical protein
Cj0256	2.37	Conserved hypothetical integral membrane protein
serB	2.30	3-phosphoserine phosphatase
chew[a]	2.92	Purine-binding chemotaxis
cheA[a]	2.75	Chemotaxis protein
cheV[a]	2.84	Chemotaxis protein
cmeC	3.69	RND efflux system, outer membrane lipoprotein
cmeB	4.40	RND efflux system, inner membrane transporter
cmeA	4.01	RND efflux system, membrane fusion protein
Cj0391c[a,c]	2.77	Conserved hypothetical protein
flaD[a,b]	2.74	Flagellin family protein
Cj0420	3.52	Conserved hypothetical protein
flgB[a]	2.05	Flagellar basal body rod protein
flaG[a,b]	2.06	Possible flagellar protein
fliD[a,c]	2.48	Flagellar hook-associated protein 2
Cj0561c	5.97	Conserved hypothetical periplasmic protein

*peb*4	2.14	Cell binding factor 2 precursor, major antigenic peptide
fba	2.78	Fructose-bisphosphate aldolase
*peb*2	2.25	Peb2 accessory colonization factor AcfC (acfC)
Cj0805	2.08	Putative peptidase/protease (M16 family)
dapA	2.98	Dihydrodipicolinate synthase
Cj0807	2.18	Putative short chain dehydrogenase
flgI [a,b]	5.63	Flagellar P-ring protein
Cj1463 [a]	2.94	Conserved hypothetical protein
flgM [a,c]	2.00	Conserved hypothetical protein
Cj1465 [a]	2.07	Conserved hypothetical protein
flgK [a,b]	3.02	Flagellar hook-associated protein
Cj1656c	5.78	Unknown
leuA	2.36	2-isopropylmalate synthase

[a] Indicates flagellar biosynthesis-associated gene.

[b] Indicates σ^{54} regulated gene.

[c] Indicates σ^{28} regulated gene.

Table 4. Transcripts detected in greater levels in the *C. jejuni* CS isolate when compared to the *C. jejuni* Turkey isolate

Gene	Fold Change	Description
Cj0091	2.05	Conserved hypothetical lipoprotein
Cj0203	2.04	Unknown
Cj0264c	2.04	Unknown
Cj0265c	2.19	Unknown
Cj0413	2.03	Probable periplasmic protein
Cj0414	5.34	Conserved hypothetical protein
Cj0415	4.50	Putative gluconate dehydrogenase
Cj0444	2.22	Unknown
thiC	2.01	Thiamine biosynthesis protein
Cj0501	2.61	Unknown
clpB	2.60	ATP-dependent Clp protease, ATP-binding subunit
hypB	2.10	Hydrogenase expression/formation protein
Cj0653c	2.37	Aminopeptidase (M24 family)
Cj0654c	3.02	Unknown
glnA	2.49	Glutamine synthetase
putP	2.04	Sodium/proline symporter
Cj1514c	2.01	Conserved hypothetical protein
nuoC	2.30	NADH-ubiquinone oxidoreductase, subunit C
nuoB	2.32	NADH-ubiquinone oxidoreductase, subunit B
hisG	2.53	ATP phosphoribosyltransferase
hisD	2.28	Histidinol dehydrogenase
hisH	2.35	Glutamine amidotransferase
hisA	2.19	Imidazolecarboxamide isomerase
metA	2.96	Homoserine O-succinyltransferase
metB	3.57	O-acetylhomoserine sulfhydrylase

Figure 1. Some *C. jejuni* isolates recovered from poultry do not secrete the Cia proteins. *C. jejuni* isolates 81-176 (Lane 1), CS (Lane 2), S1 (Lane 3), S2B (Lane 4), S3 (lane 5), SC (Lane 6), and Turkey (Lane 7) were incubated in MEM minus methionine containing 1% fetal bovine serum and labeled with [^{35}S]-methionine for 3 h. The supernatant fluids were analyzed by SDS-PAGE and autoradiography as outlined in "Materials and Methods". Molecular mass standards, in kDa, are indicated on the left.

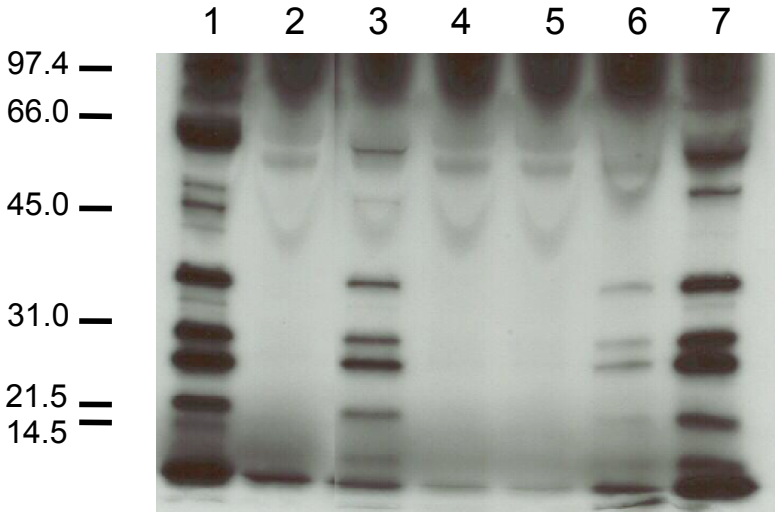

1
2
3
4
5
6
7
97.4
66.0
45.0
31.0
21.5
14.5

Figure 2. Macrorestriction enzyme pulsed field gel electrophoresis profiles of *C. jejuni* isolates. Lane 1 in panels A and B contains a PFGE lambda ladder (New England Biolabs, Beverly, MA). The sizes of the molecular mass markers are shown on the left of each panel in kilobase pairs. Panel A: MRP-PFGE of SmaI digested DNA from 81-176 (lane 2), CS (lane 3), S1 (lane 4), S2B (lane 5), S3 (lane 6), SC (lane 7), and Turkey (lane 8). Panel B: MRP-PFGE of SalI digested DNA from 81-176 (lane 2), Turkey (lane 3), and CS (lane 4).

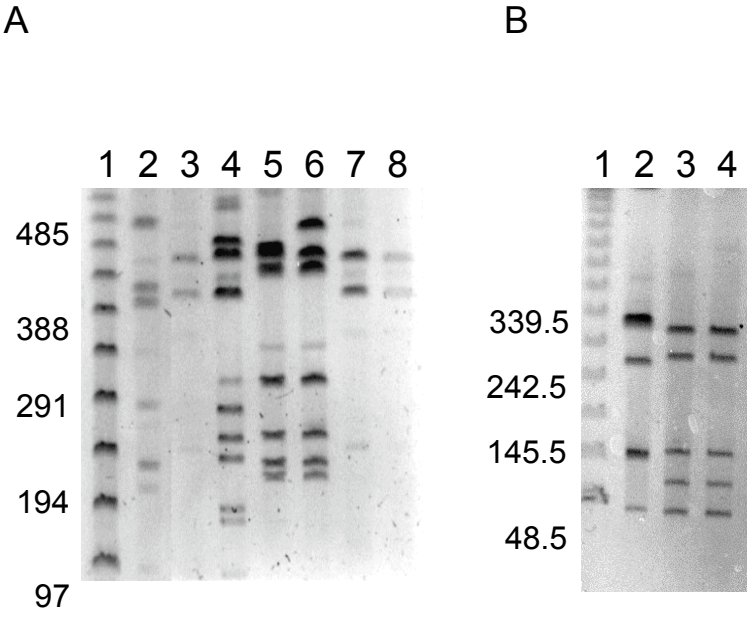

A
B
1 2 3 4 5 6 7 8
485
388
291
194
97
1 2 3 4
339.5
242.5
145.5
48.5

Figure 3. Electrophoretic and immunoblot analysis of *C. jejuni* Turkey (T) and CS whole cell lysates (WCL), outer membrane protein (OMP), and flagellin preparations. Proteins were separated in SDS-12.5% polyacrylamide gels and either stained with Coomassie brilliant blue R-250 (panel A) or transferred to polyvinylidene fluoride membranes and reacted a 1:250 dilution of a *C. jejuni* α–flagellin serum that contains antibodies reactive with both the FlaA and FlaB proteins (panel B). The arrows indicate the FlaA flagellar protein. Molecular mass standards, in kDa, are indicated on the left**.**

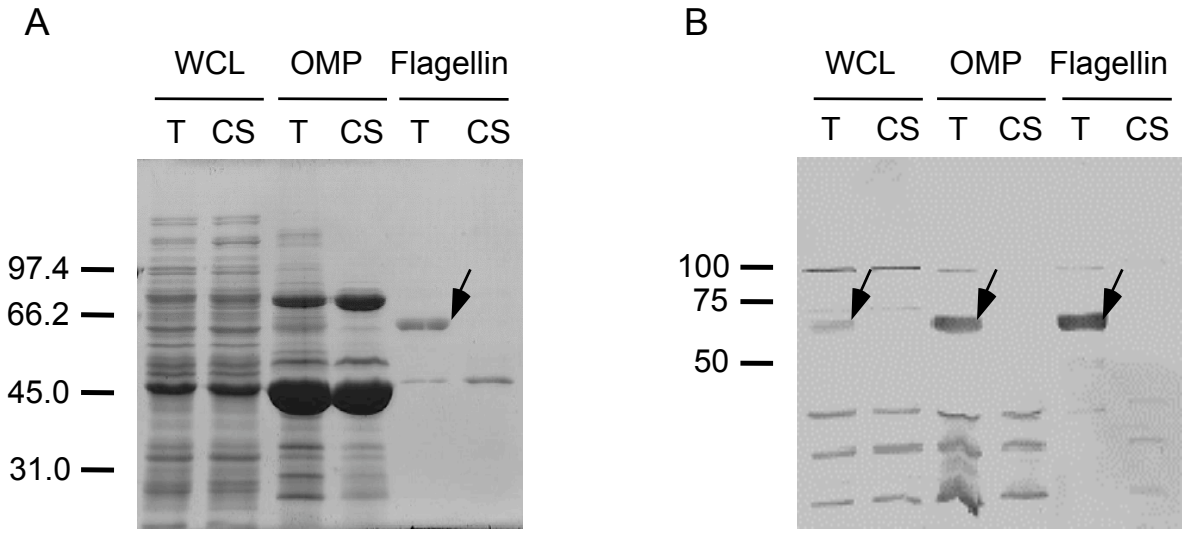
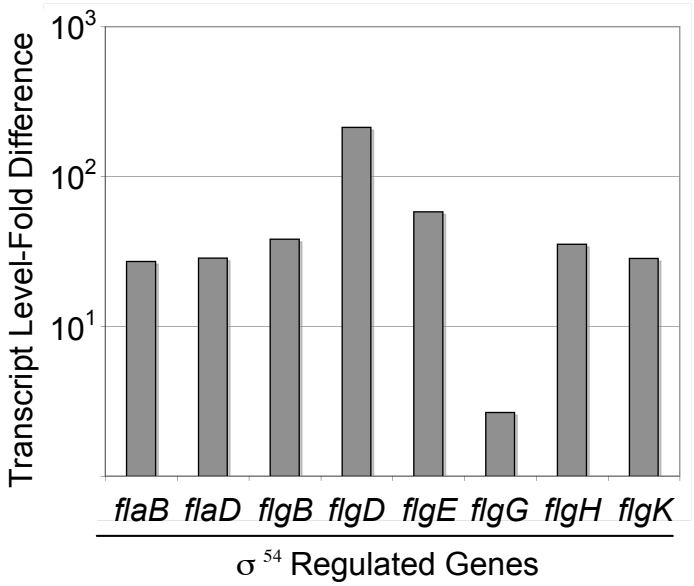

A
WCL
OMP
Flagellin
T
CS
T
CS
T
CS
97.4
66.2
45.0
31.0
B
WCL
OMP
Flagellin
T
CS
T
CS
T
CS
100
75
50

Figure 4 A. The transcript levels of σ^{54} regulated genes in the *C. jejuni* Turkey isolate are increased relative to that of the *C. jejuni* CS isolate. The *flaB, flaD, flgB, flgD, flgE, flgG, flgH,* and *flgK* transcripts were measured by real time RT-PCR using total RNA extracted from the *C. jejuni* CS and Turkey isolates grown to mid-log phase in MH broth. The fold change was measured using the comparative C_T method, where *glnA* was used as the internal control and ΔC_T of the *C. jejuni* CS isolate was used as the calibrator.

Figure 4 B. The *flaB* gene, which is σ^{54} regulated, is not expressed in the *C. jejuni* CS isolate. β-galactosidase activity in Miller units was measured from the *C. jejuni* CS and F38011 isolates harboring the P*metK*-pMW10 and P*flaB*-pMW10 constructs. The bacteria were grown in MH broth for 16 h. The values are averages of three independent experiments, with the error bars representing the standard deviations.

A


Transcript Level-Fold Difference
10^3
10^2
10^1
flaB flaD flgB flgD flgE flgG flgH flgK
σ 54 Regulated Genes

B

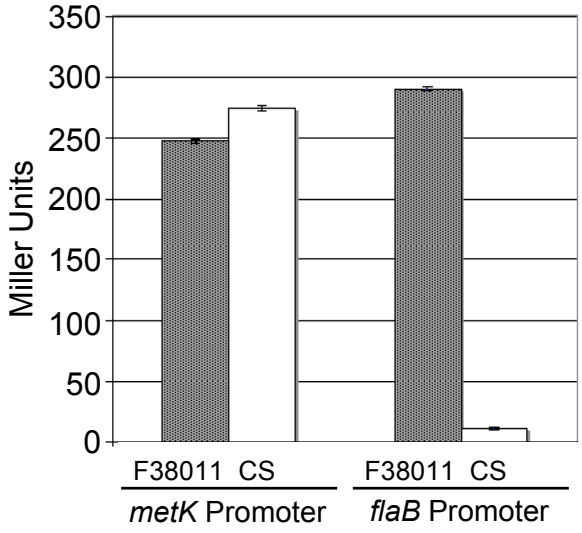
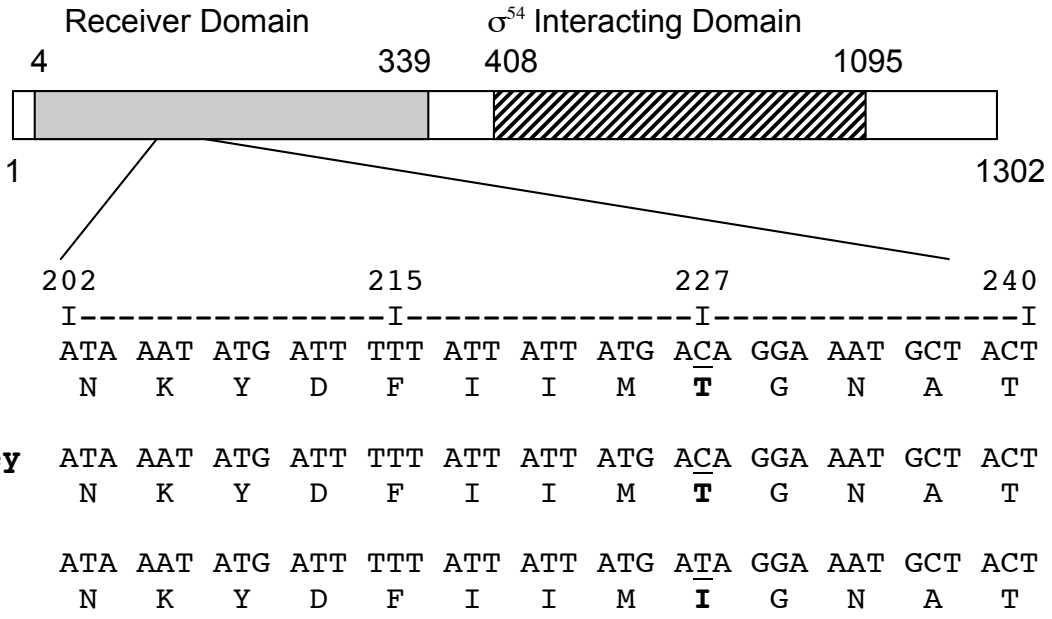

Miller Units
350
300
250
200
150
100
50
0
F38011 CS
F38011 CS
metK Promoter
flaB Promoter

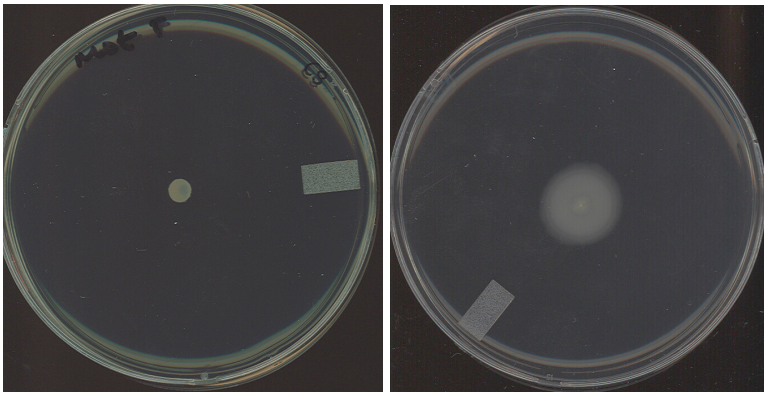
Figure 5 A. Multiple sequence alignment of the *flgR* gene from *C. jejuni* 11168, Turkey, and CS isolates. Shown is a segment of the nucleotide sequence of the *flgR* gene and its predicted translation from *C. jejuni* strains NCTC 11168 (11168), Turkey (Turkey), and CS (CS). The FlgR receiver and σ^{54} interacting domains are indicated. Also highlighted (underlined) is the residue that differed in the *C. jejuni* CS isolate when compared to the Turkey isolate.

Figure 5 B. Complementation of the *C. jejuni* CS isolate with a wild-type copy of *flgR* gene restores the isolate's motility. Both the *C. jejuni* CS wild-type and CS *flgR* pRY107 transformed isolates were spotted onto MH plates supplemented with 0.4% agar and motility assessed after 48 h of incubation.

A



B



C. jejuni CS isolate

C. jejuni CS isolate complemented with pRY107 *flgR* construct

Figure 6. Growth of the *C. jejuni* Turkey and CS isolates in MH broth. MH broth was inoculated with the *C. jejuni* Turkey and CS isolates to an OD_{540} of 0.01, and the cultures incubated at 37°C, 150 rpm, under microaerobic conditions. The points (closed circles and squares) represent the time points at which the optical density of each bacterial culture was determined at a wavelength of 540 nm. The closed circles represent the *C. jejuni* CS isolate and the closed squares represent the *C. jejuni* Turkey isolate.

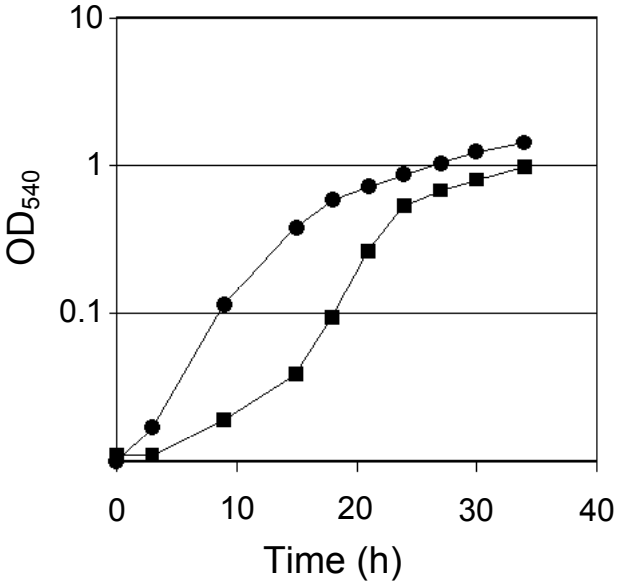

10
1
0.1
OD_{540}
0
10
20
30
40
Time (h)

Figure 7. The *C. jejuni* CS isolate and the *C. jejuni* Turkey *flgR* mutant display greater resistance to sodium deoxycholate than the *C. jejuni* Turkey isolate. The *C. jejuni* CS isolate (open bar), *C. jejuni* Turkey isolate (gray bar), and *C. jejuni* Turkey *flgR* mutant (black bar) were incubated in MH broth with 0.1%, 0.05%, 0.025%, and 0% sodium deoxycholate under microaerobic conditions for 48 h at 37°C with shaking. The Y-axis represents the optical density of bacterial culture relative to the growth achieved without deoxycholate (0% sodium deoxycholate). The error bars represent the standard deviations.

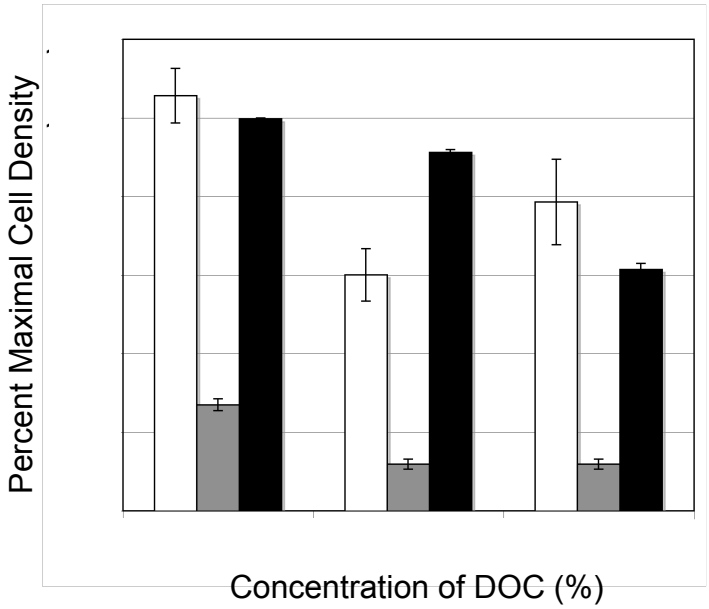
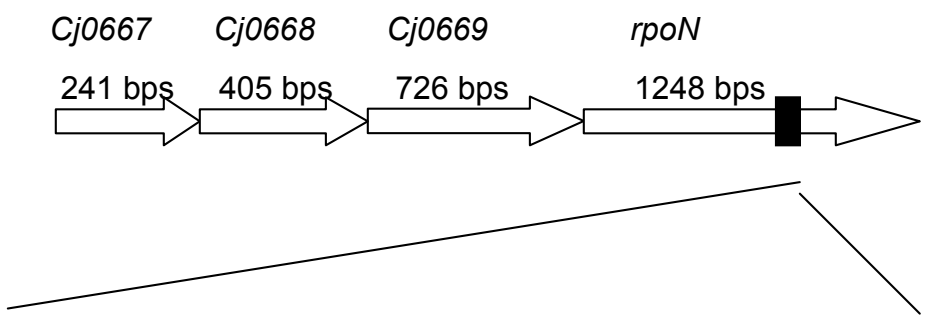

Percent Maximal Cell Density
Concentration of DOC (%)

Figure 8 A. The *rpoN* operon of the *C. jejuni* S2B isolate was sequenced and a single thymine base insertion was identified between bp 1,004 and 1,005. This base insertion results in a termination codon at bp 1,011 as apposed to the normal termination codon at bp 1,248. Shown is a segment of the nucleotide sequence of the *rpoN* gene and its predicted translation from the *C. jejuni* NCTC 11168 (11168) and S2B (S2B) isolates. Also shown (bottom) is the number of bp from the start codon of *rpoN*. The (*) indicates a termination codon.

Figure 8 B. Complementation of the *C. jejuni* S2B strain with a wild-type copy of *rpoN* restored the motility phenotype. The *C. jejuni* S2B wild-type and S2B *rpoN* pRY111 transformed isolates were spotted onto MH plates supplemented with 0.4% agar and motility assessed after 48 h of incubation.

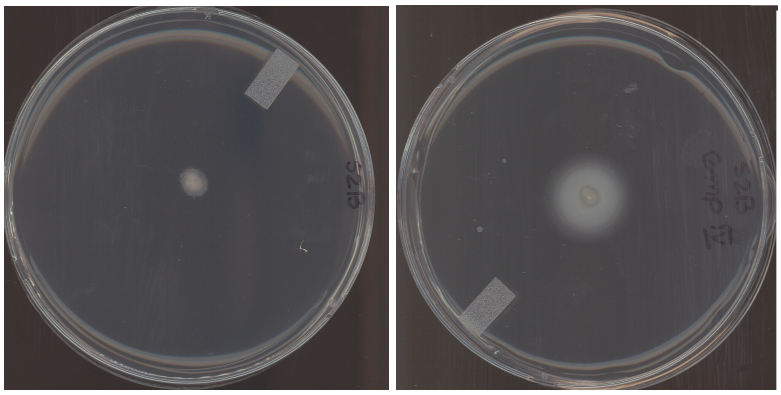
A




```
          982        990           1000          1011          1250
           I---------I-------------I-----------I-----------I
11168      TGT GAA AGA GGA CTT ATT CCC TTG CGT GAT.........TAA
            C   E   R   G   L   I   P   L   R   D .........*

S2B        TGT GAA AGA GGA CTT ATT CCC TTT GCG TGA
            C   E   R   G   L   I   P   F   A   *
```

B



C. jejuni S2B isolate

C. jejuni S2B isolate complemented with pRY111 *rpoN* construct

CHAPTER THREE

Culture of *Campylobacter jejuni* with Sodium Deoxycholate induces Virulence Gene Expression

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ABSTRACT

C. jejuni, a spiral-shaped gram-negative bacterium, is a leading bacterial cause of human foodborne illness. Acute disease is associated with *C. jejuni* invasion of the intestinal epithelium. Further, maximal host cell invasion requires the secretion of proteins termed *Campylobacter* invasion antigens (Cia). As bile acids are known to alter the pathogenic behavior of other gastrointestinal pathogens, we hypothesized that the virulence potential of *Campylobacter* may be triggered by the bile acid deoxycholate (DOC). In support of this hypothesis, culturing *C. jejuni* with a physiologically relevant concentration of DOC significantly altered the kinetics of cell invasion as evidenced by gentamicin-protection assays. In contrast to *C. jejuni* harvested from Mueller-Hinton (MH) agar plates, *C. jejuni* harvested from MH agar plates supplemented with DOC demonstrated Cia secretion as judged by metabolic labeling experiments. DOC was also found to induce the expression of the *ciaB* gene as judged by β-galactosidase reporter assays, real-time RT-PCR and microarray analysis. Further, microarray analysis revealed that DOC induced the expression of virulence genes (i.e., *ciaB, cmeABC, dccR,* and *tlyA*). In summary, we demonstrate that it is possible to enhance the pathogenic behavior of *C. jejuni* by modifying the culture conditions. These results provide a foundation to identify genes expressed by *C. jejuni* in response to *in vivo*-like culture conditions.

INTRODUCTION

Campylobacter jejuni is recognized as one of the leading bacterial causes of gastrointestinal disease in humans (1, 2). An estimated 2.4 million persons are infected by *C. jejuni* each year in the United States. Infection with *C. jejuni* results in symptoms that range from mild watery diarrhea to more severe diarrhea with blood and leukocytes. The most notable complication of campylobacteriosis is the development of Guillain-Barré syndrome (GBS), an acute demyelinating polyneuropathy. Approximately 1 in 1000 diagnosed *C. jejuni* infections result in Guillain-Barré syndrome (6).

Bile is a digestive secretion that plays a major role in fat dispersion and absorption. Approximately 50% of organic bile consists of bile acids, which are synthesized in the liver from cholesterol by a multi-enzyme process. Bile acids, including cholates and deoxycholates, are amphipathic molecules that act as detergents and possess potent antimicrobial activity. The average concentration of bile acids in the human intestine ranges from 0.2 to 2%, of which deoxycholic acid constitutes about 15% (7).

Bile has been shown to regulate virulence gene expression in several gastrointestinal pathogens (5, 12, 32-35, 38-40, 46). For example, *Shigella* spp. grown in the presence of bile show an increase in the secretion of invasion plasmid antigens (Ipa) and enhanced invasion potential (36). Specifically, deoxycholate (DOC) stimulates the localization of IpaB to the tip of the type-three secretion apparatus needle where IpaB, in association with IpaD, is hypothesized to act as a sensor of host cell contact (32). In *V.*

parahaemolyticus, bile acids enhance the production of thermostable direct hemolysin (33, 34), capsule, and adherence to epithelial cells (35).

The ability of *Campylobacter* to cause illness is related to its ability to invade epithelial cells lining the intestinal tract (3, 9, 13, 48, 51). The proteins known to promote entry of the bacteria into eukaryotic cells are different from those that facilitate binding (15). In contrast to cellular adhesion, *C. jejuni* must be metabolically active to invade human epithelial cells. *C. jejuni* synthesizes and secretes a set of proteins when cultured with epithelial cells that are required for maximal invasion of host epithelial cells (15, 16, 18, 44, 45). These proteins are termed *Campylobacter* invasion antigens (Cia). The secretion of the Cia proteins is dependent on a functional flagellum, indicating a dual function of this organelle in motility and as a type III secretion system (T3SS) (17). To date only one Cia, termed CiaB, has been identified. In contrast to a *C. jejuni* wild-type isolate, a *ciaB* null mutant is impaired in host cell invasion and is secretion deficient (44). Further, the severity and time of onset of disease in piglets inoculated with a *C. jejuni ciaB* null mutant is retarded when compared with a *C. jejuni* wild-type isolate. Piglets inoculated with a *C. jejuni ciaB* null mutant develop diarrhea 3 days post inoculation whereas piglets inoculated with a *C. jejuni* wild-type isolate develop diarrhea within 24 h (19).

Although a number of studies highlight the resistance mechanism of *Campylobacter* to bile (20, 21, 24, 41), little is known about the effect of bile on *Campylobacter* virulence determinants. Bile acids, including DOC, cholate and chenodeoxycholate, have

previously been shown to induce the synthesis of the Cia proteins (44). This study was undertaken to determine the role bile plays in the temporal expression of *ciaB* and its effect on *Campylobacter* pathogenesis. More specifically, we studied the effect of a physiologically relevant concentration of DOC on *Campylobacter* invasion potential, which is an important virulence determinant and contributes to the development of severe disease. Here we demonstrate that in comparison to bacteria grown on MH agar, *C. jejuni* grown in the presence of DOC show: 1) an increase in the kinetics of host cell invasion; 2) an increase in *ciaB* gene expression; and 3) an alteration in the expression of genes that play a role in *Campylobacter* pathogenesis. In summary, we demonstrate that it is possible to enhance the pathogenic behavior of *C. jejuni* in the laboratory by culturing them under conditions that resembles the *in vivo* environment.

MATERIALS AND METHODS

Bacterial strains and growth conditions

C. jejuni F38011 was recovered from an individual with clinical signs of campylobacteriosis. The *C. jejuni* F38011 strain was cultured on Mueller-Hinton (MH) agar plates supplemented with 5% bovine citrated blood (MH-blood) under microaerobic conditions (5% O_2, 10% CO_2, 85% N_2) at 37ºC, and subcultured to a fresh plate every 48 h. MH-blood agar plates were supplemented with 200 μg/ml of kanamycin (Kan) when appropriate. Where indicated, *C. jejuni* was cultured on MH agar plates and MH agar plates supplemented with 0.1% sodium deoxycholate (DOC; Sigma, St. Louis, MO) (1 mg/ml). *E. coli* INVαF' (Invitrogen, Carlsbad, CA) were cultured in Luria-Bertani (LB) broth and on LB agar plates at 37ºC. LB plates were supplemented with 50 μg/ml Kan when appropriate.

Tissue Culture

Stock cultures of INT 407 epithelial cells (Human embryonic intestinal; ATCC CCL 6) were grown in Minimal Essential Medium (MEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone Laboratories Inc., Logan, UT) and maintained at 37ºC in a humidified, 5% CO_2 incubator.

Binding Assay

For experimental assays, a 24-well tissue culture tray was seeded with 2 x 10^5 INT 407 cells per well and incubated for 18 h at 37ºC in a humidified, 5% CO_2 incubator. *C. jejuni* F38011 was cultured on MH and MH agar supplemented with 0.1% sodium

deoxycholate (MHD) plates for 18 h. The bacteria were harvested in MEM supplemented with 1% FBS and washed twice. The bacterial suspension was adjusted to an OD_{540} of 0.2. Ten-fold serial dilutions of the initial suspension were used to inoculate INT 407 cells as described previously (29). Briefly, the INT 407 cells were washed with MEM and 1 ml of a bacterial suspension was added to each well. The bacteria were centrifuged at 600 x *g* to facilitate bacteria-host cell interaction. After 1 h incubation, the non-adherent cells were removed by rinsing with PBS. The INT 407 cells were lysed with 0.1% Triton X-100 solution in PBS. The suspensions were 10-fold serially diluted and the number of viable, adherent bacteria determined by counting the resultant colonies on MH-blood agar plates. The initial inoculums were also plated to determine multiplicity of infection (MOI). The reported values represent the mean counts $\pm$ standard deviations derived from quadruplicate wells. The assay was repeated 3 times to ensure reproducibility.

Secretion Assay

C. jejuni F38011 was cultured for 18 h on MH agar and MHD agar plates. The bacteria were harvested in MEM without methionine (labeling medium; ICN Biomedicals, Inc., Aurora, OH), pelleted by centrifugation at 6,000 x *g* and washed twice in MEM. For metabolic labeling, approximately 5 x 10^8 colony forming units (CFU) were suspended in MEM without methionine. Six ml of the bacterial suspension was then used for labeling with [^{35}S]-methionine (Perkin Elmer Life Sciences, Inc., Boston, MA) at a concentration of 50 µCi/ml (15). Both cultures were incubated for 30 min at 37ºC under microaerobic conditions to allow incorporation of [^{35}S]-methionine. To each bacterial suspension

(harvested from MH and MHD agar plates), chloramphenicol (Cm) was added at a final concentration of 128 μg/ml. The flasks were then incubated for 30 min at 37ºC under microaerobic conditions. The suspensions were divided into two flasks and incubated with and without 1% FBS (Hyclone Laboratories Inc., Logan, UT) for 30 minutes at 37ºC under microaerobic conditions to stimulate *C. jejuni* protein secretion. Prior to each secretion assay, the albumin was removed from the FBS using a SwellGel Blue Albumin Removal Kit (Pierce, Rockford, IL). Following an additional 30 min incubation, the supernatant fluids were harvested and samples were processed as described previously (15). The bacterial pellets were resuspended in water and mixed with an equal volume of double strength sample solubilization buffer. The secreted proteins and whole cell lysates (WCL; OD_{540} equivalent = 0.1) were electrophoretically transferred to polyvinylidene fluoride (PVDF) membranes (Immobilon P; Millipore Corp., Bedford, MA). The membranes were exposed to phosphorimaging screens to detect secreted proteins and WCL proteins.

The *C. jejuni* secreted proteins and WCL were also probed with goat α-CadF serum. Membranes were washed 3 times in PBS and incubated for 18 h at 4°C with a 1:500 dilution of the goat α-*C. jejuni* CadF antibody in PBS pH 7.4/0.01% Tween-20 containing 5% dried milk. Bound antibodies were detected using peroxidase-conjugated rabbit anti-goat IgG (Sigma; St. Louis, MO) at a 1:1,000 dilution and 4-chloro-1-napthol (Sigma) as the chromogenic substrate.

Internalization kinetics

The wells of the 24-well tissue culture trays were seeded with INT 407 cells as described for the binding assay. For the internalization assay, each well of the tray was inoculated with 1 ml of a bacterial suspension. The tissue culture tray was centrifuged at 600 x *g* for 5 min and incubated at 37ºC in a humidified, 5% CO_2 incubator for various periods of time. After 15, 30, 60, 90, and 180 min of incubation, the *C. jejuni* infected-INT 407 cells were washed with MEM and incubated with MEM supplemented with 1% FBS containing gentamicin at a concentration of 250 µg/ml. After 3 h of incubation, the cells were lysed and the number of internalized bacteria determined as outlined above.

*Construction of P*ciaB-*pMW10 and P*porA-*pMW10 reporter vector*

The *ciaB* and *porA* promoter regions were PCR amplified from the *C. jejuni* NCTC 11168 strain using primers designed with a BglII and BamHI site, respectively, at the 5' end and cloned into pCR2.1 using the TOPO® TA Cloning Kit (Invitrogen, Carlsbad, CA). The ligated vectors were electroporated into *E. coli* INVαF' and the transformants were selected on LB agar supplemented with Kan (50 µg/ml). The P*ciaB*-pCR2.1 and P*porA*-pCR2.1 vectors were digested with BglII and BamHI to give a 568 bps and 377 bps fragments, respectively. The fragments were gel purified using the QIAquick gel extraction kit (Qiagen, Valencia, CA). The promoter shuttle vector pMW10 (52) was digested with BamHI, gel purified, and ligated with the BglII P*ciaB* and P*porA* fragments. The ligation mixtures were electroporated into *E. coli* INVαF'. Transformants were selected on LB agar supplemented with Kan using blue white screening. Positive clones were sequence verified using gene specific primers as

described elsewhere (52). The P*ciaB*-pMW10 and P*porA*-pMW10 reporter vectors were electroporated into *C. jejuni* F38011 and the resultant colonies were screened on MH-blood agar plates with Kan (200 μg/ml). The presence of each vector was confirmed by PCR using primers designed to amplify a portion of the *aphA-3* kanamycin gene.

β-galactosidase assay

To determine the level of *ciaB* and *porA* promoter activities in the presence and absence of DOC, overnight cultures of the *C. jejuni* F38011 harboring the P*ciaB*-pMW10 and P*porA*-pMW10 constructs were subcultured in MH broth at an OD_{540} of 0.05. The cultures were then incubated and allowed to reach an OD_{540} of 0.2. Five ml of the log phase cultures were then used to inoculate MH and MHD agar plates. The plates were incubated for 9, 12, 15 and 20 h. At each time-point the bacteria were harvested in cold PBS and washed twice. The β-galactosidase assays were performed as described previously (28). To determine the effect of DOC concentration on the promoter activities of *ciaB* and *porA*, 5 ml log phase cultures were used to inoculate MH agar and MH agar plates supplemented with 0.05%, 0.1%, 0.2% and 0.4% DOC. The plates were incubated for 15 h after which the bacteria were harvested in cold PBS, washed twice and β-galactosidase assays were performed.

RNA Isolation

Total cellular RNA was isolated from *C. jejuni* F38011 cultured on MH and MHD agar plates at 3, 6, 9, 12, 15 h using the RiboPure™-Bacteria Kit (Ambion, Austin, TX) according to the manufacturer's instructions. The extracted RNA was treated twice with

DNase at 37ºC for 30 min to remove genomic DNA. The absence of genomic DNA was confirmed via PCR using *C. jejuni ciaB* gene-sequence specific primers (CiaB-F: CTATGCTAGCCATACTTAGGC and CiaB-R: GCCCGCCTTAGAACTTAC).

Real-Time RT-PCR

To determine the temporal expression of *ciaB,* real-time quantitative reverse transcription-PCR (real-time RT-PCR) was performed on RNA isolated from the *C. jejuni* F38011 strain cultured on MH agar and MHD agar plates for 3, 6, 9, 12, and 15 h. The cDNA was synthesized using the ThermoScript™ RT-PCR system (Invitrogen, Carlsbad, CA) according to the manufacturer's directions using 500 ng of RNA. Real-time RT-PCR amplification of 2.5 µl of cDNA (1:100 dilution) was performed in a reaction mix containing Power SYBR® Green PCR master mix (Applied Biosystems, Foster city, CA), 300 nM concentration of forward and reverse primers and diethylpyrocarbonate-treated water. Real-time RT-PCR analysis was performed using Gene Amp 7000 thermocycler (Applied Biosystems), with PCR parameters of 2 min at 50°C, 40 cycles of denaturation at 95°C for 15 s, and annealing at 55°C for 1 min. Threshold (C_t) values were determined using Prism SDS software version 1.0 (Applied Biosystems). The comparative threshold cycle ($\Delta\Delta C_t$) method was used to calculate fold change where samples were normalized to *glyA*, as this is a housekeeping gene that is not differentially expressed in the presence of DOC. We also performed real-time RT-PCR analysis to determine the fold change in *porA* in the presence of DOC as a control. The primer sets used for the analyses are indicated in Table 1. Reactions were done in duplicate and three biological replicates were performed for each sample.

Microarray analysis

For the expression profiling arrays, an indirect comparison of gene expression was performed, where the expression profile of the *C. jejuni* F38011 cultured in the presence and absence of DOC was measured separately on different slides as described previously (26). Briefly, Cy5 labeled reference DNA from the *C. jejuni* F38011 strain was mixed with Cy3 labeled test cDNA (*C. jejuni* F38011 cultured in the presence and absence of DOC) and hybridized to the *Campylobacter* cDNA array (26) on separate slides. DNA microarrays were scanned using an Axon GenePix 4000B microarray laser scanner (Axon Instruments, Union City, CA) and the data for spot and background intensities were processed using the GenePix 4.0 software.

Microarray data analysis

Normalized data that passed the quality controls were analyzed using GENESPRING 7.3 software (Silicon Genetics, Palo Alto, CA). For the comparison of genes differentially expressed in the presence and absence of DOC, at least four hybridization measurements were generated per biological experiment (two technical replicate arrays and two replicate features per array) and the experiment was repeated two times (biological replicate). Significance of the centered data at $P \leq 0.05$ was determined using a parametric-based statistical t-test adjusting the individual P value with the Benjamini and Hochberg false discovery rate multiple test correction within the GeneSpring analysis package.

The microarray results were confirmed by real-time RT-PCR analysis. The genes upregulated in the presence of DOC were categorized into functional classes as described on the Sanger center website (http://www.sanger.ac.uk/Projects/C_jejuni/). Genes from each functional class (19 genes total) were selected and the fold change in gene expression of *C. jejuni* F38011 strain cultured in the presence and absence of DOC was confirmed using real-time RT-PCR analysis as described previously. The primer sets used for the analyses are indicated in Table 1.

RESULTS

Culture with sodium deoxycholate does not alter adherence or motility of C. jejuni

To determine if DOC affects the ability of *C. jejuni* to adhere to INT 407 cells, a binding assay was performed with bacteria harvested from a MH agar plate alone or a MH agar plate supplemented with DOC. DOC did not alter the ability of *C. jejuni* to bind to the INT 407 cells (Fig. 1A). In addition, DOC did not alter the motility of the *C. jejuni* F38011 strain as judged by motility assays (Fig. 1B). Collectively, these results indicate that culturing *C. jejuni* in the presence of DOC does not alter the adherence potential or motility of the bacteria.

Deoxycholate stimulates the synthesis but not secretion of Campylobacter invasion antigens

Invasion of the host cell is required for maximal disease. To determine if DOC alters the phenotypic behavior of *C. jejuni* with respect to invasion, we initially examined the effect of DOC on the secretion of the Cia proteins (Fig. 2). The bacteria were harvested from MH and MHD plates and labeled with [^{35}S]-methionine for 30 min. Following the metabolic labeling of proteins, Cm was added to inhibit protein synthesis. After an additional 30 min incubation period, FBS was added to the labeling medium. Previous studies have demonstrated that it is necessary to add FBS to the medium to induce the secretion of the Cia proteins from *C. jejuni* (44). In contrast to the supernatants of the bacteria harvested from MH agar plates, the Cia proteins were clearly visible in the supernatants of bacteria harvested from MHD agar plates (Fig. 2A, lanes 1 and 2). To ensure that this finding was due to active protein secretion and reflected a bona fide

difference in the phenotypic behavior of *C. jejuni* harvested from MH versus MHD plates, several controls were performed in parallel. First, samples were included in the assay for which FBS was not added to the labeling medium. Consistent with previous work, the Cia proteins were absent from the supernatants of bacteria harvested from both MH and MHD agar plates in the absence of FBS (Fig. 2A, lanes 3 and 4). Second, we harvested the bacteria after the labeling assay and assessed their metabolic state by SDS-PAGE analysis coupled with autoradiography of the whole cell lysates (Fig. 2B). As expected, the intensities of the protein bands were similar for the bacterial samples, indicating that the bacteria were metabolically active (Fig. 2B, lanes 1-4). Third, we probed the secreted proteins with an antibody prepared against the *C. jejuni* CadF outer membrane protein to ensure that the proteins detected in the supernatants were not due to bacterial cell lysis (Fig. 2C). In contrast to the *C. jejuni* whole cell lysates, the CadF protein was not detected in the supernatants of any of the samples. Based on these results we conclude that DOC is capable of stimulating the synthesis of the Cia proteins.

Deoxycholate alters the invasion kinetics of the C. jejuni *F38011 strain*

We tested the effect of DOC on the ability of *C. jejuni* F38011 to invade INT 407 cells as judged by a gentamicin protection assay. Here, the bacteria were incubated with INT 407 cells for 3 h, after which medium containing gentamicin was added for a 3 h period to kill the extracellular bacteria. When compared to *C. jejuni* harvested from MH agar plates, *C. jejuni* harvested from MHD agar plates were more invasive for the INT 407 cells (not shown). To further assess the specificity of DOC in stimulating the synthesis of the *C. jejuni* Cia proteins, an additional gentamicin assay was performed whereby the bacteria

harvested from MH and MHD agar plates were suspended in medium containing Cm prior to inoculation of the INT 407 cells. The concentration of Cm used in these assays immediately halts *C. jejuni* protein synthesis, thereby preventing additional protein synthesis by *C. jejuni* in the presence of epithelial cells (not shown). In the presence of Cm, an eleven-fold increase was observed in the number of intracellular *C. jejuni* harvested from MHD agar plates as compared with bacteria harvested from MH agar plates. Collectively, these data suggest that culture of *C. jejuni* with DOC induces the synthesis of proteins that facilitate the organism's invasion of epithelial cells.

Since culture with deoxycholate stimulates Cia protein synthesis and enhances the ability of *C. jejuni* to invade INT 407 cells, an invasion assay was performed to determine the effect of DOC on the kinetics of invasion. Based on the observation that DOC stimulates the bacterium to synthesize the Cia proteins, we hypothesized that culturing *C. jejuni* on MHD agar plates would increase their invasive potential relative to bacteria harvested from MH agar plates. As predicted, *C. jejuni* F38011 harvested from MHD agar plates were able to maximally invade INT 407 cells within 15 min of infection (Fig. 3). An increase in INT 407 cell invasion was also observed at 15 min post-infection with *C. jejuni* F38011 harvested from MHD agar plates and suspended in medium with Cm when compared to the invasion efficiency of *C. jejuni* F38011 harvested from MH agar plates alone. For *C. jejuni* cultured on MH agar plates, the number of intracellular bacteria increased over time, and reached a level similar to that observed for the bacteria harvested from MHD plates after a 3 h period. As expected, the invasion of INT 407 cells was significantly reduced for bacteria that were harvested from MH agar plates and

re-suspended in medium containing Cm. Based on these results, we concluded that culturing *C. jejuni* in the presence of DOC alters the invasion kinetics of *C. jejuni,* such that maximal invasion is achieved as early as 15 min post-infection.

Deoxycholate induces the ciaB *promoter*

To determine if deoxycholate induces *ciaB* gene expression, the *ciaB* promoter was cloned upstream of the β-galactosidase gene (*ciaB*$_{\textbf{promoter}}$- β-galactosidase) in the pMW10 vector. A log phase culture of *C. jejuni* F38011 harboring the P*ciaB*-pMW10 construct was then used to inoculate MH and MHD agar plates, and β-galactosidase assays were performed. The *ciaB* promoter activity gradually increased in the presence of 0.1% DOC reaching a maximum at 15 h post inoculation, after which it decreased (Fig. 4a). The ability of DOC to induce the promoter activity of *porA* was also measured. In contrast to the activity of the *ciaB* promoter, DOC did not significantly alter *porA* promoter activity over time.

We also measured the activity of the *ciaB* and *porA* promoters in response to different concentrations of DOC. Here, MH and MH agar plates supplemented with varying concentrations of DOC were inoculated with a log phase culture of *C. jejuni* F38011 harboring the P*ciaB*-pMW10 and P*porA*-pMW10. The β-galactosidase activity was measured at 15 h post inoculation. In contrast to the *porA* promoter, the activity of the *ciaB* promoter was influenced directly by the concentration of DOC. Specifically, a maximum 2-fold increase in *ciaB* promoter activity was observed when the *C. jejuni* F38011 strain was cultured on MH agar plate supplemented with 0.2% DOC (Fig. 4b).

Similar to previous experiments, *ciaB* promoter activity was also found to be maximal at 15 h post inoculation with 0.2% DOC (not shown). These results indicate that DOC stimulates *ciaB* promoter activity in a dose and time-dependent manner.

Exposure to deoxycholate stimulates the expression of ciaB

To identify genes that are differentially expressed in response to DOC, we performed microarray experiments with RNA extracted from *C. jejuni* cultured in the presence and absence of DOC. Real-time RT-PCR was performed on RNA isolated at 3, 6, 9, 12 and 15 h to determine the difference in the *ciaB* transcript levels in *C. jejuni* when cultured with DOC. Maximal fold increase in the *ciaB* transcript level was observed upon culture of *C. jejuni* with 0.1% DOC for 12 h (Fig. 5). Based on this result, we performed microarray experiments with RNA extracted from *C. jejuni* cultured with 0.1% DOC for 12 h to determine if those genes were co-expressed with *ciaB*. In total, 202 genes were differentially expressed in response to culturing *C. jejuni* F38011 with 0.1% DOC for 12 h. One hundred and fifty-six genes, including *ciaB* were upregulated $\geq$ 1.5–fold (Table 2), while 46 genes were down-regulated $\geq$ 1.5–fold (Table 3) in the presence of DOC. To confirm the microarray data, genes representative from each functional class (shown in Fig. 6) were selected and real-time RT-PCR was performed (Table 4). The genes found to be upregulated in the presence of DOC by microarray analyses were also found to be upregulated by real-time RT-PCR analyses.

Not surprisingly the *cmeABC* operon, which encodes an efflux pump that participates in the resistance of *C. jejuni* to the deleterious effects of bile salts (21-24), was upregulated

in response to culture with DOC. Moreover, approximately 20 percent of the genes upregulated in the presence of DOC were genes whose products were associated with the cell envelope of *C. jejuni*. Twelve percent of the genes upregulated in the presence of DOC were involved in synthesis and modification of RNA and DNA molecules. Ten percent of the genes upregulated in the presence of DOC were those that encode ABC transporters [i.e., enterochelin (*ceuE*), molybdenum (*modB*), biopolymers (*exbB1*) and receptor proteins like *tonB* and *chuA*]. Perhaps most relevant to this study, genes known to play an important role in *C. jejuni* pathogenesis were upregulated (i.e., *ciaB*, *dccR, flgS, tlyA*). Finally, a large number of genes upregulated in response to DOC included conserved hypothetical genes (~20%) and genes of unknown function (11%). Overall, the results of the microarray experiments indicated that *C. jejuni* displays an increase in expression of virulence genes in the presence of DOC.

DISCUSSION

Invasion is an important virulence determinant in *C. jejuni* pathogenesis. Newell *et al*. (30) reported that clinical isolates are more invasive than environmental isolates. Further, Everest *et al*. (8) observed that *C. jejuni* isolates recovered from individuals with colitis were significantly more invasive than isolates recovered from individuals with non-inflammatory diarrhea. Evidence from a number of animal studies also support the hypothesis that disease development is related to invasive potential of the *C. jejuni* strain (3, 9, 13, 48, 51). Here we report that culturing *C. jejuni* with the bile acid DOC triggers its invasive potential by stimulating the expression/synthesis of the Cia proteins. Specifically, we monitored the effect of DOC on the expression of *ciaB* and found its expression upregulated by three independent assays, namely β-galactosidase reporter assay, real-time RT-PCR assay, and microarrays.

The ability of *C. jejuni* to invade epithelial cells is dependent on several bacterial properties, including motility, adherence, and protein secretion. Thus, the effect of DOC on all three of these virulence factors was investigated. We found that culturing *C. jejuni* in the presence of DOC did not alter the organism's motility or adherence potential. The zone of migration measured for the *C. jejuni* F38011 strain on semi-solid MH and MHD agar was comparable. In addition, bacteria harvested from MH and MHD agar plates did not show differences in their ability to bind to epithelial cells; this finding was reproducible regardless of the multiplicity of infection used (MOI). However, a marked difference was seen in the amount of the Cia proteins secreted by *C. jejuni* when cultured in the presence of DOC.

To determine if DOC plays a role in Cia synthesis, the bacteria were labeled with [^{35}S]-methionine and incubated in the presence of Cm prior to inducing Cia secretion. Addition of Cm helped distinguish the proteins synthesized in the presence of DOC from those synthesized subsequently in presence of FBS, the latter of which was added to stimulate Cia protein secretion. While the Cia proteins were detected in the supernatants of *C. jejuni* harvested from MHD agar plates, we did not detect any secreted proteins in the supernatants of *C. jejuni* harvested from MH agar plates. This finding indicated the presence of pre-synthesized Cia proteins in the bacteria cultured in the presence of DOC.

The Cia proteins are required for maximal invasion of host cells. Thus, bacteria cultured in the presence of DOC are "primed" to invade epithelial cells as they harbor pre-synthesized Cia proteins. This conclusion is supported by the difference in the invasion kinetics of bacteria harvested from MH and MHD agar plates. While bacteria harvested from MH agar plates require 3 h to achieve maximal invasion, those harvested from MHD agar plates are able to invade INT 407 cells within 15 min. Taken together, the data indicate that culturing *C. jejuni* in the presence of physiological concentration of DOC results in global changes in gene expression and an alteration in the bacterium's phenotype whereby its invasive potential is significantly enhanced.

A number of *in vitro* studies have been performed to better understand how bacteria modulate gene expression upon encountering host-like conditions, and to determine the growth conditions that alter an organism's invasive behavior (4, 11, 14, 37, 47, 49, 50).

We hypothesized that we could use the expression of the *ciaB* gene as a marker to better define the kinetics of *cia* induction by DOC, and use the data to identify when the other *cia* genes are maximally expressed. Using a *ciaB* promoter-*lacZ* fusion construct, we found that DOC was able to induce *ciaB* promoter activity. The *ciaB* promoter activity increased moderately over time; however, a higher level of induction was observed with increasing concentrations of bile. We saw maximal induction of the *ciaB* promoter in the presence of 0.2% DOC at 15 h. Noteworthy is that bile has been reported to have a similar effect on the promoter that drives the expression of the genes encoding the multi-drug efflux pump CmeABC (21), which is inducible by bile acids in a dose and time dependent manner.

Based on the data from the β-galactosidase assays, we performed real-time RT-PCR analysis with the *C. jejuni* F38011 strain cultured with DOC for 3, 6, 9, 12 and 15 h to determine when the *ciaB* gene was maximally expressed. RT-PCR analysis revealed maximal expression of *ciaB* following a 12 h incubation period with DOC, which is complementary with that of the β-galactosidase assay. Although the temporal expression of the *ciaB* gene by DOC was slower than one might predict, it is similar to the induction kinetics of the *cmeABC* operon in response to bile (21). Not known is the kinetics of *ciaB* induction *in vivo*, and whether other factors contribute to trigger its expression.

Because the genes co-regulated with *ciaB* might encode for Cia proteins and other unidentified virulence factors, RNA was extracted from *C. jejuni* cultured with DOC and microarray experiments were performed to determine the entire transcriptome. DOC

differentially regulated a total of 202 genes in the *C. jejuni* F38011 strain, 150 of which were upregulated while 46 were downregulated. A number of *C. jejuni* genes predicted to play a role in signal transduction were upregulated by DOC, including *flgS* and *dccR*. The FlgS sensor kinase, is a part of the FlgSR two-component system known to play an important role in *C. jejuni* motility (53). The *dccRS* two-component system has been shown to play a role in the *in vivo* colonization of immunocompetent limited flora (I-LF) mice, severe combined immunodeficient limited flora (SCID-LF) mice, and 1-day-old chicks (25). Taken together, the evidence indicated that DOC is acting as a stimulus to trigger a global regulatory response. Whether DOC is acting directly, or indirectly, to induce these signal transduction pathways requires additional study.

Since bile salts are surface active, amphipathic molecules that act as detergents primarily exerting their effect on cell membranes (5), it is not surprising that 31 genes encoding cell envelope-associated proteins were significantly upregulated when *C. jejuni* was cultured with DOC. In addition, genes encoding members of the ABC transporter family, like *exbB1* and *modB,* along with genes involved in iron transport like *chuA* and *ceuE* (42, 43) were upregulated. The gene encoding hemolysin A (TlyA), a member of a family of contact dependent hemolysins found in *Helicobacter*, *Serpulina,* and *Mycobacterium,* was also found to be upregulated. In *H. pylori*, TlyA is homologous to a pore-forming cytolysins and a *tlyA* defined mutant shows reduced *in vitro* hemolytic activity, reduced adherence to human gastric adenocarcinoma cells, and failed to colonize the gastric mucosa of mice (27). Also of interest was the finding that culturing *C. jejuni* with DOC resulted in the upregulation of a number of conserved hypothetical genes and genes of

unknown function. Some of these genes may encode for virulence factors including the other Cia proteins.

Noteworthy is that Fox *et al*. (10) studied the effect of culturing *C. jejuni* with a concentration of bile that exceeds the concentration normally found in human and chicken intestinal tracts. Using a proteomic approach, they found that culturing *C. jejuni* with 2.5% ox-bile for 18 h increased the synthesis of GroEL, GalU, and bacterioferritin proteins (10). These proteins are indicative of a bacterial stress response. We did not observe these genes to be upregulated by *C. jejuni* in response to DOC as judged by microarray analysis, indicating that the conditions used in this study did not induce a bacterial stress response.

We show that the bile acid DOC acts as a signal for *C. jejuni*, triggering its pathogenic behavior as evidenced by its ability to invade epithelial cells. More specifically, we show that culture with DOC "primes" *C. jejuni* to invade epithelial cells by stimulating the synthesis of the Cia proteins. This is a significant finding as it highlights the effect of *in vivo*-like culture conditions on virulence factors of *C. jejuni*. Harvesting *C. jejuni* from MHD agar plates will help shorten invasion assays currently performed from three hours to 15 min. This will also allow researchers to synchronize the infection and to dissect the early events in *Campylobacter* invasion of host cells. Studies are currently being performed to characterize the proteins encoded by the genes whose expression mirrors that of *ciaB,* a known virulence factor. These genes may encode for unidentified

virulence factors that may play a role in bile resistance and/or invasion of the intestinal tract.

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TABLE 1. Primers used for real-time RT-PCR analysis

Primer Name	Gene Name	Sequence
Cj0181 RT-F	*tonB*	5'CTCAAGAAAAATCAAGTGGTGTTG
Cj0181 RT-R	*tonB*	5'CGATAGGAAACTCTGATACCATC
Cj0323 RT-F	Cj0323	5'TATACTCAAATAACTTCAAATCATAGTG
Cj0323 RT-R	Cj0323	5'CTCTTCTTGATTCTGTTCTAAAATTG
Cj0367c RT-F	*cmeA*	5'GTGTTGATTCGGCTTACGGAC
Cj0367cRT-R	*cmeA*	5'TCTAGCACTTGCTAGACTAGC
Cj0402 RT-F	*glyA*	5'CGATGGAACGGATAATCACC
Cj0402RT-R	*glyA*	5'AATACCTGCATTTCCAAGAGC
Cj0561 RT-F	Cj0561	5'TTGGCAAACAGTGATTATCTAAGC
Cj0561 RT-R	Cj0561	5'TAAAGTTCTGCACCGATAAAAGG
Cj0588 RT-F	*tlyA*	5'AATTTATGTTTCAAGAGCAGCTTT
Cj0588 RT-R	*tlyA*	5'CGTACTAGATCCTATATCAAGAC
Cj0706 RT-F	Cj0706	5'AATTAGACGCTGCAAATGATGAG
Cj0706 RT-R	Cj0706	5'CTTACTCTAATCTCGTTAATATTTTGC
Cj0786 RT-F	Cj0786	5'GGTGTTATTTTTGGAATTGATTATGTG
Cj0786 RT-R	Cj0786	5'CTATATATTCTTTTTTTTCTTTCTCTAAGC
Cj0793 RT-F	*flgS*	5'TGTTGCCTAGTGCGCTTTGG
Cj0793 RT-R	*flgS*	5'ATAAAACCTACCTTCAAATTCAAGC
Cj0862c RT-F	*pabB*	5'AAATGATACAAAAAATCTGAGTGAAAATG
Cj0862c RT-R	*pabB*	5'TTTGGTTTTTAAGCTTTTTTTTGTGATG
Cj0863c RT-F	*xerD*	5'AAGCAAAATGAAGAAGATGAAAAAGC
Cj0863c RT-R	*xerD*	5'TAATTTTACCCCTTTAGAACCTGC
Cj0914c RT-F	*ciaB*	5'AGACAAAGAAGATGTGGGTGA
Cj0914c RT-R	*ciaB*	5'AATCAATCAAACGCCTAAGTATGG
Cj0989 RT-F	Cj0989	5'TCTTTATCATCGTTACTCGCTATG
Cj0989 RT-R	Cj0989	5'TATCTTCTTTCATATTTTGTATGTTTTGG
Cj1212c RT-F	*rbn*	5'AGCAGCGCTTAGTTTTTATACTG
Cj1212c RT-R	*rbn*	5'GGAAATTTGCGTAAAAACAGAAAAAC
Cj1223 RT-F	*dccR*	5'GATATTTTGATCTTTGGATTTTAGATGT
Cj1223 RT-R	*dccR*	5'GGAGTTTGCTTTCCGCTTTTTC
Cj1224 RT-F	Cj1224	5'CAAAATGGGATAACAGCTATAGTG
Cj1224 RT-R	Cj1224	5'CTTCATTTTTACTTACAGATCTATCTG
Cj1314c RT-F	*hisF*	5'AATGCACGCAATGTTGATGAGC
Cj1314c RT-R	*hisF*	5'GCAGCCCTTGAGCCATCG

TABLE 1. continues

Gene Name	Primer Name	Sequence
Cj1458c RT-F	*thiL*	5'GAACAAAGAAGATTTTATTATCAAAGC
Cj1458c RT-R	*thiL*	5'AAATCCTTACTAAAACACCAATCATC
Cj1530 RT-F	*coaA*	5'AACCGCTTCAATTGCTTGTGG
Cj1530 RT-R	*coaA*	5'CGATTTTGTCTGCGCTAATGC
Cj1531 RT-F	*dapF*	5'AGGTGCGGATGGCTTTATCG
Cj1531 RT-R	*dapF*	5'GCAGCCCTTGAGCCATCG

TABLE 2. Transcripts upregulated in the *C. jejuni* strain F38011 in the presence of 0.1% sodium deoxycholate.

Gene	Common Name	Fold change	Functional classification
Cj0002	*dnaN*	1.6	DNA replication
Cj0008	Cj0008	1.6	Conserved Hypotheticals
Cj0037c	Cj0037c	1.9	Electron transport
Cj0040	Cj0040	2.9	Unknown
Cj0073c	Cj0073c	1.7	Conserved Hypotheticals
Cj0080	Cj0080	5.1	Cell envelope (Membranes, lipoproteins and porins)
Cj0100	Cj0100	2.2	Cell division
Cj0114	Cj0114	1.6	Cell envelope (Misc. periplasmic proteins)
Cj0155c	*rpmE*	1.5	Ribosomal protein synthesis and modification
Cj0179	*exbB1*	1.6	Transport/binding proteins (Others)
Cj0181	*tonB1*	5.1	Transport/binding proteins (Others)
Cj0188c	Cj0188c	1.5	Conserved Hypotheticals
Cj0189c	Cj0189c	1.6	Conserved Hypotheticals
Cj0190c	Cj0190c	2.2	Miscellaneous
Cj0193c	*tig*	1.9	Chaperones, chaperonins, heat shock
Cj0199c	Cj0199c	7.5	Cell envelope (Misc. periplasmic proteins)
Cj0207	*infC*	1.9	Protein translation and modification
Cj0237	*cynT*	1.5	Central intermediary metabolism
Cj0246c	Cj0246c	2.0	Signal transduction
Cj0247c	Cj0247c	3.0	Unknown
Cj0295	Cj0295	2.0	Miscellaneous
Cj0301c	*modB*	1.8	Transport/binding proteins (Anions)
Cj0309c	Cj0309c	3.3	Drug sensitivity
Cj0323	Cj0323	1.6	Unknown
Cj0324	*ubiE*	1.8	Biosynthesis of cofactors, prosthetic groups and carriers

Gene	Common Name	Fold Change	Functional classification
Cj0346	*trpD*	1.7	Amino acid biosynthesis
Cj0352	Cj0352	1.6	Cell envelope (Membranes, lipoproteins and porins)
Cj0356c	Cj0356c	2.0	Conserved Hypotheticals
Cj0365c	*cmeA*	1.9	Antibiotic resistance
Cj0366c	*cmeB*	1.6	Antibiotic resistance
Cj0367c	*cmeC*	2.1	Antibiotic resistance
Cj0381c	*pyrF*	2.1	Pyrimidine biosynthesis
Cj0382c	*nusB*	1.5	RNA synthesis, RNA modification and DNA transcription
Cj0395c	Cj0395c	2.2	Unknown
Cj0397c	Cj0397c	1.9	Conserved Hypotheticals
Cj0413	Cj0413	2.8	Cell envelope (Misc. periplasmic proteins)
Cj0484	Cj0484	1.9	Transport/binding proteins (Others)
Cj0512	*purC*	1.5	Purine biosynthesis
Cj0526c	*fliE*	2.0	Cell envelope (Surface structures)
Cj0539	Cj0539	1.7	Unknown
Cj0561c	Cj0561c	3.5	Cell envelope (Misc. periplasmic proteins)
Cj0573	Cj0573	3.9	Conserved Hypotheticals
Cj0579c	Cj0579c	1.6	Conserved Hypotheticals
Cj0580c	Cj0580c	1.5	Miscellaneous
Cj0588	*tlyA*	1.7	Pathogenicity
Cj0609c	Cj0609c	1.6	Cell envelope (Misc. periplasmic proteins)
Cj0618	Cj0618	2.2	Unknown
Cj0659c	Cj0659c	2.4	Cell envelope (Misc. periplasmic proteins)
Cj0667	Cj0667	1.6	Conserved Hypotheticals
Cj0679	*kdpD'*	2.2	Transport/binding proteins (Cations)
Cj0683	Cj0683	2.7	Cell envelope (Misc. periplasmic proteins)
Cj0690c	Cj0690c	2.1	DNA replication

Gene	Common Name	Fold Change	Functional classification
Cj0692c	Cj0692c	1.5	Cell envelope (Membranes, lipoproteins and porins)
Cj0705	Cj0705	1.8	Conserved Hypotheticals
Cj0706	Cj0706	1.9	Conserved Hypotheticals
Cj0707	*kdtA*	1.5	Cell envelope (Surface polysaccharides, lipopolysaccharides and antigens)
Cj0713	*trmD*	1.7	Aminoacyl tRNA synthetases and their modification
Cj0724	Cj0724	1.5	Unknown
Cj0729	Cj0729	2.7	Unknown
Cj0733	Cj0733	1.7	Conserved Hypotheticals
Cj0734c	*hisJ*	2.5	Transport/binding proteins (Amino acids and amines)
Cj0742	Cj0742	1.9	Cell envelope (Membranes, lipoproteins and porins)
Cj0777	Cj0777	1.6	DNA replication
Cj0778	*peb2*	1.9	Cell envelope (Misc. periplasmic proteins)
Cj0784	Cj0784	1.5	Cell envelope (Misc. periplasmic proteins)
Cj0785	*napD*	2.3	Electron transport
Cj0786	Cj0786	14.0	Unknown
Cj0787	Cj0787	1.6	Conserved Hypotheticals
Cj0788	Cj0788	1.8	Conserved Hypotheticals
Cj0789	Cj0789	1.5	RNA synthesis, RNA modification and DNA transcription
Cj0793	*flgS*	1.5	Signal transduction
Cj0798c	*ddlA*	1.7	Cell envelope (Murein sacculus and peptidoglycan)
Cj0837c	Cj0837c	2.0	Unknown
Cj0838c	*metS*	1.5	Aminoacyl tRNA synthetases and their modification

Gene	Common Name	Fold Change	Functional classification
Cj0842	Cj0842	2.6	Cell envelope (Membranes, lipoproteins and porins)
Cj0843c	Cj0843c	1.6	Protein degradation
Cj0848c	Cj0848c	3.3	Conserved Hypotheticals
Cj0852c	Cj0852c	1.7	Cell envelope (Membranes, lipoproteins and porins)
Cj0862c	*pabB*	1.5	Biosynthesis of cofactors, prosthetic groups and carriers
Cj0863c	*xerD*	2.0	DNA replication
Cj0881c	Cj0881c	1.5	Conserved Hypotheticals
Cj0884	*rpsO*	1.6	Ribosomal protein synthesis and modification
Cj0914c	*ciaB*	1.5	Pathogenicity
Cj0926	Cj0926	1.5	Cell envelope (Membranes, lipoproteins and porins)
Cj0960c	*rnpA*	1.6	RNA synthesis, RNA modification and DNA transcription
Cj0962	Cj0962	1.5	Miscellaneous
Cj0963	Cj0963	1.7	Conserved Hypotheticals
Cj0967	Cj0967	1.8	Cell envelope (Misc. periplasmic proteins)
Cj0983	Cj0983	1.7	Cell envelope (Membranes, lipoproteins and porins)
Cj0987c	Cj0987c	1.6	Cell envelope (Membranes, lipoproteins and porins)
Cj0989	Cj0989	4.4	Cell envelope (Membranes, lipoproteins and porins)
Cj1006c	Cj1006c	3.0	Conserved Hypotheticals
Cj1028c	Cj1028c	1.8	Miscellaneous nucleoside/nucleotide reactions
Cj1038	*ftsW*	2.4	Cell division

Gene	Common Name	Fold Change	Functional classification
Cj1053c	Cj1053c	6.3	Cell envelope (Membranes, lipoproteins and porins)
Cj1056c	Cj1056c	1.5	Conserved Hypotheticals
Cj1070	*rpsF*	1.7	Ribosomal protein synthesis and modification
Cj1071	*Ssb*	1.5	DNA replication
Cj1079	Cj1079	2.1	Cell envelope (Miscellaneous periplasmic proteins)
Cj1103	*csrA*	2.0	Broad regulatory function
Cj1180c	Cj1180c	2.0	Transport/binding proteins (Others)
Cj1181c	*Tsf*	1.7	Protein translation and modification
Cj1191c	Cj1191c	1.5	Signal transduction
Cj1201	*metE*	2.3	Amino acid biosynthesis (Aspartate family)
Cj1204c	*atpB*	1.7	ATP-proton motive force
Cj1212c	*Rbn*	1.8	Aminoacyl tRNA synthetases and their modification
Cj1217c	Cj1217c	2.5	Conserved Hypotheticals
Cj1223c	Cj1223c	1.5	Signal transduction
Cj1224	Cj1224	5.3	Transport/binding proteins (Cations)
Cj1242	Cj1242	5.2	Unknown
Cj1289	Cj1289	1.8	Cell envelope (Miscellaneous periplasmic proteins)
Cj1349c	Cj1349c	2.2	Pathogenicity
Cj1355	*ceuE*	1.5	Transport/binding proteins (Cations)
Cj1384c	Cj1384c	1.6	Unknown
Cj1385	*katA*	1.6	Detoxification
Cj1388	Cj1388	1.5	Conserved Hypotheticals
Cj1416c	Cj1416c	1.7	Cell envelope (Surface polysaccharides, lipopolysaccharides and antigens)
Cj1417c	Cj1417c	1.5	Miscellaneous

Gene	Common Name	Fold Change	Functional classification
Cj1418c	Cj1418c	1.8	Miscellaneous
Cj1442c	Cj1442c	1.6	Unknown
Cj1450	Cj1450	1.7	Unknown
Cj1457c	Cj1457c	1.6	Conserved Hypotheticals
Cj1458c	*thiL*	1.5	Biosynthesis of cofactors, prosthetic groups and carriers
Cj1463	Cj1463	2.4	Conserved Hypotheticals
Cj1472c	Cj1472c	2.3	Cell envelope (Membranes, lipoproteins and porins)
Cj1473c	Cj1473c	1.7	Unknown
Cj1475c	Cj1475c	2.2	Unknown
Cj1484c	Cj1484c	1.5	Cell envelope (Membranes, lipoproteins and porins)
Cj1492c	Cj1492c	1.7	Signal transduction
Cj1495c	Cj1495c	1.5	Conserved Hypotheticals
Cj1530	Cj1530	1.6	Conserved Hypotheticals
Cj1531	*dapF*	1.8	Amino acid biosynthesis (Aspartate family)
Cj1540	Cj1540	1.5	Cell envelope (Miscellaneous periplasmic proteins)
Cj1547	Cj1547	2.3	Conserved Hypotheticals
Cj1556	Cj1556	2.8	Conserved Hypotheticals
Cj1581c	*dppD*	1.8	Transport/binding proteins (Others)
Cj1589	Cj1589	2.8	Conserved Hypotheticals
Cj1603	*hisF*	6.9	Amino acid biosynthesis (Histidine)
Cj1611	*rpsT*	1.6	Ribosomal protein synthesis and modification
Cj1614	*chuA*	3.0	Transport/binding proteins (Cations)
Cj1621	Cj1621	5.9	Cell envelope (Miscellaneous periplasmic proteins)
Cj1640	Cj1640	2.0	Conserved Hypotheticals
Cj1646	*iamB*	1.9	Transport/binding proteins (Others)

Gene	Common Name	Fold Change	Functional classification
Cj1675	*fliQ*	2.4	Cell envelope (Surface structures)
Cj1679	Cj1679	1.8	Unknown
Cj1695c	*rplE*	1.5	Ribosomal protein synthesis and modification
Cj1724c	Cj1724c	1.7	Conserved Hypotheticals
CJE1111	CJE1111	2.7	Miscellaneous
CJE1278	CJE1278	1.5	Miscellaneous
CJE1470	CJE1470	2.5	Miscellaneous
CJE1472	CJE1472	1.7	Miscellaneous
ORF00215	CJE0220	2.0	Miscellaneous
ORF00225	CJE0230	1.5	Miscellaneous
ORF00226	CJE0231	1.9	Miscellaneous
ORF00236	CJE0241	3.4	Miscellaneous
ORF00237	CJE0243	3.8	Miscellaneous

TABLE 3. Transcripts downregulated in the *C. jejuni* strain F38011 in the presence of 0.1% sodium deoxycholate.

Gene	Common Name	Fold Change	Functional Classification
Cj0056c	Cj0056c	1.6	Unknown
Cj0069	Cj0069	1.6	Unknown
Cj0076c	*lctP*	1.8	Transport/binding proteins
Cj0105	*atpA*	1.6	Respiration
Cj0107	*atpD*	1.5	Respiration
Cj0136	*infB*	1.5	Synthesis and modification of macromolecules
Cj0146c	*trxB*	2.1	Biosynthesis of cofactors, prosthetic groups and carriers
Cj0153c	Cj0153c	1.7	Synthesis and modification of macromolecules
Cj0453	*thiC*	3.5	Biosynthesis of cofactors, prosthetic groups and carriers
Cj0471	*rpmG*	2.3	Synthesis and modification of macromolecules
Cj0472	*secE*	2.4	Protein and peptide secretion
Cj0473	*nusG*	2.9	Synthesis and modification of macromolecules
Cj0475	*rplA*	1.7	Synthesis and modification of macromolecules
Cj0499	Cj0499	1.6	Conserved hypothetical proteins
Cj0537	*oorB*	1.7	Respiration
Cj0698	*flgG*	1.7	Cell envelope
Cj0720c	*flaC*	2.0	Cell envelope
Cj0855	*folD*	1.5	Biosynthesis of cofactors, prosthetic groups and carriers
Cj0864	*dsbA*	1.5	Cell envelope

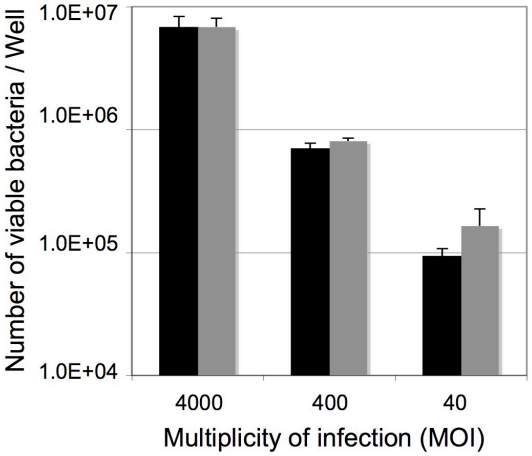
Gene	Common Name	Fold Change	Functional Classification
Cj0913c	*hup*	1.8	Synthesis and modification of macromolecules
Cj0982c	Cj0982	1.5	Transport/binding proteins
Cj0998c	Cj0998c	2.2	Cell envelope
Cj1014c	*livF*	1.6	Transport/binding proteins
Cj1015c	*livG*	1.6	Transport/binding proteins
Cj1060c	Cj1060c	1.5	Unknown
Cj1068	Cj1068	1.5	Cell envelope
Cj1163c	Cj1163c	1.7	Transport/binding proteins
Cj1168c	Cj1168c	1.7	Cell envelope
Cj1198	*luxS*	1.7	Conserved hypothetical proteins
Cj1229	*cbpA*	1.5	Chaperones, chaperonins, heat shock
Cj1274c	*pyrH*	1.6	Purines, pyrimidines, nucleosides and nucleotides
Cj1290c	*accC*	1.5	Fatty acid biosynthesis
Cj1291c	*accB*	1.8	Fatty acid biosynthesis
Cj1293	*flmA*	1.8	Cell envelope
Cj1309c	Cj1309c	1.5	Unknown
Cj1364c	*fumC*	1.5	Energy metabolism
Cj1400c	*fabI*	1.5	Fatty acid biosynthesis
Cj1403c	*gapA*	1.6	Energy metabolism
Cj1502c	*putP*	1.8	Transport/binding proteins
Cj1548c	Cj1548c	1.8	Misc
Cj1567c	*nuoM*	1.6	Respiration
Cj1628	*exbB2*	1.8	Transport/binding proteins
Cj1658	Cj1658	1.6	Cell envelope
Cj1659	Cj1659	2.0	Cell envelope
Cj1682c	*gltA*	1.7	Energy metabolism
Cj1719c	*leuA*	1.8	Amino acid biosynthesis

TABLE 4. Real-time RT-PCR analysis of transcripts upregulated in the *C. jejuni* strain F38011 in the presence of 0.1% sodium deoxycholate.

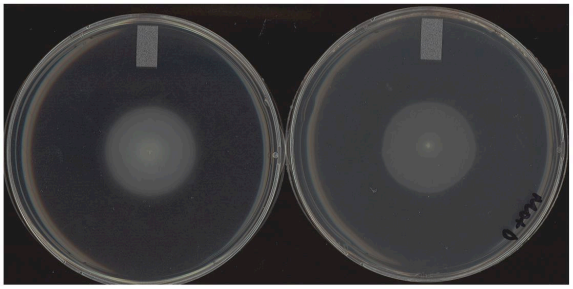
Gene Name	Fold Increase
Pathogenesis	
ciaB	3.4
tlyA	2.2
Antibiotic resistance	
cmeA	4
Amino acid biosynthesis	
dapF	1.7
hisF	2
Biosynthesis of cofactors, prosthetic groups and carriers	
pabB	2
thiL	1.7
Cell envelope	
Cj0989	1.9
Cj0561	4.5
Conserved hypothetical proteins	
coaA	3.2
Cj0706	2.9
Signal transduction	
Cj0246	2.5
dccR	1.7
Synthesis and modification of macromolecules	
xerD	1.7
rbn	2.1
Transport/binding proteins	
Cj1224	2.4
tonB1	1.8
Unknown	
Cj0786	3.3
Cj0323	3.8

Figure 1. Sodium deoxycholate does not alter adherence (A) or motility (B) of *C. jejuni* F38011 strain. Ten-fold serial dilution of the *C. jejuni* F38011 strain, cultured on Mueller Hinton (MH) and MH supplemented with 0.1% sodium deoxycholate (DOC) agar plates for 18 h, were used to inoculate INT 407 cells. Shown is the number of bacteria from a MH agar plate (black) and a MHD agar plate (gray) that bound to INT 407 cells, 30 min post inoculation. Values represent the mean $\pm$ standard deviation of viable bacteria recovered per well of a 24-well tissue culture tray. *C. jejuni* cultured on MH and MHD plates with 0.4% agar displayed equivalent zones of migration.

A


1.0E+07
1.0E+06
1.0E+05
1.0E+04
Number of viable bacteria / Well
4000
400
40
Multiplicity of infection (MOI)

B



MH MHD

Figure 2. Sodium deoxycholate stimulates the synthesis of *Campylobacter* invasion antigens (Cias). Panel A shows the proteins secreted by *C. jejuni* as determined by SDS-PAGE coupled with autoradiography of the supernatants. Panel B shows the proteins synthesized by *C. jejuni* as judged by SDS-PAGE coupled with autoradiography of the whole cell lysates (WCL; OD_{540} equivalent = 0.1). Panel C represents a control, where the proteins secreted by *C. jejuni* were probed with an antibody prepared against the *C. jejuni* CadF outer membrane protein. The metabolic labeling assays, preparation of supernatants and whole cell lysates, and autoradiography were performed as outlined in "Materials and Methods." Panel A, B and C, Lanes: 1, *C. jejuni* F38011 harvested from a MH agar plate, and radioactively labeled in medium supplemented with 1% FBS; 2, *C. jejuni* F38011 harvested from a MHD agar plate, and radioactively labeled in medium supplemented with 1% FBS; 3, *C. jejuni* F38011 harvested from a MH agar plate, and radioactively labeled in medium without 1% FBS; and 4, *C. jejuni* F38011 harvested from a MHD agar plate, and radioactively labeled in medium without 1% FBS. Panel C, additional samples, Lanes: 5, 1:4 dilution of *C. jejuni* WCL; 6, 1:2 dilution of *C. jejuni* WCL; 7, 1:1 dilution of *C. jejuni* WCL. The migration of CadF as two protein species, indicated by the arrow (37 kDa species) and arrowhead (32 kDa species), is caused by the protein's heat-modifiable property.

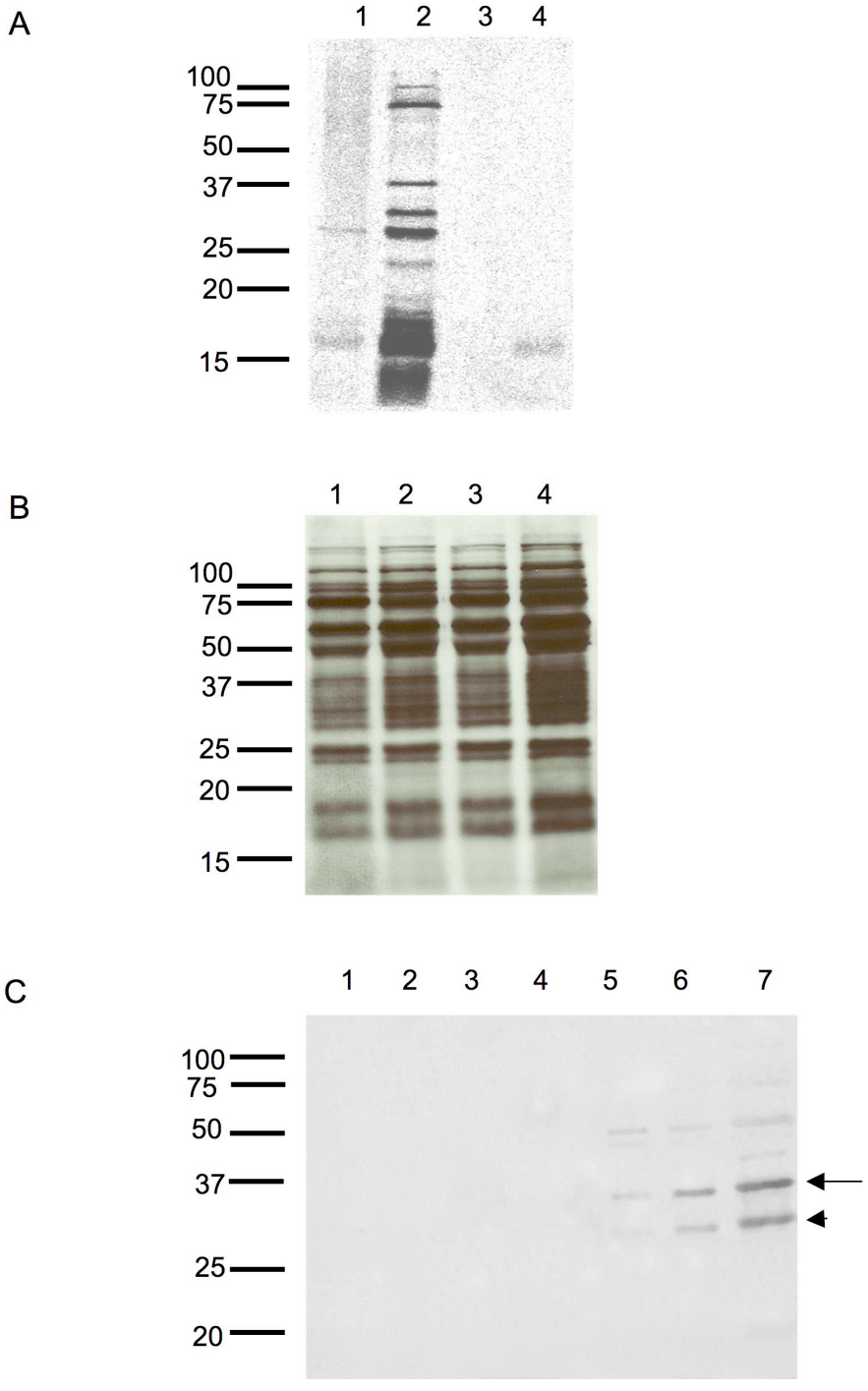

A
1 2 3 4
100
75
50
37
25
20
15
B
1 2 3 4
100
75
50
37
25
20
15
C
1 2 3 4 5 6 7
100
75
50
37
25
20

Figure 3. Culturing the *C. jejuni* F38011 with sodium deoxycholate alters the kinetics of invasion of INT 407 cells. Gentamicin protection assays were performed as described in "Materials and Methods" with *C. jejuni* F38011 cultured on Mueller-Hinton (MH) and MH supplemented with 0.1% sodium deoxycholate (DOC) agar plates for 18 h. The bacteria were incubated with INT 407 cells in the absence and presence of 128 μg/ml of chloramphenicol (Cm) to inhibit protein synthesis. The number of internalized bacteria is shown for *C. jejuni* F38011 cultured on a MH agar plate (open circle); *C. jejuni* F38011 cultured on a MH agar plate supplemented with 0.1% DOC (open square); *C. jejuni* F38011 cultured on a MH agar plate followed by 30 min incubation with Cm (closed circle); *C. jejuni* F38011 cultured on MH agar plate supplemented with 0.1% DOC followed by 30 min incubation with Cm (closed square). Values represent the mean $\pm$ standard deviation of viable bacteria recovered per well of a 24-well tissue culture tray.

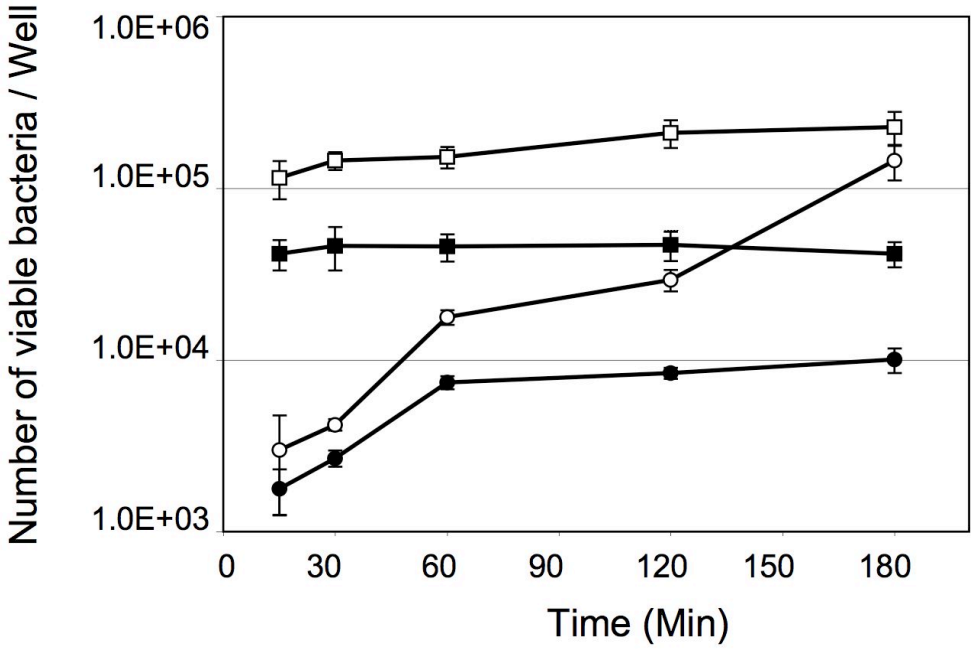
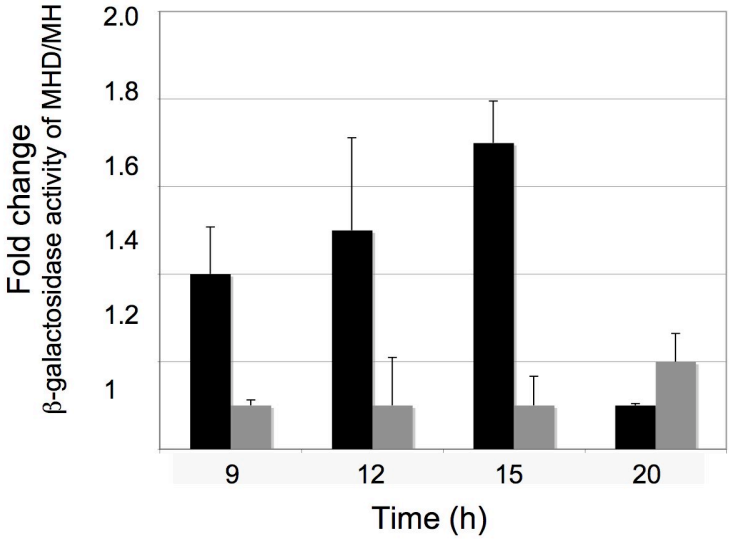

Number of viable bacteria / Well
1.0E+06
1.0E+05
1.0E+04
1.0E+03
0
30
60
90
120
150
180
Time (Min)

Figure 4. Stimulation of *ciaB* promoter activity by sodium deoxycholate is time (a) and dose (b) dependent. These data are presented as the ratio of β-galactosidase activity of *ciaB* (black) and *porA* (gray) between the *C. jejuni* F38011 strain cultured on a Mueller-Hinton (MH) agar plate supplemented with 0.1% sodium deoxycholate (DOC) and a MH agar plate alone. Each bar represents the mean $\pm$ standard deviation of two separate experiments, where each experiment was comprised of triplicate samples.

A


Fold change
β-galactosidase activity of MHD/MH
2.0
1.8
1.6
1.4
1.2
1
9
12
15
20
Time (h)

B

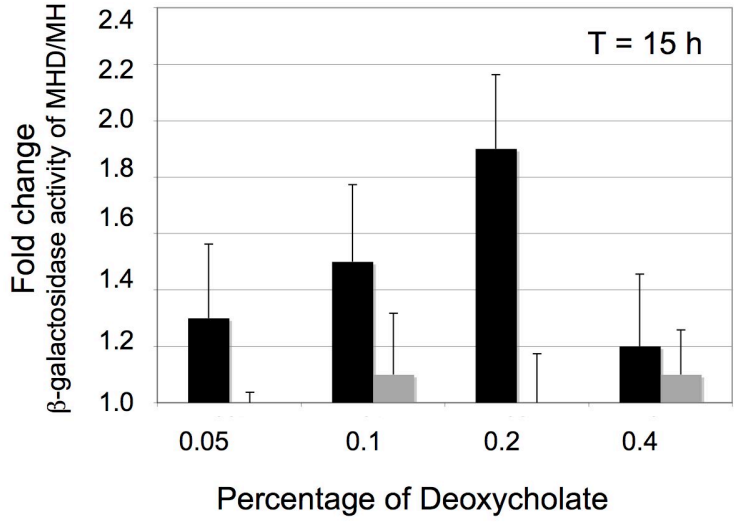

Fold change
β-galactosidase activity of MHD/MH
T = 15 h
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.05
0.1
0.2
0.4
Percentage of Deoxycholate

Figure 5. Temporal expression of *ciaB* and *porA* in *C. jejuni* F38011 cultured in the presence of deoxycholate (DOC). Real-time RT-PCR analysis of total RNA extracted from the *C. jejuni* F38011 strain grown on MH agar and MH agar plates supplemented with 0.1% DOC for 3, 6, 9, 12 and 15 h. The fold change in *ciaB* (black) and *porA* (gray) transcript level was measured using the comparative $\Delta\Delta C_T$ method, where *glyA* was used as the internal control

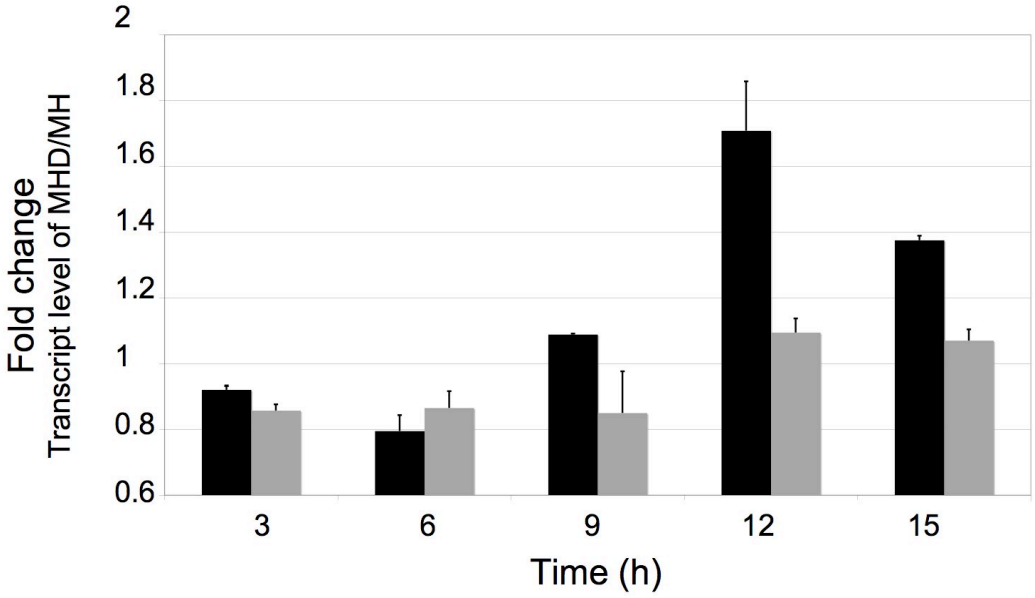
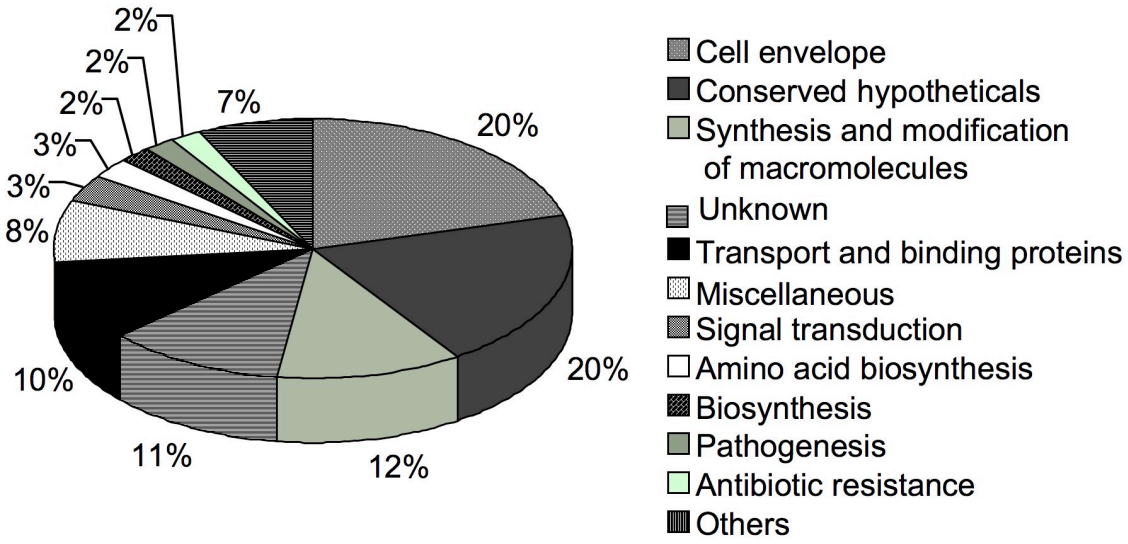

Fold change
Transcript level of MHD/MH
2
1.8
1.6
1.4
1.2
1
0.8
0.6
3
6
9
12
15
Time (h)

Figure 6. The functional classification of the *C. jejuni* F38011 genes upregulated $\geq$ 1.5 fold in the presence of deoxycholate (DOC). The pie chart represents the percentage of genes belonging to a functional class to the total number of genes upregulated in the presence of DOC. The percentages shown here are those of functional categories representing more than three percent of the total number of genes upregulated. Functional categories with 2 or less genes were grouped together and represented as 'Others' on the pie chart.


2%
2%
2%
7%
20%
3%
3%
8%
10%
20%
11%
12%
Cell envelope
Conserved hypotheticals
Synthesis and modification of macromolecules
Unknown
Transport and binding proteins
Miscellaneous
Signal transduction
Amino acid biosynthesis
Biosynthesis
Pathogenesis
Antibiotic resistance
Others

CHAPTER FOUR

CONCLUSIONS AND FUTURE DIRECTIONS

The high incidence of *Campylobacter*-mediated enteritis in the developed world has instigated a significant amount of research in the field of *Campylobacter* pathogenesis. Although our knowledge of the genus *Campylobacter* has grown exponentially over the past decade, a better understanding of its disease causing ability is required. One common theme that has emerged over time is the mutifactorial nature of the disease caused by *Campylobacter*. While the host immune system is important, the virulence potential of the infecting strain plays a significant role in the disease outcome. A number of virulence factors such as adhesions, invasions, toxins and motility have been identified. The research presented in this dissertation highlights the ability of *Campylobacter* to modulate its virulence potential by altering its gene content and gene expression.

Campylobacteriosis is an acute diarrheal illness accompanied by abdominal cramps and in some cases by a nonspecific syndrome of fever, headache, chills and myalgia. The array of symptoms and severity of disease observed vary from one individual to another. This is attributed in part to the well-documented variability observed among *Campylobacter* strains. Further, the variable phenotypes observed among strains may help the organism survive in unique ecological niches.

A unique characteristic of *Campylobacter* ecology is the ability of the organism to exhibit divergent lifestyles within different hosts. While *C. jejuni* exhibits a commensal

relationship with its poultry host, it causes disease in humans. Although motility and adhesins are known to play an important role in both chicken colonization and disease in humans, virulence determinants like invasins and toxins are commonly associated with disease. With no apparent differences in the genetic composition of poultry and clinical isolates, the ability of the bacteria to exhibit these different lifestyles may be due to differential gene expression in response to its microenvironment. It is possible that the different stimuli sensed by the bacteria govern the final outcome of the host pathogen interaction.

The research outlined in Chapter 2 clearly shows that genetically matched isolates with varying virulence potential can be used as a tool to better understand *Campylobacter* pathogenesis. This approach may help identify novel virulence factors or define the function of known virulence associated genes.

Of the seven poultry isolates studied, two of the isolates were genetically identical but varied in their virulence potential. While the *C. jejuni* Turkey isolate was found to be virulent in *in vitro* and *in vivo* assays the *C. jejuni* CS isolate was avirulent. Further analysis of the molecular mechanism behind the non-motile, and thus the avirulent phenotype of the *C. jejuni* CS isolate led to the identification of a point mutation in the *flgR* gene, resulting in a nonfunctional product. FlgR is the response regulator required for the transcription of σ^{54}-regulated genes in conjunction with the sensor kinase FlgS.

Flagellar biosythesis is an energy consuming process and shutting off this process when not required is highly favorable for *Campylobacter*. It is therefore not surprising that non-motile bacteria were isolated from the environment. Further analysis of non-motile isolates indicated that they grew at a faster rate and reached higher cell densities under laboratory condition. Another interesting observation was that the non-motile *C. jejuni* isolate CS exhibited higher resistance to bile salts than its genetically matched motile counterpart, the *C. jejuni* Turkey isolate. Because CS exhibited a mutation in the response regulator *flgR* it is hypothesized that this response regulator may play a role in bile resistance in *Campylobacter*. We also observed that the genes differentially regulated in a *rpoN* mutant were different from those differentially regulated in a *flgR* mutant, indicating that σ^{54} may play a role in regulating other biological processes in *Campylobacter*. Further studies designed to determine the role of the FlgS/FlgR two-component system and RpoN in processes other than flagellar biosynthesis are needed.

Because all poultry isolates studied in chapter two were non-motile and secretion deficient, it was difficult to differentiate the contribution of the secreted proteins from that of motility to the virulence potential of the bacteria. During this study a motile and secretion deficient revertant was isolated from the non-motile *C. jejuni* isolate S3. The molecular mechanism behind this observation is under investigation. Both the motile (S3-Revertant) and non-motile (S3-Original) isolates were unable to invade epithelial cells *in vitro*. Thus, comparison of the S3-revertant with a genetically matched motile and invasive strain like RM1221 will help identify virulence factors other than motility responsible for the *Campylobacter* pathogenesis.

Identification of the *Campylobacter* invasion antigens (Cias) has been a long-standing question in the field. The research outlined in Chapter 3 uses an environmental stimulus to upregulate the expression of the Cia proteins thus helping in their identification via microarrray analysis. *Campylobacter* when cultured in the presence of bile salt sodium deoxycholate was able to upregulate its virulence potential as assessed its ability to invade epithelial cells. Because *Campylobacter* encounters bile in the mammalian gut it may have evolved mechanisms not only to overcome its antibacterial capabilities, but also to use bile as an indicator of its anatomical location within the host.

Deoxycholate specifically stimulated the synthesis of the Cias, while no effect on bacterial motility and adhesion was observed. Further, maximum stimulation of the Cias was achieved over a period of 12 h in the presence of deoxycholate as judged by the expression levels of *ciaB*. Additionally, microarray analysis showed a number of genes previously known to play an important role in *C. jejuni* pathogenesis to be upregulated when cultured in the presence of deoxycholate.

Interestingly, a number of genes encoding proteins of unknown function and conserved hypotheticals were also found to be upregulated in *C. jejuni* cultured with deoxycholate. It is hypothesized that some of these genes may encode for the Cia proteins. Additional work in the Konkel laborartory has shown that some of the genes upregulated by deoxycholate encode for proteins that harbor a Type III secretion signal at their N-

terminus. Mutational analysis of these genes will help determine if they encode for the Cia proteins and/or play a role in *C. jejuni* pathogenesis.

A subset of the genes upregulated in the presence deoxycholate harbors an ATP/GTP binding motif. This motif is preset in a number of eukaryotic proteins involved in various cellular functions. Interestingly, this motif is present in the Rho GTPase proteins including Rac, Rho and Cdc42. A number of enteric pathogens secrete effector proteins that mimic Rho GTPase and alter the host actin cytoskeleton to either enhance internalization as in the case of *Salmonella* and *Shigella,* or prevent internalization as in the case of *Yersinia*. Because *Campylobacter* is known interact with Rac1 and Cdc42 it is hypothesized that proteins encoded by these genes may be involved.

In conclusion, the work described in this dissertation has provided a significant increase in the understanding of the mechanisms by which *C. jejuni* is able to alter its virulence potential. While genetic changes, such as a point mutation in motility genes, subsequently decreases its virulence, altered gene expression in response to bile salts help *C. jejuni* enhance its pathogenic potential. Further, this dissertation also describes two approaches; comparison of genetically matched isolates and stimulation of virulence factors by deoxycholate to help investigators further the knowledge of *C. jejuni* pathogenesis.

APPENDIX

ATTRIBUTIONS

CHAPTER 2

'Characterization of genetically-matched isolates of *Campylobacter jejuni* reveals mutations in genes involved in flagellar biosynthesis alter the organism's virulence potential'. Dr. Monteville generated the data presented in Table 1. Dr. Rivera-Amill performed the secretion assays shown in Figure 1. The piglet study (Table 2) was performed in Dr. Joens laboratory, Inversity of Arizona, Tucson, Arizona. The RNA samples used for the microarray analyses were generated by me. Dr. Parker and Dr. Quinones performed the labeling, hybridization, and data analyses. Dr. Raphael generated Figure 2 and 3, and the *flaB* promoter construct. Dr. Klena identified the genetically matched pairs used in this study. Ms. Keech performed 2-D analyses on *C. jejuni* Turkey and CS isolates. Dr. Christensen generated the *flgR* knockout construct and the *rpoN* complementation construct. My personal contribution to this manuscript was in performing the experiments and generation of Figures 4 through 8. I assisted Dr. Konkel in writing and formatting the manuscript.

CHAPTER 3

"**Culture of *Campylobacter jejuni* with Sodium Deoxycholate induces Virulence Gene Expression**". My contribution to this manuscript was in performing many of the experiments as well as generating each of the tables and figures. Dr. Parker performed the microarray hybridization and data analyses. Dr. Raphael generated the *ciaB*-β galactosidase promoter construct. Dr. Konkel and I wrote, edited and formatted the entire manuscript.