A specificity determinant for phosphorylation in a response regulator prevents in vivo cross-talk and modification by acetyl phosphate

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Bacterial two-component systems (TCSs) sense stimuli and transduce signals intracellularly through phosphotransfer between cognate histidine kinases (HKs) and response regulators (RRs) to alter gene expression or behavioral responses. Without high phosphotransfer specificity between cognate HKs and RRs, cross-phosphorylation or cross-talk between different TCSs may occur and diminish responses to appropriate stimuli. Some mechanisms to reduce cross-talk involve HKs controlling levels of cognate RR phosphorylation. Conceivably, some RRs may have evolved HK-independent strategies to insulate themselves from cross-talk with acetyl phosphate (AcP) or other small phosphodonor metabolites. Initial steps in flagellar biosynthesis in *Campylobacter jejuni* stimulate phosphotransfer from the FlgS HK to the FlgR RR to promote σ^{54}-dependent flagellar gene expression. We discovered that the FlgR C-terminal domain (CTD), which commonly functions as a DNA-binding domain in the NtrC RR family, is a specificity determinant to limit in vivo cross-talk from AcP. FlgR lacking the CTD ($FlgR_{\Delta CTD}$) used FlgS or AcP as an in vivo phosphodonor and could be reprogrammed in Δ*flgS* mutants to respond to cellular nutritional status via AcP levels. Even though exclusive AcP-mediated activation of $FlgR_{\Delta CTD}$ promoted WT flagellar gene expression, proper flagellar biosynthesis was impaired. We propose that the FlgR CTD prevents phosphotransfer from AcP so that FlgR is solely responsive to FlgS to promote proper flagellar gene expression and flagellation. In addition to mechanisms limiting cross-talk between noncognate HKs and RRs, our work suggests that RRs can possess domains that prevent in vivo cross-talk between RRs and the endogenous metabolite AcP to ensure signaling specificity.

Cellular signal transduction systems link extracellular or intracellular stimuli to appropriate output responses. Prokaryotic organisms often are useful models for understanding aspects of signal transduction that may be applicable to higher organisms. Bacteria commonly use two-component systems (TCSs) to mediate responses to specific conditions. A basic TCS consists of a sensor histidine kinase (HK) that autophosphorylates upon detection of a specific signal (1). The phosphorylated histidine of the HK serves as a phosphodonor for autophosphorylation by the cognate response regulator (RR). The phosphorylated RR can then alter gene expression or a behavioral response. TCSs generally do not exist in isolation in a bacterial cell, but among a signaling network of up to as many as 200 different TCSs, depending on the species. Despite structural similarity between many TCSs, phosphotransfer specificity between cognate HK and RR pairs is high (2). In vivo cross-phosphorylation, or cross-talk, between noncognate HK and RRs is usually maintained at a minimum. If cross-talk between two TCSs occurs, correct responses to specific signals may be diminished or inhibited.

Mechanisms exist to insulate a TCS from cross-talk and ensure that intrasystem signal transduction fidelity is preserved. In many TCSs, specific amino acids mediate molecular recognition between cognate HK and RR pairs (3–6). In addition, some HKs are bifunctional with a phosphatase activity to reduce levels of phosphorylated cognate RRs. These bifunctional HKs control activity of the RR by reducing phosphorylation that may occur via cognate HKs, noncognate HKs, or low molecular-weight phosphodonors such as acetyl phosphate (AcP) (1, 7–10). Together, these mechanisms contribute to phosphotransfer specificity in some TCSs.

The FlgSR TCS of *Campylobacter jejuni* is required for expression of the σ^{54} regulon, which mainly includes flagellar rod and hook genes (11, 12). Initiation of signal transduction through FlgSR is dependent upon components of the flagellar type III secretion system (T3SS) (13, 14). After autophosphorylation of H141 of the cytoplasmic FlgS HK, FlgR autophosphorylates on D51 of the receiver domain, using the phosphohistidine of FlgS as a substrate. Phosphorylation of FlgR is required for σ^{54}-dependent gene expression. Because the flagellar T3SS exports rod and hook proteins out of the cytoplasm, FlgSR links T3SS formation to expression of genes encoding substrates secreted by the T3SS to synthesize the organelle (14).

Most NtrC-like RRs possess an essential C-terminal DNA-binding domain (CTD) that interacts with target promoters to activate gene expression. Mutant RRs lacking the CTD fail to stimulate WT levels of σ^{54}-dependent gene expression under normal conditions, even in the presence of the cognate HK (15–17). In contrast, FlgR lacking its CTD ($FlgR_{\Delta CTD}$) activated WT levels of σ^{54}-dependent gene expression in the presence of FlgS (13). However, the activities of WT FlgR and $FlgR_{\Delta CTD}$ in Δ*flgS* mutants differed. Whereas WT FlgR without FlgS did not stimulate σ^{54}-dependent gene expression, $FlgR_{\Delta CTD}$ in a Δ*flgS* mutant activated expression of the σ^{54} regulon. However, the level of gene expression in Δ*flgS* $flgR_{\Delta CTD}$ was ∼20% of that of *C. jejuni* with a WT FlgSR TCS. Because $FlgR_{\Delta CTD}$ in the Δ*flgS* mutant required the phosphorylated D51 residue to activate gene expression (13), $FlgR_{\Delta CTD}$ must have autophosphorylated via a noncognate phosphodonor in this mutant. Thus, these results question the role of the FlgR CTD in DNA binding and suggest that the CTD may serve an alternative function to limit phosphotransfer specificity or cross-talk to FlgR.

We explored the role of the CTD of FlgR in activation of σ^{54}-dependent flagellar gene expression. By conducting genetic, biochemical, and physiological studies, we discovered that unlike most NtrC-like RRs, the CTD of FlgR has a DNA-binding activity that is not essential for expression of target genes under physiological conditions. Instead, we show that the CTD is a specificity determinant for phosphorylation that expressly limits the ability of FlgR to autophosphorylate in vivo using the small molecular-weight phosphodonor AcP. Without the CTD, metabolic processes that alter AcP levels influence FlgR activation, but complete reliance of FlgR on AcP for WT levels of gene

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expression hindered flagellation. As a result of the CTD limiting cross-talk of FlgR with AcP, FlgR activation is coupled to FlgS and a step in flagellar biosynthesis to promote proper gene expression and flagellation for optimal fitness. Whereas previously known mechanisms to eliminate intersystem cross-talk mostly involved activities of HKs, our work identified a domain within a RR that specifically prevents cross-talk with the endogenous central metabolite AcP.

Results

DNA Binding by FlgR Is Not Required for σ^{54}-Dependent Gene Expression. *C. jejuni* FlgR is a member of the NtrC family of RRs. As such, FlgR contains an N-terminal receiver domain modified by phosphorylation on D51 and a central ATPase domain necessary for oligomerization and interactions with σ^{54} (Fig. 1*A*). Within the CTD of most NtrC-like RRs is a helix-turn-helix (HTH) motif essential for DNA binding and function as a transcriptional regulator (15, 16). This HTH binds enhancer-like sites upstream of target promoters to promote WT levels of gene expression under normal conditions. Unlike most other NtrC-like RRs, the FlgR CTD lacks a strongly predicted HTH motif. Compared with WT *C. jejuni* with an intact FlgSR, a *flgR*$_{\Delta CTD}$ mutant (which produces WT FlgS) promoted similar or slightly higher levels of expression of σ^{54}-dependent genes (Table S1) (13). These results suggested either that the FlgR CTD does not bind DNA, or that any DNA-binding activity of the CTD is not essential for FlgR-dependent gene expression. Therefore, we explored the role of the CTD in the function of FlgR as a transcriptional regulator.

We analyzed FlgR interactions with the σ^{54}-dependent *flgDE2* promoter (P_{flgDE2}) by EMSAs. Purified WT FlgR bound promoter DNA encompassing –302 to +79 bases relative to the *flgDE2* transcriptional start site in a dose-dependent manner (Fig. 1*B*) (18). Binding by WT FlgR was specific, because excess unlabeled P_{flgDE2} DNA, but not P_{gyrA} DNA (a FlgR-independent promoter), competed for binding (Fig. 1*C*). In contrast, FlgR$_{\Delta CTD}$ only bound P_{flgDE2} at the highest protein concentration examined, but binding was nonspecific, because both unlabeled P_{flgDE2} and P_{gyrA} DNA reduced residual binding of FlgR$_{\Delta CTD}$ to labeled P_{flgDE2} DNA (Fig. 1*C*).

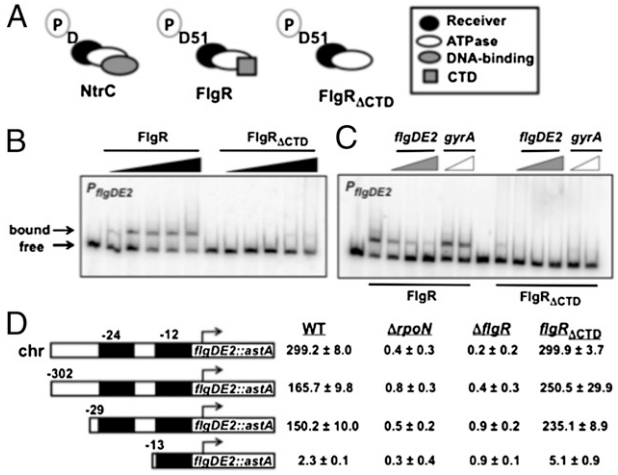

Fig. 1. Analysis of the requirement of FlgR/DNA interactions for σ^{54}-dependent flagellar gene expression. (*A*) Domain organization of *E. coli* NtrC, *C. jejuni* WT FlgR, and *C. jejuni* FlgR$_{\Delta CTD}$. The phosphorylated aspartic acids (D or D51; P denotes phosphoryl group) in receiver domains are indicated. (*B* and *C*) EMSAs to analyze P_{flgDE2} DNA binding by FlgR proteins. (*B*) WT FlgR and FlgR$_{\Delta CTD}$ were used (from left to right) at 0, 0.1, 0.25, 0.5, 0.75, and 1 μM. (*C*) Increasing ratios of unlabeled P_{flgDE2} DNA (1:1, 5:1, or 10:1) or P_{gyrA} DNA (1:1 or 10:1) to labeled P_{flgDE2} DNA were incubated with 1 μM of FlgR proteins. (*D*) Expression of *flgDE2*::*astA* with 5′ promoter truncations in WT *C. jejuni* or *flgR*$_{\Delta CTD}$, Δ*rpoN* (Δσ^{54}), or Δ*flgR* mutants. *flgDE2*::*astA* expression levels are reported as arylsulfatase units ± SD. All transcriptional fusions were on plasmids, except for the fusion at the native chromosomal *flgDE2* locus (chr). The 5′ ends of fusions on plasmids are indicated relative to the transcriptional start site (arrow). Black boxes indicate the –24 and –12 σ^{54} binding sites essential for σ^{54}-dependent gene expression.

Because our results suggested that the FlgR CTD binds DNA but is not essential for expression of the σ^{54} regulon, we hypothesized that the 5′ end of a *C. jejuni* FlgR- and σ^{54}-dependent promoter may only need to begin with the –24 and –12 σ^{54} binding sites. Thus, we tested if upstream promoter DNA, which usually contains binding sites for a NtrC-like RR, may be removed and not alter expression of *C. jejuni* σ^{54}-dependent genes. Therefore, we analyzed the ability of *C. jejuni* strains producing WT FlgS and either WT FlgR or FlgR$_{\Delta CTD}$ to express *flgDE2*::*astA* transcriptional fusions with 5′ truncations of P_{flgDE2}. Because the flagellar gene *fliK* overlaps P_{flgDE2}, and chromosomal deletions upstream of P_{flgDE2} would create *fliK* mutants that alter expression of the σ^{54} regulon in *C. jejuni* (19), we analyzed expression of *flgDE2*::*astA* with 5′ promoter truncations on plasmids in *C. jejuni* strains.

Because WT FlgR, but not FlgR$_{\Delta CTD}$, specifically bound P_{flgDE2} DNA from –302 to +79 (Fig. 1 *B* and *C*), we considered this promoter fragment potentially sufficient for *flgDE2* expression. For all analyses in this work, WT *flgR* and *flgR*$_{\Delta CTD}$ were expressed from the native chromosomal *flgR* locus. As shown in Fig. 1*D*, WT *C. jejuni* and the *flgR*$_{\Delta CTD}$ mutant, which both produced WT FlgS, expressed chromosomal- or plasmid-borne *flgDE2*::*astA* with base –302 as the 5′ end of P_{flgDE2}. More expression was noted in the *flgR*$_{\Delta CTD}$ mutant than in WT *C. jejuni* for plasmid-borne *flgDE2*::*astA*, which may indicate some DNA conformational changes that artificially promote slightly more expression with FlgR$_{\Delta CTD}$. Regardless, expression of P_{flgDE2} was dependent on both a FlgR protein and σ^{54} (encoded by *rpoN*; Fig. 1*D*). Removal of DNA up to base –29 (six bases before the essential σ^{54} binding sites) did not significantly reduce expression of *flgDE2*::*astA* in WT *C. jejuni* or the *flgR*$_{\Delta CTD}$ mutant. However, deletion of DNA up to base –13 (which removes the essential –24 site for σ^{54} binding) eliminated expression of *flgDE2*::*astA* in all strains regardless of the FlgR protein produced (Fig. 1*D*). Therefore, we concluded that a minimal σ^{54}-dependent promoter in *C. jejuni* likely includes only σ^{54} binding sites at the 5′ end. Furthermore, the FlgR CTD bound DNA in σ^{54}-dependent promoters, but DNA binding by FlgR was not essential for gene expression.

The Acetogenesis Pathway Influences in Vivo Activation of FlgR$_{\Delta CTD}$ in the Absence of FlgS. In strains producing FlgS, FlgR$_{\Delta CTD}$ promoted equal to modestly higher expression of most σ^{54}-dependent flagellar genes relative to WT FlgR (Figs. 1*D* and 2 and Table S1). In a Δ*flgS* mutant, WT FlgR did not activate σ^{54}-dependent flagellar gene expression (Fig. 2) (11). In contrast, FlgR$_{\Delta CTD}$ activated expression of σ^{54}-dependent genes without FlgS (Fig. 2) (13). However, the level of gene expression promoted by FlgR$_{\Delta CTD}$ without FlgS was ~20% of the level of *C. jejuni* with a WT FlgSR TCS (Fig. 2). Furthermore, FlgR$_{\Delta CTD}$ required phosphorylation of D51 in the receiver domain to activate gene expression in a Δ*flgS* mutant (13). These results indicated that the FlgR CTD may limit in vivo phosphotransfer to FlgR by noncognate HKs or other phosphodonors, in addition to a nonessential DNA-binding activity.

To search for in vivo phosphodonors that activate FlgR$_{\Delta CTD}$ in the absence of FlgS, we performed transposon (Tn) mutagenesis with the *darkhelmet* Tn in *C. jejuni* Δ*flgS flgR*$_{\Delta CTD}$ *flaB*::*astA* and identified seven of ~3,750 Tn mutants with seven- to 180-fold reductions in expression of the σ^{54}-dependent *flaB*::*astA* transcriptional reporter (Fig. 2). All mutants contained a Tn in genes likely affecting the acetogenesis pathway, a multistep process that converts pyruvate to ATP and acetate (reviewed in ref. 20). An intermediate in this pathway is AcP, a low molecular-weight

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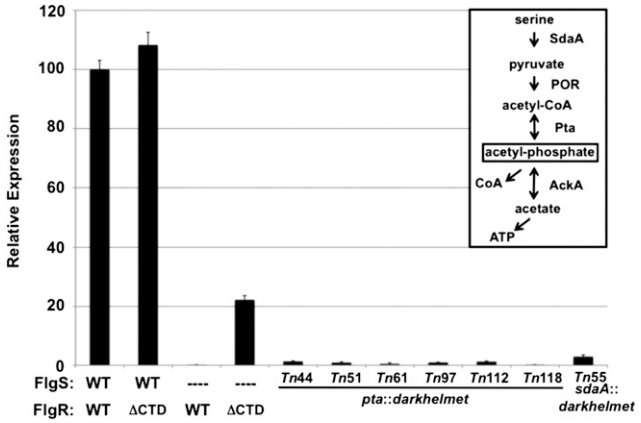

Fig. 2. Identification of *darkhelmet* Tn mutants with reduced $FlgR_{\Delta CTD}$ activity in Δ*flgS* mutants. Arylsulfatase assay examining expression of the σ^{54}-dependent *flaB*::*astA* transcriptional fusion in *C. jejuni* strains grown on MH agar. The level of *flaB*::*astA* expression in each strain is relative to WT *C. jejuni* producing the WT FlgSR TCS, which was set to 100 units. The FlgS and FlgR proteins produced in each strain are indicated. ΔCTD indicates $FlgR_{\Delta CTD}$. Dashes indicate deletions of respective genes. Tn mutants are in the *C. jejuni* Δ*astA* Δ*flgS* $flgR_{\Delta CTD}$ *flaB*::*astA* background. Error bars indicate SDs. (*Inset*) Acetogenesis pathway as outlined in *E. coli* with minor modifications (20), and predicted to be intact in *C. jejuni* (24, 25).

phosphodonor often used to autophosphorylate many RRs in vitro, and a few RRs in vivo (8, 21–23). Six independent Tn insertions were within *pta*, encoding phosphotransacetylase, which reversibly converts acetyl-CoA (Ac-CoA) and inorganic phosphate to CoA and AcP (Fig. 2). One mutant contained a Tn in *sdaA*, encoding serine dehydratase, which converts serine to pyruvate upstream of the pathway. Immediately downstream of *pta* on the *C. jejuni* chromosome is *ackA*, encoding acetate kinase, which reversibly converts AcP and ADP to acetate and ATP in the acetogenesis pathway. Thus, Tn insertions in *pta* may have had polar effects on *ackA* expression and eliminated AcP production altogether. Mutants with Tn insertions in genes encoding HKs were not identified in this screen. These results suggested that fluctuations in AcP biosynthesis from the acetogenesis pathway may have directly influenced in vivo phosphorylation of $FlgR_{\Delta CTD}$ and σ^{54}-dependent flagellar gene expression.

Initial studies suggested that the acetogenesis pathway is intact in *C. jejuni* as in *Escherichia coli* (20, 24, 25). To analyze possible in vivo AcP-mediated activation of $FlgR_{\Delta CTD}$, *C. jejuni* mutants predicted to produce different intracellular levels of AcP when grown on Mueller–Hinton (MH) agar were made: Δ*ackA*, high AcP; Δ*pta*, low AcP; and Δ*pta* Δ*ackA*, negligible AcP. Although we could not directly measure AcP levels in *C. jejuni*, our data described below suggested that the *C. jejuni* mutants produced similar trends in AcP levels as respective *E. coli* mutants (22, 26, 27). When *ackA* was deleted in the Δ*flgS* $flgR_{\Delta CTD}$ mutant, *flaB*::*astA* expression increased 4.5-fold and was 70% of that observed in WT *C. jejuni* with an intact FlgSR TCS (Table 1). Stepwise decreases in AcP levels by mutating *pta* alone and both *pta* and *ackA* reduced *flaB*::*astA* expression to negligible levels in the Δ*flgS* $flgR_{\Delta CTD}$ mutant (Table 1). Mutation of the phosphorylated D51 residue in $FlgR_{\Delta CTD}$ abolished all effects of the acetogenesis pathway on *flaB*::*astA* expression in the absence of FlgS (Table 1). In contrast to the Δ*flgS* $flgR_{\Delta CTD}$ mutant, *flaB*::*astA* expression in *C. jejuni* producing WT FlgR in a Δ*flgS* mutant was only slightly increased by deleting *ackA* (Table 1). We next examined if the acetogenesis pathway could influence FlgR or $FlgR_{\Delta CTD}$ activity in the presence of FlgS. Deletion of *ackA* did not increase *flaB*::*astA* expression in WT *C. jejuni* (Table 1). However, $FlgR_{\Delta CTD}$-stimulated *flaB*::*astA* expression in the presence of FlgS increased 18% when *ackA* was deleted (Table 1). These results verified that the acetogenesis pathway significantly modulated $FlgR_{\Delta CTD}$ activity for promoting σ^{54}-dependent gene expression, both in the presence and absence of FlgS.

The CTD Is a Specificity Determinant That Limits FlgR Autophosphorylation via AcP. Our results suggested that in vivo $FlgR_{\Delta CTD}$ autophosphorylation via AcP is likely enhanced relative to WT FlgR. Thus, we monitored the in vitro ability of FlgR proteins to autophosphorylate with AcP as the sole phosphodonor. In our assays, we observed modification of both proteins with Ac[^{32}P], with at least a two- to threefold greater level of autophosphorylation of $FlgR_{\Delta CTD}$ compared with WT FlgR (Fig. 3*A* and Fig. S1). Autophosphorylation of both RRs via AcP was increased as protein levels increased and was inhibited by mutation of the phosphorylated D51 residue. Enhanced modification of $FlgR_{\Delta CTD}$ was specific for AcP, because phosphotransfer from FlgS to $FlgR_{\Delta CTD}$ was ~25–70% less than WT FlgR over time (Fig. 3*B*). Therefore, the CTD specifically limited cross-talk and phosphotransfer from AcP to FlgR.

Reprogramming Activation of $FlgR_{\Delta CTD}$ by AcP Through Metabolism. AcP-mediated activation of $FlgR_{\Delta CTD}$ suggested that deletion of the CTD allowed FlgR to directly respond to the nutritional status of the cell through the acetogenesis pathway, in addition to responding to steps in flagellar biosynthesis via signal transduction through FlgS. Therefore, we tested if altering physiology of *C. jejuni* Δ*flgS* mutants would stimulate $FlgR_{\Delta CTD}$ more than WT FlgR to augment both flagellar gene expression and flagellar biosynthesis. In addition, we analyzed whether FlgR or $FlgR_{\Delta CTD}$ activity could be modulated by altering physiology even in the presence of FlgS.

To determine if FlgR could be reprogrammed to respond to metabolic cues, we grew *C. jejuni* strains on *Campylobacter*

Table 1. Effect of the acetogenesis pathway on activation of FlgR proteins and σ^{54}-dependent gene expression

	FlgS/FlgR protein produced		*pta ackA* genotype			
flgS flgR genotype	FlgS	FlgR	WT	Δ*ackA*	Δ*pta*	Δ*ptaA* Δ*ackA*
WT	WT	WT	100 ± 6.9*	96.4 ± 3.8	ND†	ND
Δ*flgR*	WT	–	0.3 ± 0.1	ND	ND	ND
$flgR_{\Delta CTD}$	WT	$FlgR_{\Delta CTD}$	98.3 ± 3.2	116.0 ± 5.9	ND	ND
Δ*flgS*	–	WT	0.3 ± 0.0	1.8 ± 0.6	0.3 ± 0.1	0.1 ± 0.0
Δ*flgS* $flgR_{\Delta CTD}$	–	$FlgR_{\Delta CTD}$	15.3 ± 2.3	70.6 ± 14.4	4.3 ± 0.5	0.3 ± 0.1
Δ*flgS* $flgRD51A_{\Delta CTD}$	–	FlgR D51A$_{\Delta CTD}$	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.2

*Arylsulfatase assay examining *flaB*::*astA* expression after growth on MH agar. The level of *flaB*::*astA* expression (±SD) in each strain is relative to WT *C. jejuni* producing the WT FlgSR TCS, which was set to 100 units.
†Not determined.

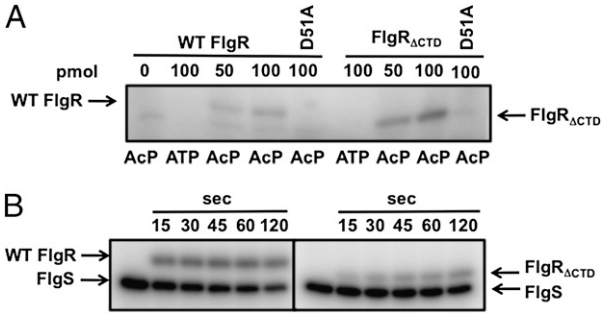

Fig. 3. In vitro phosphorylation of FlgR proteins. (*A*) Autophosphorylation of FlgR proteins with Ac[^{32}P] or [γ^{32}P]ATP. WT FlgR or FlgR$_{\Delta CTD}$ (50 and 100 pmol) or respective D51A mutants (100 pmol) are indicated. (*B*) Phosphotransfer from FlgS to FlgR proteins. Six picomoles of WT FlgR or FlgR$_{\Delta CTD}$ were mixed with autophosphorylated ^{32}P-FlgS and removed after 15–120 s.

defined media (CDM) with increasing pyruvate concentrations to increase intracellular AcP levels via the acetogenesis pathway. No changes in *flaB*::*astA* expression were observed in WT *C. jejuni* with an intact FlgSR TCS by deleting *ackA* or by increasing pyruvate levels in the media (Fig. 4). However, we observed a 9–25% increase in *flaB*::*astA* expression when strains producing WT FlgS and FlgR$_{\Delta CTD}$ were grown in increasing concentrations of pyruvate to stimulate AcP biosynthesis (Fig. 4). These results suggested that even in the presence of WT FlgS, FlgR$_{\Delta CTD}$, but not WT FlgR, is responsive to AcP, which results in augmented levels of gene expression.

We next examined *flaB*::*astA* expression upon modulating the physiology of *C. jejuni* strains lacking FlgS. As a result of deletion of *flgS*, AcP is presumably the only possible phosphodonor for FlgR proteins. *flaB*::*astA* expression in Δ*flgS flgR*$_{\Delta CTD}$ after growth on CDM alone was only 6% of that of WT *C. jejuni* with an intact FlgSR TCS grown on the same media (Fig. 4). However, upon increasing pyruvate concentrations, *flaB*::*astA* expression increased ∼14-fold (Fig. 4); this enhanced FlgS-independent, pyruvate-stimulated *flaB*::*astA* expression was 81–88% of that of WT *C. jejuni* grown in similar levels of pyruvate. When *ackA* was deleted, the baseline level of *flaB*::*astA* expression in *C. jejuni* Δ*flgS flgR*$_{\Delta CTD}$ grown in CDM increased (Fig. 4). In higher pyruvate concentrations, *flaB*::*astA* expression in the Δ*flgS flgR*$_{\Delta CTD}$ Δ*ackA* mutant equaled and even surpassed WT *C. jejuni* with an intact FlgSR TCS by 26% (Fig. 4). Deletion of both *pta* and *ackA* prevented activation of FlgR$_{\Delta CTD}$ and *flaB*::*astA* expression regardless of pyruvate concentrations, presumably due to low AcP levels in this mutant (Fig. S2).

In contrast, *C. jejuni* producing WT FlgR without FlgS was not responsive to increasing pyruvate levels as measured by *flaB*::*astA* expression (Fig. 4). However, by presumably increasing intracellular AcP levels through deletion of *ackA*, an appreciable increase in FlgS-independent gene expression by WT FlgR was observed, which was further augmented two- to threefold by increasing pyruvate levels (Fig. 4). However, *flaB*::*astA* expression levels were dramatically lower with AcP-dependent activation of WT FlgR than FlgR$_{\Delta CTD}$.

We next determined if levels of flagellation could be changed via AcP-mediated activation of WT FlgR or FlgR$_{\Delta CTD}$ by altering *C. jejuni* physiology. We monitored the number of flagella on individual bacteria after growth on CDM alone or CDM with excess pyruvate. Approximately 93–97% of WT *C. jejuni* cells produced a single flagellum at one or both poles when grown on CDM or CDM with pyruvate or with deletion of *ackA* (64–75% with a flagellum at each pole, 21–29% with a flagellum only at one pole; Table 2 and Table S2). Similar levels of flagellation were observed in the *flgR*$_{\Delta CTD}$ mutant producing WT FlgS, regardless of pyruvate levels in media or the presence of *ackA* (Table S2). Considering that pyruvate augmented FlgR$_{\Delta CTD}$-dependent flagellar gene expression but not flagellation in the presence of FlgS (Fig. 4 and Table S2), these findings suggested that other factors besides expression of flagellar rod and hook proteins are rate-limiting steps in flagellation. We next analyzed if growth on pyruvate could stimulate WT FlgR or FlgR$_{\Delta CTD}$ in Δ*flgS* mutants to result in maximal flagellation, despite lacking the ability to use FlgS to link expression of the σ^{54} regulon to flagellar T3SS formation. Even though we observed pyruvate-stimulated activation of WT FlgR in the absence of FlgS that slightly increased flagellar gene expression (Fig. 4), this strain was aflagellated under all growth conditions, with or without *ackA* mutation. For the Δ*flgS flgR*$_{\Delta CTD}$ mutant, most cells were aflagellated after growth on MH or CDM agar. However, growth on CDM with excess pyruvate resulted in ∼30% of cells producing flagella (Table 2). Upon deletion of *ackA*, the population of flagellated Δ*flgS flgR*$_{\Delta CTD}$ cells increased to 56% with pyruvate supplementation (Table 2). Despite increases in flagellation, the number of Δ*flgS flgR*$_{\Delta CTD}$ cells producing two flagella (the predominant WT phenotype) was always lower than WT *C. jejuni*. Thus, removal of the CTD reprogrammed FlgR to respond to the nutritional status of *C. jejuni* via AcP levels and resulted in similar levels of gene expression as WT *C. jejuni* with an intact FlgSR TCS (Fig. 4). However, coupling FlgR activation

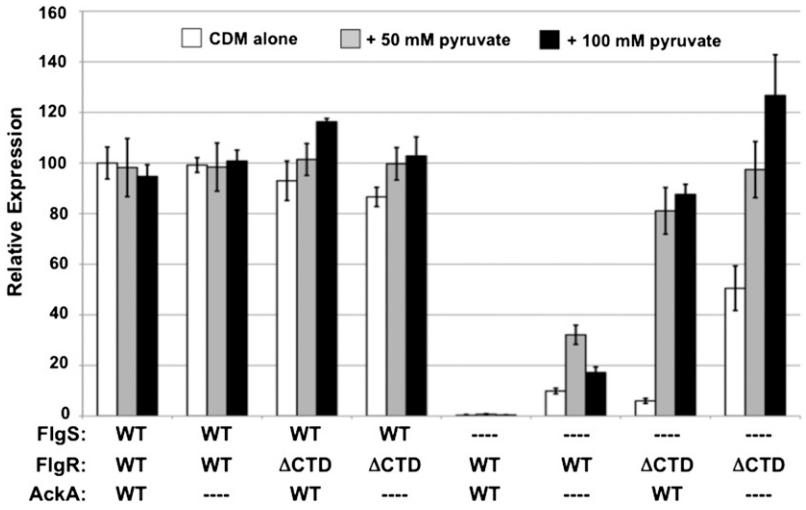

Fig. 4. FlgS-independent activation of FlgR$_{\Delta CTD}$ by altering *C. jejuni* physiology. Arylsulfatase assay examining *flaB*::*astA* expression after growth on CDM alone (white bars), CDM with 50 mM (gray bars), or 100 mM (black bars) excess sodium pyruvate. The level of *flaB*::*astA* expression in each strain is relative to WT *C. jejuni* producing the WT FlgSR TCS grown on CDM alone, which was set to 100 units. The FlgS, FlgR, and AckA proteins produced in each strain are indicated. ΔCTD indicates FlgR$_{\Delta CTD}$. Dashes indicate deletions of respective genes. Error bars indicate SDs.

Table 2. Flagellation due to AcP-dependent activation of FlgR proteins by altering *C. jejuni* physiology

Strain	Media	No. of flagella per bacterium*		
		2	1	0
WT	CDM	64 ± 7	29 ± 7	6 ± 0
WT	CDM + pyruvate	67 ± 1	27 ± 6	6 ± 5
Δ*flgS* $flgR_{\Delta CTD}$	CDM	0 ± 0	1 ± 0	99 ± 0
Δ*flgS* $flgR_{\Delta CTD}$	CDM + pyruvate	4 ± 1	26 ± 4	71 ± 3
Δ*flgS* $flgR_{\Delta CTD}$ Δ*ackA*	CDM	0 ± 0	5 ± 3	95 ± 3
Δ*flgS* $flgR_{\Delta CTD}$ Δ*ackA*	CDM + pyruvate	8 ± 3	48 ± 8	44 ± 11

*The percentage of the population producing a single flagellum at both poles (two flagella), a single flagellum at one pole (one flagellum), or no flagella (zero) after growth on CDM or CDM with 100 mM excess sodium pyruvate are indicated. Data are the average of two experiments ± SD.

to the flagellar T3SS through the cognate FlgS HK promoted optimal flagellation.

In Vivo Activation of $FlgR_{\Delta CTD}$ by AcP Is Not Reduced by a Potential FlgS Phosphatase Activity. In addition to functioning as kinases, many HKs are bifunctional with a phosphatase activity for their cognate RRs. If conditions favor the HK possessing a net phosphatase activity, the level of the phosphorylated cognate RR decreases. The HK can dephosphorylate the RR regardless of the original phosphodonor used to autophosphorylate the RR. To date, a potential FlgS phosphatase activity has not directly been examined. If one exists, then in vivo AcP-mediated activation of FlgR may be prevented both by the CTD and FlgS.

Flagellar T3SS components such as FlhA are required to stimulate FlgSR signal transduction and transcription of σ^{54}-dependent flagellar genes (11, 14). A $flgR_{\Delta CTD}$ mutant was able to express *flaB*::*astA* in the absence of FlhA and/or FlgS, due to AcP-mediated activation of $FlgR_{\Delta CTD}$ (Fig. 5) (14). More importantly, the level of *flaB*::*astA* expression in the Δ*flhA* $flgR_{\Delta CTD}$ mutant (which produces FlgS with low autokinase activity) was at least the same as the Δ*flgS* $flgR_{\Delta CTD}$ mutant. Thus, FlgS when inactive as a kinase failed to protect $FlgR_{\Delta CTD}$ from cross-talk with AcP and reduce gene expression. Furthermore, these findings demonstrate the importance of the CTD in eliminating cross-talk of FlgR with AcP and influences from metabolism. As such, the CTD prevents AcP-mediated activation of FlgR and unnecessary flagellar gene expression and protein production in a bacterium unable to synthesize flagella due to a deficiency in producing a flagellar T3SS. Thus, the CTD is a key specificity determinant in the FlgSR TCS to prevent cross-talk between FlgR and endogenous metabolic phosphodonors.

Discussion

Maintaining signaling fidelity within a TCS is essential for promoting the correct type and degree of response to particular stimuli. In many TCSs, HKs serve as phosphodonors for cognate RRs to result in proper output responses. If mechanisms do not exist to limit specificity of communication of each HK to a cognate RR, intersystem cross-talk between two TCSs may cause signaling interference and obstruct responses to activating stimuli. Mechanisms identified so far that promote intrasystem signaling fidelity and limit intersystem cross-talk include molecular recognition between cognate HK and RR pairs and phosphatase activities of some HKs that control levels of phosphorylation of cognate RRs (1, 3–6).

We identified a domain in a RR that limits in vivo cross-talk specifically from the metabolite AcP, rather than from a noncognate HK. This specificity determinant in *C. jejuni* FlgR is located in the CTD, which in most other NtrC-like RRs functions in an essential DNA-binding activity (15–17). Without the

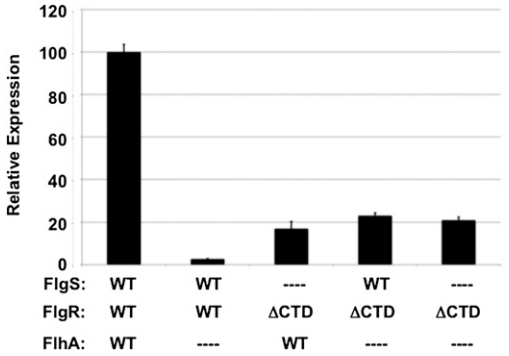

Fig. 5. Examination of an in vivo FlgS phosphatase activity for AcP-activated $FlgR_{\Delta CTD}$. Arylsulfatase assay examining *flaB*::*astA* expression after growth on MH agar. The level of *flaB*::*astA* expression in each strain is relative to WT *C. jejuni* producing the WT FlgSR TCS, which was set to 100 units. The FlgS, FlgR, and FlhA proteins produced in each strain are indicated. ΔCTD indicates $FlgR_{\Delta CTD}$. Dashes indicate deletions of respective genes. Error bars indicate SDs.

CTD, FlgR used AcP as an in vivo phosphodonor for autophosphorylation to stimulate gene expression, even in the presence of the cognate FlgS HK. Furthermore, $FlgR_{\Delta CTD}$ could be reprogrammed to respond to the nutritional status of the cell by altering the acetogenesis pathway and presumably AcP levels to result in levels of expression of the σ^{54} regulon that equaled and exceeded WT *C. jejuni* with an intact FlgSR TCS. AcP serves as an in vivo phosphodonor for autophosphorylation of many RRs (9), but AcP-dependent activation of RRs is often negated by phosphatase activities of some cognate HKs. However, AcP has been shown to be the sole in vivo phosphodonor of a few RRs that appear to lack cognate HKs, including *Borrelia burgdorferi* Rrp2, which is also an NtrC-like RR (22, 23). Though our data strongly suggests that AcP directly serves as a phosphodonor for $FlgR_{\Delta CTD}$, an alternative, yet remote, possibility is that high AcP levels stimulate a noncognate HK that weakly promotes phosphotransfer to FlgR. However, we have not identified another HK that influences FlgR activity in various genetic screens we have conducted in *C. jejuni*.

A major question arising from this work is how the CTD limits in vivo cross-talk between AcP and FlgR. Barbieri et al. (28) recently suggested a possible explanation for how phosphotransfer from small phosphodonors to some RRs may be influenced by interdomain interactions within a RR. By examining members of the OmpR/PhoB RR family, correlations between the in vitro ability of a protein to autophosphorylate and the degree of interdomain interactions were found. More extensive contacts between the receiver and effector domains of a RR were proposed to stabilize an inactivate state that is a barrier to autophosphorylation using small phosphodonors. Thus, interdomain interactions may naturally reduce substantial phosphotransfer from small phosphodonors to many RRs in TCS.

In light of this study, we attempted to determine structural differences between WT FlgR and $FlgR_{\Delta CTD}$ that may explain how the CTD reduces or prevents AcP-dependent $FlgR_{\Delta CTD}$ autophosphorylation. However, like many NtrC family members, the FlgR proteins were refractory to crystallization for structural analyses. Docking of atomic models of individual domains of *Salmonella typhimurium* NtrC have provided the most complete structure possible for this family of RRs (29). In this model, the CTD does not appear to be near to or interact with the N-terminal domain. Therefore, considerable doubt exists that the CTD of FlgR may directly obstruct the receiver domain, thereby limiting its ability to use AcP as a phosphodonor. It is possible that deletion of the CTD of FlgR may cause small structural changes in the protein, which results in a receiver domain with a conformation that more readily accepts AcP as a phosphodonor.

Even though we created physiological conditions that promoted AcP-dependent activation of $FlgR_{\Delta CTD}$ to result in levels of flagellar gene expression equivalent to WT *C. jejuni* with an intact FlgSR TCS, linking gene expression to the nutritional status of the cell did not result in WT flagellation. An ordered expression of subsets of flagellar genes is required for proper flagellar biosynthesis. Linking FlgR activation via FlgS to the flagellar T3SS appears to allow for correct temporal expression of σ^{54}-dependent genes for flagellation. Because the σ^{54} regulon encodes rod and hook proteins, expression of these genes is necessary only after the T3SS has formed so that ordered protein secretion occurs for efficient flagellar biosynthesis. AcP-dependent activation of FlgR does not allow for precise temporal gene regulation relative to T3SS formation, and consequently reduced flagellation in *C. jejuni*. Whereas *C. jejuni* would rely on environmental nutrient composition for AcP-mediated activation of FlgR for motility, FlgS activation of FlgR allows the bacterium to produce flagella independently of exogenous factors, which likely enhances in vivo fitness of *C. jejuni* in hosts. Furthermore, the CTD allows FlgR to remain insensitive to AcP and influences from metabolism, preventing unnecessary flagellar gene expression and protein synthesis when flagella cannot form due to incomplete initial stages of flagellar biosynthesis.

We also found that FlgR activated WT levels of gene expression in the absence of DNA binding by the CTD or without DNA upstream of a σ^{54}-dependent promoter. These results support the hypothesis that FlgR may initiate transcription of some σ^{54}-dependent genes without binding DNA. We propose that FlgR may interact in a soluble state with σ^{54} in a RNA polymerase holoenzyme that is bound to target promoters, negating the need for FlgR to be tethered directly to DNA. A similar mechanism has been proposed for a FlgR homolog in *Helicobacter pylori*, which naturally lacks a CTD (30). These features of FlgR proteins in different bacteria may be significant in expanding mechanisms of transcriptional initiation by the NtrC family of RRs, because many of these proteins promote only limited transcription in the absence of DNA binding under physiological conditions (16, 17).

Some HKs are bifunctional, with net autokinase and phosphatase activities that control the level of cognate RR phosphorylation, depending upon different conditions. In a flagellar T3SS mutant, the autokinase activity of FlgS is low, but AcP-mediated activation of $FlgR_{\Delta CTD}$ was not reduced. In the absence of a significant in vivo phosphatase activity of FlgS, these data demonstrate the importance of the CTD as a major specificity determinant to limit cross-talk between FlgR and AcP when conditions are not conducive for flagellation. Because this specificity determinant resides in the common DNA-binding domain of other NtrC family members, the possibility exists that this domain reduces AcP-mediated activation in these RRs to maintain intrasystem signaling fidelity. Furthermore, our findings suggest that phosphorylated regulators in other bacteria and more complex eukaryotic systems may possess domains with similar activities in providing insulation from cross-talk with small phosphodonor metabolites.

Materials and Methods

Information regarding growth of *C. jejuni* 81–176 strains is provided in *SI Materials and Methods*. Methods for generating chromosomal mutants and transposon mutants, plus a list of strains and plasmids used in this study, are located in *SI Materials and Methods* and Tables S3 and S4. A detailed description of experimental methods is also provided in *SI Materials and Methods*.

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