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Heterogeneity of a *Campylobacter jejuni* Protein That Is Secreted through the Flagellar Filament[▽]

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Cj0859c, or FspA, is a small, acidic protein of *Campylobacter jejuni* that is expressed by a σ^{28} promoter. Analysis of the *fspA* gene in 41 isolates of *C. jejuni* revealed two overall variants of the predicted protein, FspA1 and FspA2. Secretion of FspA occurs in broth-grown bacteria and requires a minimum flagellar structure. The addition of recombinant FspA2, but not FspA1, to INT407 cells in vitro resulted in a rapid induction of apoptosis. These data define a novel *C. jejuni* virulence factor, and the observed heterogeneity among *fspA* alleles suggests alternate virulence potential among different strains.

Campylobacter jejuni is among the leading causes of bacterial diarrhea worldwide (12, 40). *Campylobacter* enteritis can present with a range of clinical symptoms, and this variability has been attributed to either differences in the host or differences in virulence among strains. The molecular pathogenesis of *C. jejuni* has proven particularly refractory to analysis. This may be due, in part, to the absence of virulence factors in the genome that are analogous to those of better-characterized pathogens. Thus, the only virulence factor in *C. jejuni* that is shared with other pathogens is cytolethal distending toxin (CDT). There are no genes encoding other known toxins or specialized secretion systems common to all *C. jejuni* strains.

Flagella are considered to be virulence factors for *C. jejuni* for numerous reasons. The motility imparted by the locomotory organelle is critical to intestinal colonization of both animals and humans (3, 31). Motility and chemotaxis also modulate the invasion of intestinal epithelial cells in vitro (7, 20, 47–49). The flagellin subunits in the *Campylobacter* flagellar filament are heavily glycosylated with pseudaminic acid and legionaminic acid (29, 44). This glycosylation is required for filament assembly (15), and changes in glycan composition can affect both the immunogenicity of flagellin and microcolony formation (17, 28). *Campylobacter* flagella also appear to function as a type III secretion system (T3SS) in the absence of a specialized T3SS in this pathogen. Thus, strains of *C. jejuni* secrete the Cia (*Campylobacter* invasion antigen) proteins (24, 25, 39) and FlaC (41) through the filament.

There is limited information about the flagellar regulon of *C. jejuni*. However, it appears that most genes encoding the structural components of the basal body, hook, and minor flagellin, FlaB, are regulated by σ^{54} promoters, and the major flagellin, FlaA, is regulated by a σ^{28} promoter (16) and possibly a σ^{70} promoter as well (21). Carrillo et al. previously performed expression profiling studies on two variants of NCTC 11168 with different virulence levels (6). Those workers observed that a number of nonflagellar genes that were controlled by σ^{54} or σ^{28} promoters displayed distinct levels of expression, suggesting that some of these flagellum-coregulated genes may contribute to virulence (6). We have recently shown that mutation of one of these σ^{28}-regulated genes, Cj0977, affected the virulence of *C. jejuni* 81-176 in vitro and in vivo (14). Another of these putative σ^{28}-regulated genes, Cj0859c, which encodes a protein with no homology to known proteins, maps in a region of the chromosome that has been reported to be variable among strains in a recent microarray study (33). Analysis of this region in the *C. jejuni* genomes deposited in GenBank indicated that the putative σ^{28} promoter was conserved but that there was variation in the Cj0859c gene. Here, we demonstrate that the predicted proteins encoded by the Cj0859c genes in isolates of *C. jejuni* from different geographical areas show considerable allelic variation. We have characterized two variants of the protein and demonstrate that both forms of the protein are secreted into the supernatant in a process that requires a minimum flagellar structure. Moreover, we demonstrate significant biological differences in two variants of the protein.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* strain 81-176 and *Campylobacter coli* strain VC167 have been described previously (3, 19). Table 1 summarizes mutants of 81-176 used in this study. All of the flagellar mutants, except PG2157 (*flgI*) and PG2158 (*flgK*), were previously described (14). PG2157 and PG2158 were generated by transposon mutagenesis of clones of the *flgI* and *flgK* genes in *Escherichia coli*, respectively, from 81-176 using Tn*5*-based in vitro transposition (15, 18), followed by electroporation into 81-176. The insertion into *flgI* occurred at bp 363 of the 1,047-bp open reading frame; the insertion into *flgK* occurred at bp 113 of the 1,827-bp open reading frame. Both mutants were nonmotile and lacked all flagellar structure by negative-stain electron microscopy. Other strains of wild-type *C. jejuni* are shown in Table 2. *Campylobacter* strains were grown routinely on Mueller-Hinton (MH) agar supplemented with 50 μg/ml kanamycin and/or 15 μg/ml chloramphenicol as needed.

RT-PCR analysis. To confirm the regulation of *fspA* by σ^{28}, the relative expression of this gene was determined by real-time PCR (RT-PCR) in wild-type 81-176 and DRH311, which is a σ^{28} deletion of 81-176 (Δ*fliA*) (21). RNAs were extracted from mid-log-phase cultures of *C. jejuni* 81-176 and the Δ*fliA* strain grown in biphasic MH medium. Synthesis of cDNA was performed using an iScript cDNA synthesis kit (Bio-Rad, La Jolla, CA). RT-PCR was performed with the ABI Prism 7000 DNA analyzer (Applied Biosystems, Foster City, CA) using a QuantiTect SYBR green RT-PCR kit (QIAGEN, Valencia, CA) accord-

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TABLE 1. *C. jejuni* 81-176 mutants used in this study

Strain	Gene mutated	Parent strain	Genotype	Reference or source
PG2572	Cj0859c	81-176	*fspA*$_{81\text{-}176}$::*cat*	This study
DRH311	Cj0061	81-176	Δ*fliA*	19
PG2662	None	81-176	*astA*::*fspA*$_{8486}$ *aphA3*	This study
PG2573	Cj0859c	PG2572	*fspA*$_{81\text{-}176}$::*cat* (pCPE2575)	This study
PG2663	Cj0859c	PG2572	*fspA*$_{81\text{-}176}$::*cat* *astA*::*fspA*$_{8486}$ *aphA3*	This study
PG2453	Cj1729c	81-176	*flgE*::*cat*	13
PG2139	Cj0548	81-176	*fliD*::*cat*	13
PG2157	Cj1462	81-176	*flgI*::*cat*	This study
PG2158	Cj1466	81-176	*flgK*::*cat*	This study

TABLE 3. Primer pairs used in RT-PCR studies

Locus	Primer	Primer sequence (5′–3′)
fspA1	RT-01	AAAACGCTTTAGCGCAAGAT
	RT-02	TTGTTGCGTGTTGTTTTGTG
fspA2	RT-03	TGTTCATAGCGACAATGTGGT
	RT-04	AAGCCTCTAATGCGGGATTT
flaA	RT-05	TCGTATTAACACCAATGTTGCAG
	RT-06	GCTTGAGATCTTAAACTATCTGCTATC
ilvC	RT-07	AAAAAGTAGCAATTATAGGCTTTGG
	RT-08	TTGCTTTAACAGCACTTACACTACC
Cj0977	RT-09	ACCACAAGCGAAATGGTAGC
	RT-10	TCGTCAAAAAGTGCATGAGC
23S RNA	RT-11	GGTAGGAGAGCGTTCTATTTGC
	RT-12	CGACTTAGGACCCGACTAACC
16S RNA	RT-13	GGGAAAGTTTTTCGGTGTAGG
	RT-14	GTTGCTGCGTCAGGGTTT

ing to the manufacturer's protocol. Primer sequences are given in Table 3. RT-PCR was followed by a melting curve analysis in order to confirm amplification of specific PCR products. The expression of *fspA* in each strain was normalized to 16S and 23S RNA. The relative *fspA* expression level was obtained using the $\Delta\Delta C_T$ method as recommended in the Applied Biosystems manual. Results are based on at least three independent experiments.

PCR amplification and sequence analysis of different Cj0859c alleles. The Cj0859c-Cj0860 region of the chromosome of *C. jejuni* strains was PCR amplified using primers pg06.14 (5′-CCTATTTATGGATTGCAATTTCACCCCG-3′) and pg06.15 (5′-CTTGAAACGATCAAGGGTAGGGCAGC-3′). Primer

TABLE 2. Strains used in the characterization of Cj0859c variant frequency in *C. jejuni*

C. jejuni strain	Alternative strain name	Origin	Penner serotype	GenBank accession no.
NCTC 11168[a]		United Kingdom	2	NC_002163.1
84-25[b]		United States		ZP_01100726
RM1221[b]		United States	53	NC_003912.7
81-176[b]		United States	23/36	ZP_01087888
HB93-13[b]		China	19	ZP_01071420
CF93-6[b]		Japan		ZP_01068377
260.94[b]		South Africa	41	ZP_01070269
MK19		Kuwait		EF058200
MK50		Kuwait		EF058201
MK52		Kuwait		EF058202
MK53		Kuwait		EF058203
MK59		Kuwait		EF058204
ATCC 43429	MSC 57360	Canada	1	EF058205
ATCC 43430	PC 72	Canada	2	EF058206
ATCC 43432	MK 7	Canada	4	EF058207
ATCC 43438		Canada	10	EF058208
ATCC 43446	MK 104	Canada	19	EF058209
ATCC 43449	MK 198	Canada	23	EF058210
ATCC 43456	MK 290	Canada	36	EF058212
ATCC 43431	TGH 9011	Canada	3	EF058211
LAN749		Egypt		EF058213
HS000128		Egypt		EF058214
HS001578		Egypt		EF058215
HS008516		Egypt		EF058216
OH 4384		Japan	19	EF058217
PG836		Puerto Rico	10	EF058218
CG-99-8013		Thailand		EF058219
CG-99-8023		Thailand		EF058220
CG-99-8071		Thailand		EF058221
CG-99-8087		Thailand		EF058222
CG-99-8109		Thailand	4/13/64/66	EF058223
CG-99-8131		Thailand		EF058224
CG-99-8153		Thailand	4/10/13/16/64/66	EF058225
CG-99-8245		Thailand		EF058226
CG-99-8261		Thailand	13	EF058227
CG-99-8265		Thailand	13	EF058228
CG-99-8289		Thailand		EF058229
CG-99-8431		Thailand		EF058230
CG-99-8437		Thailand		EF058231
CG-99-8486		Thailand	4/13/64/66	EF058232
HC37		United States	27	EF058233

[a] See reference 34.
[b] TIGR *Campylobacter* Genome Projects (D. E. Fouts and K. E. Nelson, unpublished data).

pg06.14 bound to the *pabA* gene (Cj0861), and pg06.15 bound to *murA* (Cj0858c). Amplicons were subjected to DNA sequence analysis using BigDye chemistry on an Applied Biosystems model 3100 DNA sequencer, and the sequences were assembled using Sequencher 4.5 (Gene Codes). Each consensus sequence was manually inspected for quality assessment of the assembly. The Cj0859c gene sequence was identified for each consensus using Aretemis software (http://www.sanger.ac.uk/Software/Artemis/). Biological sequences were compared using the SIM alignment tool (http://ca.expasy.org/tools/sim-prot.html). The phylogenetic tree was made using CLC Free Workbench 3 software (http://www.clcbio.com/index.php?id=28).

Expression of Cj0859c in *E. coli*. The $fspA_{81\text{-}176}$ gene was amplified from 81-176 by PCR using Easy-A High Fidelity PCR master mix (Stratagene, La Jolla, CA). The template was DNA from strain 81-176, and the primers used were pg05.53 (5′-GACGACGACAAGATGCAAATTAACAATTCCTTAAATAGC-3′) and pg05.54 (5′-GAGGAGAAGCCCGGTTCAAGCTTGTTGGCTTTGGAGTTC-3′). These primers included sequences for cloning into pET41-EK LIC, a vector that allows fusion to both a glutathione *S*-transferase (GST) tag and a hexahistidine tag (EMD Bioscience, Madison, WI). The 429-bp amplicon was purified using QIAquick PCR purification columns (QIAGEN, Valencia, CA), ligated into pET41-EK LIC as directed by the manufacturer, and transformed into NovaBlue GigaSingles cells (EMD Biosciences). Several resulting clones were sequenced, and one clone, which showed the predicted sequence, was selected for protein purification. The GST-His-Cj0859c protein was purified by nickel chromatography (QIAGEN).

The FspA protein from 81-176 was also expressed as a histidine-tagged protein in *E. coli* for biological experiments as follows. The gene was PCR amplified from 81-176 using primers DR101 (5′-CCATATGCAAATTAACAATTCCTTAAATAGC-3′) and DR102 (5′-GGGATCCTCAAGCTTGTTGGCTTTGGAGTTC-3′) and HF2 DNA polymerase (Clontech). The resulting amplicon was digested with NdeI and BamHI and cloned into NdeI-BamHI-digested pET-19b (Novagen, San Diego, CA) in *E. coli* DH5α. Following confirmation of the correct construction by DNA sequencing, one resulting clone was transformed into BL21(DE3), and the protein was overexpressed and purified on Ni-nitrilotriacetic acid resin as recommended by the supplier (QIAGEN).

FspA from CG8486 was expressed as a histidine-tagged protein in pET-19b using primers DR103 (5′-CCATATGAAAATAGATACTTTGACAAAAAATTTTAGC-3′) and DR102 (shown above), as described above for 81-176.

Antibodies against different forms of FspA. Rabbit polyclonal antiserum against the GST-His-FspA from 81-176 and from the His-tagged FspA protein from CG8486 described above were generated by Harlan Biosciences (Madison, WI).

SDS-PAGE and immunoblotting. Proteins were separated on 12.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels and detected with Gel Code Blue (Pierce, Rockford, IL) or transferred onto nitrocellulose and immunoblotted using the indicated rabbit polyclonal antisera. Proteins were immunodetected with either anti-GST-His-$FspA_{81\text{-}176}$ or anti-His-$FspA_{8486}$ polyclonal rabbit antiserum at a final dilution of 1:5,000. For assays to measure the binding of both recombinant proteins to INT407 cells, a mouse monoclonal antibody (Novagen) that recognizes the histidine tag was used at a final dilution of 1:5,000. The secondary antibody was horseradish peroxidase-labeled goat anti-rabbit immunoglobulin G or goat anti-mouse antibody (Immunopure). The reactions were detected using Supersignal West Pico detection kits (Pierce, Rockford, IL). Chemiluminescence was detected using a Kodak (Rochester, NY) Image Station 2000R.

Purification of supernatants from *C. jejuni*. Bacteria were grown in MH biphasic flasks (50 ml of MH broth over 20 ml MH agar in T75 tissue culture flasks) for 18 h. Growth from two flasks (or 100 ml of MH broth) was centrifuged, and the supernatants were filtered through 0.2-μm polyvinylidene difluoride membrane filters (Millipore, Billerica, CA). Trichloroacetic acid was added to a final concentration of 10%, and the sample was iced for 45 min. The samples were centrifuged in a Sorvall RC5C refrigerated centrifuge at 10,000 × *g* for 15 min, and the pellets were washed twice in 20 ml acetone. The pellets were air dried and resuspended in 64 μl of phosphate-buffered saline per 56 ml of culture. An equal volume of 2× solubilization buffer (1 M Tris [pH 6.8], 0.001% bromophenol blue, 5% glycerol, 3% SDS, 5% β-mercaptoethanol) was added, and the samples were boiled for 10 min prior to electrophoresis. Equal loading of samples was confirmed by silver staining of SDS-PAGE gels.

Generation of an *fspA* mutant of 81-176. A mutant in $fspA_{81\text{-}176}$ was constructed using Tn*5*-based in vitro transposition (Epicenter, Madison, WI) with a *cat* cassette as previously described (18). The in vitro reaction was performed according to the manufacturer's instructions with a clone from a partially Sau3AI-digested ordered genomic library of 81-176 (L. Holder and P. Guerry, unpublished data), called pLCH8-63, as the target DNA. This clone contained the region of the 81-176 chromosome that corresponded to genes Cj0856c to Cj0860. The reaction products were transformed into *E. coli* DH5α, and plasmid DNAs from individual transformants were sequenced using primers that read out from within the *cat* cassette to determine the insertion point and the orientation of the transposon within the gene. A plasmid in which the *cat* cassette had inserted 259 bp from the translational start of *fspA* in the same orientation as *fspA* was selected. This plasmid was used to electroporate *C. jejuni* 81-176 with selection on MH agar supplemented with chloramphenicol (15 μg/ml). The successful mutation of *fspA* was verified by PCR with primers bracketing the Cm^r insertion point to confirm that the DNA had undergone a double crossover. This mutant was called PG2572.

Complementation of the Cj0859c mutant in *trans*. The *fspA* gene and its σ^{28} promoter were PCR amplified from 81-176 using the following primers: pg05.133 (5′-CGGGATCCCACCGCTAATAGCCCAAAAAATACCTCCC-3′) and pg05.134 (5′-GGAATTCCGCAAGTATACTTGAAACGATCAAGGGTAGGG-3′). These primers added BamHI and EcoRI sites, respectively. The resulting 744-bp amplicon was digested with BamHI and EcoRI (New England Biolabs, Beverly, MA) and cloned into similarly digested pRY107 (50). After confirmation that the clone was correct by DNA sequence analysis, the plasmid was transformed into DH5α cells carrying the conjugative plasmid RK212.2. The resulting cells were used as donors to conjugatively transfer the complementing plasmid, pCE2575, into PG2166, the Cj0859c mutant, with selection on kanamycin and chloramphenicol, as previously described (17, 18).

Insertion of $fspA_{8486}$ into the *astA* gene of 81-176 and selected mutants. The $fspA_{8486}$ gene and its σ^{28} promoter were PCR amplified from CG8486 using primers C360.07 F (5′-TGCGGATCCCCGAAGCGGTTTTAACTCAA-3′) and C360.07 R (5′-ATGCGAATTCAAGGGTAGGGCAGCATTTTT-3′), which included BamHI and EcoRI restriction sites. The resulting amplicon was digested with these restriction enzymes and cloned into pBluescript. A SmaI-ended *aphA3* cassette was cloned into the EcoRV site of the resulting clone. DNA sequence analysis indicated that the *aphA3* gene was inserted 3′ to $fspA_{8486}$ and was in the same orientation as $fspA_{8486}$. This plasmid was digested with BamHI and XhoI, which released $fspA_{8486}$ and the adjacent *aphA3* gene. This fragment was blunted with Klenow polymerase (New England Biolabs, Beverly, MA) and cloned into the EcoRV site of plasmid pYG660 containing the *astA* (arylsulfatase) gene of 81-176 (51). This plasmid was used to electroporate 81-176 and selected flagellar mutants to kanamycin resistance, as shown in Table 1. The resulting Km^r colonies were screened for a loss of arylsulfatase activity using MH agar supplemented with a chromogenic substrate (X-S; Sigma, St. Louis, MO), as previously described (17, 51).

Adherence and invasion assays. Adherence and invasion assays were done as previously described (2, 32, 49). Briefly, about 2×10^6 bacteria were added to a monolayer of about 3×10^5 INT407 cells. After centrifugation at 200 × *g* for 5 min, the assay mixtures were incubated at 37°C for 2 h. For the determination of adherence, the cells were washed four times with Hanks' balanced salt solution (HBSS) for 1 min before lysing the monolayer with 0.01% Triton X-100 and enumerating the total bacteria by plate counting on MH agar. For the determination of invasion, the monolayer was washed twice with HBSS, and fresh prewarmed modified Eagle's medium (MEM) supplemented with 100 μg/ml gentamicin was added to wells for an additional 2 h to kill extracellular bacteria. The monolayer was washed four times in HBSS and lysed with 0.01% Triton X-100 for 30 min. Released intracellular bacteria were enumerated by plate count. Invasion was expressed as the percentage of the inoculum surviving the gentamicin treatment, and adherent bacteria were expressed as the total number of bacteria enumerated without antibiotic treatment.

Binding of recombinant histidine-tagged *C. jejuni* proteins to INT407 cells. INT407 cells were seeded into 24-well tissue culture plates at about 5×10^5 cells per well in MEM plus 10% fetal bovine serum. Following incubation at 37°C for about 19 h, culture medium was removed and replaced with fresh MEM plus 10% fetal bovine serum prewarmed at 37°C. Aliquots of 5, 10, 25, and 50 μg of histidine-tagged $FspA_{8486}$ and 50 μg of histidine-tagged $FspA_{81\text{-}176}$ were added, and the cells were incubated for 2 h at 37°C. The monolayer was washed five times with phosphate-buffered saline and lysed in 200 μl of gel loading buffer (10% glycerol, 3% SDS, 0.01% bromophenol blue, 5% β-mercaptoethanol, 1 M Tris, and Halt protease inhibitor cocktail, EDTA free, from Pierce [Rockford, IL]). The samples were boiled for 10 min, and 10-μl aliquots were loaded onto a 12.5% SDS-PAGE gel for each sample. The gel was transferred onto nitrocellulose and immunodetected with a mouse anti-His-tag monoclonal antibody, as described above.

Apoptosis assays. Monolayers of INT407 cells in 24-well flat-bottom tissue culture dishes were treated with increasing amounts of recombinant $FspA_{8486}$ or 50 μg of $FspA_{81\text{-}176}$ and incubated for 4 h. Control wells were treated with 10 μg of membrane proteins from 81-176 containing CDT (8, 13, 26) or 10 μg

of membrane proteins from DS104, an isogenic *cdtA* mutant of 81-176 (22, 23). Cells were harvested and stained using the Guava Nexin assay kit according to the manufacturer's instructions (Guava Technologies, Hayward, CA). Basically, the cells were stained with annexin V-phycoerythrin and Nexin 7-amino-actinomycin D (7-AAD) in cold 1× Nexin buffer in a 50-μl reaction mixture and analyzed in a Guava Technologies personal cytometer. Cells that stained positive with annexin V-phycoerythrin but negative with Nexin 7-AAD were scored as being early apoptotic.

Statistical analyses. Statistical analyses of the RT-PCR results were calculated with a Wilcoxon two-group test. Statistical analyses of adherence, invasion, and apoptosis assays were done with two-tailed *t* tests.

RESULTS

Cj0859c is expressed by a σ^{28} promoter in 81-176. Carrillo et al. previously demonstrated reduced expression of Cj0859c in a *fliA* mutant of NCTC 11168 by microarray, suggesting that this gene is σ^{28} regulated, and the putative promoter sequence is consistent with a σ^{28} recognition sequence (6). Table 4 shows the results of quantitative RT-PCR analysis of Cj0859c expression in 81-176 and DRH311 (Δ*fliA*) (Table 1) (21). The data indicate that there is a >30-fold reduction in the expression of Cj0859c in DRH311 compared to that of wild-type 81-176. This reduction is comparable to that seen for two other σ^{28}-regulated genes, *flaA* and Cj0977. In contrast, there was no significant change in the expression of the σ^{70}-regulated *ilvC* (Cj0632) in DRH311 (Δ*fliA*) compared to that of 81-176.

Characterization of the Cj0859c locus in multiple strains of *C. jejuni*. Analysis of the predicted proteins encoded by the homologs of Cj0859c in three *C. jejuni* genomes deposited in GenBank, 260.94, CF93-6, and 84-25, revealed that the predicted proteins, which are highly homologous to each other, show considerable divergence with the Cj0859c protein encoded by both NCTC 11168 and 81-176. The gene corresponding to Cj0859c has been called *fspA* (flagellar secreted protein) for reasons explained below. We studied the variation of *fspA* by DNA sequence analysis of PCR products generated from a collection of isolates from diverse geographical locations, as shown in Table 2. The results, summarized in Fig. 1, revealed that there were two overall clusters of Cj0859c alleles. The FspA1 cluster, which included 81-176, NCTC 11168, and RM1221, comprised 32% of the total strains (13/41). The FspA2 cluster comprised 68% of the total strains (28/41). The FspA2 cluster includes TGH9011, which was originally reported to lack Cj0859c based on a microarray analysis using the NCTC 11168 allele as a probe (36). The allele found in TGH9011, however, is truncated and is not detected by immunoblot (see below). The FspA1 proteins share a minimum of 81.7% identity within the group, and the FspA2 proteins share a minimum of 95.1% identity. There appeared to be no association of allele type and geographical isolation, although all of the Thai isolates examined contained the FspA2 allele.

The PCR primers used to amplify Cj0859c from these strains mapped in homologs of Cj0858 and Cj0861 (see Materials and Methods). Curiously, it appeared that the FspA2 allele is always associated with the absence of the Cj0860 gene, which encodes a putative integral membrane protein. Thus, the PCR product obtained from all strains in the FspA1 cluster was 1,626 bp, and that obtained from all the strains in FspA2 cluster was 799 bp. Although adjacent on the chromosome, Cj0859c and Cj0860 are on opposite strands and are controlled by distinct promoters.

TABLE 4. Confirmation of σ^{28} regulation of Cj0859c by RT-PCR[a]

Gene	Avg fold down-regulation in Δ*fliA* ± SD	*P* value
Cj0859c	31.00 ± 17.90	≤0.028
Cj0977	28.64 ± 8.62	≤0.028
flaA	32.20 ± 14.73	≤0.028
ilvC	0.77 ± 1.40	≤0.88

[a] The average down-regulation (*n*-fold) of each gene in DRH311 (Δ*fliA*) compared to 81-176 is shown. Statistical significance (*P* value) was calculated using a Wilcoxon two-group test.

We further characterized two of the variant forms of Cj0859c. The Cj0859c gene from 81-176 (GenBank accession no. NZAANY01000003.1; locus tag CJJ81176_0875) encodes a predicted soluble, cytoplasmic protein of 15.5 kDa (pI 4.4). The Cj0859c gene from CG8486, a clinical isolate from Thailand (38), encodes a predicted soluble, cytoplasmic protein of 16.0 kDa (pI 5.62). These two variants are 41.6% identical at the protein level (Fig. 2) and 58% identical at the DNA level. Both share identical σ^{28} promoters (data not shown). The Cj0859c gene from 81-176 (from the FspA1 group) will be called $fspA_{81\text{-}176}$, and that from GC8486 (from the FspA2 group) will be called $fspA_{8486}$.

$FspA_{81\text{-}176}$ is secreted into the supernatant. Whole cells of *C. jejuni* 81-176 and an isogenic insertional mutant of $fspA_{81\text{-}176}$ were immunoblotted with rabbit polyclonal antiserum against a recombinant form of the protein (GST-His-$FspA_{81\text{-}176}$). The results, shown in Fig. 3A, revealed the presence of a band in wild-type 81-176 with an apparent molecular mass of approximately 18 kDa, slightly larger than the predicted molecular mass of $FspA_{81\text{-}176}$. A histidine-tagged version of this protein expressed in *E. coli* also migrated aberrantly in SDS-PAGE (data not shown). This band was missing in the $fspA_{81\text{-}176}$ mutant and appeared to be overexpressed when the mutation was complemented in *trans*. Since *C. jejuni* is known to secrete multiple proteins through flagella, we examined supernatants for the presence of Cj0859c. $FspA_{81\text{-}176}$ was found in supernatants of MH agar-grown 81-176 cells and the complement of the mutant but not in supernatants of the *fspA* mutant (Fig. 3A). Additional cross-reacting bands were also observed in some supernatant preparations and may represent processing events, degradation products, or cross-reacting proteins. To control for possible cell leakage, whole-cell and supernatant preparations of 81-176 were immunoblotted with antibodies to recombinant forms of three other proteins, as shown in Fig. 3B. These were Cj0977, another σ^{28}-regulated cytoplasmic protein that is not secreted (14); Omp18, a lipoprotein (5); and Omp50, an outer membrane protein (4). These proteins were readily detected in whole cells but not in the supernatant preparations.

***Campylobacter* strains that lack *fspA* are capable of secretion of the protein when the gene is supplied in *trans*.** The $FspA_{8486}$ protein was also found in the supernatant of cultures of CG8486, as shown in Fig. 3C, but did not show the aberrant migration observed with $FspA_{81\text{-}176}$. CG8486 is difficult to manipulate genetically (38). The *fspA* gene and its σ^{28} promoter from CG8486 were inserted into the *astA* gene of 81-176 to generate PG2662. The two forms of FspA can be visualized in the supernatant of this strain by immunodetection with anti-

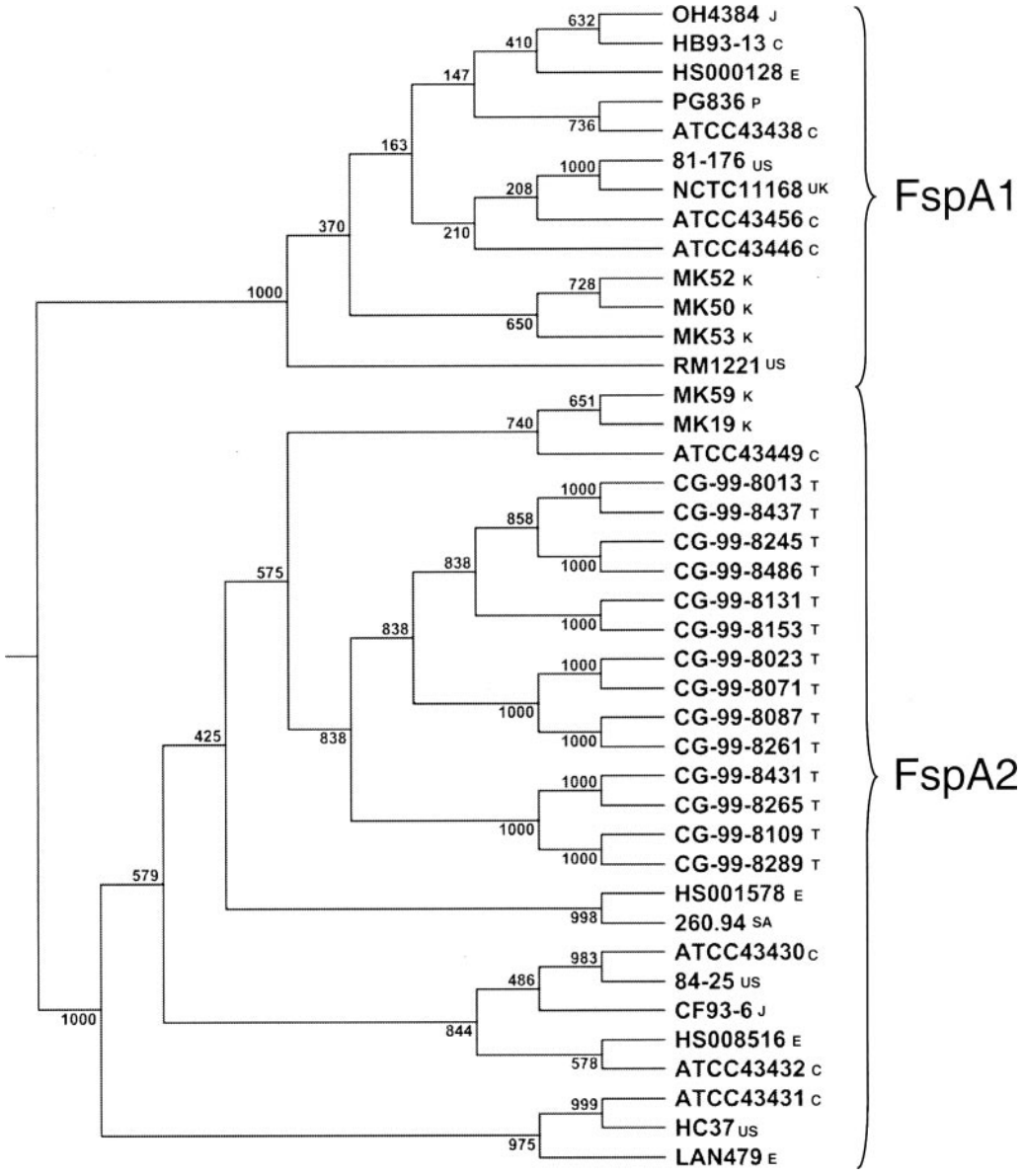

FIG. 1. Phylogenetic tree based on protein sequences encoded by alleles of the *fspA* gene. The tree is based on 1,000 bootstraps; scores are shown for all nodes. The tree clearly separates the two versions of Cj0859c, FspA1 and FspA2. The letters following the strain name refer to the country of isolation. C, Canada; CH, China; E, Egypt; J, Japan; K, Kuwait; PR, Puerto Rico; SA, South Africa; T, Thailand; UK, United Kingdom; US, United States. All the strains, with the exception of *C. jejuni* RM1221, which was isolated from a chicken carcass (30), were clinical isolates.

```
FspA1  MQINNSLNSLSQYVKVNSNEENQNSKNQEQNALAQDPAVEVNISKEAKEKSNTSNQNNSQ
       *:*:.  :.:*:* .  .:::: *: :.:*.:  : : *..  .:::.*.:.. : : .
FspA2  MKIDTLTKNFSNYQTQINKDNDLVSNVHSDNVVKIQISDEARSLSQTNSKNEDEYEIKVS

FspA1  APAQALNAQNNTQQDSSSNSEDKLTELTQKLAEIQAKIVELTAKMSKANEDQIKSIESQI
             : ::*: *:*... :  *  *  ***** ***.*** **::***:  .::::.**
FspA2  QEKIENHKDQNSIQNSKGGVNPALEALIAKLAEILAKIAELTQKMTNANEQMKTTFQKQI

FspA1  ATLNAQASTIQAQIQELQSQQA
       .*::**..**************
FspA2  DVLKSQADVIQAQIQELQSQQA
```

FIG. 2. ClustalW alignment (45) of $FspA_{81\text{-}176}$ (FspA1) and $FspA_{8486}$ (FspA2). *, identical amino acids; :, high-similarity amino acids; ., low-similarity amino acids.

$FspA_{8486}$ antiserum (Fig. 3C). Only $FspA_{8486}$ can be visualized in the supernatant of strain PG2663, the $fspA_{81\text{-}176}$ mutant of 81-176 containing $fspA_{8486}$ inserted into *astA*.

Cj0859c, or *fspA*, has been reported to be missing in several strains of *C. jejuni* by microarray analysis, including ATCC 43431 (TGH9011) (see below) (33, 36, 37). The gene was also absent in sequenced strains of *Campylobacter coli*, *Campylobacter upsaliensis*, *Campylobacter lari*, and *Helicobacter* spp. (1, 11, 42, 46). Cj0859c was not detectable in whole-cell immunoblots (data not shown) or supernatants of either *C. jejuni* TGH9011 or a *C. coli* strain, VC167 T2 (Fig. 3D). However, when pCE2575 carrying $fspA_{81\text{-}176}$ was transferred conjugatively into these strains, the $FspA_{81\text{-}176}$ protein could readily be

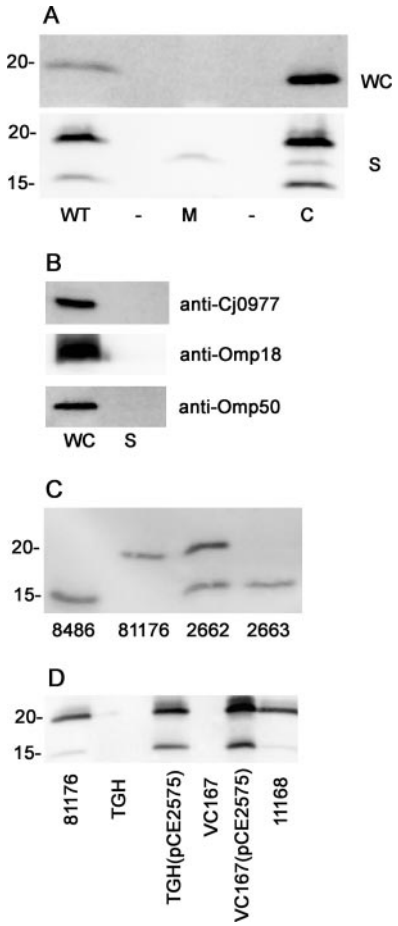

FIG. 3. Secretion of FspA. (A) Detection of $FspA_{81\text{-}176}$ in whole cells or supernatants. WT, wild-type 81-176; M, 81-176 *fspA*$_{81\text{-}176}$::*cat* (strain PG2572); C, 81-176 *fspA*$_{81\text{-}176}$::*cat* (pCPE2575) (strain PG2573); WC, whole cells; S, supernatants. (B) Controls for bacterial cell leakage. Whole cells and supernatants of wild-type 81-176 were detected with antibodies against recombinant forms of Cj0977, Omp18, and Omp50 (4, 5, 13). WC, whole cells; S, supernatants. (C) Detection of $FspA_{8486}$ in wild-type CG8486 and strain PG2662 (wild-type 81-176 containing $FspA_{8486}$ inserted into *astA*) and PG2663 (81-176 *fspA*$_{81\text{-}176}$::*cat* containing $FspA_{8486}$ inserted into *astA*). (D) Secretion of $FspA_{81\text{-}176}$ campylobacter strains with and without plasmid pCPE2575 carrying *fspA*$_{81\text{-}176}$. TGH, *C. jejuni* strain TGH9011.

detected in both whole cells (data not shown) and cell supernatants (Fig. 3D), indicating that the requisite machinery was present in these other *Campylobacter* strains to secrete the $FspA_{81\text{-}176}$ protein. Doublets of the $FspA_{81\text{-}176}$ protein were also observed in supernatants from these strains, as seen in Fig. 3D.

Secretion of $FspA_{81\text{-}176}$ requires a minimum flagellar structure. Song et al. (41) previously showed that FlaC secretion to the supernatants was not observed in *C. jejuni* TGH9011 mutants lacking hook (*flgE*) and basal body (*flgF*) structures. We examined the secretion of $FspA_{81\text{-}176}$ from similar mutants, as shown in Fig. 4. The results indicate that neither $FspA_{81\text{-}176}$ nor FlaC was secreted in 81-176 mutants in *flgE*, *flgI*, encoding the P ring subunit, or *flgK*, encoding the hook-filament junction protein. Both proteins were secreted from a mutant in *fliD* lacking the filament cap protein.

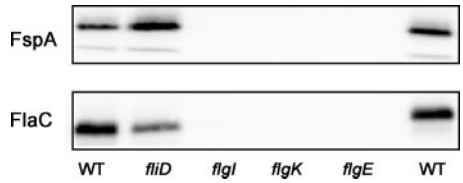

FIG. 4. Secretion of $FspA_{81\text{-}176}$ requires a minimum flagellar structure. $FspA_{81\text{-}176}$ or FlaC was detected in supernatants from 81-176 and mutants. Gels were immunodetected with anti-$FspA_{81\text{-}176}$ antibody. WT, wild type.

Mutation of *fspA*$_{81\text{-}176}$ does not affect 81-176 adherence or invasion of INT407 cells. There was no significant difference between wild-type 81-176 and an *fspA*::*cat* mutant, which was fully motile, in terms of adherence to (4.12% ± 0.74% and 4.75% ± 1.1%, respectively) ($P = 0.56$) or invasion of (2.30% ± 0.09% and 1.80% ± 0.92%, respectively) ($P = 0.61$) INT407 cells.

$FspA_{8486}$, but not $FspA_{81\text{-}176}$, binds to INT407 cells. Recombinant His-tagged $FspA_{8486}$ and $FspA_{81\text{-}176}$ proteins were added to a monolayer of INT407 cells, incubated for 2 h, and washed extensively. The monolayer was lysed, and the proteins were separated in 12% SDS-PAGE gels and immunodetected. The results shown in Fig. 5 indicated that $FspA_{8486}$ remained associated with the monolayer, while $FspA_{81\text{-}176}$ could not be detected in the lysate.

$FspA_{8486}$ but not $FspA_{81\text{-}176}$ causes induction of apoptosis of INT407 cells. Recombinant His-tagged $FspA_{8486}$, but not recombinant His-tagged $FspA_{81\text{-}176}$, caused an induction of apoptosis in INT407 cells after 4 h of incubation, as shown in Fig. 6. Moreover, there was a clear dose response to the recombinant $FspA_{8486}$ protein. The addition of 5 μg of recombinant $FspA_{8486}$ resulted in 11.9% ± 4.7% of the cells in the early apoptotic stage ($P < 0.05$ compared to untreated INT407 controls). The addition of increasing amounts of $FspA_{8486}$ from 10 μg to 50 μg resulted in a range of 15.4% ± 2.4% to 30.9% ± 4.8% of the cells in early apoptosis by 4 h ($P < 0.001$). In contrast, the addition of 50 μg of $FspA_{81\text{-}176}$ to the monolayer showed no differences compared to untreated controls. The kinetics of induction of apoptosis were markedly quicker than those caused by CDT (8, 13, 23). Membrane preparations (a total of 10 μg total protein) from 81-176 (CDT positive)

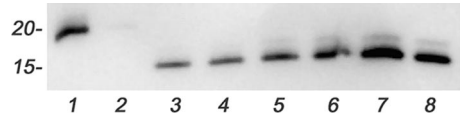

FIG. 5. Interactions of FspA proteins with INT407 cells. Hexahistidine-tagged recombinant $FspA_{81\text{-}176}$ and $FspA_{8486}$ proteins were added to a monolayer of INT407 cells, and the cells were incubated for 4 h at 37°C. The monolayer was washed five times and lysed as described in Materials and Methods, and an aliquot (10 μl, or about 750 ng protein) of each lysate was electrophoresed on a 12.5% SDS-PAGE gel, transferred onto a nitrocellulose membrane, and probed with anti-histidine-tagged mouse monoclonal antibody (Novagen). Lane 1, recombinant $FspA_{81\text{-}176}$ (40 ng); lane 2, INT407 cells plus 50 μg of $FspA_{81\text{-}176}$; lane 3, INT407 cells plus 5 μg $FspA_{8486}$; lane 4, INT407 cells plus 10 μg $FspA_{8486}$; lane 5, INT407 cells plus 25 μg $FspA_{8486}$; lane 6 INT407 cells plus 50 μg $FspA_{8486}$; lane 7, recombinant $FspA_{8486}$ (80 ng); lane 8, recombinant $FspA_{8486}$ (40 ng).

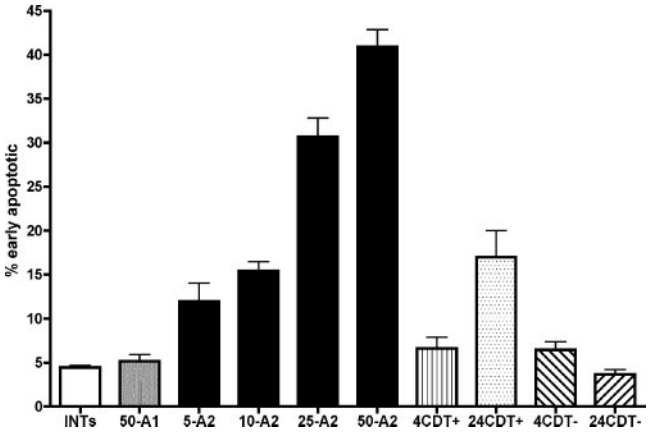

FIG. 6. Induction of apoptosis. Monolayers of INT407 cells were treated with 5 μg to 50 μg of recombinant $FspA_{8486}$ or 50 μg recombinant $FspA_{81\text{-}176}$ for 4 h and stained with a Guava Nexin kit, and the percentage of cells in early stages of apoptosis (annexin V positive and 7-AAD negative) was determined using a Guava Technologies personal cytometer. Bars are labeled as follows: INTs, untreated INT407 cells; 50-A1, 50 μg $FspA_{81\text{-}176}$; 5-A2, 5 μg $FspA_{8486}$; 10-A2, 10 μg $FspA_{8486}$; 25-A2, 25 μg $FspA_{8486}$; 50-A2, 50 μg $FspA_{8486}$. Additional controls included 10 μg of total protein from wild-type 81-176, which has been shown to contain CDT (21, 22), and 10 μg of total protein from a *cdtA* mutant of 81-176. These membrane preparations were incubated for both 4 h (4 CDT+ and 4 CDT−) and 24 h (24 CDT+ and 24 CDT−). The data represent the means and standard deviations of four to seven individual experiments done in duplicate. The *P* value for 5 μg of $FspA_{8486}$ compared to INT407 cells alone was <0.05; the *P* values for 10, 25, and 50 μg of $FspA_{8486}$ compared to INT407 cells alone were <0.001; the *P* value for the CDT-positive membranes of 81-176 compared to INT407 cells at 24 h of incubation (not shown) was <0.05.

caused apoptosis in only 6.6% ± 3.0% of the cells in 4 h and 17% ± 8% by 24 h. Membranes from a *cdtA* mutant of 81-176 had no effect, as previously reported (22, 23).

DISCUSSION

C. jejuni has proven to be remarkably recalcitrant to molecular pathogenic analysis. Genome sequencing of multiple strains has failed to identify any virulence factors shared with better-characterized pathogens other than CDT. However, the role of flagella and motility in virulence has long been recognized as critical to the pathogenesis of *C. jejuni* for a variety of reasons (3, 10, 17, 20, 24, 41, 47–49). *C. jejuni* flagella have been recognized previously to function as a T3SS to secrete both FlaC and the Cia proteins (24, 25, 39, 41). These secreted proteins modulated the invasion of some strains of *C. jejuni* but not 81-176 (14, 24, 25, 39, 41). Similarly, mutation of *fspA* in 81-176 had no effect on invasion. Despite isolation from a dysentery case, strain CG8486 is noninvasive in vitro (38), so it is unlikely that $FspA_{8486}$ plays a role in invasion. Consistent with this, the transfer of $fspA_{8486}$ into 81-176 (strain PG2662) or into 81-176 $fspA_{81\text{-}176}$ (strain PG2663) did not affect adherence or invasion compared to the wild type (data not shown).

Carrillo et al. (6) previously compared transcription profiles of virulent and nonvirulent variants of *C. jejuni* NCTC 11168 and observed that several σ^{28}- and σ^{54}-regulated genes were among those that showed differential expression. These genes had no known role in flagellar biogenesis despite apparent regulation by the two "flagellar" promoters. This led to the hypothesis that these genes might play a role in virulence, particularly since motility and virulence are strongly associated in *C. jejuni*. We have previously confirmed a role for the σ^{28}-regulated gene Cj0977 in virulence (14), and here, we have demonstrated that a second σ^{28}-regulated gene, Cj0859c, or FspA, also appears to be a virulence determinant, at least in some strains. We have confirmed expression by a σ^{28} promoter for both of these genes by RT-PCR. Cj0977 and Cj0859c (FspA) proteins could not be detected by immunoblot in the Δ*fliA* mutant, consistent with the RT-PCR results (14) (data not shown). Moreover, no FspA protein could be detected by immunoblot when $fspA_{8486}$ was transferred into DRH311 (81-176 Δ*fliA*) on a plasmid, indicating that both alleles are regulated by σ^{28} (data not shown). In contrast, there is no evidence that either *flaC* or *ciaB* is coregulated with the flagellar regulon.

The protein encoded by Cj0859c, FspA, unlike Cj0977, is secreted into the supernatant. Song et al. previously showed that FlaC was not secreted in *C. jejuni* mutants in either *flgE* or *flgF* (41); here, we have examined the expression of both FlaC and $FspA_{81\text{-}176}$ in additional flagellar mutants, and their secretion patterns appear to be identical. Thus, neither FlaC nor $FspA_{81\text{-}176}$ was secreted in mutants defective in basal body/hook structures, but both proteins were secreted in an *fliD* mutant. Mutants in *fliD*, which lack the filament cap protein, cannot assemble a filament but retain an open channel that is capable of secretion (27). However, *flaC* does not appear to be expressed by either a σ^{28} or σ^{54} promoter and is synthesized in an *fliA* mutant, unlike FspA (data not shown). The secretion of both FlaC and FspA occurs in broth-grown bacteria, unlike the Cia proteins, which require an exogenous signal from eukaryotic cells for secretion to occur (24, 25, 39).

The heterogeneity of FspA in different *C. jejuni* strains is striking, based on the limited numbers of strains examined. The form of FspA found in strain CG8486, but not that found in 81-176, binds tightly to eukaryotic cells and induces apoptosis. It is interesting that all of the isolates from Thailand examined, where *C. jejuni* is hyperendemic, contained similar forms of FspA. However, these clinical isolates all came from the same region of Thailand in 1999 and may not be representative of the entire region. In contrast, 81-176, NCTC 11168, and other strains contain an alternate form of FspA that has no observable phenotype as yet. It remains to be determined if the differences between the two proteins are in their ability to bind to epithelial cells, to induce apoptosis, or both, but binding and induction of apoptosis both show a dose dependence. The amount of FspA2 delivered during *C. jejuni* infection remains to be determined. However, recent observations that *C. jejuni* can form microcolonies on intestinal epithelial cells in vitro (17) suggest a mechanism by which the effective dose of FspA2, and perhaps other secreted proteins, could be concentrated near the cell surface. Clearly, there is considerable research remaining to understand the mechanism of action and the biological significance of $FspA_{8486}$. However, the ability of FspA2 to induce apoptosis in epithelial cells suggests a mechanism by which some strains of *C. jejuni* could disrupt the epithelial cell barrier.

The clinical spectrum of *C. jejuni* disease can range from a

mild, watery diarrhea to a dysentery-like disease. This is likely due, in part, to the immune status of the host, although it has been speculated that genomic differences among strains may also contribute. Complete genome analysis and comparative microarrays have identified gross differences among strains, largely in surface carbohydrates (9, 33, 34–37, 43). A recent microarray study has also reported heterogeneity in the Cj0859c-Cj0860 region, consistent with our data (33). The data reported here indicate that changes among FspA proteins have major effects on toxicity for epithelial cells in vitro. Clearly, there are virulent strains of *C. jejuni* that contain both *fspA1* and *fspA2* alleles, reflecting the multifactorial nature of pathogenicity. However, the observed heterogeneity among *fspA* alleles may modulate *C. jejuni* virulence. The in vitro and in vivo roles of the different forms of FspA in pathogenesis are under investigation.

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