Published in final edited form as:
Mol Microbiol. 2012 April ; 84(2): 352–369. doi:10.1111/j.1365-2958.2012.08027.x.

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

Angelica M. Barrero-Tobon and **David R. Hendrixson***
Department of Microbiology, University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Room NL 4.138A Dallas, TX 75390-9048

Summary

The flagellum of *Campylobacter jejuni* provides motility essential for commensal colonization of the intestinal tract of avian species and infection of humans resulting in diarrheal disease. Additionally, the flagellar type III secretion system has been reported to secrete proteins such as CiaI that influence invasion of human intestinal cells and possibly pathogenesis. The flagellar regulatory system ultimately influences σ^{28} activity required for expression of the FlaA major flagellin and other flagellar filament proteins. In this work, we discovered that transcription of *ciaI* and four genes we propose annotating as *feds* (for flagellar co-expressed determinants) is dependent upon σ^{28}, but these genes are not required for motility. Instead, the Feds and CiaI are involved in commensal colonization of chicks, with FedA additionally involved in promoting invasion of human intestinal cells. We also discovered that the major flagellin influences production, stability or secretion of σ^{28}-dependent proteins. Specific transcriptional and translational mechanisms affecting CiaI were identified and domains of CiaI were analyzed for importance in commensalism or invasion. Our work broadens the genes controlled by the flagellar regulatory system and implicates this system in coordinating production of colonization and virulence determinants with flagella, which together are required for optimal interactions with diverse hosts.

Keywords

Campylobacter jejuni; σ^{28}; flagellar motility; commensalism; colonization

Introduction

Campylobacter jejuni colonizes both animal and human hosts to result in different outcomes of infection. *C. jejuni* is a natural commensal organism of the intestinal tract of many wild and domesticated animals, especially avian species. In chickens, *C. jejuni* promotes a prolonged asymptomatic colonization of the ceca and large intestines, which leads to contamination of poultry meats for human consumption (Beery *et al*., 1988; Friedman *et al*., 2004). During infection of humans, *C. jejuni* invasion of intestinal and colonic epithelial cells is thought to contribute to inflammation and destruction of the epithelium (van Spreeuwel *et al*., 1985). Both invasion and the host inflammatory response likely contribute to pathogenesis of disease, which ranges from mild, watery diarrhea to a severe, bloody diarrheal syndrome.

*To whom correspondence should be addressed: David R. Hendrixson, Ph.D. University of Texas Southwestern Medical Center Department of Microbiology Room NL 4.138A 5323 Harry Hines Boulevard Dallas, TX 75390-9048 david.hendrixson@utsouthwestern.edu Phone: (214) 633-1385 Fax: (214) 648-5905.

To understand factors required by *C. jejuni* to colonize hosts, we previously used a 1-day old chick model of commensalism to identify 29 *C. jejuni* genes necessary for wild-type levels of colonization of the chick ceca (Hendrixson & DiRita, 2004). Other studies revealed that *C. jejuni* requires specific transport systems, metabolic pathways, cytochrome c peroxidases, protein glycosylation systems, capsular polysaccharide production and fibronectin-binding proteins to promote commensalism (Jones *et al*., 2004; Karlyshev *et al*., 2004; Velayudhan *et al*., 2004; Bingham-Ramos & Hendrixson, 2008; Davis *et al*., 2009; Flanagan *et al*., 2009; Ribardo & Hendrixson, 2011). An ideal *in vivo* virulence model that mimics *C. jejuni* diarrheal disease in humans remains elusive. Instead, cell culture models of infection are available to assess the ability of *C. jejuni* to invade and survive within human small intestinal or colonic epithelial cells. One transposon mutant screen revealed the importance of oxidative stress resistance and fumarate metabolism in promoting *C. jejuni* entry into or survival within colonic epithelial cells (Novik *et al*., 2010).

Flagellar motility is one factor of *C. jejuni* required for both commensal colonization of poultry and infection of human volunteers (Black *et al*., 1988; Nachamkin *et al*., 1993; Wassenaar *et al*., 1993; Hendrixson & DiRita, 2004; Wosten *et al*., 2004). Furthermore, flagella and flagellar motility are required for interactions with and invasion of human intestinal epithelial cells (Wassenaar *et al*., 1991; Grant *et al*., 1993; Yao *et al*., 1994). In addition to secreting proteins for flagellar biosynthesis, the *C. jejuni* flagellar type III secretion system (T3SS) has been implicated in secretion of proteins required for interactions with eukaryotic cells (Konkel *et al*., 1999; Konkel *et al*., 2004; Song *et al*., 2004; Poly *et al*., 2007). Some of these proteins have been annotated as the *Campylobacter* invasion antigens (Cias) (Konkel *et al*., 1999). Production of Cia proteins has been reported to be induced by bile salts, with flagellum-dependent secretion of these proteins requiring either a factor produced by intestinal cells or components of serum (Rivera-Amill *et al*., 2001; Konkel *et al*., 2004; Malik-Kale *et al*., 2008). *C. jejuni* mutants lacking different Cia proteins (CiaB, CiaC, and CiaI) are reduced in their ability to invade or survive within eukaryotic cells (Konkel *et al*., 1999; Christensen *et al*., 2009; Buelow *et al*., 2011). However, conflicting reports exist on the universal requirement of Cia proteins for cell invasion among different *C. jejuni* strains (Goon *et al*., 2006; Novik *et al*., 2010). Other proteins of *C. jejuni*, such as FlaC and the FspA proteins, are secreted by the flagellum but are not dependent on bile salts for expression or serum for secretion. FlaC, a flagellin-like protein involved in motility, is required for full level of invasion of eukaryotic cells (Song *et al*., 2004). Two related FspA proteins are produced by different *C. jejuni* strains (Poly *et al*., 2007). Of these proteins, FspA2, but not FspA1 promotes apoptosis of eukaryotic cells.

As in many motile bacteria, regulation of flagellar gene expression and biosynthesis is a complex process in *C. jejuni* (Lertsethtakarn *et al*., 2011). Two alternative σ factors in *C. jejuni* control expression of flagellar genes encoding extracytoplasmic components of the flagellar organelle (Hendrixson *et al*., 2001). σ^{54} is required for expression of most flagellar rod and hook genes, whereas σ^{28} is required for expression of *flaA*, encoding the major flagellin, and other filament genes (Hendrixson & DiRita, 2003; Carrillo *et al*., 2004; Wosten *et al*., 2004; Wosten *et al*., 2010). The flagellar T3SS components (including FlhA, FlhB, FliP, and FliR), the FlgSR two-component regulatory system, and the FlhF GTPase form a regulatory system required to initiate transcription by σ^{54}-RNA polymerase holoenzyme (Hendrixson & DiRita, 2003; Joslin & Hendrixson, 2008; Balaban *et al*., 2009; Joslin & Hendrixson, 2009; Boll & Hendrixson, 2011). A current model proposes that the FlgS histidine kinase may sense the formation or activity of the flagellar T3SS to result in activation of the FlgR response regulator for σ^{54}-dependent expression of flagellar rod and hook genes (Joslin & Hendrixson, 2008, 2009). The FlhF GTPase is also essential for σ^{54}-dependent flagellar gene expression, but specific details regarding its requirement are not yet known (Balaban *et al*., 2009). Like in other motile bacteria, activity of σ^{28} in *C. jejuni* is

repressed by the FlgM anti-σ factor until the flagellar rod and hook are synthesized (Wosten *et al*., 2004; Wosten *et al*., 2010). After rod and hook formation, FlgM is secreted from the cytoplasm to relieve σ^{28} from repression, which results in *flaA* expression necessary for filament synthesis. Additional mechanisms involving FlhF and the putative FlhG ATPase spatially and numerically regulate flagellar biosynthesis so that only one flagellum is produced per bacterial pole (Balaban *et al*., 2009; Balaban & Hendrixson, 2011). These two proteins, along with the flagellar MS ring and switch complex also influence a mechanism to spatially control cell division (Balaban & Hendrixson, 2011).

A previously reported microarray analysis provided information regarding potential members of the σ^{28} regulon (Carrillo *et al*., 2004). This analysis not only identified genes encoding proteins known to be involved in motility, but also genes with functions other than in flagellar motility that potentially may be dependent on σ^{28} for expression (Carrillo *et al*., 2004; Goon *et al*., 2006). Genes encoding the FspA proteins described above are dependent on σ^{28} for expression (Poly *et al*., 2007). *Cj0977* (encoded by *Cjj81176_0996* in the *C. jejuni* 81-176 genome; for the remainder of this work, this gene will be referred to as *0996* to be consistent with the annotation of the 81-176 genome) is another member of the σ^{28} regulon initially not thought to be required for motility (Goon *et al*., 2006). However, further analysis revealed that 0996 is required for flagellar motility in liquid media, but not in semi-solid motility agar (Novik *et al*., 2010). This motility defect may explain the reduced invasion and pathogenicity observed with a *C. jejuni 0996* mutant (Goon *et al*., 2006).

In this work, we provide an extensive analysis that establishes the σ^{28} regulon of *C. jejuni* and identifies a new class of *C. jejuni* virulence and colonization factors. We identified five σ^{28}-dependent genes (including *ciaI*) that are not required for motility, but are required for wild-type levels of commensal colonization of poultry. In addition to CiaI, we found another σ^{28}-dependent factor involved in invasion of epithelial cells. Further exploration of CiaI analyzed potential domains for a role in commensal colonization or invasion and revealed an influence of bile salts on translation of a specific *ciaI* mRNA. Due to their co-expression with flagellar genes and their requirement for wild-type levels of colonization, and in some cases virulence, we propose annotating four of these previously uncharacterized proteins as Feds (for flagellar co-expressed determinants). Furthermore, this work establishes the flagellar system of *C. jejuni* as a regulatory system required for expression of genes not only required for motility, but also for genes with broader functions than previously realized that include commensalism and virulence.

Results

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

A previous analysis of flagellated and aflagellated *C. jejuni* NCTC11168 strains revealed that expression of *fliA*, encoding σ^{28}, was partially reduced in the aflagellated mutant (Carrillo *et al*., 2004). In addition, a potential σ^{54}-binding site was identified within *flhG*, which is two genes upstream of *fliA*. Therefore, we hypothesized that *fliA* may be a member of the σ^{54} regulon and examined if factors belonging to the σ^{54} regulatory pathway influence *fliA* expression.

For all analyses in this work, we examined *C. jejuni* 81-176, a strain capable of infecting human volunteers and promoting commensal colonization of the chick cecum (Black *et al*., 1988; Hendrixson & DiRita, 2004). We compared expression of the *fliA*::*astA* transcriptional reporter in wild-type *C. jejuni* 81-176 Sm^R Δ*astA* and isogenic mutants lacking σ^{54} (Δ*rpoN*), the FlgSR two-component system (Δ*flgS* or Δ*flgR*), flagellar T3SS components (Δ*flhA* or Δ*flhB*), or the FlhF GTPase (Δ*flhF*). Expression of *fliA*::*astA* was reduced approximately ~25-80% in these mutants relative to wild-type *C. jejuni* (Figure 1A), suggesting that *fliA* is

partially dependent on the σ^{54} regulatory pathway for expression. We identified two transcriptional start sites for *fliA* by primer extension analysis (Figures 1B and 1C). The transcriptional start site for the σ^{54}-independent promoter (P1) was located 27 nucleotides upstream of the *fliA* start codon (Figure 1C and 1D). The transcriptional start site for a σ^{54}-dependent promoter (P2) was located 382 nucleotides upstream of *fliA* and within the 3' end of *flhG* (Figure 1B and 1D). Consistent with this observation, a highly conserved σ^{54}-binding site was found directly upstream of the P2 transcriptional start site (Figure 1D).

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

Initial data from a previous microarray analysis suggested involvement of σ^{28} and FlhA, a flagellar T3SS protein and component of the σ^{54} regulatory pathway, in the expression of approximately 30 genes, with some genes encoding proteins not previously associated with a role in flagellar motility in other motile bacteria (Carrillo *et al*., 2004). Therefore, we analyzed expression of these genes to determine if they indeed compose part of the *C. jejuni* σ^{28} regulon.

Expression of potential σ^{28}-dependent genes in wild-type *C. jejuni* 81-176 Sm^R and an isogenic σ*fliA* mutant (for the remainder of this report, *fliA* will be referred to as σ^{28} for clarity and to avoid confusion with *flaA* encoding the major flagellin) was compared by semi-quantitative real-time RT-PCR (qRT-PCR) (Figure 2A). Of 27 genes examined, expression of 12 genes was reduced 4- to over 100-fold in the $\Delta\sigma^{28}$ mutant (Figure 2A and Table S1). Transcription of these genes increased upon complementation with a plasmid expressing Δ^{28} *in trans*. In addition, expression of *fliS*, encoding a putative flagellin chaperone, was reduced 25% in the $\Delta\sigma^{28}$ mutant and complementation with σ^{28} *in trans* resulted in two-fold overexpression of the gene (Table S1). Transcription of the remaining 14 genes was not affected by deletion of σ^{28}, and these genes were not further analyzed.

Of the 13 σ^{28}-dependent genes, seven encode proteins that either have been verified for a role in flagellar motility or are predicted to be involved in motility (Figure 2A). These genes include *flaA* (encoding the major flagellin; (Wassenaar *et al*., 1991; Grant *et al*., 1993; Nachamkin *et al*., 1993)), *flaG* (encoding a filament length control protein; (Kalmokoff *et al*., 2006)), *fliD* (encoding the putative filament cap; (Golden & Acheson, 2002; Konkel *et al*., 2004)), *fliS* (encoding the putative flagellin chaperone; (Golden & Acheson, 2002)), *flgM* (encoding the anti-σ factor that regulates σ^{28} activity; (Hendrixson & DiRita, 2003; Wosten *et al*., 2004; Wosten *et al*., 2010)), *Cjj81176_1458* (encoding a possible chaperone for flagellar hook-associated proteins), and *0996* (encoding a protein required for motility in low viscosity media; (Novik *et al*., 2010)). However, six genes encode proteins with no homology to any known flagellar proteins. These genes and our proposed annotation based on additional findings described below include: *fspA1* (Poly *et al*., 2007), *ciaI* (Buelow *et al*., 2011), *Cjj81176_0083* (*fedA*), *Cjj81176_0414* (*fedB*), *Cjj81176_1053* (*fedC*), and *Cjj81176_1647* (*fedD*) (Figure 2A). Similar to expression of *fspA1*, expression of the *fed* genes and *ciaI* was reduced 5- to 100-fold in the $\Delta\sigma^{28}$ mutant compared to wild-type *C. jejuni* (Figure 2B and Table S1; (Poly *et al*., 2007)). Transcription of these genes was largely restored by complementation with σ^{28} *in trans*, with no statistically-significant differences in transcription of *0996*, *fspA1*, *fedB*, or *fedD* between wild-type and complemented strains. Although expression of *fedA*, *fedC*, and *ciaI* did not reach wild-type levels upon complementation with σ^{28}, transcription of these genes in the complemented strain was significantly higher than in the $\Delta\sigma^{28}$ mutant with or without the empty vector (Figure 2B).

Considering that the *fed*s and *ciaI* were dependent on σ^{28} for expression, we predicted that expression of these genes would decrease in σ^{54}-regulatory pathway mutants. These mutants have reduced expression of σ^{28} and lack rod and hook biogenesis, which can inhibit residual

σ^{28} activity due to cytoplasmic retention of the FlgM anti-σ factor (Figure 1A; (Hendrixson & DiRita, 2003; Wosten *et al*., 2004; Joslin & Hendrixson, 2008; Balaban *et al*., 2009; Joslin & Hendrixson, 2009; Wosten *et al*., 2010)). In addition, we predicted that transcription of these genes would increase in a Δ*flgM* mutant, due to derepression of σ^{28} activity (Wosten *et al*., 2004; Wosten *et al*., 2010). As expected, expression of *fspA1*::*astA* and *fedD*::*astA* transcriptional fusions was reduced approximately 3- to 62-fold (Figure 2C). Expression of *ciaI*::*astA* was more modestly reduced about 2- to 4-fold in σ^{54}-regulatory pathway mutants and the $\Delta\sigma^{28}$ mutant (Figure 2C). In addition, expression of these transcriptional fusions increased 67 to 123% in a Δ*flgM* mutant, which possesses augmented σ^{28}-dependent activity due to lack of FlgM-mediated repression (Figure 2C).

We characterized the promoter of *fedC* since a previous bioinformatic analysis did not identify a potential σ^{28}-binding site upstream of this gene (Carrillo *et al*., 2004). In addition, we characterized the *ciaI* promoter because expression of this gene was not entirely dependent upon σ^{28}. By primer extension analysis, we identified a single transcriptional start site 61 nucleotides upstream of the *fedC* start codon that was absent in the $\Delta\sigma^{28}$ mutant and restored upon complementation with σ^{28} *in trans* (Figure 3A). Immediately upstream of the σ^{28}-dependent *fedC* transcriptional start site and within the 3' end of the upstream gene *Cjj81176_1054*, a potential σ^{28} consensus-binding site was identified (Figure 3A). Similarly, we identified a σ^{28}-dependent transcriptional start site for *ciaI* 28 nucleotides upstream of the start codon of the gene (Figure 3B). As with the *fedC* promoter, a potential σ^{28}-binding site upstream of this transcriptional start site was identified (Figure 3B; (Carrillo *et al*., 2004)). We also identified one or two other potential transcriptional start sites for *ciaI* further upstream and into the coding sequence of *Cjj81176_1442* that were not dependent on σ^{28} (Figure 3B). These alternative transcriptional start sites may contribute to residual expression of *ciaI* in the $\Delta\sigma^{28}$ mutant (Figure 2B and 2C and Table S1). These data are strong evidence that the *fed* genes and *ciaI* are members of the σ^{28} regulon and dependent on the flagellar regulatory system for expression.

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

Although dependent on σ^{28} for expression, the *fed* genes and *ciaI* do not encode proteins with homology to any known motility proteins. FedA is most homologous to single-domain hemerythrins, which are diiron- and oxygen-binding proteins primarily found in anaerobic and microaerobic bacteria and some invertebrates (French *et al*., 2008). FedC contains a putative DnaJ-domain, suggesting the protein may be part of the Hsp70 chaperone machinery and involved in protein folding or degradation (Genevaux *et al*., 2007). FedB and FedD are not homologous to any proteins with known functions.

To characterize a role for the Fed proteins, CiaI, and other σ^{28}-dependent proteins in the biology of *C. jejuni*, we constructed in-frame chromosomal deletions of each gene in *C. jejuni* strain 81-176 Sm^R. All strains grew similarly to wild-type *C. jejuni* in Mueller-Hinton (MH) broth at 37 °C in microaerobic conditions (Figure S1). We also examined the *C. jejuni* $\Delta\sigma^{28}$ mutant as a control. We noted that the $\Delta\sigma^{28}$ mutant demonstrated a growth defect in standing broth cultures, which may be due to the reduced motility of the mutant (Figure S1 and Figure 4A and 4B). As observed in semi-solid motility agar and in liquid broth by dark-field microscopy, no significant motility defects were observed in any of the Δ*fed* mutants or the Δ*ciaI* mutant (Figure 4A and 4B; data not shown). In fact, deletion of *fedD* and *ciaI* appeared to cause a slight, but statistically-significant, increase in motility (Figure 4B). As controls, we examined motility in the Δ*rpoN* mutant, which lacks σ^{54} and expression of many flagellar rod and hook genes, and the $\Delta\sigma^{28}$ mutant, which expresses reduced levels of *flaA* (Table S1). Both mutants were defective for motility in motility agar (Figure 4A and 4B). Similar to previous observations (Novik *et al*., 2010), the *C. jejuni* Δ*0996* mutant was

non-motile in liquid broth, but motile in semi-solid media (Figure 4A and 4B; data not shown).

We previously found that a *C. jejuni* 81-176 Sm^R $\Delta\sigma^{28}$ mutant was defective for commensal colonization of chicks (Hendrixson & DiRita, 2004). The colonization defect of this mutant was thought to be due solely to its greatly reduced motility phenotype. However, we considered if the *fed* genes or *ciaI*, which require σ^{28} for expression, may be necessary for colonization and contribute to the reduced colonization capacity of the $\Delta\sigma^{28}$ mutant. For these experiments, we infected 1-day old chicks orally with either 10^4 or 10^2 cfu of wild-type *C. jejuni* or mutants lacking one of the *fed*s or *ciaI*. At seven days post-infection, the levels of wild-type *C. jejuni* in the ceca of chicks given an oral inoculum of 10^4 cfu averaged 2.8×10^8 cfu per gram of cecal content (Figure 5A). In contrast, the Δ*fedA*, Δ*fedC*, Δ*fedD*, and Δ*ciaI* mutants colonized at 4- to 16-fold lower levels than the wild-type strain in the ceca of chicks, which were statistically-significant differences (Figure 5A). However, the Δ*fspA1* and the Δ*fedB* mutants did not show a colonization defect when administered at this inoculum. As expected, the Δ*0996* mutant was reduced 42-fold for colonization compared to wild-type *C. jejuni*, which is likely due to its non-motile phenotype under certain conditions. When the inoculum was lowered to 10^2 cfu, all mutants except for the Δ*fspA1* mutant showed statistically-significant colonization defects relative to wild-type *C. jejuni*. These colonization defects ranged from 14-fold lower for the *C. jejuni* Δ*fedB* mutant to over 1,000-fold lower for the Δ*fedD* mutant (Figure 5B). These data indicate that many of the σ^{28}-dependent genes not required for motility are required for optimal commensal colonization of chicks.

FedA is involved in invasion of epithelial cells

Because a *ciaI* mutation in another *C. jejuni* strain was previously shown to possess a two-fold invasion defect for human intestinal epithelial cells (Buelow *et al*., 2011), we examined the *fed* mutants for defects in invasion of T84 colonic epithelial cells at six hours post-infection by using a standard gentamicin-protection assay. In these assays, approximately 2.2% of the wild-type *C. jejuni* inoculum was found intracellularly at the end of the assay (Table 1). Consistent with previous reports, the Δ*ciaI* and Δ*0996* mutants demonstrated 2- and 10-fold reductions in invasion, respectively (Goon *et al*., 2006; Buelow *et al*., 2011). Upon examination of each *fed* mutant, only the Δ*fedA* mutant showed a significantly reduced invasion capacity, which was approximately 10-fold lower than the wild-type strain. This invasion defect was similar to the levels of the $\Delta\sigma^{28}$ mutant, which is minimally motile and expresses reduced levels of *fedA* (Figure 2B and Figure 4A and 4B). Thus, in addition to being a determinant for commensal colonization of chicks, FedA is also a virulence determinant required for invasion of human colonic epithelial cells.

A *ciaI* transcript is dependent on DOC for translation

Previous analysis in *C. jejuni* strain F38011 suggested that transcription of *ciaI* and production of Cia proteins are augmented in the presence of the bile salt sodium deoxycholate (DOC) (Malik-Kale *et al*., 2008). However, our results presented above indicated that approximately 60 - 85% of *ciaI* transcription originates from the σ^{28}-dependent promoter in *C. jejuni* 81-176 (Figure 2B and Table S1). Therefore, we analyzed if expression of *ciaI* from σ^{28}-independent and -dependent promoters in *C. jejuni* 81-176 strains was increased in the presence of 0.1% DOC, a concentration previously shown to be required for CiaI production (Buelow *et al*., 2011). When wild-type and $\Delta\sigma^{28}$ mutant strains were grown in the presence of DOC, we actually observed a 13 to 30% decrease in *ciaI*::*astA* expression, respectively (Figure S2). Growth in higher concentrations of DOC resulted in similar decreased levels of *ciaI*::*astA* expression (data not shown). Therefore, contrary to a previous report analyzing CiaI production in a different strain of *C. jejuni*, we

were unable to link DOC to a mechanism regulating transcription of *ciaI* in *C. jejuni* 81-176 (Rivera-Amill *et al*., 2001).

To determine if production of CiaI was influenced by DOC, proteins from whole-cell lysates of wild-type and $\Delta\sigma^{28}$ mutant strains grown on media with and without 0.1% DOC were examined by immunoblot analysis. We observed an increase in CiaI levels in wild-type *C. jejuni* 81-176 Sm^R grown in the presence of DOC (Figure 6A). Furthermore, we discovered almost complete dependence on DOC for CiaI production in the $\Delta\sigma^{28}$ mutant. Levels of RpoA, the α component of RNA polymerase, or other σ^{28}-dependent proteins such as FspA1, FedB, and 0996 did not increase with growth in the presence of DOC (Figure 6A and data not shown), suggesting that the effect of DOC was specific for translation of CiaI. These findings suggest a DOC-dependent post-transcriptional mechanism that influences production of CiaI specifically from a σ^{28}-independent transcript.

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

Secretion of CiaI and other Cias from *C. jejuni* strain F38011 was previously found to be dependent on both flagella and serum (Rivera-Amill *et al*., 2001; Buelow *et al*., 2011). In contrast, a previous investigation found that the FspA proteins, which are other σ^{28}-dependent proteins, are secreted by the flagellum of *C. jejuni* 81-176 in the absence of serum (Poly *et al*., 2007). To analyze secretion of FspA1, FedB, CiaI and 0996, we generated or obtained antisera specific for these proteins. We were unable to generate antisera to FedA, FedC, and FedD as these proteins were refractory to purification or antisera generation. In addition, these proteins could not be detected with antiserum specific for a 6XHis tag as addition of this epitope to the N- or C-terminus of these proteins made the proteins unstable in *C. jejuni* (data not shown).

After growth of wild-type and mutant *C. jejuni* strains in MH broth alone, proteins from whole bacteria and supernatants were analyzed. We also analyzed an 81-176 Sm^R Δ*flaA* mutant, which lacks the major flagellin. Secretion of Cia proteins has been shown to be reduced in a *C. jejuni* F38011 *flaA* mutant (Konkel *et al*., 2004). As a negative control, we analyzed a $\Delta\sigma^{28}$ mutant, which lacks these proteins or produces the proteins at greatly reduced levels (Figure 6B). As previously reported, FspA1 was secreted from wild-type *C. jejuni* 81-176 Sm^R in MH broth without addition of serum, but not from the Δ*flaA* mutant (Figure 6B; (Poly *et al*., 2007)). RpoA was only observed in the whole-cell lysates of these strains, suggesting that our procedures were adequate for recovering secreted proteins. In addition, the 0996 protein remained associated with bacteria as previously reported (Goon *et al*., 2006). We also noted that FspA1 and 0996 were absent in whole-cell lysate of the Δ*flaA* mutant (Figure 6B).

Contrary to a previous report, we observed that CiaI was secreted abundantly in MH broth alone without serum addition (Figure 6B; (Buelow *et al*., 2011)). Secretion of CiaI was dependent on flagella as this protein was absent from supernatants of the Δ*flaA* mutant. In addition, we found FedB to be secreted in MH broth alone in a flagellum-dependent manner (Figure 6B). In the absence of FlaA and secretion, both CiaI and FedB were stable in *C. jejuni* (Figure 6B). In addition, deletion of any σ^{28}-dependent gene analyzed with the exception of *flaA* did not impair secretion or stability of other σ^{28}-dependent proteins. These results identified FedB as a new flagellum-dependent secreted protein and suggest that serum-dependent secretion of CiaI is not a universal feature among *C. jejuni* strains. Furthermore, our results suggest that FlaA directly or indirectly plays a role in the production, stability or secretion of σ^{28}-dependent proteins.

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

A previous study indicated that CiaI contains a dileucine motif that may be important for *C. jejuni* to promote invasion of eukaryotic cells (Buelow *et al*., 2011). This domain has been suggested to function as an endosomal-targeting motif. Analysis of CiaI-GFP ectopically produced in HeLa cells revealed a punctate distribution, but a GFP fusion to CiaI with a mutated dileucine motif appeared diffuse (Buelow *et al*., 2011). Together, these data suggested that the dileucine motif of CiaI may be important in localizing the protein to endosomal vesicles and influencing the biology or survival of intracellular *C. jejuni*. However, the effect of CiaI with a mutation of the dileucine motif on invasion when produced from *C. jejuni* was not examined (Buelow *et al*., 2011). In addition, bioinformatic analysis indicates that CiaI may also contain a putative ATP- or GTP-binding motif. We tested if either of these domains are required for CiaI to function as a commensal colonization factor or a virulence determinant for *C. jejuni*. Therefore, we replaced wild-type *ciaI* on the chromosome of *C. jejuni* 81-176 Sm^R with $ciaI_{K42A}$, which is predicted to disrupt the nucleotide-binding motif, or $ciaI_{LL153\text{-}154AA}$, which disrupts the dileucine motif. We attempted to analyze potential ATP- or GTP-binding activity or hydrolysis with purified wild-type CiaI, but the recombinant protein did not show either activity *in vitro* (data not shown).

Immunoblot analysis revealed that the mutant proteins were produced and secreted from *C. jejuni*, with perhaps a slight decrease in production or stability of the $CiaI_{LL153\text{-}154AA}$ protein (Figure 7A). Both mutants demonstrated a slight 4- to 5-fold decreased colonization capacity for chicks relative to wild-type *C. jejuni* 81-176 Sm^R when administered at an inoculum of 10^4 cfu (Figure 7B). However, these decreases were not significant compared to the level of colonization promoted by the wild-type strain. When administered at an inoculum of 10^2 cfu, the $ciaI_{K42A}$ mutant demonstrated a 17-fold decrease in commensal colonization, which was a statistically-significant difference from wild-type *C. jejuni* (Figure 7C). The $ciaI_{LL153\text{-}154AA}$ mutant only showed a 6-fold decrease in colonization when administered at an inoculum of 10^2 cfu, which was not statistically significant. Neither mutant was as defective for commensal colonization as the Δ*ciaI* mutant at either inoculum.

We also tested the *C. jejuni ciaI* mutants for invasion of T84 cells. Whereas the Δ*ciaI* mutant was reduced approximately two-fold for invasion, the invasion capacity of the $ciaI_{K42A}$ mutant was reduced only about 30%, which was not statistically significant (Table 2). Despite a previous report attributing the CiaI dileucine motif as being important for vesicular localization and possibly invasion, we did not detect a significant invasion defect of the *C. jejuni* $ciaI_{LL153\text{-}154AA}$ mutant *in vitro*. These results suggest that the dileucine motif is likely not important for CiaI to promote invasion of *C. jejuni*. Instead, our results suggest that the putative nucleotide-binding domain of CiaI may have a limited role in commensal colonization or invasion by *C. jejuni*.

Discussion

Flagella and flagellar motility are well-established virulence and colonization factors of *C. jejuni* required for infection of humans to promote diarrheal disease and natural commensal colonization of avian species (Black *et al*., 1988; Nachamkin *et al*., 1993; Wassenaar *et al*., 1993; Hendrixson & DiRita, 2004; Wosten *et al*., 2004). Flagella not only provide chemotactic motility required for bacterial migration to proper replicative niches in hosts, but also likely facilitate initial contact of *C. jejuni* with human intestinal and colonic epithelial cells for subsequent invasion (Wassenaar *et al*., 1991; Grant *et al*., 1993; Yao *et al*., 1994). Furthermore, the flagellum and its secretory system have been implicated in secretion of some proteins that are not required for motility, including the Cia proteins,

FspA proteins, and FlaC, which may modulate either interactions with or the biology of eukaryotic cells (Konkel *et al*., 2004; Song *et al*., 2004; Poly *et al*., 2007).

The intricate regulatory pathway that governs σ^{54}-dependent gene expression in *C. jejuni* involves the flagellar T3SS, the FlgSR two-component system, and the FlhF GTPase (Hendrixson & DiRita, 2003; Wosten *et al*., 2004; Joslin & Hendrixson, 2008; Balaban *et al*., 2009; Joslin & Hendrixson, 2009). Because the σ^{54} regulon largely includes flagellar rod and hook genes (Boll & Hendrixson, 2011), disruption of the σ^{54} regulatory pathway results in an aflagellated and non-motile phenotype, which has consequences for the ability of *C. jejuni* to promote commensalism or disease in multiple hosts. In this work, we established a more direct role for the flagellar regulatory system in commensalism and pathogenesis of disease. We found that *fliA*, encoding σ^{28}, is a member of the σ^{54} regulon of *C. jejuni* 81-176. Furthermore, in addition to σ^{28} being required for expression of flagellin and other filament genes, we discovered that σ^{28} is directly involved in expression of *ciaI* and four genes we annotated as *fed*s. We found that CiaI and each Fed protein are required for wild-type levels of commensal colonization of poultry, and that CiaI and FedA are involved in invasion of colonic epithelial cells. Because expression of the *fed*s and *ciaI* is largely dependent on σ^{28} and the σ^{54} regulatory pathway, our results broaden the number of genes whose expression is influenced by the flagellar regulatory system to include those involved in motility and also genes directly involved in commensalism and invasion.

Our findings indicate that a substantial proportion of genes within the *C. jejuni* σ^{28} regulon have functions other than in flagellar motility. Previous analysis revealed that *C. jejuni fspA1* is a σ^{28}-dependent gene, but a mutant lacking *fspA1* did not demonstrate a noticeable *in vitro* motility defect (Poly *et al*., 2007). Similarly, we did not detect any *in vitro* motility defects in mutants lacking CiaI or any Fed proteins. Instead, we found these proteins are required for wild-type levels of commensal colonization of the chick ceca. Mutants lacking a single Fed protein or CiaI demonstrated 4- to over a 1000-fold commensal colonization defects. Furthermore, we confirmed the previously noted slight invasion defect of the Δ*ciaI* mutant and found that the Δ*fedA* mutant possessed a 10-fold defect in invasion of T84 colonic cells (Buelow *et al*., 2011). The only σ^{28}-dependent factor not found to be involved in motility, commensalism, or invasion was FspA1.

Through our analysis, we propose that the Feds and CiaI are a new collection of colonization and virulence factors co-expressed with flagella. Currently, we do not know how CiaI or each Fed functions for *C. jejuni in vivo* to promote optimal levels of commensal colonization of chicks. FedA, which we found to be involved in both commensalism and invasion, shares most homology to hemerythrins. These proteins are found mainly in anaerobic and microaerobic bacteria and some invertebrates, but the biological function of many bacterial hemerythrins is unknown (French *et al*., 2008). FedA contains a conserved domain that has been shown in a few characterized bacterial hemerythrins to bind iron and oxygen (Karlsen *et al*., 2005; Kao *et al*., 2008). As such, hemerythrins of anaerobes and microaerobes are predicted to function in biological processes involving iron or oxygen. Thus, we hypothesize that FedA may be involved in one or more iron- or oxygen-dependent activities for *C. jejuni* during commensalism or invasion. FedC contains a C-terminal region with homology to a DnaJ domain. Proteins with DnaJ domains often interact with DnaK or similar proteins to serve as a cochaperone in the Hsp70 chaperone machine, which assists in protein folding or degradation in bacteria (Genevaux *et al*., 2007). Mutants lacking the DnaJ-like cochaperone component of an Hsp70 chaperone system are often sensitive to thermal or oxidative stress. Due to the increased body temperature of avian species compared to humans (42 °C versus 37 °C), one possibility is that FedC may be required for folding or maintaining stability of one or more specific proteins essential for colonization of poultry. FedB and FedD do not share homology with any proteins of known function. Although we have ruled out *in vitro*

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

Our work uncovered some significant findings for CiaI that differed from prior investigations regarding its expression, production, and importance in *C. jejuni* biology. Previous work suggested that transcription of *ciaI* and production of the encoded protein are induced by DOC in *C. jejuni* strain F38011 (Malik-Kale *et al*., 2008; Buelow *et al*., 2011). Furthermore, secretion of CiaI was previously determined to be both flagellum- and serum-dependent (Buelow *et al*., 2011). A defect in invasion of human intestinal epithelial cells of a *ciaI* mutant was hypothesized to be due to CiaI localizing to and influencing development of *C. jejuni*-containing vacuoles for intracellular survival (Buelow *et al*., 2011). Ectopic production of CiaI in HeLa cells suggested that a dileucine motif in CiaI is essential for the protein to localize to vesicles and perhaps *C. jejuni*-containing vacuoles.

In this study, we discovered specific details regarding the regulated transcription and production of CiaI. First, we found that σ^{28} and the flagellar regulatory system are responsible for approximately 60 - 85% of the transcription of *ciaI* in *C. jejuni* 81-176. Consistent with this finding, we identified a σ^{28}-dependent transcriptional start site and at least one possible σ^{28}-independent transcriptional start site. Expression from neither promoter was induced upon growth in the presence of DOC, which counters a previous study that indicated *ciaI* transcription was increased when *C. jejuni* strain F38011 was grown with DOC (Rivera-Amill *et al*., 2001). Instead, we discovered that translation of CiaI was induced by DOC specifically from the σ^{28}-independent transcript. These results suggest that a majority of CiaI is dependent on σ^{28} and the flagellar regulatory system for production, but not DOC. In addition, residual CiaI is produced by a DOC-dependent mechanism that influences translation of a *ciaI* mRNA that originates independently of σ^{28} and the flagellar regulatory system. It is interesting to speculate that DOC-dependent production of CiaI may be important for the bacterium *in vivo* when the flagellar regulatory cascade may be inactive and not promote expression of the σ^{28} regulon. Whether translation, rather than transcription of other Cia proteins, such as CiaB and CiaC, are induced by DOC remains to be determined. In additional analysis, we did not observe DOC to be required for production of any other σ^{28}-dependent protein. Consistent with previous analysis of secretion of FspA1, we found that CiaI and FedB also did not require serum to be secreted in a flagellum-dependent manner in *C. jejuni* 81-176 (Poly *et al*., 2007). These results suggest that serum is not universally required for flagellum-dependent secretion among *C. jejuni* strains.

Our work also provides new insights into the role of CiaI in the biology of *C. jejuni*. We found that CiaI is a commensal colonization determinant as the Δ*ciaI* mutant displayed 16- to over a 100-fold defects in cecal colonization of chicks. We were able to verify a mild, two-fold defect at an early step in invsion previously reported for the Δ*ciaI* mutant in a different strain of *C. jejuni* (Buelow *et al*., 2011). However, the $ciaI_{LL153\text{-}154AA}$ mutant did not demonstrate an invasion defect at 6 h post-infection, which suggest that the dileucine motif of CiaI previously determined to be required for vesicular-localization of the protein may not influence invasion. In addition, alteration of the dileucine motif did not influence the ability of *C. jejuni* to promote commensal colonization of chicks. We did note a mild invasion and colonization defect when the putative nucleotide-binding motif of CiaI was altered, but these defects of the $ciaI_{K42A}$ mutant were not as severe as the Δ*ciaI* mutant. As a note, we were unable to verify an *in vitro* nucleotide-binding or -hydrolysis activity of recombinant CiaI. Even though CiaI appears to be involved in invasion of human intestinal cells, we believe that CiaI is likely required for a different function in commensalism. During colonization of chicks, *C. jejuni* primarily localizes to the mucosa layer atop the cecal and intestinal epithelium, with little invasion of epithelial cells evident (Beery *et al*.,

1988). As a commensal in the intestinal tract of the natural avian host, we do not expect CiaI to be influencing an invasion mechanism of invasion for *C. jejuni*. The role of CiaI in colonization of poultry remains to be fully elucidated.

We also noticed a curious requirement for FlaA, the major flagellin of the flagellar filament of *C. jejuni*, in the production, stability or secretion of multiple proteins encoded by σ^{28}-dependent genes. We found that both CiaI and FedB are dependent on FlaA for secretion. In the absence of FlaA, both proteins remain stably associated with the bacterium. We also found that FspA1 requires FlaA for either production or stability. As a result, FspA1 is not found in the whole-cell lysate or in the secreted protein fraction in the absence of FlaA. Lastly, the 0996 protein, which we and others did not find to be secreted (Goon *et al*., 2006), requires FlaA for either production or stability in *C. jejuni*. These results suggest an additional function of FlaA outside of its role as a flagellin composing the filament for flagellar motility. Currently, it is unknown if the effect of FlaA on these proteins is direct or indirect. For instance, it is possible that FlaA may need to be secreted first to alter the flagellar secretory system for secretion of other σ^{28}-dependent proteins. Alternatively, these proteins may directly complex with FlaA for increased stability or secretion or FlaA may be involved in a mechanism influencing translation of the proteins. It is intriguing to speculate that in addition to being a secreted flagellin, FlaA may have some chaperone activity for other σ^{28}-dependent proteins. This latter possibility may provide a reason why these proteins are part of the σ^{28} regulon and co-expressed with FlaA.

Previous work from our group identified a requirement of flagellar components for proper spatial regulation of division (Balaban & Hendrixson, 2011). In this work, we continued to expand our understanding of the requirements of flagella and the flagellar regulatory system in the biology of *C. jejuni*. This study demonstrates that the flagellar regulatory system of *C. jejuni* is directly required for expression of genes essential for broad biological functions, such as motility, commensalism and virulence. Furthermore, we established the Fed proteins and CiaI as a new class of colonization factors co-expressed with flagella. Due to the dependence of many of these proteins on FlaA for stability or secretion, our findings suggest a possible new function for the major flagellin in *C. jejuni*. Continued exploration will undoubtedly further contribute to our understanding of the global requirement of flagella for many diverse aspects of *C. jejuni* biology.

Materials and Methods

Bacterial strains and plasmids

All strains and plasmids used in this study are listed in Tables S2 and S3 in Supporting Information. *C. jejuni* strain 81-176 was originally isolated from a patient with gastroenteritis (Korlath *et al*., 1985). Subsequent studies verified the capacity of this strain to infect human volunteers and promote commensal colonization of chicks (Black *et al*., 1988; Hendrixson & DiRita, 2004). *C. jejuni* was typically grown in microaerobic conditions (85% N_2, 10% CO_2, 5% O) on Mueller-Hinton (MH) agar or in MH broth at 37 °C. As required, antibiotics were added to MH media at the following concentrations: 10 μg/ml trimethoprim (TMP), 20 μg/ml chloramphenicol, 50 μg/ml kanamycin, 30 μg/ml cefoperazone or 0.5, 1, 2, or 5 mg/ml streptomycin. All *C. jejuni* strains were stored at -80 °C in a 85% MH broth and 15% glycerol solution. For routine growth to perform most experiments, *C. jejuni* strains were grown from frozen stocks for 48 h in microaerobic conditions at 37 °C, then streaked on MH agar and grown for additional 16 h in identical conditions. *Escherichia coli* DH5α, XL1-Blue and BL21 strains were grown on Luria-Bertani (LB) agar or in LB broth containing 100 μg/ml ampicillin, 50 μg/ml kanamycin or 15 μg/ml chloramphenicol as appropriate. All *E. coli* strains were stored at -80 °C in a 80% LB broth and 20% glycerol solution.

Construction of mutants

C. jejuni mutants were constructed by electroporation following previously described methods (Hendrixson *et al*., 2001). For cloning of each gene to be deleted from the *C. jejuni* 81-176 Sm^R (DRH212) chromosome, DNA fragments containing approximately 750 bases upstream and downstream of each gene were amplified by PCR using primers containing 5' BamHI restriction sites. Each fragment was then cloned into the BamHI site of pUC19 to create the following plasmids: pDRH3022 (pUC19::*fspA1*), pABT253 (pUC19::*Cjj81176_0996*), pABT115 (pUC19::*ciaI*), pABT249 (pUC19::*fedA*), pABT113 (pUC19::*fedB*), pABT328 (pUC19::*fedC*), and pABT111 (pUC19::*fedD*) (Table S3). To create restriction sites within the coding sequence of some genes, point mutations were introduced into plasmids by PCR-mediated mutagenesis (Makarova *et al*., 2000). These reactions created an EcoRV site in *fspA1* (pDRH3025), a PmeI site in *fedA* (pABT322), and StuI sites in *Cjj81176_0996* (pABT329), *fedC* (pABT355), and *fedD* (pABT152). A SmaI-digested *cat-rpsL* cassette was obtained from pDRH265 and ligated into plasmids in the appropriate restriction sites to interrupt each gene (Table S3; (Hendrixson *et al*., 2001)).

Each plasmid generated above was electroporated into *C. jejuni* 81-176 Sm^R (DRH212) to interrupt each respective gene on the chromosome with the *cat-rpsL* cassette. Transformants were recovered on MH agar containing chloramphenicol. Mutations were verified by colony PCR and the following isogenic mutants of 81-176 Sm^R were obtained: ABT103 (*fspA1*::*cat-rpsL*), ABT366 (*Cjj81176_0996*::*cat-rpsL*), ABT214 (*ciaI*::*cat-rpsL*), ABT353 (*fedA*::*cat-rpsL*), ABT261 (*fedB*::*cat-rpsL*), ABT370 (*fedC*::*cat-rpsL*), and ABT233 (*fedD*::*cat-rpsL*) (Table S3).

PCR-mediated mutagenesis was employed to construct in-frame deletions of genes that were originally cloned into pUC19 (see above and Table S3; (Makarova *et al*., 2000)). After sequencing to verify correct construction of in-frame deletions, the following plasmids were obtained: pABT205 (pUC19::Δ*fspA1*), pABT325 (pUC19::Δ*Cjj81176_0996*), pABT173 (pUC19::Δ*ciaI*), pABT280 (pUC19::Δ*fedA*), pABT357 (pUC19::Δ*fedB*), pABT356 (pUC19::Δ*fedC*), and pABT164 (pUC19::Δ*fedD*) (Table S3). These plasmids were electroporated into strains containing *cat-rpsL* interruptions of the respective genes on the chromosome. Transformants were recovered on MH agar with 0.5, 1, 2 or 5 mg/ml of streptomycin and then screened for chloramphenicol sensitivity. Deletion of each gene was verified by colony PCR, which resulted in creation of the following 81-176 Sm^R mutant strains: ABT361 (Δ*fspA1)*, ABT501 (Δ*Cjj81176_0996*), ABT279 (Δ*ciaI*), ABT477 (Δ*fedA*), ABT473 (Δ*fedB*), ABT472 (Δ*fedC*), and ABT278 (Δ*fedD*).

Creation of 81-176 Sm^R Δ*astA* Δ*flgM* was accomplished by electroporation of 81-176 Sm^R Δ*astA* (DRH461) with pDRH552 to interrupt *flgM* on the chromosome with a *cat-rpsL* cassette (Hendrixson & DiRita, 2003). Transformants were recovered on MH agar containing chloramphenicol. Colony PCR verified interruption of *flgM* with the *cat-rpsL* cassette, resulting in 81-176 Sm^R Δ*astA flgM*::*cat-rpsL* (DRH557). This strain was then electroporated with pDRH565 to replace *flgM*::*cat-rpsL* with the in-frame Δ*flgM* mutation (Hendrixson & DiRita, 2003). Transformants were recovered on MH agar containing 0.5, 1, 2 or 5 mg/ml of streptomycin and then screened for chloramphenicol sensitivity. Deletion of *flgM* on the chromosome was verified by colony PCR to result in creation of 81-176 Sm^R Δ*astA* Δ*flgM* (DRH604).

To create plasmids containing *astA* transcriptional fusions to genes of interest, a SmaI-digested *astA-kan* cassette from pDRH580 was ligated into the EcoRV site of *fspA1* in pDRH3025, the EcoRV site of *ciaI* in pABT115, the HpaI site of *fliA* in pDRH263, and the StuI site of *fedD* in pABT152 (Hendrixson & DiRita, 2003). As a result, *astA* transcriptional fusions were created in the following plasmids: pABT405 (*fliA*::*astA-kan*), pDRH3027

(*fspA1*::*astA-kan*), pABT119 (*ciaI*::*astA-kan*), and pABT236 (*fedD*::*astA-kan*). These plasmids were then electroporated into 81-176 Sm^R Δ*astA* (DRH461) and isogenic strains lacking σ^{28}, FlgM, or components of the σ^{54} regulatory pathway. Transformants were recovered on MH agar containing kanamycin and acquisition of the *astA* transcriptional reporter at the native locus on the chromosome of each gene was verified by colony PCR (Table S2).

PCR-mediated mutagenesis was used to introduce point mutations in the coding sequence of *ciaI* in pABT115 to result in pABT673 (pUC19::$ciaI_{K42A}$) and pABT674 ($ciaI_{LL153\text{-}154AA}$) (Makarova *et al*., 2000). These plasmids were electroporated into ABT214 to replace *ciaI*::*cat-rpsL* on the chromosome with genes encoding the mutant proteins. Transformants were recovered on MH agar containing 0.5, 1, 2 or 5 mg/ml of streptomycin and screened for chloramphenicol sensitivity. Putative transformants were verified by colony PCR and sequencing to result in 81-176 Sm^R $ciaI_{K42A}$ (ABT704) and 81-176 Sm^R $ciaI_{LL153\text{-}154AA}$ (ABT706).

Gene expression analysis

Arylsulfatase assays were employed to measure the level of expression of *astA* transcriptional fusions located on the chromosome of *C. jejuni* strains as previously described (Henderson & Milazzo, 1979; Yao & Guerry, 1996; Hendrixson & DiRita, 2003). Each strain was analyzed in triplicate and each assay was performed three times. The level of expression of each transcriptional fusion in mutant strains was calculated relative to the expression in wild-type *C. jejuni* 81-176 Sm^R Δ*astA*, which was set to 100 units.

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

Primer extension analyses

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

and analyzed using a Storm 820 Phosphorimager according to manufacturer's instructions (Amersham Biosciences).

Motility Assays

C. jejuni strains were suspended from plates in MH broth and diluted to an OD_{600} 0.8. Each bacterial strain was stabbed into semisolid MH motility media containing 0.4% agar using an inoculation needle. The motility of each strain was tested six times. The plates were incubated for 30 h at 37 °C in microaerobic conditions and the area of the motility zone for each strain was calculated and averaged. For darkfield microscopy, the cultures were further diluted 1:10 in MH broth. Strains were immediately analyzed for motility by applying 3 μl of culture between a glass slide and glass coverslip.

Analysis of *in vitro* growth

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

Chick colonization assays

The ability of wild-type or mutant *C. jejuni* 81-176 Sm^R strains to colonize the ceca of chicks after oral inoculation was determined as previously described (Hendrixson & DiRita, 2004). Briefly, fertilized chicken eggs (SPAFAS) were incubated for 21 d at 37.8 °C with appropriate humidity and rotation in a Sportsman II model 1502 incubator (Georgia Quail Farms Manufacturing Company). Approximately 12 to 36 h after hatching, chicks were orally infected with 100 μl of PBS or MH broth containing approximately 10^2 or 10^4 cfu of a single wild-type *C. jejuni* or mutant strain. To prepare strains for infection, *C. jejuni* strains were suspended from plates after growth at 37 °C in microaerobic conditions and diluted in PBS or MH broth to obtain the appropriate inoculum for oral gavage of chicks. Dilutions of the inocula were spread on MH agar to determine the number of bacteria in each inoculum. Seven days post-infection, chicks were sacrificed and the cecal contents were recovered and suspended in PBS or MH broth. Serial dilutions were spread on MH agar containing TMP and cefoperazone. Bacteria were grown for 72 h at 37 °C in microaerobic conditions and then counted to determine the cfu per gram of cecal contents.

In vitro invasion assays

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

trypsin and the cells were disrupted by repeated pipetting. Serial dilutions were then spread on MH agar. After incubation for 72 h at 37 °C in microaerobic conditions, the number of internalized bacteria were determined. Percent invasion was determined by dividing the number of internalized bacteria by the number of bacteria in the inoculum.

Generation of antisera

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

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

For purification of recombinant CiaI, primers containing 5' in-frame BamHI restriction sites were used. In addition, one of the primers contained an in-frame 6XHis-tag to the last codon of *ciaI*. The BamHI-digested PCR product was cloned into pMal-C2x to create pABT613, which encoded a MBP-CiaI-6XHis tag protein. The plasmid was transformed into *E. coli* BL21 (DE3) cells for protein production and purification from the soluble fraction with amylose resin according to manufacturer's instructions (New England Biolabs). Each protein was then used to immunize five mice for production of polyclonal antisera (Cocalico Biologicals, Inc).

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

C. jejuni strains were suspended from MH agar plates into MH broth to an OD_{600} of 0.6. For each strain, 20 ml of diluted culture were incubated at 37 °C in microaerobic conditions without shaking for 4 h. At the end of the incubation period, final OD_{600} measurements were obtained. For preparation of proteins from whole-cell lysates (WCL), 1 ml of culture for each strain was pelleted in a microcentrifuge at full speed for 3 min, washed once with PBS and resuspended in 25 μl of PBS and 25 μl of 2X SDS-PAGE loading buffer for a total volume of 50 μl. For recovery of supernatant proteins, the remaining 19 ml of culture were centrifuged for 30 min at 13,000 rpm. The supernatants were recovered and the centrifugation step was repeated to ensure removal of all bacteria. Proteins present in the supernatant were precipitated by combining 18 ml of supernatant with 2 ml of trichloroacetic acid (TCA; 10% final concentration) followed by a 30 min incubation on ice. Precipitated proteins were recovered by centrifugation for 15 min at 10,000 rpm. The protein pellets were rinsed with 0.5 ml of cold acetone and dried. Precipitated proteins were resuspended in 20 μl of 1M Tris, pH 8.0 and 20 μl of 2X SDS-PAGE loading buffer.

All protein samples were boiled for 5 min prior to loading on 12.5% SDS-PAGE gels. For wild-type *C. jejuni* WCL, 10 μl of WCL (representing 200 μl of culture) were analyzed for detection of all proteins except FspA1 and FedB. For these latter proteins, 15 μl (for analysis of FspA1) and 2.5 μl (for analysis of FedB) of WCL were loaded onto 12.5% SDS-PAGE

gels. The volumes of WCL of mutant strains loaded onto gels were normalized based on the final OD_{600} readings of wild-type and mutant strains to ensure analysis of equal amounts of proteins between strains. For supernatant samples, 10 μl of precipitated proteins were separated by 12.5% SDS-PAGE. Duplicate 12.5% SDS-PAGE gels were analyzed by silver staining to verify equal loading of proteins. For immunoblot analysis, primary murine antisera was used at the following concentrations to detect proteins: α-FspA1 M140, 1:1500 and 1:2000 for WCL and supernatant proteins, respectively; α-Cjj81176_0996 M151, 1:2000; α-CiaI M154, 1:2000; and α-RpoA M59, 1:2500 (Sommerlad & Hendrixson, 2007). For detection of FedB, polyclonal rabbit antiserum donated by Dr. Patricia Guerry (Naval Medical Research Center) was used at a dilution of 1:10,000 or 1:7500 for WCL and supernatant proteins, respectively. A 1:10,000 dilution of a horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit antiserum (Bio-Rad) was used as the secondary antibody. Immunoblots were developed by using the Western Lightning Plus ECL kit (Perkin-Elmer).

Statistical analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Deborah Ribardo for assistance in chick colonization experiments. This work was supported by NIH grant R01AI065539 and associated research supplements 3R01AI065539-05S1 and 3R01AI065539-06S1.

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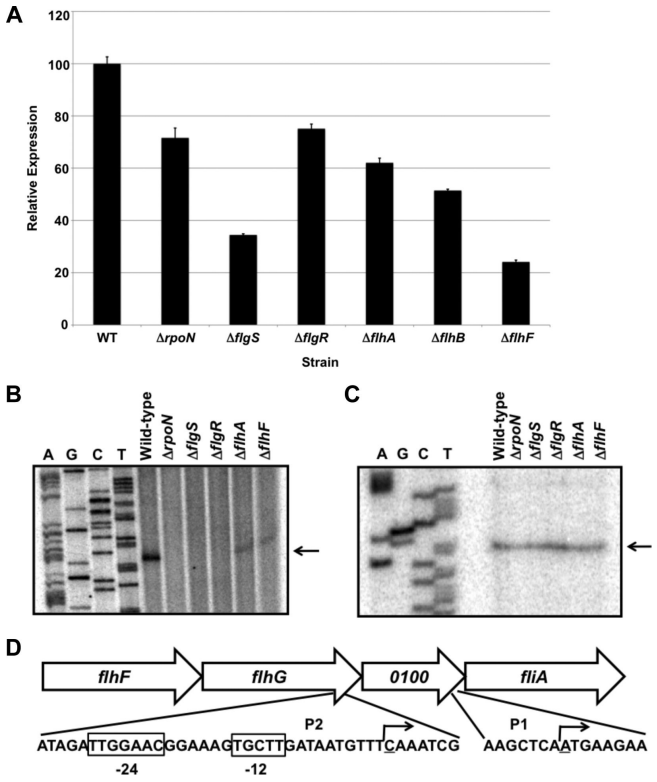

Figure 1. Analysis of *fliA* expression and transcriptional start sites in wild-type and mutant *C. jejuni* strains

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

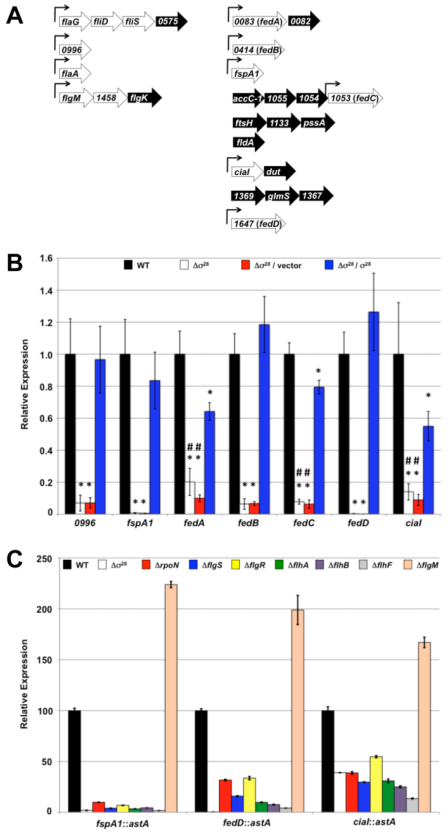

Figure 2. Identification and analysis of expression of the *C. jejuni* σ^{28} regulon

(A) Individual genes and operons examined for potential dependence on σ^{28} for expression. Genes and operons were selected for investigation based on a previous microarray analysis that suggested at least partial dependence on σ^{28} and FlhA, a flagellar T3SS protein and component of the σ^{54} regulatory pathway, for expression (Carrillo *et al*., 2004). Numerical annotation of each gene and location of genes in potential operons are based on the *C. jejuni* 81-176 genome (Fouts *et al*., 2005). Genes in the left column are homologous to known flagellar genes or have been shown to be involved in flagellar motility of *C. jejuni*. Genes to the right are not homologous to any known flagellar genes. Genes in white were verified in this work to be at least partially dependent on σ^{28} for expression in *C. jejuni* 81-176 Sm^{R}. Genes in black were not found to depend on σ^{28} for wild-type levels of expression. Bent arrows indicate location of consensus σ^{28}-binding sites in the promoter for each gene or operon. (B) Semi-quantitative real time RT-PCR (qRT-PCR) analysis of expression of *0996*, *fspA1*, the *fed* genes, and *ciaI* in wild-type *C. jejuni* 81-176 Sm^{R} (black) and an isogenic Δσ^{28} mutant (white). Also included for analysis were the Δσ^{28} mutant strains complemented with vector alone (pECO102; red) or pECO102 expressing σ^{28} *in trans* (blue). Expression of each gene in each mutant is relative to the expression in wild-type *C. jejuni*, which was set to 1. All strains were examined in triplicate and the error bars indicate the standard error. Statistically-significant differences in gene expression between wild-type *C. jejuni* and Δσ^{28} mutant strains with or without σ^{28} *in trans* are indicated (* *P*-value < 0.05). Statistically-significant differences in gene expression between *C. jejuni* Δσ^{28} with σ^{28} expressed *in trans* and the Δσ^{28} mutants alone or with empty vector are indicated (# *P*-value < 0.05). (C) Arylsulfatase assays measuring expression of *fspA1::astA*, *fedD*::*astA*, and *ciaI*::*astA* transcriptional fusions in wild-type *C. jejuni* or mutant strains lacking σ^{28}, FlgM, or a component of the σ^{54} regulatory pathway. Strains used in analysis include: wild-type *C. jejuni* 81-176 Sm^{R} (black) and isogenic mutants lacking σ^{28} (white), *rpoN* (encoding σ^{54};

red), *flgS* (blue), *flgR* (yellow), *flhA* (green), *flhB* (purple), *flhF* (grey), and *flgM* (peach). The amount of expression of the transcriptional reporter in each mutant is relative to wild-type *C. jejuni*, which was set to 100 units. Error bars indicate the standard error of the average arylsulfatase activity analyzed from three samples. The reporter activities in each mutant were significantly different from the activity in the wild-type strain (*P*-value < 0.05).

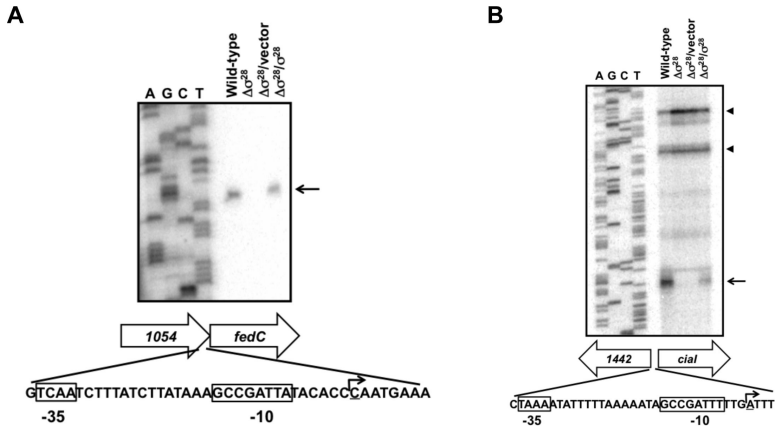

Figure 3. Identification of transcriptional start sites for *fedC* and *ciaI*

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

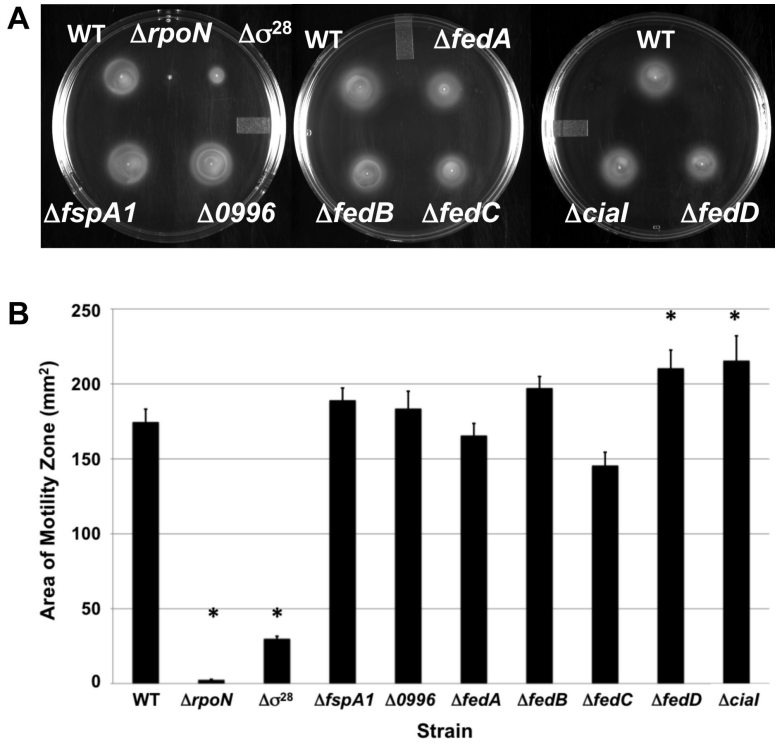

Figure 4. Motility phenotype of mutants lacking σ^{28}-dependent genes

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

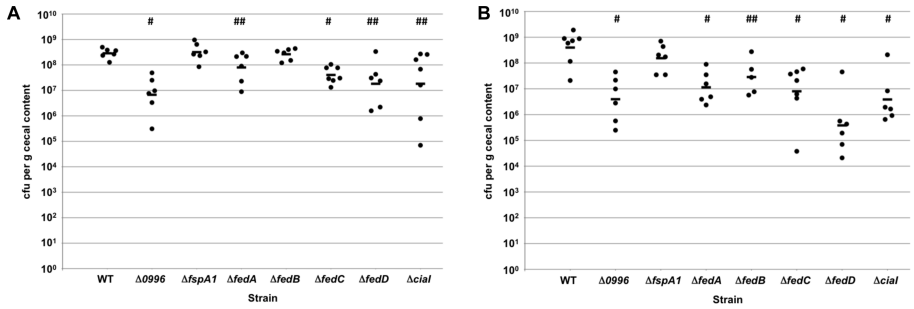

Figure 5. Commensal colonization capacity of wild-type *C. jejuni* and mutant strains

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

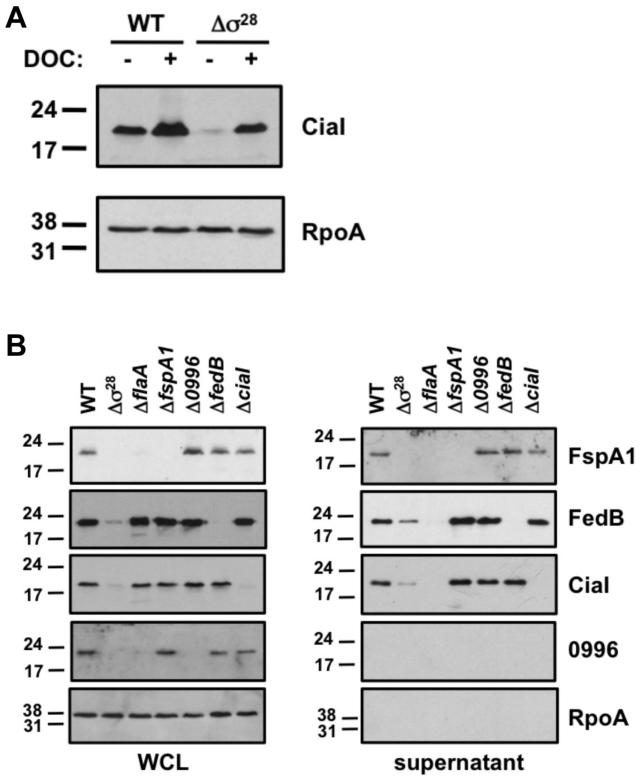

Figure 6. Analysis of production and secretion of CiaI and other σ^{28}-dependent proteins

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

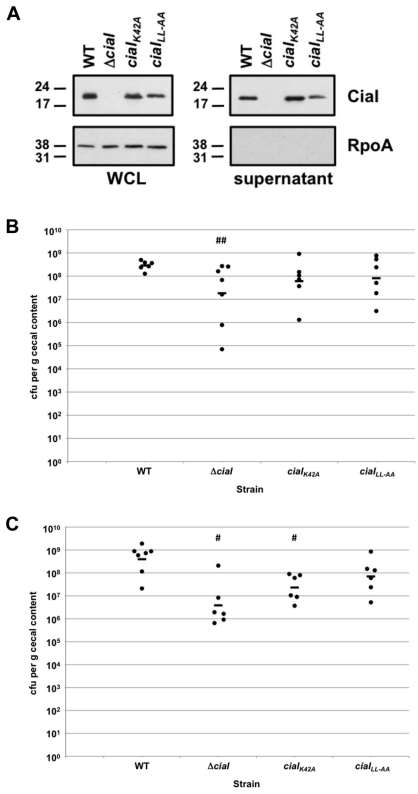

Figure 7. Analysis of the commensal colonization capacity of *C. jejuni ciaI* mutants

(A) Production of CiaI proteins in bacteria and supernatants after growth in MH broth. Wild-type *C. jejuni* 81-176 Sm^R and isogenic mutants lacking *ciaI*, or containing $ciaI_{K42A}$ or $ciaI_{LL153\text{-}153AA}$ ($ciaI_{LL\text{-}AA}$) were inoculated in MH broth and grown for 4 h at 37 °C in microaerobic conditions. Proteins from whole-cell lysates (WCL) and supernatants were recovered and examined by immunoblot analysis using antiserum specific CiaI or RpoA. For both WCL and supernatants, equal amount of proteins were loaded across strains. Molecular weight markers are indicated in kDa. (B and C) Commensal colonization capacity of wild-type *C. jejuni* 81-176 Sm^R and *ciaI* mutants. One-day old chicks were orally inoculated with 10^4 cfu (B) or 10^2 cfu (C) of *C. jejuni* strains. Each dot represents the amount of *C. jejuni* recovered from the ceca of each chick seven days post-infection. The geometrical mean for each group is depicted by the horizontal bar. Statistical analysis was performed using the Mann-Whitney U test (## $P < 0.05$; # $P < 0.01$). For wild-type *C. jejuni* and the Δ*ciaI* mutant, the same data is shown as in Figure 5A and 5B.

Table 1

Invasion capacity of wild-type *C. jejuni* 81-176 Sm^R and mutant strains for T84 colonic cells.

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

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

*P-value < 0.05.

Table 2

Invasion capacity of wild-type *C. jejuni* 81-176 Sm^R and *ciaI* mutant strains for T84 colonic cells.

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

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

* *P*-value < 0.05.