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Amino-terminal residues dictate the export efficiency of the *Campylobacter jejuni* filament proteins via the flagellum

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Summary

Bacterial flagella play an essential role in the pathogenesis of numerous enteric pathogens. The flagellum is required for motility, colonization, and in some instances, for the secretion of effector proteins. In contrast to the intensively studied flagella of *Escherichia coli* and *Salmonella typhimurium*, the flagella of *Campylobacter jejuni*, *Helicobacter pylori*, and *Vibrio cholerae* are less well characterized and composed of multiple flagellin subunits. This study was performed to gain a better understanding of flagellin export from the flagellar type III secretion apparatus of *C. jejuni*. The flagellar filament of *C. jejuni* is comprised of two flagellins termed FlaA and FlaB. We demonstrate that the amino-termini of FlaA and FlaB determine the length of the flagellum and motility of *C. jejuni*. We also demonstrate that protein-specific residues in the amino-terminus of FlaA and FlaB dictate export efficiency from the flagellar T3SS of *Yersinia enterocolitica*. These findings demonstrate that key residues within the amino-termini of two nearly identical proteins influence protein export efficiency, and that the mechanism governing the efficiency of protein export is conserved amongst two pathogens belonging to distinct bacterial classes. These findings are of additional interest because *C. jejuni* utilizes the flagellum to export virulence proteins.

Keywords

flagellum; motility; protein secretion; virulence determinants; T3SS

Introduction

C. jejuni is a leading food-borne cause of bacterial gastroenteritis. An estimated 400-500 million cases of campylobacteriosis occur each year worldwide (Ruiz-Palacios, 2007). Infection with *C. jejuni* usually occurs from the mishandling and consumption of undercooked poultry products (Konkel *et al.*, 2001). *C. jejuni* infections are usually self-limiting, with symptoms including abdominal cramps, fever, and diarrhea with blood or leukocytes. Certain serotypes of *C. jejuni* are linked to the development of Guillain-Barré syndrome, an acute demyelinating neuropathy characterized by flaccid paralysis (Konkel *et al.*, 2001).

C. jejuni possesses a flagellum that functions in both motility and protein secretion (Wassenaar *et al.*, 1991, Grant *et al.*, 1993, Konkel *et al.*, 2004, Guerry, 2007). The flagellum consists of a basal body, hook, and filament (Konkel *et al.*, 2004). The basal body consists of a conduit spanning the inner and outer membranes of the cell, as well as the motor that drives flagellar rotation. The hook section of the flagellum is composed primarily of the protein FlgE, and connects the flagellar filament to the basal body. The flagellar

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filament is composed of two proteins, FlaA and FlaB, which are exported through the flagellum and polymerize to form a helical conduit 1-3 μm in length (Guerry *et al.*, 1991). Expression of the flagellar apparatus components are regulated by three sigma factors: σ^{70} (RpoD), σ^{54} (RpoN), and σ^{28} (FliA) (Jagannathan *et al.*, 2001). σ^{70} controls expression of some basal body components, while σ^{54} regulates the other components needed for a functional flagellar secretory apparatus, including the filament protein FlaB. σ^{28} regulates expression of the FlaA filament protein and other flagellar-related secreted proteins (Jagannathan *et al.*, 2001, Carrillo *et al.*, 2004).

Structural analysis of the *Salmonella typhimurium* filament protein FliC by electron microscopy and X-ray crystallography enabled the 495 residue protein to be divided into four linearly connected domains designated D₀ - D₃ (Samatey *et al.*, 2001, Yonekura *et al.*, 2003). The D₀ domain consists of the amino- (residues 1-45) and carboxy-terminal (residues 456-495) ends that fold into a coiled-coil tertiary structure. The amino-terminal portion of the D₀ domain facilitates protein secretion, whereas the entire domain is important for filament polymerization (Gugolya *et al.*, 2003, Vegh *et al.*, 2006). FliS, the chaperone for FliC, binds to 40 amino acids in the carboxy-terminal D₀ domain (Ozin *et al.*, 2003, Muskotal *et al.*, 2006). Domain D₁ consists of residues 46-180 and 408-455, and together with the D₀ domain, forms the inner core of the flagellar conduit (Namba *et al.*, 1989, Yonekura *et al.*, 2003). Domains D₂ (residues 181-190 and 285-407) and D₃ (residues 191-284) form the surface-exposed outer regions of the filament (Namba *et al.*, 1989, Yonekura *et al.*, 2003). Deletions within the D₂ and D₃ region of the *S. typhimurium* FliC and *E. coli* FliC filament proteins have revealed that it is unnecessary for the formation of full-length flagella and bacterial motility (Kuwajima, 1988, Yoshioka *et al.*, 1995). Because the structure of *C. jejuni* flagellin has not yet been determined, comparisons of the primary sequence of *S. typhimurium* FliC with those of *C. jejuni* FlaA and FlaB were used in this study to predict the location and function of domains in *C. jejuni* flagellin.

Type III secretion systems (T3SS) allow bacteria to secrete proteins from the cytosol to the extracellular environment, through a hollow conduit that spans both the inner and outer membranes (Cornelis, 2006). The bacterial flagellum is a T3SS, and is the only T3SS present in *C. jejuni* (Konkel *et al.*, 2004, Minamino & Namba, 2004, Desvaux *et al.*, 2006). *C. jejuni* uses its flagellar T3SS to secrete virulence proteins, termed *Campylobacter* invasion antigens (Cia), that are required for invasion of epithelial cells (Konkel *et al.*, 1999, Konkel *et al.*, 2004, Larson *et al.*, 2008). Previous research has revealed that both flagellar and classical T3SS recognize secreted proteins through a common mechanism involving the amino-terminal amino acid sequence (Sory *et al.*, 1995, Cornelis, 2006, Vegh *et al.*, 2006, Badea *et al.*, 2009). Information about the secretion of flagellin through the flagellar T3SS of *C. jejuni* can thus be used to further our understanding of virulence protein secretion through other T3SS.

Previous work has revealed that FlaA is the major constituent of the flagellar filament (Guerry *et al.*, 1991, Wassenaar *et al.*, 1991). A *C. jejuni* *flaB* mutant (*flaA*⁺*B*⁻) produces a filament that is equivalent in length to a wild-type isolate, and these bacteria are motile. In contrast, a *C. jejuni* *flaA* mutant (*flaA*⁻*B*⁺) produces a severely truncated filament, and these bacteria are non-motile (Guerry *et al.*, 1991, Wassenaar *et al.*, 1991, Konkel *et al.*, 2004). The goal of this study was to determine why *C. jejuni* *flaB* mutants produce full-length flagellar filaments while *C. jejuni* *flaA* mutants produce truncated filaments. Based on the fact that expression of *flaA* is regulated by a σ^{28} promoter and *flaB* is regulated by a σ^{54} promoter (Jagannathan *et al.*, 2001, Carrillo *et al.*, 2004), we hypothesized that the contrasting phenotypes of *C. jejuni* *flaA* or *flaB* mutants were due to differences in gene expression levels. However, we found that filament length and motility were only partially dependent on flagellin gene expression levels. Amino acid sequence analysis of *C. jejuni*

flagellar filament proteins revealed that the D₀ domain of the FlaA and FlaB proteins contain highly-conserved residues. Additional experiments were then performed to determine whether these unique residues could affect the secretion efficiency of flagellin monomers. Our results revealed that flagellar filament length is determined by flagellin gene expression levels and the amino-terminal sequences of FlaA and FlaB. The latter finding was supported using an alternative approach whereby we examined FlaA and FlaB protein export from *Yersinia enterocolitica*. Thus, the amino-terminal sequence of proteins dictates the efficiency of export through the flagellar T3SS.

Results

***C. jejuni* FlaA and FlaB share homology with *S. typhimurium* FliC in the amino- and carboxy-termini**

Sequence analysis of the filament proteins from 8 different *C. jejuni* strains revealed that each strain possesses two filament proteins, ranging in size from 1716 nucleotides (572 amino acids) to 1740 nucleotides (580 amino acids). At the amino acid level, the two filament proteins share greater than 90% identity within a strain (Supplemental Table 1). Alignments of the *C. jejuni* NCTC 11168 FlaA and FlaB amino acid sequences with *S. typhimurium* LT2 FliC were conducted to estimate the spatial organization of domains in FlaA and FlaB (Supplemental Figure 1). Putative domain boundaries were assigned solely to approximate the size of the amino- and carboxy-terminal domains, which are more homologous than the central domains. The amino acid identity was greatest in the D₀ domain (> 40% identity), followed by the D₁ domain (> 20% identity), with the D₂ and D₃ domains being highly variable (< 20% identity) (Table 1). Using the domain boundaries determined in FliC by X-ray crystallography, putative domain boundaries were assigned to FlaA and FlaB based upon the amino acid alignment. Comparisons of *C. jejuni* FlaA (Table 2A) and FlaB (Table 2B) amino acid sequences revealed the greatest sequence conservation is within the D₀ and D₁ domains (> 90% identical). The D₃ domain is less conserved (70% to 100% identical) than the D₀ and D₁ domains, and the D₂ domain is the most variable (50% to 100% identical). These results indicate that the D₀ and D₁ terminal domains, which function in secretion and subunit polymerization, are more conserved than the surface-exposed central domains, which are targets of adaptive immune defenses.

Considerations of plasmid copy number and flagellar gene phase variation

A *C. jejuni* F38011 *flaAB* mutant was generated via homologous recombination to replace the *flaA* and *flaB* genes with a tetracycline resistance marker. To study the individual roles of the flagellin genes and promoters, the *flaAB* mutant was transformed with the *E. coli*-*C. jejuni* shuttle vector pRY111 harboring *flaA* or *flaB* expressed from the *flaA* or *flaB* promoters. Because pRY111 is a multi-copy plasmid of unknown copy number, we compared the flagellin levels in the *C. jejuni* F38011 wild-type strain, F38011 *flaAB* mutant, and F38011 *flaAB* mutant harboring pRY111 containing *flaA* under control of the *flaA* promoter (Supplemental Figure 2). Similar levels of flagellin were detected in the whole cell lysates of the *C. jejuni* F38011 wild-type strain and *flaAB* mutant harboring *flaA*, whereas flagellin was not detected in the whole cell lysate of the *flaAB* mutant (Supplemental Figure 2A). Examination of the supernatants from both the *C. jejuni* F38011 wild-type strain and *flaAB* mutant harboring *flaA* revealed that most of the exported FlaA and FlaB proteins were incorporated into the filament. While neither the FlaA and FlaB proteins were detected in the supernatant fraction of the *C. jejuni* F38011 wild-type strain and *flaAB* mutant, a faint band was observed in the lane containing the *flaAB* mutant harboring *flaA* (Supplemental Figure 2B). These results demonstrate that similar amounts of flagellin are produced whether the flagellin genes are expressed from a single copy on the chromosome or the multi-copy plasmid pRY111. Collectively, these findings suggested that expression of the

flagellin genes from the multi-copy plasmid did not significantly alter the amount of flagellin produced, and did not alter the composition or function of the flagellar filament.

Another factor that could potentially influence the amount of flagellin produced and the composition of the flagellar filament is phase variation of flagellar regulatory and structural genes. Phase variation can occur via strand slippage, recombination, or gene inversion, resulting in activation/inactivation of genes that would affect the regulation of flagellar assembly or motility. To determine the frequency at which wild-type and the *flaAB* mutant harboring pRY111 spontaneously undergo phase variation to become non-motile, cultures of *C. jejuni* were diluted and plated in MH soft agar (0.4% agar), such that each plate contained ~50 CFUs (Supplemental Figure 3). After 48 h, 500 individual colonies were examined for motility. The percentage of non-motile colonies observed for F38011 wild-type and the *flaAB* mutant harboring pRY111 containing *flaA* expressed from the *flaA* promoter were 2% and 1.4%, respectively. These results demonstrated that the rate of phase variation in wild-type and the *flaAB* mutant harboring pRY111 was low enough to allow us to accurately examine motility, filament length, and flagellin synthesis.

Expression of *flaA* or *flaB* from the *flaA* promoter results in increased motility and filament length compared to the *flaB* promoter

After confirming that expression of *flaA* in trans from pRY111 in the *flaAB* mutant did not overtly affect flagellin synthesis or phase variation compared to F38011 wild-type, we sought to determine why full-length flagella are produced by *C. jejuni* strains that possess a *flaA* gene, while *C. jejuni* strains that encode only *flaB* produce severely truncated filaments and are non-motile (Guerry *et al.*, 1991, Wassenaar *et al.*, 1991, Konkel *et al.*, 2004). We hypothesized that gene expression, dictated by the promoter, was responsible for the phenotypic differences between *flaA* and *flaB* mutants. To determine the effects of the promoter on flagellar filament length, a *C. jejuni* F38011 *flaAB* mutant was transformed with shuttle vectors harboring *flaA* and *flaB* expressed from the *flaA* and/or *flaB* promoter. Motility assays were performed using the *C. jejuni* wild-type strain, *flaAB* mutant, and *flaAB* mutant transformed with vectors containing the *flaA* or *flaB* genes (Figure 1A). The wild-type strain displayed the greatest zone of motility, measuring 13.4 ± 2.6 mm, while the *C. jejuni flAAB* mutant was non-motile. When the *C. jejuni flAAB* mutant was transformed with the shuttle vector harboring *flaA* or *flaB* expressed from the *flaA* promoter, the width of the motility zones were 8.3 ± 0.6 mm and 3.6 ± 0.8 mm, respectively. When the *C. jejuni flAAB* mutant was transformed with the shuttle vector harboring *flaA* or *flaB* expressed from the *flaB* promoter, the width of the motility zones were reduced to 1.1 ± 0.3 mm and 1.1 ± 0.2 mm, respectively. TEM was also performed to determine the length of the flagellar filament in each of the different strains (Figure 1B). The number of flagellated bacteria observed varied depending on the sample preparation, and not the genotype being examined. Results were recorded only from sample preparations in which the majority of bacteria were flagellated. The length of the flagellum was very similar among flagellated bacteria, while a small population of flagellated bacteria displayed shortened flagella, presumably due to partial shearing of the flagellum during sample preparation (many intact flagella were observed detached from cells). Hundreds of individual bacteria from multiple sample preparations were observed, and the trends described were highly consistent. The length of intact flagella from ten individual bacteria were measured from each sample. The *C. jejuni* wild-type strain had a filament length of 3.2 ± 0.2 μ m, while the *C. jejuni flAAB* mutant did not have an observable flagellum. Transformation of the *C. jejuni flAAB* mutant with *flaA* or *flaB* driven by the *flaA* promoter resulted in longer flagellar filaments (3.6 ± 0.6 and 1.8 ± 0.2 μ m, respectively) versus when *flaA* or *flaB* were driven by the *flaB* promoter (0.1 ± 0.03 and 0.2 ± 0.08 μ m, respectively). The motility and filament length phenotypes of the *flaAB* mutant containing pRY111 expressing *flaA* or *flaB* from their native promoters are in

agreement with the results obtained by others for *flaB* and *flaA* mutants, respectively (Guerry *et al.*, 1991, Wassenaar *et al.*, 1991). Taken together, the motility assays and TEM data indicate that expression of the filament protein genes from the *flaA* promoter is required for maximal motility and filament length. However, it was clear that the promoter was not the only factor determining filament length and motility, as *flaB* expressed from the *flaA* promoter did not result in a full-length flagellar filament.

The amino-terminal D₀ domain of FlaA results in increased motility and filament length compared to the D₀ domain of FlaB

Previous studies have revealed that the amino-terminus of a protein exported via a T3SS harbors the export sequence (Sory *et al.*, 1995, Ramamurthi & Schneewind, 2002, Vegh *et al.*, 2006). To determine whether the D₀ domains of FlaA and FlaB affect bacterial motility and filament length, three domain swap constructs were generated in which the amino- and carboxy-termini of FlaB were substituted with those of FlaA (Figure 2A). The amino-termini and carboxy-termini were swapped both individually and together to generate three modified versions of *flaB*. The *flaB* domain-swap genes were expressed from the *flaA* promoter, to allow for maximum filament length. The domain swap constructs were transformed into a *C. jejuni flaAB* mutant and assayed for motility and filament length.

When the *C. jejuni flaAB* mutant was transformed with a shuttle vector containing either the amino-terminal D₀ domain swap construct (*PflaA-flaA*_{D₀}*B*_{D₁₂₃}*B*_{D₀}) or the amino and carboxy-terminal D₀ domain swap construct (*PflaA-flaA*_{D₀}*B*_{D₁₂₃}*A*_{D₀}), the width of the motility zones were 5.9 ± 0.8 mm and 7.2 ± 0.7 mm, respectively (Figure 2B). Replacement of the carboxy-terminal D₀ domain with that of *flaA* (*PflaA-flaB*_{D₀}*B*_{D₁₂₃}*A*_{D₀}) resulted in a zone of motility 1.3 ± 0.6 mm in width, while the *C. jejuni flaAB* mutant transformed with *PflaA-flaB* had a motility zone width of 3.6 ± 0.8 mm. The filament length of the *C. jejuni flaAB* mutant was also increased when the isolate was transformed with a vector containing either the *PflaA-flaA*_{D₀}*B*_{D₁₂₃}*B*_{D₀} (3.4 ± 0.4 μm) or *PflaA-flaA*_{D₀}*B*_{D₁₂₃}*A*_{D₀} (3.2 ± 0.2 μm) constructs (Figure 2C). Both the *PflaA-flaB* and *PflaA-flaB*_{D₀}*B*_{D₁₂₃}*A*_{D₀} constructs resulted in intermediate length filaments of 1.8 ± 0.2 μm and 1.8 ± 0.3 μm, respectively. In summary, replacement of the amino-terminal D₀ domain of *flaB* with that of *flaA* results in a more motile bacterium with a longer filament versus a *C. jejuni flaAB* mutant expressing a wild-type copy of the *flaB* gene.

To ensure that the level of protein synthesis was dependent only on the promoter and was not affected by the sequence of *flaA* or *flaB*, immunoblot analysis was performed using a polyclonal antibody that recognizes both FlaA and FlaB. Whole cell lysates of each *C. jejuni flaAB* mutant transformed with a vector harboring *flaA*, *flaB*, or one of the three domain-swap constructs, which were all expressed from the *flaA* promoter, contained similar amounts of flagellin protein (Figure 3A). Immunoblots performed using serial two-fold dilutions of *C. jejuni* whole-cell lysates confirmed that the difference in protein concentrations between the samples was less than two-fold (not shown). A reactive band was sometimes observed below the flagellin band; we believe this is a flagellin degradation product, as it was observed in multiple lanes in different immunoblots. The immunoblot analysis data also confirmed that replacement of the *flaB* amino- and carboxy-terminal D₀ domains did not significantly affect protein synthesis. Culture supernatants were also probed for the presence of flagellin (Figure 3B). The *flaAB* mutant strains harboring the three domain swap constructs had more flagellin in the supernatant fraction than the *flaAB* mutant strains harboring *flaA* or *flaB*. However, the quantity of flagellin in the supernatants was equal among the three domain swap construct strains, despite the reduced filament length and motility phenotype seen in the strain harboring the *PflaA-flaB*_{D₀}*B*_{D₁₂₃}*A*_{D₀} construct. Collectively, these results demonstrate that flagellin synthesis is similar in each of the strains

examined, and that the majority of flagellin produced is found in the whole-cell lysate and not the supernatant fractions.

The D₀ domain of FlaA and FlaB contain protein-specific conserved residues

Based on the results obtained from the promoter and domain swapping experiments, we hypothesized that differences in the amino acid sequence between the two proteins resulted in different filament lengths. To investigate this possibility, alignments were performed using the FlaA and FlaB sequences from the eight *C. jejuni* strains. While the central domains of the filament proteins are variable, the terminal D₀ and D₁ domains are more conserved (Table 2A and 2B). Despite the high degree of conservation in the D₀ domain, certain residues within this domain were unique to FlaA or FlaB, but they were identical among the eight strains (Figure 4). The seven amino-terminal protein specific residues include 9, 10, 15, 19, 20, 24, and 27, which are V, A, K, D, L, S, and A in FlaA, and I, G, H, V, V, E, and K in FlaB, respectively. We also noted that the five carboxy-terminal protein specific residues include 543, 546, 558, 562, and 569, which are Y, A, A, S, and R in FlaA, and F, Y, S, A, and K in FlaB, respectively. These twelve residues within the amino- and carboxy-terminal D₀ domain are specific to FlaA or FlaB. Regardless, the domain swap experiments indicated that the difference in length of the flagellum is due to specific residues in the amino-terminus of the FlaA protein.

The amino-terminal D₀ domain of FlaA facilitates enhanced secretion of a YplA fusion protein from *Yersinia enterocolitica* compared to the D₀ domain of FlaB

We chose to utilize a *Yersinia enterocolitica* phospholipase (YplA) reporter secretion assay to directly examine the efficiency of T3 secretion conferred by the amino-termini of FlaA and FlaB, as this system was not affected by flagellin polymerization, flagellar regulation/assembly, or the sequence upstream of the flagellin gene (*i.e.*, promoter and 5' untranslated region). This assay is based on the principle that the amino-terminal sequence of a protein is required for its recognition and export by the flagellar apparatus (Christensen *et al.*, 2009). *Y. enterocolitica* also recognizes and exports *C. jejuni* proteins that contain T3S amino-terminal sequences (Christensen *et al.*, 2009). Secretion of the phospholipase YplA into TYE media containing Tween 80 results in a zone of fatty-acid precipitation surrounding the bacterial colony that can be easily quantified.

A *Y. enterocolitica* *yplA* mutant transformed with pCSP50 harboring a 5' truncated *yplA* gene did not secrete YplA, as the T3S amino-terminal sequence was removed (Figure 5A). When the end of the *flaA* or *flaB* genes (*i.e.*, which encode the first 36 amino acids) were fused to the 5' truncated *yplA* gene, secretion zone widths of 2.6 ± 0.12 mm and 1.7 ± 0.06 mm were observed end of *cysM*, which encodes a cytoplasmic at 24 h, respectively (Figure 5B and C). The 5' protein from *C. jejuni*, served as a negative control and did not result in secretion of YplA (not shown). Immunoblot analysis of the *Y. enterocolitica* strains with an α -YplA antibody confirmed that the amino-terminal sequences did not affect YplA synthesis (not shown). Different mixtures (ratios) of the *Y. enterocolitica* YplAB mutant transformed with the *flaA-yplA* fusion gene and the *Y. enterocolitica* YplAB mutant transformed with empty pCSP50 vector were assayed for YplA secretion to generate a standard curve of relative percent secretion efficiency (*i.e.*, 100% secretion efficiency was defined by a culture containing only the *Y. enterocolitica* YplAB mutant transformed with FlaA-YplA fusion gene vector). Based on the standard curve, the FlaB-YplA fusion protein was secreted only 40% as efficiently as the FlaA-YplA protein. The increased zone of precipitation resulting from the FlaA-YplA fusion relative to the FlaB-YplA fusion demonstrated that the amino-terminus of FlaA is secreted more efficiently. These results are consistent with the observed increase in filament length of the *C. jejuni* *flaAB* mutant transformed with the *PflaA*-

*flaA*_{D0}*B*_{D123}*B*_{D0} or *PflaA-flaA*_{D0}*B*_{D123}*A*_{D0} vectors relative to *C. jejuni flaAB* mutant harboring the *PflaA-flaB* vector.

Mutation of specific residues in the amino-terminus of FlaA and FlaB alters secretion efficiency

To determine the effect of individual amino-terminal protein-specific conserved residues on secretion efficiency, seven of the residues within the FlaA amino-terminal sequence were mutated to the respective residues found within FlaB (Figure 5D). The mutated residues were V9I, A10G, K15H, D19V, L20V, S24E, and A27K. While the mutations V9I, K15H, S24E, and A27K did not significantly affect secretion (P-value > 0.01), mutation D19V reduced secretion by 43%. Surprisingly, the mutation of L20V completely abolished secretion of the fusion protein, and the A10G mutation increased secretion by 57%. To investigate the role of the three residues at positions 10, 19, and 20 in determining secretion efficiency of FlaB, these residues were mutated in the FlaB-YplA fusion protein to the corresponding amino acids in FlaA. Interestingly, the G10A and V19D mutations did not significantly affect the secretion efficiency relative to the FlaB-YplA fusion protein, while the V20L mutation reduced secretion efficiency nearly 50%. To determine whether the alteration of codon 20 disrupts secretion by altering the RNA sequence, FlaA-YplA fusion constructs were created using the 6 different codons for leucine. While the alternative codons alter the predicted RNA structure (RNAfold, <http://rna.tbi.univie.ac.at/>), secretion of the FlaA-YplA fusion proteins was not abolished, and all were secreted at levels greater than 80% of the native *flaA* sequence (not shown). Collectively, these data indicate that the T3S amino-terminal sequences of FlaA and FlaB contain protein-specific residues that alter the efficiency of protein secretion.

Discussion

It has been previously reported that mutations in *flaA* or *fliA* (σ^{28}) result in severely truncated flagellar filaments, indicating that *flaA* is the predominant constituent of the flagellar filament (Guerry *et al.*, 1991, Wassenaar *et al.*, 1991, Jagannathan *et al.*, 2001). In this study, we sought to determine whether the phenotypic differences between the *C. jejuni flaA* and *flaB* mutants were due to the regulation of *flaA* and *flaB* (*i.e.*, the filament of a *C. jejuni flaA* mutant is truncated due to low level *flaB* expression from the σ^{54} promoter). Due to the fact that FlaA and FlaB are greater than 90% identical within a strain, it appeared unlikely that they differed other than in regulation of expression. Our initial experiments revealed that expression of *flaB* from the *flaA* promoter did not form a full-length filament. The fact that FlaA and FlaB are nearly identical, yet appeared to be secreted at different levels, allowed us the unique opportunity to compare the proteins and investigate their differences in secretion. Additionally, the flagellum is the only T3SS in *C. jejuni*, and to date there have been no other studies comparing two T3S proteins that are so similar in sequence. Therefore, we performed domain swap experiments to locate the region of the filament proteins that alter protein secretion. Replacement of the amino-terminus of FlaB with that of FlaA resulted in increased filament length and motility, while replacement of the carboxy-terminus did not appear to increase filament length or motility. Secretion assays performed with FlaA-YplA and FlaB-YplA fusion proteins in *Y. enterocolitica* confirmed the results seen in *C. jejuni*, and also allowed us to identify residues that affect secretion efficiency. In summary, these data indicate that the protein-specific residues within the amino-termini of FlaA and FlaB affect the level of protein secretion through the flagellum. As previously mentioned, the flagellum is a T3SS used to secrete flagellar structural components as well as effector proteins (Konkel *et al.*, 1999, Konkel *et al.*, 2004, Minamino & Namba, 2004, Desvaux *et al.*, 2006). The mechanisms of T3S protein recognition and export are conserved between classical “injectisome” T3SS and flagellar T3SS, as it has been demonstrated that

several proteins can be secreted through both systems. *Salmonella typhimurium* uses the *Salmonella* Pathogenicity Island 1 (SPI-1) T3SS to secrete flagellin into the host cell cytosol (Sun *et al.*, 2007), and enteropathogenic *Escherichia coli* utilizes the locus of enterocyte effacement (LEE) encoded T3SS to secrete flagellin in the absence of a functional flagellar T3SS (Badea *et al.*, 2009). *S. typhimurium* normally secretes SptP and SopE from the SPI-1 T3SS, however, when the chaperone binding domains of these proteins are deleted, they are instead secreted through the flagellar T3SS (Lee & Galan, 2004). Thus, it is expected that the mechanism by which the amino-terminus of FlaA and FlaB modulate secretion efficiency through the flagellar T3SS may apply to non-flagellar T3SS.

C. jejuni motility is dependent on the length of the flagellar filament, and motility assays can be used to indirectly observe changes in the filament length of a large population of bacteria. Wild-type *C. jejuni* F38011 are highly motile and possess full-length flagellar filaments. *C. jejuni* F38011 *flaA* mutants have extremely truncated flagella, and are only weakly motile (not shown). When the *C. jejuni* F38011 *flaA* mutant is complemented with *flaA* under control of the native promoter, filament length and motility are restored to that of wild-type (not shown). The *C. jejuni* *flaAB* mutant used in this study did not produce a flagellar filament, and was non-motile. Transformation of a *C. jejuni* *flaAB* mutant with a vector harboring *flaB* under control of the native promoter resulted in a phenotype equivalent to the *C. jejuni* *flaA* mutant. When the *C. jejuni* *flaAB* mutant was transformed with a vector containing the *flaA* gene under control of the native promoter, a filament was produced that was similar in length to wild-type isolate, but the motility was still reduced compared to wild-type. These contrasting results imply that both the FlaA and FlaB filament proteins are necessary for full motility. One possible reason for this observation is that the heterogeneity of flagellin monomers results in a flagellar structure that generates more thrust.

To examine the effects of flagellin gene expression on the function and length of the flagellar filament, TEM and motility assays were performed with promoter swap constructs. Expression of *flaB* from the *flaA* promoter resulted in an intermediate length flagellar filament, which was approximately half the length of a filament comprised solely of FlaA. Based on this finding, it was evident that regulation of gene expression was not the only factor affecting the length and motility of flagellar filaments. Other factors that we explored to explain the differences in flagellar filament length and motility included levels of protein synthesis and protein export efficiency.

In silico analyses were performed to determine whether FlaA and FlaB contain sequence variations in specific functional/structural domains. The domains of the *C. jejuni* FlaA and FlaB proteins were assigned by alignment with the *S. typhimurium* FliC protein. The purpose of determining the location of these domains in FlaA and FlaB was to be able to compare conservation or divergence within a domain to the function of that domain. The amino- and carboxy-terminal D₀ and D₁ domains of FlaA and FlaB were found to be highly conserved among *C. jejuni* strains, and shared the greatest similarity/identity with the *S. typhimurium* FliC protein. Despite the similarity of the three flagellin proteins in the D₀-D₁ region, one noteworthy difference is that *S. typhimurium* FliC is a potent activator of Toll-like receptor 5 (TLR5), while *C. jejuni* FlaA and FlaB have evolved such that they do not activate TLR5 signaling (Andersen-Nissen *et al.*, 2005, Galkin *et al.*, 2008).

We hypothesized that the D₀ domain played a role in determining secretion efficiency because it is known to affect flagellin secretion in *S. typhimurium* (Vegh *et al.*, 2006). Moreover, the amino-terminus of a protein secreted from a T3SS is known to harbor a sequence for export (Sory *et al.*, 1995, Lloyd *et al.*, 2001, Ramamurthi & Schneewind, 2002). We conducted the domain swap experiments with both the amino- and carboxy-termini of the filament proteins because they combine to form a coiled-coil structure

(Yonekura *et al.*, 2003). We chose to modify the *flaB* gene, when expressed from the *flaA* promoter, because the phenotype produced by this strain allowed us to observe an increase or decrease in filament length and motility versus the *C. jejuni* wild-type strain and *flaAB* mutant. The amino- and carboxy-terminal portions of the D₀ domain of FlaB were replaced individually and in combination with the termini of FlaA to generate three new flagellin constructs: *PflaA-flaA*_{D₀B_D123B_D0}, *PflaA-flaB*_{D₀B_D123A_D0}, or *PflaA-flaA*_{D₀B_D123A_D0}. Motility assays and TEM of the *C. jejuni flaAB* mutant transformed with a vector containing the *PflaA-flaA*_{D₀B_D123B_D0}, *PflaA-flaB*_{D₀B_D123A_D0}, or *PflaA-flaA*_{D₀B_D123A_D0} constructs revealed that only the amino-terminus is required for increased motility and filament length relative to *PflaA-flaB*. This is consistent with previous research, which indicates that the sequence required for flagellar secretion is located in the amino-terminus (Sory *et al.*, 1995, Lloyd *et al.*, 2001, Ramamurthi & Schneewind, 2002, Vegh *et al.*, 2006). Immunoblot analysis confirmed that the protein levels among the strains were similar, and that the sequence of the flagellin gene does not affect protein synthesis. Interestingly, there was more flagellin detected in the supernatant fractions of the *flaAB* mutant strains containing the domain swap constructs, possibly due to issues with polymerization or flagellum stability in the recombinant proteins. However, similar levels of flagellin were seen in the supernatant fractions for strains expressing the three domain swap-constructs; thus flagellar breakage or deficient polymerization of flagellin monomers cannot account for the shortened flagella produced by strains expressing the *PflaA-flaB*_{D₀B_D123A_D0} construct. The slight increase in motility observed for the *PflaA-flaA*_{D₀B_D123A_D0} construct relative to the *PflaA-flaA*_{D₀B_D123B_D0} construct could be due to the mismatching of the FlaA amino-terminus with the FlaB carboxy-terminus in the *PflaA-flaA*_{D₀B_D123B_D0} construct.

Knowing that the amino-terminus of the filament protein dictates protein secretion, we sought to more closely examine this region of the FlaA and FlaB proteins. While others have used a *flgK* mutant to examine secretion of the FliC flagellin from *Salmonella typhimurium*, we chose not to use this approach, as regulation of flagellar gene expression (including *fliC*) is altered in a *flgK* mutant (Komoriya *et al.*, 1999, Brown *et al.*, 2008). Thus, the secretion efficiency of the amino-terminal sequences of FlaA and FlaB were determined using the *Y. enterocolitica* YpIA secretion assay. The advantages of using the YpIA secretion assay are: 1) All flagellin genes are expressed from the *cat* promoter for equivalent expression and protein synthesis; 2) The 5' untranslated regions of *flaA* and *flaB* have been removed, so they cannot confound the results; 3) The FlaA-YpIA and FlaB-YpIA fusion proteins cannot polymerize into the flagellum; and 4) The secretion of FlaA-YpIA and FlaB-YpIA are not affected by regulation of flagellar assembly. Equivalent levels of protein synthesis amongst each of the strains was confirmed by immunoblot analysis performed using an anti-YpIA antibody (not shown).

The amino-termini of both FlaA and FlaB facilitated export of the YpIA-fusion protein through the flagellum of *Y. enterocolitica*, although the secretion efficiency of the FlaB-YpIA fusion was reduced by 60% compared to FlaA-YpIA. Interestingly, previous work revealed that the FlaA-YpIA fusion protein is secreted at levels greater than wild-type YpIA (Christensen *et al.*, 2009). Collectively, these findings support the hypothesis that the amino-termini of FlaA and FlaB affect secretion efficiency and not gene expression/protein synthesis or filament polymerization. Additionally, the mechanism governing flagellar secretion efficiency is conserved between *C. jejuni* and *Y. enterocolitica*, as the amino-terminus of FlaA results in enhanced secretion relative to FlaB in both species.

We noted that the amino-termini of FlaA and FlaB contained residue differences specific to FlaA or FlaB. Moreover, certain residues, such as V9 in FlaA and I9 in FlaB have similar biochemical properties, while other residues, such as 19D in FlaA and 19V in FlaB have different biochemical properties. To determine if these residues affected secretion

efficiency, seven protein-specific conserved residues located within the first 36 residues of the FlaA-YplA fusion protein were mutated individually to the corresponding amino acid in FlaB. The terminal domains of flagellin are known to be disordered in solution, and form coiled-coil tertiary structures composed of alpha helices when polymerized into the filament (Vonderviszt *et al.*, 1989, Yonekura *et al.*, 2003). Amino-terminal coiled-coil structures are common among T3S proteins, and have been suggested to play a role in secretion (Gazi *et al.*, 2009). Interestingly, mutations in the amino-terminus of FlaA that incorporate residues favoring beta sheets (D19V, L20V) reduce secretion efficiency. The V19D mutation in the amino-terminus of FlaB had no effect on secretion, while V20L reduced secretion efficiency. Substitution of a helix-favoring alanine with glycine, which disrupts secondary structure, greatly enhanced secretion efficiency of the FlaA-YplA fusion protein. However, in the amino-terminus of FlaB, the reciprocal mutation did not affect secretion. Collectively, these results suggest that residues favoring alpha helix formation do not enhance secretion. Silent mutations in codon 20 of the FlaA-YplA fusion did not significantly reduce secretion, despite altering the RNA sequence and the predicted structure. These results support the hypothesis that secretion of FlaA from the flagellar T3SS is dependent on the peptide sequence and not the RNA sequence, but do not rule out the possibility that RNA structure may influence secretion. While additional studies are required to determine how sequence parameters affect secretion efficiency, the secretion data suggest that multiple residues work in concert to facilitate protein secretion.

The novelty of this study lies in the fact that the secretion efficiency of T3S proteins is modulated by the amino-terminal sequence, demonstrating an additional level of regulation of protein secretion. This finding was demonstrated both in *C. jejuni* as well as *Y. enterocolitica*. Thus, the difference in the amino-termini of secreted proteins provides one possible explanation for how a pathogen exports various toxins and other proteins at different levels, even when the proteins are synthesized at similar levels. Further studies will need to be performed to determine how the efficiency of protein export can influence the assembly of the T3SS export apparatus or alter the virulence of a pathogen. In the case of *C. jejuni*, the differences in secretion of the two filament proteins likely influence the composition of the filament. Additional work is in progress, using *C. jejuni* as a model, to dissect the amino-terminal secretion signal and the mechanism of T3S protein recognition to gain a better understanding of protein export, the T3SS, and bacterial pathogenesis.

Experimental procedures

In silico analyses

All genomic sequences, except for *C. jejuni* F38011, were obtained from microbesonline.org, compiled by the Virtual Institute for Microbial Stress and Survival. The sequences for *C. jejuni* F38011 FlaA and FlaB have been deposited in GenBank, accession numbers (pending) and (pending), respectively. All sequence analyses were performed using the DNASTAR Lasergene software suite, version 7.1.0. Amino acid alignments were performed with the Megalign program, using the CLUSTAL W algorithm. The gap penalty was set to 10.0, the gap length penalty was 0.2, and a Gonnet series was used for the protein weight matrix.

Bacterial strains and culture methods

C. jejuni strains F38011 (Raphael *et al.*, 2005) and NCTC 11168 (ATCC type isolate) were used in this study. Bacteria were cultured either on Mueller-Hinton agar plates supplemented with 5% citrated bovine blood (MHB), or in Mueller-Hinton (MH) broth with constant shaking. Cultures were incubated in a microaerobic environment (85% N₂, 10% CO₂, 5% O₂), at 37°C. The *C. jejuni* cultures were passaged onto fresh media every 48 h. The *E. coli*

Inv- α (Invitrogen) and S17-1 λ -*pir* (Tascon *et al.*, 1993) strains were used in this study. Both strains were cultured on Luria-Bertani agar plates (LB) or in LB broth with constant shaking. The cultures were incubated at 37°C in an aerobic environment. All cloning steps were performed in *E. coli* Inv- α , and the complementation vectors were transformed into *E. coli* S-17 for conjugation into *C. jejuni*.

Generation of *C. jejuni* flagellar mutant

The experiments described were all performed in *C. jejuni* F38011, a human clinical isolate. The *FlaA* and *FlaB* genes of *C. jejuni* F38011 were PCR amplified using HiFi *Taq* DNA polymerase (Invitrogen) with primers derived from the genome sequence of *C. jejuni* NCTC 11168, and sequenced using dideoxynucleotide sequencing. The final sequences were assembled using the Seqman program from the Lasergene software suite. A *flaAB* mutant of *C. jejuni* F38011 was generated via double crossover recombination and subsequent insertion of a tetracycline resistance cassette. Approximately 1.5 kilobases of the upstream and downstream regions flanking *flaA* and *flaB* were PCR amplified using the primer pairs MEK 1011 / MEK 1001 and MEK 1851 / MEK 1844, respectively (Supplemental Table 2). The two fragments were cloned sequentially into pBSK-Kan2, using the sites *Sst*I, *Sst*II, *Bam*HI and *Xho*I. The *tetO* tetracycline resistance gene was inserted between the two homologous flanking regions using the *Sst*II and *Bam*HI sites. pBSK-Kan2 is identical to the pBluescript vector (Stratagene), except that the original kanamycin resistance cassette was replaced with the *aphA-3* kanamycin resistance cassette, which functions in both *E. coli* and *C. jejuni*. The plasmid was introduced into the *C. jejuni* F38011 strain via electroporation. Transformants were selected for on MHB agar plates supplemented with 2 μ g/ml tetracycline or 50 μ g/ml kanamycin (MHB Tet2 or MHB Kan50). Tetracycline resistant colonies were streaked onto MHB Tet2 and MHB Kan50 plates to confirm that the transformants were kanamycin sensitive. Deletion of *flaA* and *flaB* was confirmed by PCR (not shown) and immunoblot analyses.

Generation of *C. jejuni* flagellar strains

The *flaA* and *flaB* genes of *C. jejuni* NCTC 11168 were used to transform the *C. jejuni* F38011 *flaAB* mutant. Both *flaA* and *flaB* were PCR amplified using HiFi *Taq* DNA polymerase (Invitrogen) from NCTC 11168 chromosomal DNA using the primer pairs MEK 1332 / MEK 2015 and MEK 1334 / MEK 2025, respectively. Each gene was cloned into the *E. coli*-*C. jejuni* shuttle vector pRY111 (Yao *et al.*, 1993), using the *Sst*I and *Eco*RI sites present in the MCS. The promoter regions for *flaA* and *flaB* were PCR-amplified using the primer pairs MEK 1611 / MEK 1612 and MEK 1613 / MEK 1614, respectively. Both promoters were inserted upstream of each filament protein gene to produce four different flagellin complementation constructs.

Domain swap constructs were generated by removing the amino- and carboxy-terminal portions of *flaB* and replacing them with the complementary portions of *flaA*. The amino-termini, spanning residues 1-114, and the carboxy-termini, spanning residues 495-572, were swapped both individually and together to generate three modified versions of *flaB*. Cloning was performed using a native *Eco*RV site at +342 basepairs (residue 114), and a recombinant *Xba*I site at +1,484 basepairs (residue 495). The *Xba*I site was generated by inverse PCR of the *PflaA-flaB* pRY111 construct using the primer pairs MEK 1858 / MEK 1859 and MEK 1860 / MEK 1861, followed by blunt-ended ligation of the PCR product. Although the portions of the gene that were exchanged extended beyond the D₀ domain, the amino acid sequences external to the D₀ domain are identical in *FlaA* and *FlaB*. The flagellin gene constructs were transformed into *E. coli* S-17 via electroporation. The inserts contained within each vector were sequenced using the dideoxynucleotide method for

confirmation. The vectors were then introduced into the *C. jejuni* F38011 *flaAB* mutant by conjugation.

Phase variation motility assays

C. jejuni F38011 wild-type and the *flaAB* mutant harboring *flaA* expressed from the *flaA* promoter were grown for 48 h on MHB agar plates and resuspended in MH broth. The OD₅₄₀ was determined, and the sample was diluted in MH soft agar (0.4% agar) to a density that would yield ~30 colony-forming units (CFU) when plated. After 48 h incubation, the motility of 500 individual CFU was observed for each strain.

Motility assays

Cultures of *C. jejuni* were grown to mid-log phase overnight in MH broth, supplemented with 8 µg/ml of chloramphenicol for strains containing pRY111 derivatives. The cultures were normalized to an OD₅₄₀ = 1.0 by centrifugation and resuspension in MH broth, and 5 µl of each culture was applied to the center of a MH plate containing 0.4% agar, and incubated for 48 h. The zone of motility was determined by measuring from the edge of the inoculation spot to the end of the visible migration. The image analysis program ImageJ (www.nih.gov) was used to measure scanned images of each motility assay. Six replicates of each assay were performed.

Transmission electron microscopy

C. jejuni were harvested from 48 h MHB plates supplemented with the appropriate antibiotics and resuspended in distilled water to an OD₅₄₀ = 0.1. Four µl of each suspension was applied to a formvar-coated nickel grid, and combined with 4 µl of 1% phosphotungstic acid. After 60 seconds, the liquid was removed from each grid via capillary action using Whatman filter paper. Transmission electron microscopy was performed using a Jeol 1200EX electron microscope, at a magnification of 25,000x. Images were taken of bacteria possessing full-length flagella, as determined by observing the flagellated population to approximate maximal flagellar length. Bacteria lacking visible flagella or with severely truncated flagella were not included in the measurements, as it was obvious that sample preparation results in flagellar shearing. We know flagellar shearing is occurring due to the fact that many intact flagella are observed detached from cells, and the phase variation motility assay demonstrates that 98% of the bacteria are motile. Only sample preparations in which the majority of bacteria were flagellated were used for measurements. The lengths of the flagella were determined using the segmented line tool of ImageJ (Abramoff, 2004).

Yersinia enterocolitica YplA fusion protein secretion assays

To determine whether FlaA and FlaB were exported at different efficiencies in a heterologous system, we utilized the YplA fusion protein secretion assay (Christensen *et al.*, 2009). In this assay, the first 108 bp of the amino terminal regions of both *flaA* and *flaB* genes were PCR amplified from *C. jejuni* NCTC 11168 chromosomal DNA with primers containing 5' NdeI and 3' BglII restrictions sites for directional cloning into the *E. coli* - *Y. enterocolitica* shuttle vector pCSP50. The pCSP50 vector includes a tetracycline resistance gene (*tetO*), a constitutive promoter (*cat*), a 5' truncated phospholipase gene (*yplA*) lacking 150 nucleotides encoding the native T3S signal, and *yplB*, which is located immediately downstream of *yplA* (Schmiel *et al.*, 1998). The NdeI and BglII sites facilitate directional cloning of *C. jejuni* T3S amino-terminal sequences as fusions with the truncated *yplA*. The pCSP50 derived vectors were transformed into *E. coli* S17-1 λ -*pir*, and confirmed by PCR fragment size and sequence analysis. The vectors were conjugated into a *Y. enterocolitica* *yplAB* mutant, and confirmed by agarose gel electrophoresis of restriction digested plasmid preparations.

Secretion of the YplA fusion proteins was assayed by inoculation onto phospholipase assay (PLA) media. Briefly, *Y. enterocolitica* strains were grown overnight in LB broth with shaking at 26°C. Secretion via the flagellar T3SS was induced by spotting 1.5 µl of culture on TYE PLA medium (1% tryptone, 0.5% yeast extract, 1.5% agar, 1% Tween 80, and 1 mM CaCl₂), and incubation at 26°C. Each isolate was tested for secretion a minimum of three times from two independent PLA plate assays to ensure reproducible phenotypes. Sixteen conjugates were inoculated on each PLA plate, in addition to the *Y. enterocolitica* *yplAB* mutant expressing wild-type YplA as a positive control. All plates were scanned at 300 dpi resolution (12, 24, and 48 h), and secretion zone widths were measured manually from digital images using select tools in Adobe Photoshop CS2 version 9.0.2 (Adobe Systems Incorporated, USA).

Immunoblots

Whole cell lysates of *C. jejuni* were probed for FlaA and FlaB using a polyclonal antibody that recognizes both of the filament proteins. Broth cultures of *C. jejuni* were grown to mid-exponential growth phase, and an equivalent amount of cells (OD₅₄₀ = 2) were harvested by centrifugation. The cell pellet was resuspended in 200 µl 1X sample buffer and boiled for 5 min to prepare whole-cell lysates (WCL). Culture supernatants were collected and filtered through a 0.22µm filter to remove any cells. 10 µl of each WCL was separated by SDS-PAGE (12% polyacrylamide) at 100 volts for 1 h. The gel was stained in Coomassie Blue dye for 20 min, and de-stained overnight. A second 12% SDS-polyacrylamide gel was loaded with 10 µl of a 1:10 dilution of each WCL and electrophoresed at 100 V for 1 h. A third 12% SDS-polyacrylamide gel was loaded with volumes of the supernatant fractions normalized to the culture density, so they could be directly compared to the whole-cell lysate fractions. After electrophoresis, the proteins were transferred to a polyvinylidene fluoride PVDF membrane (Immobilon P; Millipore Corp., Bedford, MA) by electrophoresis. FlaA and FlaB were detected with a 1:1000 dilution of a rabbit α-FlaA/FlaB antibody K6932, and bound primary antibodies were detected with 1:1000 α-rabbit IgG conjugated to horseradish peroxidase. The immunoblots were developed by chemiluminescence (Western Lightning, PerkinElmer Life Sciences, Inc.) and images taken using a FujiFilm LAS-2000 multi-imager.

Mutation of residues in the FlaA-YplA and FlaB-YplA fusion proteins

Seven residues in the amino-terminus of the FlaA-YplA (9, 10, 15, 19, 20, 24 and 27) and three residues in the FlaB-YplA fusion proteins (10, 19, and 20) were mutated to the corresponding amino acid in the other filament protein. The mutations were introduced into the pCSP50 based FlaA-YplA and FlaB-YplA vectors by inverse PCR using 5' phosphorylated primers to facilitate blunt-ended ligation of the inverse PCR product. The mutations in the FlaA-YplA (9, 10, 15, 19, 20, 24 and 27) and FlaB-YplA (10, 19, and 20) vectors were performed using the primer pairs MEK 1655/1656, 1657/1658, 1659/1660, 1661/1662, 1663/1664, 1665/1666, 1667/1668, 2255/2256, 2257/2258, and 2259/2260, respectively (Table 4). The nucleotide sequence of each codon was mutated to match the corresponding codon in the other filament protein gene. The constructs were sequence confirmed, transformed into *E. coli* S-17, and conjugated into the *Y. enterocolitica* *yplAB* mutant.

Silent mutation of leucine 20 in the FlaA-YplA fusion construct

Codon 20 of the FlaA-YplA fusion construct, which encodes leucine (TTA), was mutated to the five alternative codons (CTT, TTG, CTA, CTC, and CTG) using the inverse PCR technique described above. The primer pairs used for inverse PCR were MEK 1664/2391 (CTT), 1664/2392 (TTG), 1664/2393 (CTA), 1664/2394 (CTC), and 1664/2395 (CTG). The

constructs were sequence confirmed, transformed into *E. coli* S-17, and conjugated into the *Y. enterocolitica* *yplAB* mutant. Secretion efficiency was determined as describe above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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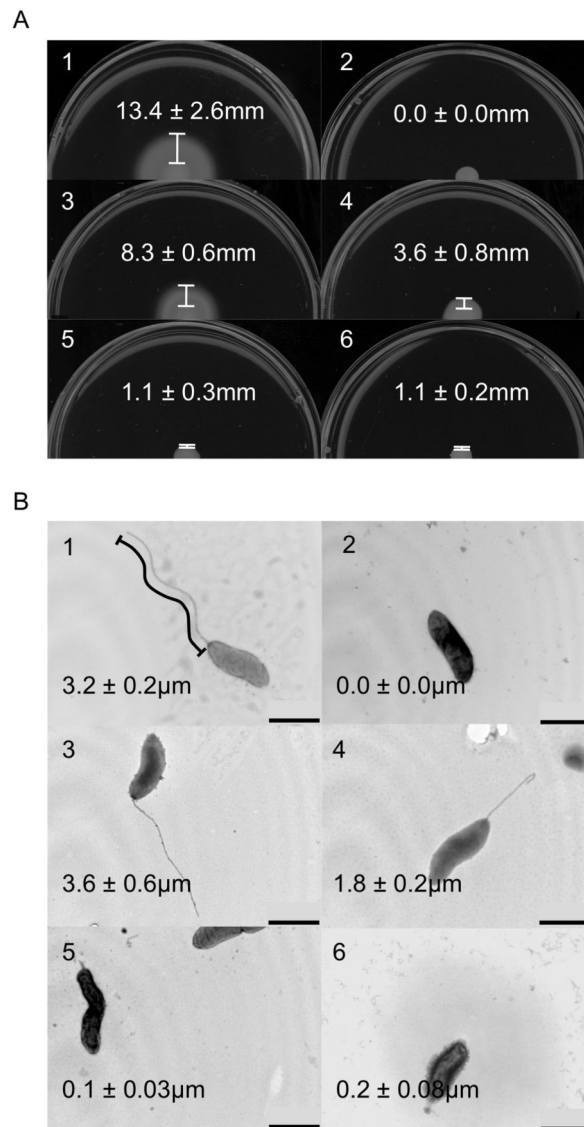


Figure 1. Motility assays and TEM of promoter experiments

Motility assays and TEM of *C. jejuni* F38011 wild-type and a flagellar *flaAB* mutant transformed with a vector harboring either the *flaA* and *flaB* genes. In this experiment, the *C. jejuni* NCTC 11168 *flaA* and *flaB* genes were expressed from both the *flaA* and *flaB* promoters (*PflaA* or *PflaB*). *C. jejuni* strains were cultured on MH 0.4% agar plates, and the distance from the edge of the culture spot to the haze of motility was determined (Panel A). Bacteria were stained with 1% phosphotungstic acid, TEM performed, and the length of the flagellum was measured (Panel B). The images are of the: 1) *C. jejuni* F38011 wild-type strain; 2) *C. jejuni* F38011 *flaAB* mutant; 3) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaA*; 4) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaB*; 5) *C. jejuni* F38011 *flaAB* mutant harboring *PflaB-flaA*; and 6) *C. jejuni* F38011 *flaAB* mutant harboring *PflaB-flaB*. The values shown represent the mean \pm standard deviation of 6 motility assays and 10 TEM images. One representative image is shown for each strain. Bar = 1 μm .

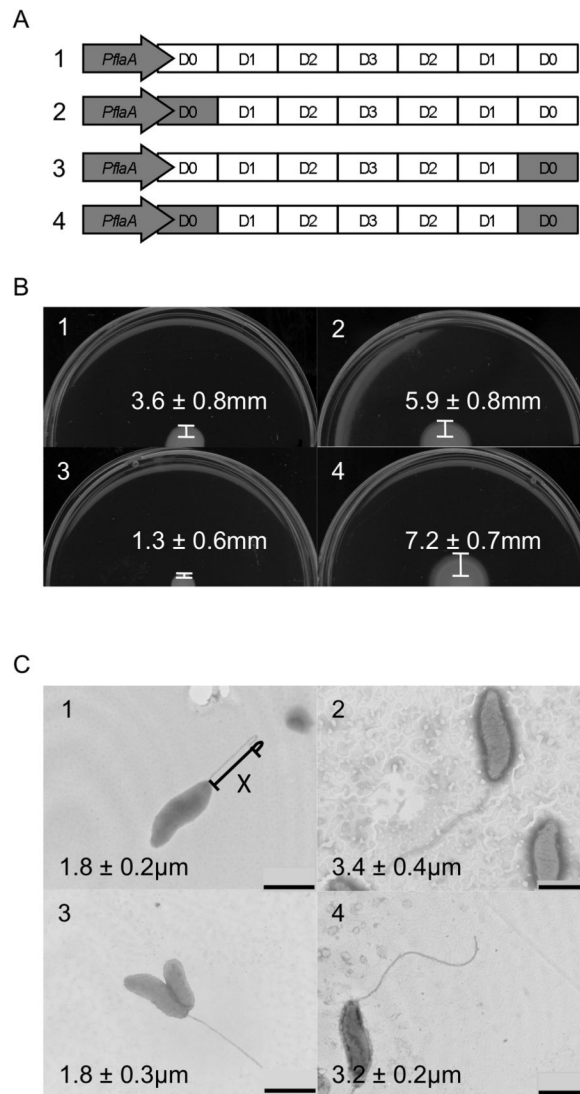


Figure 2. Motility assays and TEM of domain swaps

Panel A shows a linear schematic of the domain organization of the *C. jejuni flaB* gene and three constructs containing the domain swaps (not drawn to scale). Regions in gray correspond to *C. jejuni flaA* gene, while regions in white correspond to the *C. jejuni flaB* gene. Motility assays and TEM of flagellar mutants transformed with a vector harboring *flaB*, which had been modified to include the D₀ domain of FlaA. *C. jejuni* strains were cultured on MH 0.4% agar plates, and the distance from the edge of the culture spot to the haze of motility was determined (Panel B). Bacteria were stained with 1% phosphotungstic acid, TEM performed, and the length of the flagellum was measured (Panel C). The images are of the: 1) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaB*; 2) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaA_{D0}B_{D123}B_{D0}*; 3) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaB_{D0}B_{D123}A_{D0}*; and 4) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaA_{D0}B_{D123}A_{D0}*. The values shown represent the mean \pm standard deviation of 6 motility assays or 10 TEM images. One representative image is shown for each strain. X = Example of line drawn to measure flagellar length. Bar = 1 μm .

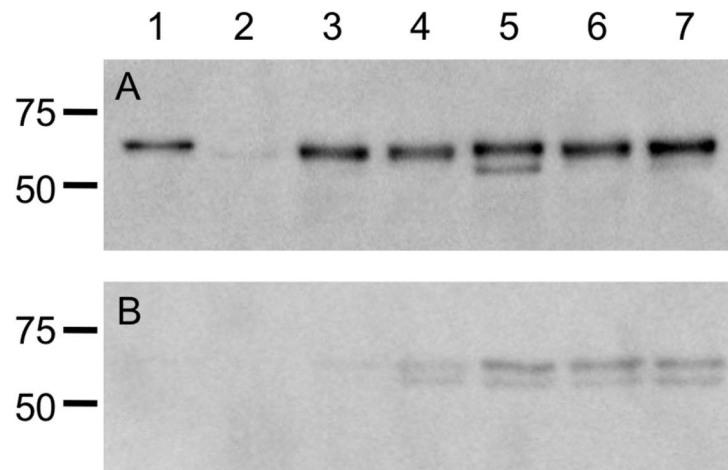


Figure 3. Immunoblots of mutants and transformed strains

Analysis of FlaA and FlaB protein synthesis in the *C. jejuni* F38011 wild-type strain, F38011 *flaAB* mutant, and transformed mutants. Whole cell lysates were prepared from broth cultures of *C. jejuni* in mid-exponential growth phase, and separated in 12% SDS-polyacrylamide gels. Panels: A) Whole cell lysates; and B) Supernatants were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane and the immunoblots were probed with a polyclonal serum that recognizes both FlaA and FlaB. The samples in each lane are the: 1) *C. jejuni* F38011 wild-type strain; 2) *C. jejuni* F38011 *flaAB* mutant; 3) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaA*; 4) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaB*; 5) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaA_{D0}B_{D123}B_{D0}*; 6) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaB_{D0}B_{D123}A_{D0}*; and 7) *C. jejuni* F38011 *flaAB* mutant harboring *PflaA-flaA_{D0}B_{D123}A_{D0}*. The proteins loaded in the immunoblots (Panels A and B) are normalized according to culture density, so the levels of flagellin in the whole cell lysates and supernatants can be directly compared.

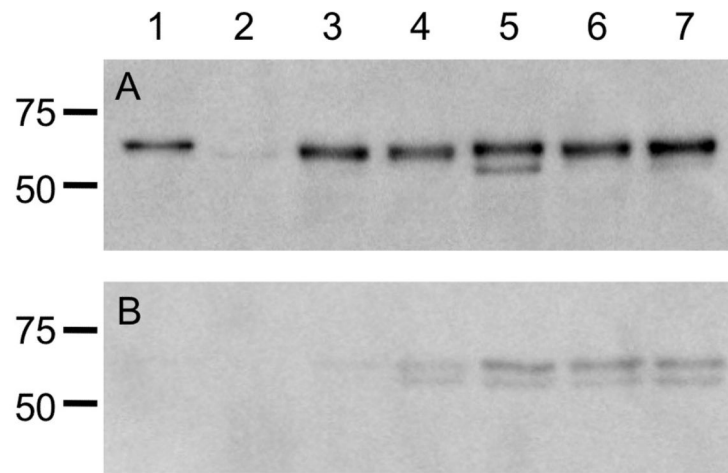


Figure 4. Alignment of FlaA and FlaB D₀ domains

Amino acid sequence alignment of the D₀ domain of FlaA and FlaB from 8 different strains of *C. jejuni* performed using MegAlign and the ClustalW algorithm. The amino- (Panel A) (residues 1-45) and carboxy-terminal (Panel B) (residues 533-572) D₀ domain sequences of FlaA (above black line) and FlaB (below black line) are shown. Residues shaded in gray/black are conserved, while residues in white are divergent.

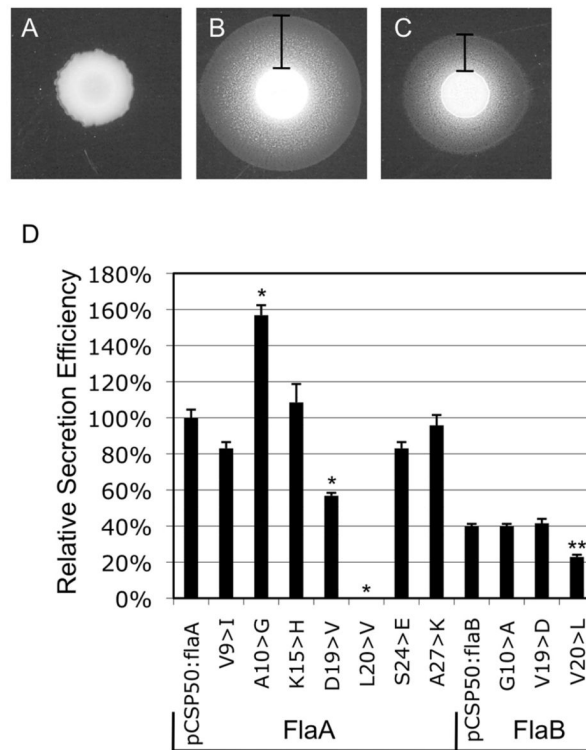


Figure 5. YplA secretion assays

Secretion of flagellin-YplA fusion proteins from *Y. enterocolitica*. The 5' end of the *flaA* or *flaB* genes, gene. Panels: A) which encode the first 36 residues, were fused to the truncated *yplA* Secretion assay performed using *Y. enterocolitica yplA* mutant harboring pCSP50 empty vector; B) Secretion assay performed using *Y. enterocolitica yplA* mutant harboring *flaA-yplA*; C) Secretion assay performed using *Y. enterocolitica yplA* mutant harboring *flaB-yplA*; and D) Relative secretion efficiency of *Y. enterocolitica yplA* mutant harboring modified *flaA-yplA* and *flaB-yplA* fusion gene constructs. The values shown represent the mean zone of secretion from six individual assays, and the error bars represent \pm one standard deviation. The width of the zone of secretion was determined by measuring from the edge of colony growth to the outer edge of precipitation. A standard curve was created by assaying varying combinations of pCSP50:*flaA* and pCSP50 containing *Y. enterocolitica*.

Table 1Alignment of *S. typhimurium* FliC with *C. jejuni* NCTC 11168 FlaA and FlaB

Domain	Residues within domain ^a		% Identity with FliC ^b	
	FliC	FlaA/FlaB	FlaA	FlaB
D ₀ (N)	1-45	1-45	48.9	48.9
D ₁ (N)	46-180	46-181	27.2	28.9
D ₂ (N)	181-190	182-192	18.2	30.0
D ₃	191-284	193-335	21.5 ^c	24.2 ^c
D ₂ (C)	285-407	336-473	19.5 ^c	18.7 ^c
D ₁ (C)	408-455	474-532	31.2 ^c	29.2 ^c
D ₀ (C)	456-495	533-572	55.0	50.0

^aThe domain boundaries within the *C. jejuni* FlaA and FlaB proteins were assigned based on alignment with *S. typhimurium* FliC.

^bPercent identity was determined using Megalign version 7.1.0, Clustal W algorithm with a gap penalty of 10.0 and a gap length penalty of 0.2.

^cThe values shown were based on a sub-alignment of a specific domain, and not on the alignment of the entire protein.

Table 2

In silico analysis of FlaA and FlaB amongst *C. jejuni* strains

A. FlaA amino acid % identity compared with <i>C. jejuni</i> NCTC 11168										
Strain	D ₀ (N)	D ₁ (N)	D ₂ (N)	D ₃	D ₂ (C)	D ₁ (C)	D ₀ (C)	Whole		
NCTC 11168	100	100	100	100	100	100	100	100		
81116	95.6	94.9	63.6	71.3	62.8	98.3	95.0	81.1		
81-176	95.6	94.9	63.6	71.3	62.8	98.3	95.0	81.1		
Rml221	100	99.3	81.8	93	77.7	100	100	92.1		
F38011	91.1	93.4	63.6	76.2	58.1	93.2	95.0	81.2		
CF93-6	100	100	100	100	99.3	100	100	99.8		
260.94	91.1	93.4	63.6	73.4	56.1	96.6	95.0	79.2		
84-25	100	100	100	100	99.3	100	100	99.8		

B. FlaB amino acid % identity compared with <i>C. jejuni</i> NCTC 11168										
Strain	D ₀ (N)	D ₁ (N)	D ₂ (N)	D ₃	D ₂ (C)	D ₁ (C)	D ₀ (C)	Whole		
NCTC 11168	100	100	100	100	100	100	100	100		
81116	95.6	95.6	54.5	73.4	60.7	98.4	97.5	81.5		
81-176	95.6	94.1	54.5	73.4	60.7	98.4	97.5	81.1		
Rml221	100	98.5	90.9	97.2	77.9	100	100	93.4		
F38011	97.8	91.9	45.5	73.4	56.6	91.9	97.5	80.2		
CF93-6	100	100	100	100	100	100	100	100		
260.94	97.8	92.6	54.5	74.1	53.8	98.4	97.5	79.5		
84-25	100	100	90.9	100	100	100	100	99.8		