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Activation of the *Campylobacter jejuni* FlgSR Two-Component System Is Linked to the Flagellar Export Apparatus[▽]

Stephanie N. Joslin and David R. Hendrixson*

Department of Microbiology, University of Texas Southwestern Medical Center, Dallas, Texas 75390

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Activation of σ^{54}-dependent gene expression essential for formation of flagella in *Campylobacter jejuni* requires the components of the inner membrane-localized flagellar export apparatus and the FlgSR two-component regulatory system. In this study, we characterized the FlgS sensor kinase and how activation of the protein is linked to the flagellar export apparatus. We found that FlgS is localized to the *C. jejuni* cytoplasm and that His141 of FlgS is essential for autophosphorylation, phosphorelay to the cognate FlgR response regulator, motility, and expression of σ^{54}-dependent flagellar genes. Mutants with incomplete flagellar export apparatuses produced wild-type levels of FlgS and FlgR, but they were defective for signaling through the FlgSR system. By using genetic approaches, we found that FlgSR activity is linked to and downstream of the flagellar export apparatus in a regulatory cascade that terminates in expression of σ^{54}-dependent flagellar genes. By analyzing defined *flhB* and *fliI* mutants of *C. jejuni* that form flagellar export apparatuses that are secretion incompetent, we determined that formation of the apparatus is required to contribute to the signal sensed by FlgS to terminate in activation of expression of σ^{54}-dependent flagellar genes. Considering that the flagellar export apparatuses of *Escherichia coli* and *Salmonella* species influence σ^{28}-dependent flagellar gene expression, our work expands the signaling activity of the apparatuses to include σ^{54}-dependent pathways of *C. jejuni* and possibly other motile bacteria. This study indicates that these apparatuses have broader functions beyond flagellar protein secretion, including activation of essential two-component regulatory systems required for expression of σ^{54}-dependent flagellar genes.

Responding to changing environmental and intracellular conditions in cells requires efficient communication networks that can rapidly receive and integrate signals. Two-component regulatory systems, which are distributed almost ubiquitously among prokaryotic organisms, allow bacteria to monitor their intracellular and extracellular environments and react by altering the expression of appropriate genes. These systems are typically comprised of a sensor histidine kinase and a response regulator protein (reviewed in references 46 and 65). The sensor protein contains a domain usually in the N-terminal portion that detects a specific signal, commonly through an interaction with another protein or a small effector molecule. Activation includes autophosphorylation of the sensor kinase and a conformational change that allows the transmitter domain, usually in the C-terminal portion, to activate a cognate response regulator via phosphotransfer. Some histidine kinases also have the ability to function as a phosphatase to remove a phosphate group from either themselves or their cognate response regulators when activity of the regulatory system is not favored.

The largest group of sensor histidine kinases includes those that are anchored to the cytoplasmic membrane and receive signals from the extracellular environment, allowing a cell to respond to external factors such as pH, temperature, or the presence of specific compounds (reviewed in reference 46). Since the monitoring of intracellular conditions is also vital to basic cellular processes, sensor kinases that are activated by alterations within bacteria have also evolved. These kinases include a relatively small group of kinases that are membrane anchored but respond to signals in the cytoplasm or periplasm and a larger group of soluble, cytoplasmic sensor kinases. Several members of the latter group have been characterized, such as NtrB, a kinase involved in nitrogen metabolism, whose activity is controlled by the PII protein (33). Nitrogen starvation results in uridylylation of PII, which blocks interaction with NtrB and causes the sensor protein to function as a kinase to initiate phosphorelay, culminating in phosphorylation of its cognate response regulator, NtrC. Under nitrogen-replete conditions, PII is deuridylylated and interacts with NtrB, allowing it to function as a phosphatase instead of as a kinase. Another example of a cytoplasmic histidine kinase that responds to intracellular conditions is KinA of *Bacillus subtilis*. Through interactions with two different proteins that inhibit the function of the kinase, KinA is responsive to the energy state of the bacterium or the ability of the cell to initiate replication (9, 58, 63). Activation of KinA begins a complex regulatory cascade leading to expression of genes essential for sporulation.

Flagellar assembly and chemotaxis systems also rely on two-component signaling systems to properly regulate bacterial motility (6, 62). The CheA kinase receives signals from a number of membrane-bound methyl-accepting chemotaxis protein (MCP) receptors (reviewed in references 3, 17, and 18). Motile bacteria respond via chemotaxis to small molecules that are attractants or repellants, and many of these effectors are bound by the periplasmic domains of MCPs. Through interactions of the cytoplasmic domains of MCPs with the CheA kinase, CheA is able to integrate and transmit these signals via phosphorelay

* Corresponding author. Mailing address: University of Texas Southwestern Medical School, Department of Microbiology, 5323 Harry Hines Boulevard, Dallas, TX 75390-9048. Phone: (214) 648-5949. Fax: (214) 648-5907. E-mail: david.hendrixson@utsouthwestern.edu.

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to CheY, ultimately influencing the decision to continue swimming in a single direction or tumble and change direction.

Campylobacter jejuni is a gram-negative, microaerophilic bacterium commonly associated with a number of animals of agricultural significance, especially fowl. While the relationship between *C. jejuni* and avian species develops into commensalism, infection of humans causes gastroenteritis that can range from very mild enteritis to severe, bloody diarrheal episodes (7, 8). In both the developed and developing regions of the world, *C. jejuni* is responsible for a substantial percentage of cases of bacterial gastroenteritis (13, 53). In the United States, this bacterium is believed to be the leading single-species cause of diarrheal disease, resulting in significant loss in economic productivity (11).

C. jejuni is a highly motile organism owing to the presence of a single flagellum elaborated from one or both poles of the bacterium. Motility is critical for promoting optimal interactions between *C. jejuni* and avian or human hosts. Nonmotile variants of *C. jejuni* colonize the gastrointestinal tract of chicks at levels significantly lower than wild-type motile strains (25, 29, 50, 64, 66), and only motile strains can be recovered after coinfection of human volunteers with motile and nonmotile strains (5). In this organism, motility is a highly organized, regulated, and complex process, relying on the coordination of over 40 proteins to assemble a complete organelle (26). Although there are some similarities with the well-characterized regulatory cascades described for *Escherichia coli* and *Salmonella* species (12), genetic screens and in silico analyses indicate that there are several differences that distinguish the flagellar gene transcription and assembly processes in *C. jejuni*. While *C. jejuni* utilizes σ^{28} to activate transcription of the major flagellin (encoded by *flaA*) and other minor flagellum-associated proteins (10, 22, 23, 28, 30, 66), σ^{54} has been shown or proposed to be involved in transcription of the bulk of flagellar genes, including those encoding the hook, basal body, and minor flagellin (26, 28, 30, 66). The use of both σ^{28} and σ^{54} in these pathways indicates that flagellar gene transcription in *C. jejuni* is more similar to the regulatory cascades of species of *Vibrio*, *Pseudomonas*, and *Helicobacter* than to those of *E. coli* or *Salmonella* species (2, 16, 34, 39, 40, 47, 51, 56, 60).

In a transposon mutagenesis screen, a number of gene products were found to be required for σ^{54}-dependent flagellar gene transcription in *C. jejuni* (30). These proteins include members of the flagellar export apparatus (FEA), FlhF (a putative GTPase), and the FlgSR two-component system, comprised of the FlgS sensor kinase and the FlgR response regulator (30). It was hypothesized that these proteins may act separately or in concert to integrate signals required to initiate transcription of σ^{54}-dependent flagellar genes. Previous work characterized the unusual NtrC-like response regulator FlgR to understand the means by which this protein functions (35). Our group has also found that FlgR and FlgS are targets of phase variation, making FlgSR the only known two-component regulatory system in which both proteins are subject to this form of control (25, 27). However, the mechanism by which FlgS is activated and functions as a sensor kinase remains to be characterized. Sequence analyses indicate that this protein appears to contain domains common to many sensor histidine kinases, such as the ATP-binding catalytic domain and the histidine-containing phosphotransfer domain (61, 65). Although the homology is somewhat weaker in the N-terminal region of the protein, FlgS is similar to the flagellum-associated histidine kinases that are required for σ^{54}-dependent flagellar gene expression and motility in species of *Vibrio*, *Pseudomonas*, and *Helicobacter* (14, 40, 51, 57). However, the signals that activate any of these kinases for positively influencing flagellar gene expression are uncharacterized.

In this work, we characterized FlgS and the activating signals that influence its ability to positively regulate flagellar gene expression and motility in *C. jejuni*. We first identified the histidine in the phosphotransfer domain that is autophosphorylated upon activation by FlgS. Through extensive experimentation, we characterized the origin of the signal that influences FlgS activation. Our research has led us to believe that (i) activation of FlgSR is dependent on the FEA and (ii) the signal for FlgS autophosphorylation may lie within the FEA, as formation of this apparatus appears to be necessary to promote expression of σ^{54}-dependent flagellar genes. Our work expands previous models of *Campylobacter* flagellar gene regulation and motility by characterizing the FlgS sensor kinase and introducing potential mechanisms for activating this protein. Furthermore, our work suggests that the FEAs of a subset of motile bacteria that use σ^{54} to control expression of flagellar genes have broader functions than flagellar protein secretion, including influencing signaling pathways through two-component regulatory systems to activate flagellar gene expression.

MATERIALS AND METHODS

Bacterial strains. *C. jejuni* strain 81-176 is a clinical isolate from a patient presenting with gastroenteritis and has been shown to promote commensal colonization of the chick gastrointestinal tract and to cause disease in human volunteers (5, 29). *C. jejuni* was routinely grown on Mueller-Hinton (MH) agar containing 10 μg/ml trimethoprim (TMP) under microaerobic conditions (85% N_2, 10% CO_2, and 5% O_2) at 37°C. When necessary, strains were grown on MH agar containing 50 μg/ml kanamycin, 15 μg/ml chloramphenicol, or 0.5, 1, 2, or 5 mg/ml streptomycin. All *C. jejuni* strains were stored at −80°C in a solution of 85% MH broth and 15% glycerol. *E. coli* strains DH5α, XL1-Blue, and BL21(DE3)/pLysE were cultured with Luria-Bertani (LB) agar or broth containing 100 μg/ml ampicillin or 15 μg/ml chloramphenicol when required. All *E. coli* strains were stored at −80°C in a solution of 80% LB broth and 20% glycerol.

Construction of mutants. All strains were constructed by using previously described protocols (28). To construct *flgS*(*H141*) mutants, pDRH310 (30) was subjected to PCR-mediated mutagenesis (45) to mutate the histidine codon at position 141 to a codon for alanine and then was verified by DNA sequence analysis. One plasmid, pDRH1276, was recovered and introduced into 81-176 Sm^r *flgS*::*cat-rpsL* (DRH441 [30]) and 81-176 Sm^r Δ*astA flgS*::*cat-rpsL* (DRH460 [30]) by electroporation. Mutants were recovered on MH agar containing streptomycin and verified by PCR analysis and DNA sequencing. Mutants used for further analysis were designated DRH1323 [81-176 Sm^r *flgS*(*H141A*)] and SNJ947 [81-176 Sm^r Δ*astA flgS*(*H141A*)].

We replaced native *flgR* with the $flgR_{\Delta receiver}$ and $flgR_{\Delta CTD}$ alleles (where receiver indicates the N-terminal receiver domain and CTD indicates the C-terminal domain) in FEA mutants. For Δ*fliP*, Δ*flhA*, and Δ*flhB* mutants, *flgR*::*kan-rpsL* (pDRH443) was electroporated into strains 81-176 Sm^r Δ*astA* Δ*fliP* (DRH1016), 81-176 Sm^r Δ*astA* Δ*flhA* (DRH979), and 81-176 Sm^r Δ*astA* Δ*flhB* (DRH1734) (30). The resultant strains, 81-176 Sm^r Δ*astA* Δ*fliP* *flgR*::*kan-rpsL* (SNJ158), 81-176 Sm^r Δ*astA* Δ*flhA* *flgR*::*kan-rpsL* (DRH1765), and 81-176 Sm^r Δ*astA* Δ*flhB* *flgR*::*kan-rpsL* (DRH1830), were electroporated with pDRH1855 containing the $flgR_{\Delta receiver}$ allele and pDRH1856 containing the $flgR_{\Delta CTD}$ allele (35). All transformants were selected on MH agar with streptomycin and verified by PCR and DNA sequencing.

C. jejuni Δ*fliI* mutants were constructed by first cloning the *fliI* locus into pUC19 (to generate pDRH1453) and then cloning an SmaI-digested *kan-rpsL* cassette (from pDRH427 [30]) into a PmeI site within the *fliI* coding sequence to generate pDRH1506. pDRH1506 was introduced into 81-176 Sm^r Δ*astA* (DRH461 [30]) by electroporation, generating 81-176 Sm^r Δ*astA fliI*::*kan-rpsL*

(DRH2246), which was recovered on MH agar with kanamycin. pDRH1453 was then used in PCR-mediated mutagenesis (45) to delete a large portion of the coding sequence of the gene by fusing codon 4 to codon 453, creating pDRH1843. DRH2246 was then electroporated with pDRH1843 to replace *fliI*::*kan-rpsL* with the Δ*fliI* allele to create 81-176 Sm^r Δ*astA* Δ*fliI* (DRH2257).

Generation of *flhB* mutants first involved PCR-mediated mutagenesis (45) to create a point mutation, generating an StuI site in the coding sequence of *flhB* in pDRH666 (30) to create pSNJ355. This plasmid was then digested with StuI so that a *cat-rpsL* cassette generated by digestion of pDRH265 (28) with SmaI could be inserted with *flhB*. The resulting plasmid, pSNJ360, was then introduced into DRH461 (81-176 Sm^r Δ*astA* [(30]) by electroporation, replacing *flhB* with *flhB*::*cat-rpsL* to generate SNJ404 (81-176 Sm^r Δ*astA flhB*::*cat-rpsL*). PCR-mediated mutagenesis (45) with pDRH666 was used to generate point mutations and in-frame deletions within *flhB*. These mutations and the resulting plasmids included *flhB*(*N267A*) (pSNJ238), $flhB_{\Delta 214\text{-}218}$ (pSNJ243), $flhB_{\Delta 224\text{-}228}$ (pSNJ236), and $flhB_{\Delta 244\text{-}253}$ (pSNJ237). These plasmids were introduced into SNJ404 by electroporation to replace the *flhB*::*cat-rpsL* allele with the different *flhB* alleles. Mutants were recovered on MH agar with streptomycin. The resulting strains included SNJ438 [81-176 Sm^r Δ*astA flhB*(*N267A*)], SNJ464 (81-176 Sm^r Δ*astA* $flhB_{\Delta 214\text{-}218}$), SNJ428 (81-176 Sm^r Δ*astA* $flhB_{\Delta 224\text{-}228}$), and SNJ475 (81-176 Sm^r Δ*astA* $flhB_{\Delta 244\text{-}253}$). Mutants were verified by PCR and DNA sequencing.

To construct strains containing transcriptional reporters, plasmids pDRH532 (containing *flgDE2*::*nemo*), pDRH608 (containing *flaA*::*astA*), pDRH610 (containing *flaB*::*astA*), and pDRH669 (containing *flgD*::*astA*) were electroporated into *C. jejuni* to replace the native *flgDE2*, *flaA*, *flaB*, and *flgD* loci on the chromosome as previously described (30, 59). All mutants were recovered on MH agar containing kanamycin and were verified by PCR analysis.

Generation of polyclonal antiserum against *C. jejuni* proteins. Generation of polyclonal murine antiserum against the RNA polymerase subunit A (RpoA) protein of *C. jejuni* involved first constructing primers with 5′ BamHI sites to amplify the coding sequence from codon 2 through the stop codon of *rpoA* from *C. jejuni* strain 81-176 (31). Ligation of this DNA fragment into the BamHI site of pQE30 (Qiagen) and transformation into *E. coli* XL1-Blue allowed recovery of pDRH2907, which encodes a His_6-RpoA fusion protein. To purify the protein, a 1-liter culture in LB broth was grown to an optical density at 600 nm (OD_{600}) of 0.5, and then the culture was induced for 4 h with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The bacteria were disrupted by two passages through an EmulsiFlex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2. The protein was purified under native conditions from the soluble fraction with Ni-nitrilotriacetic acid agarose according to the manufacturer's instructions. Polyclonal murine antiserum was generated in mice by standard procedures using a commercial vendor (Cocalico Biologicals).

Detection of FlhB in *C. jejuni* required generation of rabbit polyclonal antiserum against the cytoplasmic domain of the protein. Because this portion of FlhB in *Salmonella enterica* serovar Typhimurium undergoes autoproteolytic processing between the asparagine and proline residues at positions 269 and 270 (19, 21, 48), we attempted to create a soluble, more stable protein to immunize rabbits for antiserum generation. We first used PCR-mediated mutagenesis (45) with pDRH666 (containing the wild-type *flhB* allele [30]) to change the codons for asparagine and proline at positions 267 and 268, respectively, to codons for alanines to generate pDRH2339. After construction of pDRH2339, we amplified a portion of the *flhB*(*N267A P268A*) sequence encoding amino acids 209 through 369 that encompasses the predicted entire unprocessed cytoplasmic domain of the protein. Primers were used in PCR so that in-frame BamHI sites were added to the 5′ end of the amplified product. The DNA was then cloned into BamHI-digested pGEX-4T-2 (GE Healthcare) in the correct orientation to produce a glutathione *S*-transferase (GST)-$FlhB_{cyto}$(N267A P268A) fusion protein. The resulting plasmid was designated pDRH2367 and used to transform BL21(DE3). The resulting strain was grown in 3 liters of LB broth to mid-log phase and then induced with 25 mM IPTG for 3 h at 37°C. The bacteria were harvested and disrupted with an EmulsiFlex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2. The soluble fraction was obtained by removing the insoluble material by centrifugation at 13,000 rpm for 2 h at 4°C. The soluble material was rocked with 2.4 ml of glutathione Sepharose 4B (GE Healthcare) for 30 min at room temperature. The protein was then purified according to the manufacturer's instructions. Despite our attempt to create a more stable, unprocessed version of the cytoplasmic domain of FlhB fused to GST, about one-third of the recovered purified protein had a molecular mass of approximately 29-kDa after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis, which correlates with the size of GST rather than 46 kDa, the predicted size of the full-length fusion protein. Therefore, this protein was only partially stable. The purified products were used to immunize rabbits by standard procedures for antiserum generation using a commercial vendor (Cocalico Biologicals).

Immunoblot analyses of FlgS, FlgR, FlhB, and FlaA proteins. *C. jejuni* strains were grown from frozen stocks on MH agar containing appropriate antibiotics at 37°C for 48 h and restreaked 16 h prior to use. SDS-PAGE and immunoblotting of FlgS and FlgR proteins were performed as previously described with anti-FlgS Rab11 and anti-FlgR Rab13 rabbit polyclonal antisera, respectively (25). Briefly, cells were resuspended from 16-h growth plates in MH broth and diluted to an OD_{600} of 0.8. One-milliliter samples were harvested by centrifugation and washed once with phosphate-buffered saline. For whole-cell lysates (WCLs), the pellet was resuspended in 50 μl 1× Laemmli buffer, and 4 μl (for FlgR analysis) or 7 μl (for FlgS analysis) of each resuspended pellet was loaded onto 10% SDS-PAGE gels.

For FlgS localization studies, 5-ml portions of cultures of wild-type and mutant strains at an OD_{600} of 0.8 were prepared as described above, resuspended in 10 mM HEPES (pH 7.4), and broken by sonication. Unbroken cells were removed by centrifugation at 13,000 × *g* for 5 min at 4°C, and the supernatant was transferred to a new tube and centrifuged at 13,000 × *g* for 30 min at 4°C to pellet the total membrane fraction (outer and inner membrane proteins). The supernatant contained soluble proteins (cytoplasmic and periplasmic proteins). Volumes representing equivalent cell numbers for the membrane and soluble proteins were analyzed by 10% SDS-PAGE after resuspension and boiling in 1× Laemmli buffer. For detection of FlgS, anti-FlgS Rab11 antiserum was used at a dilution of 1:10,000 (25). To detect proteins representative of the cytoplasmic fraction or inner membrane fraction, we analyzed the location of the RpoA cytoplasmic protein and the AtpF inner membrane protein by using anti-RpoA M59 antiserum at a dilution of 1:2,000 and anti-AtpF M3 antiserum at a dilution of 1:1,000 (4), followed by a goat anti-mouse secondary antibody.

To monitor the stability and location of FlhB proteins, bacteria were grown and 5-ml samples of cultures of wild-type and mutant strains were prepared and sonicated as described above. The total membrane fraction, containing inner and outer membrane proteins, was recovered by centrifugation at 13,000 rpm for 30 min at 4°C. The recovered pellet was suspended in 50 μl of 1× Laemmli buffer and loaded onto a 10% SDS-PAGE gel for immunoblot analysis. Primary anti-FlhB Rab476 antiserum was used at a dilution of 1:1,000 and was rocked with the membrane overnight at 4°C. The blot was then washed and incubated with a 1:10,000 dilution of goat anti-rabbit secondary antibody for 4 h at room temperature.

For analysis of FlaA secretion in *C. jejuni* strains, bacteria were grown and resuspended from plates as described above. WCLs from 1-ml portions of cultures of wild-type and mutant strains were prepared as described above. For recovery of outer membrane proteins, 5-ml cultures of each bacterial strain were prepared and sonicated, and the unbroken cells were removed by centrifugation at 13,000 rpm for 5 min at 4°C. Each supernatant was recovered and spun at 13,000 rpm for 30 min at 4°C. The pellet containing insoluble material representing the total membrane proteins (inner and outer membrane proteins) was resuspended in 1% *N*-laurylsarcosine (sodium salt) and incubated for 30 min at room temperature to solublize the inner membrane proteins. The outer membrane proteins were recovered as the insoluble pellet after centrifugation at 13,000 rpm for 30 min at 4°C. Volumes of sample corresponding to 200 μl and 700 μl of bacteria were loaded for analysis of WCLs and outer membrane proteins, respectively. Immunoblot analysis was performed with a 1:10,000 dilution of anti-FlaA LLI antiserum (42) and a 1:10,000 dilution of goat anti-rabbit secondary antibody.

Motility assays and transmission electron microscopy. To analyze relative levels of motility, strains were grown on MH agar with TMP from freezer stocks for 48 h at 37°C under microaerobic conditions and then restreaked and grown for 16 h prior to use. Cells were resuspended in MH broth to an OD_{600} of 0.8, and a sterile needle was used to inoculate semisolid MH motility agar as described previously (28). Plates were incubated under microaerobic conditions for 24 to 36 h at 37°C and photographed. For transmission electron microscopy, 1-ml samples of bacteria in MH broth at an OD_{600} of 1.0 were centrifuged at 13,000 rpm for 3 min and then resuspended in 2% glutaraldehyde. After incubation for 1 h on ice, samples were stained with 1% uranyl acetate and visualized with an FEI Technai G2 Spirit BioTWIN transmission electron microscope.

Arylsulfatase reporter assays. Strains were grown from frozen stocks for 48 h at 37°C under microaerobic conditions on MH agar with TMP or kanamycin and restreaked and grown for 16 h prior to the assay. Strains were analyzed for arylsulfatase activity by a previously described method (30), which was based on previously established methods (24, 67). Briefly, all strains were resuspended in phosphate-buffered saline to an OD_{600} of 0.8 to 1.0, washed in arylsulfatase assay buffer, and incubated with 10 mM nitrophenylsulfate and 1 mM tyramine for 1 h at 37°C. NaOH was added to terminate the assays, and the amount of nitrophe-

nol present in each sample was determined spectrophotometrically at OD_{410}. The number of arylsulfatase units produced by each strain was calculated by comparing the OD_{410} value of each sample to a standard curve obtained using known nitrophenol concentrations. One arylsulfatase unit is defined as the amount of enzyme catalyzing the release of 1 nmol of nitrophenol per h per OD_{600} unit. Each strain was tested in triplicate, and each assay was performed three times.

Purification of FlgS and FlgR proteins. Wild-type His_6-FlgR protein was purified as previously described (35). *flgS*(*H141A*) from pDRH1276 was amplified from codon 2 to the stop codon by PCR using primers that added in-frame 5′ and BamHI restriction sites to facilitate cloning into BamHI-digested pQE30 to generate pSNJ960. This plasmid was then transformed into XL1-Blue for induction and purification of the protein. Wild-type His_6-FlgS and His_6-FlgS(H141A) were purified by using previously described protocols (25).

Autophosphorylation of FlgS. FlgS autophosphorylation assays were performed as described previously using purified His_6-FlgS or His_6-FlgS(H141A) in the presence of [γ-^{32}P]ATP (35, 66). Briefly, 6 pmol His_6-FlgS or His_6-FlgS(H141A) was added to a buffer containing 50 mM Tris-HCl (pH 8.0), 75 mM KCl, 2 mM $MgCl_2$, and 1 mM dithiothreitol. Ten microcuries of [γ-^{32}P]ATP was then added. At each time point, a sample was removed and the reaction was stopped by addition of an equal amount of 2× SDS-PAGE loading buffer. Proteins were resolved by 10% SDS-PAGE, and the gels were dried and exposed to a phosphorimager screen. The screen was read with a Storm 820 phosphorimager (Amersham Biosciences), and the data were analyzed using the manufacturer's software.

FlgR phosphorylation. In vitro phosphotransfer from His_6-FlgS proteins to His_6-FlgR was monitored as previously described (35, 66). For each reaction, 6 pmol of His_6-FlgR was added to 6 pmol of His_6-FlgS or His_6-FlgS(H141A) that had been allowed to autophosphorylate for 2 min as described above. Reactions were stopped by addition of an equal volume of 2× SDS-PAGE loading buffer, and the samples were analyzed by SDS-PAGE. After drying, polyacrylamide gels were analyzed with a phosphorimager.

Real-time RT-PCR. *C. jejuni* strains 81-176 Sm^r (DRH212), 81-176 Δ*flhA* (DRH946), 81-176 Δ*flhB* (SNJ471), and 81-176 Δ*fliP* (DRH1065) were grown from frozen stocks on MH agar containing appropriate antibiotics at 37°C for 48 h and restreaked 16 h prior to use (28, 30). Bacteria were suspended from the agar plates in MH broth, and total RNA was extracted from the bacteria with Trizol reagent (Invitrogen). The RNA was then treated with DNase prior to analysis. The final concentration of RNA used in a Sybr green PCR master mixture was 50 ng/μl. Real-time reverse transcription (RT)-PCR was performed using a 7500 real-time PCR system (Applied Biosystems). Detection of mRNA for *gyrA*, encoding DNA gyrase, served as an endogenous control, and the transcript levels of *flgS* and *flgR* in mutants (lacking *flhA*, *flhB*, or *fliP*) were compared to those in the wild-type strain (DRH212). The following primer pairs were used for real-time RT-PCR analysis: *flgS* RT#1 (5′-GCTACAGATATTA GCGATGAAAAACG-3′) and *flgS* RT#2 (5′-TAGGATTTCTTATCTCATGT GCCAAAT-3′), *flgR* RT#3 (5′-TCAAGCCAAACTTTTAAGAGCTTTG-3′) and *flgR* RT#4 (5′-CTATTTTGATGCTTTTCGTACTTCCA-3′), and *gyrA* F (5′-CGACTTACACGGCCGATTTC-3′) and *gyrA* R (5′-ATGCTCTTTGCAG TAACCAAAAAA-3′).

Transposon mutagenesis. Chromosomal DNA from *C. jejuni* 81-176 Δ*astA* Δ*flhA flgDE2*::*nemo* (DRH1021 [30]), 81-176 Δ*astA* Δ*flhB flgD*::*astA* (SNJ331), and 81-176 Δ*astA* Δ*fliP flaB*::*astA* (DRH1178 [30]) was purified and subjected to in vitro random transposon mutagenesis with the *darkhelment* transposon by using previously published protocols (27–30). Twelve in vitro transposon mutagenesis reactions were performed with DNA from each strain. Each reaction mixture contained 2 μg of chromosomal DNA, 1 μg of pSpaceball1, and 250 ng of *Himar1* C9 transposase purified from DH5α/pMalC9 (1). After transposition, the mutagenized DNA was repaired and transformed into each strain as previously described (28). Transposon mutants were recovered after growth on MH agar containing chloramphenicol and 5-bromo-4-chloro-3-indolyl sulfate and then examined for blue or white colony phenotypes.

RESULTS

FlgS is a cytoplasmic protein. Bioinformatic analysis suggests that unlike most sensor kinases that are localized to the bacterial inner membrane, the *C. jejuni* FlgS sensor kinase is a cytoplasmic protein. This protein lacks both a predicted signal sequence that would target it for secretion and hypothetical spans of hydrophobic residues that would be indicative of a protein associated with the inner membrane. To determine if FlgS is localized to the cytoplasm, we fractionated wild-type *C. jejuni* 81-176 Sm^r (DRH212 [28]) to obtain a soluble fraction containing cytoplasmic and periplasmic proteins and an insoluble fraction containing proteins associated with the outer and inner membranes. As shown in Fig. 1, FlgS was found only in WCLs and the soluble fraction of wild-type bacteria. In a comparison with the control proteins, FlgS was present in the same fraction as the soluble cytoplasmic protein RpoA and absent in the fraction containing the insoluble inner membrane protein AtpF. Considering both the bioinformatic and biochemical analyses, we concluded that FlgS is a cytoplasmic protein (Fig. 1).

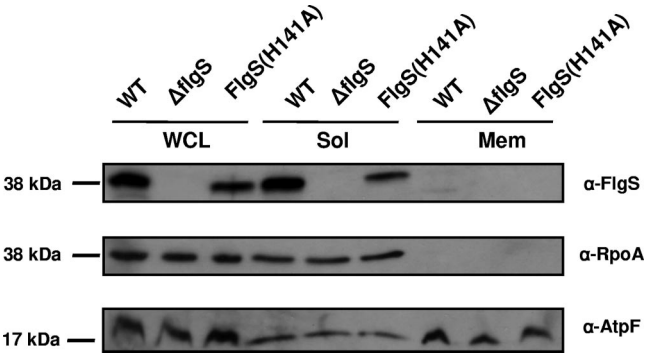

FIG. 1. Localization and stability of FlgS proteins in *C. jejuni*. Wild-type strain *C. jejuni* 81-176 Sm^r (DRH212) (WT), 81-176 Sm^r Δ*flgS* (DRH460), and 81-176 Sm^r *flgS*(*H141A*) (DRH1323) were grown, and protein samples were obtained from the WCL, the soluble fraction (Sol), and the insoluble membrane fraction (Mem) after sonication. Anti-FlgS Rab11 antiserum (α-FlgS) was used to detect FlgS proteins (25). Anti-RpoA M59 antiserum (α-RpoA) and anti-AtpF M3 antiserum (α-AtpF) were used to detect the soluble cytoplasmic RpoA protein and the insoluble inner membrane protein AtpF, respectively (4).

Autophosphorylation of residue H141 is required for FlgS activity. It has been shown that the autophosphorylation site of the NtrB sensor kinase is residue H139 (52). Alignment of FlgS to NtrB indicated that this phosphorylated residue likely corresponds to H141 of FlgS, an amino acid located within the putative phosphotransfer domain (spanning amino acids 131 to 195) that receives the phosphate group upon autophosphorylation of other kinases. To determine if H141 is essential for FlgS activity as a kinase and for flagellar gene expression, the wild-type *flgS* allele of *C. jejuni* was replaced with *flgS*(*H141A*), which results in production of FlgS with alanine at position 141 instead of histidine. The resulting mutant was first examined for a potential defect in FlgS stability. We found that while FlgS(H141A) appears to lack any detectable degradation products, the levels of the FlgS(H141A) protein present in WCLs and the soluble fraction were about one-half the levels of the wild-type FlgS (Fig. 1). By comparing the phenotypes of the wild-type and mutant strains, we found that the *flgS*(*H141A*) mutation affected motility, flagellar biosynthesis, and σ^{54}-dependent flagellar gene expression (Fig. 2 and data not shown). The nonmotile phenotype of the *flgS*(*H141A*) mutant on semisolid agar plates at 24 h after inoculation was similar to that observed for a Δ*flgS* strain in which *flgS* had been deleted from the chromosome (Fig. 2A and data not shown) (30). This lack of motility in the *flgS*(*H141A*) mutant correlated with a com-

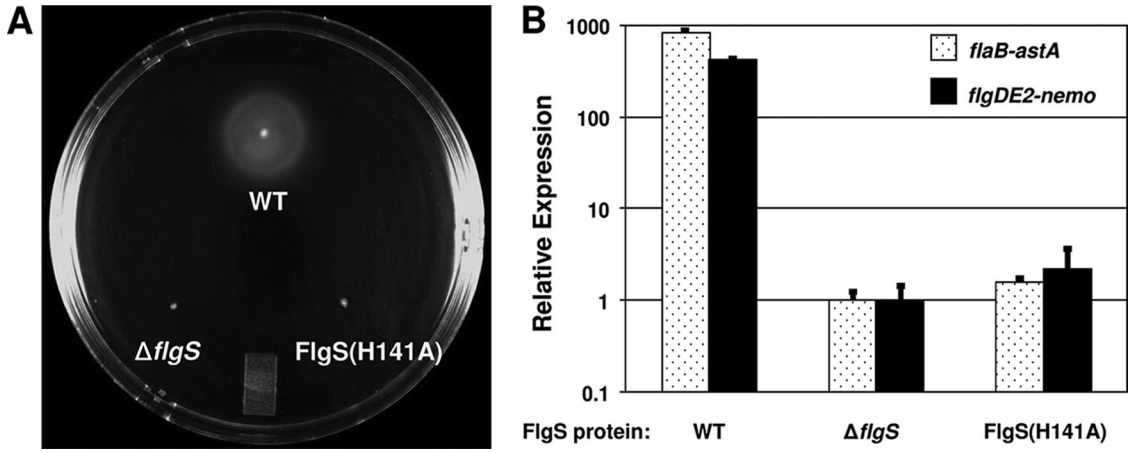

FIG. 2. Phenotypic analyses of *C. jejuni* wild-type and *flgS*(*H141A*) mutant strains. (A) Motility phenotypes of *C. jejuni* strains producing wild-type or mutant FlgS proteins in MH semisolid agar 24 h after inoculation. The strains used included wild-type strain 81-176 Sm^r (DRH212) (WT), 81-176 Sm^r Δ*flgS* (DRH460), and 81-176 Sm^r *flgS*(*H141A*) (DRH1323). (B) Arylsulfatase assays for analysis of expression of *flaB*::*astA* and *flgDE2*::*nemo* in *C. jejuni* 81-176 derivatives producing wild-type and FlgS mutant proteins. The results are the results of a typical assay in which each strain was tested in triplicate. The values reported for each strain are the average arylsulfatase activity ± standard deviation relative to the level of expression of each transcriptional fusion in 81-176 Sm^r Δ*astA* Δ*flgS*, which was defined as 1 arylsulfatase unit. For expression of *flaB*::*astA*, the strains used included wild-type strain DRH665 (81-176 Sm^r Δ*astA flaB*::*astA*) (WT), DRH939 (81-176 Sm^r Δ*astA* Δ*flgS flaB*::*astA*), and SNJ958 [81-176 Sm^r Δ*astA flgS*(*H141A*) *flaB*::*astA*]. For expression of *flgDE2*::*nemo*, the strains used included wild-type strain DRH533 (81-176 Sm^r Δ*astA flgDE2*::*nemo*) (WT), DRH936 (81-176 Sm^r Δ*astA* Δ*flgS flgDE2*::*nemo*), and SNJ956 [81-176 Sm^r Δ*astA flgS*(*H141A*) *flgDE2*::*nemo*]. The FlgS proteins produced by the strains are indicated below the graph.

plete absence of flagella as analyzed by transmission electron microscopy (data not shown). We then analyzed expression of *flgDE2*- and *flaB-astA* transcriptional fusions in strains producing the FlgS(H141A) protein and found that the level of σ^{54}-dependent flagellar gene expression in an *flgS*(*H141A*) mutant was equivalent to that in a Δ*flgS* mutant (Fig. 2B), indicating that H141 is critical for proper function of FlgS in *C. jejuni*.

Since H141 is the predicted site of phosphorylation, we performed autophosphorylation assays with purified His_6-tagged versions of FlgS and FlgS(H141A). Whereas FlgS autophosphorylated and accumulated radiolabeled phosphate over time, FlgS(H141A) remained unphosphorylated (Fig. 3A and 3B). In previous work, we showed that the FlgR response regulator is modified by phosphorylation in the presence of purified FlgS in vitro (35). We performed similar experiments to determine if phosphotransfer to FlgR was abolished in the presence of FlgS(H141A). In these experiments, we observed phosphorelay to FlgR in the presence of wild-type FlgS but not in the presence of the FlgS(H141A) protein (Fig. 3C), consistent with the hypothesis that autophosphorylation of FlgS on H141 contributes to phosphotransfer to FlgR. Thus, we believe that H141A is the most likely site of autophosphorylation and is essential for proper function of the protein.

Production of FlgS and FlgR is not dependent on the presence of the FEA. The FEA is a multiprotein complex that translocates flagellar subunits across the inner membrane for incorporation into a functional organelle (for a review, see reference 44). As mentioned above, many of the FEA components (e.g., FlhA, FlhB, FliP, and FliR) in addition to FlgS and FlgR are required for σ^{54}-dependent flagellar gene expression in *C. jejuni* (30). We next performed experiments to determine if the FEA and FlgSR systems are linked together in a regulatory cascade that terminates in activation of expression of σ^{54}-dependent flagellar genes. More specifically, we investi-

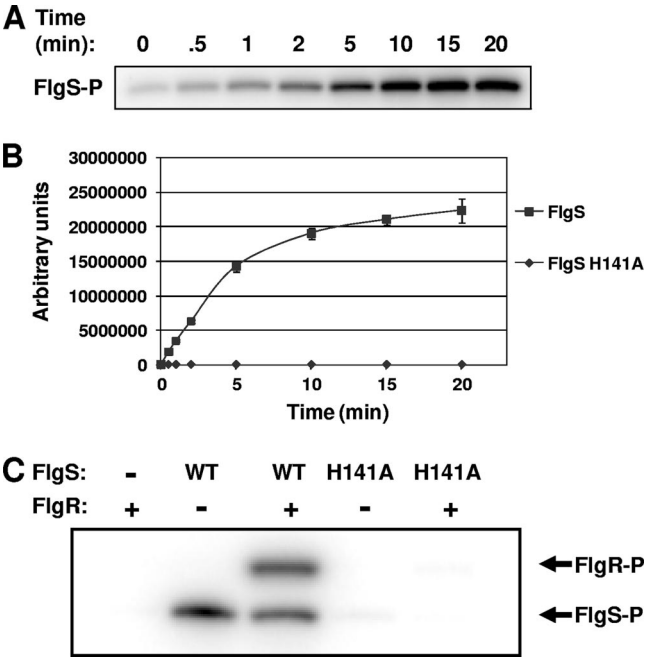

FIG. 3. Autophosphorylation of FlgS proteins and phosphorelay to FlgR. (A and B) Analysis of autophosphorylation of His_6-FlgS and His_6-FlgS(H141A) over time after incubation of proteins with [γ-^{32}P]ATP. (A) Representative gel analyzed by autoradiography from an FlgS autophosphorylation assay. (B) Relative quantification of autophosphorylation of FlgS proteins as determined by densitometry after autoradiography of gels. Three separate FlgS and FlgS(H141A) autophosphorylation assays were performed, and the results of these assays were averaged. The amount of incorporation of ^{32}P is expressed in arbitrary units based on the densitometric analysis. (C) Analysis of phosphorelay to His_6-FlgR from His_6-tagged FlgS or FlgS(H141A) protein. FlgS proteins were preincubated with [γ-^{32}P]ATP before addition of His_6-FlgR. A representative gel analyzed by autoradiography from a phosphotransfer assay is shown. The presence (+) or absence (−) of FlgR and the FlgS protein used in each reaction are indicated above the lanes. WT, wild type.

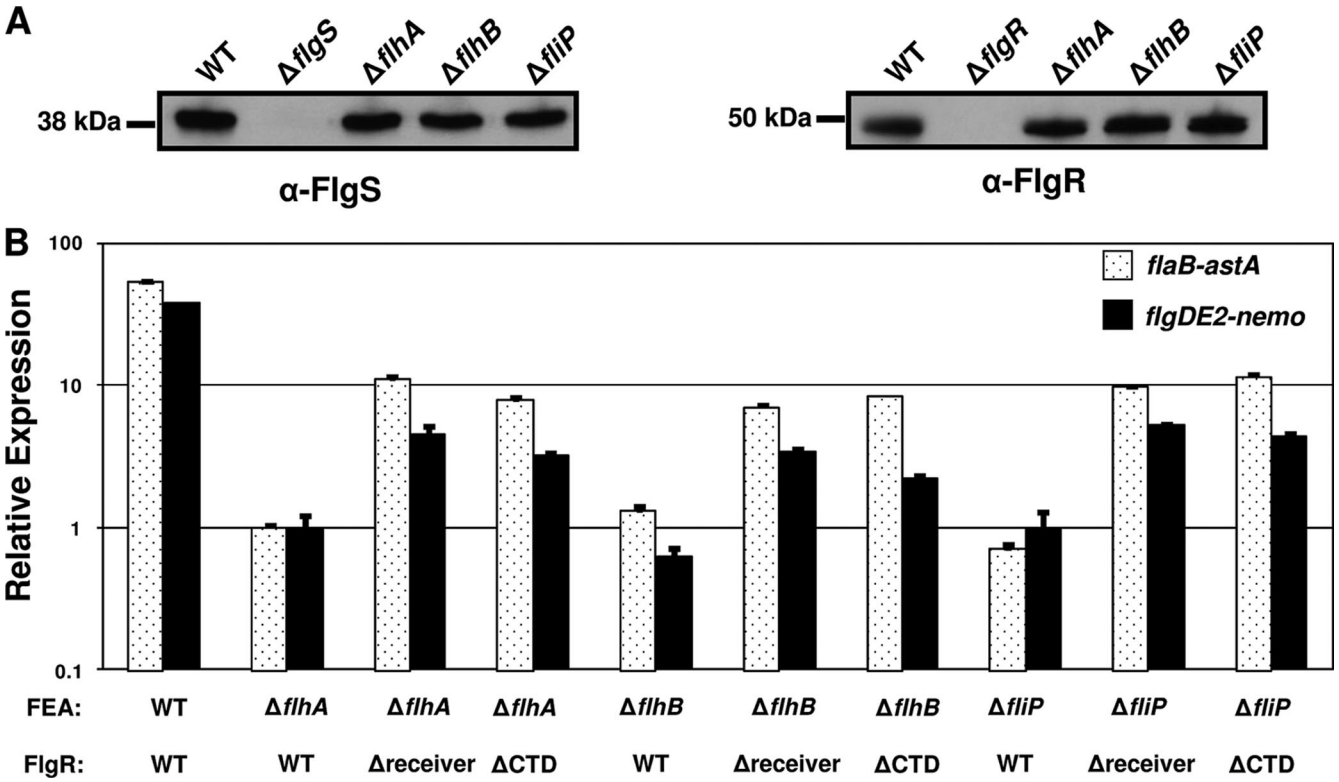

FIG. 4. Production of FlgS and FlgR and activity of FlgR proteins in FEA mutants of *C. jejuni*. (A) Production of FlgS and FlgR proteins in mutants of *C. jejuni* lacking one component of the FEA. WCLs of wild-type and *C. jejuni* mutant strains were prepared for immunoblot analysis. Anti-FlgS Rab11 (α-FlgS) and anti-FlgR (α-FlgR) Rab13 antisera were used to detect the FlgS (left gel) and FlgR (right gel) proteins (25). The strains used for analysis included wild-type strain DRH212 (81-176 Sm^r) (WT), DRH460 (81-176 Sm^r Δ*flgS*), DRH737 (81-176 Sm^r Δ*flgR*), DRH946 (81-176 Sm^r Δ*flhA*), SNJ471 (81-176 Sm^r Δ*flhB*), and DRH1065 (81-176 Sm^r Δ*fliP*). (B) Arylsulfatase assays for analysis of expression of *flaB*::*astA* and *flgDE2*::*nemo* in the *C. jejuni* 81-176 Sm^r wild-type strain and mutant strains lacking a component of the FEA and producing wild-type and FlgR mutant proteins. The results are the results of a typical assay in which each strain was tested in triplicate. The values reported for each strain are the average arylsulfatase activity ± standard deviation relative to the level of expression of each transcriptional fusion in 81-176 Sm^r Δ*astA* Δ*flhA*, which was defined as 1 arylsulfatase unit. For expression of *flaB*::*astA*, the strains used included (from left to right) wild-type strain DRH665, DRH1049, SNJ112, SNJ273, DRH1827, SNJ109, SNJ1021, DRH1178, SNJ261, and SNJ1015. For expression of *flgDE2*::*nemo*, the strains used included (from left to right) wild-type strain DRH533, DRH1021, SNJ115, SNJ274, DRH1827, SNJ113, SNJ1017, DRH1204, SNJ358, and SNJ1012. The FEA mutation and the type of FlgR protein produced in each strain are indicated below the graph. WT, wild type.

gated whether the FEA influences the production or activity of the FlgSR two-component system.

To examine if production of FlgS or FlgR is dependent on the FEA, we performed an immunoblot analysis of cell lysates from the wild-type strain and mutant strains lacking *flhA*, *flhB*, and *fliP*, which encode some of the proteins comprising the FEA. We observed similar levels of FlgS and FlgR in the wild-type strain and the FEA mutants (Fig. 4A), indicating that production of FlgS and FlgR is independent of the FEA. As additional verification that the FEA does not affect production of FlgS and FlgR, we compared the levels of the *flgS* and *flgR* mRNA transcripts in mutants lacking *flhA*, *flhB*, and *fliP* to the levels in the wild-type strain by real-time RT-PCR analysis. We did not detect significant changes in the levels of the *flgS* or *flgR* mRNAs in the mutant strains compared to wild-type bacteria (data not shown). Therefore, FEA mutants of *C. jejuni* appear to produce normal levels of the FlgS and FlgR proteins but have defects in signaling pathways for stimulation of σ^{54}-dependent flagellar gene expression.

We next analyzed *C. jejuni* to determine if the FlgSR system functions downstream of the FEA in a regulatory cascade to activate expression of σ^{54}-dependent flagellar genes. Previous work in our laboratory generated *flgR* alleles encoding proteins lacking the N-terminal receiver or C-terminal domain of the response regulator (35). These proteins were shown to have partial constitutive activity in the absence of the FlgS sensor kinase, indicating that FlgR functions downstream of FlgS (35). We used these *flgR* alleles ($flgR_{\Delta receiver}$ and $flgR_{\Delta CTD}$) to replace wild-type *flgR* on the chromosome of mutants lacking *flhA*, *flhB*, or *fliP* to determine if these partially constitutively active FlgR proteins suppress the phenotype of the FEA mutants for expression of flagellar genes. As shown previously (30) and in Fig. 4B, *flhA*, *flhB*, or *fliP* mutants containing wild-type *flgR* and producing the wild-type protein expressed 40- to 50-fold less of the σ^{54}-dependent *flaB*- and *flgDE2-astA* transcriptional fusions. When *flgR* in these FEA mutants was replaced with the *flgR* alleles encoding $FlgR_{\Delta receiver}$ and $FlgR_{\Delta CTD}$, partial restoration of σ^{54}-dependent flagellar gene expression was observed (Fig. 4B). Although the levels of expression were not restored to wild-type levels, they were approximately 5- to 10-fold higher than those in the FEA mutants that produced wild-type FlgR. These analyses suggest that

FlgSR functions downstream of the FEA and that activation of FlgSR is dependent in some manner on the FEA of *C. jejuni*.

Formation of the FEA likely initiates activation of the FlgSR system. Considering our data, we speculated that the FEA may contribute an essential signal to activate the FlgSR system to terminate in expression of σ^{54}-dependent flagellar genes. We hypothesized that either formation of the FEA or the secretory activity of the FEA may comprise the signal to activate the FlgS sensor kinase. If the former hypothesis is correct, it is possible that positioning one component of the FEA or the whole FEA complex in the inner membrane may directly provide the signal sensed directly by the cytoplasmic FlgS protein, leading to autophosphorylation of the kinase. Alternatively, formation of the FEA may be required for production of a downstream signal sensed by FlgS. The latter hypothesis includes the possibility that the secretory activity of a formed FEA may influence activation of FlgS. For instance, a negative regulator that represses activity of FlgS may be present in the cell before the FEA is competent for secretion, and the secretory activity of the FEA may be required to inactivate or remove this protein from the cytoplasm, relieving FlgS from repression and allowing autophosphorylation and phosphorelay to FlgR to occur.

To distinguish between these possibilities, we generated mutants with FEA complexes that are predicted to assemble in the inner membrane but are hindered for secretion of flagellar substrates. For this approach, we targeted *fliI* and *flhB* for mutation. FliI functions as an ATPase that dissociates export substrates (e.g., flagellins) from their chaperones in *S. enterica* serovar Typhimurium (49, 55). While FliI is not absolutely required for secretion of flagellar substrates, its absence substantially reduces the efficiency of this process. Due to the significant homology between the FliI proteins of *C. jejuni* and *S. enterica* serovar Typhimurium strain LT2 (43% identity and 62% similarity over 424 amino acids), we hypothesize that FliI serves a similar function in *C. jejuni* in increasing the efficiency of secretion of flagellar proteins. Therefore, we deleted *fliI* from the *C. jejuni* genome to create a mutant with possibly impaired efficiency of FEA-mediated secretion of flagellar proteins.

Previous analysis with *S. enterica* serovar Typhimurium revealed that defined mutations can also be made in *flhB* so that the FEA assembles in the inner membrane, but secretion of substrates through the FEA is reduced or blocked (21). These mutations include mutations that result in small, in-frame deletions and point mutations in the FlhB protein. By aligning the sequences of the *S. enterica* serovar Typhimurium and *C. jejuni* strain 81-176 proteins (which are 36% identical and 60% similar across 351 amino acids), we identified regions of the FlhB protein of *C. jejuni* that may be deleted or mutated, resulting in FEA mutants that form but do not secrete efficiently. To this end, we constructed *flhB* mutant alleles that encoded $\text{FlhB}_{\Delta 214\text{-}218}$, $\text{FlhB}_{\Delta 224\text{-}228}$, $\text{FlhB}_{\Delta 244\text{-}253}$, and FlhB(N267A) mutant proteins. The deletions and mutations in the *C. jejuni* FlhB protein correspond to types of domain deletions and point mutations resulting in the FlhBΔ2, FlhBΔ4, FlhBΔ8-9, and FlhB(N269A) proteins of *S. enterica* serovar Typhimurium constructed by Fraser et al. (21), respectively.

After construction of *fliI* and *flhB* mutants of *C. jejuni*, we first analyzed the strains to determine stability of the FlhB protein produced in each mutant by immunoblot analysis. FlhB is produced as a 42-kDa protein in *S. enterica* serovar Typhimurium that is cleaved to a 31-kDa protein by autoproteolysis of the peptide bond between positions N269 and P270 (19, 21, 48). Although *flhB* of *C. jejuni* appears to encode a 37-kDa protein, we predict that similar processing may occur between N267 and P268, resulting in a 30-kDa FlhB protein. Immunoblot analysis of the total membrane fraction of wild-type *C. jejuni* revealed that FlhB appeared as the processed 30-kDa protein (Fig. 5A). In three of the four *fliI* and *flhB* mutants, we observed similar levels of processed FlhB proteins, indicating that the mutant FlhB proteins were stable. The *flhB*(*N267A*) mutant was expected to produce an FlhB protein that is not able to undergo autoproteolytic processing. Indeed, we observed only the full-length 37-kDa protein in this mutant (Fig. 5A). In the $flhB_{\Delta 244\text{-}253}$ mutant, we could not detect any mutant FlhB protein. The reason for the lack of detection of this mutant form of FlhB remains unknown, but it may be due to the method used to generate the anti-FlhB antiserum. The antigen that was used to make the anti-FlhB antiserum contained amino acids 209 to 367 of FlhB, which form the complete cytoplasmic domain of the protein before processing. Due to predicted processing of FlhB at position 267 in *C. jejuni*, ultimately only a maximum of 58 amino acids (amino acids 209 to 267) in processed FlhB proteins are the same as the amino acids in the antigen that was used to generate the anti-FlhB antiserum. Since $\text{FlhB}_{\Delta 244\text{-}253}$ lacks 10 of the 58 amino acids of the antigen, the epitope that the anti-FlhB antiserum recognizes may have been destroyed or deleted in this protein, resulting in its lack of detection. Because the mutant producing $\text{FlhB}_{\Delta 244\text{-}253}$ stimulated expression of σ^{54}-dependent flagellar genes (see below), we believe that this protein is made and is stable but is undetectable with current reagents.

We next determined if the secretion of the *flhB* and *fliI* mutants was impaired. To do this, we performed two different analyses. We first determined if motility was reduced since motility is directly dependent on FEA-mediated secretion of flagellar proteins out of the cytoplasm to construct a flagellar organelle. For all the *flhB* and *fliI* mutants, we observed that the level of motility was $\leq$10% of that of the wild-type strain, indicating that flagellar motility and presumably secretion through the FEA had been severely impaired (Fig. 5A).

We next performed a more direct analysis of the secretion competence of the FEA in the derived mutants by monitoring FEA-dependent secretion of the FlaA flagellin protein to the outer membrane of *C. jejuni* strains. Unlike the situation in *S. enterica* serovar Typhimurium, the complete regulatory pathways that govern *flaA* expression in *C. jejuni* are not completely understood. In *S. enterica* serovar Typhimurium, σ^{28}-dependent expression of *fliC* encoding the major flagellin is repressed in FEA mutants due to cytoplasmic retention of the anti-σ^{28} factor FlgM (32, 38). In *C. jejuni*, *flaA* is expressed by a σ^{28}-dependent promoter (10, 28, 30, 66). However, evidence for expression of *flaA* and secretion of the encoded protein via the FEA to form a truncated flagellum with partial motility has been obtained for an *fliA* (encoding σ^{28}) mutant, indicating that a σ^{28}-independent promoter likely exists (28, 30, 37). Also unlike the situation in *S. enterica* serovar Typhimurium, there is evidence that *flaA* expression is only moderately decreased

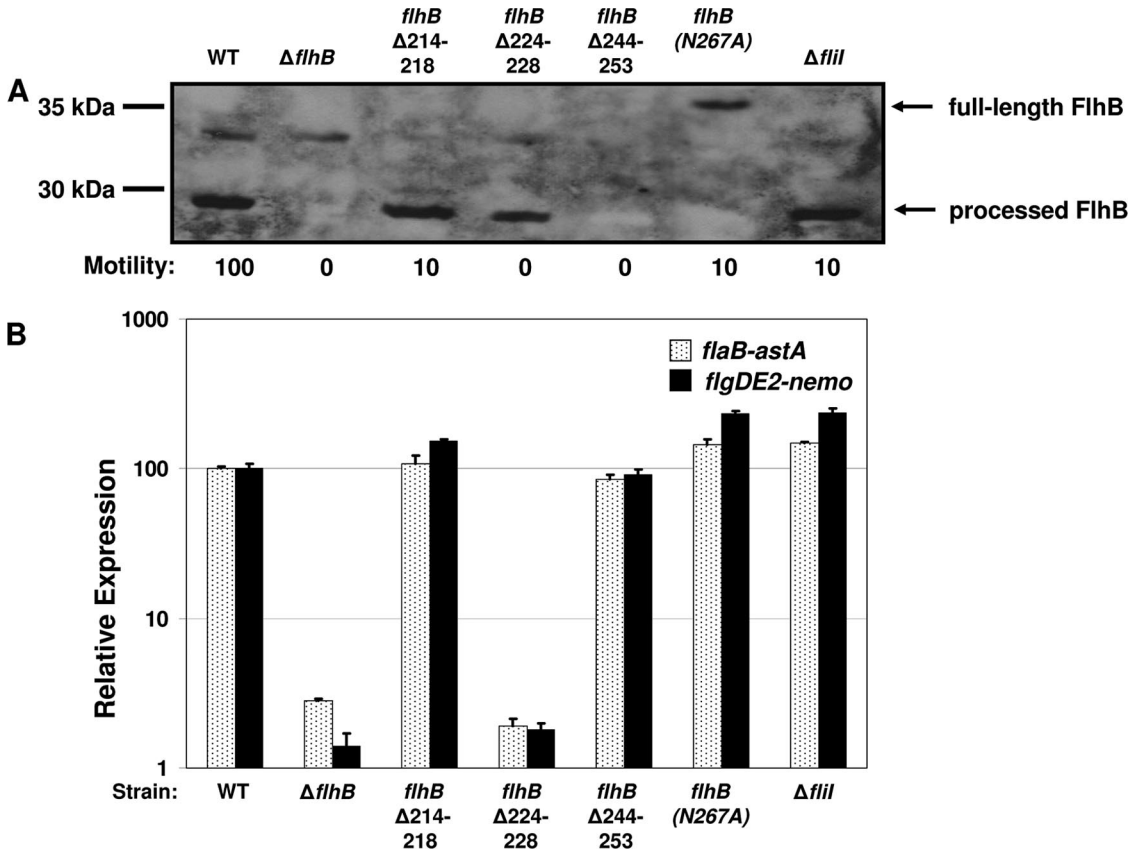

FIG. 5. Phenotypic analyses of *C. jejuni* strains with formed but secretion-impaired FEA complexes. (A) Immunoblot analysis of FlhB proteins and motility phenotypes of *C. jejuni* wild-type and *flhB* or *fliI* mutant strains. Total membrane proteins were isolated from wild-type and mutant strains of *C. jejuni*. Equal amounts of proteins from the strains were analyzed. Anti-FlhB Rab476 antiserum was used to detect the FlhB proteins. The arrows indicate the positions of the 37-kDa full-length, unprocessed FlhB protein and the 30-kDa processed FlhB protein. The motility phenotypes of wild-type and mutant strains are indicated below the blot. The diameter of the motile ring around the point of inoculation in MH semisolid agar was measured after 36 h of incubation at 37°C under microaerobic conditions. The level of motility of each mutants is expressed relative to the level of motility of the wild-type strain, which was defined as 100%. The strains used for both analyses included (from left to right) wild-type strain DRH461 (WT), DRH1734, SNJ464, SNJ428, SNJ475, and SNJ438. (B) Arylsulfatase assays for analysis of expression of *flaB*::*astA* and *flgDE2*::*nemo* in *C. jejuni* 81-176 Sm^r wild-type or mutant strains containing a secretion-impaired FEA. The results are the results of a typical assay in which each strain was tested in triplicate. The values reported for each strain are the average arylsulfatase activity ± standard deviation relative to the level of expression of each transcriptional fusion in wild-type strain 81-176 Sm^r Δ*astA*, which was defined as 100 arylsulfatase units. For expression of *flaB*::*astA*, the strains used included (from left to right) wild-type strain DRH665 (WT), DRH1830, SNJ467, SNJ434, SNJ508, SNJ442, and SNJ422. For expression of *flgDE2*::*nemo*, the strains used included wild-type strain DRH533 (WT), DRH1827, SNJ466, SNJ433, SNJ504, SNJ439, and SNJ457. The type of mutation in the FEA of each strain is indicated below the graph.

in certain FEA mutants of *C. jejuni* 81-176, indicating that some expression of *flaA* is independent of the FEA status of the bacterium (30). Furthermore, any existing translation controls for *flaA* mRNAs in *C. jejuni* have not been characterized. Since evidence that *flaA* expression and FlaA production are not entirely dependent on the status of the FEA in *C. jejuni*, as they are in other bacteria, we analyzed FEA-dependent secretion of FlaA in our defined *flhB* and *fliI* mutants.

We first ensured that *flaA* was expressed in the mutants by monitoring expression of *flaA*::*astA* in the *flhB* and *fliI* mutants. We found that *flaA*::*astA* expression was not defective in three of the mutants [$flhB_{\Delta 214\text{-}218}$, *flhB*(*N267A*), and Δ*fliI*]. Rather, the expression of *flaA*::*astA* in these mutants was approximately twofold higher than that in the wild-type strain (Fig. 6A). Expression of *flaA*::*astA* was slightly reduced in the $flhB_{\Delta 244\text{-}253}$ mutant, to approximately 75% of that in the wild-type strain. The remaining mutant, $flhB_{\Delta 224\text{-}228}$, expressed *flaA*::*astA* at a level that was 50% less than the level in the wild-type strain (Fig. 6A). The level of expression of *flaA*::*astA* in this mutant was similar to that in Δ*flhB* or Δ*fliA* (lacking σ^{28}) mutants. With the exception of the expression in the $flhB_{\Delta 224\text{-}228}$ muant, *flaA*::*astA* expression in the mutants was mostly intact or the level was higher than the level in the wild-type strain.

We next monitored FEA-mediated secretion of FlaA by comparing the levels of FlaA associated with outer membranes of wild-type and mutant strains of *C. jejuni*. As shown in Fig. 6B, the $flhB_{\Delta 214\text{-}218}$, *flhB*(*N267A*), and Δ*fliI* mutants produced comparable levels of FlaA in WCLs, but reduced levels of the protein were associated with the outer membrane compared to the outer membrane of wild-type bacteria. The most severe mutation was *flhB*(*N267A*), which caused complete lack of FlaA in the outer membrane. The other two mutants, the $flhB_{\Delta 214\text{-}218}$ and Δ*fliI* mutants, had approximately two- to five-fold reductions in the level of of FlaA associated with the outer

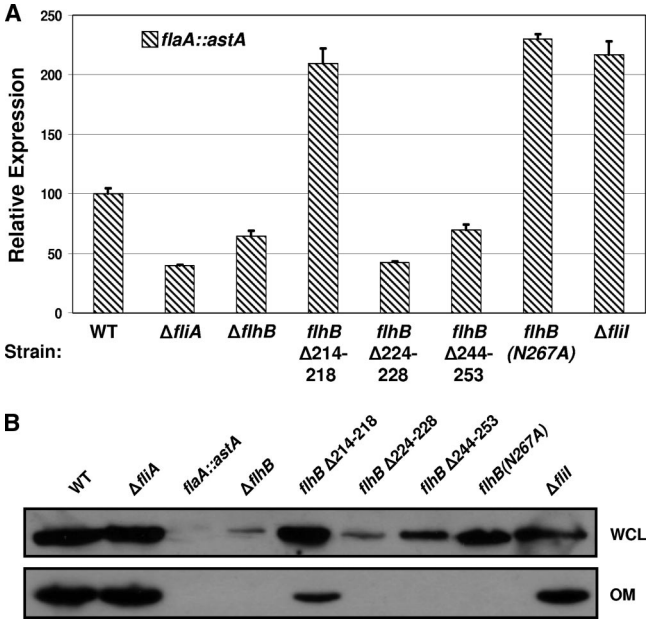

FIG. 6. Analysis of *flaA* expression and FlaA secretion mediated by the FEA. (A) Arylsulfatase assays for analysis of expression of *flaA*::*astA* in the *C. jejuni* 81-176 Sm^r wild-type strain or strains with a secretion-impaired FEA. The results are the results of a typical assay in which each strain was tested in triplicate. The values for each strain are the average arylsulfatase activity ± standard deviation relative to the level of expression of each transcriptional fusion in wild-type strain 81-176 Sm^r Δ*astA*, which was defined as 100 arylsulfatase units. For expression of *flaA*::*astA*, the strains used included (from left to right) wild-type strain DRH655 (WT), DRH1070, SNJ365, SNJ427, SNJ1033, SNJ1034, SNJ1038, and SNJ1042. The type of mutation in each strain is indicated below the graph. (B) Immunoblot analysis of FlaA production in WCLs and secretion to the outer membrane of wild-type and FEA mutant strains. WCL and outer membrane (OM) fractions were isolated from wild-type and mutant strains of *C. jejuni*. Anti-FlaA LL-1 antiserum was used to detect the FlaA proteins (42). The strains used included (from left to right) wild-type strain DRH212 (WT), DRH724, DRH655, SNJ471, SNJ464, SNJ428, SNJ475, SNJ438, and DRH2257.

membrane, suggesting that secretion had been impaired in these mutants. For the $flhB_{\Delta 244\text{-}253}$ mutant there was about threefold less FlaA in WCLs, but this mutant completely lacked FlaA in the outer membrane. Only in one mutant, the $flhB_{\Delta 224\text{-}228}$ mutant, did FlaA production appear to be greatly hindered, similar to a Δ*flhB* mutant.

Considering that four of the five mutants that we created appeared to have FEAs with greatly diminished secretion abilities, we then analyzed expression of σ^{54}-dependent flagellar genes in these mutants. In the same four mutants [$flhB_{\Delta 214\text{-}218}$, $flhB_{\Delta 244\text{-}253}$, *flhB*(*N267A*), and Δ*fliI*], expression of the *flaB*- and *flgDE2-astA* transcriptional fusions was equal to or slightly higher than the expression in the wild-type strain (Fig. 5B). These results indicate that completely blocking or hindering secretion through the FEA did not affect expression of σ^{54}-dependent flagellar genes. This analysis provided evidence that formation of the FEA, rather than secretory activity of the apparatus, is required and may be the key element to activate the FlgSR system for expression of σ^{54}-dependent flagellar genes.

Only in the mutant that produced the $FlhB_{\Delta 224\text{-}228}$ protein did we observe reduced expression of *flaB*::*astA* and *flgDE2*::*nemo* comparable to that of the Δ*flhB* mutant (Fig. 5B). Considering that this mutant also behaved similar to the Δ*flhB* mutant in terms of expression of *flaA*::*astA* and secretion of the FlaA protein, we believe that, like the Δ*flhB* mutant, this mutant may not form a complete FEA. Thus, this mutant may not actually be germane to our goal of creating secretion-incompetent but correctly formed FEAs. However, if an FEA forms in this mutant, then our alternative hypothesis that a negative regulator may be active and inhibit the FlgSR system in a nonsecreting bacterium may have some credence. To investigate this hypothesis, we performed transposon mutagenesis with the *darkhelment* transposon (27) in *C. jejuni* 81-176 Δ*astA* Δ*flhA flgDE2*::*nemo*, 81-176 Δ*astA* Δ*flhB flgD*::*astA*, and 81-176 Δ*astA* Δ*fliP flaB*::*astA*. These mutants do not express the σ^{54}-dependent transcriptional *astA* fusions due to the lack of a complete FEA. Disruption of a gene encoding a putative repressor would allow expression of the transcriptional reporters in the FEA mutants. Such a transposon mutant could be identified by recovering mutants on media containing a chromomeric substrate for arylsulfatase and observing a switch from a white colony phenotype to a blue colony phenotype. Despite screening over 65,000 transposon mutants, we were unable to identify any mutant with a transposon that disrupted a gene for such a negative regulator, suggesting that such a gene may not exist or is an essential gene. Considering these data as a whole, we propose that FlgSR activation likely depends on proper assembly of the FEA. While we cannot entirely exclude the possibility that the secretory activity is required for FlgSR activation, our results indicating that four of five *flhB* or *fliI* mutants were impaired for secretion but had mutations that did not affect expression of σ^{54}-dependent flagellar genes, coupled with the results of our transposon mutagenesis screen, weaken this hypothesis.

DISCUSSION

Previous studies in our laboratory have found that the proteins of the FEA, the putative FlhF GTPase, and the FlgSR two-component system are required for full expression of σ^{54}-dependent flagellar genes in *C. jejuni* (30, 35). In the current study, we obtained evidence that links the FEA to stimulation of the FlgSR two-component regulatory system. We found that activation rather than production of the FlgSR system is dependent on the FEA. Furthermore, we believe that formation of the apparatus rather than the secretory function of the apparatus is key to producing the signal detected by FlgS leading to its activation and subsequent expression of σ^{54}-dependent flagellar genes. Analysis of the genomic sequences of various *C. jejuni* strains indicates that the consensus σ^{54}-binding site is in the promoters of most genes that encode the flagellar proteins that are external to the cytoplasm and likely secreted by the FEA (20, 31, 54). Because gene expression and protein production are energetically expensive processes, it is likely that the introduction of a level of transcriptional control by the FEA allows *C. jejuni* to ensure that σ^{54}-dependent flagellar genes are expressed and the secreted proteins are produced only after the apparatus has formed.

The flagellar regulatory cascade of *C. jejuni* appears to bear some resemblance to the cascades utilized by species of *Heli-*

cobacter, *Vibrio*, and *Pseudomonas* (2, 16, 34, 39, 40, 47, 51, 56, 60). First, all the cascades are known to require σ^{54} and a two-component regulatory system with functional similarity to FlgSR for expression of a subset of flagellar genes. In addition, *Vibrio* and *Pseudomonas* species require the activity of a master regulator protein to initiate transcription of genes encoding FEA proteins and these flagellar two-component regulatory systems (2, 15, 16, 36, 40, 56). However, in *C. jejuni* and *Helicobacter pylori*, no master regulator of flagellar biosynthesis has been found, and one current hypothesis is that the expression of genes encoding components of the FEA and FlgSR is largely constitutive (26, 51). In all these bacteria, activation of the flagellar two-component regulatory system leads to the σ^{54}-dependent expression of genes encoding flagellar proteins that are secreted by the FEA (16, 25, 27, 30, 35, 40, 51, 56). Considering the similarity of the compositions of these flagellar regulatory cascades, our findings may suggest that the formation of the FEA could influence σ^{54}-dependent flagellar gene expression in a number of bacterial species. Further analysis of each of these organisms is required to determine if this relationship is shared across multiple genera of motile bacteria.

The analysis presented in this work allowed us to more precisely clarify the relationship between the FEA and the FlgSR system in σ^{54}-dependent flagellar gene expression. We constructed *C. jejuni* mutants whose mutations impaired FEA-mediated secretion to determine if formation of the export apparatus or its secretory activity was required for FlgS activation. Based on our finding that three of four *flhB* mutations and a *fliI* mutation reduced or blocked secretion of the FlaA flagellin but did not negatively affect σ^{54}-dependent gene expression, we concluded that the formation of the FEA in the inner membrane could be the signal detected by FlgS that directly leads to activation of the kinase. Alternatively, formation of the FEA may be indirectly involved by being required for the production of a downstream activating signal. Although the data alone do not define the nature of the communication between the FEA and FlgSR, we have provided a foundation for future studies to understand activation of the system. Characterization of additional FEA proteins and structures such as the inner membrane MS ring and the cytoplasmic C ring that are associated with the FEA (43, 44), along with better reagents to detect complete FEA formation, may allow us to further define the activating signal emanating from this secretory apparatus.

If our hypothesis that FlgS detects formation of the FEA for autoactivation is correct, the cytoplasmic localization of FlgS may provide insight into the origin of the signal relative to the FEA structure. Since FlgS is a cytoplasmic protein, FlgS may detect a signal originating on the cytoplasmic face of the inner membrane-localized FEA complex. For instance, FlgS may detect a completed FEA structure by monitoring whether certain proteins with large cytoplasmic domains are in the FEA. Possible candidates for this type of signal include the cytoplasmic domains of FlhA and FlhB. To find evidence supporting this hypothesis, we attempted to use numerous approaches to directly detect interactions that may occur between FlgS and FEA proteins, including affinity chromatography, affinity blotting, and in vivo chemical cross-linking. However, the results of these assays were inconsistent and inconclusive. New and better reagents and protocols have to be developed to extend these types of analyses. In vivo detection of an FlgS interaction with a member of the FEA may be difficult, due to the fact that flagellated *C. jejuni* assembles only one or two of these secretory apparatuses per bacterium. Thus, the number of interactions of FlgS with the FEA or an FEA component may be small and the interactions may be temporally transient.

As mentioned above, our results strongly support the hypothesis that formation of the FEA either directly comprises the signal or is required to produce the signal to activate FlgSR and expression of σ^{54}-dependent flagellar genes. An alternative hypothesis that we considered suggested that the secretory activity of the FEA could be the activating signal, with a cytoplasmic repressor hindering the FlgSR regulatory cascade prior to formation of and secretion by the FEA. However, four of the five *flhB* or *fliI* mutants whose mutations were shown to hinder or block secretion of flagellar proteins were not affected for σ^{54}-dependent expression of flagellar genes. Only the $flhB_{\Delta 226\text{-}230}$ mutant showed decreased expression of these genes, but analysis of this mutant suggested that it behaved most like a Δ*flhB* mutant, which does not form a complete FEA. Thus, we cannot confidently conclude that the $flhB_{\Delta 224\text{-}228}$ mutant makes a fully formed but secretion-incompetent apparatus. Second, our transposon mutagenesis screen did not reveal any transposon insertions in FEA mutants that relieved repression of expression of σ^{54}-dependent flagellar genes. These combined results greatly weaken the hypothesis that the secretory activity of the FEA alone forms the FlgS-activating signal. Thus, the results of this study strongly favor the hypothesis that that formation of the FEA is a requirement for and quite possibly a component of the essential signal for activating the FlgSR system that results in expression of σ^{54}-dependent flagellar genes.

Our work also suggests a new function in the signaling mediated by the FEA in flagellar regulatory cascades. In the well-characterized pathways observed in *E. coli* and *Salmonella*, formation of the FEA ultimately controls the activity of the alternative sigma factor σ^{28} involved in expression of genes encoding the major flagellins and some motor proteins (41). The FEA is responsible for secretion of flagellar proteins and the anti-σ factor, FlgM, which represses the activity of σ^{28} until the cell has completed formation of the FEA, basal body, and hook structures required to secrete flagellins to build a filament (32, 38). In this study, we found that the FEA is intimately involved in creating a signal that activates the FlgSR two-component system, leading to activation of σ^{54}. Therefore, the FEA plays a different role in influencing signaling for σ^{54}-dependent expression of flagellar genes in *C. jejuni*. This finding may also be applicable to other motile bacteria that utilize σ^{54} in flagellar gene regulation and biosynthesis, including species of *Vibrio*, *Pseudomonas*, and *Helicobacter*. This work expands the known mechanisms of regulating flagellar gene expression and suggests that there are more complex functions associated with the FEA beyond protein secretion.

Future analyses of FlgS will involve determining the domain and residues of the protein required for sensing an autoactivating signal. In analyzing the sequence of FlgS, we found that the central and C-terminal portions of the protein contain the histidine-containing phosphotransfer domain and the ATP-catalytic domain (61, 65). These domains are required for accepting a phosphate group on a conserved histidine and for

ATP hydrolysis, respectively, for autophosphorylation. Indeed, we found that H141 in the phosphotransfer domain is required for modification by phosphorylation and for functioning of the active FlgS to stimulate expression of σ^{54}-dependent flagellar gene expression. In a comparison of the amino acid sequence of FlgS to those of other sensor kinases, the predominant homology with the latter kinases is localized almost exclusively to the phosphoacceptor and ATP hydrolysis domains. Only limited homology between the initial 130 amino acids of FlgS and other sensor kinases is apparent. The sensor kinases that share the most homology to this region of the *C. jejuni* FlgS protein are other FlgS homologues in *Campylobacter* species (almost 100% identity), the FlgS orthologue in *Helicobacter* species (31 to 37% identity and 57 to 66% similarity), and the FlrB sensor kinase of *Vibrio cholerae* (26% identity and 54% similarity). The N-terminal regions of these proteins have no obvious motifs that suggest a function or how they may sense a specific factor. Since these N-terminal domains are unique to the group of FlgS orthologues, it is likely that this region of these proteins may function in specifically recognizing the signal necessary to culminate in expression of σ^{54}-dependent flagellar genes. Future studies will focus on further characterizing this domain of the protein.

Previous work in our laboratory focused on understanding the activation and function of the FlgR response regulator (25, 30, 35). In this study, we describe work that provides a foundation for understanding the activation of the cognate sensor kinase, FlgS, and how the FEA influences activation of this two-component regulatory system. To date, we have linked activation of the FlgSR system to the FEA and have characterized a previously undescribed mechanism for controlling activation of flagellar gene expression. In addition, FlgSR appears to be an unusual two-component system in which expression of both components is controlled by phase-variable mechanisms (25, 27), a trait unique among well-characterized bacterial two-component systems. Thus, there appears to be at least two mechanisms for controlling σ^{54}-mediated expression via the FlgSR proteins. Future analyses will focus on further defining the nature of the activating signal emanating from the FEA and how it influences expression of σ^{54}-dependent flagellar genes.

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