Analysis of the Campylobacter jejuni FlgR Response Regulator Suggests Integration of Diverse Mechanisms To Activate an NtrC-Like Protein

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Flagellar motility in Campylobacter jejuni mediates optimal interactions with human or animal hosts. σ^54 and the FlgSR two-component system are necessary for the expression of many C. jejuni flagellar genes. The FlgR response regulator is homologous to the NtrC family of transcriptional activators. These regulators usually contain an N-terminal receiver domain, a central domain that interacts with σ^54 and hydrolyzes ATP, and a DNA-binding C-terminal domain. Most often, phosphorylation of the receiver domain influences its inherent ability to either positively or negatively control the activity of the regulator. In this study, we performed genetic and biochemical analyses to understand how FlgR activity is controlled to culminate in the expression of σ^54-dependent flagellar genes. Our data suggest that the FlgR receiver domain has the capacity for both positive and negative regulation in controlling the activation of the protein. Analysis of the C-terminal domain of FlgR revealed that it lacks a DNA-binding motif and is not required for σ^54-dependent flagellar gene expression. Further analysis of FlgR lacking the C-terminal domain indicates that this protein is partially functional in the absence of the cognate sensor kinase, FlgS, but its activity is still dependent on the phosphorylated residue in the receiver domain, DS1. We hypothesize that the C-terminal domain may not function to bind DNA but may ensure the specificity of the phosphorylation of FlgR by FlgS. Our results demonstrate that FlgR activation mechanisms are unusual among characterized NtrC-like proteins and emphasize that various means are utilized by the NtrC family of proteins to control the transcription of target genes.

Flagella are produced by diverse bacterial species to aid in processes, including motility and adhesion, that allow bacteria to occupy an environmental niche or maintain a relationship with a host. Flagellar biosynthesis requires coordinating both the expression of over 40 flagellar genes and assembly of the encoded flagellar components into the organelle. Several mechanisms of flagellar gene regulation have evolved, with the best-understood system exemplified by Escherichia coli and Salmonella species. Flagellar genes in these bacteria are grouped into three classes based on their temporal expression (reviewed in reference 10). Briefly, global regulatory signals activate the transcription of the class I (early) genes flhD and flhC, which encode the master regulator of flagellar gene transcription. FlhDC activates the transcription of class II (middle) flagellar genes, which include those encoding the hook and basal body components and the alternative sigma factor, σ^28. σ^28-dependent class III (late) genes include those for the flagellins and the motor complex.

Species of the Vibrio and Pseudomonas genera employ a four-tiered regulatory cascade utilizing σ^25 and another, alternative sigma factor, σ^54, to control the expression of flagellar genes (14, 39, 49). In Vibrio cholerae, the class I master regulator, FlrA, interacts with σ^54 for the transcription of class II genes, including the flrBC operon which encodes a two-component regulatory system (31). The transcription of class III genes, such as those encoding the hook, basal body, and major flagellin, is activated by FlrC and the σ^54-RNAP polymerase (RNAP) holoenzyme (12, 49). Class IV genes are σ^28-dependent and include those encoding the minor flagellin and motor proteins. Similar genetic regulators and pathways exist in Pseudomonas aeruginosa to control the transcription of flagellar genes (2, 14, 29, 50). As in Vibrio and Pseudomonas species, the regulatory system employed by Helicobacter pylori also requires σ^28 and σ^54, but a master regulator of flagellar gene transcription has not been described and may be absent in this bacterium (28, 43).

Many bacteria utilize σ^54 to transcribe genes required for such diverse activities as nitrogen fixation, root nodule formation during plant symbiosis, and flagellar motility (reviewed in reference 30, 35). Unlike other σ factors, σ^54-RNAP holoenzyme alone cannot mediate the opening of DNA at target promoters. Instead, it requires interaction with a regulator (also termed “enhancer-binding protein”) to mediate this process. NtrC is one such, well-characterized, σ^54-dependent response regulator, consisting of a phosphorylatable N-terminal regulatory (or receiver) domain, a central σ^54 interaction domain, and a C-terminal domain (CTD) that contains dimerization determinants and is also indispensable for DNA binding in vivo (16; reviewed in reference 45). Under nitrogen-limiting conditions, the NtrB histidine kinase autophosphorylates and donates its phosphate residue to NtrC at residue D54 (32, 44, 51), which activates the protein to promote its oligomerization.

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DNA binding, and interactions with $\sigma^{54}$ in the RNAP holoenzyme. In addition, the phosphorylation of NtrC stimulates the hydrolysis of ATP by the central domain, providing the energy necessary for open DNA complex formation at target promoters, such as those for genes necessary for the utilization of alternative nitrogen sources. The NtrC family proteins often activate transcription by binding to upstream activation sequences (UAS) and are able to directly contact the $\sigma^{54}$-RNAP holoenzyme. ATP hydrolysis is accomplished directly by the NtrC-like proteins so that remodeling of the closed DNA complex can occur, allowing for transcriptional initiation.

*Campylobacter jejuni* is a highly motile, gram-negative bacteria responsible for a substantial percentage of food-borne illness in both industrialized and developing nations. Currently, this organism is a leading cause of bacterial gastroenteritis in the United States (9). *C. jejuni* is commonly found as a commensal organism in the gastrointestinal tracts of many animals and birds, including those of agricultural significance. Flagellar motility is a major determinant for *C. jejuni* colonization of animal hosts and in promoting human disease. Nonmotile *C. jejuni* mutants are able to colonize the lower gastrointestinal tracts of chickens but at greatly reduced levels compared to the levels of wild-type, motile strains (21, 23, 42, 55, 56). Similarly, in human challenge studies, the presence of flagella is required to promote diarrheal disease; nonmotile strains cannot be recovered from infected volunteers (3).

_C. jejuni_ elaborates a single flagellum at one or both poles, and the synthesis of this organelle is accomplished through both $\sigma^{28}$- and $\sigma^{54}$-dependent transcriptional pathways (20, 22, 27, 54, 56). $\sigma^{28}$ is involved in the transcription of the major flagellin flaA; other minor flagellar components; and C9977, encoding a nonflagellar protein that is involved in virulence (7, 19, 24, 56). $\sigma^{54}$, FlhF (a putative GTPase), the flagellar export apparatus, and the FlgSR two-component system are required for the transcription of the middle and late flagellar genes necessary for the formation of the hook, basal body, and the minor flagellin FlaB (7, 22, 24, 27, 56). The FlgS sensor kinase has been hypothesized to sense an unidentified signal, possibly emanating from the flagellar export apparatus and FlhF, which initiates a signal transduction cascade. FlgS can phosphorylate the FlgR response regulator in vitro (56), presumably activating FlgR so that it may productively interact with $\sigma^{54}$ to mediate the transcription of target flagellar genes. FlgR is highly homologous to NtrC and the NtrC-like proteins utilized by *Vibrio* (FlrC) (12, 31), *Pseudomonas* (FlrE) (50), and *Helicobacter* (FlgR) (5, 52) species to regulate the transcription of $\sigma^{54}$-dependent flagellar genes, but characterization of the molecular mechanisms leading to its activation is lacking. In addition, recent work has revealed that _C. jejuni_ FlgR is unusual as a response regulator of a two-component system in that the translation of the protein is subjected to an additional level of control by a mechanism involving phase variation (21).

In this study, we constructed a series of FlgR mutants in _C. jejuni_ and performed both genetic and biochemical analyses to understand how FlgR activation is controlled for the proper expression of $\sigma^{54}$-dependent flagellar genes. Initial studies with point mutations allowed us to determine the likely site of phosphorylation within the N-terminal receiver domain which is essential for FlgR function. Analyses with FlgR mutants lacking either the receiver domain or the CTD led us to discover previously undescribed mechanisms utilized to control FlgR activity. We found that residue D51 in the receiver domain appears to be phosphorylated by FlgS, leading to the activation of the protein and transcription of $\sigma^{54}$-dependent flagellar genes. These results suggest that the phosphorylation of this domain functions to activate FlgR. However, we also report that both the receiver domain and the CTD can inhibit FlgR activity in the absence of an appropriate signal from FlgS. While the CTDs of NtrC-like proteins usually contain a DNA-binding domain essential for their function as transcriptional activators in vivo, the CTD of FlgR appears to lack this DNA-binding motif. Instead, we found that this domain may possess a regulatory function in preventing the phosphorylation of FlgR by non-FlgS phosphodonors, so that FlgR is kept inactive during noninducing conditions. Our data provide new insights into the diversity of NtrC family response regulators and continue to reveal atypical mechanisms by which _C. jejuni_ controls flagellar gene transcription.

**MATERIALS AND METHODS**

**Bacterial strains.** _C. jejuni_ strain 81-176 is a clinical isolate from a patient presenting with gastroenteritis that has since been shown to promote disease in humans and commensal colonization of the chick gastrointestinal tract (3, 21, 23, 34). _C. jejuni_ cells were routinely grown on Mueller-Hinton (MH) agar containing 10 mg/ml of trimethoprim (TMP) at 37°C under microaerobic conditions (85% N2, 10% CO2, and 5% O2). As required, strains were grown on MH agar containing 50 mg/ml kanamycin, 20 mg/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 mg/ml chloramphenicol as necessary. All _E. coli_ strains were stored at −80°C in a solution of 80% LB broth and 20% glycerol.

**Construction of strains.** All strains were constructed as previously described (22). To create the flgR mutations, pDHHR428 (24) was subjected to PCR-mediated mutagenesis (38) to generate in-frame deletions of the receiver and CTD of FlgR. The *flgR*Δreceiver, mutation was made by fusing the start codon to codon 132, removing the intervening 131 codons, to create pDHHR855. pDRH1856 contains *flgR*ΔCTD, which lacks the terminal 51 codons of the gene. This plasmid was subsequently used in PCR-mediated mutagenesis to create pSNJ711, which contains the *flgR*ΔCTDΔmutant. pDHHR865, containing *flgR*ΔCTD, was generated by two steps of PCR mutagenesis to remove the N-terminal 131 codons and the C-terminal 52 codons. PCR-mediated mutagenesis was also performed with pDRH428 to generate plasmid derivatives containing single-base mutations in the CTD of FlgR. These point mutations include D46A (in pSNJ511), D51A (in pSNJ512), and D58A (in pSNJ513). All plasmid constructs were verified by DNA sequence analysis.

All plasmids containing *flgR* mutations were electroporated into strains 81-176 Sm^r^*flgR*:kan-rpsL and 81-176 Sm^r^ΔastA*flgR*:kan-rpsL (DRH473 and DRH475, respectively) (24), replacing *flgR*:kan-rpsL with the specific *flgR* mutant at the native locus. To introduce the *flgR* mutants into the *flgR* background, strains 81-176 Sm^r^ΔastA (DRH4480 and 81-176 Sm^r^ΔastA*ΔΔ*flgR*::kan-rpsL (DRH9011) (24) were electroporated with pDHHR443 (containing *flgR*:kan-rpsL) (24). The resulting strains, 81-176 Sm^r^ΔΔ*flgR*::kan-rpsL (SNJ767) and 81-176 Sm^r^ΔastA*ΔΔ*flgR*:kan-rpsL (DRH1763), were then electroporated with the plasmids containing specific *flgR* mutations described above. All mutants were recovered on MH agar containing streptomycin and verified by PCR and DNA sequencing. The transcriptional reporter strains were constructed as previously described (24).

Briefer, plasmids pDRH532 (containing *flgDE2*:nemo) and pDRH610 (containing *flbDE2*:Xta) were electroporated into the _C. jejuni_ strains to replace *flgDE2* and *flbDE2*:nemo and *flbDE2*:Xta on the chromosome. Transformants were recovered on MH agar containing kanamycin and were verified by PCR.

Strains 81-176 Sm^r^ΔastA*flgR*ΔDS1 (DS1) *flgDE2*:nemo (SNJ3062) and 81-176 Sm^r^ΔastA*flgR*ΔDS1 *flbDE2*:Xta (SNJ6056) were complemented in trans with wild-type *flgR* on plasmid pDRH18 or *flbDE2* by conjugation via _E. coli_ strain DH5αpRK2222 (24). Transconjugants were selected on MH agar containing chloramphenicol and kanamycin and verified by PCR. *flgR* from *Helicobacter pylori* strain 26695 was amplified from chromosomal DNA (a kind gift from D. Scott Merrell, Uniformed Services University of the
Health Sciences) by PCR. The primers were designed so that in-frame BamHI sites were fused to the second codon and to the stop codon. The PCR product was digested with BamHI and ligated into BamHI-digested pECo102 (24) so that \( H. pylori \) flgR (FlgRhap) is expressed from the constitutive chloramphenicol acetyltransferase (cat) promoter. Clones were verified by sequencing and used to transform \( E. coli \) strain DH5α/pRK221.2 for conjugation into strains 81-176 Sm'\( \Delta astA \) sf16X flgDE2::momo and 81-176 Sm'\( \Delta astA \) sf16X flaB::zastA (DRH830 and DRH832, respectively) (24) and strains 81-176 Sm'\( \Delta astA \) sf16X flgDE2::momo (SNJ307) and 81-176 Sm'\( \Delta astA \) sf16X flaB::zastA (SNJ308). Transconjugants were selected on MH agar containing TMP, kanamycin, and chloramphenicol and were verified by PCR.

Bioinformatic analyses. Protein homology searches and analyses were performed using the BLASTP and BLAST2 programs (www.ncbi.nlm.nih.gov/BLAST). Alignments were preformed with ClustalW. Helix-turn-helix (HTH) motifs were identified by using PBL-IBCP-Geerland (http://phil.ibcp.fr/index.php).

Immunoblotting analyses of FlgR proteins. \( C. jejuni \) strains were grown from frozen stocks on MH agar under microaerobic conditions for 48 h and then restreaked and grown for 16 h prior to use. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting for the detection of FlgR proteins were performed as described previously (21). Briefly, cells were resuspended in MBH broth to an optical density at 600 nm (OD_{600}) of 0.8, and 1 ml of this suspension was harvested by centrifugation. The pellet was washed once with PBS and then resuspended in 50 \( \mu \)l 1× Laemmli buffer. Four micro-liters of each sample was separated by 10% SDS-PAGE and then transferred to an Immobilon-P membrane (Millipore). The immunoblot was developed with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad).

Arylsulfatase reporter assays. Arylsulfatase transcriptional reporter assays were performed as previously described (24). \( C. jejuni \) strains were grown from frozen stocks on MH agar containing TMP or kanamycin and chloramphenicol as necessary for 48 h under microaerobic conditions and then restreaked and grown for 16 h prior to the assay. The strains were resuspended from agar plates and diluted to an OD_{600} of 0.7 to 1.0, washed in arylsulfatase assay buffer, and then incubated with 10 mM nitrophenol sulfate and 1 mM tricine for 1 h at 37°C. The assay was terminated with 0.2 N NaOH, and the amount of nitrophenol released in each sample was determined by measuring the absorbance of the sample at OD_{410}. The amount of nitrophenol in each sample was determined by comparing the OD_{410} readings of each sample to those from a standard curve of flavoharbin. Transconjugants were selected on MH agar containing TMP for 48 h and then restreaked and grown for 16 h prior to use. Each strain was tested in triplicate, and each assay was performed three times.

Motility assays. To compare the respective motility phenotypes of our \( C. jejuni \) 81-176 derivatives, strains were grown from freezer stocks on MH agar containing TMP for 48 h and then restreaked and grown for 16 h prior to use. Each strain was resuspended in MBH broth to an OD_{600} of 1.0, and a sterile platinum wire was used to inoculate semisolid MH motility medium containing TMP. Plates were incubated at 37°C under microaerobic conditions for 24 to 72 h prior to analysis.

Transmission electron microscopy. To examine wild-type and \( fglR \) mutant strains for the production of flagella, strains from freezer stocks were grown on MH agar containing TMP for 48 h and then restreaked and grown for 16 h prior to study. Strains from plates were resuspended in MBH broth to an OD_{600} of 1.0, pelleted, and then resuspended in a 2% (vol/vol) glutaraldehyde solution for fixation. Fixation was completed by incubating strains for 1 h on ice. All samples were stained with 1% (wt/vol) uranyl acetate and visualized with a JOEL 1210 transmission electron microscope at 80 kV.

Purification of FlgR and FlgS proteins. Wild-type \( fglR \) and \( fglR \) derepressed, \( fglBCEDTS \), \( flgS_{cont} \), and \( flgS_{D51A} \) were amplified by PCR. The primers for each \( fglR \) mutant were designed so that a 5′ BamHI site was added in-frame. The other primer to amplify each mutant contained codons for an in-frame six-His tag followed by a stop codon and a BamHI site. After amplification, the DNA fragments were digested with BamHI and ligated into BamHI-digested pT7-7. For purification, 500 ml LB broth was inoculated with BL21(DE3)PlyesE bacteria containing each of the expression constructs and the bacteria were grown at 37°C to an OD_{600} of 0.4, induced with 1 mM isopropyl-\( \beta \)-thiogalactopyranoside, and incubated at 30°C for an additional 4 h. The bacteria were harvested and passed through an Empus/Miflex-C5 cell disruptor (Avestin) at 15,000 to 20,000 lb/in². Ni-nitrilotriacetic acid-agarose beads were used to purify the proteins under native conditions according to the manufacturer's instructions (Qiagen). The eluted fractions were analyzed by 10% SDS-PAGE, and the eluate fractions containing the highest purity of FlgR proteins were combined for dialysis against a buffer containing 10 mM Tris-HCl, 50 mM KCl, 5% (vol/vol) glycerol, 0.1 mM EDTA, and 1 mM dithiobitol (DTT). FlgS was purified as previously described (21), and the eluate fractions were dialyzed against a buffer containing 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 25% (vol/vol) glycerol, and 1 mM DTT. Following dialysis, the concentration of each protein was determined by using a Bradford assay and aliquots of each were frozen at −80°C. To minimize contamination with free phosphates, we used ultrapure water (Fluka) in all buffers and reagents for purification.

In vitro assays of phosphorylation of FlgR by FlgS. To analyze the ability of FlgS to phosphorylate wild-type and mutant FlgR proteins, we adapted a previously published protocol (56). Six micromoles FlgS–six-His was incubated with 10 \( \mu \)Ci [γ-32P]ATP for 15 min in a buffer containing 30 mM Tris-HCl, pH 8.0, 75 mM KCl, 2 mM MgCl₂, and 1 mM DTT. Six micromoles of each FlgR–six-His construct was added to the reaction mixture; the mixture was incubated for 2 min, and the reaction stopped by the addition of 2× Laemmli buffer. Samples were analyzed by 10% SDS-PAGE. Following drying of the gels, they were autoradiographed by using a Storm 820 phosphoinager (Amersham Biosciences). The data were analyzed using the manufacturer's software.

DNA-binding assays. Mobility-shift assays were based on the protocols of Porter et al. (40) and McEver et al. (40). Briefly, DNA fragments corresponding to base pairs 1 to 250 or 250 to 500 upstream of the start codon of \( C. jejuni \) genes \( flgR \), \( flaB \), and \( flgS \) were used in each binding reaction mixture. Either phosphorylated or unphosphorylated wild-type FlgR protein was used in concentrations of up to 10 μg per reaction. The phosphorylation of FlgR was performed with 5 μg FlgS with unlabeled ATP as described above. The phosphorylation reaction mixture was added to a tube containing labeled probe and DNA-binding buffer. Reaction mixtures were incubated for 20 min at either 4°C or 37°C before electrophoresis on 5% acrylamide gels at 110 V for 3 h.

RESULTS

FlgR activation is dependent on phosphorylation of residue D51. The members of the NtrC family of response regulators are generally modular in structure, with each domain contributing to transcriptional activation under the proper conditions. Based on its homology to this class of proteins, FlgR can be divided into three putative domains (data not shown). The N-terminal domain (amino acids 1 to 131) resembles a typical response regulator receiver domain, which is often phosphorylated at a specific aspartic acid residue to result in the activation of the protein. The central domain of FlgR is homologous to the highly conserved central domain of NtrC family members that hydrolyzes ATP and interacts with the σ^54, RNP holoenzyme (reviewed in reference 53). Most NtrC family members also possess a CTD containing an HTH motif, necessary for the DNA binding to activate the transcription of target genes (11, 26). Bioinformatics analyses comparing the NtrC proteins of \( E. coli \) and Salmonella enterica serovar Typhimurium and the FlgR homologues in \( P. aeruginosa \) (FlrE) and \( V. cholerae \) (FlrC) demonstrate a highly predicted HTH in the CTDs of these proteins (data not shown). However, the results of analyses of \( C. jejuni \) FlgR and these proteins suggest an absence of homology between its CTD and those of other NtrC-like proteins. Furthermore, the FlgR CTD does not appear to contain a predicted HTH structure or any other DNA-binding motif, which may imply that this domain of FlgR does not function in DNA interactions. Similar to the results of a previous study, alignment of the FlgR protein of \( H. pylori \) with these proteins shows that it is prematurely truncated and completely lacks the CTD and, presumably, a DNA-binding motif (5 and data not shown).

Based on the known properties of NtrC family proteins and...
the putative features of *C. jejuni* FlgR, we analyzed alterations of the N-terminal receiver domain and the CTD to better understand how the activity of the protein is controlled. Alignment of the FlgR receiver with other σ^54^-dependent response regulators suggested that D51 may be the site of phosphorylation (data not shown). We performed site-directed mutagenesis to individually change this residue to an alanine (Fig. 1). We noticed two other aspartic acids, D46 and D58, present in the same region of the protein that, due to their vicinity to D51, could be alternative sites of phosphorylation. Mutants were constructed that changed these residues to alanines to eliminate the possibility that amino acid substitutions in this subregion of the receiver domain could cause nonspecific structural changes that may result in the inactivation of FlgR. The *flgR* mutants were used to replace wild-type *flgR* on the chromosome of *C. jejuni* strains 81-176Sm^t*(DRH212) (22) and 81-176 Sm^t* Δast4*(DRH461) (24). This latter strain is used as the genetic background for analysis of the expression of two σ^54^-dependent flagellar genes: flaB, encoding a minor flagellin, and the *flgDE2* operon, encoding flagellar hook-associated proteins. In *C. jejuni*, these mutant FlgR proteins are stable and produced at levels similar to the levels of wild-type FlgR (Fig. 2A). The examination of mutants producing FlgR(D46A) or FlgR(D58A) revealed no effect on motility, flagellar biosynthesis, or the expression of σ^54^-dependent flagellar genes (Fig. 2B to D). However, the alteration of residue D51 of FlgR to an alanine results in a *C. jejuni* mutant that is incapable of promoting motility (Fig. 2B), producing flagella (Fig. 2C), or activating σ^54^-dependent flagellar gene expression (Fig. 2D). These phenotypes of the *flgR*(D51A) mutant are similar to those of the Δ*flgR* mutant with a deletion of codons 17 to 408 of the gene (Fig. 2B to D) (24). We were able to complement all deficiencies of the *flgR*(D51A) mutant by introducing wild-type *flgR* on a multicopy plasmid (Fig. 2D and data not shown), indicating that FlgR(D51A) was responsible for all observed phenotypes.

A previous study has shown that FlgS can mediate the in vitro phosphorylation of FlgR, but the phosphorylated residue was not identified (56). Our analyses described above strongly support D51 as the site of phosphorylation. In vitro phosphorylation assays were performed and demonstrated that wild-type FlgR, but not FlgR(D51A), is phosphorylated in the presence of FlgS (Fig. 3). This in vitro result confirms the results of our in vivo genetic analyses and supports the hypothesis that D51 is the phosphorylated residue of FlgR. However, until more-extensive experimentation can be performed with purified phosphorylated FlgR protein from *C. jejuni*, we cannot definitively conclude that D51 is the phosphorylated residue. For simplicity, we will assume for purposes of discussion for the remainder of this work that D51 is the phosphorylated residue.

Upon the identification of D51 as the site of FlgR phosphorylation, we attempted to create a constitutively activated FlgR protein by substituting a glutamic acid for this residue, as NtrC can be constitutively activated by a similar mutation at its phosphorylated residue, D54 (32). However, this type of substitution does not activate all NtrC family proteins, as the D55E mutation of *Sinorhizobium meliloti* DctD inactivates the protein (41). We constructed an *flgR*(D51E) allele and used it to replace wild-type *flgR* on the chromosome of *C. jejuni*. The resultant strain produces a stable protein, but σ^54^-dependent flagellar gene expression and motility are both fully ablated (Fig. 2D and data not shown). Thus, unlike NtrC, FlgR cannot be constitutively activated by glutamic acid substitution at the site of phosphorylation.

**Evidence for dual activating and inhibitory functions of the FlgR receiver domain.** To further understand how the domains of FlgR influence its activation as a transcriptional regulator, we performed a more global domain analysis of the FlgR protein. An N-terminal receiver domain is often necessary to influence the activity of NtrC family proteins under appropriate conditions. Within the NtrC subfamily of response regulators, the receiver domain can employ either positive or negative modes of regulation, which are influenced by the...
phosphorylation state of the domain (reviewed in reference 18). For example, the unphosphorylated receiver domains of *S. meliloti* DctD and *Aquifex aeolicus* NtrC1 prevent intermolecular interactions between the central domains that would result in protein activity (36, 37). Thus, the removal of the receiver domain of these proteins results in a constitutively active protein in the absence of phosphorylation, while the full-length, unphosphorylated protein is held inactive. The phosphorylation of the receiver domain removes this inhibition, allowing for oligomerization and ATPase activity, requirements for the initiation of target gene transcription. In contrast, the receiver domain of NtrC of *S. enterica* serovar Typhimurium positively regulates protein activation upon phosphorylation, as this modification induces conformational changes necessary for the function of the response regulator (15). In NtrC, the receiver domain is essential for activation and its removal results in an inactive protein.

As described above, we have shown that the phosphorylation
of residue D51 is essential for the full activation of FlgR to mediate the expression of $\sigma^{54}$-dependent flagellar genes. Based on the shared homology between the FlgR proteins of C. jejuni and H. pylori and the domain architecture of the FlgR<sub>ctd</sub> protein described in Brahmachary et al. (5), we have designated amino acids 1 to 131 as the receiver domain. To characterize the means by which this domain influences FlgR activity, we generated an in-frame deletion of the N-terminal 131 amino acids of the protein and used the gene encoding this construct (flgR<sub>receiver</sub>) to replace flgR at the native locus. As shown in Fig. 4A, FlgR<sub>receiver</sub> is stable and produced at levels comparable to the level of wild-type FlgR in C. jejuni bacteria. Analysis of C. jejuni bacteria producing FlgR<sub>receiver</sub> revealed that their expression of $\sigma^{54}$-dependent reporter genes is approximately 10% of the level observed in wild-type bacteria (Fig. 4B). However, the expression of these genes in the flgR<sub>receiver</sub> mutant is nearly 100-fold greater than in a ΔflgR mutant. The initial observation of the flgR<sub>receiver</sub> mutant showed a nonmotile phenotype (Fig. 4C). However, with prolonged incubation for up to 3 days, slight motility could be observed (data not shown), although this level of motility is of questionable significance. In addition, the examination of over 100 bacteria by electron microscopy did not reveal any signs of flagellar biosynthesis (Fig. 4D). Despite the motility and flagellar biosynthesis phenotypes, our observation that the level of $\sigma^{54}$-dependent flagellar gene expression in strain 81-176 Sm<sup>+</sup> ΔflgR<sub>receiver</sub> is higher than in strain 81-176 Sm<sup>+</sup> ΔflgR indicates that the FlgR<sub>receiver</sub> protein is partially active despite lacking the phosphorylated N-terminal receiver domain.

As previously shown, a ΔflgS mutant producing wild-type FlgR is nonmotile and incapable of activating $\sigma^{54}$-dependent flagellar gene expression, demonstrating that wild-type FlgR is inactive in the absence of FlgS and presumably requires phosphorylation to function as a transcriptional regulator (24, 56). Our data shown above strengthen this hypothesis, as FlgR(D51A) is not phosphorylated in the presence of FlgS (Fig. 3) and this protein does not support the expression of $\sigma^{54}$-dependent flagellar genes (Fig. 2D). Given that FlgR<sub>receiver</sub> lacks the phosphorylated residue, D51, but is still partially active when the cognate sensor kinase is present, we hypothesize that this activity is likely independent of phosphorylation. Indeed, in an in vitro phosphorylation assay, FlgR<sub>receiver</sub> is not phosphorylated in the presence of FlgS (Fig. 3). Thus, the in vivo ability of FlgR<sub>receiver</sub> to activate flagellar gene expression is likely due to the constitutive activity of this protein. If this is the case, then we would expect that, unlike wild-type FlgR, FlgR<sub>receiver</sub> would activate the expression of $\sigma^{54}$-dependent flagellar genes in a ΔflgS mutant. Therefore, we introduced flgR<sub>receiver</sub> into a ΔflgS background and compared the resulting mutant to a ΔflgS mutant which produces wild-type FlgR. The level of $\sigma^{54}$-dependent flagellar gene expression in the ΔflgS flgR<sub>receiver</sub> mutant was 20- to 80-fold greater than in the ΔflgS mutant (Fig. 4E), demonstrating that the removal of the N-terminal receiver domain of FlgR increases its activity compared to that of wild-type FlgR in the absence of FlgS. However, the expression of flagellar genes in the ΔflgS flgR<sub>receiver</sub> mutant is 20- to 30-fold less than in wild-type C. jejuni bacteria (producing both FlgS and FlgR) (Fig. 4E). In addition, this flgR deletion in the ΔflgS background does not support motility (data not shown). Considering these results, we propose that one function of the receiver domain is to inhibit FlgR activity in the absence of phosphorylation and that this inhibition is partially relieved when the receiver domain is artificially removed. Ultimately, we believe the primary function of the receiver domain is to positively regulate the protein upon phosphorylation, since wild-type levels of expression of $\sigma^{54}$-dependent flagellar genes are only observed when the receiver domain of FlgR is intact and phosphorylated by FlgS. Thus, the receiver domain appears to have both stimulatory and inhibitory activities based on the state of phosphorylation.

**Analysis of an alternative role for the CTD in controlling FlgR activity.** The results of bioinformatics analyses using software to identify DNA-binding motifs, such as HTH motifs, suggest that the FlgR CTD lacks the strongly predicted HTH found in other NtrC family response regulators that is required in vivo for the expression of NtrC target genes (16 and data not shown). To begin characterizing the putative functions of the CTD, we removed the C-terminal 52 amino acids, generating FlgR<sub>CTD</sub>. FlgR<sub>ctd</sub> naturally lacks these residues, and presumably, DNA-binding activity (5). This construct was used to replace wild-type flgR on the chromosome of C. jejuni. FlgR<sub>CTD</sub> is produced by C. jejuni (Fig. 4A) but at levels that are substantially lower than the levels of FlgR or FlgR<sub>receiver</sub> indicating that the CTD may be necessary for FlgR stability. Despite low observed protein levels, the deletion of the CTD had no apparent effects on the expression of $\sigma^{54}$-dependent flagellar genes (Fig. 4B), motility (Fig. 4C), or the production...
FIG. 4. Phenotypic analyses of *C. jejuni* strains producing in-frame deletion of domains within FlgR. (A) Immunoblot analysis of whole-cell lysates of *C. jejuni* wild-type (WT) and mutant strains to detect FlgR proteins. The FlgR protein produced by each strain is listed above the blot. Arrow indicates full-length FlgR protein, arrowhead indicates FlgR_{receiver}, filled dot indicates FlgR_{CTD}, and empty circle indicates FlgR_{central}. Molecular sizes in kDa are shown at the left. (B) Results of arylsulfatase assays for analysis of expression of *flgDE2::nemo* and *flaB::astA* in *C. jejuni* 81-176 derivatives producing WT and FlgR mutant proteins. Results are from a typical assay with each strain tested in triplicate. Value reported...
of flagella (Fig. 4D), indicating that in the presence of FlgS, FlgR<sub>ACTD</sub> behaves similarly to wild-type FlgR. This finding is consistent with expectations for FlgR<sub>Hp</sub>, which naturally lacks a CTD but functions to transcribe σ<sup>54</sup>-dependent flagellar genes (5). We performed DNA-binding assays using the full-length FlgR protein and the promoter regions of flgDE2 and flaB. Although multiple approaches were used, including different phosphorylation states of FlgR and altered incubation conditions, we could not demonstrate any DNA-binding activity. This finding was not entirely surprising, however, as there is evidence that FlgR<sub>Hp</sub> does not bind DNA to activate transcription (5). We believe our following observations support the hypothesis that C. jejuni FlgR lacks a DNA-binding requirement: (i) the deletion of the CTD does not affect the expression of σ<sup>54</sup>-dependent flagellar genes; (ii) a DNA-binding motif cannot be found via bioinformatics analysis of the isolated CTD or full-length FlgR; and (iii) the full-length FlgR protein does not appear to bind DNA upstream of σ<sup>54</sup>-dependent promoters in vitro.

Considering our findings and those of the published reports analyzing FlgR<sub>Hp</sub>, we initially suspected that the CTD of C. jejuni FlgR is not necessary for DNA binding and that it may be vestigial. However, by introducing flgR<sub>ACTD</sub> into a ΔflgS background, we discovered that the CTD has an inhibitory function. Similar to our findings with the FlgR<sub>receiver</sub> protein, σ<sup>54</sup>-dependent reporter gene expression mediated by FlgR<sub>ACTD</sub> in a ΔflgS background is 10- to 100-fold greater than that in the ΔflgS mutant that produces wild-type FlgR (Fig. 4E). Additionally, the production of FlgR<sub>ACTD</sub> in the ΔflgS background restores the motility to 20 to 50% of the wild-type levels (data not shown). These results indicate that, like the receiver domain, the CTD may also inhibit FlgR activity in the absence of phosphorylation. In vitro phosphorylation assays show that FlgR<sub>ACTD</sub> is phosphorylated in the presence of FlgS and that this modification is dependent on residue D51, as it is in the wild-type protein (Fig. 3B). To determine if the in vivo activity of FlgR<sub>ACTD</sub> for the expression of flagellar genes in the absence of FlgS is dependent on the phosphorylation of D51, we introduced flgR(D51A)<sub>ACTD</sub> into the ΔflgS background. In this mutant, the expression of σ<sup>54</sup>-dependent reporter genes is reduced compared to their expression levels in the ΔflgS flgR<sub>ACTD</sub> mutant (Fig. 4E), indicating that the phosphorylation of FlgR<sub>ACTD</sub> on residue D51 is required in part for its constitutive transcriptional activity. Although FlgR<sub>ACTD</sub> activity still appears to be largely dependent on the phosphorylation of D51 in a mutant lacking FlgS, FlgS cannot be responsible for this modification. Thus, we hypothesize that FlgR<sub>ACTD</sub> may be phosphorylated by non-FlgS kinases or phosphodonor(s). This finding stands in contrast to the results for the wild-type FlgR that is produced in the ΔflgS mutant, as it remains completely inactive without FlgS and likely is not phosphorylated in vivo by another phosphodonor.

Considering our findings that both the receiver domain and CTD can inhibit FlgR activity in the absence of phosphorylation by FlgS and that the removal of each domain creates a protein with partial constitutive activity, we proposed that the deletion of both domains from FlgR may be additive to produce a protein with high constitutive activity. We generated a C. jejuni mutant that produced only the central domain of FlgR (flgR<sub>central</sub>, encoding amino acids 132 to 381). The examination of this mutant revealed that the FlgR<sub>central</sub> protein was produced at very low levels, similar to the FlgR<sub>ACTD</sub> protein (Fig. 4A). Regardless of the presence of FlgS, the FlgR<sub>central</sub> domain only afforded a very low level of flagellar gene expression (Fig. 4B) that did not result in flagellar biosynthesis or motility (Fig. 4C and D), suggesting that this version of the protein is inactive. Currently, we do not understand why the removal of both the receiver domain and CTD does not activate the protein. However, a relatively simple explanation is that this severely truncated FlgR protein may have an altered conformation or may not fold properly, resulting in complete inactivation.

FlgR<sub>ACTD</sub> activity is controlled differently than that of an FlgR orthologue that naturally lacks the CTD. Unlike most members of the NtrC family of transcriptional regulators that contain a CTD to bind DNA, evidence suggests that FlgR<sub>Hp</sub> naturally lacks this domain and does not bind DNA (5). The work described above also suggests that the CTD of C. jejuni FlgR is not required for the expression of flagellar genes and inhibits FlgR activity in the absence of phosphorylation. Not using an HTH-containing CTD to interact with DNA in vivo distinguishes these FlgR proteins from other NtrC family members; thus, they may represent a subfamily of NtrC-like proteins that do not require DNA interaction to activate transcription.

The FlgR proteins of C. jejuni strain 81-176 and H. pylori strain 26695 are 56% identical and 73% similar, with a major difference being that FlgR<sub>Hp</sub> lacks the CTD observed in C. jejuni FlgR. As C. jejuni FlgR<sub>ACTD</sub> is partially active in the absence of FlgS, we investigated whether FlgR<sub>Hp</sub> would be

for each strain is average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in strain 81-176 Sm<sup>+</sup> astA<sub>a</sub> ΔflgR, which was set to 1 arylsulfatase unit. For expression of flgDE2::astA, strains include DRH533, DRH830, SNJ118, SNJ120, and SNJ126. For expression of flaB::nemo, strains include DRH665, DRH842, SNJ145, SNJ138, and SNJ142. The FlgR protein produced by each strain is listed below the graph. (C) Motility phenotypes in MH semisolid agar of C. jejuni strains producing WT FlgR or FlgR in-frame domain deletions. WT, DRH212 (81-176 Sm<sup></sup>); ΔflgR, DRH737 (81-176 Sm<sup></sup> ΔflgR); Δreceiver, DRH1901 (81-176 Sm<sup></sup> flgR<sub>Δreceiver</sub>); ΔCTD, DRH1925 (81-176 Sm<sup></sup> flgR<sub>ACTD</sub>); central, DRH1928 (81-176 Sm<sup></sup> flgR<sub>central</sub>). Level of motility after 24 h of incubation is shown. (D) Electron micrographs of C. jejuni WT and mutant strains for analysis of flagellar biosynthesis. All electron micrographs are at ×16,000 magnification. The bar for each micrograph represents 0.5 μm. WT, DRH212 (81-176 Sm<sup></sup>); ΔflgR, DRH737 (81-176 Sm<sup></sup> ΔflgR); Δreceiver, DRH1901 (81-176 Sm<sup></sup> flgR<sub>Δreceiver</sub>); ΔCTD, DRH1925 (81-176 Sm<sup></sup> flgR<sub>ACTD</sub>); central, DRH1928 (81-176 Sm<sup></sup> flgR<sub>central</sub>). (E) Results of arylsulfatase assays for analysis of expression of flgDE2::nemo and flaB::nemo in C. jejuni 81-176 Sm<sup></sup> ΔflgS derivatives producing WT and FlgR mutant proteins. Results are from a typical assay with each strain tested in triplicate. Value reported for each strain is average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in strain 81-176 Sm<sup></sup> ΔflgS, which was set to 1 arylsulfatase unit. For expression of flaB::astA, strains include DRH665, DRH939, SNJ136, SNJ268, and SNJ730. For expression of flgDE2::nemo, strains include DRH533, DRH936, SNJ123, and SNJ264, and SNJ728. The status of flgS and flgR in each strain is shown below the graph.
constitutively active without FlgS. First, we examined the ability of FlgR<sub>Hp</sub> to complement a C. jejuni ΔflgR mutant in the presence of FlgS. When introduced into strain 81-176 Sm<sup>v</sup> Δflg<sup>S</sup> on a multicopy plasmid and expressed from the constitutive chloramphenicol acetyltransferase (cat) promoter, FlgR<sub>Hp</sub> is able to fully restore the expression of σ<sup>54</sup>-dependent flagellar genes (Fig. 5), indicating that the activation of FlgR<sub>Hp</sub> and C. jejuni FlgR by FlgS is conserved in the two bacteria. However, when FlgR<sub>Hp</sub> is produced in a Δflg<sup>S</sup> Δflg<sup>R</sup> mutant, it is unable to activate the expression of σ<sup>54</sup>-dependent flagellar genes (Fig. 5). While FlgR<sub>Hp</sub> displays partial constitutive activity in a Δflg<sup>S</sup> background (Fig. 4E and 5), FlgR<sub>Hp</sub> is entirely inactive in the absence of C. jejuni FlgS. These data suggest that a non-FlgS kinase or phosphodonor may exist to phosphorylate FlgR<sub>A<sub>CTDD</sub></sub>, but not the FlgR<sub>Hp</sub> protein. Alternatively, FlgR<sub>Hp</sub> may utilize another means of inhibiting its activity in the absence of phosphorylation. Thus, this subfamily of NtrC-like proteins that may not interact with DNA appear to possess diverse mechanisms to control their activation. This work illustrates that C. jejuni FlgR is an unusual member of the well-studied NtrC family of proteins.

**DISCUSSION**

**Mechanism of action of NtrC-like proteins and other response regulators.** A common mechanism for controlling the activity of response regulators in bacteria is through phosphorylation of the N-terminus of the receiver domain of these proteins (18). Approximately 10% of all known bacterial response regulators are within the NtrC family of transcriptional activators (18). Within this class, two means by which phosphorylation of the receiver domains ultimately influence the activity of the protein have been described. The archetypal member of this family, NtrC, is positively regulated by phosphorylation (16), while other members, such as S. meliloti DctD and A. aeolicus NtrC1, utilize phosphorylation to remove inhibition mediated by the receiver domain (36, 37). These positive and negative regulatory mechanisms both ultimately influence the ability of the regulator to hydrolyze ATP and interact with the σ<sup>54</sup>-RNAP holoenzyme and DNA via the C-terminal HTH to transcribe target genes under appropriate conditions.

A few characterized response regulators outside of the NtrC family have receiver domains that utilize both positive and negative regulation, depending on the state of phosphorylation. One of the best-characterized examples of this dual regulation is CheB, a protein involved in chemotaxis in motile bacteria (reviewed in reference 4). It is comprised of two major domains, an N-terminal receiver that is phosphorylated by the CheA kinase and a C-terminal methylesterase domain that dephosphorylates methyl-accepting chemotaxis proteins. Deletion of the receiver domain results in a mutant with increased methylesterase activity compared to that of the unphosphorylated wild-type protein, indicating that the receiver domain inhibits methylesterase activity in an unphosphorylated state (1). However, phosphorylation of the receiver domain enhances methylesterase activity to a greater extent than does CheB with a deletion of the entire domain. Thus, the receiver domain has dual roles in inhibition and activation, depending on the state of phosphorylation. The receiver domain of FixJ, a Sinorhizobium meliloti protein utilized in nitrogen fixation and microaerobic respiration, is also believed to exert both positive and negative control, which is dependent on phosphorylation, over the activity of the output domain (13). In this case, phosphorylation removes inhibition by opening the structure of the protein and activates the protein by inducing dimerization.

**Comparative models of the activation of FlgR and other NtrC-like proteins.** By analyzing the domains of FlgR, we have developed a model of FlgR activation. We hypothesize that the
receiver domain likely activates the protein when phosphorylated and inhibits it in an unphosphorylated state. The highest level of in vivo FlgR activity observed occurs when the wild-type protein is phosphorylated in the presence of FlgS. In the $\Delta$flgR and flgR(D51A) mutants, as well as the $\Delta$flgS mutant (where wild-type FlgR is present but inactive, presumably due to lack of phosphorylation in the absence of FlgS), there is a complete loss of $\sigma^{54}$-dependent gene expression. These results suggest that phosphorylation of the receiver domain positively influences FlgR activity. When we analyzed FlgR$_{\text{receiver}}$ in C. jejuni, we noticed an intermediate phenotype between strains with wild-type phosphorylated and unphosphorylated FlgR. While FlgR$_{\text{receiver}}$ activates flagellar gene expression at a level that is 10-fold lower than the activation by wild-type FlgR in the presence of FlgS, the expression is increased 100-fold compared to the expression levels in the $\Delta$flgR, flgR(D51A), and $\Delta$flgS mutants. Hence, the removal of the receiver domain appears to eliminate an inhibitory function mediated by this domain of the protein. Thus, we believe the receiver domain can exert both positive and negative regulatory effects. We hypothesize that wild-type, phosphorylated FlgR and FlgR$_{\text{receiver}}$ will have one or more biochemical activities (e.g., oligomerization, ATP hydrolysis, or interactions with $\sigma^{54}$) that are enhanced compared to these activities of wild-type FlgR in an unphosphorylated state. Future studies using purified FlgR and FlgR$_{\text{receiver}}$ proteins will focus on characterizing differences in the biochemical properties of these proteins which may elucidate the precise mechanisms by which the receiver domain prevents FlgR activity under noninducing conditions and how phosphorylation influences FlgR activation.

Generally, the CTDs of NtrC-like proteins contain an HTH motif necessary for interacting with a specific UAS to mediate transcription from target promoters in vivo. The necessity of DNA interactions by these motifs has been studied in several NtrC-like proteins. NtrC, the prototypical member of the NtrC family of proteins, requires the CTD for transcriptional activation in vivo (46). However, some forms of NtrC with mutations in the CTD are capable of promoting a lower level of transcription in vitro (46). While deletion of the CTD of the Rhizobium leguminosarum DetD regulator results in partial expression from the dctA promoter in E. coli, the level of expression observed is 100-fold lower than that in DetD containing the CTD (26). Similarly, R. meliloti producing NifA lacking its CTD results in a 10-fold reduction in the expression of nifH relative to the expression level in a strain containing the wild-type protein (25). The reduced gene expression due to mutation of the CTD in the respective proteins is believed to be largely due to the lack of specific DNA binding to target promoters. Thus, while not always an absolute requirement, DNA binding to appropriate sites is necessary for optimal transcriptional activation by these proteins.

Bioinformatics does not indicate any type of DNA-binding motif located within the CTD of FlgR or in any other portion of the protein. Furthermore, deletion of the FlgR CTD has no observable effect on the expression of $\sigma^{54}$-dependent genes or motility in C. jejuni when the FlgS sensor kinase is present. Thus, FlgR likely does not need to interact with DNA to mediate the expression of target genes. DNA-binding assays were performed with full-length FlgR in the presence and absence of FlgS. In both cases, we could not observe any binding to the promoter regions of flgDE2 or flaB. Although this negative result alone cannot be used to state conclusively that FlgR activates transcription without binding DNA, these data taken in combination with our finding that the CTD is not required for in vivo expression of target genes and the lack of any DNA-binding motif suggest that FlgR may not bind DNA to activate transcription. The FlgR orthologues in H. pylori and Chlamydia trachomatis also do not have a CTD, but the means by which its activity is induced and controlled require further characterization (33). Rrp-2 of Borrelia burgdorferi, another NtrC-like protein, appears to contain a standard CTD but does not need to interact with an enhancer upstream of $\sigma^{54}$-dependent promoters to activate the expression of target genes (6).

Further exploration into a possible role for the CTD of FlgR allowed us to observe that this portion of the protein may have an alternative function in preventing the receiver domain of FlgR from being modified by non-FlgS kinases or phosphodonsor. This hypothesis is supported by the observation that in the absence of FlgS, the expression of $\sigma^{54}$-dependent flagellar genes mediated by FlgR$_{\text{CTD}}$ is principally dependent on D51, the phosphorylated residue. An increased susceptibility for phosphorylation by such phosphodonors or kinases may account for the ability of FlgR$_{\text{CTD}}$ to promote greater flagellar gene expression in the absence of FlgS. Further studies will compare the abilities of wild-type FlgR and FlgR$_{\text{CTD}}$ to be phosphorylated by small phosphodonors or non-FlgS kinases. With the CTD identified as an important domain for putatively controlling the phosphorylation of FlgR, future work will more specifically analyze this domain to determine specific amino acid residues or subregions of this domain that are required for its activity.

NtrC-like proteins with atypical CTDs. Two challenges are created for a bacterium that produces a $\sigma^{54}$-dependent response regulator that does not need to bind DNA for the transcription of target genes: (i) a means of interaction of the response regulator with $\sigma^{54}$ at target promoters when not anchored to DNA and (ii) maintenance of response regulator specificity for activating transcription at the correct regulons to accomplish a specific biological function. The most common mechanism for $\sigma^{54}$-dependent response regulators to interact with $\sigma^{54}$ in the RNAP holoenzyme at target promoters involves the binding of the regulator to the UAS and then bending of the DNA to allow the regulator to interact with $\sigma^{54}$. The only requirement that these DNA-independent regulators must fulfill is that they interact with $\sigma^{54}$ bound to the target promoter. While the exact mechanism of the interaction of FlgR and other DNA-independent regulators with $\sigma^{54}$ in RNAP is unknown, these proteins may directly interact with the $\sigma^{54}$ component of RNAP in solution. Subsequent studies will focus on how FlgR interacts with $\sigma^{54}$ in the RNAP holoenzyme and whether phosphorylation enhances its ability to complex with $\sigma^{54}$.

Considering that these DNA-independent regulators could activate transcription at any $\sigma^{54}$-dependent promoter, maintaining specificity for the expression of only their target genes would seem to present a problem. In most bacteria that have
two or more σ^{54}-dependent response regulators, the specificity of activation at the proper promoters is maintained by UAS binding. For example, Pseudomonas putida must ensure that the activation of the NtrC-like protein FleR (involved in flagellar motility) does not result in the transcription of the genes that are normally regulated by activated XylR (involved in xylene and toluene catabolism) (reviewed in reference 8). One level of specificity is maintained by the FleR-specific UAS being upstream of flagellar genes, whereas the XylR-specific UAS is upstream of the appropriate catabolic genes. Analysis of genome sequences of species of Campylobacter, Helicobacter, and Chlamydia reveals that these bacteria contain only one σ^{54}-dependent response regulator and in each case, the regulator lacks a CTD entirely or expresses one that does not appear to have a DNA-binding motif (5, 33). Therefore, σ^{54} can only be activated by the sole regulator in these bacteria. In addition, all σ^{54}-dependent promoters in these bacteria are used for the expression of genes for a single activity (i.e., flagellar motility in Campylobacter and Helicobacter species). Twelve σ^{54}-dependent promoters are predicted in the C. jejuni genome (17, 47), and the gene products of these promoters are almost all predicted to function in motility. Thus, there is not a need for the extra layer of specificity provided by the regulators binding to specific DNA sequences.

Considering our findings and those from work performed by others, we propose that the FlgR proteins of C. jejuni and H. pylori, CtcC of chlamydial species, and Rep2 of B. burgdorferi may represent a subfamily of NtrC-like proteins that do not interact with DNA at target promoters to activate gene expression. We have demonstrated that within this subfamily, previously undescribed mechanisms to control the activation of the FlgR proteins in C. jejuni and H. pylori may exist. Both of these wild-type proteins can function with C. jejuni FlgS to express σ^{54}-dependent flagellar genes in C. jejuni. However, when we compared the activities of C. jejuni FlgR_{ACSTD} and FlgR_{Ht} (which naturally lacks the CTD) in the C. jejuni ΔflgS mutant, the C. jejuni protein exhibited partial activation but the H. pylori protein was completely inactive. Whereas the function of the CTD of C. jejuni FlgR has been adapted to prevent inappropriate activity in the absence of FlgS and an activating signal, the lack of a CTD in FlgR_{Ht} does not result in partial constitutive activity. Unlike C. jejuni FlgR, FlgR_{Ht} may not interact with an alternative phosphodonor when produced in C. jejuni. Alternatively, FlgR_{Ht} may have a different mechanism for keeping the protein inactive under noninducing conditions that does not depend on a CTD. It remains to be determined how the activation of FlgR_{Ht} is controlled in the absence of a CTD.

Conclusions. As with most motile bacteria, the control of flagellar gene expression in C. jejuni bacteria is important for the proper construction of the flagellum. Combining the results of our studies with those from previous works, we have revealed that C. jejuni has complex and unusual mechanisms for controlling flagellar gene expression that distinguish it from other well-characterized bacteria. Not only is the essential FlgR response regulator subjected to a phase-variable mechanism controlling the production of the protein (21), but it is posttranslationally controlled by a signaling cascade through its cognate sensor kinase FlgS (24, 56 and the present work). Our current analysis of FlgR reveals that C. jejuni has evolved alternative mechanisms of controlling flagellar gene expression that differ from those of the mechanistically characterized NtrC-like proteins. First, it appears to have a receiver domain that can exert both positive and negative influence over controlling the activation of the protein, depending on its phosphorylation state. Second, it has adapted the CTD of FlgR to limit the specificity of phosphorylation to the cognate sensor kinase FlgS. The signal that is sensed by FlgS to culminate in FlgR activation is hypothesized to originate from the flagellar export apparatus, presumably in conjunction with FlhF, a putative GTPase. Future analysis will provide insight into how FlhF, the export apparatus, and the FlgSR two-component system communicate with each other, ultimately activating the FlgR response regulator to stimulate σ^{54}-dependent flagellar gene expression.

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