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Secretion of Virulence Proteins from *Campylobacter jejuni* Is Dependent on a Functional Flagellar Export Apparatus

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***Campylobacter jejuni*, a gram-negative motile bacterium, secretes a set of proteins termed the *Campylobacter* invasion antigens (Cia proteins). The purpose of this study was to determine whether the flagellar apparatus serves as the export apparatus for the Cia proteins. Mutations were generated in five genes encoding three structural components of the flagella, the flagellar basal body (*flgB* and *flgC*), hook (*flgE2*), and filament (*flaA* and *flaB*) genes, as well as in genes whose products are essential for flagellar protein export (*flhB* and *fliI*). While mutations that affected filament assembly were found to be nonmotile (Mot$^-$) and did not secrete Cia proteins (S$^-$), a *flaA* (*flaB*$^+$) filament mutant was found to be nonmotile but Cia protein secretion competent (Mot$^-$, S$^+$). Complementation of a *flaA flaB* double mutant with a shuttle plasmid harboring either the *flaA* or *flaB* gene restored Cia protein secretion, suggesting that Cia export requires at least one of the two filament proteins. Infection of INT 407 human intestinal cells with the *C. jejuni* mutants revealed that maximal invasion of the epithelial cells required motile bacteria that are secretion competent. Collectively, these data suggest that the *C. jejuni* Cia proteins are secreted from the flagellar export apparatus.**

Campylobacter jejuni, a gram-negative motile bacterium, is a frequent cause of human gastrointestinal infections (39). The spectrum of disease observed in *C. jejuni*-infected individuals ranges from asymptomatic to severe enteritis characterized by fever, severe abdominal cramping, and diarrhea with blood and mucus (2, 4). By analogy with other more extensively characterized bacterial pathogens, the mechanism of *C. jejuni*-mediated enteritis is proposed to be multifactorial. Previous work has indicated that motility as well as the presence of the flagellum contributes to the ability of *C. jejuni* to colonize the intestinal tract of animals (33, 36, 42).

The flagellum of *C. jejuni* is composed of a basal body, hook, and filament. The flagellar filament is comprised of two proteins, FlaA and FlaB, although it appears that FlaA is the preferred subunit (3). While the *C. jejuni* FlaA and FlaB flagellin proteins are transcribed concomitantly (16), the *flaA* gene is regulated by σ^{28} and the *flaB* gene is regulated by σ^{54} (3, 16). Hendrixson et al. (16) noted that a *C. jejuni* isolate deficient in σ^{28}, which is encoded by the *fliA* gene, is able to assemble a truncated filament composed exclusively of the flagellin protein FlaB. This result indicates that the regulation of flagellar gene expression within *C. jejuni* differs from the regulation in more intensely studied systems such as that of *Salmonella enterica*. Unlike flagellar gene expression in *C. jejuni*, flagellar gene expression in *S. enterica* is initiated by a master regulator, while late gene expression and motility require σ^{28} (1).

Previous work in our laboratory has demonstrated that *C. jejuni* synthesizes a set of proteins during coculture with epithelial cells, a subset of which are secreted. The secreted proteins have been collectively referred to as *Campylobacter* invasion antigens (Cia proteins) (22). The functions of the secreted proteins are not known; however, insertional mutagenesis of the gene (*ciaB*) encoding a 73-kDa secreted protein (CiaB) results in a significant reduction in the number of *C. jejuni* cells internalized compared to a *C. jejuni* wild-type isolate. The absence of Cia protein secretion in the *C. jejuni ciaB* mutant is specific, as the invasive phenotype of this organism is restored by complementation in *trans* with the *ciaB* gene (37). CiaB lacks an identifiable signal sequence (22). In addition, an environmental stimulus is required to induce Cia protein secretion (37). While these characteristics are reminiscent of virulence-associated type III secretion systems, translation of the complete genome of *C. jejuni* NCTC 11168 failed to reveal proteins with identity to virulence-associated type III secretory systems (http://www.sanger.ac.uk/Projects/C.jejuni).

Some proteins that have the classical type III secretory apparatus show amino acid sequence similarity with flagellar structural proteins (26, 29). Moreover, evidence is beginning to accumulate that components of the flagellar apparatus participate in the export of virulence determinants in several pathogens. For example, experiments by Young et al. (45) demonstrated that *Yersinia* secretes flagellar outer proteins (Fops) via the flagellar apparatus. More recently, secretion of virulence-associated proteins from *Bacillus thuringiensis* has been found to be dependent on *flhA*, an essential component of the flagellar export apparatus (13). Consistent with the notion that components of the flagellar export apparatus can play a role in the export of virulence-associated proteins in some organisms, we noted that a *C. jejuni flhB* export mutant was deficient in secretion of the Cia proteins. The purpose of this study was to determine whether the flagellar protein export apparatus is required for secretion of the Cia proteins.

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Related Organisms, in Freiburg, Germany, 1 to 5 September 2001; and at the 82nd annual meeting of the Conference of Research Workers in Animal Diseases, St. Louis, Mo., 11 to 13 November 2001.)

MATERIALS AND METHODS

Bacterial isolates and growth conditions. Human clinical *C. jejuni* strains F38011 and 81116 were cultured as described previously (38). Plates were supplemented with kanamycin sulfate (200 μg/ml) as appropriate. *Escherichia coli* InvαF′, MRF XL-1 Blue (Tet^r), and DH5α were cultured in Luria-Bertani broth or solid medium, supplemented with kanamycin (50 μg/ml) or tetracycline (15 μg/ml) as appropriate, in a 37°C incubator.

Isolation of *C. jejuni* flagellar mutants. All of the suicide vectors described below were introduced into *C. jejuni* F38011 by electroporation. *C. jejuni* F38011 mutants were identified by acquisition of kanamycin or chloramphenicol resistance or kanamycin sensitivity, and specific gene disruption was confirmed by PCR.

C. jejuni strain NCTC 11168 *flaA*, *flaB*, *flhB*, *flgB*, *flgC*, *flgE2*, *fliD*, and *fliI* gene sequences were obtained from the Sanger Center website (http://www.sanger.ac.uk/Projects/C.jejuni). Two *flgE* genes have been identified in NCTC 11168 (Cj0043 and Cj1729c); however, based on previously published work showing sequence similarity of the *flgE2* product to other hook proteins (18, 25), *flgE2* (Cj1729c) was targeted. The *flhB* (Cj0335), *flgB* (Cj0528c), *flgC* (Cj0527c), *flgE2* and *fliD* (Cj0548), and *fliI* (Cj0195) genes in *C. jejuni* F38011 were disrupted by recombination via a single crossover event between the chromosomal gene and an internal fragment of the homologous gene in a suicide vector harboring the *aphA3′* gene encoding kanamycin resistance (21). The *C. jejuni* *flhB* (forward primer, 5′-AAA AAA CAG AAG AAC CCA CG; reverse primer, 5′-GTG CAA CCA TAG TAT AAA GCT C), *flgB* (forward primer, 5′-CAT TTA AAT CAA AAG AAC TGG; reverse primer, 5′-TCC ATC AAG AGC TGT TAT C), *flgC* (forward primer, 5′-AAG TGA TTT TGA TAT TAG TGG; reverse primer, 5′-GTA GCT TCA ATC AAA TCT GC), *flgE2* (forward primer, 5′-AGT GGT TTA AAT ATA GGA ACT TCA AG; reverse primer, 5′-AAA GAA TCA TAA ATT TCA AGC C), *fliD* (forward primer, 5′-TGC GAA AAG AAA AGT TGT AGG; reverse primer, 5′-ATT TGC ATC TTC AGT AGT TCC), and *fliI* (forward primer, 5′-CTC GAG GCT ACA AGT ATA GAA ATT CGT GG; reverse primer, 5′-GAG CTC GTT GCA CTT GCA CTT CCT AC) internal gene fragments were amplified with *Taq* polymerase (Invitrogen) and gene-specific primers as part of a PCR.

In addition to the single-crossover insertion mutants generated above, a deletion-insertion mutation was constructed in the *fliA* (Cj0061c) gene by allelic replacement via a double crossover event. With a similar strategy, a filament-negative *C. jejuni* F38011 mutant was generated by deletion of the 3′ end of the *flaA* (Cj1338c) gene and the 5′ end of the *flaB* (Cj1339c) gene. Target genes in *C. jejuni* F38011 were disrupted by allelic replacement via a double-crossover event between the target chromosomal gene and a plasmid-borne copy of the target gene containing an internal deletion. To aid in the selection of the double-crossover mutations, the deletions were marked by the *cat* gene, encoding chloramphenicol resistance. The final vectors were constructed with standard molecular biology techniques. Briefly, the 5′ end of the *fliA* gene with flanking DNA was amplified with the F1 and R1 primer set (F1, 5′-TT*G GAT CC*T TGG AAG ACA TTT TAA TAG AAG; R1, 5′-AA*C CGC GG*A AA*G CTA GC*C ACA AGC TCA TCT TGC TCT TTC). The italics represent restriction sites that were used to facilitate the cloning and construction of the final vector.

The *fliA* F1 primer contained a BamHI restriction site, and the *fliA* R1 primer contained NheI and SacII restriction sites. The 3′ end of the *fliA* gene with a flanking region was amplified with a second set of primers (F2, 5′-TT*G CTA GC*C ACG AAG TGC TAG ATG ATC TTA AAG; R2, 5′-AA*C CGC GG*A TTT CTT TGA TTT CAT CTT TAT C). The *fliA* F2 primer contained an NheI restriction site and the *fliA* R2 primer contained a SacII restriction site. Following ligation of DNA fragments harboring the 5′ and 3′ regions of the *fliA* gene, the *cat* gene was ligated into the NheI restriction site. Portions of the *flaA* and *flaB* genes were amplified with the primer sets FlaAF1/FlaAR1 and FlaBF1/FlaBR1 (FlaAF1, 5′-*GGA TTC* CCG TTA AAG CAA CTA TAG GTG CAA; FlaAR1, 5′-*GCT AGC* CCT GAT ACT GAA CTA ATA GAA TTG; FlaBF2, 5′-CT *A GCT AGC* GGT CAA GCT ATA AAC AAT GGT AAT GAT C; and FlaBR2, 5′-TT*G GAT CC*T TAT TGA AGA AGT TTT AAA ACA TTT TGC TGC). The italics represent restriction sites that were used to facilitate the cloning and construction of the final vector. The *flaA*F1 and *flaB*R2 primers contained a BamHI restriction site, and the *flaA*R1and *flaB*F2 primers contained an NheI restriction site. Amplification with FlaAF1/FlaAR1 resulted in deletion of the 3′ end of *flaA*, whereas amplification with FlaBF2/FlaBR2 resulted in deletions at both the 5′ and 3′ ends of *flaB*. Following ligation of DNA fragments harboring the truncated *flaA* and *flaB* genes, the *cat* gene was ligated into the NheI restriction site, and the deletion-insertion mutation was crossed onto the F38011 chromosome.

Phenotypic analysis of the *C. jejuni* flagellar mutants. Motility assays were performed with Mueller-Hinton (MH) medium supplemented with 0.4% Select Agar (Gibco-BRL). A 10-μl suspension of each bacterial isolate was spotted on the surface of semisolid medium. Motility plates were incubated at 37°C under microaerophilic conditions for 48 h. *C. jejuni* strains were also analyzed by transmission electron microscopy (TEM). Bacterial suspensions prepared from MH agar plates with phosphate-buffered saline were added dropwise to Formvar-coated copper grids. Bacteria were stained with 1% phosphotungstic acid. Samples were analyzed with a 1200 EX transmission electron microscope (JEOL).

Complementation analysis. A 2,032-bp fragment containing the entire *flaA* gene and flanking DNA sequences was amplified from *C. jejuni* NCTC 11168 by PCR with the primers 5′-*GGA TCC* TAA AAC GCA TTT CAT CAC AGC (forward primer; BamHI linker is italic in each case) and 5′-*GGA TCC* GAT TAA AGC AAA AAG TGT TC (reverse primer). The forward primer is 199 bp upstream of the AUG methionine initiation codon, and the reverse primer extends 114 bp beyond the UAG stop codon. Following an intermediate cloning step into pCR2.1 (Invitrogen), the gel-purified insert was ligated into the BamHI site of pMEK80 (37). The resultant shuttle plasmid, designated pMEK3502, was introduced into a *C. jejuni* strain 81116 *flaA flaB* mutant (GRK7) by electroporation (15). Transformants were identified as described above. The *C. jejuni* pMEK3503 shuttle vector (*flaB*$^+$) was generated in a similar fashion with the primers 5′-*GGA TCC* CAA AAT GTT TTA AGA TTA CTA CAG (forward primer) and 5′-*GGA TCC* TTT TTG CTT GGG TTT ATG CAC (reverse primer). The forward primer is 172 bp upstream of the AUG methionine initiation codon, and reverse primer extends 161 bp beyond the UAA stop codon. The amplified PCR product containing the *flaB* gene and flanking DNA sequences was 2,052 bp.

Analysis of *C. jejuni* secreted proteins. *C. jejuni* cells were metabolically labeled with [^{35}S]methionine as described elsewhere (19). For each sample, the supernatant fluid was treated in an identical manner. Briefly, the supernatant fluids were concentrated fourfold by the addition of 5 volumes of ice-cold 1 mM HCl-acetone. The pellets were air dried and resuspended in an equal volume of water. Equal volumes of the concentrated samples were then loaded in the wells of a sodium dodecyl sulfate–12.5% polyacrylamide gel. Proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with the discontinuous buffer system described by Laemmli (24). In most instances, gels were treated with Amplify (Amersham Life Sciences) according to the manufacturer's instructions, and autoradiography was performed with Kodak BioMax MR film at −70°C. Alternatively, the proteins were electrophoretically transferred to polyvinylidene fluoride membranes (Immobilon P; Millipore Corp., Bedford, Mass.) for immunoblot analyses.

RNA isolation and reverse transcription-PCR. Bacteria were grown in Mueller-Hinton broth with 0.05% deoxycholate under microaerobic conditions overnight, pelleted, and resuspended in 200 μl of TE (10 mM Tris Cl, 1 mM EDTA, pH 7.4) buffer. The bacteria were then treated with 100 μl of 50 mM glucose–25 mM Tris-Cl–10 mM EDTA–200 μl of 0.1 M NaOH–1% SDS–150 μl of 5 M potassium acetate and centrifuged for 5 min at 13,000 × *g*. All solutions were pretreated with 0.1% diethyl pyrocarbonate (Sigma). The supernatant was treated with an equal volume of phenol-chloroform (1:1). The resulting supernatant was added to an equal volume of 90% isopropanol and centrifuged for 5 min at 13,000 × *g*. The pellet was then washed in 70% isopropanol, pelleted by centrifugation as above, dried by vacuum aspiration, and incubated with 50 U of RNase-free DNase (Roche, Indianapolis, Ind.) for 15 min at 37°C. After DNase treatment, an equal volume of 7.5 M sodium acetate was added as well as 2 volumes of 90% isopropanol, centrifuged for 20 min at 4°C, washed in 70% isopropanol, and recentrifuged. The pellet was resuspended in 50 μl of RNase-free water (27).

Ten micrograms of RNA was used for cDNA preparation with 3 μl of the random primers provided with the ProSTAR First Strand reverse transcription-PCR kit (Stratagene, La Jolla, Calif.) according to the manufacturer's instructions. Then, 2.5 μl of the cDNA was subjected to PCR amplification with primers within the coding region of the *ciaB* primers (forward primer, 5′-CTA TGC TAG CCA TAC TTA GGC; reverse primer, 5′-GCC CGC CTT AGA ACT TAC) as well as primers within the *asp* gene (asp-S3 forward primer, 5′-CCA ACT GCA AGA TGC TGT ACC; asp-S6 reverse primer, 5′-TTA ATT TGC GGT AAT ACC ATC) (12). The cycling conditions were as follows: one cycle of 5 min at 94°C; five cycles of 30 s at 94°C, 30 s at 45°C, and 1 min at 72°C; 20 cycles of 30 s

TABLE 1. Phenotypes displayed by wild-type isolates and isogenic flagellar protein export and filament mutants

Background strain	Relevant characteristic	FlaA synthesis	Filament assembly	Motility	Secretion	Adherence[a]	Invasion[b]	I/A[c]
F38011	Wild type	+	+	+	+	100 ± 5	100 ± 20	5.3
	flhB	−	−	−	−	47 ± 9	0.24 ± 0.04	0.03
	flgB	−	−	−	−	40 ± 6	0.46 ± 0.1	0.06
	flgC	−	−	−	−	73 ± 2	2.1 ± 0.5	0.13
	flgE2	−	−	−	−	13 ± 2	0.26 ± 0.1	0.11
	fliD	+	+	+	+	59 ± 8	44 ± 4	4.0
	ciaB	+	+	+	−	223 ± 15	1.9 ± 0.7	0.02
81116	Wild type	+	+	+	+	100 ± 9	100 ± 27	2.7
	flaA flaB	−	−	−	−	191 ± 27	10 ± 3	0.14
	flaA	−	Truncated	−	+	145 ± 19	70 ± 7	1.3
	flaA flaB/pMEK3502 (*flaA*$^+$)	+	+	+	+	127 ± 18	29 ± 1	0.63
	flaA flaB pMEK3503 (*flaB*$^+$)	−	Truncated	−	+	255 ± 27	43 ± 7	0.46

[a] As a percentage of the wild-type value, which was $9.4 \times 10^5 \pm 0.5 \times 10^5$.
[b] As a percentage of the wild-type value, which was $5.0 \times 10^4 \pm 1 \times 10^4$.
[c] I/A, [(number of internalized bacteria)/(number of adherent bacteria)] × 100.

at 94°C, 30 s at 49°C, and 1 min at 72°C; and a final extension of 5 min at 72°C. The resulting products were resolved by electrophoresis through 1% agarose in Tris-borate-EDTA buffer, and bands were visualized by UV light after ethidium bromide staining.

Examination of the interactions of *C. jejuni* with INT 407 cells. Adherence and internalization assays were performed with INT 407 cells (human embryonic intestine cells; ATCC CCL 6) as previously described (20). Briefly, each well of a 24-well tissue culture tray was seeded with 10^5 cells/well and incubated for 18 h at 37°C in a humidified, 5% CO_2 incubator. The cells were rinsed with Eagle's minimal essential medium–1% fetal bovine serum and inoculated with approximately 5×10^7 CFU of a bacterial suspension. Tissue culture trays were centrifuged at 600 × *g* for 5 min and incubated at 37°C in a humidified, 5% CO_2 incubator. For binding, the infected monolayers were incubated for 30 min and rinsed three times with phosphate-buffered saline, and the epithelial cells were lysed with a solution of 0.25% (wt/vol) sodium deoxycholate. The suspensions were serially diluted, and the number of viable, adherent bacteria was determined by counting the resultant colonies on MH/blood plates.

To measure bacterial internalization, the infected monolayers were incubated for 3 h, rinsed three times with Eagle's minimal essential medium–1% fetal bovine serum, and incubated for an additional 3 h in Eagle's minimal essential medium–1% fetal bovine serum containing a bactericidal concentration (250 μg ml^{-1}) of gentamicin (31). The number of internalized bacteria was determined as outlined above. The number of adherent bacteria for *C. jejuni* F38011 was typically 10^6 CFU and the number of internalized bacteria was 5.0×10^4 CFU; for actual values, refer to Table 1. Because adherence is a prerequisite for *C. jejuni* invasion, the values reported were transformed with the following equation: [(number of internalized bacteria)/(number of adherent bacteria)] × 100. Significance between samples was determined with Student's *t* test following $\log_{10}$ transformation of the data. Two-tailed *P* values were determined for each sample, and a *P* value of <0.01 was considered significant. All assays were repeated a minimum of three times.

Other analytical procedures. Freshly prepared serum against the *C. jejuni* CiaB protein was generated in a New Zealand White rabbit as described elsewhere (22). Immunoblot analyses were performed with a 1:50 dilution of a rabbit anti-*C. jejuni* flagellin serum or a rabbit anti-CiaB protein serum as described elsewhere (15). Bound antibodies were detected with peroxidase-conjugated goat anti-rabbit immunoglobulin G and 4-chloro-1-naphthol (Sigma) as the chromogenic substrate.

RESULTS

Generation of *C. jejuni* flagellar mutants. Experiments were performed to determine if the Cia proteins are secreted from the flagellar apparatus. A *C. jejuni* F38011 *flhB* flagellar export apparatus mutant was found to be nonmotile (Mot$^-$) and also failed to secrete the Cia proteins (S$^-$) (Table 1), which is consistent with the notion that the flagellar export apparatus is required for Cia protein secretion. Additional experiments were undertaken to test this preliminary finding. Insertion mutations were generated in the *C. jejuni* F38011 genes encoding the flagellar basal body (*flgB* and *flgC*) and hook (*flgE2*) and in the putative filament cap protein (*fliD*). In addition, we used previously published strains of *C. jejuni* 81116 with disruptions in either *flaA* (GRK17) or *flaA* and *flaB* (GRK7) (15). For clarity, we will refer to the *C. jejuni* 81116 *flaA* mutant throughout the remainder of the text as a *flaA* (*flaB*$^+$) mutant. After confirming each mutation by gene-specific PCR, we assessed whether each mutant was motile, synthesized the FlaA filament protein, and assembled a filament.

Phenotypically, the *C. jejuni flgB*, *flgC*, *flgE2* and *flaA flaB* flagellar mutants exhibited a Mot$^-$ phenotype on motility agar, failed to synthesize FlaA, as determined by immunoblot analysis, and did not assemble a filament, as judged by TEM (Table 1 and data not shown). However, a 62-kDa immunoreactive band of decreased intensity, likely corresponding to the FlaB protein, was detected in whole-cell lysate preparations of *C. jejuni* F38011 *flgB*, *flgC*, and *flgE2* mutants as well as in the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant with a flagellin polyclonal serum. The 62-kDa immunoreactive band was not detected in the *C. jejuni* 81116 *flaA flaB* mutant. Collectively, these data indicate that synthesis of the FlaA protein was greatly reduced in the *C. jejuni* F38011 *flgB*, *flgC*, and *flgE2* flagellar export mutants, which is consistent with the findings of Matz et al. (28), who determined that there was a significant decrease in the level of *flaA* gene expression in a *C. jejuni* strain containing a defective flagellar export apparatus. A truncated filament was observed in the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant. These results are consistent with the findings of Wassenaar et al. (41), who showed that motility correlated with the synthesis and assembly of a FlaA but not a FlaB filament. Wassenaar et al. (41) also observed truncated filaments in a *flaA* (*flaB*$^+$) mutant.

A mutation in the *C. jejuni fliD* gene resulted in bacteria with a motility-impaired phenotype. Although TEM examination revealed that the *C. jejuni fliD* mutant displayed full-length filaments when harvested directly from motility agar plates, the same strain displayed truncated filaments when harvested from broth cultures (not shown). In fact, the *C. jejuni fliD* mutant

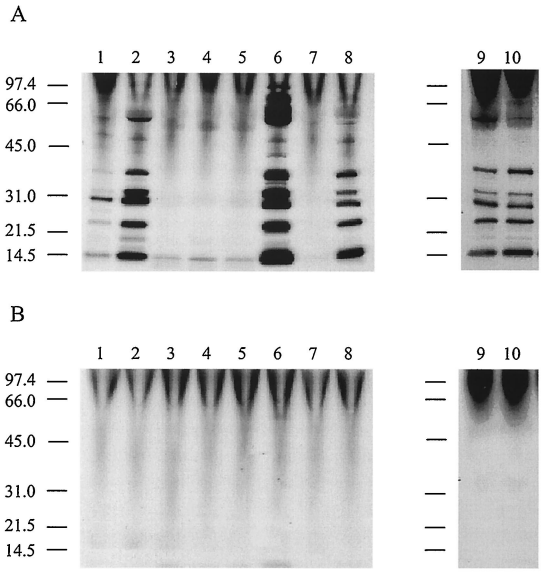

FIG. 1. *C. jejuni* Cia protein secretion requires a functional flagellar apparatus. *C. jejuni* cells were precultured on Mueller-Hinton plates (lane 1) and Mueller-Hinton plates supplemented with deoxycholate (lanes 2 to 10), and labeled in minimal essential medium in the presence (A) and absence (B) of FBS. Lanes: 1, *C. jejuni* F38011 (Mueller-Hinton plate); 2, *C. jejuni* F38011 (Mueller-Hinton deoxycholate plate); 3, *C. jejuni* F38011 *flgB* mutant; 4, *C. jejuni* F38011 *flgC* mutant; 5, *C. jejuni* F38011 *flgE2* mutant; 6, *C. jejuni* 81116; 7, *C. jejuni* 81116 *flaA flaB* (GRK7) mutant; 8, *C. jejuni* 81116 *flaA* (*flaB*$^+$) (GRK17) mutant; 9, *C. jejuni* F38011; and 10, *C. jejuni* F38011 *fliD* mutant. *C. jejuni* strains were deemed positive for protein secretion based on the presence of multiple bands in a lane (i.e., panel A, lanes 1, 2, 6, 8, 9, and 10). The positions of molecular mass standards are indicated on the left (in kilodaltons).

grown in broth resembled the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant with respect to filament structure. Yokoseki et al. (44) found that an *S. enterica* serovar Typhimurium *fliD* mutant also formed minute swarms on motility agar plates but did not produce filaments in liquid medium. The investigators proposed that the motility of the *S. enterica* serovar Typhimurium *fliD* mutant was due to flagellin monomers which could not freely diffuse in the motility agar being assembled into filaments (44).

Secretion of the Cia proteins requires the intact flagellar apparatus. Secretion assays were performed in the presence and absence of fetal bovine serum (FBS) to determine whether the *C. jejuni* mutants were capable of Cia protein export. FBS serves as an artificial signal to stimulate the synthesis and secretion of the Cia proteins (37). The Cia proteins were readily identifiable in the supernatant fluids of *C. jejuni* F38011 and 81116 wild-type strains as well as the *C. jejuni* F38011 *fliD* mutant and the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant (Fig. 1A). However, the Cia proteins were not detected in the supernatant fluids from the *flgB*, *flgC*, and *flgE2* mutants or the *flaA flaB* mutant. In agreement with previous work indicating that a stimulus is required to induce Cia secretion, the Cia proteins were not detected in the supernatant fluids when FBS was omitted from the labeling medium (Fig. 1B).

To determine if the *ciaB* gene was expressed in the *flaA flaB* mutant, reverse transcription-PCR analysis was performed

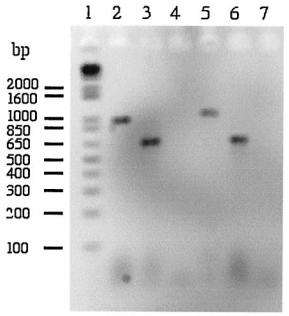

FIG. 2. Detection of a *ciaB* transcript in the *C. jejuni* 81116 wild-type isolate and *C. jejuni* 81116 *flaA flaB* mutant by reverse transcription-PCR. *C. jejuni* were cultured on Mueller-Hinton plates supplemented with deoxycholate. PCR was performed with gene-specific primers and a cDNA that was generated with RNA extracted from the *C. jejuni* 81116 wild-type isolate (lanes 2 to 4) and the *C. jejuni* 81116 *flaA flaB* (GRK7) mutant (lanes 5 to 7). Lanes: 1, DNA ladder (Invitrogen, Carlsbad, Calif.); 2, PCR performed with the *ciaB* gene-specific primers; 3, PCR performed with the *asp* gene-specific primers; 4, PCR performed with the *ciaB* gene-specific primers where the reverse transcriptase enzyme was omitted from the cDNA synthesis step; 5, PCR performed with the *ciaB* gene-specific primers; 6, PCR performed with the *asp* gene-specific primers, and 7, PCR performed with the *ciaB* gene-specific primers where the reverse transcriptase enzyme was omitted from the cDNA synthesis step.

(Fig. 2). A band representing the *ciaB* transcript was clearly evident in this mutant, demonstrating that *ciaB* is expressed. To demonstrate that the Cia protein is indeed one of the proteins secreted from the *C. jejuni* wild-type isolate, whole-cell lysates and supernatant fluids of the *C. jejuni* wild-type isolate and the *C. jejuni flaA flaB* mutant were analyzed by immunoblotting with a CiaB antibody (Fig. 3). A band corresponding in size to the CiaB protein was detected in whole-cell lysates of both the *C. jejuni* F38011 and 81116 wild-type strains and the *C. jejuni* 81116 *flaA flaB* mutant, but not in the whole-cell lysate of the *C. jejuni* F38011 *ciaB* mutant (Fig. 3A). In addition, CiaB was detected in the supernatant fluids of the *C. jejuni* wild-type F38011 and 81116 isolates, but only when FBS was added to the medium (Fig. 3, Panels B and C). However, CiaB was not detected in the supernatant fluids of the *C. jejuni ciaB* or *flaA flaB* mutants even when FBS was added to the media. These data show that the CiaB protein is synthesized, but is not secreted, in the *C. jejuni flaA flaB* mutant.

To address whether the Cia proteins are secreted from a *C. jejuni* 81116 *flaA*$^+$ *flaB* strain, the *C. jejuni flaA flaB* mutant was transformed with a *Campylobacter* shuttle plasmid harboring an intact *flaA* gene. As a control, the *C. jejuni flaA flaB* mutant was also transformed with the same *C. jejuni* shuttle plasmid harboring an intact *flaB* gene. Immunoblot analysis with anti-flagellin serum revealed that both the *C. jejuni flaA flaB*/*flaA*$^+$ and *C. jejuni flaA flaB*/*flaB*$^+$ transformants synthesized a filament protein, presumably FlaA and FlaB, respectively (Table 1). In addition, assays with metabolic labeling coupled with autoradiography revealed that the Cia proteins were secreted from the *C. jejuni flaA flaB*/*flaA*$^+$ transformant, although not to the same extent as from the wild-type strain (Fig. 4). Similarly, a reduced amount of Cia proteins was detected in the supernatant fluid from the *flaB*$^+$ transformant versus the *C. jejuni flaA* (*flaB*$^+$) mutant (Fig. 4). Based on these data, it is

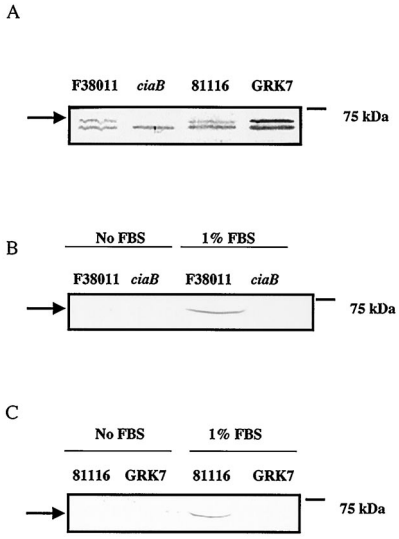

FIG. 3. Detection of the CiaB protein in *C. jejuni* wild-type isolates. Secretion assays were performed as outlined in Materials and Methods. Bacterial whole-cell lysates (A) and supernatant fluids (B and C) were analyzed by SDS-PAGE coupled with immunoblot analysis with the CiaB antibody. (A) Lanes: 1, *C. jejuni* F38011 wild-type strain; 2, *C. jejuni* F38011 *ciaB* mutant; 3, *C. jejuni* 81116 wild-type strain; 4, *C. jejuni* 81116 *flaA flaB* (GRK7) mutant. (B) Lanes: 1, *C. jejuni* F38011 wild-type strain minus FBS; 2, *C. jejuni* F38011 *ciaB* mutant minus FBS; 3, *C. jejuni* F38011 wild-type strain with FBS added to the medium to induce Cia protein export; 4, *C. jejuni* F38011 *ciaB* mutant with FBS added to the medium. (C) Lanes: 1, *C. jejuni* 81116 wild-type strain minus FBS; 2, *C. jejuni* 81116 *flaA flaB* (GRK7) mutant minus FBS; 3, *C. jejuni* 81116 wild-type strain with FBS added to the medium to induce Cia protein export; 4, *C. jejuni* 81116 *flaA flaB* (GRK7) mutant with FBS added to the medium to induce Cia protein export. The arrows indicate the position of the CiaB protein.

clear that the lack of CiaB export from the *C. jejuni flaA flaB* mutant is not due to the absence of *ciaB* gene expression or CiaB protein synthesis. These results further suggest that an intact flagellar structure (containing the basal body, hook, and at least a partial filament) is required for Cia protein secretion.

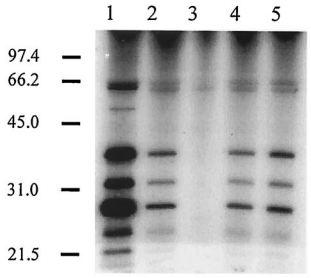

FIG. 4. Transformation of a *C. jejuni flaA flaB* strain with a recombinant plasmid harboring either *flaA* or *flaB* restores Cia secretion. *C. jejuni* cells were precultured on Mueller-Hinton plates supplemented with deoxycholate and labeled in minimal essential medium in the presence of FBS. Lanes: 1, *C. jejuni* 81116 wild-type strain; 2, *C. jejuni* 81116 *flaA flaB* (GRK7) mutant harboring pMEK3502 (*flaA*$^+$); 3, *C. jejuni* 81116 *flaA flaB* (GRK7) mutant; 4, *C. jejuni* 81116 *flaA flaB* (GRK7) mutant harboring pMEK3503 (*flaB*$^+$); 5, *C. jejuni* 81116 *flaA* (*flaB*$^+$) (GRK17) mutant. The positions of molecular mass standards are indicated on the left (in kilodaltons).

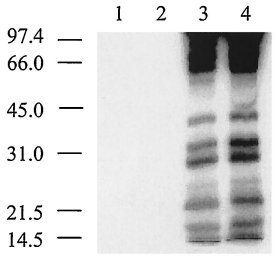

FIG. 5. *C. jejuni* protein secretion is independent of σ^{28}. *C. jejuni* cells were harvested from Mueller-Hinton agar plates labeled in minimal essential medium in the absence (lanes 1 and 2) and presence (lanes 3 and 4) of FBS as outlined in Materials and Methods. Lanes: 1, *C. jejuni* F38011 (without FBS); 2, *C. jejuni fliA* mutant (without FBS); 3, *C. jejuni* F38011 (with FBS); 4, *C. jejuni fliA* mutant (with FBS). The positions of molecular mass standards are indicated on the left (in kilodaltons).

Cia protein export is independent of σ^{28}. The *C. jejuni flaA* gene is transcribed from a σ^{28}-specific promoter (35), and the *C. jejuni flaB* gene is transcribed from a σ^{54}-specific promoter (16). A mutation was generated in the *fliA* gene of *C. jejuni* F38011, which encodes σ^{28}, to determine whether this sigma factor is responsible for *cia* gene transcription. As predicted, the *C. jejuni fliA* mutant was nonmotile, most likely due to the lack of *flaA* transcription. Indeed, an immunoreactive band of decreased intensity, corresponding in mass to the FlaB protein, was detected in the mutant, as judged by immunoblot analysis of whole-cell lysates with antiflagellin polyclonal serum (not shown). The *C. jejuni fliA* mutant also resembled the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant with respect to filament structure, as judged by TEM analysis. Finally, secretion assays revealed that the *C. jejuni fliA* mutant was secretion competent (Fig. 5). This result demonstrates that a sigma factor other than σ^{28} is required for transcription of the *cia* genes.

Maximal *C. jejuni* invasion requires Cia protein secretion and motility. In vitro assays were performed to assess the contribution of motility and Cia protein secretion in *C. jejuni* uptake (Table 1). Because *C. jejuni* binding is a prerequisite for invasion (19), *C. jejuni*-INT 407 cell contact was promoted by centrifugation. Despite this effort to minimize motility-dependent effects on *C. jejuni*-host cell association, a reduction was noted in binding of all *C. jejuni* flagellar mutants (*flhB*, *flgB*, *flgC*, *flgE2*, and *fliD*) to the INT 407 cells compared to the *C. jejuni* wild-type strain. Therefore, the data are presented as the percentage of adherent bacteria that were internalized to more readily identify those organisms with deficiencies in invasive potential. With the exception of the *C. jejuni fliD* mutant, a significant ($P < 0.01$) decrease was noted in internalization efficiency of the *C. jejuni flhB*, *flgB*, *flgC*, and *flgE2* flagellar mutants compared to the *C. jejuni* wild-type strain. Noteworthy is that the *C. jejuni ciaB* mutant displayed a Mot$^+$ S$^-$ phenotype but exhibited an invasion phenotype that was similar to that of the *flhB*, *flgB*, *flgC*, and *flgE2* flagellar export and structure mutants. Based on these results, it appears that motility, in the absence of Cia protein secretion, is not sufficient for maximal *C. jejuni* invasion of epithelial cells.

Binding and internalization assays were also performed with the *C. jejuni* 81116 wild-type strain, isogenic *flaA flaB* (Mot$^-$, S$^-$), and *C. jejuni* 81116 *flaA* (*flaB*$^+$) (Mot$^-$, S$^+$) mutants

TABLE 2. Phenotypes displayed by F38011 wild-type isolates and isogenic filament cap mutants

Relevant characteristic	FlaA synthesis	Filament assembly	Motility	Secretion	Adherence[a]	Invasion[a]	I/A
Wild type	+	+	+	+	100 ± 14	100 ± 1	5.5
fliD mutant (plate cultured)	+	+	+	+	85 ± 6	64 ± 5	4.1
fliD mutant (broth cultured)	+	Truncated	ND[b]	ND	77 ± 6	29 ± 3	2.1
flgC mutant	−	−	−	−	52 ± 14	0.58 ± 0.2	0.06

[a] Percentage of the value for the wild type.
[b] ND, not determined.

(Table 1). Significant ($P < 0.01$) differences were noted in the invasive potentials of the *C. jejuni* 81116 *flaA flaB* and *flaA* (*flaB*$^+$) mutants compared to the wild-type strain. A significant difference was also noted in the invasiveness of the *C. jejuni flaA flaB* and *flaA flaB*$^+$ mutants compared to one another, with a greater number of the *flaA* (*flaB*$^+$) mutant organisms internalized. Wassenaar et al. (41) also reported that a *flaA* (*flaB*$^+$) strain was capable of invading INT 407 epithelial cells as long as cell-to-cell contact was promoted by centrifugation. Complementation of the *flaA flaB* mutant with a shuttle vector, in *trans*, harboring either the *flaA* or *flaB* gene resulted in transformants that displayed characteristics similar to that of the *C. jejuni* 81116 wild-type strain or the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant, respectively. However, transformation of the *C. jejuni flaA flaB* mutant with a shuttle plasmid harboring a functional *flaA* or *flaB* gene did not fully restore Cia secretion or the percentage of bacteria internalized to the expected levels. A possible explanation for the diminished secretion rates and corresponding reduction in internalization efficiency is that an increase in the amount of FlaA or FlaB within a cell may interfere with Cia secretion. In agreement with the results presented in Table 1, these data suggest that the secretion of the Cia proteins from the flagellar export apparatus contributes significantly to *C. jejuni* host cell invasion.

The *C. jejuni* F38011 *fliD* mutant displayed a filament structure similar to that of the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant. Given this finding, we hypothesized that the *C. jejuni* F38011 *fliD* mutant grown in liquid medium should be internalized at a level comparable to that of the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant, given that both mutants displayed truncated flagellar filaments. Invasion assays were performed with the *C. jejuni* F38011 *fliD* mutant grown in liquid medium and the *C. jejuni* F38011 *fliD* mutant grown on solid medium as an appropriate control (Table 2). Consistent with the results shown in Table 1 with the *C. jejuni* 81116 *flaA* (*flaB*$^+$) mutant, a twofold difference in the ratio of internalized to adherent cells was obtained with the F38011 *fliD* mutant cultured in broth compared to the wild-type strain. Based on this finding as well as that obtained with the *C. jejuni fla* mutants, it appears that organisms that have a filament and are Mot$^+$ are 1.5- to 2.5-fold more invasive than organisms that have a filament and are Mot$^-$. However, the *C. jejuni* F38011 wild-type strain (S$^+$, Mot$^+$) was found to be approximately 50-fold more invasive than the *C. jejuni ciaB* isogenic mutant (S$^-$, Mot$^+$), which highlights the importance of the secretion-competent phenotype.

DISCUSSION

Gram-negative bacteria possess at least six different mechanisms to actively transport proteins across the bacterial membranes (reviewed in reference 5). Of these six pathways, protein secretion induced upon contact of the bacteria with host cells has been referred to as the type III secretion pathway (10). Requirements of type III secretion pathways include the absence of a cleavable, hydrophobic amino-terminal signal sequence in the secreted protein, export of the protein across the bacterial inner and outer membranes without a periplasmic intermediate, and a signal to induce secretion (23). Most but apparently not all type III secreted proteins require chaperones (7, 8). We have demonstrated elsewhere that *C. jejuni* synthesize a novel set of proteins upon coculture with epithelial cells, some of which are secreted (22, 37). The secreted proteins were termed the *Campylobacter* invasion antigens (Cia proteins) because they were found to be required for maximal invasion of intestinal epithelial cells by *C. jejuni* (22, 30, 37). Because the Cia proteins are synthesized and secreted in response to an environmental stimulus and the secreted CiaB protein is not processed, the Cia proteins appear to conform to the criteria for type III proteins. However, a BLAST search of the *C. jejuni* genome revealed that the only apparent type III export system in *C. jejuni* is the flagellar apparatus.

A considerable amount of evidence exists that motility is essential for the maximal colonization of animals by *C. jejuni* (32, 33, 34, 36, 42). In parallel with these studies, additional work has been done to dissect the importance of motility versus the actual flagellum in the interaction of *C. jejuni* with cultured epithelial cells (15, 41, 43). Investigators have targeted genes encoding various flagellar structural components, and while discrepancies have been reported with respect to the phenotypes of particular mutants (14, 43), there appears to be a consensus among investigators that motility plays a role in *C. jejuni* pathogenesis. Moreover, motility and the expression of the *flaA* gene are clearly necessary for maximal invasion of eukaryotic cells and for the translocation of *C. jejuni* across polarized cells (15, 41). Perhaps more relevant to this study, differences in the invasive potential of *C. jejuni flaA* (*flaB*$^+$) and *C. jejuni flaA flaB* strains were noted in earlier studies; *C. jejuni flaA* (*flaB*$^+$) strains have been reported to be more invasive than a *C. jejuni flaA flaB* strain (15, 41). Also noteworthy is that the invasiveness of a *C. jejuni flaA* (*flaB*$^+$) strain is enhanced 10-fold by promoting bacterium-host cell contact via centrifugation; in contrast, the centrifugation step did not change the invasive potential of the *C. jejuni* wild-type strain

(40). Based on the difference observed in the invasive potential of the *C. jejuni flaA* (*flaB*$^+$) strain versus the *C. jejuni flaA flaB* strain, Grant et al. (15) concluded that the flagellar structure played a role in internalization that was independent of motility. Prior to this study, it was unclear how the flagellum could have any effect on *C. jejuni* host cell invasion other than by conferring motility or acting directly as an adhesin.

Given our previous work suggesting that the Cia proteins are secreted in a type III-dependent manner and the absence of a type III secretion system dedicated to the export of virulence proteins in the *C. jejuni* genome, experiments were performed to determine if the flagellum serves as the Cia export apparatus. Mutations that abolished flagellin export (*flhB*, *flgB*, *flgC*, and *flgE2*), filament structure (*fliD*, *flaA*, *flaB*), and filament synthesis (*fliA*) were generated. With these mutants, we have shown that *C. jejuni* motility and virulence are linked. Specifically, we demonstrate that the *C. jejuni* Cia proteins are secreted via the flagellar export apparatus. The secretion system utilized by *C. jejuni* appears to be unique in that either one of the filament proteins is required for Cia protein secretion.

To test whether components of the flagellar apparatus serve as the *C. jejuni* Cia export apparatus, two separate experiments were performed. Mutations were generated in several flagellar structural genes in *C. jejuni* strain F38011 to determine if the loss of an operational flagellar apparatus resulted in the loss of Cia export. In addition, with two mutants of *C. jejuni* strain 81116 that were defective in expression of either one or both flagellin filaments as well as Cia protein export, we tested whether restoration of a flagellar filament also restored Cia protein synthesis. Mutations that affected either the export of flagellar components (*flhB*) or the nonfilament structural components (*flgB*, *flgC*, and *flgE2*) resulted in an S$^-$ phenotype. Comparable results were obtained with a second *C. jejuni* strain, 81116, in which the genes encoding the flagellin filament (*flaA* and *flaB*) were mutated. Complementation of the flagellar filament defect in 81116 with either *flaA* or *flaB* restored the organism's ability to secrete the Cia proteins. To ensure that the S$^-$ phenotype exhibited by the *C. jejuni* 81116 *flaA flaB* mutant was not unique to a particular strain, a *C. jejuni* F38011 *flaA flaB* mutant was generated. The *C. jejuni* F38011 *flaA flaB* mutant also exhibited an S$^-$ phenotype (not shown). Therefore, the genetic evidence presented is consistent with Cia protein secretion through the flagellar export system.

Insertion mutagenesis of *fliD* (Cj0548) and *flgC* (Cj0527c) was expected to have a polar effect on downstream gene expression. As the downstream genes in these putative operons are also expected to be associated with flagellar biosynthesis, we predicted that the phenotype associated with polarity on the downstream genes would be similar to that of the targeted gene. With regard to *flgB* and *flgC*, *fliE* (Cj0525c) and *pbpB* (Cj0524) would also be affected. In the case of *fliD*, *fliS* (Cj0549) and a hypothetical open reading frame of no known function (Cj0550) would have been affected.

The fact that the amount of FlaA protein was reduced in the whole-cell lysates of the *C. jejuni flgB*, *flgC*, and *flgE2* mutants, as judged by immunoblot analysis with a flagellin antiserum, raised the possibility that *C. jejuni* may possess the FlgM anti-sigma factor. In bacteria such as *Salmonella*, *Yersinia*, and *Helicobacter pylori*, the negative regulator FlgM inhibits flagellin transcription in response to a defective hook-basal body complex (6, 9, 11). A protein corresponding to a putative FlgM homolog that shows similarity with the recently identified FlgM protein from *H. pylori* (9) has been identified in the genome of *C. jejuni* NCTC 11168 (Cj1464). In *S. enterica* serovar Typhi, σ^{28} is involved in regulating gene expression of type III proteins (13). Given this fact, it is clear that the expression of virulence genes in *S. enterica* serovar Typhi is affected, albeit indirectly, by FlgM and the assembly of the flagellar export apparatus. Regardless, the Cia proteins are secreted in a *C. jejuni fliA* (σ^{28}) mutant. Therefore, the *cia* genes cannot be subject to transcriptional regulation via a mechanism involving the anti-sigma factor FlgM. Our results are in agreement with those of Jagannathan et al. (17), who observed that a *C. jejuni fliA* mutant displayed truncated flagella; this finding indicates that σ^{28} is not responsible for the transcription of the genes encoding the flagellar export apparatus in *C. jejuni*.

The ATPase FliI plays an essential role in flagellar apparatus assembly and in flagellar protein export. To address whether the S$^-$ phenotype of some of the *C. jejuni* mutants was due to regulatory effects, the expression of *ciaB* was analyzed in a *C. jejuni* F38011 *fliI* mutant. After the mutant was generated, RNA was extracted from bacteria that were cultured in Mueller-Hinton broth with 0.05% deoxycholate under microaerobic conditions. Importantly, *ciaB* was transcribed in the *C. jejuni* F38011 *fliI* mutant, as judged by reverse transcription-PCR. In addition, the CiaB protein was synthesized in the *C. jejuni* F38011 *fliI* mutant, as judged by immunoblot analysis with a CiaB-specific antibody.

The results of this study are consistent with the hypothesis that the flagellar type III secretion pathway is required for Cia protein export. Secretion of the Cia proteins requires a functional basal body and hook and at least one of the filament proteins. Coupled with the metabolic labeling experiments in which the *C. jejuni* strains were examined for protein secretion, the adherence and internalization data indicate that the difference in the invasiveness of the *C. jejuni flaA flaB*$^+$ and *C. jejuni flaA flaB* strains is a result of Cia secretion. Based on the phenotypes of the *C. jejuni ciaB* mutant (Mot$^+$, S$^-$), it is also evident that motility, in the absence of Cia protein secretion, is not sufficient for *C. jejuni* invasion of epithelial cells. We believe that the data presented here reveal what had formerly been unclear about the Cia protein export apparatus and the relationship between *C. jejuni* motility and host cell invasion.

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