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Analysis of the Roles of FlgP and FlgQ in Flagellar Motility of *Campylobacter jejuni*[▽]

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Flagellar motility is an important determinant of *Campylobacter jejuni* that is required for promoting interactions with various hosts to promote gastroenteritis in humans or commensal colonization of many animals. In a previous study, we identified a nonmotile mutant of *C. jejuni* 81-176 with a transposon insertion in Cj1026c, but verification of the role of the encoded protein in motility was not determined. In this study, we have determined that Cj1026c and the gene immediately downstream, Cj1025c (here annotated as *flgP* and *flgQ*, respectively), are both required for motility of *C. jejuni* but not for flagellar biosynthesis. FlgP and FlgQ are not components of the transcriptional regulatory cascades to activate σ^{28}- or σ^{54}-dependent expression of flagellar genes. In addition, expression of *flgP* and *flgQ* is not largely dependent on σ^{28} or σ^{54}. Immunblot analyses revealed that the majority of FlgP in *C. jejuni* is associated with the outer membrane. However, in the absence of FlgQ, the amounts of FlgP in the outer membrane of *C. jejuni* are greatly reduced, suggesting that FlgQ may be required for localization or stability of FlgP at this location. This study provides insight into features of FlgP and FlgQ, two proteins with previously undefined functions that are required for the larger, multicomponent flagellar system of *C. jejuni* that is necessary for motility.

Campylobacter jejuni is the second leading cause of bacterial gastroenteritis in humans in the United States and a leading cause of diarrheal disease throughout the world (7). In contrast, *C. jejuni* is able to promote a natural, commensal colonization of the gastrointestinal tracts of many domesticated and agriculturally important animals, including poultry (18, 23). These commensal relationships, while harmless to the animal hosts, create large reservoirs of the bacterium that can result in contamination of the human food supply, leading to a high incidence of *C. jejuni* gastroenteritis (9).

The production of flagella and flagellar motility are important determinants of *C. jejuni* for promoting interactions with various hosts. Nonmotile flagellar mutants of *C. jejuni* colonize chicks at reduced levels in a 1-day-old chick model of commensalism (14, 20, 26, 28). In addition, flagellar motility is required for interactions with the human intestinal tract to promote infection which can culminate in a productive gastroenteritis (3).

C. jejuni produces a single flagellum at one or both poles of the bacterium. To produce functional flagella, bacteria must coordinate both the temporal expression of over 40 flagellar genes and the ordered production and secretion of the encoded proteins. *C. jejuni* uses two alternative σ factors, σ^{28} and σ^{54}, to mediate transcriptional regulation of specific flagellar genes (6, 13, 15, 16, 28). σ^{28} is involved in the transcription of a small subset of genes, including the major flagellin gene, *flaA*. σ^{54} is required for transcription of many genes encoding the flagellar rod, basal body, and hook components and a minor flagellin. Previous studies have determined that activation of transcription of σ^{54}-dependent flagellar genes is dependent on the FlgSR two-component system and the proteins of the flagellar export apparatus, including FlhA, FlhB, FliP, and FliR (15). We have proposed that these two systems may constitute a regulatory cascade where formation or the secretory activity of the flagellar export apparatus may activate the FlgS sensor kinase. Through a phosphorelay system, FlgS would be predicted to then phosphorylate the FlgR σ^{54}-dependent response regulator (28), activating it for transcription of σ^{54}-dependent flagellar genes.

In a previous screen to identify genes of *C. jejuni* strain 81-176 that are required for flagellar motility, we used the *mariner*-based *solo* transposon to randomly mutagenize the bacterium and then isolated 28 mutants defective for motility (13). One nonmotile mutant was found to have a *solo* insertion in Cj1026c/Cjj81176_1045 (gene designations are based on the previously annotated *C. jejuni* 11168 genome [22] and the more recently sequenced *C. jejuni* 81-176 genome [GenBank accession number AANY01000004], respectively) but this mutant was not investigated further. Because this gene is located two genes upstream of *flgR*, which is required for motility and transcription of σ^{54}-dependent flagellar genes, the possibility existed that the *solo* insertion in Cj1026c may have a polar effect on expression of *flgR*.

In this study, we have characterized Cj1026c and the downstream gene Cj1025c/Cjj81176_1044 and have found that both genes are required for motility. (Due to simplicity in communicating our findings, we propose to annotate Cj1026c and Cj1025c as *flgP* and *flgQ*, respectively, and will refer to these genes as such hereafter.) Mutants lacking either of these genes are nonmotile but possess flagella of normal appearance. In addition, the Δ*flgP* and Δ*flgQ* mutants are not defective for transcription of σ^{28}- or σ^{54}-dependent flagellar genes. Localization studies revealed that a large proportion of FlgP in *C. jejuni* is associated with the outer membrane. Furthermore,

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association of FlgP with the outer membrane is dependent on FlgQ, suggesting that FlgQ may be necessary for localization and subsequent stabilization of FlgP to contribute to the larger macromolecular flagellar complex to promote motility.

MATERIALS AND METHODS

Bacterial strains and plasmids. *C. jejuni* 81-176 is a clinical isolate from a patient with gastroenteritis that has since been shown to promote gastroenteritis in humans and commensal colonization of the chick gastrointestinal tract (3, 14, 17). *C. jejuni* was routinely grown on Mueller-Hinton (MH) agar containing 10 μg/ml trimethoprim (TMP) in microaerobic conditions (10% CO_2, 5% O_2, and 85% N_2) at 37°C. Antibiotics for *C. jejuni* growth were added to MH agar when necessary at the following concentrations: kanamycin, 50 μg/ml; chloramphenicol, 15 μg/ml; cefoperazone, 30 μg/ml; or streptomycin, 0.5, 1, 2, or 5 mg/ml. All *C. jejuni* strains were stored at −80°C in a 85% MH broth–15% glycerol solution. *Escherichia coli* DH5α and XL1-Blue were grown in Luria-Bertani (LB) agar or broth. Antibiotics were used in the following concentrations: kanamycin, 100 μg/ml; chloramphenicol, 15 μg/ml; or ampicillin, 100 μg/ml. All *E. coli* strains were stored at −80°C in a 80% LB–20% glycerol solution.

Construction of bacterial strains. PCR was used to amplify a 2.4-kb fragment from the chromosome of *C. jejuni* 81-176 containing the *flgPQ* locus with approximately 700 nucleotides of upstream and downstream DNA sequence. Primers were used with BamHI restriction sites to facilitate cloning into BamHI-digested pUC19 to create pDRH1954.

Defined deletion mutagenesis was used to create in-frame deletions of *flgP* and *flgQ* from the chromosome of *C. jejuni* (13). To generate a construct with insertion of a *cat-rpsL* cassette in *flgP*, pDRH1954 was digested with NheI, which cleaves the DNA within the coding sequence of *flgP*. Blunt-end fragments were generated with T4 DNA polymerase and the DNA was then ligated to SmaI-digested *cat-rpsL* from pDRH265. The resulting plasmid was designated pDRH1955. Because of the lack of a convenient restriction site in *flgQ*, PCR-mediated mutagenesis was used to generate an MscI site in *flgQ* by making A-to-G and T-to-C mutations at nucleotides 188 and 189 of the *flgQ* coding sequence to generate pDRH1981 (19). This DNA was then digested with MscI and ligated to SmaI-digested *cat-rpsL* to create the plasmids pDRH2028 and pDRH2029, which differed by the orientation of the *cat-rpsL* cassette. PCR-mediated deletion mutagenesis was also used to create in-frame deletions of *flgP* or *flgQ* from pDRH1954 (19). For *flgP*, primers were designed and used in a reaction with pDRH1954 to fuse in frame the start codon to the last 23 codons of the gene, deleting the intervening 148 codons to create pDRH1957. Similarly, the first 10 codons of *flgQ* were fused to the stop codon of *flgQ* to delete the intervening 139 codons to create pDRH1958.

Strains DRH212 (81-176 Sm^r) and DRH461 (81-176 Sm^r Δ*astA*) were electorporated with pDRH1955, pDRH2028, or pDRH2029 and then grown on MH agar with chloramphenicol to select for transformants with *cat-rpsL* insertions in *flgP* or *flgQ* (25). The resulting mutants were then electroporated with the appropriate plasmid, pDRH1957 or pDRH1981, and grown on MH agar with streptomycin to select for transformants that replaced the *flgP*::*cat-rpsL* or *flgQ*::*cat-rpsL* mutations with the in-frame deletion constructs on the chromosome of *C. jejuni*. The resulting mutants were designated DRH2064 (81-176 Sm^r Δ*astA* Δ*flgP*), DRH2070 (81-176 Sm^r Δ*flgP*), DRH2071 (81-176 Sm^r Δ*flgQ*), and DRH2118 (81-176 Sm^r Δ*astA* Δ*flgQ*).

For generation of *flgP*::*astA* or *flgQ*::*astA* transcriptional fusions in *C. jejuni*, NheI-digested pDRH1954 that had been filled in by T4 DNA polymerase to create blunt ends and MscI-digested pDRH1981 were ligated to a SmaI-digested *astA-kan* cassette from pDRH580. The resulting plasmids, pDRH2047 containing *flgQ*::*astA-kan* and pDRH2080 containing *flgP*::*astA-kan*, were electroporated into the appropriate Δ*astA* deletion strains to replace native *flgP* or *flgQ* with the transcriptional fusions on the chromosome of *C. jejuni* strains. For creation of DRH2316 (81-176 Sm^r Δ*astA* Δ*flgQ flgP*::*astA-kan*), pDRH1958 containing the *flgPQ* locus with the Δ*flgQ* deletion was digested with NheI, treated with T4 DNA polymerase, and ligated to the SmaI-digested *astA-kan* cassette to create a transcriptional fusion of *flgP* to *astA*. The resulting plasmid, pDRH2308, was then electroporated into DRH2118 to generate DRH2316.

To create *astA* transcriptional fusions to *flgD* or *motA*, PCR was used with chromosomal DNA from *C. jejuni* 81-176 to amplify a 2.1-kb fragment containing *flgD* and a 2.7-kb fragment containing the *motAB* locus. These DNA fragments were then digested with BamHI to facilitate cloning into pUC19 to generate pDRH325 (containing *flgD*) and pDRH2312 (containing *motAB*). MscI-digested pDRH325 and SpeI-digested pDRH2312 were then ligated to the SmaI-digested *astA-kan* cassette to create pDRH669 and pDRH2322, respectively.

For generation of *flaA*-, *flaB*-, *flgD*-, *flgE2*-, and *motA-astA* transcriptional fusions on the chromosomes of *C. jejuni* strains, appropriate Δ*astA* mutants were electroporated with pDRH608, pDRH610, pDRH669, pDRH532, or pDRH2322, respectively. These constructs replaced the native loci with the respective *astA* transcriptional fusions.

To complement Δ*flgP* or Δ*flgQ* mutants with plasmids expressing *flgP* or *flgQ*, primers with in-frame 5′ BamHI restriction sites were designed to amplify the coding sequence of *flgP* or *flgQ* from the second codon to the stop codon. After PCR, the amplified DNA was digested with BamHI and ligated to BamHI-digested pECO102 to generate plasmids expressing *flgP* (pSMS265) or *flgQ* (pSMS269) from the chloramphenicol acetyltransferase (*cat*) promoter (27). These plasmids were then transferred to DH5α/RK212.1 to facilitate conjugation into wild-type *C. jejuni* 81-176 Sm^r or the Δ*flgP* or Δ*flgQ* mutants (10).

Protein homology and domain analyses. Homology searches were performed with BLASTP and PSI-BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Analyses of N-terminal signal sequences and lipoprotein sorting signals were performed with the SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/) and the LipoP 1.0 server (http://www.cbs.dtu.dk/services/LipoP/), respectively.

Preparation of antisera. The *flgP* coding sequence from the second codon to the stop codon was cloned into the BamHI site of pQE30 (QIAGEN) to generate pSMS511 in *E. coli* XL1-Blue for induction of His_6-tagged FlgP. For induction of a His_6-tagged FlgQ protein, the coding sequence from codon 30 to the stop codon of *flgQ* was amplified and inserted into the BamHI site of pQE30 to generate pSMS506. For purification of proteins, XL1-Blue containing pSMS511 or pSMS506 was grown in 500 ml LB broth with ampicillin to mid-log phase, induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and then incubated for another 4 h at 37°C. Bacteria were harvested and disrupted by passage twice through an EmulsiFlex-C5 cell disruptor (Avesin) at 15,000 to 20,000 lb/in^2. Insoluble matter was separated from lysates by centrifugation for 2 h at 20,000 × *g* at 4°C. Protein was purified with Ni-nitrilotriacetic acid agarose under denaturing conditions in the presence of 8 M urea. After purification, the urea concentration was gradually reduced to below 100 mM urea through dialysis. Anti-FlgP (α-FlgP) and α-FlgQ antisera were generated by immunizing five and eight mice, respectively.

α-RpoA antiserum was generated by synthesizing a peptide with an N-terminal cysteine followed by the sequence of amino acids representing residues 207 to 229 of the *C. jejuni* RpoA protein (N′-CITPNEAFQNALEAMYKQLSVFD K-C′) at the Protein Synthesis Core at the University of Texas Southwestern Medical Center. This peptide was conjugated to keyhole limpet hemocyanin and then used with TiterMax to immunize BALB/c mice for production of antisera.

Fractionation of *C. jejuni* strains. *C. jejuni* strains were grown from frozen stocks on MH agar containing 10 μg/ml TMP or 15 μg/ml chloramphenicol for 48 h at 37°C under microaerobic conditions. Strains were then restreaked on MH agar containing TMP or chloramphenicol and grown at 37°C under microaerobic conditions for 16 h. Strains were suspended from agar plates and diluted to an optical density at 600 nm (OD_{600}) of 0.8. These bacterial cultures were used for all fractionation procedures described below.

For whole-cell lysates, 1 ml of bacteria was pelleted, washed once with phosphate-buffered saline (PBS), and then resuspended in 50 μl 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis loading buffer. Outer and inner membrane proteins were prepared and separated based on Sarkosyl insolubility by the method of Carlone et al. (5). Briefly, 5-ml aliquots of bacteria were pelleted, washed once in 10 mM HEPES (pH 7.4), and resuspended in 1 ml of the same buffer. Sonication to disrupt bacteria was achieved with a Branson Sonifier 450 with a microtip attachment. Unbroken bacteria were removed by brief centrifugation at 16,000 × *g* for 2 min. To recover total membranes, bacteria were then centrifuged for 16,000 × *g* for 30 min at 4°C. Membrane pellets were resuspended in 400 μl 1% sodium lauryl sulfate in 10 mM HEPES (pH 7.4) and incubated at room temperature for 30 min. Sarkosyl-insoluble outer membrane proteins were then removed by centrifugation for 30 min at 16,000 × *g* at 4°C. The supernatant contained Sarkosyl-soluble inner membrane proteins.

Periplasmic and cytoplasmic proteins were collected by pelleting 20-ml aliquots of bacteria at 4,200 × *g* for 10 min. To isolate periplasmic proteins, we modified the procedure of Donohue-Rolf and Keusch (8). The bacterial pellets were washed twice with 2 ml of PBS containing 0.1% gelatin by centrifugation at 4,200 × *g* at 4°C. The pellets were then suspended in 200 μl of PBS containing 0.1% gelatin, and polymixin B sulfate was added to a final concentration of 2 mg/ml. The bacteria were agitated for 30 min at 4°C. The preparation was then centrifuged at 16,000 × *g* for 30 min at 4°C to separate the spheroplasts in the pellet from the periplasmic proteins in the supernatant. The supernatants were removed and centrifuged again at 16,000 × *g* for 30 min at 4°C to remove any contaminating spheroplasts.

The spheroplasts obtained from the periplasmic preparations were suspended

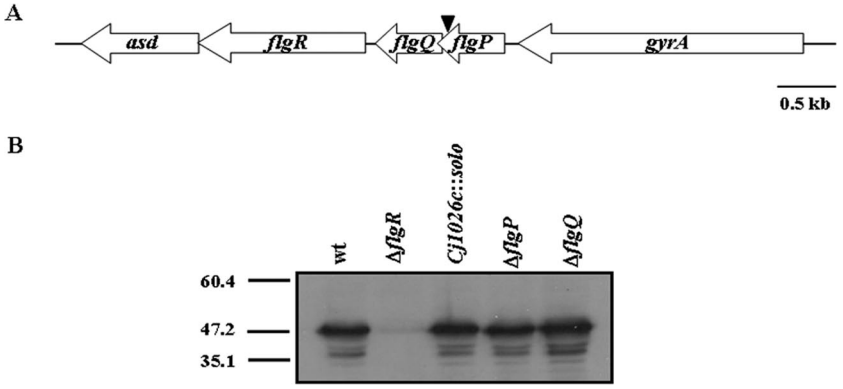

FIG. 1. Deletion of *flgP* or *flgQ* does not affect FlgR production in *C. jejuni*. (A) Organization of the *flgPQ* locus and surrounding genes. *flgP* and *flgQ* are immediately upstream of *flgR*, and their coding sequences overlap by 27 nucleotides. The arrowhead indicates the location of the *solo* transposon in strain 81-176 *flgP*::*solo* (DRH241). The annotations of *flgP* and *flgQ* in the genomic sequences of *C. jejuni* 11168 and 81-176, respectively, are Cj1026c and Cjj81176_1045 for *flgP* and Cj1025c and Cjj81176_1044 for *flgQ*. (B) Immunoblot analysis of FlgR production in *C. jejuni flgP* and *flgQ* mutants. Whole-cell lysates of wild-type (wt) 81-176 Sm^r (DRH212), 81-176 Sm^r Δ*flgR* (DRH737), 81-176 *flgP*::*solo* (DRH241), 81-176 Sm^r Δ*flgP* (DRH2070), and 81-176 Sm^r Δ*flgQ* (DRH2071) were used for analysis.

in 1 ml of 10 mM HEPES (pH 7.4) and then disrupted by sonication as described above. Insoluble matter and unbroken bacteria were removed by centrifugation at 16,000 × *g* for 2 min at 4°C. The supernatant was removed and centrifuged again at 16,000 × *g* for 30 min at 4°C. The resulting supernatants represented the proteins from the cytoplasm of the bacteria.

Immunoblot analyses. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% acrylamide gels. For whole-cell lysates, protein samples from each bacterial preparation were loaded to represent the proteins recovered from 200 μl of bacterial cultures that had been equilibrated to the same density by OD_{600} readings. For outer membrane proteins and cytoplasmic proteins, protein samples from each bacterial preparation were loaded to represent those obtained from 750 μl of culture. To detect FlgP or RpoA, a 1:1,500 dilution of murine α-FlgP M1 antiserum or murine α-RpoA M1 antiserum was used, respectively. Anti-major outer membrane protein (α-MOMP) rabbit antiserum diluted 1:1000 was used to detect MOMP in outer membrane preparations (30). For immunoblot analysis of FlgR production, 5 μl of whole-cell lysates from *C. jejuni* strains (representing 100 μl of bacterial culture) were used. Rabbit α-FlgR antiserum Rab13 was used at a dilution of 1:3,500 as the primary antibody for detection of FlgR (12). Horseradish peroxidase-conjugated goat α-mouse or α-rabbit immunoglobulin G antiserum was used as the secondary antibody.

Motility assays. *C. jejuni* strains from frozen stocks were grown on MH agar with TMP for 48 h in microaerobic conditions at 37°C. After growth, strains were streaked on MH agar with TMP or chloramphenicol and grown for an additional 16 h in microaerobic conditions at 37°C. Strains were suspended from agar plates and diluted to an OD_{600} of 1.0. For agar-based assays for motility, bacterial strains were stabbed into semisolid MH agar plates containing 0.4% agar as previously described (13). Motility phenotypes were assessed approximately 32 h after inoculation and incubation at 37°C in microaerobic conditions. For direct observation of motile bacteria, bacterial cultures were grown as described above but diluted to an OD_{600} of 0.75. The bacteria were then diluted 1:10 in MH broth, and 10 μl of each sample was placed on a glass slide and topped with a glass coverslip. Bacteria were then visualized under a magnification of ×400 using an Olympus BH2 dark-field microscope.

Transmission electron microscopy. *C. jejuni* strains were grown from frozen stocks on MH agar with TMP for 48 h in microaerobic conditions at 37°C. After growth, strains were streaked on MH agar with TMP and grown for an additional 16 h at 37°C in microaerobic conditions. Strains were suspended from agar plates and diluted to an OD_{600} of 0.4. Bacteria were pelleted and then resuspended in 2% glutaraldehyde for 1 hour at room temperature. Samples were stained with 1% uranyl acetate and visualized with a JOEL 1200 EX transmission electron microscope at 80 kV.

Arylsulfatase reporter assays. Arylsulfatase assays were performed as previously described (15), which was based on previously published procedures (11, 29). *C. jejuni* strains were grown from frozen stocks on MH agar with TMP or chloramphenicol in microaerobic conditions at 37°C for 48 h. Strains were then restreaked on MH agar and grown again for 16 h in microaerobic conditions at 37°C. Bacteria were suspended from agar plates to an appropriate dilution, washed, and then incubated with 10 mM nitrophenylsulfate and 1 mM tyramine at 37°C for 1 h. Reactions were terminated with NaOH, and the amount of nitrophenol released in each sample was determined by reading the OD_{410} of the sample with a spectrophotometer. Values were compared to a standard curve of OD_{410} readings of known nitrophenol concentrations to determine the number of arylsulfatase units produced by each strain. One arylsulfatase unit is defined as the amount of enzyme catalyzing the release of 1 nmol of nitrophenol per hour per OD_{600} unit. Each strain was tested in triplicate, and each assay was performed three times.

RESULTS

Deletion of *flgP* or *flgQ* causes defects in flagellar motility. We previously identified a nonmotile mutant of *C. jejuni* 81-176 with an insertion of the *solo* transposon in *flgP* (DRH241) (13). However, this mutant was not further characterized to verify that the nonmotile phenotype is actually due to interruption of *flgP* by the transposon. The 3′ end of *flgP* overlaps the 5′ end of the uncharacterized downstream gene *flgQ* by 27 nucleotides. These genes are positioned on the *C. jejuni* chromosome immediately upstream of *flgR* (Fig. 1A), which we and others have also shown is required for flagellar motility and transcription of σ^{54}-dependent flagellar genes (15, 16, 28). The *solo* transposon in the original transposon mutant is located 34 bases upstream of the stop codon of *flgP* and 10 bases upstream of the start codon of *flgQ*.

Due to the location of the *solo* transposon and the organization of *flgP*, *flgQ*, and *flgR* on the *C. jejuni* chromosome, it is possible that the motility defect of the *solo* transposon mutant is due to either interruption of *flgP* or polar effects on expression of downstream genes such as *flgQ* or *flgR*. Immunoblot analysis with antiserum against FlgR revealed that the original transposon mutant (81-176 *flgP*::*solo*) produces normal levels of FlgR compared to the wild-type strain 81-176 Sm^r (DRH212), demonstrating that the *solo* insertion in *flgP* does not cause a defect in FlgR production (Fig. 1B).

We then constructed mutants of 81-176 Sm^r with in-frame deletions of large portions of *flgP* or *flgQ* to determine if either one of these genes is required for motility. To construct a Δ*flgP* mutant, we deleted from the chromosome of 81-176 Sm^r codons 2 to 149, fusing the start codon of *flgP* to the last 23

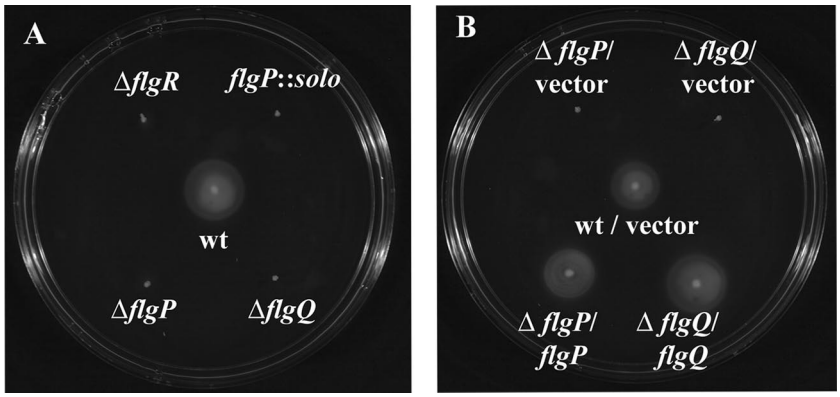

FIG. 2. FlgP and FlgQ are required for flagellar motility of *C. jejuni*. Strains were inoculated into MH motility agar. Motility was assessed 32 h after inoculation. Strains are (A) wild-type (wt) 81-176 Sm^r (DRH212), 81-176 Sm^r Δ*flgR* (DRH737), 81-176 *flgP*::*solo* (DRH241), 81-176 Sm^r Δ*flgP* (DRH2070), and 81-176 Sm^r Δ*flgQ* (DRH2071) and (B) wild-type 81-176 Sm^r/pECO102 (DRH837), 81-176 Sm^r Δ*flgP*/pECO102 (SMS304), 81-176 Sm^r Δ*flgQ*/pECO102 (SMS306), 81-176 Sm^r Δ*flgP*/pSMS265 (SMS308), and 81-176 Sm^r Δ*flgQ*/pSMS269 (SMS312). pECO102 is the empty vector control, and pSMS265 and pSMS269 are pECO102 derivatives that express *flgP* and *flgQ*, respectively.

codons. For the Δ*flgQ* mutant, we fused the first 10 codons of *flgQ* to the stop codon, deleting the intervening 139 codons. Deletion of either of these genes does not affect levels of FlgR (Fig. 1B), demonstrating that the deletions in *flgP* and *flgQ* that we created do not have polar effects on FlgR production. Analysis of the Δ*flgP* and Δ*flgQ* mutants in semisolid motility agar revealed that both mutants display a nonmotile phenotype (Fig. 2A), similar to that of a Δ*flgR* mutant. Complementation of each mutant with a plasmid expressing only the respective gene deleted from each strain restores the motility phenotype to wild-type levels (Fig. 2B), confirming that *flgP* and *flgQ* are both required for motility. Since each *flgP* or *flgQ* mutant could be complemented with just the respective gene supplied in *trans*, these findings indicate that the *flgP* deletion construct is nonpolar with respect to *flgQ* and vice versa. Dark-field microscopy was also used to examine the motile phenotypes of individual bacterial mutants. Wild-type bacteria were observed to migrate rapidly across the field, whereas mutants lacking *flgP* or *flgQ* were unable to move (data not shown), suggesting that the flagella of these mutants may have a paralyzed phenotype. These results combined indicate that both *flgP* and *flgQ* are required for motility of *C. jejuni*.

Domain predictions and homologues of FlgP and FlgQ proteins. *flgP* is predicted to encode a precursor protein of 171 amino acids with a size of 18.5 kDa containing an N-terminal signal peptide cleaved after amino acid 23. Alternatively, a lipoprotein sorting signal peptide contained in the first 16 residues is also predicted. PSI-BLAST analysis suggests that FlgP is encoded in the genomes of other *Campylobacter* species, with 59 to 84% identity and 73 to 91% similarity across the lengths of the homologous proteins of *C. coli* (CCO1093), *C. lari* (CLA1520), *C. upsaliensis* (CUP0109), and *C. fetus* (CFF8240_1669). FlgP is also homologous to proteins of *Helicobacter pylori* (JHP_0775 and HPAG1_0822) (1, 21) and *Helicobacter hepaticus* (HH0979) (24), with 36 to 38% identity and 77% similarity across approximately 77% of the lengths of the proteins. In addition, *Wolinella succinogenes* encodes a protein (WS1756) (2) with 42% identity and 66% similarity to FlgP. The only predicted motif found in FlgP by BLAST analysis is a DUF400 domain contained within amino acids 73 to 171 of the protein. However, the DUF400 domain is undefined, with no known function. This domain is present in a series of functionally uncharacterized proteins of many other bacteria. As such, these bacterial proteins, which include the ones of pathogenic species of *Vibrio*, contain approximately 25% identity and 50% similarity to FlgP, but this homology is primarily limited to the DUF400 domains with little to no homology to the first 72 amino acids of FlgP.

flgQ is predicted to encode a precursor protein of 149 amino acids and 17.4 kDa with an N-terminal signal peptide predicted to be cleaved after the first 17 amino acids. PSI-BLAST analysis predicts only FlgQ homologues with 56 to 79% identity and 75 to 91% similarity to proteins of *C. coli* (CCO1092), *C. lari* (CLA1519), and *C. upsaliensis* (CUP0110). *C. fetus* possesses a FlgQ homologue (CFF8240_1668) with less homology (32% identity and 51% similarity). No other bacteria are predicted to have a protein with similarity to FlgQ.

FlgP and FlgQ are not required for flagellar biosynthesis or expression of σ^{54}- or σ^{28}-dependent flagellar genes. To determine the possible roles of FlgP and FlgQ in motility, we analyzed the Δ*flgP* and Δ*flgQ* mutants for defects in flagellar biosynthesis and transcription of known regulated flagellar genes. As could be determined from visualization of strains by transmission electron microscopy, Δ*flgP* and Δ*flgQ* mutants construct a single flagellum at one or both bacterial poles that is similar in length and appearance to those produced by a wild-type strain (Fig. 3). These phenotypes are different than those of a Δ*flgR* mutant, which fails to produce any flagella (Fig. 3).

C. jejuni uses two alternative σ factors, σ^{28} and σ^{54}, to transcribe many flagellar genes. Whereas σ^{28} is involved in the transcription of *flaA*, encoding the major flagellin, σ^{54} and FlgR are required for transcription of many genes encoding the basal body, rod, hook, and minor flagellin proteins (6, 13, 15, 16, 28). Through analysis of expression of *flaA*- and the σ^{54}-dependent flagellar gene *flgD*-, *flgE2*-, and *flaB-astA* transcriptional fusions, we found that expression of these genes was not reduced in the Δ*flgP* or Δ*flgQ* mutants (Table 1). These results are in contrast to the defects in transcription of *flaA* in the σ^{28} mutant (Δ*fliA*) or of *flgD*, *flgE2*, and *flaB* in the σ^{54} (Δ*rpoN*) or *flgR* mutants. In fact, we observed a slight elevation

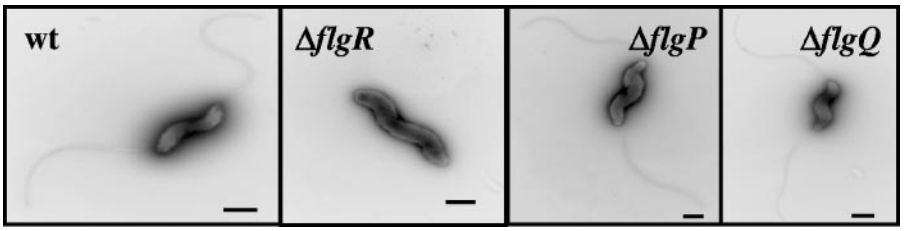

FIG. 3. FlgP and FlgQ are not required for flagellar biosynthesis. Bacteria were stained with 1% uranyl acetate. All electron micrographs are at a magnification of ×20,000. The bar for each micrograph represents 0.5 μm. Strains shown are wild-type (wt) 81-176 Sm^r (DRH212), 81-176 Sm^r Δ*flgR* (DRH737), 81-176 Sm^r Δ*flgP* (DRH2070), and 81-176 Sm^r Δ*flgQ* (DRH2071).

of expression (less than two- to threefold) of *flaA* and *flgE2* in mutants lacking *flgP* or *flgQ*.

Since the Δ*flgP* and Δ*flgQ* mutants are nonmotile but produce flagella, we examined the possibility that these mutants may be defective for expression of one or more of the genes required for production of the flagellar motor, such as *motA* and *motB*. These two genes are separated by two nucleotides, with *motA* 5′ to *motB* (22). This type of organization suggests that *motA* and *motB* may be in an operon and cotranscribed. We constructed a *motA*::*astA* transcriptional fusion to replace native *motA* on the chromosomes of Δ*flgP* and Δ*flgQ* mutants. Via arylsulfatase assays, we were unable to detect a defect in expression of *motA* in these mutants or any other mutants lacking σ^{28}, σ^{54}, or FlgR (Table 1). Thus, the flagellated, nonmotile phenotype of the Δ*flgP* or Δ*flgQ* mutants cannot be explained by a defect in expression of the motor component encoded by *motA*.

Considering that many flagellar genes in *C. jejuni* are under regulation by the σ^{54}-dependent or σ^{28}-dependent regulatory cascades, we explored whether expression of *flgP* or *flgQ* is a target of one of these cascades. *flgP*::*astA* and *flgQ*::*astA* transcriptional fusions were used to replace the native *flgP* or *flgQ* loci on the chromosomes of mutants lacking σ^{28} or σ^{54}. Expression of *flgP* or *flgQ* is reduced only approximately two- to threefold, respectively, in the σ^{54} mutant (Δ*rpoN*) compared to wild-type strains (Table 2). Expression levels could be partially restored upon complementation of the Δ*rpoN* mutants with a plasmid expressing *rpoN*, suggesting that *flgP* and *flgQ* may be partially dependent either directly or indirectly on σ^{54} for expression.

FlgP requires FlgQ for localization to or stability in the outer membrane of *C. jejuni*. To better understand the roles of FlgP and FlgQ in flagellar motility, we constructed His_6-tagged fusion proteins of FlgP and FlgQ for generation of polyclonal murine antisera to determine the locations of the proteins in *C. jejuni*. Whereas the generated antisera were reactive to the respective purified recombinant proteins, the α-FlgQ antiserum was not reactive to protein preparations from wild-type *C. jejuni* with normal FlgQ production (data not shown); thus, analysis of FlgQ localization in *C. jejuni* could not be done.

To determine the location of FlgP in *C. jejuni*, we separated wild-type and mutant strains of *C. jejuni* into fractions containing outer membrane, periplasmic, inner membrane, or cytoplasmic proteins. Immunoblot analysis revealed the presence of a protein of approximately 16 kDa in whole-cell lysates (total protein) of wild-type *C. jejuni* (Fig. 4). Examination of the cytoplasmic and outer membrane proteins derived from an equivalent number of wild-type bacteria revealed that more FlgP is located in the outer membrane than in the cytoplasm (Fig. 4). Thus, the outer membrane appears to represent the terminal destination for FlgP. The band corresponding to FlgP was absent in fractions from a Δ*flgP* mutant but could again be observed when the Δ*flgP* mutant was complemented with a plasmid expressing *flgP* in *trans*. No bands representing FlgP were found in the periplasm or inner membrane (data not shown). To ensure that our fractionation procedures were appropriate and represented the proper subcellular compartments, immunoblot analyses were performed with antiserum

TABLE 1. Arylsulfatase activities of *flaA*-, *flgD*-, *flgE2*-, *flaB*-, and *motA*-*astA* transcriptional fusions in Δ*flgP* and Δ*flgQ* mutants

Strain	Arylsulfatase units[a] (mean ± SD)				
	flaA::*astA*[b]	*flgD*::*astA*[c]	*flgE2*::*astA*[d]	*flaB*::*astA*[e]	*motA*::*astA*[f]
Wild type	133.2 ± 2.7	196.4 ± 7.3	33.5 ± 1.5	175.5 ± 6.8	174.6 ± 6.7
Δ*fliA*	64.5 ± 7.0	ND[g]	ND	ND	179.9 ± 13.6
Δ*rpoN*	ND	0.6 ± 0.1	<0.1 ± 0.1[h]	<0.2 ± 0.1[h]	185.9 ± 10.6
Δ*flgR*	139.1 ± 4.7	<0.4 ± 0.3[h]	<0.1 ± 0.1[h]	0.2 ± 0.1	181.5 ± 11.7
Δ*flgP*	340.6 ± 8.5	275.2 ± 20.5	78.5 ± 5.0	225.7 ± 4.1	189.7 ± 16.9
Δ*flgQ*	341.8 ± 15.3	275.5 ± 18.9	78.0 ± 1.0	245.5 ± 9.1	190.4 ± 7.7

[a] Results are from a typical assay with determinations for each sample performed in triplicate. One unit equals the amount of arylsulfatase required to generate 1 nmol of nitrophenol per hour per OD_{600} unit.
[b] Strains used were DRH655, DRH1070, DRH1122, SMS360, and SMS407.
[c] Strains used were SMS354, SMS401, SNJ230, SNJ316, and SNJ322.
[d] Strains used were DRH533, DRN536, DRH830, SMS357, and SMS404.
[e] Strains used were DRH665, DRH667, DRH842, SMS364, SMS410.
[f] Strains used were DRH2329, DRH2331, DRH2333, DRH2335, DRH2337, and DRH2342.
[g] ND, not determined.
[h] At least one of the three tested samples had a value below the limit of detection of the assay, which was 0.06 arylsulfatase unit.

TABLE 2. Arylsulfatase activities of *flgP*- and *flgQ*-*astA* transcriptional fusions in σ^{28} and σ^{54} mutants

Strain	Arylsulfatase units[a] (mean ± SD)	
	flgP::*astA*[b]	*flgQ*::*astA*[c]
Wild type	282.7 ± 40.0	29.3 ± 1.1
Δ*fliA*	308.1 ± 22.9	29.0 ± 0.7
Δ*rpoN*	163.9 ± 1.5	10.6 ± 0.9
Δ*rpoN*/vector	155.6 ± 6.3	11.4 ± 0.8
Δ*rpoN*/*rpoN*	215.4 ± 4.2	19.1 ± 1.2

[a] Results are from a typical assay with determinations for each sample performed in triplicate. One unit equals the amount of arylsulfatase required to generate 1 nmol of nitrophenol per hour per OD_{600} unit.
[b] Strains used were SMS341, SMS350, SMS420, SMS524, and SMS526.
[c] Strains used were SMS281, SMS323, SMS335, SMS532, and SMS534.

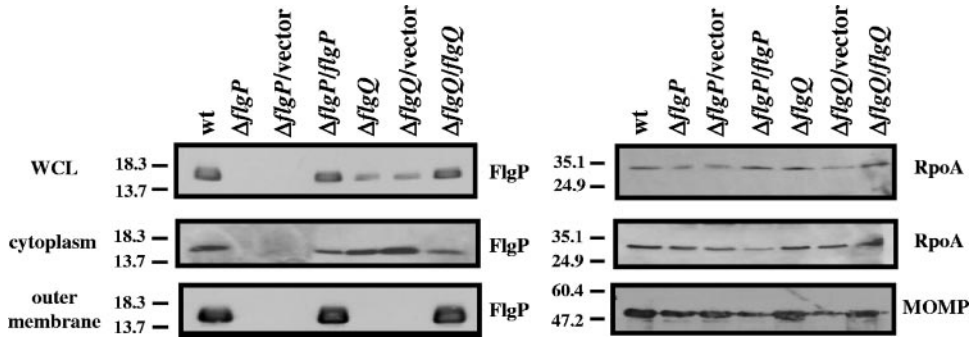

FIG. 4. FlgP requires FlgQ for localization to or stability in the outer membrane. Proteins from whole-cell lysates representing 200 μl of bacterial culture or proteins from outer membrane preparations or cytoplasmic preparations representing 750 μl of bacterial culture were used for immunoblotting. Blots were developed with α-FlgP mouse antiserum M1, α-RpoA mouse antiserum M1, or α-MOMP rabbit antiserum (30). Blots on the left are detecting FlgP. The top and middle blots on the right are detecting RpoA, whereas the bottom blot is detecting MOMP. Strains are wild-type (wt) 81-176 Sm^r (DRH212), 81-176 Sm^r Δ*flgP* (DRH2070), 81-176 Sm^r Δ*flgP*/pECO102 (SMS304), 81-176 Sm^r Δ*flgP*/pSMS265 (SMS308), 81-176 Sm^r Δ*flgQ* (DRH2071), 81-176 Sm^r Δ*flgQ*/pECO102 (SMS306), and 81-176 Sm^r Δ*flgQ*/pSMS269 (SMS312). pECO102 is the empty vector control, and pSMS265 and pSMS269 are pECO102 derivatives that express *flgP* and *flgQ*, respectively. WCL, whole-cell lysates.

against RpoA, a component of the cytoplasmic RNA polymerase, or MOMP of *C. jejuni* (30). As expected, RpoA was found in the whole-cell lysates and cytoplasm, whereas the outer membrane contained a protein of 48 kDa representing MOMP. In addition, these markers served as loading controls to ensure that proteins from equivalent amounts of bacteria were analyzed for each sample.

We then determined if the amount or location of FlgP was affected in the Δ*flgQ* mutant. Analysis of the Δ*flgQ* mutant revealed that the amount of FlgP from whole-cell lysates was noticeably decreased compared to that in wild-type bacteria (Fig. 4). Further analysis revealed that roughly equivalent levels of FlgP are present in the cytoplasms of the Δ*flgQ* mutant and the wild-type strain. However, no FlgP is present in the outer membrane of the Δ*flgQ* mutant. Wild-type levels of FlgP in the whole-cell lysates and outer membrane are restored when the Δ*flgQ* mutant is complemented with a plasmid expressing *flgQ*. These results suggest that FlgQ may be required for localizing FlgP to the outer membrane and perhaps maintaining its stability at this location. We also investigated whether FlgP accumulates in the periplasm of the Δ*flgQ* mutant, but we could not detect FlgP in this compartment in wild-type *C. jejuni* or any mutant (data not shown). Since the Δ*flgQ* mutants have less FlgP in the whole-cell lysates, a reasonable hypothesis is that FlgQ may be required for transcription or translation of FlgP. However, the cytoplasms of the Δ*flgQ* mutants have levels of FlgP similar to those of the wild-type strain, but the outer membranes of the *flgQ* mutants lack FlgP. The most likely possibility for the decrease in total FlgP in the whole-cell lysates is due to the failure of FlgP to localize to and be stable in the outer membrane in the absence of FlgQ. Thus, FlgQ is unlikely to influence the transcription of *flgP* or its initial production. To ensure that FlgQ is not required for expression of *flgP*, *flgP*::*astA* transcriptional fusions were analyzed in the wild-type and Δ*flgQ* strains. No differences in the levels of arylsulfatase were observed (data not shown), indicating that *flgQ* is not required for expression of *flgP*.

DISCUSSION

In this work, we have identified two additional proteins of *C. jejuni* required for flagellar motility. Deletion of either *flgP* or *flgQ* from *C. jejuni* resulted in mutants that produce a single flagellum of normal appearance at the poles of the bacterium. These mutants, however, are nonmotile, as they are unable to migrate in motility agar or swim as visualized by dark-field microscopy, indicating that the lack of FlgP or FlgQ in *C. jejuni* may create paralyzed flagella. Localization studies revealed that the terminal location of FlgP is the outer membrane and that FlgQ is required for localizing FlgP to the outer membrane and possibly stabilizing FlgP at this location. These results suggest that FlgP may play a direct role in motility, whereas the requirement of FlgQ may be indirect in aiding FlgP to localize or be stable at or near the flagellar structure so that it can function in motility. Considering our findings, our leading hypothesis for the role of FlgP in motility is that it may be an outer membrane protein required for the actual process of flagellar rotation.

It is currently unclear how FlgP may contribute to flagellar rotation if the lack of these proteins does indeed cause a paralyzed flagellar phenotype. Visualization of individual bacteria by dark-field microscopy gives credence to this phenotype, since individual bacteria lacking *flgP* are unable to move. One possible role for FlgP is that it could be required for stabilization of parts of the flagellar motor complex, such as the MotA or MotB stators. Whereas MotA is an integral membrane protein, MotB is localized to the inner membrane with a large periplasmic domain (4), which could conceivably interact with an outer membrane-localized FlgP protein if it is oriented appropriately at this location. Future experiments will be directed at analyzing the fate of MotA and MotB in the presence and absence of FlgP.

Since our immunoblot analyses were performed by examining cytoplasmic or outer membrane proteins extracted from the same amount of bacteria, we were able to determine that more FlgP is located in the outer membrane than in the cytoplasm in wild-type bacteria. In addition, we were also able to

determine that the levels of FlgP in the cytoplasms of the strains are similar in wild-type and Δ*flgQ* mutant strains, but no FlgP was found associated with the outer membrane of the Δ*flgQ* mutant. Thus, we hypothesize that the outer membrane is the terminal destination of FlgP and that FlgQ is required to localize FlgP to this compartment. For this to occur, FlgQ may interact with FlgP at some point in the secretion, localization, or stabilization of FlgP. Thus, FlgQ may have some type of chaperone function for FlgP. In the absence of FlgQ and proper localization, FlgP may be highly unstable and degraded in the periplasm; we were unable to detect FlgP in the periplasms of the wild-type and Δ*flgQ* mutant strains. Like FlgP, FlgQ is also predicted to have an N-terminal signal sequence, suggesting that it could be secreted across the inner membrane as well. However, we were unable to monitor the localization of FlgQ in *C. jejuni* since the antisera we generated did not recognize the native protein. Once in the periplasm, FlgQ may be able to interact with FlgP to target it to the outer membrane, possibly close to the flagellar basal body and rod complex. If FlgQ possesses a chaperone function for FlgP, FlgQ may form a physical interaction with FlgP and we may be able to capture interacting complexes. Thus, future experiments will be focused on developing methods to detect FlgQ and performing experiments with *C. jejuni* or with a heterologous system such as *E. coli* to attempt to detect a complex of FlgP and FlgQ by a combination of cross-linking and immunoprecipitation experiments or two-hybrid experiments.

We do not favor the hypothesis that FlgQ is required for the actual secretion of FlgP out of the cytoplasm for two reasons. First, FlgP is predicted to possess an N-terminal signal sequence or lipoprotein sorting signal; each would presumably function to transport the protein via the Sec system across the inner membrane, independent of FlgQ. Second, if FlgQ is required for secretion of FlgP, we would have expected to see an accumulation of FlgP in the cytoplasm of a Δ*flgQ* mutant. Instead, similar levels of FlgP were found in the cytoplasms of wild-type and Δ*flgQ* mutant strains. However, we cannot entirely exclude the possibility that FlgQ could be required for secretion of FlgP. A series of pulse-chase experiments in combination with induction of FlgP and FlgQ proteins would allow us to follow the fate of FlgP in the presence and absence of FlgQ to better analyze a possible role for FlgQ in FlgP transport, localization, and stability. However, these types of experiments require induction strategies that are currently unavailable for *C. jejuni*; we are actively pursuing the development of such technologies.

Further analysis will also allow us to more precisely determine whether FlgP is located on the surface of *C. jejuni* or associated with the periplasmic face of the outer membrane. Currently, we know only that FlgP is associated with the outer membrane. FlgP is predicted to have an N-terminal lipoprotein sorting signal, which may indicate that the protein could be associated with the periplasmic face of the outer membrane. We attempted to localize FlgP to the outer surface of *C. jejuni* by using whole-cell dot immunoblotting and immunogold labeling with transmission electron microscopy, but the results were inconclusive. These methods could have been limited by the α-FlgP antiserum that is currently available. Since Kyte-Doolittle analysis predicts a strong hydrophobic region only at the N terminus of FlgP where the predicted N-terminal signal sequence or lipoprotein sorting sequence is located, we do not expect that FlgP is an integral outer membrane protein. Additional studies with site-directed mutagenesis of *flgP* and improved reagents and methods will be required to better determine the nature of the association of FlgP with the outer membrane.

Considering our findings with *C. jejuni*, the role of FlgP in motility of other bacteria is unclear. Both FlgP and FlgQ are required for motility in *C. jejuni*, and we suspect that the same will be true in other *Campylobacter* species, as they contain genes to encode homologues of both proteins. However, other bacteria such as *Helicobacter* species and *W. succinogenes* contain a highly similar FlgP homologue but lack a gene encoding FlgQ. Meanwhile, other bacteria appear to encode a protein homologous only to the portion of FlgP that contains the uncharacterized DUF400 domain (composing the last 60% of FlgP), while also lacking a FlgQ homologue. Since FlgQ is required for motility in *C. jejuni* and appears to be necessary for proper levels and localization of FlgP, it is unclear whether FlgP-like proteins of other bacteria would function in motility without a cognate FlgQ protein. Alternatively, these other bacteria may have FlgP-like proteins that are independent of FlgQ or are partnered with an unknown protein that can substitute for FlgQ to promote proper localization and function of FlgP. Determining if these FlgP-like proteins of other bacteria function in motility warrants further investigation.

In summary, we have identified a pair of proteins required for flagellar motility that may be unique to *C. jejuni* and other campylobacters. Further characterization of these proteins may reveal a better understanding of the role of FlgQ in the localization and stability of FlgP and the role of FlgP in motility. In addition, analysis of these proteins may reveal new insights regarding membrane proteins of bacteria that are possibly involved in the physical rotation of flagella. Due to the uniqueness of these two proteins in *Campylobacter* species, further analysis of these proteins may reveal new understandings in protein-protein interactions required for the process of motility in campylobacters.

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