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Genome-wide Expression Analyses of *Campylobacter jejuni* NCTC11168 Reveals Coordinate Regulation of Motility and Virulence by *flhA**[S]

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We examined two variants of the genome-sequenced strain, *Campylobacter jejuni* NCTC11168, which show marked differences in their virulence properties including colonization of poultry, invasion of Caco-2 cells, and motility. Transcript profiles obtained from whole genome DNA microarrays and proteome analyses demonstrated that these differences are reflected in late flagellar structural components and in virulence factors including those involved in flagellar glycosylation and cytolethal distending toxin production. We identified putative σ^{28} and σ^{54} promoters for many of the affected genes and found that greater differences in expression were observed for σ^{28}-controlled genes. Inactivation of the gene encoding σ^{28}, *fliA*, resulted in an unexpected increase in transcripts with σ^{54} promoters, as well as decreased transcription of σ^{28}-regulated genes. This was unlike the transcription profile observed for the attenuated *C. jejuni* variant, suggesting that the reduced virulence of this organism was not entirely due to impaired function of σ^{28}. However, inactivation of *flhA*, an important component of the flagellar export apparatus, resulted in expression patterns similar to that of the attenuated variant. These findings indicate that the flagellar regulatory system plays an important role in campylobacter pathogenesis and that *flhA* is a key element involved in the coordinate regulation of late flagellar genes and of virulence factors in *C. jejuni*.

Campylobacter jejuni is a significant food- and water-borne pathogen (1, 2). Campylobacter infection in humans occurs primarily through the consumption of contaminated poultry products (3). Although little is known about how the organism causes disease, it is evident that the bipolar flagella are one of the most important virulence factors (4). *C. jejuni* is able to move through viscous environments at speeds up to 75 μm/s and demonstrates an increased ability to adhere and invade intestinal cells under these conditions (5). Flagellin is also the immunodominant antigen during human and animal infection and is absolutely required for colonization *in vivo* (4).

Recently, it was demonstrated that campylobacters glycosylate their flagellar filaments with *O*-linked pseudaminic acid monosaccharides and modified derivatives (6, 7). Complete loss of the glycosyl moieties causes intracellular accumulation of the flagellin monomeric subunits resulting in loss of motility (8), whereas variation in the pseudaminic acid derivatives enables immune evasion (4). Several genes involved in the biosynthesis of these unusual sugars have been identified in the 50-kb flagellar gene locus in *C. jejuni* NCTC11168 (4, 6, 7, 9) and have been recently reviewed (10).

The production of flagella in bacteria requires significant energy expenditure; thus, regulation of flagellar structural gene expression is important. The initiation of transcription by sigma factors is a key step in bacterial gene regulation. Unlike *Bacillus subtilis* with 14 sigma factors or *Escherichia coli* with seven sigma factors, only three sigma factors (σ^{28}, σ^{54}, and σ^{70}) were identified in the genome sequence of *C. jejuni* NCTC11168 (11, 12), suggesting that certain pathways in this organism may be coordinately regulated. *rpoD* encodes σ^{70}, which is involved in regulating expression of housekeeping genes in *C. jejuni* (13), whereas a number of flagellar genes are regulated by the alternative sigma factors, *rpoN* (σ^{54}) and *fliA* (σ^{28}) (14–17).

In Gram-negative bacteria, flagellar biosynthesis is regulated in a hierarchical cascade, with genes expressed in the order in which they are required for the assembly of the flagellum (reviewed in Refs. 18 and 19). In peritrichous organisms, such as those belonging to the family of *Enterobacteriaceae*, *flhC* and *flhD* are the master regulators of the flagellar cascade, acting on class II promoters. Class II genes encode proteins forming part of the hook and basal body complex, the flagellar export complex, and σ^{28}. Class III genes encode flagellin proteins required for extension of the flagellar filament. Within this hierarchy, there is feedback regulation to ensure that the order of flagellar gene expression is maintained.

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[S] The on-line version of this article (available at http://www.jbc.org) contains six additional tables.

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In polar flagellated organisms, such as *C. jejuni*, *flhCD* is absent. In *C. jejuni* and *Helicobacter pylori*, *flgR* (an NtrC homologue) is required to activate class II flagellar genes with σ^{54} promoters (14, 20, 21). As in the enterobacteriaceae, the major flagellin, *flaA*, belongs to class III and is controlled with a σ^{28} promoter in both *C. jejuni* and *H. pylori*, and in both of these organisms, the minor flagellin, *flaB*, is controlled by σ^{54}. Additional flagellar biosynthetic and modification genes regulated by these alternate sigma factors in *C. jejuni* include the σ^{28}-regulated *flaG* (22, 23) and the σ^{54}-regulated *flgD*, *flgE*, and Cj1293 (8, 15–17, 24). In some organisms, including *H. pylori*, *flgM* homologues provide negative feedback on σ^{28} (25). In contrast, *flgM* does not appear to be a strong repressor of σ^{28}-regulated genes in *C. jejuni* (21). To date, the regulation and components of the *C. jejuni* flagellar transcriptional hierarchy have not been well characterized.

An integral part of flagellar biosynthesis is the ability to export flagellar structural components. A key component of the flagellar export apparatus is FlhA, which belongs to the FHIPEP (flagellar/Hr/invasion proteins export pore) family of bacterial export proteins involved in flagellar assembly and type III secretion. FlhA has been shown to coordinately regulate both motility and virulence in organisms such as *Bacillus*, *Helicobacter*, and *Pseudomonas* (26–28). Recent transposon mutagenesis studies in *C. jejuni* demonstrate that inactivation of *flhA* leads to loss of FlaA expression, motility, autoagglutination, and invasion (29). Another key component involved in flagellar protein export is FlhB, which associates with FlhA and is involved in determining substrate specificity (30). In *C. jejuni*, FlhB has also been shown to be required for production of flagella and motility (29, 31).

In this study, we compare two sources of the genome-sequenced strain, NCTC11168, which exhibit very different virulence properties including motility. Gene expression analyses indicate that these differences are largely due to changes in expression of both σ^{28}- and σ^{54}-regulated genes. We now provide evidence that *flhA* plays an important role in the regulation of both classes of genes. Furthermore, we provide a model for the unique flagellar regulatory network of *C. jejuni* and demonstrate that flagellar biosynthesis is coordinately regulated with virulence in this organism.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—*C. jejuni* NCTC11168 (HS:2) was originally isolated from a case of human enteritis in 1977 (32) and later sequenced by Parkhill *et al.* (12). NCTC11168 V1 was purchased from ATCC (catalog no. 700819) in 2000 and is representative of the original clinical isolate. NCTC11168 V26 was obtained in 1977 and has been a laboratory-passaged strain since its initial isolation. *E. coli* DH10B (Invitrogen) was used as the host for the cloning experiments. Plasmid pPCR-Script Amp (Stratagene) or pGEM®-T Easy (Promega) was used as the cloning vector.

C. jejuni NCTC11168 V1 and V26 were routinely grown using Mueller Hinton agar (Difco) under microaerophilic conditions (10% CO_2, 5% O_2, 85% N_2) at 37 °C. *E. coli* DH10B clones were grown on S-Gal™/LB agar (Sigma) or Mueller Hinton agar at 37 °C. When appropriate, antibiotics were added to the following final concentrations: 30 μg/ml kanamycin and 150 μg/ml ampicillin.

Construction and Characterization of Insertional Mutations—For construction of the *fliA* mutant, genes *fliM* (Cj0060c) to Cj0062c were PCR-amplified from V1 (primer sequences available upon request) and cloned as described previously (33). A blunt-ended kanamycin resistance cassette from pILL600 (34) was inserted into the HpaI restriction site of *fliA*, generating pRV*fliA*. For the *flhA* mutant, genes Cj0881c to Cj0883c were amplified from V1. The kanamycin resistance cassette was inserted into the NcoI site, generating pPL*flhA*. The orientation of both cassettes was determined to be nonpolar by sequencing. pRV*fliA* and pPL*flhA* were electroporated into V1 (35), and the kanamycin-resistant transformants were characterized by PCR to confirm that the incoming plasmid DNA had integrated by a double crossover recombination event. It has previously been shown that gene insertion of the campylobacter kanamycin resistance cassette in a nonpolar orientation has no effect on transcription of downstream genes (36). Furthermore, transcript profiling by microarray analysis (see below) showed transcripts for the downstream genes: *fliM* (Cj0060c) in the *fliA* mutant and Cj0881c in the *flhA* mutant.

Sequencing—DNA was sequenced using terminator chemistry and AmpliTaq cycle sequencing kits (Applied Biosystems) and analyzed on an Applied Biosystems 373 DNA sequencer. Primers used for sequencing are available upon request.

Colonization of 1-Day-old Chicks—Colonization of 1-day-old chicks was done as described by Stern *et al.* with some modifications (37). Leghorn chicks were obtained from the hatchery at the Department of Animal and Poultry Science, University of Saskatchewan on the day of hatch. They were randomly assigned into groups of 20–25 birds and provided with feed and water *ad libitum*. Birds were cared for in accordance with the approved guidelines of the Canadian Council for Animal Care.

In the standard model, five birds in each group of 25 birds were tested for colonization by *C. jejuni* before the challenge. Then all birds in the group were orally challenged with the indicated dose of *C. jejuni* in 0.5 ml of normal saline. Inocula for challenge experiments were produced by harvesting cells grown for 18 h into cold 0.85% (w/v) NaCl, diluting to the indicated concentration in normal saline, and maintaining on ice until immediately before use. The viable cell count was determined by plating serial dilutions onto Mueller Hinton agar, while colonization of the birds was monitored by culturing cloacal swabs on Karmali agar (Bacto). Birds were maintained for 7 days after challenge and then were euthanized by cervical dislocation. In most experiments cloacal swabs were done on day 7. Ceca were aseptically collected for qualitative as well as quantitative assessment of colonization.

To assess the ability of *C. jejuni* to colonize unchallenged birds that were placed in contact with orally challenged birds, we challenged 20% of the birds that were then cohabited with the remaining birds in the group. The challenged birds were marked so they could be readily identified. All birds were treated as described.

Caco-2 Cell Infection Assays—The human Caco-2 cell line was grown in Eagle's minimal essential medium (Sigma) supplemented with 1% (v/v) nonessential amino acids (Sigma) and 20% (v/v) fetal bovine serum (Sigma) at 37 °C with 5% CO_2. Cells were trypsinized and seeded at a density of 5×10^5 according to Oelschlaeger *et al.* (38). The next day, the Caco-2 cells were infected with 5 μl of an overnight culture of V1 and V26 adjusted to an $A_{600} = 0.2$ ($\sim 1 \times 10^8$ cells, multiplicity of infection = 200) with and without 5 min centrifugation at $200 \times g$. The plates were incubated for 2 h at 37 °C with 5% CO_2. In order to determine the total number of bacteria that adhered and invaded, Caco-2 cells were lysed with 0.01% Triton X-100 (J.T. Baker Chemical Inc.), and dilutions from each well were plated. To determine the levels of invasion, the Caco-2 cells were first treated with 100 μg/ml gentamicin for 2 h followed by Triton X-100 treatment. Under the latter conditions, only internalized bacteria would survive gentamicin treatment, and when this number is subtracted from the total count, the number of bacteria that adhered could be determined.

Electron Microscopy—A copper grid covered with formvar film and coated with carbon (Electron Microscopy Sciences) was floated on a drop of bacterial cells resuspended in phosphate-buffered saline for 5 min. The grids were then stained with 1% (w/v) ammonium molybdate for 1 min, air-dried, and examined with a Zeiss EM902 transmission electron microscope operated at an accelerating voltage of 80 kV. Images were recorded on 70-mm fine grain release film (Eastman Kodak Co.).

Isolation and Labeling of Total RNA and Genomic DNA for Microarray Analysis—*C. jejuni* cells were harvested after 15 h of growth and homogenized in Trizol (Invitrogen) by passing the mixture through a syringe. Nucleic acids (RNA and DNA) were isolated as recommended by the manufacturer. DNA and RNA amounts were quantified using the ND-1000 Spectrophotometer (Nanodrop).

Preparation of cDNA probes was performed according to the indirect labeling protocol adapted from Hughes *et al.* (39) with some modifications. Briefly, 15 μg of total RNA was reverse transcribed with Superscript II (Invitrogen) in 40 μl of 1× first strand synthesis buffer containing 15 μg of random octamers, 10 mM dithiothreitol, 500 μM deoxynucleoside triphosphate mix including equimolar amounts of aminoallyl-dUTP (Sigma) and dTTP and 40 units of RNAseOUT (Invitrogen). The reaction was incubated at 45 °C for 120 min and then at 95 °C for 5 min. RNA was degraded by the addition of NaOH to 0.3 M and incubation at 65 °C for 15 min, followed by neutralization with an equimolar amount of HCl. cDNA was purified using Microcon YM-30 columns (Millipore Corp.). Purified products were labeled with *N*-hydroxysuccinimide esters of Cy3 or Cy5 (CyDye; Amersham Biosciences) following the manufacturer's instructions. Unincorporated dye was re-

moved by purification through Qiaquick columns (Qiagen). Labeled sample yields and dye incorporation efficiencies were quantified spectrophotometrically.

Genomic DNA was restricted to an average size of 2–5 kb by nebulization in 35% (v/v) glycerol. After isopropyl alcohol precipitation, DNA was resuspended in 10 mM EDTA (pH 8.0), and 5 μg of DNA was fluorescently labeled using direct chemical coupling with the Label-IT (Mirus) cyanine dyes as recommended by the manufacturer. Probes were purified and quantified as described above.

Data Acquisition and Analysis of Microarray Data—The microarrays used in this study contain PCR amplicons representing 1454 of the 1634 *C. jejuni* NCTC11168 open reading frames described in the annotated genomic sequence (12). Details about the construction and content of the microarray are available on the World Wide Web at http://ibs-isb.nrc-cnrc.gc.ca/ibs/immunochemistry/campychips_e.html. Equivalent amounts of Cy3- and Cy5-labeled samples were pooled, lyophilized, and then resuspended in hybridization buffer (1× DIGEasy hybridization solution (Roche Applied Science) with 0.5 μg/μl yeast tRNA (Roche Applied Science) and 0.5 μg/μl of denatured salmon sperm genomic DNA (Roche Applied Science)). Probes were denatured at 65 °C for 5 min, cooled to room temperature, and applied to the microarray. Hybridizations were performed overnight at 37 °C under 24 × 42-mm glass coverslips (Fisher) in a high humidity chamber. Microarrays were washed at 50 °C for 2 × 10 min in 2× SSC, 0.1% SDS, 2 × 5 min in 0.5× SSC, and 1 × 5 min in 0.1× SSC. Slides were spun dry (1000 × *g*, 1 min) and stored in light-tight containers until scanning using a Chipreader (Bio-Rad). Spot quantification, signal normalization, and data visualization were performed using Array Pro Analyzer 4.5 (Media Cybernetics). Net signal intensities were obtained by performing local-ring background subtraction, and spots with a signal less than 2× background in both channels were excluded from the analysis. The mean signal intensities for triplicate spots were averaged, and data from each channel were adjusted by subarray normalization using cross-channel Loess regression. ArrayStat (Imaging Research) was used for statistical analysis of the replicated data. A proportional model with offsets, for dependent data, was selected, and statistical significance was determined using the pooled common error method with the false discovery rate multiple test correction (nominal $\alpha = 0.05$). For each experiment, 3–4 biological replicates were tested, including one comparison in which the Cy3 and Cy5 dyes were swapped to compensate for biases caused by differing chemical properties of the fluorescent dye molecules. Complete results for these experiments are available at the Gene Expression Omnibus Repository (NCBI, available on the World Wide Web at www.ncbi.nlm.nih.gov/geo/). The accession number for the data set is GSE708.

Quantitative Analysis of Gene Expression by Real Time PCR—cDNA was synthesized as described above from RNA samples used in microarray experiments, except RNA was DNase-treated (1 unit of DNase; Ambion) and aminoallyl-dUTP was replaced with dTTP. Real time PCR amplification of 1 μl of cDNA was performed in a reaction mixture containing the iQ SYBR green supermix (Bio-Rad), 0.5 pM concentration of the forward and reverse primers, and diethylpyrocarbonate-treated water. Real time PCR analysis was performed using an iCycler iQ detection system (Bio-Rad), with a PCR condition consisting of 2 min at 95 °C and then 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 60 s (primer sequences available upon request). The dye-labeled PCR products were quantified with an iCycler (Bio-Rad). The relative quantitation method (63) was used to calculate fold change where samples were normalized to *cdtB*, since this gene was not differentially expressed by microarray analysis. Reactions were done in duplicate, and three biological replicates were performed for each sample.

Extract Preparation for Comparative Proteomic Studies—Overnight cultures of V1 and V26 were centrifuged and the pellets washed twice with ice-cold 20 mM HEPES buffer, pH 7.5. The cells were resuspended directly in lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS,[1] and 10 μl/ml Protease Arrest (Calbiochem) and sonicated at 4 °C with a Fisher microtip model 550 sonicator, 6 × 30 s. After sonication, Benzonase (150 units/ml; Sigma) was added, and the proteins were solubilized by incubation of the lysate on ice for 1 h. Unlysed cells and cell debris were removed by centrifugation at 14,000 × *g* for 10 min. The supernatants were subjected to ultracentrifugation at 100,000 × *g* for 1 h and stored at −20 °C. Protein concentrations of the extracts were assayed using a modified Bradford assay (40). Two-dimensional PAGE was performed on the solubilized proteins, and differentially expressed protein spots were excised and prepared for mass spectrometric analysis as previously described (33).

TABLE I
Dose response for colonization by NCTC11168 variants

	Birds with positive cloacal swabs		
	Day 3	Day 5	Day 7
	%	%	%
V1 dose			
10^2 cfu	30	0	0
10^4 cfu	40	90	95
10^6 cfu	95	100	100
10^8 cfu	100	100	100
V26 dose			
10^6 cfu	4	17	17
10^7 cfu	21	14	38
10^8 cfu	38	46	43
10^9 cfu	26	52	61

Protein Identification—The in-gel digests were analyzed by nanoliquid chromatography-MS/MS using a "CapLC" capillary liquid chromatography system (Waters) coupled to a "Q-TOF Ultima" hybrid quadrupole time-of-flight mass spectrometer (Waters). The peptide extracts were injected on a 75-μm inner diameter × 150 mm PepMap C_{18} nanocolumn (Dionex/LC-Packings) and resolved by gradient elution (5–75% acetonitrile, 0.2% formic acid in 30 min, 350 nl/min). The mass spectrometer was set to operate in automatic MS/MS acquisition mode (6-s acquisition time per precursor ion). MS/MS spectra were acquired on doubly, triply, and quadruply charged ions. Proteins were identified by matching the sequences derived from peptide MS/MS spectra with sequences in the *C. jejuni* NCTC11168 protein sequence data base using Mascot™ data base searching software (Matrix Science) and Nemesis, an algorithm generated in house to extract and tabulate the significant sequence matches from multiple Mascot result files.

RESULTS

Colonization of 1-Day-old Chicks by C. jejuni NCTC11168 V1 and V26—Comparison of the infective dose of two variants of NCTC11168 revealed a significant difference in their ability to colonize young chicks. *C. jejuni* NCTC11168 V1 was a more aggressive colonizer, which required a dose of 10^4 colony-forming units (cfu) to colonize 100% of birds by day 7 (Table I). In contrast, 10^8 cfu of *C. jejuni* NCTC11168 V26 were required to colonize all birds by day 7. To determine whether the birds could be colonized by horizontal transfer, 20 unchallenged chicks were housed with five challenged chicks that had each been given 10^8 cfu of one of the variants (Table II). V1 rapidly spread to the unchallenged contact birds, and by day 7 all birds in the group were colonized. V26 spread to the unchallenged contact birds very poorly, and only 5% of the contact birds became colonized after 7 days.

On day 7, the cecal contents were also tested, because *C. jejuni* is known to colonize the cecum at a higher level than the numbers shed in the feces (represented by cloacal swabbing). Even by this method, only 80% of the birds challenged with V26 were positive, and transfer to the contact birds occurred at a much lower level. In addition, the cecal contents of the birds challenged with V1 had a higher mean level of colonization (9.5×10^8 cfu/g of cecal contents) when compared with that of V26 (6.18×10^7 cfu/g of cecal contents, $p = 0.0001$). Challenge of 1-day-old chicks with the V1 mutants, *fliA* and *flhA*, showed significantly lower levels of colonization and horizontal transfer compared with the V1 parent (Table II; see below). The level of colonization observed for the V1 parent in this study is comparable with levels observed for other strains such as the poultry isolate, RM1221, and the human pathogenic isolate, 81–176.[2]

[1] The abbreviations used are: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; cfu, colony-forming units; MS, mass spectrometry; nt, nucleotide(s).

[2] B. Allan, unpublished observation.

TABLE II
Colonization and horizontal transfer of NCTC11168 variants and mutants

	Birds with positive cloacal swabs			Birds with positive cecal swabs
	Day 3	Day 5	Day 7	
	%	%	%	%
Challenged with V1	80–100	100	100	100
Contact birds V1	30–75	47–100	100	100
Challenged birds V26	20	80	ND[a]	80
Contact birds V26	0	0	ND	5
Challenged with V1 *fliA*	0	20	20	100
Contact birds V1 *fliA*	0	0	0	5
Challenged with V1 *flhA*	0	0	0	20
Contact birds V1 *flhA*	0	0	0	20

[a] ND, not determined.

In Vitro Infection, Motility, and Flagellar Morphology—We examined the two *C. jejuni* variants by electron microscopy and found noticeable differences between the two cell types (Fig. 1, *A–C*). The most striking difference was that most cells of V26 did not possess flagella (Fig. 1*A*), although a small number of cells were flagellated (Fig. 1*B*). In contrast, V1 had typical bipolar flagella on every cell (Fig. 1*C*). Using motility assays (41), we confirmed that V1 was more motile than V26 (results not shown).

Because the two variants of NCTC11168 demonstrated such marked differences in their abilities to colonize chickens, we were interested in determining whether such differences would also be observed *in vitro*. Human intestinal Caco-2 cells were infected with both V1 and V26, and total numbers of bacteria that had adhered and invaded were determined. Bacteria were either allowed to swim to the monolayer or were centrifuged onto the monolayer to eliminate any differences caused by motility. As expected, the less motile V26 showed a 10-fold lower infection rate (adherence and invasion) than the motile V1 without centrifugation (Table III). In contrast, no noticeable differences in infection were observed when the strains were centrifuged onto the monolayer. We then compared the abilities of the two variants to invade after centrifugation when motility is not a factor. Although both strains demonstrated no difference in adherence, V26 showed a 10-fold reduction in invasion compared with V1 suggesting that components required for invasion as well as for motility were attenuated in V26.

Levels of Genes Encoding Specific Flagellar Transcripts and Virulence Factors are Higher in V1 than in V26—The gene content of the variants was analyzed using a whole genome *C. jejuni* NCTC11168 DNA microarray. We compared fluorescently labeled genomic DNA from V1 to that of V26 by competitive hybridization. No significant differences in relative intensities were observed for any of the genes (Fig. 2*A*), indicating that no gene deletions occurred between the variants. Typically, different isolates of *C. jejuni* have highly variable gene contents, and we consistently observe differences from the sequenced strain in genotyping experiments.[3]

In contrast, a number of differences in the transcript profiles between the two variants were observed by DNA microarray analysis (Fig. 2*B*). Notably, relative spot intensities for many genes encoding flagellar structural proteins were higher in V1 (Fig. 3*A*). When the expression of all genes known to be involved in flagellar biosynthesis was examined, genes encoding proteins required late in flagellar assembly showed the greatest differences in relative intensity (Fig. 3*A*, *black bars*). It should be noted that due to sequence similarity between certain genes, some of the signals might have resulted from cross-hybridization. In particular, *flaA* and *flaB* are very similar (93.9% identity in a 572-amino acid overlap), and Cj0170 is similar to Cj1325 (73.8% identity in a 61-amino acid overlap). The reduced level of flagellar gene expression in V26 correlates with the observation that this variant produces less flagella and exhibits decreased motility. These differences are not likely to be growth-related, since comparison of growth curves, as previously described (42), demonstrated that all strains used in this study had similar growth rates (results not shown).

Transcripts encoding several virulence factors unrelated to flagellar biosynthesis were also more highly represented in V1 (Fig. 3*B*). These include *cdtC*, encoding a subunit of the cytolethal distending toxin complex, the phase-variable Cj0045c encoding a putative iron-binding protein, and the catalase gene, *katA*, which has been shown to be important for hydrogen peroxide resistance and survival within macrophages (43). Additionally, transcripts for the *pseA* gene, encoding a protein involved in flagellar glycosylation (6), were also higher in V1, as were a number of other transcripts for genes located within the flagellar carbohydrate modification locus (Cj1315c, Cj1326, Cj1342c, Cj1343c, and Cj1344c).

By transcript profiling, we also identified several genes that were more highly expressed in V26 (see supplementary data, Table 1S). Of particular note are *clpB*, a protease involved in stress response, genes encoding an iron hydrogenase involved in respiration (*hydB, hydC*, and *hydD*), and genes encoding ribosomal proteins (*rplA* and *rplB*) where *rplA* is a translational repressor. However, we do not believe that the genes with higher transcript levels in V26 contribute to its lower virulence, since we found that the expression of a number of these genes was quite variable and was not restricted to V26. For example, in one replicate experiment, we found an increase in the expression of this group of genes in V1 relative to a V1 *flhA* mutant (see below), whereas in another biological replicate, expression of these genes was higher in the mutant. Similarly, when we compared V1 with the V1 *fliA* mutant, expression of many of these genes was higher in the mutant in 3 of 4 replicates, whereas in one replicate, these genes were not differentially expressed (supplementary data, Table 3S).

Proteomic Comparison of V1 and V26—We used two-dimensional gel electrophoresis to compare the proteomes of the two variants (Fig. 4). Protein spots that exhibited an intensity difference of 1.5 or greater between the two strains were excised and identified by mass spectrometric analysis of their in-gel tryptic digests (Table IV). Proteins more highly represented in V1 can generally be classified into three groups: flagellar, respiratory (tricarboxylic acid cycle and electron transport), and chemotaxis-related, whereas proteins more highly expressed in V26 include stress-related proteins (*e.g.* ClpB and GroEL) and proteins involved in detoxification (SodB and Tpx). Note that analysis of one of the relatively weak protein spots unique to the V1 extract (molecular mass ~33 kDa, pI ~4.5) contained peptides that can be assigned to one of three chemotaxis-related proteins (Cj0144, Cj0262c, and/or

[3] E. Taboada, unpublished data.

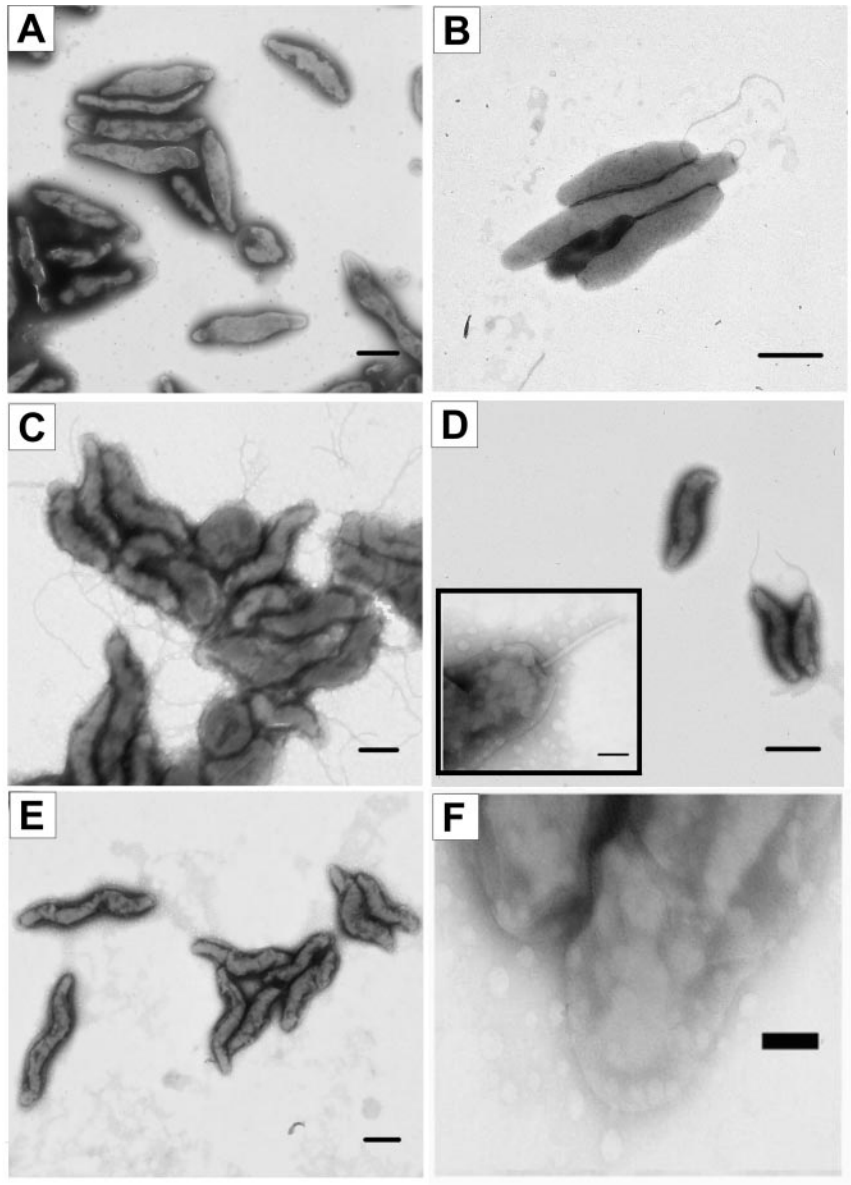

FIG. 1. **Transmission electron micrographs of negatively stained *C. jejuni* NCTC11168.** Shown are electron micrographs of *C. jejuni* V26 demonstrating no flagella (*A*) or only a single flagellum (*B*) (*bar*, 1 μm). In contrast, *C. jejuni* V1 has a typical morphology with bipolar flagella on every cell (*C*; *bar*, 0.5 μm). Shown are electron micrographs of *C. jejuni* V1 *fliA* mutant indicating truncated flagella (*D*; *bar*, 1 μm); the *inset* shows an enlargement of the V1 *fliA* mutant, demonstrating stubby flagellin phenotype (*bar*, 100 nm). *C. jejuni* V1 *flhA* mutant has no flagella (*E*; *bar*, 1 μm); a representative high magnification image of the cell pole (*F*) demonstrates the absence of both flagellar filament and hook (*bar*, 100 nm).

TABLE III
Infection of Caco-2 cells by V1 and V26

Condition	V26	V1
Total infection (adherence + invasion)		
With centrifugation	0.3247 ± 0.0950[a]	0.4303 ± 0.1371
Without centrifugation	0.0207 ± 0.0038	0.1823 ± 0.0748
Infection with centrifugation		
Adherence	0.3133 ± 0.0733	0.2165 ± 0.0135
Invasion	0.0028 ± 0.0005	0.0277 ± 0.0064

[a] Values are given as percentage of inoculum ± S.E.

Cj1564), since the amino acid sequences of the C termini are identical. Furthermore, this spot was only observed intermittently in the two-dimensional gels of the V1 strain although never in those prepared using the V26 strain. Another protein spot (molecular mass ~25 kDa, pI ~5.8) consistently observed only in the two-dimensional gels of the V1 strain appears to be the product of two genes, Cj1325 and Cj1326 (Fig. 4*B*). In the annotated genome, these genes overlap and contain a phase-variable poly(G)$_{9-10}$ in the overlapping region, where poly(G)$_{10}$ produces a stop codon at the end of Cj1325 and poly(G)$_9$ allows translation into the downstream gene, Cj1326. The 25-kDa protein observed only in V1 is predicted to represent a fused poly(G)$_9$ product; however, we cannot discount the possibility that Cj1325 and Cj1326 were found in the in gel digest of this spot due to protein interactions that were not disrupted by denaturation.

Many of the differences observed by proteome analyses corresponded with microarray results (Fig. 3*A*, *asterisks*). For example, genes known to be involved in the tricarboxylic acid cycle and electron transport are increased in V1 as observed in both the transcriptome (*sucD*, *oorC*) and the proteome (SucD, OorC), but these pathways are more highly represented in the proteome, where levels of OorB, Mdh, and SdhC were also found to be higher. Higher levels of flagellar proteins were also

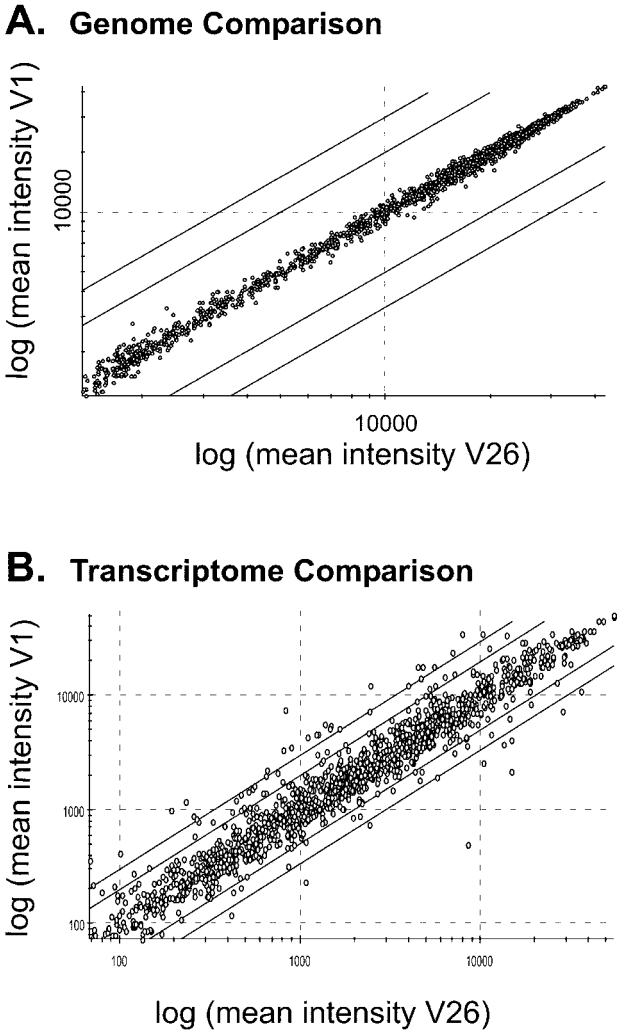

FIG. 2. ***C. jejuni* V1 and V26 have identical gene contents but differ in their transcriptomes.** Labeled genomic DNA (*A*) or cDNA (*B*) was hybridized to a whole genome *C. jejuni* NCTC11168 microarray. The log (signal intensity) V1 *versus* V26 was plotted for every gene represented on the DNA microarray. The *lines* indicate 2- or 3-fold differences in intensity. Gene content is identical in both variants, since the distribution of points is at or near a ratio of 1 (*A*); however, changes in transcript levels greater than 2-fold are observed for a number of genes (*B*).

observed in V1 in both the transcriptome and proteome (FliD, FlgG, FlaD, PtmA, FlaA, and FlgE2), although in this case, flagellar genes were more highly represented in the transcriptome. In addition, transcript levels for Cj1325 (see above) and Cj1026 were consistently higher in V1 by microarray analysis, but more replicates would be required to show that these changes were statistically significant by microarray analysis alone.

Identification of σ^{28} and σ^{54} Promoters for Differentially Expressed Genes—Since flagellar genes have been shown to be regulated by σ^{28} and σ^{54}, we attempted to identify binding motifs for these promoters within the published *C. jejuni* NCTC11168 genome to determine whether genes with these promoters were more likely to differ between the two variants. We analyzed the upstream regions of the genes that were differentially expressed between the two *C. jejuni* variants to identify conserved promoter sequences. Most of the σ^{28} and σ^{54} promoters we identified were within 100 nt of the translation start site similar to σ^{70} promoters, which are usually found within 150 nt of the start (13).

To find σ^{54} promoters, we used pattern-searching tools developed in house to screen for a minimal conserved motif of $GGN_{10}GC$ within 200 nt of the translation start site of differentially expressed genes. This low stringency search was used, since this core σ^{54} motif is highly conserved among different organisms (44). We aligned promoters identified in this search, and a more stringent motif ($TTGGAACRN_4TTGCTT$) was created for subsequent searches based on this alignment and on similarity to σ^{54} promoters previously identified for *flaB*, *flgD*, *flgE*, and Cj1293 (8,15–17, 24). We found several putative σ^{54} promoters in *C. jejuni* with conserved −12 and −24 motifs (Fig. 3*C*). Genes with σ^{54} promoters include many late flagellar genes, particularly structural genes required for the hook and basal body complex. The *C. jejuni* σ^{54} consensus sequence appears to differ somewhat from the consensus $YTGGCACGN_4TTGCWNN$ derived from σ^{54} sequences from multiple organisms (44). In particular, the first cytosine in the universal consensus is an adenine in *C. jejuni* except for in *flaG*, where a cytosine is found at this position. This latter gene is of interest, since it also has a σ^{28} promoter 71 nt upstream of the translation start site (see below).

A similar strategy was used to identify σ^{28} promoters. We used a minimal conserved sequence of $TWWWN_{13-18}CGAT$, since this core sequence is conserved for σ^{28} promoters in *E. coli* and *H. pylori* (45). We found putative σ^{28} promoters for a number of genes that have higher levels of transcripts in V1, including *flaA* and *flaG*, which have previously been shown to be σ^{28}-regulated in *C. jejuni* (22, 23) and/or *H. pylori* (45, 46). However, the σ^{28} promoter that we identified for *flaG* exhibits several differences from the consensus, particularly within the −35 motif. In addition to these flagellar genes, we found promoters for *pseA*, involved in flagellar carbohydrate modification, and a number of genes with unknown function. Lower levels of sequence conservation were observed for putative σ^{28} promoters than for σ^{54} promoters, particularly for the −35 motif (Fig. 3*D*). This latter region tended to be AT-rich with a single conserved T as seen in the sequence logo. Moreover, the 17-nt spacer region appeared to be particularly AT-rich in the promoters identified.

Real Time PCR Confirmation of Microarray Data—The greatest differences in relative abundance of transcripts in the V1 *versus* V26 comparison were observed for genes with σ^{28} promoters (Fig. 3, *A* and *B*, *blue triangles*). Furthermore, the *fliA* gene encoding σ^{28} was the only known flagellar regulator with higher levels of V1 transcripts in the microarray experiments (Fig. 3*A*, *striped bars*), although this difference was not statistically significant. To determine whether levels of *fliA* transcripts were indeed more abundant in V1, we performed real time PCR analyses. In three biological replicates, we observed greater than 2-fold higher levels of *fliA* transcripts in V1 (ratio 2.4; S.D. = 0.49). Furthermore, we confirmed microarray results for the σ^{54}-regulated *flgD* (ratio 7.0; S.D. = 1.67) and the σ^{28}-regulated *flaA* (ratio 7.0; S.D. = 1.69), which were not observed in proteome analyses, and for *cdtC* (ratio 2.0; S.D. = 0.23), which had low expression levels in proteome analyses. We decided to further investigate the role of σ^{28} in the regulation of flagellar genes.

Analysis of V1 fliA Mutant: σ^{28} Represses Expression of σ^{54}-regulated Genes—To determine the role of σ^{28} in flagellar gene expression, the V1 *fliA* gene encoding σ^{28} was disrupted by cassette insertion. The morphology of the *fliA* mutant, as determined by electron microscopy, was distinct from both V1 and V26 (Fig. 1*D*). The V1 *fliA* mutant possessed primarily stubby flagella (Fig. 1*D*, *inset*) as previously reported (14) and similar to those synthesized by *C. jejuni* mutants defective in the σ^{28}-regulated major flagellin gene, *flaA* (4). The mutant was

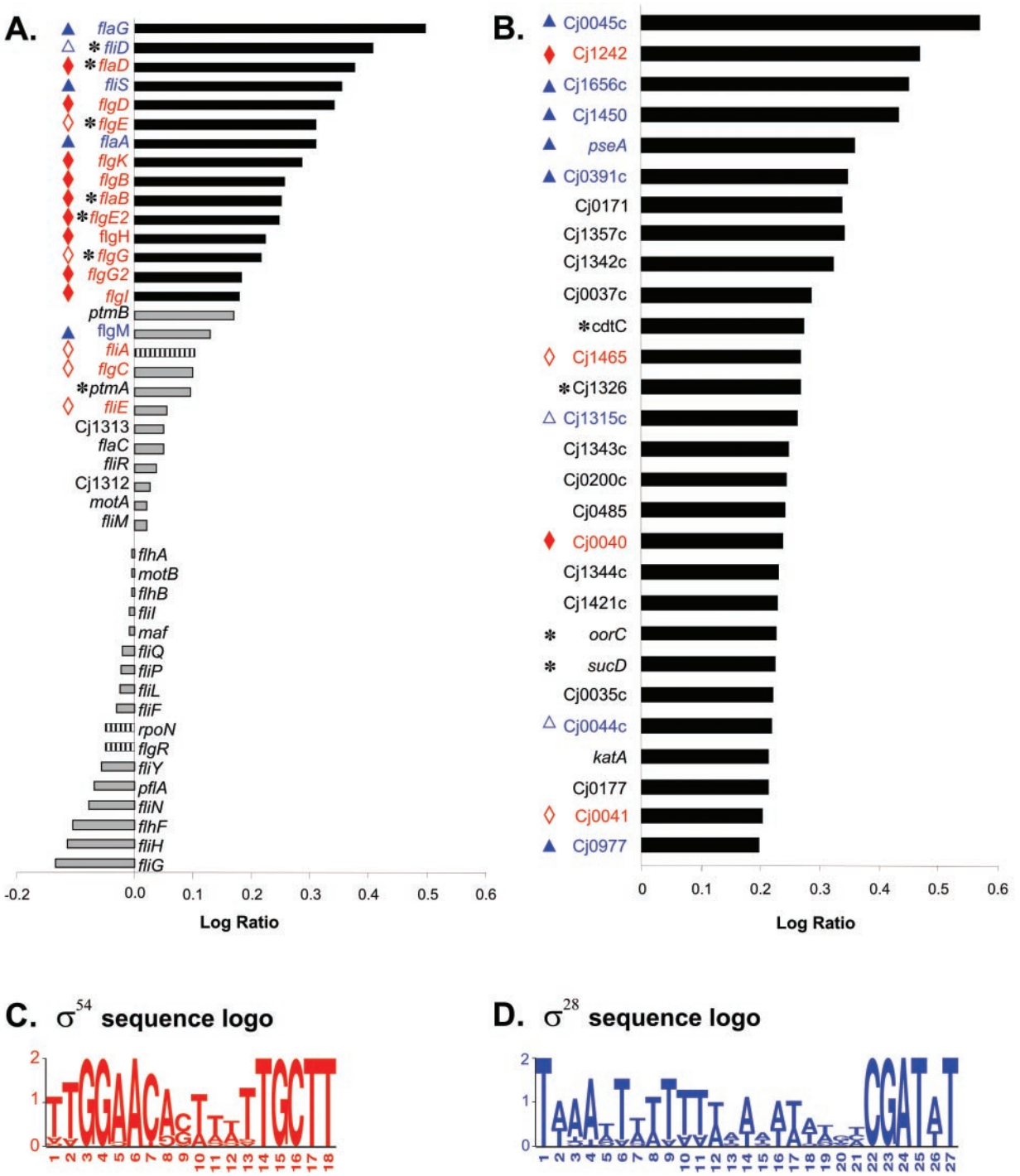

FIG. 3. **Transcripts of late flagellar genes are more abundant in V1 relative to V26.** A *bar graph* of the log(V1/V26) of flagellar gene transcripts was plotted according to magnitude of difference observed (*A*). Sequence within 200 nt upstream of genes that were differentially expressed in V1 relative to V26 were analyzed to identify conserved promoter sequences (see "Results"). σ^{28} (*blue triangles*) or σ^{54} (*red diamonds*) promoters were identified for many of the genes with differences in expression levels. The *open symbols* indicate genes located downstream of a gene with either a σ^{28} or σ^{54} promoter that are potentially co-transcribed. The *black bars* indicate statistically significant differences in expression, and *striped bars* are used to highlight genes with known flagellar regulatory functions (*fliA*, *rpoN*, and *flgR*). A number of additional genes, many of which have unknown functions, were more highly expressed in V1 relative to V26 (*B*). The *asterisks* indicate differences in expression that were confirmed by proteomics. Sequence logos (*C* and *D*) were created to indicate conservation of σ^{28} (10 sequences) and σ^{54} (16 sequences) promoters identified in *C. jejuni* (WebLogo available on the World Wide Web at www.bio.cam.ac.uk/seqlogo/logo.cgi) (62). The height of a nucleotide is proportional to its frequency in the sequence at that position. See supplementary data for alignment of promoter sequences (Tables 5S and 6S).

also nonmotile as determined by motility assays (results not shown) and a poor colonizer of chickens relative to the V1 parent (Table II).

We performed DNA microarray analyses to determine the effect of the *fliA* mutation on the V1 transcriptome. The transcript levels of genes for which we had identified σ^{28} promoters were greatly decreased in the mutant (Fig. 5*A*, *blue triangles*, and supplementary data, Table 2S). In contrast, transcript levels for genes with σ^{54} promoters were higher in the *fliA* mutant (Fig. 5*A*, *red diamonds*), indicating that σ^{28} or a gene regulated by σ^{28} represses σ^{54} activity or σ^{54}-regulated genes. *flaG* is co-regulated with the σ^{28} genes, suggesting that the σ^{54}

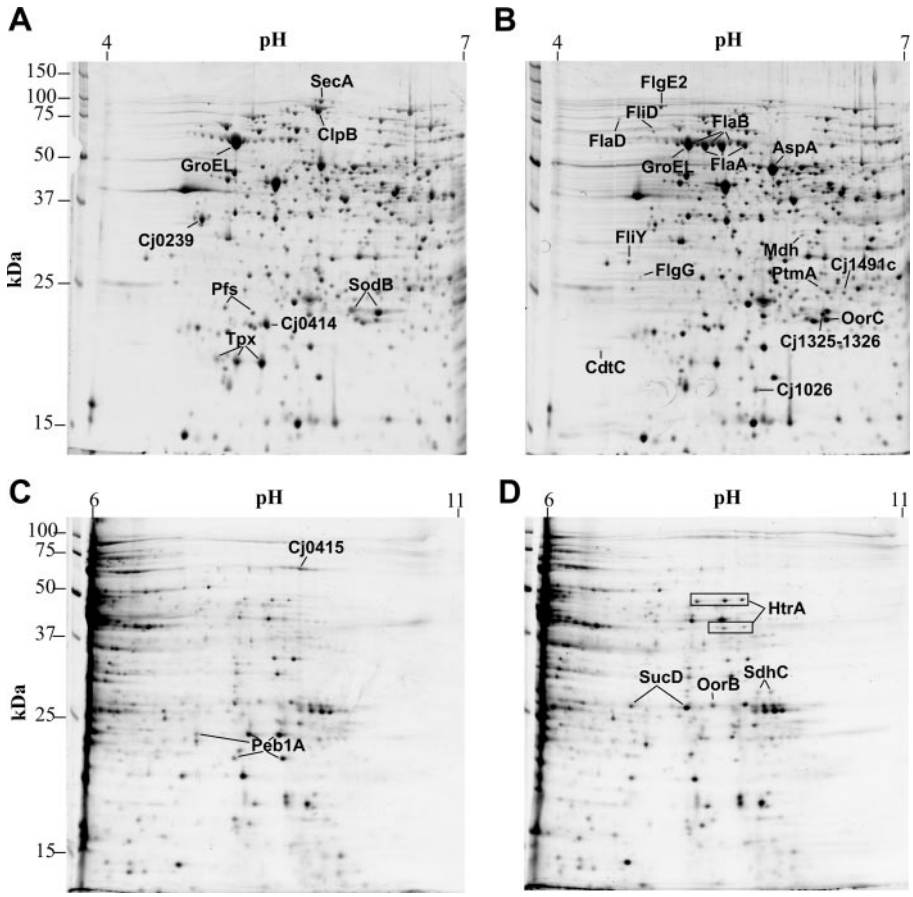

FIG. 4. **Two-dimensional gel analysis of whole-cell lysate from *C. jejuni* NCTC11168 V26 (*A* and *C*) and V1 (*B* and *D*).** Proteins were separated on two-dimensional PAGE in two pH ranges, pH 4–7 (*A* and *B*) and pH 6–11 (*C* and *D*). The gels were first stained with the fluorescent dye Sypro Ruby (Bio-Rad) for quantitative analysis and then silver-stained. The identities of the spots were determined by mass spectrometry of their tryptic digests. A full list of the identified proteins is given in Table IV.

promoter upstream of this gene is not functional. Perhaps the single nucleotide variation in the putative σ^{54} promoter sequence was sufficient to inactivate it completely, or the promoter may be less active due to the greater distance to the start codon (supplementary data, Table 5S). This gene is likely to be co-transcribed with *fliD* and *fliS*, since promoters were not identified for these genes, and levels of their transcripts were also decreased in the *fliA* mutant. *cdtC* is also co-regulated with σ^{28} genes, and we were able to identify a potential σ^{28} promoter for this gene by lowering the stringency of the search.

In the *fliA* mutant analysis, we identified a number of genes that were co-regulated with the σ^{28} class of genes but were not identified in the V1 *versus* V26 comparison (see supplementary data, Table 2S). The analysis of the mutant transcriptome was probably a more sensitive method for identifying smaller expression changes, since mutation causes a more complete inactivation of *fliA* than is observed in the comparison of the variants. Of particular interest is Cj1464, which is predicted to be a *flgM* homologue encoding an anti-σ^{28} regulator (21, 25) and for which a putative σ^{28} promoter was identified (supplementary data, Table 6S). Several genes within the flagellar locus also appear to be co-regulated with σ^{28} genes. As with *cdtC*, we were able to find putative promoters for some of these genes by lowering the stringency of our searches, but such promoters should be verified experimentally. Interestingly, the observation of increased expression of σ^{28}-regulated genes with a reduction in levels of transcripts for σ^{54}-regulated genes is not consistent with our observations for attenuated V26, suggesting that a component higher in the flagellar regulatory hierarchy may be responsible for the differences.

flhA Is Required for the Transcription of σ^{28}- and σ^{54}-regulated Genes—We sequenced all of the genes encoding known flagellar gene regulators in order to determine whether sequence variation in these regions could be responsible for differences observed in the V1 *versus* V26 comparison. These included *fliA* and *rpoN*, which encode σ^{28} and σ^{54}, respectively; *flgR*, which encodes a σ^{54} activator (14); and *flhA* and *flhB*, which have been implicated in the regulation of flagellar genes in campylobacter and in other organisms (26, 27, 31). PCR products of flagellar regulators generated from V1 and V26 were sequenced in both directions, including at least 200 nt of the upstream region. No differences were observed for *fliA*, *rpoN*, *flgR*, *flhA*, or *flhB*.

However, based on previous reports of *flhA* regulation of flagellar assembly and virulence in *Bacillus, Pseudomonas*, and *Helicobacter* (26–28), we inactivated *flhA* in V1 to determine whether we would see changes similar to those observed for the variants. The *flhA* mutant was nonmotile on swarming plates (results not shown) and produced no flagella or hooks (Fig. 1, *E* and *F*). In chicken colonization studies, the *flhA* mutant was a poor colonizer and was not efficiently transmitted by horizontal transfer (Table II).

Microarray analysis was performed to determine the effect of the *flhA* mutation on the transcriptome of V1. Transcript levels of both σ^{28}-regulated (Fig. 5*B*, *blue triangles*) and σ^{54}-regulated (Fig. 5*B*, *red diamonds*) genes were lower in the mutant relative to V1 (see supplementary data, Table 4S). Furthermore, all of the genes with decreased expression in the V1 *flhA* mutant were affected in V26 (decreased) or in the *fliA* mutant (increased or decreased). These data indicate that the transcription of both σ^{28}- and σ^{54}-regulated genes is affected by *flhA* and that inactivation at this level of the flagellar hierarchy resembles the effects observed in the two variants.

DISCUSSION

We have determined through proteome and transcriptome analysis that the attenuated virulence of *C. jejuni* NCTC11168 V26 is correlated to reduced expression of genes with σ^{28} or σ^{54}

TABLE IV

Identification of proteins with different expression levels between the V1 and V26 strains of C. jejuni NCTC11168 as determined by two-dimensional PAGE (Fig. 4) and nano-liquid chromatography-MS/MS

Protein	Name	M_r	pI	Increase
				-fold
Increased in V1				
CdtC	Cytolethal distending toxin	21.14	4.48	1.47
AspA	Aspartate ammonia-lyase	51.73	5.50	1.45
Cj0144[a]	Methyl-accepting chemotaxis signal transduction protein	72.36	4.97	+[b]
Cj0262c[a]	Putative methyl-accepting chemotaxis signal transduction protein	72.83	4.94	+
SdhC	Putative succinate dehydrogenase subunit C	31.34	8.37	+
Mdh	Malate dehydrogenase	33.49	5.60	+
SucD	Succinyl-CoA synthetase α-chain	30.02	7.65	3.98
OorB	Probable OORB subunit of 2-oxoglutarate:acceptor oxidoreductase	31.19	7.99	2.35
OorC	Probable OORC subunit of 2-oxoglutarate:acceptor oxidoreductase	20.08	5.7	1.47
FliD	Putative flagellar hook-associated protein	69.78	4.81	2.19
FlgG	Flagellar basal body rod protein	27.7	4.65	2.83
FlaD	Possible flagellin	82.00	4.53	5.17
Cj1026c	Putative lipoprotein	18.52	7.74	5.94
HtrA	Serine protease DO	51.00	9.20	3.47
Cj1325[c]	Hypothetical protein	10.05	7.85	+
Cj1326[c]	Hypothetical protein	16.27	5.17	+
PtmA	Oxidoreductase flagellin modification	28.65	5.83	2.07
FlaA	Flagellin A subunit	59.00	5.59	3.5
Cj1491c	Two-component regulator	25.80	5.90	+
Cj1564[a]	Probable methyl-accepting chemotaxis signal transduction	73.08	5.47	+
FlgE2	Flagellar hook chain protein	91.86	4.86	2.76
Increased in V26				
Pfs	Probable 5′-methyoadenosine/*S*-adenosylhomocysteine nucleosidase	25.21	5.05	1.56
SodB	Superoxide dismutase (Fe)	24.81	5.83	2.43
Cj0239c	Protein homolog *nifU*	35.47	4.76	5.04
Cj0414	Possible oxidoreductase subunit	26.91	5.5	5.18
Cj0415	Possible oxidoreductase subunit	63.65	8.76	+
ClpB	ATP-dependent CLP protease ATP-binding subunit	95.54	5.47	21.3
Tpx	Probable thiol peroxidase	18.44	5.13	3.55
Peb1A	Probable ABC-type amino acid transporter periplasmic solute-binding protein PEB1	28.18	8.94	3.28
SecA	Preprotein translocase SecA subunit	97.92	5.59	3.9
GroEL	60-kDa chaperonin	57.93	5.02	2.5

[a] The peptides identified by nano-liquid chromatography-MS/MS could arise from any of these three proteins, since they have identical C-terminal domains. Stronger solubilization conditions (5 M urea, 2 M thiourea, 2% CHAPS, 2% SB3–10, 1% dithiothreitol, and 0.2% BioLytes) were required to observe these proteins by second dimension (not shown).

[b] +, proteins that were detected in only one variant.

[c] Cj1325 and Cj1326 were observed in the same spot.

promoters involved in virulence, flagellar biosynthesis, and carbohydrate modification. This effect can be reproduced by the inactivation of *flhA*, a key component of the flagellar export apparatus. This study provides the first description of the global effects of inactivation of *flhA* on a bacterial transcriptome and provides new insight into the unique regulatory networks in *C. jejuni*.

FlhA belongs to the FHIPEP family of bacterial export proteins involved in flagellar assembly and type III secretion. Both the N-terminal and C-terminal domains of FlhA homologues have been conserved throughout evolution. The highest level of similarity exists within the N-terminal hydrophobic domain containing 6–8 transmembrane regions that are believed to be necessary for anchoring in the inner membrane (18). In contrast, the hydrophilic C-terminal domain of FlhA extends into the cytoplasm and is involved in energy-dependent secretion of flagellar proteins (47). Zhu *et al.* (48) demonstrated that the cytoplasmic domains of FlhA and FlhB form complexes with FliH (regulator of FliI), FliI (ATPase), FliJ (general chaperone), and the substrate to be exported. Then through ATP hydrolysis by FliI, the substrate is translocated across the cytoplasmic membrane through the membrane-spanning domains of FlhA and FlhB and followed by dissociation of FliH, FliI, and FliJ from the export apparatus.

In *Campylobacter coli*, high frequency phase variation of flagellin gene expression (*flaA* and *flaB*) was linked to a homopolymeric tract of thymine residues in *flhA* (49). In *C. jejuni*, there is a cytidine residue within this homopolymeric tract, and phase variation has not been observed within this gene. However, transposon mutagenesis studies in *C. jejuni* demonstrate that inactivation of *flhA* leads to loss of FlaA expression, motility, autoagglutination, and invasion (29). It has also previously been suggested that *flhA* could be a master regulator of flagellar expression and virulence in *Bacillus thuriengensis*, *H. pylori*, and *Pseudomonas aeruginosa* (26–28, 36). In *P. aeruginosa*, inactivation of *flhA* led to decreased invasion that could not be restored by centrifugation, suggesting that the defect was not entirely due to lack of motility (28). Similarly, in our studies, invasion levels were not restored with centrifugation in the less motile V26 to levels seen in V1. Transcript analyses also demonstrate that *flhA* affects more than simply flagellar assembly in *C. jejuni*. We now provide evidence that this gene also plays an important role in the regulation of motility and virulence in *C. jejuni*, where both σ^{28}- and σ^{54}-regulated genes are affected by inactivation of *flhA*. However, we have not observed any differences between the two variants in the gene sequence of *flhA* or other known regulators of flagellar biosynthesis, suggesting that another export component or class I regulator in the cascade has been affected in this system and warrants further study.

Using combined bioinformatic and experimental approaches, we have identified σ^{28} and σ^{54} promoters within the genome of *C. jejuni* NCTC11168. Many of the genes with σ^{54} promoters are known to be involved in flagellar biosynthesis and encode components of the hook and basal body complex and the flagellar filament. Many of the remaining σ^{54}-regulated genes have

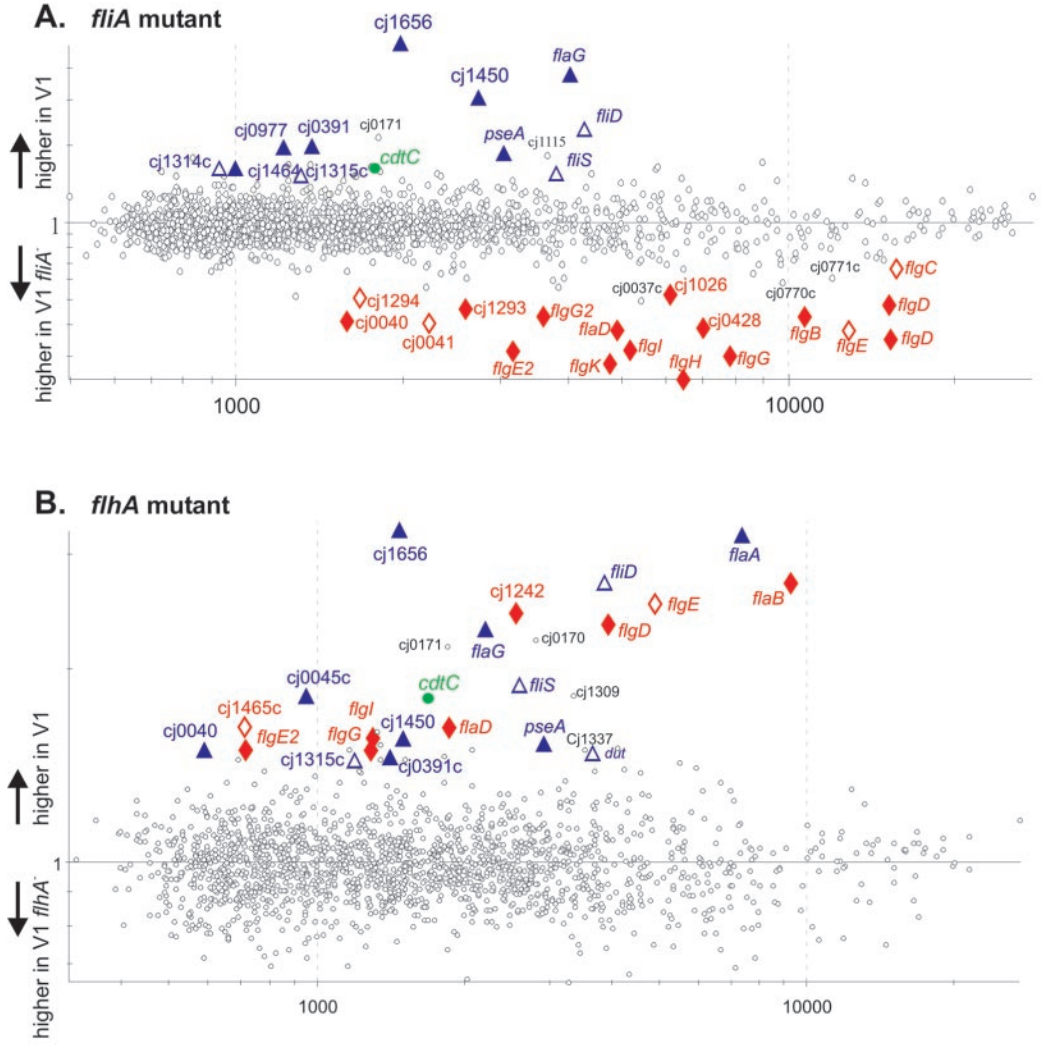

FIG. 5. **Microarray analysis of V1 *fliA*⁻ and V1 *flhA*⁻ mutants.** *A*, DNA microarrays were used to determine the effects of inactivation of σ^{28} (*fliA*) in V1. An MA plot of signal intensities for all genes (log (ratio V1/V1 *fliA*⁻) *versus* log (average V1, V1 *fliA*⁻)) indicates that signal levels of genes with σ^{28} promoters (*blue triangles*) were higher in the wild type V1, as expected. However, levels of genes with σ^{54} promoters (*red diamonds*) were lower in wild type V1, demonstrating that the presence of a functional σ^{28} gene represses transcription of σ^{54}-regulated genes. *B*, DNA microarrays were used to determine the effects of mutation of *flhA* in V1. An MA plot of signal intensities for all genes (log (ratio V1/V1 *flhA*⁻) *versus* log (average V1, V1 *flhA*⁻)) showing levels of genes with σ^{28} promoters (*blue triangles*) and σ^{54} promoters (*red diamonds*) were higher in the wild type V1 relative to the mutant, as was observed in the V1 *versus* V26 comparison (see Fig. 3). Note that *cdtC* appears to be co-regulated with σ^{28} genes (*green circle*). The *open symbols* indicate genes that are located downstream of a gene where either a σ^{28} or σ^{54} promoter was found. See Tables 2S, 3S, and 4S (supplementary data) for a complete list of genes that were differentially expressed and for statistical analysis of replicate data.

not been well characterized, although several recent transposon mutagenesis experiments have demonstrated that Cj0041, Cj0062c, and Cj1026 are required for motility (17, 29, 50). For Cj1026 and Cj0062c, these motility defects may be caused by polar effects on the transcription of the downstream genes: *flgR* (σ^{54} activator) and *fliA* (σ^{28}), respectively. However, the UDP-GlcNAc C6-dehydratase homologue, Cj1293, has recently been shown to be σ^{54}-regulated and involved in pseudaminic acid biosynthesis and proper flagellar assembly (8). Thus, only two of the σ^{54}-regulated genes identified here, Cj0428 and Cj1242, were not previously demonstrated to be important for motility. We are currently investigating these genes further.

In contrast, only a few of the σ^{28}-regulated genes that we identified are known to be required for motility. These include *flaA*, which encodes the major flagellin in campylobacter (22, 23, 51), and genes within the operon *flaG-fliD-fliS*, which have been shown to be essential for motility and colonization in *H. pylori* (*fliD*) (52), for motility in *C. jejuni* (*fliD* and *fliS*) (29), and for adherence in *Aeromonas caviae* (*flaG*) (53) and *P. aeruginosa* (*fliD* but not *flaG*) (54). The *flaG-fliD-fliS* operon has the same organization in *H. pylori*, with both σ^{28} and σ^{54} promoters (45). However, in contrast to what was observed in *H. pylori*, only the σ^{28} promoter appears to be active in *C. jejuni* under the conditions that were used in our experiments. Another σ^{28}-regulated gene potentially involved in motility is *flgM* (Cj1464). This gene is particularly noteworthy, since it may perform a role in the regulation of flagellar genes, since it is the predicted homologue of *flgM*, a σ^{28} repressor (21, 25). Extrusion of FlgM through the completed hook and basal body complex is important for regulation of late flagellar genes in other bacteria (reviewed in Ref. 18), but evidence suggests that FlgM is not a strong repressor of σ^{28}-regulated genes in *C. jejuni* (21).

Several σ^{28}-regulated genes are potential virulence factors. For example, the expression of one of the cytolethal distending toxin subunits, encoded by *cdtC*, is higher in V1 at both the transcript and protein level and was higher in microarray comparisons with the *fliA* and *flhA* mutants. Cytolethal distending toxins were first described by Johnson and Lior in 1987

(55) and are common to many Gram-negative mucosal pathogens. *cdtC* has been shown to be co-transcribed with *cdtA* and *cdtB* in *C. jejuni* 81–176 (56) and potentially regulated by σ^{70} (13). Our studies demonstrate that *cdtC* is also independently regulated by σ^{28}. This subunit may have some activity on its own, since injection of purified CdtC from *Actinobacillus actinomycetemcomitans* into the host cytosol caused distention and cell death (57), although transfection experiments with *cdtC* in *C. jejuni* did not demonstrate any changes in cell morphology (58).

Several genes within the flagellar locus of NCTC11168 (Cj1293 to Cj1342) were also more highly expressed in V1 and showed σ^{28} regulation. A number of these genes are involved in pseudaminic acid biosynthesis (including the σ^{54}-regulated Cj1293 mentioned above). These include *ptmA* and *ptmB* (post-translational modification proteins), homologues of CMP-*N*-acetylneuraminic acid synthetase and alcohol dehydrogenases, respectively (9, 59). Mutation of these genes in *C. jejuni* 81–176 caused loss of flagellar reactivity with carbohydrate-specific sera and changes in the pI of the purified protein. Other flagellar modification genes include Cj1312, which shows homology to structural genes involved in flagellar biosynthesis (10); Cj1313, a putative acetyltransferase possibly involved in pseudaminic acid biosynthesis; and Cj1316, or *pseA,* which has been demonstrated to be involved in forming the acetamidino derivative of pseudaminic acid (6). The ability to regulate carbohydrate genes that affect flagellar assembly and antigenicity is probably an important determinant of pathogenicity in *C. jejuni*. We did not find a strong consensus for σ^{28} promoters for many of the genes within the flagellar modification locus or for *cdtC*. However, due to the higher variability of this promoter, it is not surprising that it is more difficult to confidently identify these promoters without an experimentally determined transcription start site.

A number of genes encoding proteins with unknown function (Fig. 3*B*) were also found to be σ^{28}-regulated. It is interesting to speculate that unknown genes expressed with late flagellar proteins may be involved in virulence, similar to other genes within this class. Recent evidence suggests that the flagellar protein export systems may be involved in type III-like secretion of virulence factors (60). *C. jejuni* does not encode a typical type III secretion apparatus; thus, it is possible that the flagellar export system performs this function (61). If this were the case, it would be beneficial to co-regulate expression of secreted virulence factors with late flagellar genes.

Based on our analyses, we propose a model for coordinate regulation of late flagellar genes and virulence factors in *C. jejuni*. As in other bacteria, genes encoding flagellar proteins belong to three classes, corresponding to the order in which they are transcribed (Fig. 6). Class I includes products that form part of the flagellar transport apparatus and have σ^{70} promoters (13). The FlhA protein forms part of this complex and is known to be essential for secretion of flagellar proteins. Class II genes are σ^{54}-regulated and are required for the basal body, hook, and flagellar filament biosynthesis. Class III, with σ^{28} promoters, includes genes required for filament biosynthesis and capping as well as genes for flagellin post-translational modification and virulence. We have determined that inactivation of *flhA* inhibits transcription of both class II and class III flagellar genes and potential virulence factors regulated by σ^{28} and σ^{54} promoters. This regulation may occur through secretion of a regulatory protein, or may be due to inhibition from accumulated flagellar products in the cytoplasm, or *flhA* may repress transcription at a higher level by directly preventing transcription of σ^{28}- and σ^{54}-controlled genes.

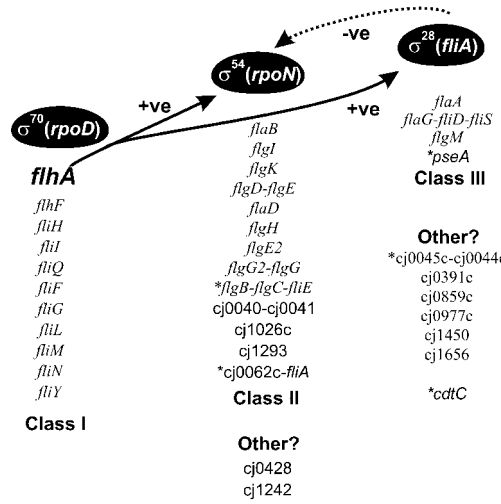

FIG. 6. **Regulation of flagellar genes in *C. jejuni*.** Campylobacter flagellar genes can be classified into three groups based on their promoters. Early genes required for assembly of the basal body and export apparatus are predicted to have σ^{70} promoters (13), whereas genes required later in flagellar assembly for completion of the hook and basal body complex and extension of the flagellum have σ^{54} and σ^{28} promoters, respectively. The *asterisks* indicate genes in class II and III with predicted σ^{70} promoters. Genes in class II with σ^{54} promoters, including several uncharacterized genes, appear to perform important roles in motility (see "Discussion"). In class III, several genes with unknown function are observed, including *cdtC* and *pseA*, that are important for virulence and host evasion in *C. jejuni*. We have shown that an intact *flhA* appears to be important for expression of both classes (*solid arrows*) and that σ^{28} represses expression of σ^{54} (*dotted arrows*).

This model demonstrates that campylobacter shares some common regulatory features with other flagellated bacteria such as the sequential expression of flagellar genes based on order of transcription and the usage of common σ^{28} and σ^{54} promoters. However, even when compared with the closely related *H. pylori* (25, 28, 45), campylobacter is unique in that *fliA* (σ^{28}) or a σ^{28}-regulated gene represses expression of σ^{54} genes. Furthermore, class III genes include flagellar modification genes not previously observed to be σ^{28}-regulated in *H. pylori* as well as some unique genes with no homology to other known bacterial genes.

Using genomic and proteomic methods, we have characterized two variants of *C. jejuni* NCTC11168 that differed in motility, Caco-2 cell invasion, and chicken colonization. Although these investigations have not identified the source for the differences between the variants, the analyses have revealed a novel pathway for the regulation of flagellar biosynthesis and virulence in campylobacter that in part may be responsible for the different virulence phenotypes observed and will form the foundation for further studies into campylobacter pathogenesis. Future work will determine the relevance of such regulatory networks in an organism that is able to adapt to multiple hosts and environments.

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