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

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*C. 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 (*flhA* and *flhB*). 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.

*C. 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 enteritis mediated by *C. jejuni* 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 σ28 and the *flaB* gene is regulated by σ26 (3, 16). Hendrixson et al. (16) noted that a *C. jejuni* isolate deficient in σ26, which is encoded by the *flaA* gene, is able to assemble a truncated filament composed exclusively of the flagellin protein FlaB. This result indicates that the regulation of flagellin gene expression within *C. jejuni* differs from the regulation in more intensely studied systems such as that of *Salmonella enterica*. Unlike flagellin gene expression in *C. jejuni*, flagellar gene expression in *S. enterica* is initiated by a master regulator, while late gene expression and motility require σ28 (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 82081 and 81116 were cultured as described previously (38). Plates were supplemented with kanamycin sulfate (200 μg/ml) as appropriate. E. coli DH5α and DH5 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 82081 by electroporation. C. jejuni 82081 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 fluk, flaB, flhB, flgB, flgC, flgE2, fld, and flh gene sequences were obtained from the Sanger Center website (http://www.sanger.ac.uk/Projects/C jejuni). Two flaB genes have been identified in NCTC 11168 (Cj0043 and Cj0045); however, published work showing sequence similarity of the flgE2 product to other hook proteins (18, 25) established that the flgE2 (Cj1729c) was targeted. The flhB (Cj0335), flgB (Cj0526c), flgC (Cj0527c), flgE2, and flhB (Cj0548), and flhC (Cj0195) genes in C. jejuni 82081 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 upaA3 gene encoding kanamycin resistance (21). The C. jejuni flaB (forward primer, 5′-AAA AAA CAG AAG AAG CCA CG; reverse primer, 5′-GGT CAA CTA TAG TATA AAT CTG C); the forward primer, 5′-CAT TTA AAT CAA AAG AAC TGG; reverse primer, 5′-ATC AGC TGA AGG TGT ACC TGT TAT TAG TGG; reverse primer, 5′-GTA GCT TAA GAT GGA ACG TCA AG; reverse primer, 5′-AAC GAG AAG AAA AGT AGG; reverse primer, 5′-ATT TGC TAT AGC AGT TCC; and flhB (forward primer, 5′-CTC GAG GCT ACA AGT ATA GAA ATT CTC CAG) gene with a BamHI restriction site, and the deletion-insertion mutation was crossed onto the chromosomal gene by allelic exchange. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion. To aid in the selection of the double-crossover mutants, the deletions were marked by the target gene containing an internal deletion.
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.

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

<table>
<thead>
<tr>
<th>Background strain</th>
<th>Relevant characteristic</th>
<th>FlaA synthesis</th>
<th>Filament assembly</th>
<th>Motility</th>
<th>Secretion</th>
<th>Adherencea</th>
<th>Invasionb</th>
<th>I/A’</th>
</tr>
</thead>
<tbody>
<tr>
<td>F38011</td>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100 ± 5</td>
<td>100 ± 20</td>
<td>5.3</td>
</tr>
<tr>
<td></td>
<td>flaB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>47 ± 9</td>
<td>0.24 ± 0.04</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>flaB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>40 ± 6</td>
<td>0.46 ± 0.1</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>flaC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>73 ± 2</td>
<td>2.1 ± 0.5</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>flaE2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13 ± 2</td>
<td>0.26 ± 0.1</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>flaD</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>59 ± 8</td>
<td>44 ± 4</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>criaB</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>223 ± 15</td>
<td>1.9 ± 0.7</td>
<td>0.02</td>
</tr>
</tbody>
</table>

81116

Wild type

flaA flaB | + | + | + | + | + | 100 ± 9 | 100 ± 27 | 2.7  |
flaA | - | Truncated | - | + | 145 ± 9 | 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 × 10⁶ ± 0.5 × 10⁶.

b As a percentage of the wild-type value, which was 5.0 × 10⁶ ± 1 × 10⁶.

I/A, [(number of internalized bacteria)/(number of adherent bacteria)] × 100.

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 flaB 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 flaB, 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 filament, was detected in whole-cell lysate preparations of C. jejuni F38011 flaB, flgC, and flgE2 mutants as well as in the C. jejuni 81116 flaA flaB⁻ mutant with a flagellin polycytoplasmic domain. 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 flaB, flgC, and flaE2 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 flaD gene resulted in bacteria with a motility-impaired phenotype. Although TEM examination revealed that the C. jejuni flaD 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 flaD mutant...
grown in broth resembled the \textit{C. jejuni} 81116 \textit{flaA} (\textit{flaB}⁺) mutant with respect to filament structure. Yokoseki et al. (44) found that an \textit{S. enterica} serovar Typhimurium \textit{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 \textit{S. enterica} serovar Typhimurium \textit{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 \textit{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 \textit{C. jejuni} F38011 and 81116 wild-type strains as well as the \textit{C. jejuni} F38011 \textit{flID} mutant and the \textit{C. jejuni} 81116 \textit{flaA} (\textit{flaB}⁺) mutant (Fig. 1A). However, the Cia proteins were not detected in the supernatant fluids from the \textit{flGB}, \textit{flGC}, and \textit{flGE2} mutants or the \textit{flaA} \textit{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 \textit{ciaB} gene was expressed in the \textit{flaA} \textit{flaB} mutant, reverse transcription-PCR analysis was performed (Fig. 2). A band representing the \textit{ciaB} transcript was clearly evident in this mutant, demonstrating that \textit{ciaB} is expressed. To demonstrate that the Cia protein is indeed one of the proteins secreted from the \textit{C. jejuni} wild-type isolate, whole-cell lysates and supernatant fluids of the \textit{C. jejuni} wild-type isolate and the \textit{C. jejuni} \textit{flaA} \textit{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 \textit{C. jejuni} F38011 and 81116 wild-type strains and the \textit{C. jejuni} 81116 \textit{flaA} \textit{flaB} mutant, but not in the whole-cell lysate of the \textit{C. jejuni} F38011 \textit{ciaB} mutant (Fig. 3A). In addition, CiaB was detected in the supernatant fluids of the \textit{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 \textit{C. jejuni} \textit{ciaB} or \textit{flaA} \textit{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 \textit{C. jejuni} \textit{flaA} \textit{flaB} mutant.

To address whether the Cia proteins are secreted from a \textit{C. jejuni} 81116 \textit{flaA}⁺ \textit{flaB} strain, the \textit{C. jejuni} \textit{flaA} \textit{flaB} mutant was transformed with a \textit{Campylobacter} shuttle plasmid harboring an intact \textit{flaA} gene. As a control, the \textit{C. jejuni} \textit{flaA} \textit{flaB} mutant was also transformed with the same \textit{C. jejuni} shuttle plasmid harboring an intact \textit{flaB} gene. Immunoblot analysis with anti-flagellin serum revealed that both the \textit{C. jejuni} \textit{flaA} \textit{flaB}⁺ and \textit{C. jejuni} \textit{flaA} \textit{flaB}⁻ transformants synthesized a filament protein, presumably \textit{FlaA} and \textit{FlaB}, respectively (Table 1). In addition, assays with metabolic labeling coupled with autoradiography revealed that the Cia proteins were secreted from the \textit{C. jejuni} \textit{flaA} \textit{flaB}⁺ \textit{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 \textit{flaB}⁻ transformant versus the \textit{C. jejuni} \textit{flaA} \textit{flaB}⁺ mutant (Fig. 4). Based on these data, it is
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. 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. The positions of molecular mass standards are indicated on the left (in kilodaltons). 

**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 decycholeate 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 harboring pMEK3503 (flaB·); 5, C. jejuni 81116 flaA (flaB·) (GRK7) mutant. The positions of molecular mass standards are indicated on the left (in kilodaltons).

**FIG. 5. C. jejuni protein secretion is independent of σ²⁸.** 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 flaA mutant (without FBS); 3, C. jejuni F38011 (with FBS); 4, C. jejuni flaA mutant (with FBS). The positions of molecular mass standards are indicated on the left (in kilodaltons).
TABLE 2. Phenotypes displayed by F38011 wild-type isolates and isogenic filament cap mutants

<table>
<thead>
<tr>
<th>Relevant characteristic</th>
<th>FlaA synthesis</th>
<th>Filament assembly</th>
<th>Motility</th>
<th>Secretion</th>
<th>Adherence*</th>
<th>Invasion*</th>
<th>I/A</th>
</tr>
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<tbody>
<tr>
<td>Wild type</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>100 ± 14</td>
<td>100 ± 1</td>
<td>5.5</td>
</tr>
<tr>
<td>fID mutant (plate cultured)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>85 ± 6</td>
<td>64 ± 5</td>
<td>4.1</td>
</tr>
<tr>
<td>fID mutant (broth cultured)</td>
<td>+</td>
<td>Truncated</td>
<td>ND</td>
<td>ND</td>
<td>77 ± 6</td>
<td>29 ± 3</td>
<td>2.1</td>
</tr>
<tr>
<td>flgC mutant</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>52 ± 14</td>
<td>0.58 ± 0.2</td>
<td>0.06</td>
</tr>
</tbody>
</table>

* Percentage of the value for the wild type.

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 (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 fID 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 fID 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 fID mutant grown in liquid medium and the C. jejuni F38011 fID 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 fID 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.
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 (flhD, flaA, flaB), and filament synthesis (flaA) 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 flagellin 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 flhD (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, flhE (Cj0525c) and phpB (Cj0524) would also be affected. In the case of flhD, flhS (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 flagelin 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 flagelin 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, \(\sigma^{28}\) 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 flaA (\(\sigma^{28}\)) 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 flaA mutant displayed truncated flagella; this finding indicates that \(\sigma^{28}\) 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 flhA 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 flhA mutant, as judged by reverse transcription-PCR. In addition, the CiaB protein was synthesized in the C. jejuni F38011 flhA 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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