Novel Components of the Flagellar System in Epsilonproteobacteria

Beile Gao,[a] Maria Lara-Tejero,[a] Matthew Lefebre,[a] Andrew L. Goodman,[a,b] Jorge E. Galán[a]

Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, Connecticut, USA[a]; Microbial Diversity Institute, Yale University School of Medicine, New Haven, Connecticut, USA[b]

ABSTRACT **Motility is essential for the pathogenesis of many bacterial species. Most bacteria move using flagella, which are multiprotein filaments that rotate propelled by a cell wall-anchored motor using chemical energy. Although some components of the flagellar apparatus are common to many bacterial species, recent studies have shown significant differences in the flagellar structures of different bacterial species. The molecular bases for these differences, however, are not understood. The flagella from epsilonproteobacteria, which include the bacterial pathogens *Campylobacter jejuni* and *Helicobacter pylori*, are among the most divergent. Using next-generation sequencing combined with transposon mutagenesis, we have conducted a comprehensive high-throughput genetic screen in *Campylobacter jejuni*, which identified several novel components of its flagellar system. Biochemical analyses detected interactions between the identified proteins and known components of the flagellar machinery, and *in vivo* imaging located them to the bacterial poles, where flagella assemble. Most of the identified new components are conserved within but restricted to epsilonproteobacteria. These studies provide insight into the divergent flagella of this group of bacteria and highlight the complexity of this remarkable structure, which has adapted to carry out its conserved functions in the context of widely diverse bacterial species.**

IMPORTANCE **Motility is essential for the normal physiology and pathogenesis of many bacterial species. Most bacteria move using flagella, which are multiprotein filaments that rotate propelled by a motor that uses chemical energy as fuel. Although some components of the flagellar apparatus are common to many bacterial species, recent studies have shown significant divergence in the flagellar structures across bacterial species. However, the molecular bases for these differences are not understood. The flagella from epsilonproteobacteria, which include the bacterial pathogens *Campylobacter jejuni* and *Helicobacter pylori*, are among the most divergent. We conducted a comprehensive genetic screen in *Campylobacter jejuni* and identified several novel components of the flagellar system. These studies provide important information to understand how flagella have adapted to function in the context of widely diverse sets of bacterial species and bring unique insight into the evolution and function of this remarkable bacterial organelle.**

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Address correspondence to Jorge E. Galán, jorge.galan@yale.edu.

The bacterial flagellum is a very complex nanomachine that is highly conserved across bacterial species (1–3). Despite this conservation, there is significant variation in the numbers and the locations of flagella on the bacterial body (4). While some bacteria have multiple peritrichous flagella, others have a single flagellum at one (unipolar or monotrichous) or both (bipolar or amphitrichous) poles or have flagella embedded within the periplasmic space (5–8). Numerous studies, mostly carried out in the model organisms *Salmonella enterica serovar* Typhimurium and *Escherichia coli*, have provided detailed information about the composition, structure, assembly, and function of this remarkable organelle (3, 9). These studies have shown that the bacterial flagellum consists of two main structural components, the hook basal body complex and the extracellular filament. In addition, a number of nonstructural components are required for flagellar assembly and function. The deployment of the flagellar apparatus is highly regulated by a complex regulatory network that ensures the expression of its components at the appropriate time and within the appropriate environment (3). Although the general organization of flagella is highly conserved, it has recently become apparent that there is considerable diversity in the flagellar structure itself across bacterial species (1, 10, 11). In particular, cryo-electron tomography studies have shown that flagella from members of the *Epsilonproteobacteria* are among the most structurally diverse, exhibiting several unique structural features that most likely correspond to novel, yet unidentified, flagellar components (12). Understanding the molecular bases for these differences would be important to understand the evolution and adaptation of this bacterial organelle across bacterial species.

All members of the *Epsilonproteobacteria* have their flagella located at either one or both cellular poles (13–19). *Campylobacter jejuni* and *Helicobacter pylori* are the most studied epsilonproteobacteria because they are important human pathogens. *C. jejuni* is a major cause of food-borne illness (20, 21), while *H. pylori* is an important cause of stomach ulcers and gastric cancer (22, 23). Motility is essential for these bacteria to invade cultured cells,

colonize animals, and cause disease in susceptible hosts (24–27). In addition to their unique structural features revealed by cryo-electron tomography (12), *Campylobacter* and *Helicobacter* flagella exhibit unique aspects in the regulation of the expression of their flagellar genes and in the assembly of their flagellar structure (28, 29). For example, *C. jejuni* flagellar assembly, which occurs at the poles, requires the posttranslational glycosylation of the flagellin subunits and is specifically coordinated with cell division (30–33). Regulation of flagellar gene expression in *Campylobacter* and *Helicobacter* is also unique, involving a two-component system (FlgRS), the FlhF GTPase, and the transcription factors sigma54 and sigma28 (34–36). Here we have identified several novel components of the *C. jejuni* flagellar system through a high-throughput screen. We present data that collectively indicate that these novel genes encode factors that directly influence motility. The newly identified components are highly conserved in but largely restricted to *Epsilonproteobacteria*. This report significantly enhances our understanding of the unique flagellar system of these bacterial taxa and highlights the versatility of this nanomachine with respect to its ability to adapt its design to the specific needs of diverse bacteria.

RESULTS

Construction and characterization of a *C. jejuni* transposon mutant library by INSeq: insights into essential genes. Transposon mutagenesis coupled to next-generation sequencing is a powerful tool to carry out high-throughput mutant screens in bacteria (37–39). We established a genome-wide disruption library in *C. jejuni* 81-176 with a sequencing-adapted mariner transposon derivative specifically tailored for its use in this bacterium. The transposable element has two antibiotic resistance markers flanked by modified mariner inverted repeats containing an MmeI restriction site (Fig. 1A). The two resistance genes lack transcription terminators and are arranged in opposite orientations so that their own promoters point outward of the transposable element, thus minimizing polar transcription effects on downstream genes. The transposon mutant library was constructed after *in vitro* transposition and natural transformation as previously described (40). Characterization of the mutant library by nucleotide sequencing of the transposon insertion sites indicated that the library consists of ~50,000 transposon insertion mutants with insertions well distributed across the genome at an average density of 31 insertions/kb (Fig. 1B; see also Table S1 in the supplemental material). To validate the reproducibility of high-throughput insertion sequencing (INSeq), we performed technical and biological replicates, which indicated a very high degree of reproducibility (Fig. 1C).

After filtering out insertions within the last 20% of any coding region (because such insertions could permit gene function), we found that 1,583 of the 1,758 predicted open reading frames (ORFs) in the *C. jejuni* genome and its two resident plasmids (*pTet* and *pVir*) had been directly disrupted in the mutant pool. Insertions were obtained for all but 175 predicted open reading frames, making these genes potential candidates to be identified as essential under the growth conditions used in this study (Table S2). This set of genes, however, shows little overlap with two recently reported lists of potential essential genes for the *C. jejuni* NCTC11168 strain (41, 42). Besides differences in the strains, there are significant experimental differences between the previous studies and those described here which could account for the discrepancies. The previous studies, which identified 195 and 233 potential essential genes, involved a much smaller transposon insertion library (~7,000 insertions) and used microarrays instead of next-generation sequencing to determine the transposon insertion sites. The use of microarrays does not allow the elimination of insertions close to the 3′ end of an open reading frame, which may lead to insertions resulting in a functional product. Furthermore, the transposon element used in our study minimized polar transcriptional effects, potentially allowing insertions upstream of a cotranscribed essential gene. This feature may help to account for the smaller size of the list of essential genes reported here. In summary, the transposon insertion library exhibited robust coverage of the *C. jejuni* genome and provided insight into *C. jejuni* essential genes.

Identification of *C. jejuni* motility genes by searching for mutants unable to invade cultured mammalian cells. Key structural features of the flagellar apparatus in *Epsilonproteobacteria* diverge from those of model organisms such as *E. coli* and *S.* Typhimurium (12). These differences are more than likely attributable to unidentified flagellar components specific to *Epsilonproteobacteria*. These components cannot be identified with strategies that rely on amino acid sequence homology to known flagellar proteins (43, 44). Consequently, we set out to carry out a genetic screen to identify flagellar components unique to *Campylobacter* and other *Epsilonproteobacteria*. Previous genetic screens for *C. jejuni* nonmotile mutants have relied on assays that are not amenable to high throughput and therefore have not been comprehensive (40, 45, 46). Here, we carried out a high-throughput genetic screen that comprehensively searched the virulent strain of *C. jejuni* 81-176 for nonmotile mutants. We took advantage of the observation that motility is strictly required for the ability of *C. jejuni* to invade cultured mammalian cells (26, 47) and that the examination of the cultured-cell invasion phenotype is amenable to high-throughput screening. We therefore used INSeq to screen our comprehensive transposon insertion library (see above) for mutants unable to invade cultured mammalian cells (see details in Materials and Methods) to compare the representation of insertion mutants in the bacterial inoculum with that in the bacteria obtained after the invasion assay (37, 48).

Our screen identified mutants with mutations in 36 genes with drastic defects in their ability to invade cultured mammalian cells (Table 1 and Fig. 1D; see also Table S1 in the supplemental material), which is the expected phenotype of nonmotile mutants. Four of the genes had been previously reported to be specifically involved in *C. jejuni* invasion of epithelial cells with no specific involvement in motility (49) and therefore were not considered for further analysis. Twenty-four of the identified genes encode proteins known to be involved in flagellar biosynthesis, modification, regulation, and chemotaxis, which validated the rationale for the screening protocol. Homology searches for the proteins encoded by the remaining 8 genes with a motility defect (CJJ81176_0100, CJJ81176_0198, CJJ81176_0199, CJJ81176_0240, CJJ81176_0413, CJJ81176_0891, CJJ81176_1488, and CJJ81176_1489) detected no proteins previously associated with flagellar function except for CJJ81176_1489. This particular protein exhibits amino acid sequence similarity to FliJ, a poorly conserved component of the flagellar export apparatus, which has been shown to be required for efficient flagellar assembly in *S.* Typhimurium (43, 50). Four of the genes showing a motility defect (CJJ81176_0100, CJJ81176_0198, CJJ81176_0199, and

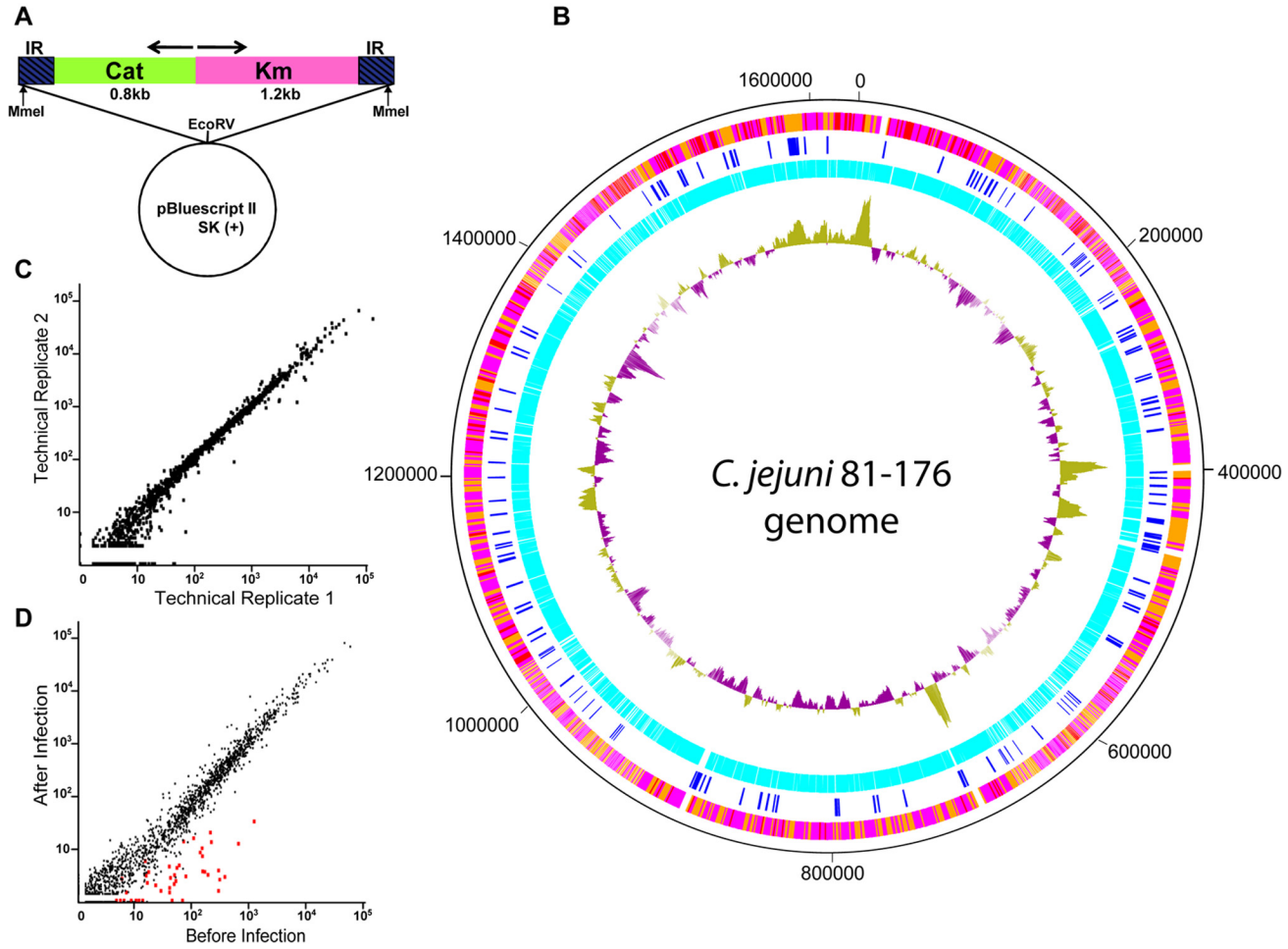

FIG 1 INSeq transposon mutagenesis of *C. jejuni*. (A) Schematic depiction of the transposable element used in this study. Arrows represent the direction of the promoters of the chloramphenicol acetyltransferase (Cat) and kanamycin (Km) antibiotic resistance genes. (B) *C. jejuni* 81-176 genome map depicting the distribution of transposon insertions in the mutant library. The outer (first) circle depicts sequence length (in bases); the second, the number of reads per kb of the genome (white, 0 reads; orange, ≤10; magenta, ≤100; red, >100); the third (blue), essential genes; the fourth (cyan), nonessential genes; and the fifth, GC skew (G−C/G+C) (khaki, values > 0; purple, values < 0). (C) Reproducibility of experimental protocols. Technical replicates were prepared and sequenced from a single transposon mutant population. Each point represents the abundance of read numbers of a single gene, which is normalized to 1 million reads. The coefficient of determination, R^2, value corresponding to the log-transformed abundance value is 0.97. (D) Relative abundances of insertion mutants before and after cultured mammalian cell infection. The relative abundances of mutations in each gene (points) in the input (before infection) and output (after infection) populations were compared. Genes that showed a statistically significant change (q <0.01) in representation in all 3 biological replicates are shown in red; the others are shown in black. The R^2 value corresponding to the log-transformed abundance value is 0.93.

CJJ81176_0413) were exclusively detected in *Epsilonproteobacteria*, including *Campylobacter*, *Helicobacter*, *Sulfurospirillum*, *Sulfuricurvum*, *Sulfurimonas*, *Wolinella*, *Caminibacter*, and *Nautilia* (Table 2) (17, 18, 51–57). Genomic localization of the identified putative motility genes revealed that two of them (CJJ81176_0100 and CJJ81176_0891) are located immediately adjacent to known motility genes (see Fig. S1), providing further support for their potential involvement in motility. Taken together, these results indicate that the genetic screen identified several novel putative motility genes.

Functional characterization of the *C. jejuni* motility mutants. To verify the phenotype of the novel motility mutants, we constructed mutants with deletions of the candidate genes in *C. jejuni* and subsequently examined their ability to invade cultured mammalian cells. We found a drastic (<100-fold) reduction in the levels of bacterial internalization in cultured mammalian cells in all mutants except for the *C. jejuni* CJJ81176_0240 mutant, which showed an ~5-fold decrease in invasion (Fig. 2). Since we previously observed that motility defects are always associated with very strong invasion phenotypes (49), it is likely that the invasion defect of the CJJ81176_0240 mutant is due to reasons other than motility and it was therefore not considered any further in our analysis. We used a soft-agar motility plate assay to examine the motility phenotype of all the mutants severely affected in invasion (Fig. 3). Consistent with their cell invasion phenotype, we found that *C. jejuni* ΔCJJ81176_0100, ΔCJJ81176_0198, ΔCJJ81176_0199, ΔCJJ81176_0413, ΔCJJ81176_0891, and ΔCJJ81176_1489 exhibited a strong motility defect. In contrast, the ΔCJJ81176_1488 mutant showed wild-type motility in this assay, indicating that the invasion defect might be due to factors

TABLE 1 Genes identified by INseq exhibiting strong cultured cell invasion phenotype

81-176 gene and encoded protein functional category	NCTC11168 gene	Symbol	Annotation	*q* value[a]
Flagellar assembly				
CJJ81176_0097	Cj0059c	*fliY*	Flagellar motor switch protein	8.39E-09
CJJ81176_0098	Cj0060c	*fliM*	Flagellar motor switch protein	1.44E-19
CJJ81176_0101	Cj0063c	*flhG*	ParaA family ATPase	0.001
CJJ81176_0226	Cj0195	*fliI*	Flagellum-specific ATP synthase	2.38E-20
CJJ81176_0357	Cj0335	*flhB*	Flagellar biosynthesis protein	6.27E-16
CJJ81176_0358	Cj0336c	*motB*	Flagellar motor protein MotB	9.00E-07
CJJ81176_0359	Cj0337c	*motA*	Flagellar motor protein MotA	6.21E-19
CJJ81176_0837	Cj0820c	*fliP*	Flagellar biosynthesis protein	6.63E-30
CJJ81176_0890	Cj0882c	*flhA*	Flagellar biosynthesis protein	3.14E-08
CJJ81176_1044	Cj1025c	*flgQ*	Hypothetical protein	0.001
CJJ81176_1045	Cj1026c	*flgP*	Putative lipoprotein	7.84E-07
CJJ81176_1194	Cj1179c	*fliR*	Flagellar biosynthesis protein	2.51E-17
CJJ81176_1459	Cj1466	*flgK*	Flagellar hook-associated protein	2.93E-07
CJJ81176_1550	Cj1565c	*pflA*	Paralyzed flagellar protein	1.22E-13
CJJ81176_1671	Cj1675	*fliQ*	Flagellar biosynthesis protein	9.94E-08
Regulators for flagellar biosynthesis				
CJJ81176_0099	Cj0061c	*fliA*	Flagellar biosynthesis sigma factor	1.14E-07
CJJ81176_0102	Cj0064c	*flhF*	Flagellar biosynthesis regulator	0.001
CJJ81176_0696	Cj0670	*rpoN*	RNA polymerase factor sigma54	5.77E-15
CJJ81176_0814	Cj1024c	*flgR*	Sensor histidine kinase	5.43E-24
CJJ81176_1043	Cj0793	*flgS*	sigma54-dependent regulator	1.16E-07
Flagellar modification				
CJJ81176_1310	Cj1293	*pseB*	Polysaccharide biosynthesis protein	8.86E-05
CJJ81176_1333	Cj1316c	*pseA*	Flagellin modification protein	0.008
Chemotaxis				
CJJ81176_0309	Cj0283c	*cheW*	Purine-binding chemotaxis protein	1.54E-06
CJJ81176_0931	Cj0924c	*cheB*	Protein-glutamate methylesterase	7.29E-04
Genes that were identified in previous studies[b]				
CJJ81176_0295	Cj0268c		SPFH domain-containing protein	0.001
CJJ81176_0479	Cj0454c		Hypothetical protein	0.001
CJJ81176_0481	Cj0456c		Hypothetical protein	4.47E-09
CJJ81176_0996	Cj0977		Hypothetical protein	6.01E-12
Other unknown function				
CJJ81176_0100	Cj0062c		Hypothetical protein	0.004
CJJ81176_0198	Cj0162c		Hypothetical protein	0.003
CJJ81176_0199	Cj0163c		Hypothetical protein	2.27E-05
CJJ81176_0240	Cj0208		DNA methyltransferase	0.003
CJJ81176_0413	Cj0390		TPR domain-containing protein	7.38E-10
CJJ81176_0891	Cj0883c		RrF2 family protein, putative	1.62E-06
CJJ81176_1488	Cj1496c		Hypothetical protein	1.38E-08
CJJ81176_1489	Cj1497c		Hypothetical protein	2.28E-12

[a] These genes have q-value <0.01.

[b] These genes were also identified from previous study (49).

other than motility. Alternatively, its motility defect may not be effectively captured by the motility agar plate assay. In fact, we have previously observed that certain *C. jejuni* mutants showing nearly wild-type motility in the agar plate assay can exhibit a motility phenotype in liquid which can result in severe cultured mammalian cell invasion defects (49). Introduction of a wild-type copy of these genes elsewhere in the chromosome restored the invasion and motility defects to wild-type levels in all but two of the mutants and partially in ΔCJJ81176_0100 (see below). The ΔCJJ81176_0891 mutant could not be complemented, indicating that its phenotype may be due to polar effects of the mutation on the downstream motility gene *flhA*. In fact, introduction of a wild-type copy of *flhA* into the ΔCJJ81176_0891 strain restored its motility (Fig. 3), indicating that the motility defect was due to a polar effect on this downstream motility gene and hence was not analyzed further.

Loss of motility could result from defects in flagellar assembly or defects in the motor that propels flagellar movement. To distinguish between these possibilities, we examined the different *C. jejuni* nonmotile mutants by transmission electron microscopy (TEM) (Fig. 4). The *C. jejuni* ΔCJJ81176_1489 mutants showed a complete absence of flagella on the bacterial surface, indicating that this gene is essential for flagellar assembly. This is consistent with its low but significant amino acid sequence similarity to FliJ, which in other systems has been shown to be essential for flagellar assembly (50). Therefore, we propose that CJJ81176_1489 is a true

TABLE 2 Genes encoding flagellar proteins specific to epsilonproteobacteria identified in this study[a]

Epsilonproteobacterium	ID of gene corresponding to *C. jejuni* gene:			
	CJJ81176_0100	CJJ81176_0198	CJJ81176_0199	CJJ81176_0413
H. pylori	–[b]	HP1358	HP1359	HP1479
Arcobacter butzleri	NA[c]	NA	NA	ABED_1764
Sulfurospirillum deleyianum	Sdel_0295	Sdel_0156	Sdel_0155	Sdel_1757
Sulfuricurvum kujiense	Sulku_0477	Sulku_0283	Sulku_0282	Sulku_1890
Sulfurimonas denitrificans	Suden_0705	Suden_1809	Suden_1810	Suden_1668
Wolinella succinogenes	WS1640	WS0360	NA	WS0873
Caminibacter mediatlanticus	CMTB2_07016	CMTB2_06076	CMTB2_06071	CMTB2_08760
Nautilia profundicola	NAMH_1348	NAMH_1408	NAMH_1409	NAMH_0076

[a] The proteins encoded by these genes are found only in epsilonproteobacteria, with no homologs present in any other bacteria. The analysis was done by PSI-BLAST. ID, identifier.
[b] –, CJJ81176_0100 is not found in *H. pylori* 26695, but it is present in other *H. pylori* strains.
[c] NA, not found in this species.

homolog of FliJ. The ΔCJJ81176_0198 mutant exhibited abnormal flagellation, showing either a lack of flagella or a single flagellum at only one of the bacterial poles (Fig. 4; see also Fig. S2 in the supplemental material), indicating that this gene is required for correct flagellar assembly. In contrast, the ΔCJJ81176_0100, ΔCJJ81176_0199, ΔCJJ81176_0413, and ΔCJJ81176_1488 mutants exhibited apparently normal flagella at their poles, suggesting that these mutations may affect motor function and not flagellar assembly (Fig. 4). In summary, the functional analysis of the candidate genes indicated that at least a subset of them encode potential novel components of the flagellar system.

Subcellular localization of putative flagellar proteins. Because of the complexity of the flagellar system and the energy burden associated with motility, many mutations affecting unrelated physiological process can indirectly affect motility (58, 59). To further ascertain a potential direct role of a subset of the identified motility gene products in flagellar biogenesis or function, we investigated their subcellular localization by fluorescence microscopy. Given the polar localization of flagella in *C. jejuni*, we reasoned that if the identified proteins play a direct role in flagellar biogenesis and/or its function, they should localized at the cellular poles. We chose to examine CJJ81176_0100, CJJ81176_0413, and

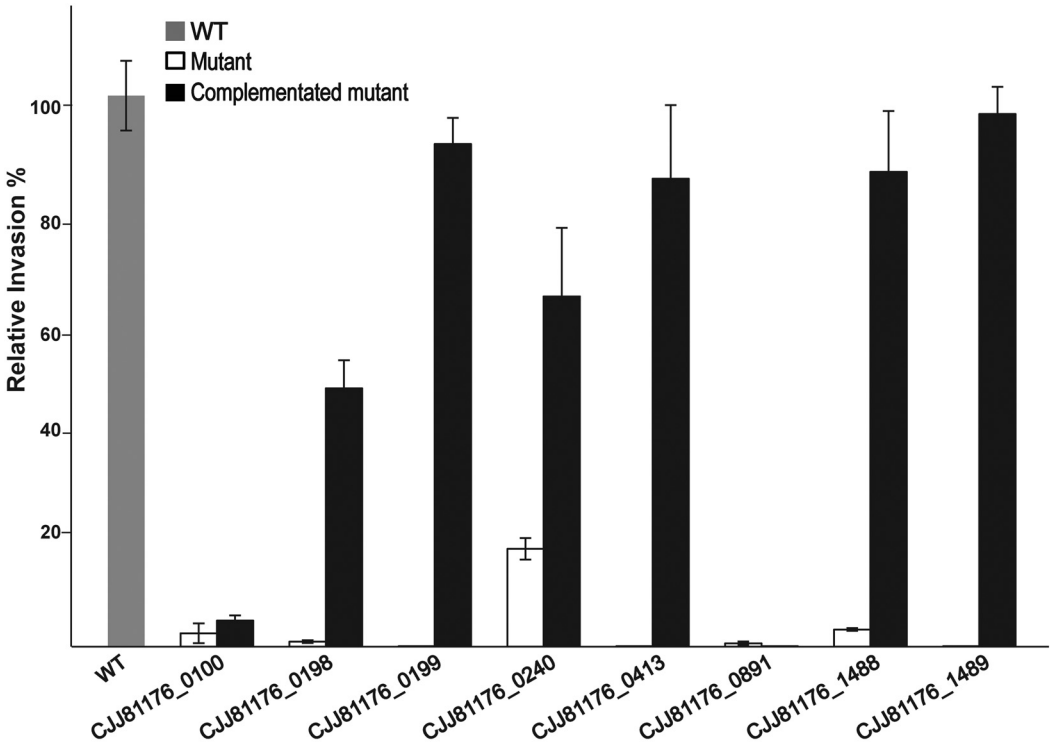

FIG 2 Ability of the *C. jejuni* 81-176 mutants to invade cultured mammalian cells. Cultured mammalian cells were infected with the *C. jejuni* 81-176 wild type (WT), the indicated mutants, or the complemented mutant strains at an MOI of 100 for 2 h, followed by 2 h of incubation in the presence of gentamicin. Levels of invasion are shown as the percentages of bacteria that survived treatment with gentamicin relative to the WT, whose level was set at 100%. Values are means ± standard errors of results of 3 independent determinations. The difference between the value for the knockout mutants and that for the WT or the complemented mutant was statistically significant ($P < 0.001$).

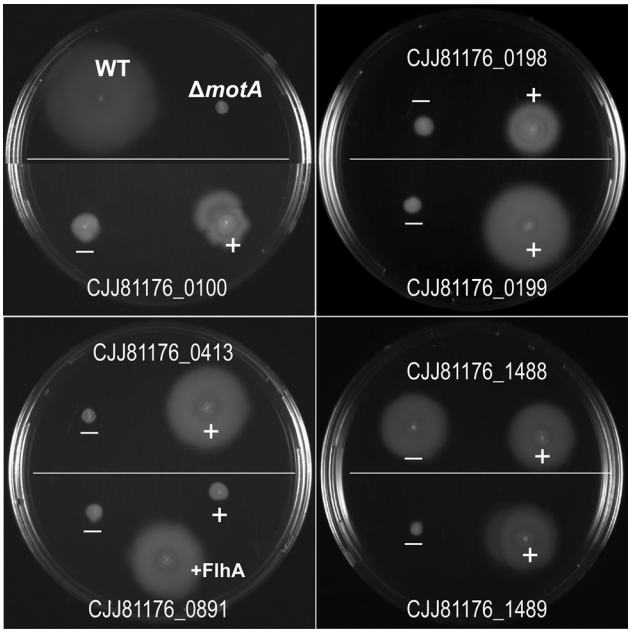

FIG 3 Motility analysis of the invasion-defective *C. jejuni* mutants and complemented strains on soft agar. WT, wild-type *C. jejuni*; −, deletion mutant; +, complemented mutant of the specified gene; +FlhA, complementation of the indicated mutant with the *flhA* gene.

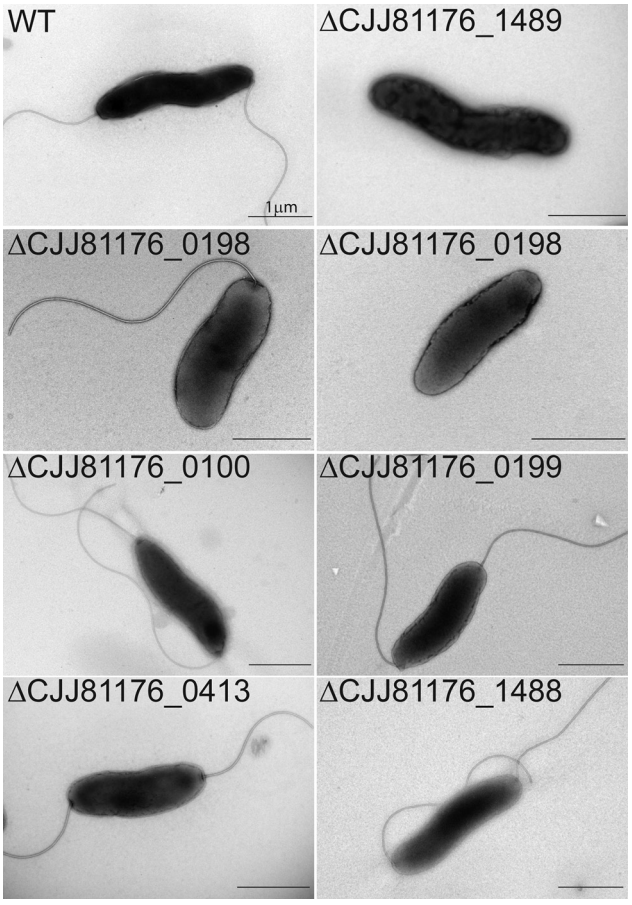

FIG 4 Transmission electron microscopy analysis of the nonmotile mutant strains of *C. jejuni*. WT, wild-type *C. jejuni*.

CJJ81176_1488 because the deletion mutants of the genes encoding these proteins exhibited apparently wild-type flagella, did not exhibit amino acid sequence similarity to other flagellar proteins, and/or are not encoded within known flagellar gene loci. We therefore reasoned that additional evidence was necessary to directly implicate these proteins in motility. We constructed *C. jejuni* strains expressing functional (see Fig. S3 in the supplemental material) green fluorescent protein (GFP)-tagged versions of a subset of the putative flagellar proteins and introduced them into the chromosome at their respective loci by homologous recombination to ensure the native level of expression. We found that the CJJ81176_0100, CJJ81176_0413, and CJJ81176_1488 GFP fusion proteins localized to the two *C. jejuni* poles (Fig. 5). This observation further supports the idea of a potential direct role of these proteins in flagellar structure, assembly, and/or function.

Interaction of putative flagellar proteins with known flagellar components. To provide additional evidence for a potential direct role in the motility of the proteins identified in our screen, we searched for interacting proteins with the goal of identifying interactions with known flagellar components. We reasoned that identifying interactions with known flagellar proteins would further support the idea of a direct role in flagellar function and/or assembly. We generated functional, FLAG-tagged fusion constructs of the identified putative flagellar proteins and introduced them into the *C. jejuni* chromosome by allelic exchange. We then identified interacting proteins by coimmunoprecipitation (co-IP) followed by liquid chromatography-mass spectrometry (LC-MS/MS) analysis. Protein interactions were further confirmed by constructing *C. jejuni* strains encoding differentially tagged versions of the interacting proteins and subsequently examining the strains by immunoprecipitation and Western blot analysis (Table 3 and Fig. 6).

We found that CJJ81176_0100 interacts with FliF (Table 3 and Fig. 6), which is a central component in the flagellar membrane and supramembrane ring located in the inner membrane (60). Although our results cannot address whether the interaction of CJJ81176_0100 and FliF is direct or indirect, it is noteworthy that CJJ81176_0100 has two putative transmembrane domains predicting its location at the inner membrane, where it could potentially interact directly with FliF. The idea of the potential physical interaction of CJJ81176_0100 with the flagellar apparatus is also supported by the observation that, although less consistently, interactions with other flagellar structural components such as FlhA, FlgC, and FlgB were also detected, suggesting that, perhaps through FliF, CJJ81176_0100 may interact with the flagellar apparatus. It is not clear how CJJ81176_0100 contributes to motility, but it is intriguing that our analysis of CJJ81176_0100-interacting proteins also identified CJJ81176_0996, a sigma28 (FliA)-regulated protein (61, 62). In fact, coimmunoprecipitation experiments using FLAG-tagged CJJ81176_0996 as an affinity probe detected CJJ81176_0100 and FliF as interacting proteins, further supporting the idea of the interaction among these flagellar proteins (Table 3 and Fig. 7). It has been reported that a mutation in CJJ81176_0996 results in a drastic defect in *C. jejuni* entry into cultured mammalian cells (61) (confirmed in our screen; see Table 1), which was later shown to be due to a motility defect manifested in liquid cultures but not on motility plates (49). These

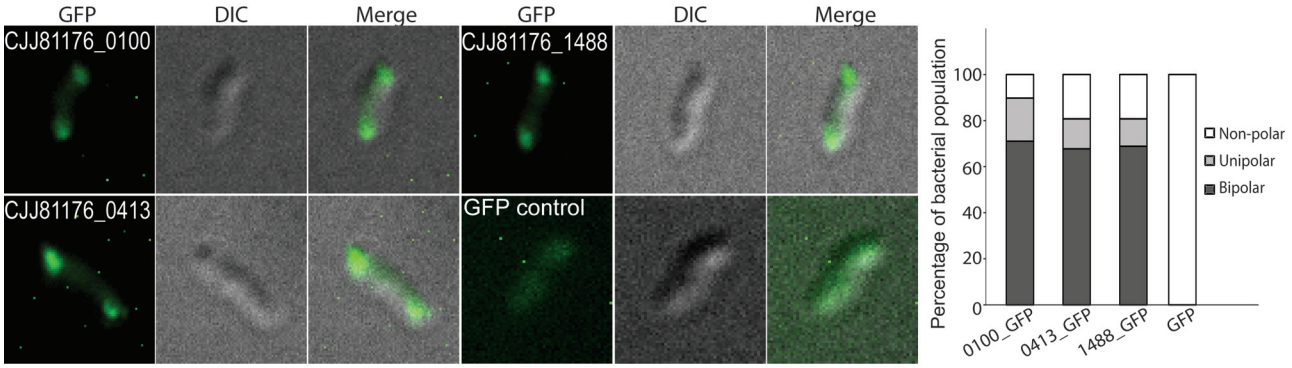

FIG 5 Subcellular localization of *C. jejuni* proteins involved in motility. *C. jejuni* strains expressing the indicated sfGFP-tagged proteins were examined by fluorescence microscopy. The quantification of the proportion of bacteria exhibiting GFP localization at one or both poles or showing no polar fluorescence is shown. CJJ81176_0100_GFP $n = 101$; CJJ81176_0413_GFP $n = 285$; CJJ81176_1488_GFP $n = 327$; sfGFP $n = 87$. sfGFP, superfolder green fluorescent protein; DIC, differential interference contrast.

observations provide support for the idea of a direct role in motility for both CJJ81176_0100 and CJJ81176_0996.

Our analysis showed that CJJ81176_0198 interacts with FliO (Table 3 and Fig. 6), a component of the flagellar type III secretion export apparatus (63), thus supporting the idea of a direct role for CJJ81176_0198 in motility. This finding is consistent with the observation that the *C. jejuni* ΔCJJ81176_0198 mutant exhibited aberrant or defective flagellar assembly (see Fig. 4).

CJJ81176_0199 showed interactions with MotA and MotB (Table 3 and Fig. 6), two components of the flagellar motor (64). Consistent with this interaction, the *C. jejuni* ΔCJJ81176_0199 mutant exhibited apparently normal flagella although it is nonmotile. Therefore, CJJ81176_0199 may exert its effect by modulating motor assembly or function.

We found that CJJ81176_0413 interacts with PflA, a protein exclusively encoded by *Epsilonproteobacteria* (65) and previously shown to be required for motility (46). Interestingly, disruption of *pflA* results in apparently normal but paralyzed flagella (46), the same phenotype observed after the disruption of CJJ81176_0413. Electron microscopy examination of the *C. jejuni* ΔCJJ81176_0413 mutant showed apparently normal flagella at both poles (Fig. 4) although the mutant was completely defective in motility (Fig. 3). These observations suggest a role for these proteins in the function and/or assembly of the flagellar motor. Despite a lack of detectable primary amino acid sequence similarity between CJJ81176_0413 and PflA, structural homology searches indicated that these proteins share structural similarities to the same O-linked N-acetylglucosaminyltransferase (PDB 1W3B [66]), suggesting that these two proteins are structurally similar. *C. jejuni* flagellin and other flagellar components are known to be glycosylated, a modification that is required for flagellar assembly (31, 67–69). It is possible that CJJ81176_0413 as well as PflA may be involved in this process. Intriguingly, both these proteins have tetratricopeptide (TPR) repeats, which previous studies have implicated in conferring substrate specificity to eukaryotic N-acetylglucosaminyltransferases (70). The presence of these repeats in CJJ81176_0413 as well as PflA is consistent with a potential role in protein glycosylation. Interestingly, CJJ81176_0413 also interacted with KdpD and CJJ81176_1442, the latter of which belong to a cluster of genes implicated in the biosynthesis of the capsular polysaccharide of *C. jejuni* (71), suggesting that its activity may not be exclusively associated with flagellar biosynthesis.

Lastly, we detected interactions of CJJ81176_1488 with FlgM and FliK (Table 3 and Fig. 6), two conserved essential components of the flagellar system. FlgM is an anti-sigma factor that controls expression of class 3 flagellar genes by directly binding the flagellum-specific transcription factor sigma28 (FliA), thus preventing the expression of genes controlled by this regulator (72, 73). FliK, on the other hand, is involved in the regulation of substrate switching during flagellar assembly (74). Although these flagellar proteins exhibit very different functions, they are both secreted through the flagellar type III secretion system. In this context, it is noteworthy that CJJ81176_1488 showed polar localization. Since FlgM would likely be cytoplasmic when interacting with sigma factors, it is likely that CJJ81176_1488 interacts with FlgM only when it is being secreted and not when it is undertaking its regulatory functions. We therefore hypothesize that CJJ81176_1488 may assist in some aspect of flagellar type III secretion. Consistent with this hypothesis, upstream of CJJ81176_1488 is CJJ81176_1489, which we propose is the homolog of FliJ, a cytoplasmic component of the flagellar type III export apparatus involved in flagellar protein export. Although the ΔCJJ81176_1488 mutant retains some motility, its subcellular localization, genomic organization, and interactome support the idea of a direct role for this protein in flagellar biology.

DISCUSSION

Flagella are widely distributed organelles among bacterial species. Although the basic architecture and core components of this organelle are highly conserved, it is becoming increasingly clear that there are significant differences among flagellar structures from different bacterial species. Among the more structurally diverse flagella are those of *Epsilonproteobacteria* such as *C. jejuni* and *H. pylori*. Indeed, previous cryoelectron tomography studies have visualized specific protein densities in the cryotomograms of *C. jejuni* and *H. pylori* flagella that must correspond to unique components of this organelle in these bacteria (12). Most of the knowledge on bacterial flagella is derived from studies done in *S.* Typhimurium and *E. coli*. However, since the flagellar structures of these bacteria are among the simplest, the annotation of flagellar genes in bacterial genomes based on homologies to these

TABLE 3 Proteins that interact with the flagellar proteins identified in this study[a]

Gene ID	Symbol	Annotation	Protein mass (Da)	No. of spectral counts[b]		
				R 1	R 2[c]	R 3
CJJ81176_0100		**Hypothetical protein**	**14221**	**17**	**1**	**1**
CJJ81176_0340	FliF	Flagellar membrane and supramembrane-ring protein	62579	153	4	136
CJJ81176_0996		Hypothetical protein	21265	44	8	30
CJJ81176_1732		Hypothetical protein	13124	31		27
CJJ81176_0890	FlhA	Flagellar biosynthesis protein	77558	11		12
CJJ81176_0552	FlgC	Flagellar basal body rod protein	18317	3		6
CJJ81176_0553	FlgB	Flagellar basal body rod protein	16055	4		2
CJJ81176_0433	FrdA	Fumarate reductase subunit	74653	2		2
CJJ81176_0996		**Hypothetical protein**	**21265**	**47**	**62**	**14**
CJJ81176_1732		Hypothetical protein	13124	21	6	8
CJJ81176_0340	FliF	Flagellar membrane and supramembrane-ring protein	62579	1	2	4
CJJ81176_0100		Hypothetical protein	14221	4	4	1
CJJ81176_0757	CjaC	CjaC protein	27838	2	3	2
CJJ81176_1205	CetA	Methyl-accepting chemotaxis protein	51156		3	5
CJJ81176_0198		**Hypothetical protein**	**20021**	**55**	**10**	**46**
CJJ81176_0376	FliO	Flagellar export apparatus protein	31089	46	6	51
CJJ81176_0289		Methyl-accepting chemotaxis protein	72901	29		22
CJJ81176_0635	MacA	Macrolide-specific efflux protein	42778	28		20
CJJ81176_0180		Methyl-accepting chemotaxis protein	72546	18		10
CJJ81176_1205	CetA	Methyl-accepting chemotaxis protein	51156	14	1	11
CJJ81176_0473		Methyl-accepting chemotaxis protein	40562	9		4
CJJ81176_0971		Carbon-nitrogen family hydrolase	34107	5		6
CJJ81176_1272		Hypothetical protein	25530	4		6
CJJ81176_0412	SerS	Seryl-tRNA synthetase	46900	2	1	2
CJJ81176_0199		**Hypothetical protein**	**7859**	**22**	**12**	**51**
CJJ81176_0359	MotA	Flagellar motor protein	28320	15		2
CJJ81176_0358	MotB	Flagellar motor protein	27880	3		1
CJJ81176_1204	CetB	Methyl-accepting chemotaxis protein	19358	5	4	
CJJ81176_0507	RplJ	50S ribosomal protein L10	17761	3	4	
CJJ81176_0920	CysK	Cysteine synthase A	27208	1	2	2
CJJ81176_1452		Hypothetical protein	42424	3		2
CJJ81176_0413		**TPR domain-containing protein**	**93554**	**609**	**367**	**444**
CJJ81176_1550	PflA	Paralyzed flagellar protein	91269	133	57	241
CJJ81176_1442		Hypothetical protein	15629	33	8	7
CJJ81176_0702	KdpD	Truncated KdpD protein	73235	3	2	1
CJJ81176_0920	CysK	Cysteine synthase A	27208	2	2	
CJJ81176_1488		**Hypothetical protein**	**19620**	**91**	**30**	**210**
CJJ81176_1457	FlgM	Anti-sigma factor protein	7139	2	1	20
CJJ81176_0079	FliK	Flagellar substrate switch protein	69078	3	2	15
CJJ81176_1205	CetA	Methyl-accepting chemotaxis protein	51156	2	3	210

[a] The bait proteins are highlighted in boldface characters. Only detected protein with spectral counts > 1 in at least 2 replicates after filtering the proteins that were also detected in control sample are presented here. Data represent the results of three independent experiments.

[b] R, replicate. For replicate 2, analysis of the Co-IP proteins was conducted under more-stringent conditions resulting in lower spectral counts.

[c] The protein encoded by the CJJ81176_0100 gene is a membrane protein which is more difficult to detect by MS/MS, therefore resulting in lower spectral counts.

model organisms is likely to miss essential flagellar components in other bacterial species with more-complex flagella. We have described here several novel *C. jejuni* flagellar genes identified through a comprehensive high-throughput genetic screening. Although some mutations could indirectly lead to lack of motility, through a variety of functional, biochemical, and *in vivo* imaging analyses we have provided strong evidence that the genes we have identified encode factors directly involved in the assembly and/or function of the flagellar apparatus. Consequently, we have assigned to these different proteins a nomenclature consistent with their newly identified role in motility (Fig. 8; see also Table S3 in the supplemental material). Thus, we have renamed CJJ881176_100 FlgV, CJJ881176_0198 FlgW, CJJ881176_0199 FlgX, CJJ881176_1448 FlgY, CJJ881176_0413 PflB, and CJJ881176_1489 FliJ.

Our analysis identified 6 new components of the flagellar system which, on the basis of the phenotypes of the mutants and their interactome, we postulate play different roles in motility or flagellar assembly. Of these proteins, only CJJ81176_0100 is located within a known flagellar gene cluster, which contains *flhF*, *flhG*, *fliA*, *fliY*, and *fliM*. Therefore, it is likely that CJJ81176_0100 is coregulated with these flagellar genes. However, none of the other

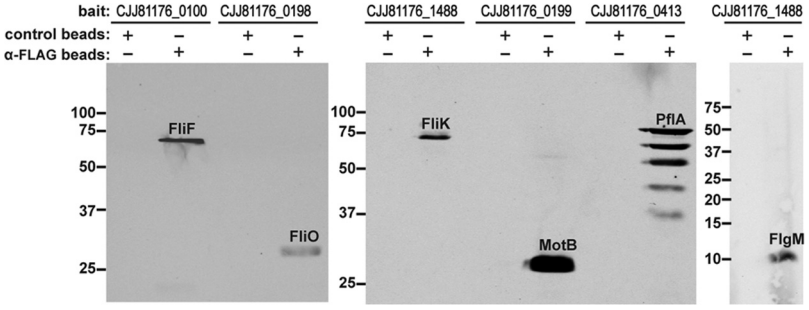

FIG 6 Confirmation of protein interactions by Western immunoblot analysis. *C. jejuni* strains carrying M45-epitope-tagged or FLAG-epitope-tagged versions of the interacting proteins (as indicated) were subjected to immunoprecipitation with anti-FLAG affinity gels (or control beads), and the immunoprecipitated materials were analyzed by Western immunoblotting using an anti-M45 antibody.

identified genes are located within known flagellar gene clusters and there are no previous reports indicating that they are coregulated by a known flagellar regulator such as RpoN, FliA, or FlgRS. One of the identified proteins, CJJ81176_1489, was shown to be essential for flagellar assembly since its mutation resulted in no flagella on the bacterial surface. This is consistent with the observation that CJJ81176_1489 exhibits low but significant amino acid sequence similarity to FliJ, a poorly conserved flagellar protein shown to be essential for flagellar assembly in *S.* Typhimurium. Another protein identified in this study, CJJ81176_0198, was also shown to be required for proper flagellar assembly, and a *C. jejuni* strain carrying a mutation in this gene exhibited either no flagella or a flagellum at only one of the poles instead of at the two poles as seen in the wild type. Consistent with a role in flagellar assembly, CJJ81176_0198 was shown to interact with FliO, a component of the flagellar export apparatus.

The rest of the identified proteins (CJJ81176_0100, CJJ81176_0199, CJJ81176_0413, and CJJ81176_1488) are not essential for flagellar assembly since wild-type flagella were identified on the surface of the respective mutant strains. Consequently, we postulate that they play a role in motor function. Consistent with this role, CJJ81176_0199 was shown to interact with the motor proteins MotA and MotB. Since no homolog of this protein exists other than in *Epsilonproteobacteria*, it is likely that this protein performs a function unique to the flagella of this group of bacteria. Cryo-electron tomography studies have shown unique protein densities in the flagellar structure of *Epsilonproteobacteria* which have been proposed to be associated in part with unique structural features of its motor. It is possible that CJJ81176_0199 and the other flagellar proteins unique to epsilon proteobacteria identified in this study may account for some of those unique protein densities. It is not clear how the other identified proteins may exert their function, but imaging analysis located them at the bacterial poles. It is intriguing that CJJ81176_1488 interacts with FlgM and FliK, two proteins that are exported by the flagellar apparatus. It is therefore possible that CJJ81176_1488 has a role in coordinating protein secretion. CJJ81176_0100, on the other hand, was shown to interact with FliF, a central component of the flagellar basal body. How CJJ81176_0100 exerts its function is not

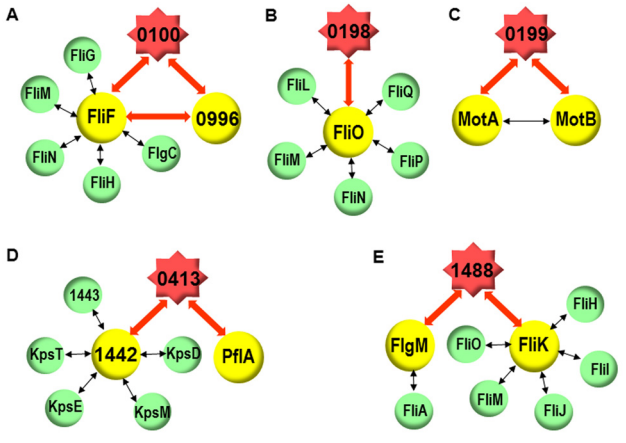

FIG 7 Interaction map of the *C. jejuni* motility proteins. The red lines with arrowheads indicate interactions confirmed by coimmunoprecipitation. Other interactions are adopted from the STRING database 9.0 (http://string-db.org/) using the highest confidence (0.9) parameters. The analyzed *C. jejuni* proteins are indicated in red.

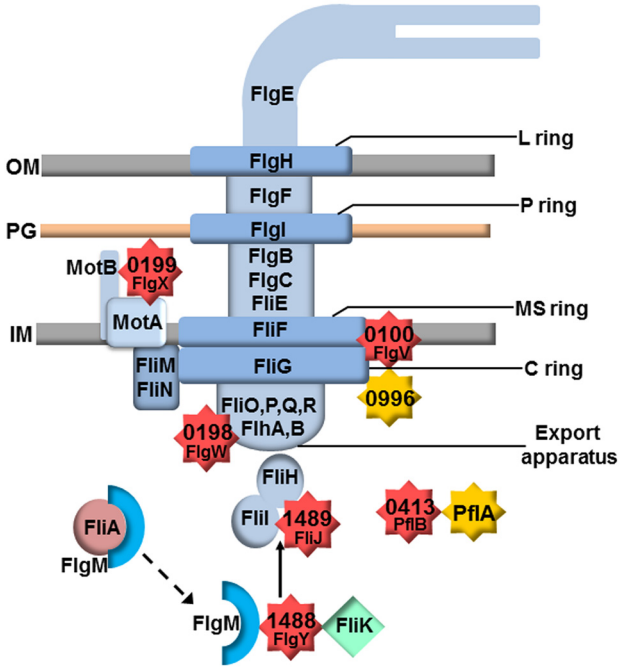

FIG 8 Schematic of the flagellar structure depicting the identified *C. jejuni* flagellar proteins and their interactions. OM, outer membrane; IM, inner membrane; PG, peptidoglycan.

clear, although its location and interactome and the phenotype of its mutation suggest a role in motility but not in flagellar assembly. In addition, our interactome studies indicate that CJJ81176_0996 may play a direct role in motility by interacting with CJJ81176_0100 and FliF.

One of the unique features of the *Epsilonproteobacteria* flagella is that some of the components are posttranslationally modified by specific glycosyltransferases. Although the role of glycosylation in flagellar function is not understood, it is clear that, at least in the case of flagellin, protein glycosylation is necessary for flagellar assembly. In this context, it is intriguing that one of the identified proteins in our study, CJJ81176_0413, exhibits amino acid sequence similarity to glycosyl transferases. Furthermore, our interactome study showed that this protein interacts with PflA, a previously identified protein required for motility. In fact, structure-based homology searches indicate that, despite their low primary amino acid sequence identity, CJJ81176_0413 and PflA share obvious structural similarities. Therefore, it is possible that both these proteins exert a related function and that this function may involve the posttranslational modification of some flagellar component. Given its interactome and structural similarity, we have renamed CJJ81176_0413 PflB.

Through the most comprehensive genetic screen yet to be performed in *C. jejuni* coupled to functional, biochemical, and imaging analysis, we have identified novel flagellar components that are widely distributed among but restricted to *Epsilonproteobacteria*. The identification of these components provides the basis to understand the specific adaptation of this remarkable bacterial organelle to function in the context of a widely diverse set of bacterial species and brings unique insight into its evolution and function.

MATERIALS AND METHODS

Bacterial strains, cell lines, and culture conditions. The complete list of strains and plasmids used in this study is shown in Table S4 in the supplemental material. The *C. jejuni* 81-176 wild-type strain was grown on brucella broth agar or on blood agar plates (Trypticase soy agar supplemented with 5% sheep blood) at 37°C in an incubator equilibrated to a 10% CO_2 atmosphere. The *C. jejuni* transformants were selected on plates supplemented with 50 μg/ml kanamycin and/or 7.5 μg/ml chloramphenicol, as indicated below. For liquid cultures, *C. jejuni* strains were grown in brain heart infusion (BHI) medium with no antibiotics added. All *C. jejuni* strains were stored at −80°C in BHI broth containing 30% glycerol.

Cos-1 (African green monkey kidney fibroblast-like cell line) cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). All cell lines were kept under a 5% CO_2 atmosphere.

Construction of *C. jejuni* transposon mutant library. The transposon used in this study is a derivative of the *Himar1* Mariner transposon with a single nucleotide change to result in an MmeI site in each inverted repeat (37). To minimize potential transcriptional polar effects of the insertions, two genes encoding kanamycin (from pILL600) and chloramphenicol (from pRY109) resistance lacking transcription terminators were cloned within the transposable element with their promoters facing outward to promote the transcription of downstream genes and minimize the possibility of polar effects (Fig. 1A). The transposon was constructed by recombinant PCR amplification of both antibiotic resistance genes using primers listed in Table S5 in the supplemental material and was introduced into pBluescript II SK plasmid, resulting in the final plasmid, pSB4118.

An *in vitro* transposon mutagenesis system was used to generate insertion mutants of *C. jejuni* as previously described (40). Briefly, the *in vitro* transposition reactions were carried out with purified MarC9 transposase, *C. jejuni* DNA, and transposon plasmid pSB4118 with reaction buffers described previously (38, 75). Reaction mixtures were incubated for 4 h at 30°C and inactivated for 20 min at 72°C, and the transposition products were transformed into naturally competent *C. jejuni* and transformants plated on brucella agar plates containing both kanamycin and chloramphenicol. After 48 h of incubation, the transformants were collected from plates and pooled into cryo tubes with approximately 5,000 transformants per tube. A total of ~50,000 transformants were collected.

Screening of *C. jejuni* transposon mutants for their ability to enter cultured cells. The screening of the *C. jejuni* transposon insertion library for mutants unable to enter cultured Cos-1 cells was carried out as previously described (49). To determine the size of the potential "bottleneck" of the assay, bacterial infections were carried out with different ratios of two differently marked strains of *C. jejuni* that have equal cultured-cell invasion capacities. This test showed that for a 10-cm-diameter dish infected with a multiplicity of infection (MOI) of 100, even a ratio of 1:16,000 allowed the detection of both strains without any measurable stochastic loss. Based on these results, a mutant pool of 5,000 mutants was used to infect each 10-cm-diameter dish infection, which ensured that no mutant would be stochastically lost during the infection assay. Ten mutant pools of 5,000 were screened as one biological replicate totaling ~50,000 mutants. A total of three independent biological replicates, each with ~50,000 mutants, were carried out to ensure the robust coverage of the library.

INseq DNA sample preparation and data analysis. The INseq DNA sample preparation and amplification were carried out as previously described (37, 48). The resulting 125-bp products from the mutant pools were sequenced on an Illumina HiSeq2000 system at the Yale Center for Genomic Analysis. The sequencing data were analyzed using the INSeq_pipeline_v2 package (48). The processed data are provided in Table S1 in the supplemental material. Essential genes were identified using the R package *Negenes* (76) as previously described (37) with two modifications: insertions represented by (i) fewer than 3 counts per million reads or (ii) insertions in the distal 20% of each gene were excluded from the analysis.

***C. jejuni* mutant strain construction.** *C. jejuni* 81-176 knockout mutant strains were constructed by PCR amplification of the flanking regions of these open reading frames (ORFs) with specific primers (see Table S5 in the supplemental material) and cloning of a kanamycin resistance cassette (*aphA3*) between the amplified flanking regions. The resulting plasmids (built on a pBluescript II SK backbone) were used to move the mutated alleles into the chromosome of *C. jejuni* 81-176 by natural transformation and allelic recombination. Complementation of the mutant strains of *C. jejuni* was achieved by introducing a wild-type copy of the gene at the *hsdM* locus as previously described (77). Briefly, the gene with its original Shine-Dalgarno sequence was cloned into pSB3313 to generate a 3× FLAG fusion protein, which is expressed from a *cat* promoter upstream, at the C terminus. The resulting 3× FLAG fusion protein was moved into pSB3021, which was then integrated into the *hsdM* locus of the chromosome where its expression is driven by a chloramphenicol resistance gene promoter (77). To confirm the protein interactions identified by LC-MS/MS, *C. jejuni* strains were constructed in which the interacting proteins were tagged with different epitopes. Briefly, the genes encoding the identified interacting proteins (*fliF*, *fliO*, *motA*, *motB*, *pflA*, *fliK*, and *flgM*) were cloned into pSB4868 to generate M45-tagged versions of these proteins. M45-tagged genes were then cloned into pSB3021-derived plasmids carrying FLAG-tagged versions of the genes encoding the corresponding interacting motility proteins identified in the mutant screen. The resulting plasmids were then integrated into the *hsdM* locus of the *C. jejuni* chromosome as described above (see Table S4).

Motility plate assay and EM imaging. The optical density at 600 nm (OD_{600}) of the bacterial cultures to be tested was adjusted to 0.4 and spotted onto soft agar (0.8% [wt/vol]). Plates were incubated for 24 h at 37°C, and the swarming diameter of the tested strain was compared to those of the wild-type strain and the nonmotile *C. jejuni* Δ*motA* mutant strain. Bacterial flagella were visualized by negative staining and transmis-

sion electron microscopy (TEM). Briefly, bacterial cells were pelleted (2,000 rpm for 2 min) and resuspended in prewarmed phosphate-buffered saline (PBS). Samples were directly applied to glow-discharged carbon-coated 200-mesh Cu grids and stained using 2% phosphotungstic acid (pH 7.0). Images were acquired using 10,000-fold to 35,000-fold magnification on a Tecnai Biotwin TEM (FEI Company) at 80 kV. Images were collected using a Morada Soft Imaging system and a 6-M-pixel charge-coupled-device (CCD) camera (Olympus).

Fluorescence microscopy. Fusions of selected *C. jejuni* proteins to the amino terminus of superfolder GFP (sfGFP) were introduced at the native loci by homologous recombination using standard recombinant DNA techniques and natural transformation. A strain expressing wild-type sfGFP was constructed by inserting the sfGFP gene into the *hsdM* locus as previously described (77). The resulting strain expresses sfGFP from a chloramphenicol resistance gene promoter. All *C. jejuni* strains were grown in BHI liquid medium to an OD_{600} of 0.4 and washed with PBS once, and 2 μl of the culture volume was loaded into a 1.5% low-melting agarose patch to trap the fast-moving bacteria for fluorescence observation on a Nikon TGE2000-U Eclipse inverted microscope fitted with a Micromax Princeton digital camera.

Immunoprecipitation of interacting proteins and LC-MS/MS analysis. *C. jejuni* strains expressing a 3× FLAG-tagged version of the different proteins were grown on blood agar, resuspended in PBS, pelleted at 6,000 rpm, and then resuspended in 2 ml of Tris-buffered saline (TBS), 1% Triton X-100, and 0.5 mM $MgSO_4$ containing protease inhibitors and 10 μg/ml DNAse. After lysis by sonication, cell debris were removed by centrifugation at 14,000 rpm, supernatants were recovered, and immunoprecipitation (IP) of 3× FLAG-bait protein was performed using anti-FLAG M2 affinity gel following the manufacturer recommendations. Bound proteins were eluted twice by acid elution with 40 μl 0.1 M glycine HCl (pH 3.5). All the elution products were pooled and loaded onto a 10% SDS-PAGE gel for LC-MS/MS. The identification of IP products by LC-MS/MS was carried out as previously described (78).

Confirmation of protein interaction by Western blot analysis. *C. jejuni* strains encoding FLAG- and M45-tagged versions of the interacting proteins were grown and lysed as indicated above. Immunoprecipitations with anti-FLAG antibodies were carried out also as indicated above, and coimmunoprecipitated proteins were analyzed by Western immunoblotting with an anti-M45 epitope monoclonal antibody. As negative controls, samples were treated with the same gel beads with nickel-nitrilotriacetic acid (Ni-NTA) instead of anti-FLAG antibodies.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01349-14/-/DCSupplemental.

Figure S1, TIF file, 9.7 MB.
Figure S2, TIF file, 8.9 MB.
Figure S3, TIF file, 9.2 MB.
Table S1, DOCX file, 1.1 MB.
Table S2, DOCX file, 0.2 MB.
Table S3, DOCX file, 0.1 MB.
Table S4, DOCX file, 0.1 MB.
Table S5, DOCX file, 0.1 MB.

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