SIGNALING SPECIFICITY IN A *CAMPYLOBACTER JEJUNI* TWO-COMPONENT SYSTEM TO MEDIATE PROPER FLAGELLAR GENE TRANSCRIPTION

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DEDICATION

To my parents, Beverly and Scott Hickey.

For your encouragement, support, and love.

To my beautiful wife, Sarah.

You make every day incredible.

ACKNOWLEDGEMENTS

I would like to thank my mentor, Dr. David Hendrixson for training and preparing me both in the lab and intellectually for future research endeavors. The tools that he has given me are invaluable and I am truly grateful. Dave has been a wonderful mentor and a true friend over the years. Working with him has been inspiring and lots of fun and I am lucky to have had such a great graduate school opportunity. Also, I would like to thank the fellow members of the Hendrixson lab including Deb Ribardo, Lacey Bingham-Ramos, Stephanie Joslin, Murat Balaban, Angelica Barrero-Tobon, C.J. Gulbronson, and Paul Luethy for their help and thoughtful suggestions throughout the years.

I would also like to thank the members of my thesis committee including Dr. Eric Hansen, Dr. Michael Norgard, and Dr. Vanessa Sperandio for the thoughtful discussions, suggestions, and much needed guidance while working on my graduate school projects.

Also, I would like to thank my family and friends for their support and belief in me, especially my parents, Beverly and Scott who have been encouraging throughout the many years I have been in school. I would also like to thank Sarah, Bob, and Katie and Vicki and David who are dear friends that have always supported me. Lastly, I would like to thank my beautiful wife Sarah, who has endured the trials and tribulations of graduate school with me. You are my best friend and I can't wait to experience the next chapter in our life together.

SIGNALING SPECIFICITY IN A *CAMPYLOBACTER JEJUNI* TWO-COMPONENT SYSTEM TO MEDIATE PROPER FLAGELLAR GENE TRANSCRIPTION

by

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DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of the Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

May, 2013

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Publication No._____

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The University of Texas Southwestern Medical Center at Dallas, 2013

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Campylobacter jejuni is a worldwide leading cause of bacterial gastroenteritis. While infection of humans leads to diarrheal disease, *C. jejuni* asymptomatically colonizes the intestinal tract of many agriculturally-significant animals, especially poultry. Flagellar motility is essential for *Campylobacter jejuni* to promote commensal colonization of avian species and for infection of humans to result in disease. Expression of flagellar genes is regulated by alternative σ factors, whose activities are controlled by a regulatory cascade. Previous genetic screens discovered the flagellar type III secretion system (T3SS), the FlgSR two-component regulatory system (TCS),

and the FlhF GTPase as requirements to positively regulate expression of σ^{54}-dependent genes that encode flagellar rod and hook proteins. Our laboratory previously proposed that signal transduction through the FlgSR TCS initiates with FlgS detecting formation of the flagellar T3SS and culminates in phosphorylation of the FlgR response regulator and expression of flagellar genes. I investigated this model by determining if any other flagellar components are required for activation of FlgSR and expression of σ^{54}-dependent flagellar genes. I found that mutants lacking the MS ring (FliF), the rotor component of the C ring (FliG), and the rod proteins (FliE, FlgB, FlgC and FlgF) expressed reduced levels of σ^{54}-dependent flagellar genes. These findings suggest a more complex flagellar structure rather than solely the flagellar T3SS is required to activate σ^{54}-dependent gene expression. Due to data generated by additional experimentation, I propose a revised model in which the *C. jejuni* flagellar T3SS facilitates polymerization of the MS ring and rotor component of the C ring, which together form a cytoplasmic domain that is likely the direct signal sensed by FlgS to activate signal transduction required for flagellar gene expression in *C. jejuni*.

Previous analysis demonstrated that activation of σ^{54}-dependent flagellar gene expression in *C. jejuni* is dependent on phosphotransfer through the FlgSR two-component system. Whereas this signaling mechanism results in specific activation of FlgR via its cognate FlgS sensor kinase, I identified a domain of FlgR that possesses an unusual activity in specifically preventing *in vivo* crosstalk with small phosphodonor metabolites. Through genetic and biochemical analysis, I demonstrated that the metabolite acetyl-phosphate (AcP) serves as an efficient phosphodonor for FlgR lacking its C-terminal domain but not for wild-type FlgR. Additionally, I could reprogram FlgR-dependent flagellar gene expression to respond to the

metabolic state of the cell and restore wild-type levels of flagellar gene expression in the absence of FlgS. However, flagellar biosynthesis was not restored to wild-type levels when AcP was the sole phosphodonor for FlgR. This study illustrates how signaling specificity in a TCS ensures a correct output response and highlights the importance of controlling proper signaling between cognate histidine kinase and response regulator pairs in bacterial TCS systems.

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AcP	acetyl phosphate
Amp	ampicillin
C. jejuni	*Campylobacter jejuni*
Cm	chloramphenicol
CTD	C-terminal domain
E. coli	*Escherichia coli*
g	grams
GI	gastrointestinal
h	hour
H. pylori	*Helicobacter pylori*
HTH	helix-turn-helix
IM	inner membrane
Kn	Kanamycin
lb	pound
LB	Luria-Bertaini
LOS	lipooligosaccharide
mg	milligram
ml	milliliter

μg	microgram
μl	microliter
MH	Mueller-Hinton
nmol	nanomol
OM	outer membrane
P. aeruginosa	*Pseudomonas aeruginosa*
pmol	picomol
PG	peptidoglycan
RNAP	RNA polymerase holoenzyme
Sol	soluble fraction
S. typhimurium	*Salmonella typhimurium*
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoreses
T3SS	type-III secretion system
TMP	trimethoprim
UAS	upstream activating sequence
*V. cholera*e	*Vibrio cholerae*
Vol	volume
WCL	whole-cell lysates
Wt	weight
WT	wild-type
xg	times gravity

CHAPTER ONE

INTRODUCTION

Campylobacter jejuni is a motile, polarly-flagellated, Gram-negative bacterium commonly found in the digestive tract of many agriculturally-significant animals. Transmission of *C. jejuni* to humans through the consumption or handling of raw or undercooked poultry often results in diarrheal disease (1). Epidemiological studies have identified *C. jejuni* as a leading cause of bacterial gastroenteritis worldwide (2). While *C. jejuni* asymptomatically colonizes the intestinal tract of many agriculturally-significant animals and infects humans to cause disease, the molecular mechanisms that regulate the bacterial-host interactions are not well understood.

Previous studies have identified flagellar motility as a requirement for *C. jejuni* to colonize chickens in a commensal relationship and to cause pathogenesis in humans (3, 4). Flagellar motility is dependent on the flagellum, a complex organelle composed of hundreds of proteins spanning from the cytoplasm of the cell, through the inner membrane, periplasm, and outer membrane into the extracellular environment (5). Proper assembly of this nanomachine requires a hierarchical progression of flagellar gene expression, which is intimately linked to the assembly of certain flagellar substructures. Linking flagellar gene transcription to flagellar biosynthesis facilitates a proximal-to-distal pattern of flagellar formation whereby flagellar substructures first form at the inner membrane (IM) and then progresses outward, ending with the polymerization of the external flagellar filament (6). Flagellar biosynthesis in bacteria

requires alternative specific σ factors, which are subunits of RNA polymerase holoenzyme that tightly control the expression of genes that encode flagellar proteins (5). In *C. jejuni*, flagellar gene expression is dependent on the alternative σ factors, σ^{54} and σ^{28}, similar to observations in other polarly-flagellated bacteria such as *Vibrio cholera*, *Pseudomonas aeruginosa*, and *Helicobacter pylori* (3, 6-11). Previous studies demonstrated that σ^{54} controls expression of genes encoding the rod and hook proteins and σ^{28} regulates expression of the genes encoding filament proteins. While many of the regulatory mechanisms that control expression of σ^{28} have been elucidated, little was known about the specific mechanisms and activating signals that regulate expression of σ^{54}–dependent rod and hook flagellar genes in polar flagellates (12).

A previous transposon mutagenesis screen identified flagellar type III secretion system (T3SS) components (FlhA, FlhB, FliP, and FliR), the FlgSR two-component system (TCS), and the FlhF GTPase as regulatory components required to activate σ^{54}-dependent gene expression in *C. jejuni* (3). Subsequent analysis showed that the formation of the flagellar T3SS is required to initiate signal transduction through the FlgSR TCS to activate σ^{54}-dependent flagellar gene expression (13). While these studies provided some insights into activation of σ^{54}-dependent gene expression, many questions remained unanswered. To better understand the regulatory mechanisms controlling expression of σ^{54}-dependent flagellar genes in *C. jejuni*, I analyzed whether additional flagellar proteins were required for expression of σ^{54}-dependent flagellar genes. After discovery of these new components necessary for signaling, I characterized the identified proteins to understand how these additional flagellar substructures regulate activation of the FlgSR TCS and gene expression. In addition, I identified a domain in the FlgR response

regulator (RR) that maintains specificity in the FlgSR signal transduction pathway to ensure flagellar gene expression only occurs in response to the proper signal, via FlgS.

The first part of this project demonstrated that the MS ring component (FliF), the rotor component of the C ring (FliG), and rod structures (FliE, FlgB, FlgC, FliF) are all required for wild-type (WT) levels of σ^{54}-dependent flagellar gene expression. Recent studies of both the flagellar T3SSs and the injectisome T3SSs of bacterial pathogens proposed a model for the biogenesis of these systems. These studies suggest that T3SS components such as FlhA, FlhB, FliP, FliQ, and FliR interact first, and then facilitate the recruitment and multimerization of FliF and FliG (or homologs) to form the MS and C rings, respectively, which surround the T3SSs (14-16). Taking this recent model of T3SS formation into account and my observation that FliF and FliG were required for activation of σ^{54}-dependent genes, I hypothesized that since FliF and FliG possess cytoplasmic domains, one or both of these proteins could be the direct activator of the cytoplasmic sensor kinase, FlgS. I discovered that FliF and FliG interact directly with FlgS *in vivo* and the T3SS is required for this interaction. Additionally, my work demonstrated that FliF and FliG directly interact with each other and this interaction is essential for their stability, an observation never before described in a motility system. Stability of both FliF and FliG required K558 of FliF, while the N-terminal 10 amino acids of FliG are required for the stability of these proteins. In addition, I demonstrated that the rod proteins FliE, FlgB, FlgC, and FlgF are required for WT levels of σ^{54}-dependent gene expression.

Prior studies of FlgR found that it is closely related to the NtrC family of transcriptional activators (13). Typical NtrC proteins contain three domains, an N-terminal receiver domain

with the phosphorylation site, a central ATPase and σ^{54}-interaction domain that directly interacts with the σ^{54}-RNAP holoenzyme, and a C-terminal DNA-binding domain with an essential helix-turn-helix motif (17, 18). FlgR contains homologous domains in its N-terminus and central domain but lacks homology in it C-terminal domain. My work suggested that like typical NtrC proteins, FlgR binds DNA specifically at σ^{54}-dependent promoters. However, unlike many NtrC proteins, DNA binding was not required for WT levels of gene expression in *C. jejuni*. In addition, previous studies showed that unlike WT FlgR, $FlgR_{\Delta CTD}$ activates σ^{54}-dependent gene expression in the absence of the cognate kinase FlgS (19). This result suggested that the CTD of FlgR is a specificity determinant to limit the phosphodonor of FlgR to only FlgS, and its absence allows FlgR to autophosphorylate using a non-cognate phosphodonor. Using transposon mutagenesis, I identified acetyl-phosphate (AcP) as a phosphodonor for $FlgR_{\Delta CTD}$, but not for WT FlgR. Using genetic and biochemical techniques I demonstrated that the CTD of FlgR limits phosphorylation using AcP as a phosphodonor and ensures that FlgR activation is specifically linked to only the cognate kinase FlgS and the flagellar system.

The work performed in these studies reveals new insights into *C. jejuni* biology and regulatory mechanisms that control flagellar gene expression. This research expands on previous studies by elucidating components the bacterium requires to activate its flagellar gene expression and describing a novel component in a response regulator that maintains proper signaling fidelity through the a TCS.

CHAPTER TWO

LITERATURE REVIEW

Historical Context and Classification of *Campylobacter*

Campylobacter was first described in 1886 by Theodore Escherich as 'cholera infantum', a small, spiral-shaped organism observed in stool samples taken from children suffering from diarrheal disease (20, 21). Despite an increase in the number of the bacteria found in stool samples, Escherich believed the bacterium played no etiological role in disease. McFadyean and Stockman in 1913 and Smith and Taylor in 1919 described similar spiral, rod-shaped bacteria associated with aborted sheep and calf fetuses, respectively (22, 23). Smith and Taylor called the organism *Vibrio fetus* (later reassigned *Campylobacter fetus*) because of its spiral-or curved-shape morphology, which was historically associated with *Vibrio* species. In 1931, a closely-related organism, *V. jejuni* (later reassigned *Campylobacter jejuni*) was isolated from the jejunum of cows with diarrheal disease (24).

The first documented cases of disease outbreak in humans where *Vibrio*-related organisms were isolated from the blood and stool occurred in 1938 when 355 inmates in two adjacent Illinois state institutions developed diarrheal disease after consuming contaminated milk (25). After isolation of the bacterium from stools, studies demonstrated that the *Vibrio*-related organism was more similar to *V. jejuni* than *V. fetus*. In his study, Levy speculated that the *Vibrio*-related organisms could promote disease in both cows and humans, but because the

organism was unculturable, he was unable to verify his hypothesis (25). Despite many attempts to isolate and culture *Vibrio*-related organisms, no group was successful until 1968. Dekeyser and Butzler published their culture methods in 1972, which was grown on blood agar medium in a microaerobic environment, and described isolation of an organism from the blood and stool of a hospitalized 20-year old female (26).

Prior to the first *in vitro* culture, Sebald and Veron analyzed the metabolic and DNA components of the *Vibrio*-related organisms and discovered they did not belong in the genus *Vibrio*. Instead, their work identified an unrelated genus of bacteria that they named *Campylobacter* (27). The bacteria were spiral-shaped rods that required a non-fermentative, microaerobic environment for growth and contained a low G-C genomic content, which are all uncharacteristic features of bacteria classified within the *Vibrio* genus. Later improvements in diagnostic technology and methodology enabled quick and accurate identification of *Campylobacter* in stool samples of diseased individuals, therefore establishing the genus as important for human health and disease (20, 28, 29). As of 2008, 17 different species of *Campylobacter* have been characterized with *C. jejuni* being the most important for human health (30).

Based on 16S rRNA gene sequencing, *C. jejuni* and other *Campylobacteraceae* are currently grouped into the ε-proteobacteria class together with the *Helicobacter* and *Wolinella* species. Many of the Gram-negative bacteria in this class are medically-relevant human pathogens, especially *C. jejuni*, which is the leading cause of acute enteritis worldwide (2). *C. jejuni* shares features common among the *Campylobacteraceae* including a spiral shape and a

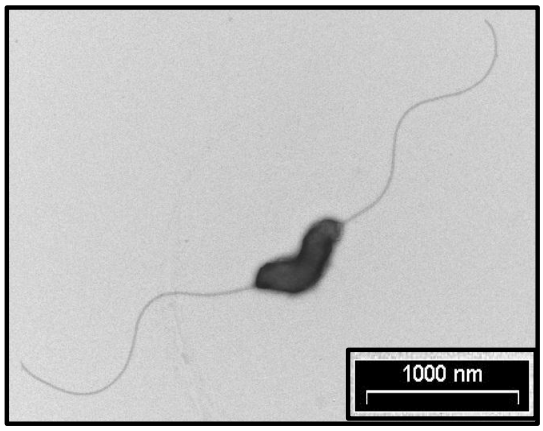

Figure 1: Electron Micrograph of *C. jejuni*. 81-176 *rpsL*^{Sm} (DRH461) is a spontaneous streptomycin-resistant isolate and is used for these studies as a wild-type strain. Magnification: 16000X. Bar represents 1.0 μm.

single flagellum located at each pole of the bacterium (Figure 1). The shape of the bacterium and its polar flagellation pattern together confer a corkscrew-like motility that is thought to aide in host colonization (6). *C. jejuni* optimally grows under microaerobic conditions due to its sensitivity to atmospheric oxygen levels. Additionally, the 1.6 Mb genome of *C. jejuni* NCTC11168 harbors a G-C content of 30.6%, including 1654 coding sequences (31). Metabolic analysis has revealed that *C. jejuni* is asaccharolytic and utilizes amino acids as carbon sources for metabolism (31). Sequence comparisons between *C. jejuni* strains revealed that the genome contains hypervariable sequences that are involved in modification of surface structures and possibly facilitate host colonization (31, 32).

Clinical Presentation of Disease

Acute Disease

While the identification of *Campylobacter* as a human pathogen took almost a hundred years, we now know that *C. jejuni* is the leading cause of acute bacterial enteritis throughout the world (2, 33, 34). Clinical presentation of campylobacteriosis, the disease resulting from *Campylobacter* infections, can occur through the ingestion of less than 500 organisms (4, 35). Disease manifests as fever, nausea, vomiting, diarrhea, abdominal pain, cramping, and often times includes bloody stools (33). Symptoms appear within two to five days after exposure and the illness is self-limiting in most individuals, with infections typically resolving within a week. However, recovery in immunocompromised patients often takes significantly longer. Chronic and recurrent infections in immunocompromised patients are treated with antibiotics that will shorten the duration of symptoms if given early (36). Shedding of *C. jejuni* in feces of infected individuals occurs for weeks after symptoms are resolved unless antibiotics are administered (37, 38). Even though most *C. jejuni* infections are self-limiting and resolve quickly, infections have been linked to secondary complications such as Guillan-Barré syndrome (GBS) and Reiter's Syndrome (39).

Secondary Sequelae

C. jejuni infections are one of the leading risk factors for GBS, which is the most common secondary complication. About one in every 1,000 infected individuals will experience GBS after a *C. jejuni* infection (40). GBS is an autoimmune disorder characterized by a flaccid paralysis of the peripheral nervous system (41). Symptoms are characterized by numbness in the periphery of the limbs and the paralysis may spread throughout the rest of the body over time.

Later symptoms of disease include loss of muscle mass and paralysis of the face and trunk. Symptoms often last for weeks to months and treatments present a severe economic cost (42).

On its surface, *C. jejuni* expresses lipooligosaccharides (LOS) molecules similar to lipopolysaccharides (LPS) that are found in the outer membrane (OM) of many enteric pathogens (43, 44). Many *C. jejuni* serotypes attach carbohydrate moieties to the LOS that mimic sugar residues found on human gangliosides. Studies demonstrated that through molecular mimicry, antibodies generated against some serotypes of *C. jejuni* LOS cross-react with gangliosides on nerves, which can result in the paralysis associated with GBS (45, 46).

Another secondary sequelae associated with *C. jejuni* infection is Reiter's syndrome, a condition characterized by reactive arthritis. This disease presents with pain and swelling in the joints, most often observed in the knee (47). However, the diagnostic criteria are not standardized and the epidemiology concerning this disorder varies significantly. Additionally, the mechanisms *C. jejuni* utilizes to cause this disease, are largely unknown (48).

Epidemiology

Prevalence and Incidence

C. jejuni poses a severe human health problem in developed countries such as the United States, where in 2010, 13 out of every 100,000 people were diagnosed with enteritis related to *C. jejuni*. Because symptoms associated with the illness often resolve without medical intervention, the true number of *C. jejuni* disease cases is likely higher. The Center for Disease control estimates that about 0.8% of the population or 2.4 million people are infected with *C. jejuni*

every year (49). The overall incidence of *Campylobacter* infections declined 30% in 2002 when compared to previous years. This decline corresponds to the United States Department of Agriculture (USDA) introducing new meat and poultry regulations to reduce contamination of poultry at the time of slaughter (33).

While *C. jejuni* colonizes humans to result in diarrheal disease, it also colonizes many agriculturally-significant animals such as poultry as a commensal without the presentation of disease. Once infected, chickens quickly spread *Campylobacter* throughout their flocks by shedding feces and by ingestion of feces and contaminated water reservoirs. At the time of slaughter, processing of colonized birds often leads to the contamination of the meat with *C. jejuni*. In 2005, the USDA found that 47% of all processed chicken meat was contaminated with *Campylobacter* (49). Campylobacteriosis most often results from improper handling or ingestion of raw poultry meats, which give rise to sporadic cases of disease. While less common, outbreak cases of human *Campylobacter* infections have been reported from consumption of unpasteurized milk and contaminated water supplies.

Distribution

Because of the implications that food-borne disease has on public heath, in 1996 the US federal government began a program called FoodNet to actively monitor food-borne disease outbreaks (including *Campylobacter*) in nine states (50, 51). In these areas, clinicians collaborate with laboratories to identify the causative agent of disease by analyzing stool

specimens. FoodNet compiles data on *Campylobacter* infections in the US population to monitor the impact of the bacterium on public health.

According to FoodNet, *Campylobacter* infections occur throughout the US, but disease is more prevalent in western regions such as California, New Mexico, and Colorado. While infections are observed in all age ranges, a increase in disease is associated with the elderly and with infants less than 1 year old, where 27 out of every 100,000 were infected in 2005 (52). The greater number of documented cases in this age group is likely a result of parents seeking medical help for their children. As young children increase in age, the incidence of disease decreases until age 20-29, when disease incidents begin to increase. Another high risk group includes the elderly (52). Infections associated with *Campylobacter* are 20% higher in men than women presumably because men engage in riskier preparation and consumption of foods when compared with women (53). Reporting of infections shows seasonal preference where higher incidence occurred from March to the fall, with peak infections occurring in the summer months (52). The CDC estimates that 124 people die each year from to *Campylobacter* infections (49).

Control of Campylobacter

While case studies have shown consumption of untreated water and unpasteurized milk have given rise to outbreak incidences of campylobacteriosis (25), the vast majority of infections are sporadic and linked to the ingestion of raw or undercooked poultry. Control of sporadic disease includes education on contamination in the kitchen, reduction of cross-contamination between infected and uninfected meats at the time of slaughter, and control of colonization in

live animals (33). Additionally, pasteurization of milk and chlorination of drinking water are important precautions to prevent disease outbreaks.

Treatment of Campylobacteriosis

Treatment for *C. jejuni* infections include administration of antibiotics such as erythromycin and fluoroquinolones, which if given at early points in infection, can shorten the duration of symptoms and fecal shedding (37, 38). However, most infections resolve with minimal supportive treatment, such as rehydration and electrolyte replacement (54). One problem associated with the use of antibiotics in *C. jejuni* is the development of antibiotic-resistant strains, especially fluoroquiolones (55-57). Many farms use antibiotic-supplemented feed to lessen the bacterial load in animals and several studies in chickens and pigs provide evidence that *C. jejuni* antibiotic-resistant isolates emerge and persist over the course of antibiotics (58-60). Presumably, the drug-resistant isolates are transmitted to people, creating a need for novel therapeutic development.

Pathogenesis and Virulence Factors

Several groups have shown that *C. jejuni* invades host intestinal epithelial cells *in vivo* and *in vitro* (61-66). However, with the exception of flagellar motility and the characterization of some secreted proteins, the direct determinants leading to cellular invasion are poorly understood (67). *C. jejuni*-associated enteritis is caused by host intestinal inflammation, which is a result of proinflammatory cytokine expression (68-76). Studies have demonstrated that *C.*

jejuni localizes to the human gut and stimulates Nf-κB to promote inflammatory cytokine production (76, 77). However, many of the bacterial signals that the host recognizes to induce a pro-inflammatory response are not known. Intestinal epithelial cells detect microbes in the lumen of the gut or during invasion and trigger an inflammatory response (78). Most often, bacterial sensing occurs through extracellular Toll-like receptors (TLRs) or intracellular NOD receptors that recognize specific pathogen-associated molecular patterns (PAMPs) on the bacterium. In contrast, studies have found that *C. jejuni* induces production of the pro-inflammatory cytokine IL-8, which occurs independently of TLRs or NOD receptors (70).

Cytolethal Distending Toxin

Presumably, TLR- and NOD-independent production of IL-8 occurs through the nuclease activity of the cytolethal distending toxin (CDT), a protein secreted to by *C. jejuni* that induces DNA damage and cell-cycle arrest (79, 80). CDT has been postulated to interfere with fluid absorption within the intestinal tract to cause inflammation and diarrheal disease (80). Additionally, CDT is produced by many other enteric pathogens such as *E. coli, Salmonella enterica* serovar Typhi, and *Shigella dysenteriae* but the direct role of this toxin in pathogenesis is not known (81).

CDT is a multi-protein complex that consists of three subunits, CdtA, CdtB, and CdtC. All three subunits are required for intoxication of target cells. Presumably CdtA and CdtC bind host ligands to act as a toxin-delivery system to promote internalization of CdtB into target cells (82). CdtB has been shown to be the enzymatically active subunit *in vitro*. CdtB exhibits DNase activity that induces fragmentation of host DNA and cell-cycle arrest in the transition from G_2 to M phase,

which causes cell death (79). These studies suggest that CDT plays a role in invasion and the intracellular survival during human infections.

Capsular Polysaccharides

Evidence of *C. jejuni* containing a capsular polysaccharide (CPS) was revealed when the first sequenced genome showed that the bacterium possessed the genetic determinants to build CPS (31, 83). The genetic arrangement of the CPS genes in *C. jejuni* is similar to other bacteria where the biosynthetic genes are directly flanked on either side by the *kps* genes, which encode proteins involved in CPS transport to the cell surface (31, 84, 85). The contribution of CPS to *C. jejuni* pathogenesis is not well understood but it is evident that CPS is required to cause disease. Insertional interruption of *kps* genes resulted in reduced invasion of intestinal epithelial cells and reduced virulence in a ferret model of diarrheal disease (86, 87). Additionally, CPS conjugate vaccines protect against diarrheal disease in non-human primates, which strongly suggests that CPS is synthesized during infection and plays a role in pathogenesis (88).

In addition to its role in virulence, *C. jejuni* relies on CPS for efficient colonization of chickens (87, 89). Interestingly, CPS minimally induces an immune response in an avian host, but produces a significant humoral immune response in the human host (83, 90, 91). This discovery suggests that capsule production could be differentially regulated in different hosts. In addition to transcriptional regulation, the CPS genes are subject to random phase variation, which is also an immune evasion strategy. However, more studies need to be performed to identify the multiple regulatory mechanisms that influence CPS production (88).

N- and O-linked Glycosylation

The first general or *N*-linked glycosylation system in a bacterium was described in *C. jejuni*. This system, encoded by the *pgl* genes, modifies proteins by addition of sugars to asparagine residues in the target protein through an amide bond (92-94). The *pgl* gene cluster consists of 13 genes encoding proteins to generate a heptasaccharide attached to undecaprenyl phosphate (Und-P) on the cytoplasmic face of the inner membrane (93). Subsequently, PglK is an ABC-transporter that translocates the sugars across the membrane into the periplasm of the cell, whereas PglB is a transferase that adds the polysaccharides to asparagine residues in the target protein (95, reviewed in 96).

C. jejuni modifies more than 65 periplasmic proteins of various functions through this pathway. The *N*-linked glycosylation system is required for intestinal colonization of mice and adherence to and invasion of an intestinal epithelial cell line (97). Additionally, *C. jejuni* requires this general glycosylation pathway to colonize its avian host (98). While these studies illustrate the importance of the *N*-linked glycosylation system in pathogenesis and commensal colonization, glycosylation of any single protein has not been found to be essential for any host interaction or *in vivo* growth.

While heptasaccharides generated by the *N*-linked glycosylation system of *C. jejuni* are attached to proteins, many more are released into the periplasmic space as free oligosaccharides (fOS). Like the *N*-linked saccharides, fOS are dependent on PglB for cleavage of the heptasaccharide from Und-P. Studies to elucidate the biological role of fOS demonstrated that the periplasmic levels of fOS fluctuate in the presence of salts, sucrose, and changing bacterial

growth phase presumably due to changes in the enzymatic activity of PglB (99). fOSs produced by the *N*-linked glycosylation pathway are hypothesized to regulate cell osmolarity in response to changing environmental conditions, similar to the role of glucans in other Gram-negative bacteria (100). Thus, the importance of the *N*-linked glycosylation system for *in vivo* growth in animal and murine species and invasion of human cells may be due to its role in providing protection to osmotic stress.

C. jejuni is dependent on motility using its polar flagella to colonize its animal hosts and to cause diarrheal disease in humans (3, 4, 98). The *O*-linked protein glycosylation system in *C. jejuni* modifies flagellar proteins with predominantly pseudaminic acid (Pse) or legionaminic acid (Leg) derivatives. These modifications are related to sialic acids and are required for a variety of flagellar-associated functions including flagellum assembly, secretion of proteins required for virulence, intestinal colonization, auto-agglutination (AAG), and biofilm formation (96). Unlike *N*-linked glycosylation, *O*-linked glycosylation substrates are not found in the periplasm, suggesting glycosylation occurs in the cytoplasm or in the flagellar basal body (101).

While there is significant variability among the *C. jejuni* strains in their *O*-linked glycosylation genes, all of the characterized genes are found in a variable region near the flagellin structural genes, *flaA* and *flaB*. The best characterized *O*-linked system is found in strain 81-176, where the bacterium glycosylates flagellar proteins using Pse and a derivative called acetamidino pseudaminic acid (PseAm). *pseB* encodes the first enzyme in the pathway to generate Pse and mutation of this gene caused a non-motile phenotype with bacteria accumulating flagellin intracellularly (102, 103). This study was the first experimental evidence

that glycosylation is required for flagellar biosynthesis. *pseA* and *pseD* encode proteins important for modification of Pse into PseAc and inactivation of either of these genes caused the mutants to produce Pse modifications instead of PseAc at glycosylation sites (104). While these mutations did not disrupt flagellar biosynthesis, the cells failed to autoagglutinate (AAG), a characteristic feature associated with adherence and invasion of eukaryotic cells in bacterial pathogens (105).

In *C. jejuni* strain 81-176, up to 19 different glycans are added to the major flagellin, FlaA, at serine or threonine residues. Functional analysis of each glycosylation site identified that five modifications were required for AAG, suggesting that the modifications were surface exposed and were important for interacting with other flagellar filaments (96, 104, 106). Additional work demonstrated that the PseAm flagellar glycan moieties interact with filaments from other *C. jejuni* cells to form microcolonies on intestinal epithelial cells. In *pseA* mutants that lack PseAm sugar modifications, *C. jejuni* cells displayed a reduced adherence and invasion phenotype when cultured with intestinal epithelial cells and the mutants displayed decreased virulence in a ferret diarrheal disease model (105). While these studies illustrate the importance of glycan moieties on flagellar formation and suggest the bacterium may use the sugar moieties to interact with epithelial cell lines, much remains to be discovered about the specific eukaryotic ligands and the biological role of the sugar modifications.

Flagella and Flagellar Motility

In bacteria, motility is dependent on a flagellum, an organelle spanning from the cytoplasm of the bacterium into the extracellular environment, which rotates to propel the bacterium in a certain direction. *C. jejuni* requires flagellar motility to colonize agricultural reservoirs such as chickens and to cause disease in humans (3, 4, 9). *C. jejuni* produces an amphitrichous flagella, meaning it has one flagellum located at each pole (Figure 1) (30). *C. jejuni* motility is thought to be important in colonization to facilitate persistence in highly viscous intestinal mucous of hosts (107). Several groups have also shown flagellar motility is required for *C. jejuni* to invade intestinal epithelial cells *in vitro* and to cause disease in human volunteers (4, 108-114).

In addition to secretion of proteins that compose the flagellum, the flagellar T3SS also secretes non-motility related proteins that are required for invasion of intestinal epithelial cells and colonization of avian hosts (66, 115, 116). While other bacterial pathogens such as *Salmonella*, *Yersinia*, and *Shigella* use an injectisome T3SS to facilitate cellular invasion by inserting bacterial effectors directly into the host cytoplasm, *C. jejuni* does not encode an injectisome T3SS (31). Thus, much attention has focused on the flagellar T3SS and its secretory substrates. The Cia, FedB, and FlaC proteins are not required for motility, but are secreted in a flagellar T3SS-dependent manner and are required for invasion of intestinal epithelial cells *in vitro* (66, 115-117). Furthermore, CiaI and FedB are required for colonization of chickens (66). Such studies demonstrated that flagellar motility and proteins secreted by the flagellum are important virulence factors for *C. jejuni* infections.

Structure and Biosynthesis of the Flagellar Organelle

The bacterial flagellum is a complex organelle that spans from the cytoplasm of the cell through the inner membrane (IM), periplasm, and outer membrane (OM) into the extracellular environment. Proper assembly requires the production and assembly of over 30 different proteins, many of which multimerize to form distinct substructures of the organelle. The flagellar proteins and many of the structures they form are fairly conserved across motile bacteria (Figure 2). The flagellum can be divided into two core structures, the basal body and the filament. The basal body is composed of the base, the surface-localized hook, and the rod that connects the two structures (reviewed in 5, 6, 118).

The flagellar base components are localized in the IM and cytoplasm and consist of the flagellar T3SS (FlhA, FlhB, FliO, FliP, FliQ, and FliR), the MS ring (FliF), motor proteins

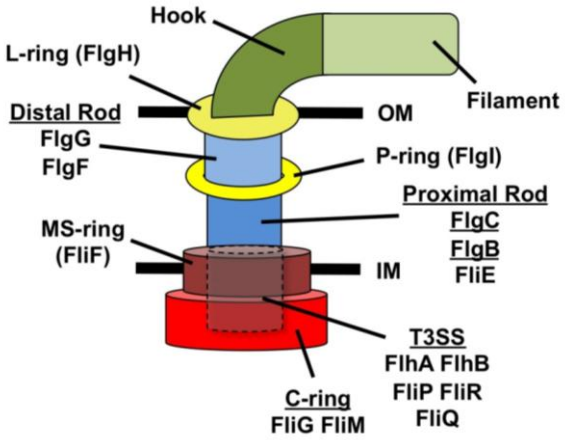

Figure 2. Structure of the Flagellar Organelle. The major components that are common to flagella from various species are shown. The flagellar components are color coordinated based on the substructures they form. The flagellar T3SS (red within the dotted line) is located in the IM and is encased by the MS ring (maroon). The C ring (red) is localized on the cytoplasmic face of the T3SS. The proximal rod (dark blue) is attached to the periplasmic face of the T3SS and the distal rod (light blue) is found at the periplasmic face of the OM. The P ring and L ring (yellow) allow the rod to penetrate the peptidoglycan and OM, respectively. The hook (dark green) is attached to the distal rod and connects the filament (light green) to the basal flagellar structure. Abbreviations: T3SS, type III secretion system; OM, outer membrane; IM, inner membrane.

(MotA and MotB), and the C ring (FliG, FliM, FliN). Additionally, some bacteria such as *C. jejuni* and other ε-proteobacteria encode another putative C ring component called FliY, which contains a putative phosphatase domain that likely affects flagellar motor function (6). The flagellar T3SS is required for the ordered secretion of many flagellar substrates beyond the IM during flagellar biosynthesis. The flagellar T3SS is encased in the IM by the MS ring, a multimeric structure composed solely of FliF subunits (Figure 2). While previous hypotheses postulated that flagellar biogenesis initiates with FliF multimerization into the MS ring in the IM with subsequent insertion of the T3SS into the center of the ring, results of recent studies illustrate an alternative order of events (119). Based on studies from the flagellar T3SS and similar injectisome T3SSs, the components of the T3SS appear to assemble first and then recruit FliF, FliG, FliM, and FliN to the site of flagellar formation, where they polymerize into the MS ring and C ring structures (14-16).

The C ring is located on the cytoplasmic face of the MS ring and forms the rotor-switch complex of the flagellar motor (Figure 2). Whereas FliG multimerizes into the rotor component that rotates the flagellum, FliM and FliN complex and interact with the chemotaxis system to mediate clockwise or counter-clockwise changes in rotation of the flagellum (120). Directional switching is dependent on the phosphorylation state of the chemotaxis RR CheY (121). MotA and MotB are the stators of the flagellar motor and form a proton channel in the IM to power motility (122). MotB is an IM protein with a periplasmic domain that binds peptidoglycan to anchor the motor complex while MotA contains a cytoplasmic loop that interacts with FliG. The

interaction of FliG with the stator complex generates torque to result in rotation powered by the proton flow (123, 124).

The flagellar rod is a tube-like structure that spans the periplasmic space and connects the flagellar hook on the surface of the OM to the periplasmic face of the MS ring and flagellar T3SS (Figure 2). The proximal rod components (FliE, FlgB, and FlgC) and distal rod components (FlgF, FlgG) compose the rod. FlgI, the component that forms the P ring and FlgH, which forms the L ring, both allow the rod to penetrate the peptidoglycan layer and the OM, respectively (Figure 2). Once completed, the rod essentially forms a conduit whereby secretion substrates translocate out of the cytoplasm into the extracellular environment while never being exposed to the periplasm. Once the MS ring, C ring, and T3SS form, the T3SS secretes the rod proteins in the correct order to form the rod. When flagellar substrates are secreted by the nascent flagellum, the substrates migrate to the most distal end of the structure and polymerize at the growing flagellar tip. (5, 6).

After formation of the rod, FlgE and FlgD are secreted to the tip of the growing flagellum where they assemble to form the hook and hook cap, respectively. FlgD is secreted first and caps the rod so that when FlgE proteins are secreted, they are trapped by FlgD and polymerize into the flagellar hook (Figure 2) (125, 126) . Without FlgD, FlgE subunits are freely released into the environment without polymerizing into a hook. Through an interaction with FlgD and the T3SS component FlhB, FliK works as a molecular ruler to regulate the length of the hook (127). Once the hook reaches it proper length, FliK initiates an autoproteolytic cleavage event in

FlhB. Cleavage of FlhB initiates a switch in the specificity of the secreted substrates, which causes the flagellum to begin secreting filament proteins instead of hook proteins (128).

Filament biosynthesis begins with the secretion of FliD, which functions as a filament cap, and the flagellins, which polymerize form into the filament (Figure 2) (129). In a *fliD* mutant, flagellins are secreted into the culture medium demonstrating that the filament cap is important for polymerization of the filament (129). Like the hook cap, FliD as the filament cap captures flagellins so that they polymerize into the filament. Several hundred flagellin proteins eventually polymerize into the flagellar filament, which can reach lengths of two microns or greater.

Regulatory Mechanisms that Control Flagellar Gene Expression

As demonstrated above, flagellar biosynthesis begins at the IM with assembly of the flagellar T3SS and in a step-wise order, specific proteins polymerize at the growing flagellar tip until the filament is assembled in the extracellular space. To accomplish this coordinated assembly, production and secretion of flagellar substrates is temporally regulated by transcription of flagellar genes in stages or classes, based on where the respective encoded proteins are required in flagellar biosynthesis. In general, motile bacteria have similar mechanisms that control the expression of flagellar genes in stages. However, among bacteria there are different regulatory proteins governing transcription of flagellar genes.

E. coli and Salmonella spp.

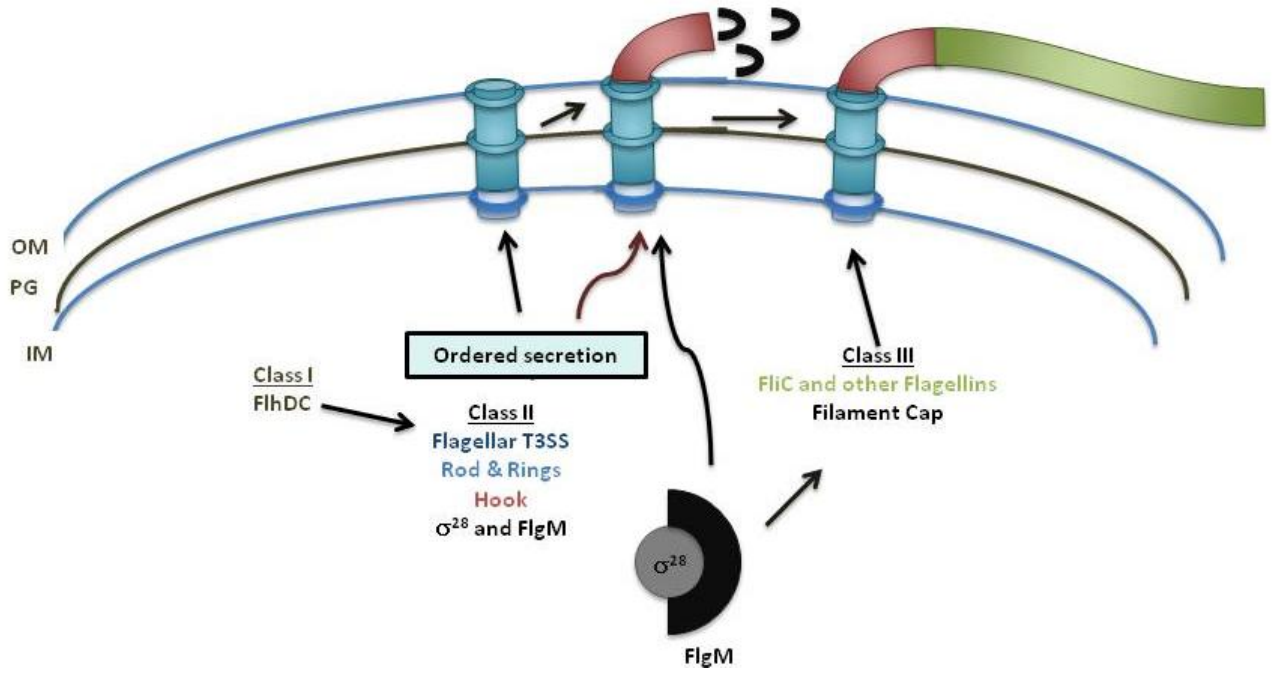

Figure 3. The Flagellar Transcriptional Regulatory Cascade in *E. coli* and *Salmonella* Species. The flagellar components and respective proteins included in flagellar transcriptional regulatory cascade are shown. The class I genes encode FlhDC, which are master regulators that activate expression of class II genes. Class II genes encode the flagellar T3SS (dark blue), the rod and rings (light blue), the hook proteins (red) that form the HBB, σ^{28} (grey), and the anti-sigma factor FlgM (black). Secretion of the anti-sigma factor, FlgM through the HBB relieves repression of σ^{28}-dependent class III genes. Class III genes encode FliC and other filament proteins. Abbreviations: T3SS, type III secretion system; HBB, hook basal body; OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

The most widely studied motility systems are those of *E. coli* and *Salmonella* species. Studies of these bacteria provide a general understanding of how some motile bacteria control flagellar gene expression. In these examples of peritrichous bacteria, flagellar gene expression can be divided into three classes based of their temporal order of expression (Figure 3). Class I genes encode the master regulator, FlhDC (130). Once produced, the regulator activates transcription of the class II genes whose products assemble and form the flagellar T3SS, hook and basal body, the alternative sigma factor, σ^{28}, and its anti-sigma factor, FlgM (131).

As the flagellar base, rod, and hook are forming, FlgM complexes with σ^{28} to prevent σ^{28}-dependent gene expression (132). Once the flagellar T3SS, rod, and hook structures are formed, FlgM is secreted out of the cell to relieve σ^{28} from inhibition so that it can then promote expression of class III genes (12). FliC and other filament products encoded by class III genes polymerize at the end of the hook structure to form the flagellar filament. These regulatory mechanisms ensure that flagellar genes are expressed only at the point in flagellar biosynthesis when the encoded proteins are required for the next step in biogenesis.

Pseudomonas and Vibrio spp.

Pseudomonas and *Vibrio spp*. also employ hierarchical transcriptional cascades during flagellar biogenesis (Figure 4). However, these pathogens utilize an additional alternative sigma factor, σ^{54}, and have a four-class tiered system to ensure proper flagellar protein production, secretion, and interactions (133, 134). The use of σ^{54} to regulate flagellar gene expression is a common theme among polarly-flagellated bacteria.

In response to global regulatory signals, class I genes in *Pseudomonas* and *Vibrio spp*. are transcribed. Class I genes include *fleQ* in *Pseudomonas sp*. and *flrA* in *Vibrio sp*., which encode master regulator proteins (135-137). These proteins function with σ^{54} to regulate transcription of class II genes. Class II genes include those that encode the flagellar T3SS components, the MS ring, the C ring, σ^{28}, FlgM, and a TCS. These TCSs include FleSR in *Pseudomonas sp*. and FlrBC in *Vibrio sp.,* which are required for the activation of σ^{54}-dependent class III genes (133,

138). The class III genes encode the hook and basal body components. Like *E. coli* and *Salmonella*, when the flagellar T3SS, rod, and hook are properly formed, FlgM is secreted, which promotes σ^{28}-dependent class IV flagellar gene expression. In *Pseudomonas* and *Vibrio spp.*, the class IV genes encode the flagellins and motors subunits that are required to form a functional flagellum.

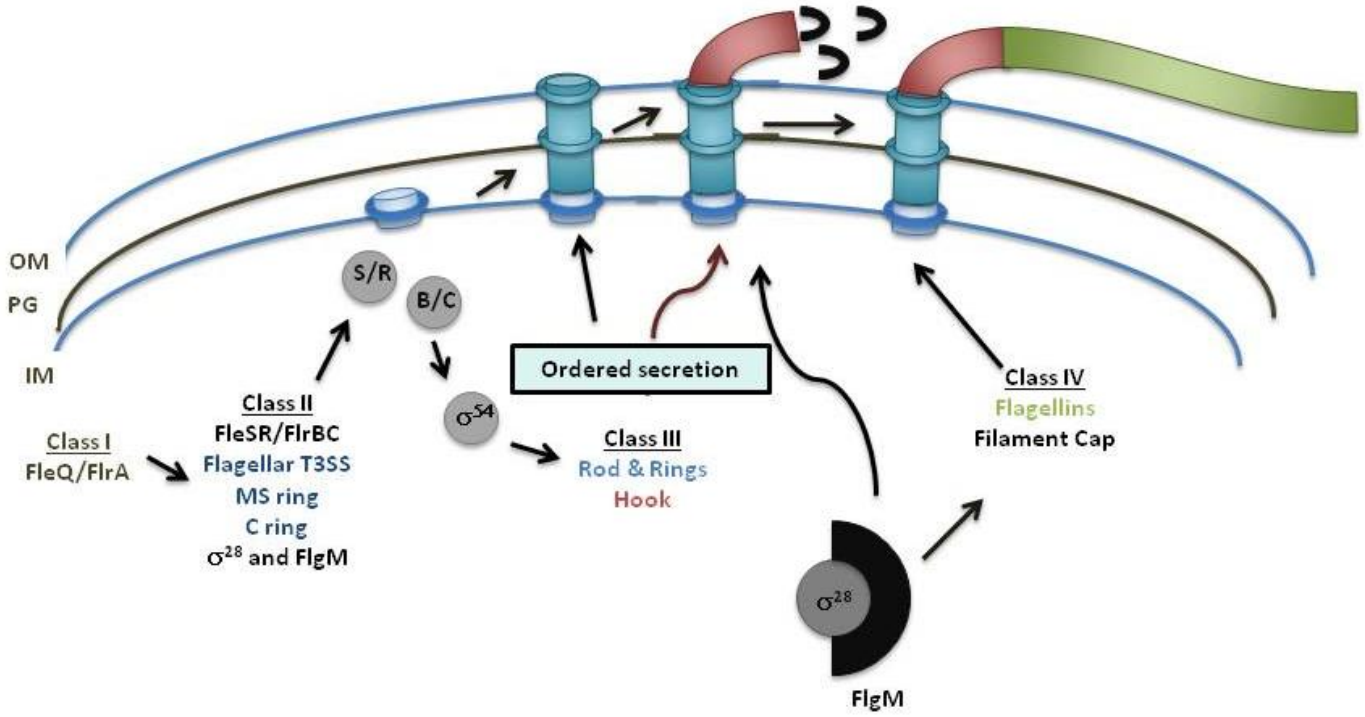

Figure 4. The Flagellar Transcriptional Regulatory Cascade in *Pseudomonas* and *Vibrio* Species. The flagellar components and respective proteins included in flagellar transcriptional regulatory cascade are shown. The class I genes encode FleQ/FlrA, which are the master regulators that activate class II genes. Class II genes encode the FleSR/FlrBC TCSs, the MS ring, C ring, and flagellar T3SS (dark blue), σ^{28} (grey), and the anti-sigma factor FlgM (black). In response to an unknown signal, the TCSs are activated to initiate expression of the class III σ^{54}-dependent flagellar genes. Class III genes encode the rod and rings (light blue) and hook (red) proteins that form the HBB. Secretion of the anti-sigma factor FlgM through the HBB relieves repression of σ^{28}-dependent class IV genes. Class IV genes encode the flagellins and other filament proteins. Abbreviations: T3SS, type III secretion system; TCS, two component system; HBB, hook basal body; OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

Flagellar Gene Expression in *C. jejuni*

Advances in genetic tools to manipulate *C. jejuni* have facilitated identification of motility-related proteins and characterization of flagellar transcriptional regulatory pathways. Like *Pseudomonas* and *Vibrio*, *C. jejuni* is a polarly-flagellated bacterium that requires σ^{54} to activate expression of flagellar genes (Figure 5) (reviewed in 139). Whereas studies in *C. jejuni* have identified components that compose a signaling pathway to activate σ^{54}-dependent flagellar gene expression, the mechanism that initiates activation of this flagellar signaling pathways and mechanisms to control signaling fidelity within this pathway, are not well understood.

The FlgSR Two-Component System Activates σ^{54}-dependent Flagellar Gene Expression

Unlike *E. coli*, *Salmonella*, *Pseudomonas*, and *Vibrio spp*., *C. jejuni* lacks a master transcriptional regulator to activate expression of flagellar genes. Therefore, class I genes are grouped together based on their requirement to activate expression of class II flagellar rod and hook genes and their expression has been proposed to be constitutive. Class I genes include those that encode the flagellar T3SS components, the FlgSR TCS, σ^{54}, and FlhF (3, 11). Upon sensing a signal, the FlgS sensor histidine kinase (HK) autophosphorylates and functions as a phosphodonor for the FlgR RR. FlgR is a NtrC-like protein that autophosphorylates at a conserved D51 residue in its N-terminal receiver domain (19). Whereas most NtrC-like RRs contain an essential C-terminal domain (CTD) with a helix-turn-helix (HTH) motif required for DNA binding and σ^{54}-dependent gene expression (18, 140, 141), *C. jejuni* does not appear to have a HTH motif in its CTD. However, in the absence of its CTD, $FlgR_{\Delta CTD}$ still activates σ^{54}-

dependent gene expression at WT levels suggesting that FlgR activates promotes transcription using a DNA-binding independent mechanism (19).

The Flagellar T3SS is Required to Activate Gene Expression

Experimental studies suggested that the flagellar T3SS is required to activate the FlgSR TCS. In addition, flagellar T3SS mutants that are not fully secretion-competent activate FlgSR. Thus, it was proposed that formation of some component of the T3SS, rather than its secretory activity may be a direct signal sensed by FlgS (13). This mode of regulatory

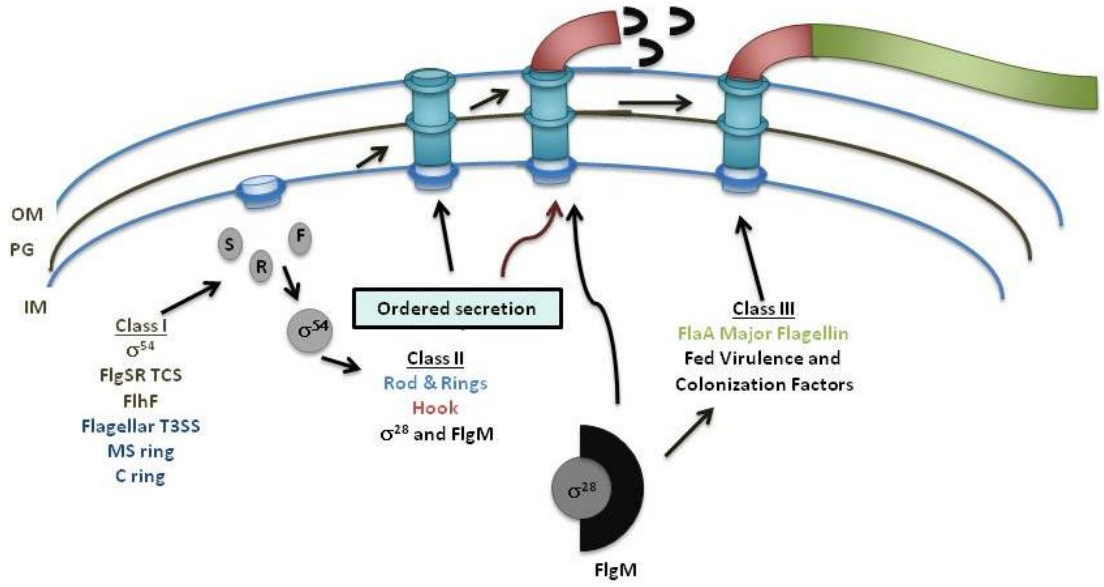

Figure 5. The Flagellar Transcriptional Regulatory Cascade in *C. jejuni*. The flagellar components and respective proteins included in flagellar transcriptional regulatory cascade are shown. The class I genes encode σ^{54}, the FlgSR TCS, FlhF, MS ring, C ring, and the flagellar T3SS components (dark blue). Formation of the flagellar T3SS has been proposed to activate the FlgSR TCS to initiate expression of the σ^{54}-dependent class II genes. Class II genes encode the rod, rings (light blue), and hook (red) that form the HBB, σ^{28} (grey), and the anti-sigma factor FlgM (black). Secretion of the anti-sigma factor, FlgM through the HBB relieves repression of σ^{28}-dependent class IV genes. Class III genes include *flaA*, which encodes the major flagellin and other filament proteins. Other class III genes include those that are not required for motility but may be involved in colonization and virulence. Abbreviations: T3SS, type III secretion system; TCS, two component system; HBB, hook basal body; OM, outer membrane; PG, peptidoglycan; IM, inner membrane.

control likely creates a checkpoint where *C. jejuni* can ensure formation of the flagellar T3SS before the FlgSR TCS activates expression of σ^{54}-dependent rod and hook genes, whose products are secreted by the T3SS. Additional studies, which were the focus of my research, are needed to elucidate how the flagellar T3SS may be involved in direct activation of FlgS.

In *C. jejuni*, expression of σ^{28} is partially dependent on σ^{54}. Once σ^{28} is produced, it is negatively regulated in the cell by FlgM. Like other motile bacteria, upon formation of the flagellar base, rod, and hook structures, FlgM is secreted out of the cell. Then σ^{28} is able to initiate expression of the flagellins that polymerize to form the filament and other virulence and colonization factors such as the Fed proteins (66).

Phase Variation Influences Expression of Flagellar Genes

In addition to a regulatory cascade to regulate flagellar gene expression, the genes encoding the FlgSR TCS are susceptible to random phase variation (8, 142). This mechanism enables motile *C. jejuni* to randomly switch from a phase-on phenotype where FlgS and FlgR are produced and σ^{54}-dependent genes are expressed, to a phase-off phenotype where the coding sequences of FlgS or FlgR are disrupted. Phase variation occurs when the length of homopolymeric adenine or thymine tracts are altered or heteropolymeric repeats are lost. Insertion or deletion of nucleotides causes a frame-shift of the coding sequence to result in premature termination during translation (8, 142). Alteration of the flagellar phenotype through phase variation may benefit *C. jejuni* by promoting adaptation to host or environmental settings.

Helicobacter pylori

Based on 16S RNA phylogenetic classification, *H. pylori* is a member of the ε-proteobacteria and closely related to *C. jejuni*. Both pathogens frequently associate with animal or human hosts and flagellar motility is required for efficient host colonization (98, 143, 144). Flagellar motility enables *H. pylori* to move within the thick mucus layers in the stomach of its host (145). Although not as well-characterized as in *C. jejuni*, early studies of the *H. pylori* flagellar transcriptional cascade suggest many similarities. Like *C. jejuni*, *H. pylori* likely does not have a master transcriptional regulator and utilizes a three-tiered gene expression cascade that requires the FlgSR homologs to activate expression of σ^{54}-dependent rod and hook genes (6).

Activation of σ^{54}-dependent Gene Transcription in Bacteria

The alternative sigma factor, σ^{54} is a subunit of the RNA polymerase (RNAP) holoenzyme used by many bacteria to control transcription of specific genes. Originally, σ^{54} was discovered to control transcription of genes involved in nitrogen assimilation in enteric bacteria, but we now know that σ^{54} is widely distributed among bacteria and involved in activating expression of genes involved in diverse processes (146, 147).

The σ^{54}-subunit of RNAP uses a C-terminal motif to bind to DNA and promoter regions of σ^{54}-dependent genes (148). σ^{54} binds highly conserved DNA sequences located at position -

24 and -12 relative to the transcriptional start site. These sites contain an invariable GG and GC nucleotide base sequence, respectively (17).

Activation of σ^{54} requires an enhancer-binding RR protein containing ATPase activity (147). Once activated, the RR forms an oligomer and binds to DNA upstream of the σ^{54}-promoter region (149). DNA bending promotes a direct interaction between the activator and the σ^{54}-subunit of the RNA polymerase holoenzyme. ATP hydrolysis forms an open DNA complex during transcriptional initiation (150). One example of an enhancer binding protein is the RR, NtrC. In *E. coli*, NtrC activates σ^{54}-RNAP to initiate expression of genes for nitrogen assimilation (146). NtrC-family proteins are synthesized in an inactive form, but switch to an active form through phosphorylation of a conserved aspartate located in the N-terminal receiver domain. Phosphorylation and activation occur in the presence of the cognate kinase, NtrB (151). Once phosphorylated, NtrC family proteins oligomerize and use an essential HTH DNA-binding motif in the C-terminal domain (CTD) to bind upstream of target σ^{54}-dependent promoters (149). Subsequent bending of the DNA promoter allows a direct interaction of the NtrC central domain and the σ^{54}-subunit of RNAP. ATPase activity promotes DNA complex formation to initiate target gene transcription (reviewed in 17, 146).

Cell Division is Dependent on Flagellar Structural Components

Genetic screens identified many of the components involved in activation of σ^{54}-dependent genes in *C. jejuni*, including the requirement for FlhF. FlhF is a GTPase that is likely involved in early steps of flagellar biosynthesis (152). While the role to understand how FlhF

activates flagellar signaling is currently under investigation, FlhF and the putative ATPase FlhG have been implicated in controlling the spatial and numerical requirements in polarly-flagellated bacteria such as *Pseudomonas*, *Vibrio*, and *C. jejuni* (152-157). From these studies, FlhF appears to influence spatial parameters of flagellar biosynthesis whereas FlhG is important for controlling the number of flagella produced by polar flagellates. FlhG is an ortholog of MinD, a component of the Min system, which is required to ensure symmetrical cell division in many bacteria (158). However, *C. jejuni* appears to lack a Min system. Recent studies to characterize FlhG demonstrated that FlhG may possess some MinD-like activities to influence where cell division occurs in *C. jejuni* (158*)*. Additional analysis found that FlhF, the MS ring component FliF, and the C ring components FliM and FliN appear to function with FlhG in determining the location of the division site during cell division. In the absence of any one component, cell division often mislocalized to polar regions and resulted in production of non-viable minicells (158).

Although the mechanism is unknown, it has been hypothesized that the localization of the MS and C ring of a nascent flagellum at the new pole of a daughter cell is important for FlhG to function in a role to regulate flagellar number and inhibit cell division at polar regions (158). This important study showed that *C. jejuni* has exploited its polar flagellation pattern to link cell division to early components of flagellar biogenesis. Considering data generated in my studies, the MS and C ring structures are central to regulation of many events in *C. jejuni*, including signaling for flagellar gene transcription, motility, and cell division.

Two Component Regulatory Systems

Bacteria sense and adapt to intracellular and environmental stimuli through two-component regulatory systems (TCSs). A typical TCS consists of a sensor histidine kinase (HK) that autophosphorylates at a conserved histidine in response to a specific stimulus and subsequently functions as a phosphodonor for a response regulator (RR) protein that autophosphorylates at a conserved aspartate (159). Phosphorylation activates the RR to induce a physiological response by the bacterium, most often by altering gene expression. Such systems are very important to bacteria and are found in nearly every sequenced bacterial genome with some encoding up to 200 TCSs.

In a bacterial cell, cognate HK and RR pairs are comprised of paralogous genes that are similar at the sequence and structural level (160). However, signaling specificity across TCSs is somehow maintained with high fidelity in a bacterial cell. Such similarities present a challenge for the bacterium to maintain each of its individual phosphotransfer signaling pathways so that specific stimuli only trigger a specific TCS and result in the desired response. Otherwise, cross-talk or cross-phosphorylation between signaling pathways could occur. Cross-talk or cross-phosphorylation is communication between two separate signaling pathways that typically function independently of each other. In native bacterial systems, cross-talk is minimized in order to ensure a specific response is generated only in the presence of an activating ligand. In the presence of crosstalk, a specific output is diminished because additional non-cognate pathways are activated, a result that is often detrimental to the cell.

Many bacterial cells contain branched-pathway TCSs where a HK may modify two RRs or a RR may be modified by two HKs (160). While such regulatory mechanisms diversify the response to increase the fitness of the bacterium, they would not be an example of detrimental cross-talk. In most bacterial systems, cross-talk is not observed in the native system because the bacterium has evolved mechanisms to prevent disadvantageous signaling (161). However, the introduction of mutations in one component of a TCS can unveil cross-talk between signaling pathways that does not occur in wild-type cells. Mutational analysis can identify cellular mechanisms to ensure signaling fidelity and to prevent cross-talk with other signaling networks in cell (reviewed in 160).

Mechanisms to Maintain Signaling Fidelity and Minimize Cross-talk

Direct interactions between small groups of specific amino acids in many HKs and RRs mediate molecular recognition between cognate HK and RR pairs that naturally ensures specificity of phosphotransfer within TCSs and minimizes crosstalk between TCSs (162, 163). The importance of molecular recognition in TCSs has been shown in a number of bacterial signaling systems. Specific changes in amino acid residues can rewire the specificity of a signaling pathway to allow a HK to alternatively interact with a noncognate RR (162). Conversely, the switch in specificity can be rescued by making corresponding mutations in the cognate RR (162-164).

Many HKs are bifunctional, possessing an autokinase activity in the presence of a ligand to function as a phosphodonor, and a phosphatase activity for a cognate RR in the absence of

signal (165). Such HKs tightly control of the phosphorylation state of the RR to ensure that the RR is only activated in the presence of the correct activating signal. This bifunctional activity has been seen in a number of bacterial systems and appears to be a general mechanism to eliminate or reduce cross-talk in TCSs (160, 166). An example of the bifunctionality of a HK that control signal specificity has been experimentally investigated with the NtrB and NtrC system (167). By removing the gene that encodes the NtrB HK in *E. coli*, its cognate RR NtrC was actively phosphorylated by two noncognate HKs, UhpB and PhoR, or the small molecule metabolite acetyl-phosphate (AcP) (167). This crosstalk and erroneous phosphotransfer to NtrC occurred in the absence of the normal activating signal and NtrB. This study demonstrated how a HK often controls the phosphorylation state of a cognate RR by functioning as a phosphodonor and by serving as a phosphatase.

Acetyl-phosphate as a Phosphodonor

Bacterial metabolism results in the production of the small molecule AcP through the acetogenesis pathway (168). This pathway is important for ATP generation, conversion of pyruvate into acetate, and recycling NAD^+ and CoA during exponential growth. In this pathway, Pta (phosphotransacetylase) reversibly converts acetyl-CoA into AcP. AckA (acetate kinase) also reversibly assimilates exogenous acetate into AcP via hydrolysis of ATP (reviewed in 168). The *C. jejuni* genome encodes *pta* and *ackA* homologs and analysis has demonstrated that

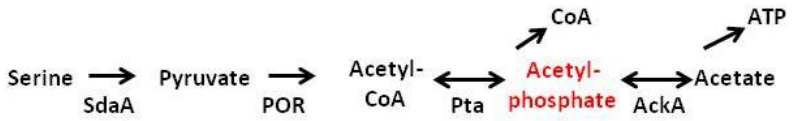

Figure 6. The Acteogenesis Pathway. The acetogenesis pathway as outlined in *E. coli* with minor modifications. Acetyl phosphate (red) is generated by Pta through generation of CoA from acetyl-CoA or by AckA through assimilation of extracellular acetate. Abbreviations: SdaA, serine dehydratase; POR, pyruvate acceptor oxoreductase; Pta, phosphotransacetylase; CoA, coenzyme A; AckA, acetate kinase.

removal of these genes resulted in a loss of cell viability at a faster rate than WT in stationary phase (169, 170). This study suggests that the acetogenesis pathway is functional in *C. jejuni* (169).

Other studies have revealed that the intracellular concentration of AcP reaches 3mM in *E. coli*, a concentration sufficient to directly activate a TCS through phosphotransfer (171). As such, the Rcs system in *E. coli* is modified by AcP *in vivo* (172). In addition, in *Borrelia burgdorferi*, the orphan RR, Rrp2, has no known cognate kinase. However Rrp2 is activated by AcP *in vivo* to induce σ^{54}-dependent gene expression (173). Additionally, the Rcs system in *E. coli* is modified by AcP *in vivo* (172). In many systems where the HK has been genetically removed, AcP has the potential to act as a phosphodonor presumably due to removal of the phosphatase activity of the cognate HK (160, 172, 174). As such, the ability of AcP to influence gene expression in a WT bacterium via a TCS is thought to be fairly minimal. However, if AcP

does influence a RR in WT cells, it is likely that the RR is an orphan RR or is in a TCS where the cognate HK lacks phosphatase activity. In summary, TCSs often employ molecular recognition and phosphatase activities to limit crosstalk from non-cognate TCS and AcP.

CHAPTER THREE

MATERIALS AND METHODS

Bacterial strains.

C. jejuni 81-176 is a clinical isolate from a young girl with diarrheal disease (175). The parent strain relevant for construction of all mutants is 81-176 *rpsL*^{Sm} (9). Previously described *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* mutant strains include: Δ*rpoN* (DRH453) (3); Δ*flgR* (DRH749) (3); Δ*flgS* (DRH911) (3); Δ*flhA* (DRH979) (3); Δ*flhB* (DRH1734) (13); Δ*fliE* (SNJ915) (158); Δ*flgG* (SNJ925) (152); Δ*fliP* (DRH1016) (3); *flgR*$_{\Delta CTD}$ (DRH1931) (19); Δ*flgS flgR*$_{\Delta CTD}$ (SNJ227) (19); Δ*flhA flgR*$_{\Delta CTD}$ (SNJ235) (13); and Δ*flgS flgR D51A*$_{\Delta CTD}$ (SNJ713) (19). *E. coli* DH5α was used for all cloning procedures and *E. coli* BL-21 cells were used for protein expression. Electroporation of *C. jejuni* and creation of insertional and in-frame deletions was performed by previously published protocols (9, 176).

Bacterial growth conditions.

For all experiments, *C. jejuni* strains were initially grown from freezer stocks on Mueller-Hinton (MH) agar containing 10 μg/ml trimethoprim for 48 h under microaerobic conditions at 37 °C. *Campylobacter* defined media (CDM) contains nutrients at concentrations to support growth (177). When appropriate, sodium pyruvate was added to CDM at 50 mM and 100 mM excess. After initial growth, strains were restreaked onto appropriate media and grown for

another 16 h for use in experiments. Chloramphenicol, kanamycin, and streptomycin were added to media at 10, 50, and 100 μg/ml, respectively, when necessary.

Construction of *C. jejuni* mutants and transcriptional reporter strains.

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliQ* was constructed by elctroporation of pSMS469 into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliQ::cat-rpsL* (DRH230) (3, 158). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. pSMS462 was electroporated into DRH2305 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliQ* (DAR125) (158). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct a strain containing a chromosomal *flaB*::*astA* fusion, pDRH610 was electroporated into DAR125 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} strain Δ*astA* Δ*fliQ flaB::astA-kan* (DAR152) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliR* was constructed by elctroporation of pDRH424 into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliR::cat-rpsL* (DRH1647) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. pDRH449 was electroporated into DRH1647 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliR* (DRH1701) (3). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin.

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* was constructed by electroporation of pDRH1814 into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliF::cat-rpsL* (DRH1876) (3, 158). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR.

PCR-mediated mutagenesis was performed with pDRH1777 to fuse codon two to 548 thereby deleting 545 amino acids of the coding sequence of *fliF* to create pDRH2073 (158). pDRH2073 was electroporated into DRH1876 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* (DRH2077) (152). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct a strain containing a chromosomal *flaB::astA-kan* fusion, pDRH610 was electroporated into DRH2077 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF flaB::astA-kan* (DRH2113) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG* was constructed by electroporation of pALU115 into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG::cat-rpsL* (JMB1161) (158) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. PCR-mediated mutagenesis was performed with pDRH2407 to create the following in-frame *fliG* deletion mutants: FliG$_{\Delta S38\text{-}F297}$ (Δ*fliG*; pJMB1204), FliG$_{\Delta K3\text{-}K110}$ (FliG$_{\Delta NTD}$, pJMB1731), FliG$_{\Delta M112\text{-}P207}$ (FliG$_{\Delta MD}$, pJMB1729), FliG$_{\Delta A209\text{-}V319}$ (FliG$_{\Delta CTD}$, pJMB1513), FliG$_{\Delta M112\text{-}V319}$ (FliG$_{\Delta MCTD}$, p JMB1512) (158). These plasmids were transformed into JMB1161 to create *C. jejuni* 81-176 *rpsL*^{Sm} strain Δ*astA* Δ*fliG* (JMB1242), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta K3\text{-}K110}$ (JMB1756), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta M112\text{-}P207}$ (JMB1748), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta A209\text{-}V319}$ (JMB1641), or *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta M112\text{-}V319}$ (JMB1517). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct strains containing a *flaB::astA-kan* transcriptional reporter fusions, pDRH610 was electroporated into JMB1242, JMB1756, JMB1748, and JMB1517 to replace native *flaB* on the chromosome

with the *flaB*::*astA*, thus creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG flaB::astA-kan* (JMB1258) , *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta I2\text{-}S111}$ *flaB::astA-kan* (JMB1779), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta S111\text{-}R208}$ *flaB::astA-kan* (JMB1777), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta R208\text{-}V320}$ *flaB::astA-kan* (JMB1669), and *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliG*$_{\Delta S111\text{-}V320}$ *flaB::astA-kan* (JMB1805) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliM* constructed by electroporation of pJMB572 into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliM::cat-rpsL* (JMB1163). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR (3, 158). PCR-SOEing was performed with pJMB531 to fuse codon 3 to codon 335 thereby deleting 332 amino acids of the *fliM* coding sequence and creating pJMB1401 (158). The plasmid pJMB1401 was electroporated into JMB1163 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliM* (JMB1415). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To replace native *flaB* on the chromosome with the *flaB*::*astA*, pDRH610 was electroporated into JMB1415, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliM flaB::astA-kan* (JMB1417) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

The *fliN* locus was amplified with 750 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primers containing 5' BamHI sites. After cloning the fragment into BamHI-digested pUC19 (creating JMB533), a SmaI-digested *cat-rpsL* cassette (from pDRH265) was cloned into the EcoRV site in *fliN* to create pJMB537 (9). The plasmid pJMB537 was electroporated into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliN::cat-*

rpsL (JMB1407) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. To construct a strain containing a chromosomal *flaB::astA-kan* transcriptional reporter, pDRH610 (containing *flaB::astA-kan)* was electroporated into JMB1407 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliN::cat-rpsL flaB::astA-kan* (JMB1741) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin and 10 μg/ml chloramphenicol.

The *fliY* locus was amplified with 750 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primer containing 5' BamHI sites. After cloning the fragment into BamHI-digested pBR322 (to create JMB1635), PCR-mediated mutagenesis was performed on JMB1635 to create a SwaI site in the *fliY* coding sequence (creating pJMB1971). A SmaI-digested *cat-rpsL* cassette (from pDRH265) was cloned into a SwaI-digested site in the *fliY* coding sequence to create pJMB1980 (3). pJMB1980 was electroporated into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliY::cat-rpsL* (JMB2008) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. To construct a strain containing a chromosomal *flaB*::*astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into JMB2008 to replace native *flaB* on the chromosome with the *flaB*::*astA* creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA fliY::cat-rpsL flaB::astA-kan* (JMB2020) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin and 10 μg/ml chloramphenicol.

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgB* was constructed by electroporation of pSNJ360 into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA flgB::cat-rpsL* (SNJ1043) (3, 13). Mutants

were recovered on MH agar containing chloramphenicol and verified by colony PCR. To construct the *flgB* deletion plasmid, PCR-mediated mutagenesis was performed with pSNJ356 to fuse start and stop codons of *flgB*, thereby creating pSNJ919 (13). The plasmid pSNJ919 was electroporated into SNJ1043 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgB* (SNJ1046). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct a strain containing a chromosomal *flaB::astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into SNJ1046 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgB flaB::astA-kan* (JMB577) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

The *flgC* locus was amplified with 750 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primer containing 5' BamHI sites. After cloning the fragment into BamHI-digested pUC19 (creating DRH275), a SmaI-digested *cat-rpsL* cassette (from pDRH265) was cloned into the StyI site in *flgC* to create pDRH2662 (3). The plasmid pDRH2662 was electroporated into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA flgC::cat-rpsL* (JMB2008) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. PCR-mediated mutagenesis was performed with DRH275 to fuse start and stop codons of *flgC*, thereby deleting the coding sequence of *flgC* and creating pSNJ1009. The plasmid pSNJ1009 was electroporated into SNJ1043 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgC* (SNJ1048). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct a strain containing *flaB::astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into SNJ1048 to replace native *flaB* on

the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgC flaB::astA-kan* (SNJ1078) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

To insert the *flaB::astA* transcriptional reporter fusion in *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliE* and *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgG*, pDRH610 (containing *flaB::astA-kan)* was electroporated into SNJ915 and SNJ925 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliE flaB::astA-kan* (JMB575) and *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgG flaB::astA-kan* (SNJ935) (3, 152, 158). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

The *flgF* locus was amplified with 750 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primer containing 5' BamHI sites. After cloning the fragment into BamHI-digested pUC19 (creating DRH1349), a SmaI-digested *cat-rpsL* cassette (from pDRH265) (3) was cloned into the EcoRV site in the *flgF* coding sequence to create pDRH2534. The plasmid pDRH2534 was electroporated into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA flgF::cat-rpsL* (SNJ931) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. PCR-mediated mutagenesis was performed with DRH1349 to fuse start and stop codons of *flgF*, thereby creating pDRH2504. The plasmid pDRH2504 was electroporated into SNJ931 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgF* (JMB1230). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct a strain containing a chromosomal *flaB::astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into JMB1230 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgF*

flaB::astA-kan (JMB1251) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

The *flgI* locus was amplified with 750 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primer containing 5' BamHI sites. After cloning the fragment into BamHI-digested pUC19 (creating DRH1348), a SmaI-digested *cat-rpsL* cassette (from pDRH265) was cloned into the EcoRV site in the *flgI* coding sequence to create pDRH1348 (3). The plasmid pDRH1348 was electroporated into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA flgI::cat-rpsL* (JMB2004) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. To construct a strain containing a chromosomal *flaB::astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into JMB2004 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA flgI::cat-rpsL flaB::astA-kan* (JMB2069) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin and 10 μg/ml chloramphenicol.

The *flgH* locus was amplified with 750 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primer containing 5' BamHI sites. After cloning the fragment into BamHI-digested pUC19 (creating DRH1878), a SmaI-digested *cat-rpsL* cassette (from pDRH265) was cloned into the PmlI site in the *flgH* coding sequence to create pALU101 (3). The plasmid pALU101 was electroporated into DRH461 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA flgH::cat-rpsL* (ALU107) (3). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. PCR-mediated mutagenesis was performed with DRH1878 to fuse

start and stop codons of *flgH*, creating pDRH2422. The plasmid pDRH2422 was electroporated into ALU107 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgH* (DRH2468). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct a strain containing a chromosomal *flaB::astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into DRH2468 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgH flaB::astA-kan* (JMB2044) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin.

In order to complement the *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* and Δ*fliG* mutants, the *fliF* and *fliG* coding sequences from codon 2 to the stop codon were amplified from the *C. jejuni* 81-176 chromosome with primers containing 5' BamHI sites and cloned into BamHI-digested pECO102 or pCE107 to create pDRH2309 and JMB1859, respectively (178, 179). The *flaA* promoter fused to the *fliG* coding sequence was amplified from pJMB1859 with NotI and XhoI sites and cloned into NotI and XhoI-digested pRY112 to create pJMB2071 (178). In parallel, the *flaA* promoter was amplified from pCE107 with NotI and XhoI sites and cloned into NotI and XhoI –digested pRY112 to create pJMB2074 (178). pECO102, pDRH2309, pJMB2071, and pJMB2074 were conjugated into DRH2077 and JMB1242 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pECO102 (JMB2104), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pDRH2309 (JMB1467), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG*/pJMB2071 (JMB2116), and *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG*/pJMB2074 (JMB2113) (178). All transconjugates were recovered on MH agar containing chloramphenicol and verified by PCR.

PCR was used to amplify the *fliF c*oding sequence from the *C. jejuni* 81-176 chromosome with different 5' endpoints or 3' endpoints. Primers were used that contained 5' BamHI sites and 3' PstI sites. Mutant *fliF* alleles lacking different domains were cloned into BamHI and PstI-digested pECO102 to create pJMB1505 ($FliF_{\Delta D2\text{ -}T18}$), pJMB1518 ($FliF_{\Delta C'\ 82aa}$), pJMB1519 ($FliF_{\Delta C'\ 6AA}$), pJMB1560 ($FliF_{E557A}$), or pJMB1561 ($FliF_{K558A}$), pJMB1927 ($FliF_{\Delta E519\text{-}E554}$), and pJMB1924 ($FliF_{\Delta K507\text{-}E554}$) (178). DH5α/pRK212.1 was transformed with the plasmids and then conjugated into *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* (DRH2077) or Δ*astA* Δ*fliF flaB::astA* (DRH2113) to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA*/pJMB1505 (JMB1767), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1518 (JMB1555), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1519 (JMB2142) , *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1560 (JMB1576), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1561 (JMB1579), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF flaB::astA*/pJMB1927 (JMB1951), and *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF flaB::astA/*pJMB1924 (JMB1948). Transconjugants were selected for by growth on MH agar containing 10 μg/ml chloramphenicol, 100 μg/ml streptomycin, and 10 μg/ml trimethoprim. To construct strains containing a *flaB::astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into JMB1767, JMB1555, JMB1519, JMB1576, and JMB1579 creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1505 (JMB2172), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1518 (JMB2163), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1519 (JMB2175), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1560 (JMB2166), and *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1561 (JMB2169) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin and 10 μg/ml chloramphenicol.

To construct other in-frame *fliF* mutants, PCR-mediated mutagenesis was performed with DRH1777 to delete the codons for A175 and S176 to create pJMB1827 and to delete the codons for L510-I521 to create pJMB1516 (158). The plasmid pJMB1827 was electroporated into DRH1876 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* *fliF*$_{\Delta A175\text{-}S176}$ (JMB1960). Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. To construct a strain containing a chromosomal *flaB::astA* transcriptional reporter fusion, pDRH610 (containing *flaB::astA-kan)* was electroporated into JMB1960 to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* FliF$_{\Delta A175\text{-}S176}$ *flaB::astA-kan* (JMB2064) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin. In addition, the mutant *fliF* coding sequence was amplified from codon 2 to the stop codon from pJMB1516 with 5' BamHI and 3' PstI sites and cloned into BamHI and PstI-digested pECO102 to create pJMB1609 (*fliF*$_{\Delta L510\text{-}I521}$) (178). DH5α/pRK212.1 was transformed with the plasmid and then used to conjugate plasmids into *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* (DRH2077) to create *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB1516 (JMB1629). Transconjugants were selected for by growth on MH agar containing 10 μg/ml chloramphenicol, 100 μg/ml streptomycin, and 10 μg/ml trimethoprim. To construct strains containing a chromosomal *flaB::astA* transcriptional reporter, pDRH610 (containing *flaB::astA-kan)* was electroporated into JMB1876 and JMB1629 to replace native *flaB* on the chromosome with the *flaB*::*astA*, creating *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* *flaB::astA-kan*/pJMB1516 (JMB1665) (3). Transformants were recovered on MH agar containing 50 μg/ml kanamycin and 10 μg/ml chloramphenicol.

To construct a vector to produce proteins with an N-terminal flag tag, primers were constructed to amplify the *cat* promoter from pRY109 (178). One primer contained a 5' XbaI site and the other contained a 5' BamHI site and codons to add a flag tag immediately after the start codon of the *cat* gene. This fragment was cloned into XbaI and BamHI digested pRY112 to generate pDAR965 (178). The *fliF* coding sequence from codon two to the stop codon was amplified from the 81-176 chromosome with primers containing 5' BamHI sites and cloned into BamHI-digested pDAR965 to generate pJMB2032 which encodes a Flag-FliG fusion protein.

In parallel, the *fliG* coding sequence from the start codon to the stop codon was amplified from the 81-176 chromosome with primers that contain 5' BamHI sites and add a C-terminal flag tag to the last codon of *fliG*. The *fliG* allele was cloned into BamHI-digested pCE107 to create pJMB1740, which encodes a FliG-Flag fusion protein (pJMB1740) (179). Plasmids were transferred to DH5α/pRK212.1 and then conjugated into *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF* (DRH2077) or 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG* (JMB1242) to form 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pJMB2032 (JMB2046) and 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG*/pJMB1740 (JMB1903). In addition, the parental vectors lacking *fliF* or *fliG* were transferred into conjugated into DH5α/pRK212.1 and conjugated into each strain to create 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliF*/pDAR965 (JMB2061) and 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliG*/pCE107 (JMB2203).

The *pta ackA* locus was amplified with 750 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primers containing 5' KpnI sites. After cloning into KpnI-digested pUC19 (creating pJMB553), a SmaI-digested *cat-rpsL* cassette (from pDRH265) was cloned into the AflII site in *ackA* (generating pJMB653) or the SpeI site in *pta* (creating

pJMB565) (9). Interruption of both *pta* and *ackA* was accomplished by inserting SmaI-digested *cat-rpsL* cassette in AflII- and SpeI-digested pJMB553 (creating pJMB955). PCR-mediated mutagenesis was performed with pJMB553 to fuse the start and stop codons, thereby deleting the remainder of the coding sequence of *ackA* and creating pJMB630.

Plasmids pJMB653, pJMB565, and pJMB955 were introduced into *C. jejuni* 81-176 *rpsL*^{Sm} strains Δ*astA* Δ*flgS* (DRH911), Δ*astA* Δ*flgS flgR*$_{\Delta CTD}$ (SNJ227), and Δ*astA* Δ*flgS flgR D51A*$_{\Delta CTD}$ (SNJ713) by electroporation, generating 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS ackA::cat-rpsL* (JMB740), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS pta::cat-rpsL* (JMB1066), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS pta ackA::cat-rpsL* (JMB971), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS flgR*$_{\Delta CTD}$ *ackA::cat-rpsL* (JMB865), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS flgR*$_{\Delta CTD}$ *pta::cat-rpsL* (JMB862), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS flgR*$_{\Delta CTD}$ *pta ackA::cat-rpsL* (JMB977), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS flgR D51A*$_{\Delta CTD}$ *ackA::cat-rpsL* (JMB732), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS flgR D51A*$_{\Delta CTD}$ *pta::cat-rpsL* (JMB825), and 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS flgR D51A*$_{\Delta CTD}$ *pta ackA::cat-rpsL* (JMB974). Additionally, pJMB653 was electroporated into 81-176 *rpsL*^{Sm} Δ*astA* (DRH461) and 81-176 *rpsL*^{Sm} Δ*astA* *flgR*$_{\Delta CTD}$ (DRH1931) to generate 81-176 *rpsL*^{Sm} Δ*astA ackA::cat-rpsL* (JMB669) *and* 81-176 *rpsL*^{Sm} Δ*astA* *flgR*$_{\Delta CTD}$ *ackA::cat-rpsL* (JMB811). Mutants were recovered on MH agar containing chloramphenicol and verified by colony PCR. Semi-quantitative real-time reverse-transcriptase PCR was used to ensure that the *pta*::*cat-rpsL* mutation did not cause a polar effect on transcription of the downstream *ackA* gene.

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS* Δ*ackA* (JMB760), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS* *flgR*$_{\Delta CTD}$ Δ*ackA* (JMB919), 81-176 *rpsL*^{Sm} Δ*astA* Δ*flgS flgR D51A*$_{\Delta CTD}$ Δ*ackA* (JMB815), and

81-176 *rpsL*^{Sm} Δ*astA* *flgR*$_{\Delta CTD}$ Δ*ackA* (JMB857) were created by electroporating JMB740, JMB865, JMB732, and JMB811 with pJMB630. Transformants were recovered on MH agar containing 0.5 – 5 mg/ml streptomycin. Deletion of *ackA* was verified by colony PCR.

To construct strains containing chromosomal promoterless *astA* transcriptional reporters, pDRH610 (containing *flaB::astA-kan)* or pDRH669 (containing *flgDE2*::*astA-kan*) was electroporated into all relevant strains to replace native *flaB* or *flgDE2* on the chromosome with the *flaB*::*astA* or *flgDE2*::*astA* transcriptional fusion (3). Mutants were recovered on MH agar containing kanamycin and verified using colony PCR.

Construction of plasmids containing *flgDE2*::*astA* transcriptional fusions.

An *astA-kan* cassette was released from pDRH580 by digestion with SmaI and cloned into the MscI site of *flgD* in the *flgDE2* locus of pDRH351 (3). One plasmid (pDRH669) was recovered with *astA-kan* in the proper orientation to create a *flgD*::*astA* transcriptional fusion, with *astA-kan* located 582 bp downstream of the *flgDE2* transcriptional start site.

PCR was used to amplify *flgDE2*::*astA* with different 5' endpoints including at bases -302, -29, and -13 relative to the start site of transcription of *flgDE2*. Primers were used that contained 5' PstI sites and 3' KpnI sites. Transcriptional fusions were cloned into PstI and KpnI-digested pRY108, an *E. coli-C. jejuni* shuttle plasmid to result in pJMB1074 (with a 5' end at base -302), pJMB1075 (with a 5' end at base -29), and pJMB1076 (with a 5' end at base -13) (178). Plasmids were transferred to DH5α/pRK212.1 and then conjugated into *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* (DRH461), *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* *flgR*$_{\Delta CTD}$ (DRH1931), *C. jejuni* 81-176

rpsL^{Sm} Δ*astA* Δ*flgR* (DRH749), and *C. jejuni* 81-176 *rpsL*^{Sm} Δ*astA* Δ*rpoN* (DRH453). Transconjugants were selected for by growth on MH agar containing 10 μg/ml chloramphenicol, 100 μg/ml streptomycin, and 10 μg/ml trimethoprim.

Bioinformatics Analysis.

Protein homology searches and analysis were performed using BLASTP and ClustalW2 programs (http:www.ncbi.nlm.nih.gov/BLAST; http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Generation of polyclonal antiserum against *C. jejuni* proteins.

Generation of the polyclonal murine antiserum against *C. jejuni* FliF involved amplifying the *fliF* coding sequence with 5' BamHI sites starting from codon 2 through the stop codon of *fliF* from 81-176. This DNA fragment was ligated into the BamHI-digested pGEX-4T-2 (GE Healthcare) and transformed into *E. coli* BL-21 (DE3) to create pDRH2267. The resulting strain was grown in 4 L of LB to mid-log phase and then induced with 1 mM IPTG for 4 h at 37ºC. The bacteria were harvested and lysed using an Emulsiflex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2. The soluble fraction was isolated using centrifugation and the incubated with 1 mL of glutathione Sepharose 4B (GE Healthcare) for overnight at 4ºC. The proteins were purified according to the manufacturer's instructions. Polyclonal murine antiserum was generated in mice by standard procedures using a commercial vendor (Cocalico Biologicals).

Generation of the polyclonal murine antiserum against *C. jejuni* FliG involved amplifying the *fliG* coding sequence with a C-terminal 6x His tag and 5' BamHI sites from 81-176. This DNA fragment was ligated into the 5' BamHI-digested pT7-7 and transformed into *E. coli* BL-21 (DE3) to create pJMB1506. The resulting strain was grown in 2 L of LB to mid-log phase and then induced with 1 mM IPTG for 4 h at 37ºC. The bacteria were harvested and lysed using an Emulsiflex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2. The soluble fraction was isolated by centrifugation and the incubated with 1 mL Ni-NTA agarose (Qiagen) for 2 h at 4ºC. The proteins were purified according to the manufacturer's instructions. Polyclonal murine antiserum was generated in mice by standard procedures using a commercial vendor (Cocalico Biologicals).

Co-Immunoprecipitation of *C. jejuni* proteins

C. jejuni strains expressing FliF or FliG flag-tagged proteins were grown on MH agar with appropriate antibiotics for 48 h at 37º C in microaerobic conditions and restreaked 16 h prior to harvest. Cells were resuspended to OD_{600} 1.0 and washed once with PBS and subjected to *in vivo* cross-linking based on previously established protocols with slight modifications (180). In brief, cells were resuspended in 20 ml of PBS with 1% w/w formaldehyde and samples were incubated at 37º C for 30 min. The cross-linking reaction was quenched with addition of 4 ml of 1 M glycine for 10 min at 25º C.

The cells were lysed by osmotic lysis and Triton X-100 was then added to solubilize membranes and insoluble material as described previously with slight modifications (181). In

brief, cells were resuspended in 500 µl of 200 mM Tris (pH 8.0). One ml of 200 mM Tris (pH 8.0) with 1 M sucrose was added along with 100 µl of 10 mM EDTA, 10 ml of 10 mg/ml lysozyme, and 3 ml of water. Additionally, 300 µl of a 100 mM stock of PMSF was added. The solution was incubated on ice for 10 min followed by addition of 5 ml of 50 mM Tris (pH 8.0) 10 mM $MgCl_2$ and 2% Triton X-100. The cells were incubated on ice for 30 min and then centrifuged at 16,000 xg for 10 min. The supernatant was subjected to additional centrifugation at 160,000 xg for 1 h. Following ultracentrifugation, the supernatant was used for CoIP experiments.

For immunoprecipitation of flag-tagged FliF and FliG proteins, α- Flag M2 affinity gel resin was used according to the manufacturer's instructions protocols (Sigma-Aldrich) with slight modifications. In brief, 1 ml of supernatant from lysed *C. jejuni* cells was incubated with 5 µl of α-flag M2 affinity gel resin at 4ºC for 3 h with rocking. After binding was complete, the resin was washed four times with RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100). The resin was resuspended in 20 µl Lamelli buffer, boiled for 5 min, and loaded onto a 10% SDS-PAGE gel for analysis.

Immunoblotting Analysis of *C. jejuni* Proteins.

C. jejuni strains were grown from frozen stocks on MH agar containing appropriate antibiotics at 37º C in microaerobic conditions for 48 h and restreaked 16 h prior to use. Cells were resuspended using MH broth from 16 h growth plates and diluted to OD_{600} 0.8. For whole-

cell lysates, one ml samples were centrifuged and washed with 1 mL of PBS. Pellets were resuspended in 50 µl of 1x Lamelli buffer and 10 µl were loaded onto a 10% SDS-PAGE gel.

For detection of FliF and FliG proteins in whole cell lysates, α-FliF M203 antiserum was used at a concentration of 1:3000 and α-FliG M161 antiserum was used at a concentration of 1:5000 followed by goat α-mouse secondary antibody at a 1:5000 dilution. Immunoblotting of FlgS, FlgR, and RpoA was performed as previously described (8, 142).

Semi-quantitative Real-time RT-PCR Analysis.

C. jejuni strains were grown from frozen stocks on agar containing appropriate antibiotics at 37 °C for 48 h under microaerobic conditions and then restreaked on MH agar and grown for another 16 h. Total RNA was extracted with Trizol (Invitrogen) and RNA was treated with DNaseI prior to analysis. RNA for analysis was used at a concentration of 50 ng/µl. Semi-quantitative real-time RT-PCR was performed using a 7500real-time PCR system (Applied Biosystems). Detection of *secD* mRNA served as an endogenous control and experimental transcript levels were compared to strains DRH461 or SNJ268, which served as WT controls in these studies.

Purification of Proteins.

Expression of *flgR* constructs for purification of proteins with C-terminal 6XHis-tags was performed as previously described (19). Expression of *flgS* with an N-terminal 6XHis-tag was performed as previously described (142). For phosphorylation experiments, FlgS and FlgR

proteins were purified as previously described (19, 142). For purification of 6XHis-tagged FlgS for phosphorylation of FlgR for EMSAs, cell lysates after production of 6XHis-tagged FlgS were loaded onto a His-TrapFF column (GE Biosciences) for affinity purification. Proteins were eluted using a linear gradient of 250 mM imidazole in 50 mM NaH_2PO_4, 300 mM NaCl and 10 mM imidazole, pH 8.0. Eluted proteins were dialyzed overnight in 20 mM Tris-HCl, pH 8.0. Dialyzed proteins were loaded onto a Hi Trap Q column HP (GE Biosciences) in 20 mM Tris-HCl, pH 8.0 and eluted by linear gradient with addition of 1 M NaCl. Proteins were dialyzed as previously described and stored at -80 °C.

Electrophoretic mobility shift assays (EMSAs).

6XHis-FlgS and -FlgR proteins were purified as described below. EMSAs were performed based on a modified protocol (182). A 381-bp DNA fragment of *flgDE2* spanning -302 to +79 relative to the transcriptional start site and a 348-bp DNA fragment of *gyrA* flanking 298 bp upstream and 50 bp downstream of the start codon were amplified. After FlgS-mediated phosphorylation of FlgR proteins (19), 0.1 to 1 μM of FlgR proteins were incubated with ^{32}P-labeled DNA at 25 ºC for 20 min. For competition experiments, unlabeled P_{flgDE2} and P_{gyrA} were added at 1:1, 5:1 or 10:1 ratios relative to ^{32}P-labeled P_{flgDE2} DNA and 1 μM of phosphorylated FlgR proteins were used. After electrophoresis, gels were analyzed with a Storm 820 phosphorimager according to manufacturer's instructions (Amersham Biosciences).

***astA* Transcriptional Reporter Assays.**

C. jejuni Δ*astA* strains were used in transcriptional reporter assays (Table S3). After growth on appropriate agar, arylsulfatase production from *flgDE2*::*astA* or *flaB*::*astA* transcriptional fusions in strains was measured as previously described (3). Each strain was tested in triplicate and each assay was performed three times.

Transposon Mutagenesis.

C. jejuni 81-176 Δ*astA* Δ*flgS* $flgR_{\Delta CTD}$ *flaB*::*astA* chromosomal DNA was used in *in vitro* transposition reactions with the *darkhelmet* Tn as previously described (3, 9, 98). Tn mutants were recovered on MH agar containing chloramphenicol, kanamycin, and 35 μg/ml 5-bromo-4-chloro-3-indolyl sulfate. The site of the Tn insertion in mutants with light blue or white colony phenotypes was determined as previously described (8).

***In vitro* FlgR autophosphorylation assays with AcP.**

Ac[^{32}P] was generated as previously described with minor modifications (183). FlgR proteins (50 and 100 pmol) were incubated with 15 μl of Ac[^{32}P]-generating reaction for 20 min at 37°C. Analysis of FlgR phosphorylation by FlgS was performed as previously described (19). Proteins were separated by 10% SDS-PAGE without boiling. Gels were analyzed as described above.

Transmission electron microscopy (TEM).

After 16 h growth on appropriate agar, strains were prepared for TEM as previously described (19) Over 220 individual cells for each strain were analyzed for flagellation. Data from two experiments were combined and averaged and standard deviations were determined. Bacteria were grouped into one of three categories: 2 flagella, producing a flagellum at each pole; 1 flagellum, producing a flagellum only at one pole; or 0 flagella, aflagellated.

CHAPTER FOUR

A Flagellar MS Ring and Rotor Involved in Signal Transduction to Activate Transcription of Flagellar Genes

Introduction

Signal detection and transduction by bacterial two-component regulatory systems (TCSs) are essential for a bacterium to link specific extracellular or intracellular stimuli to correct behavioral responses such as gene expression. In contrast to peritrichous *E. coli* and *Salmonella* species, polarly-flagellated bacteria including *Pseudomonas, Vibrio, Campylobacter*, and *Helicobacter* species require a TCS to activate transcription of σ^{54}-dependent genes (6, 133, 134). However, the precise signal sensed by these flagellar-associated TCSs to ultimately result in flagellar gene expression is not known.

One of the best characterized flagellar-associated TCSs among polar flagellates is the FlgSR TCS of *Campylobacter jejuni*, a leading cause of bacterial diarrheal disease throughout the world. In this TCS, the cytoplasmic FlgS histidine kinase autophosphorylates upon sensing a signal and then activates the cognate FlgR response regulator via phosphotransfer to a specific aspartate residue (13, 19). FlgR, which is a member of the NtrC family of transcriptional regulators, then positively influences σ^{54}-RNA polymerase holoenzyme to initiate transcription of flagellar genes (19). These flagellar genes include those encoding flagellar rod and hook

proteins and the FlaB minor flagellin (9). Furthermore, FlgSR and σ^{54} are required for full level of expression of σ^{28}, which is necessary for production of the FlaA major flagellin to complete flagellar biosynthesis and the Fed proteins (11, 66). Many of these Fed proteins are required by *C. jejuni* for optimal commensal colonization of the natural avian host and invasion of human intestinal epithelial cells, presumably to initiate diarrheal disease (66).

We previously identified other *C. jejuni* proteins required for expression of σ^{54}-dependent flagellar genes including the flagellar type III secretion system (T3SS) components (i.e, FlhA, FlhB, FliP, and FliR) and FlhF, a GTPase that functions to localize flagella to the poles in *C. jejuni* and other polar flagellates (3). Due to the apparent absence of a master transcriptional regulator for flagellar gene expression in *C. jejuni*, T3SS genes and FlhF are thought to be constitutively expressed. While the requirement of FlhF for flagellar gene expression is not known, experimental evidence suggested that the flagellar T3SS is linked to activation of the FlgSR TCS and resultant expression of σ^{54}-dependent flagellar genes (3, 13). Furthermore, a formed, but not fully secretion-competent flagellar T3SS activated FlgSR- and σ^{54}-dependent gene expression, suggesting that only formation of the T3SS is required for signal transduction through the FlgSR TCS (13). Based on these findings, we proposed that FlgS may directly sense a specific component of a fully-formed T3SS in the inner membrane to initiate signal transduction and flagellar gene expression in *C. jejuni*. However, a specific domain of the T3SS directly sensed by FlgS has not yet been discovered.

In this work, we performed a more thorough analysis of flagellar components required for FlgSR- and σ^{54}-dependent flagellar gene expression. These studies found that FliF, which forms

the homopolymeric MS-ring housing the flagellar T3SS, and the FliG rotor component found at the cytoplasmic base of the MS-ring are also required to activate flagellar gene expression. Further analysis revealed that unlike in other bacteria where FliF and FliG have been studied, *C. jejuni* FliF and FliG require each other for stability, which is mediated through unique domains absent from many FliF and FliG homologs. Domain analysis of the two proteins revealed a region of the cytoplasmic domain of FliF that appears to be essential for signal. Additionally, a mutant of FliF that is predicted to not be able to interact with the flagellar T3SS FlhA component also does not result in gene expression. Additionally, we demonstrated that FlgS interacts with FliF and FliG *in vivo*, but this interaction is dependent on components from the T3SS (FlhA). These data indicate a new function for the MS ring and rotor of flagellar systems with the capacity to be direct signals for TCSs and function in signal transduction. Considering that FliF also functions with other proteins in *C. jejuni* to control spatial aspects of division, the *C. jejuni* flagellar motor is a unique motor capable of three major biological functions: motility, division, and signal transduction.

Results

Analysis of Flagellar Proteins Required for σ^{54}-dependent Flagellar Gene Expression.

The *C. jejuni* flagellum contains standard components as the flagella of other motile bacteria (Figure 7A; (5, 6)). Through transposon mutagenesis and subsequent analysis of defined in-frame deletion mutants, we previously discovered that components of the flagellar T3SS (FlhA, FlhB, FliP, and FliR), the FlhF GTPase, and the FlgSR TCS are required for wild-

type level of expression of σ^{54}-dependent flagellar (Figure 7B; (3)). We analyzed whether *C. jejuni* mutants lacking other flagellar proteins affected expression of the σ^{54}-dependent *flaB*::*astA* transcriptional reporter (Figure 7A and 7B). In a Δ*fliQ* mutant, expression of *flaB*::*astA* was reduced approximately 50-fold, confirming previous data that T3SS components are required for σ^{54}-dependent flagellar gene transcription. Mutants lacking FliF or FliG, which form the inner membrane MS ring and rotor component of the C ring, respectively, were nearly as defective as T3SS mutants in expressing *flaB*::*astA* (Figure 7A and 7B). However, deletion of *fliM* or *fliN*, which encode switch proteins of the C ring, or *fliY*, which encodes a putative phosphatase that likely influences chemotaxis, resulted in similar or mildly increased expression of *flaB*::*astA* relative to the wild-type strain. We also observed that deletion of *fliE*, which is predicted to encode the most proximal component of the rod, caused a 50-fold reduction in *flaB*::*astA* expression. However, *flaB*::*astA* expression was only reduced 3- to 5-fold in mutants lacking proximal rod proteins FlgB, FlgC, and FlgF (Figure 7B). Mutants lacking the FlgG distal rod protein, the FlgI P ring, or the FlgH L ring were not defective for σ^{54}-dependent flagellar gene expression. These data indicate that all predicted components of the flagellar T3SS, the FliF MS ring, and the FliG rotor of the C ring and some rod proteins are required for full level of expression of σ^{54}-dependent flagellar genes in *C. jejuni*.

The *fliF* stop codon overlaps the *fliG* start codon by one base, suggesting that the two genes may be co-transcribed. Although we created an in-frame deletion of *fliF* or *fliG* so that the coding sequence of the other gene would not be disturbed, it is possible that the mutations in

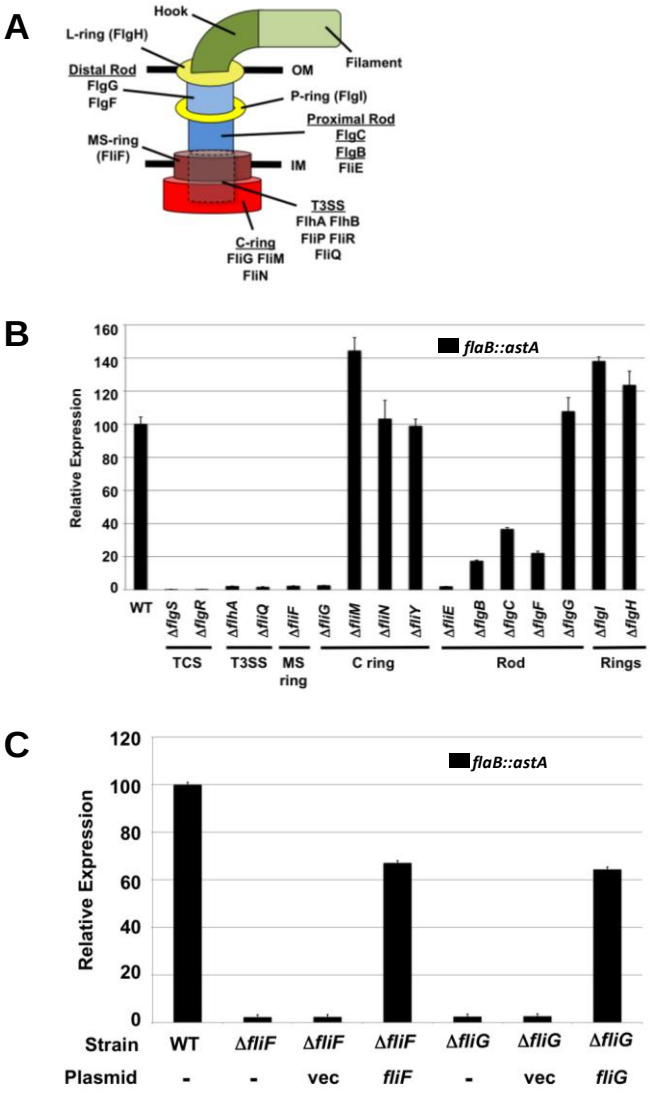

Figure 7. Analysis of Expression of the σ^{54}-dependent *flaB*::*astA* Transcriptional Reporter in *C. jejuni* Flagellar Mutants. (A) Typical structural organization of a bacterial flagellum. The T3SS (shown as a dotted cylinder) is located in the inner membrane (IM) and surrounded by the MS and C rings. OM, outer membrane. (B) Arylsulfatase assay examining *flaB*::*astA* expression in wild-type *C. jejuni* and isogenic mutants lacking different flagellar genes. The assay was performed in triplicate with the level of expression of *flaB*::*astA* in each mutant is relative to wild-type *C. jejuni*, which was set to 100 units. Error bars indicate standard deviation. (B) Arylsulfatase assay examining *flaB*::*astA* expression in wild-type *C. jejuni* and isogenic Δ*fliF* or Δ*fliG* mutants. Each Δ*fliF* and Δ*fliG* mutant was not complemented (-) or complemented *in trans* with vector alone (vec) or a plasmid to express *fliF* or *fliG*. The genotype and the plasmid used for complementation is listed for each strain.

each gene could have affected transcription of the other. However, complementation of each mutant *in trans* with a plasmid to constitutively express the gene that was deleted restored flagellar gene expression to approximately 60-65% of wild-type levels (Figure 7C). These data demonstrate that both FliF and FliG are required for σ^{54}-dependent flagellar gene expression.

FliF and FliG are Dependent on Each Other for Stability.

In flagellated bacteria, FliF forms the inner membrane MS ring with a cytoplasmic domain to interact with the FliG rotor component of the C ring (Figure 7A; (184, 185)). The biogenesis of flagella and bacterial injectisome systems with similar T3SSs is thought to begin with T3SS proteins interacting to form a complex in the inner membrane that then recruits FliF, FliG, FliM, and FliN (14-16). FliF subsequently multimerizes into the MS ring, and FliG, FliM, and FliN multimerize into the C ring located at the base of the MS ring. Both the MS and C rings form around the T3SS. Considering this proposed model for T3SS biogenesis, the requirement of FliF and FliG for σ^{54}-dependent flagellar gene expression, and the fact that FlgS is a cytoplasmic sensor kinase, we reasoned the cytoplasmic-accessible regions of FliF and FliG to be attractive candidates for forming a direct signal that may be sensed by FlgS to stimulate signal transduction through the FlgSR TCS and activate σ^{54}-dependent flagellar gene expression.

To ensure that production of FliF and FliG was not affected in any other flagellar mutants defective for σ^{54}-dependent gene expression, we performed immunoblot analysis of *C. jejuni* whole-cell lysates with specific FliF or FliG antisera. The levels of FliF and FliG were unaffected in FlgSR TCS or flagellar T3SS mutants (Figure 8A). However, in the Δ*fliG* mutant, we detected only a minor amount of the full-length 63-kDa FliF protein. Instead, a smaller protein of 25 kDa was detected, suggesting that FliF was mostly unstable in the absence of FliG

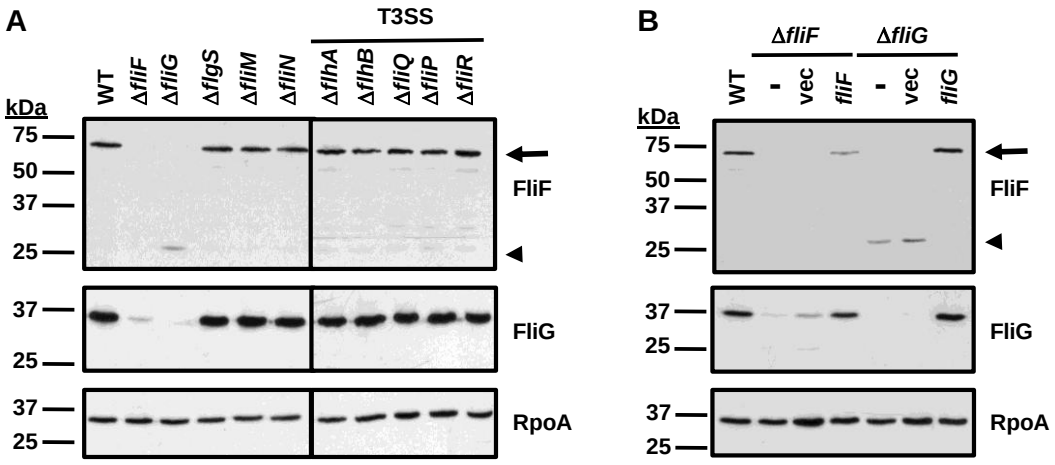

Figure 8. Examination of FliF and FliG Levels in *C. jejuni* Flagellar Mutants. (A) Whole-cell lysates of wild-type *C. jejuni* or isogenic mutants lacking different flagellar genes were analyzed by immunoblot analysis with α-FliF, α-FliG, or α-RpoA antisera. For (B), levels of FliF and FliG in wild-type *C. jejuni* and isogenic Δ*fliF* or Δ*fliG* mutants. Each Δ*fliF* and Δ*fliG* mutant was not complemented (-) or complemented *in trans* with vector alone (vec) or a plasmid to express *fliF* or *fliG*. For (A and B), arrows indicate full-length FliF and arrowheads indicate a truncated FliF protein.

(Figure 8A). Similarly, the full-length 38-kDa FliG protein was present at greatly reduced levels in *C. jejuni* Δ*fliF* (Figure 8A). In contrast, the levels of either full-length FliF or FliG were not affected in the Δ*fliM* or Δ*fliN* mutants, which each lack a switch component of the C ring. The levels of an internal standard, RpoA (the α subunit of RNA polymerase), were similar in all strains (Figure 8A). In the Δ*fliF* and Δ*fliG* mutants complemented *in trans* with the respective gene lacking in each, production of full-length FliF and FliG proteins were restored (Figure 8B). Unlike in other flagellar systems where FliF and FliG are stable in the absence of

the other protein (185), our data suggested that *C. jejuni* FliF and FliG are dependent on each other for stability.

We next analyzed domains of FliF and FliG required for stability of each protein. Furthermore, analysis of FliF and FliG mutant proteins lacking certain domains provided insight into domains required for motility and σ^{54}-dependent flagellar gene expression. In other flagellar systems, interactions between the N-terminus of FliG and the C-terminus of FliF facilitate docking of the C ring to the cytoplasmic face of the MS ring (186, 187). Similar to other FliF proteins (188), *C. jejuni* FliF is predicted to be organized into a short N-terminal cytoplasmic domain of 22 amino acids, followed by two transmembrane domains with an intervening periplasmic domain of 400 amino acids, and a C-terminal cytoplasmic domain of 100 amino acids (Figure 9A). Therefore, we examined FliF proteins lacking a major portion of the N-terminal cytoplasmic domain ($FliF_{\Delta D2\text{-}T18}$) or the C-terminal cytoplasmic region ($FliF_{\Delta C'82AA}$). Plasmids expressing wild-type *fliF* or *fliF* mutants encoding these truncated proteins were examined for their ability to complement a *C. jejuni* Δ*fliF* mutant. We discovered that WT FliF and $FliF_{\Delta D2\text{-}T18}$ supported similar levels of *flaB::astA* expression and production of FliF and FliG (Figure 9A and 9B). In contrast, $FliF_{\Delta C'82AA}$ did not restore production of FliF and FliG or flagellar gene expression (Figure 9A and 9B).

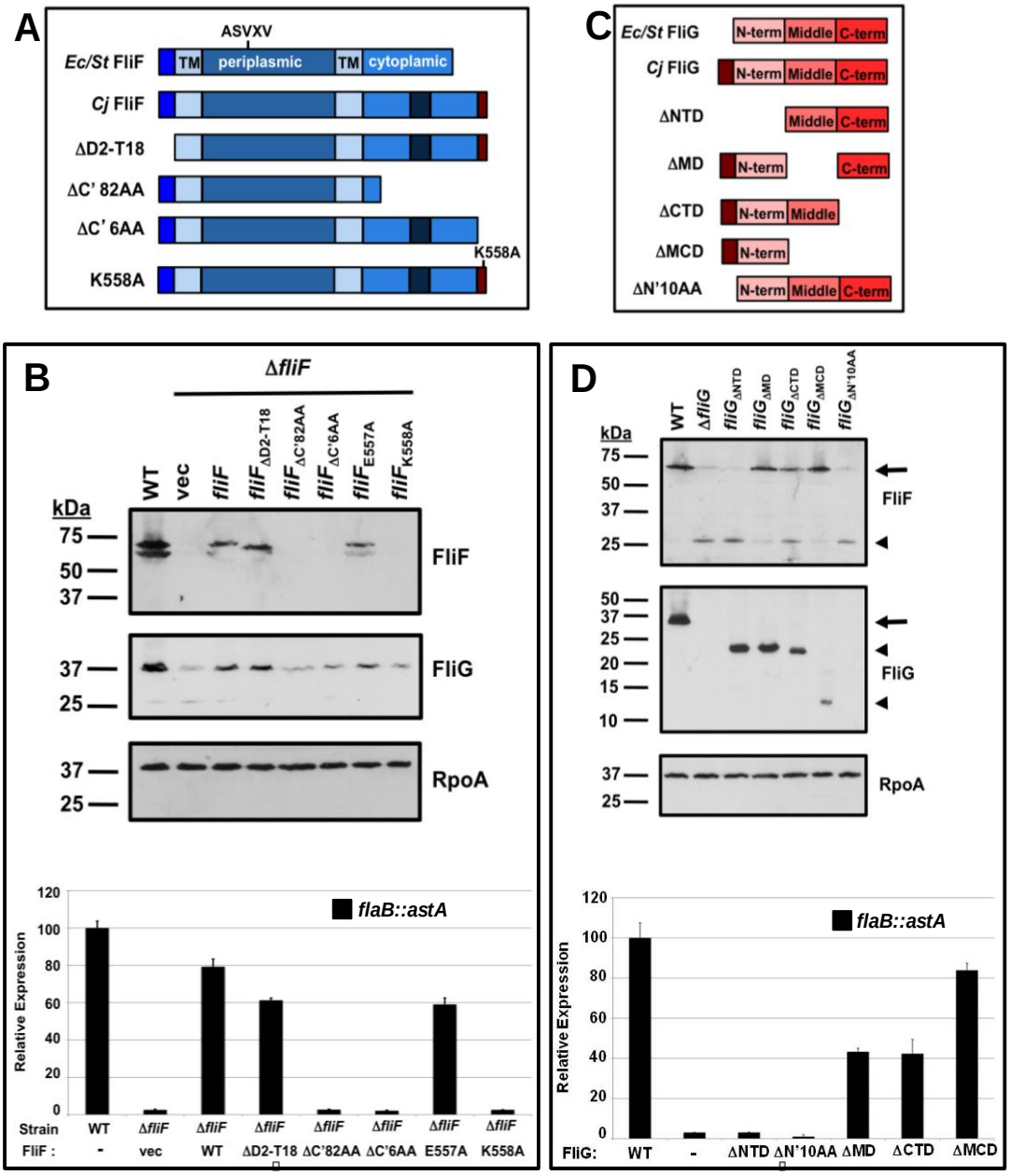

Figure 9. Analysis of Domains of FliF and FliG Required for Stability and *flaB*::*astA* Expression. (A) Organization of the *E. coli* (*Ec*), *Salmonella enterica* serovar Typhimurium (*St*), and *C. jejuni* (*Cj*) FliF proteins. The FliF proteins are predicted to possess a short N-terminal cytoplasmic domain (bright blue), two transmembrane (TM) domains, a periplasmic domain and a C-terminal cytoplasmic domain. In the C-terminal cytoplasmic domain of *C. jejuni* FliF, there is an additional domain of 12 amino acids (dark blue) and a six amino acid C'-terminal extension (red). The ASVXV motif that is conserved in FliF proteins is shown in the periplasmic domain. Shown

below the wild-type *C. jejuni* FliF proteins are mutant FliF proteins with in-frame deletions of specific regions analyzed in this study. (B) Immunoblot analysis and arylsulfatase assay examining *flaB*::*astA* expression of wild-type *C. jejuni* and isogenic *fliF* mutants. For immunoblotting analysis, whole-cell lysates of wild-type *C. jejuni* or isogenic mutants lacking different flagellar genes were analyzed with α-FliF, α-FliG, or α-RpoA antisera. The arylsulfatase assay was performed in triplicate with the level of expression of *flaB*::*astA* in each mutant is relative to wild-type *C. jejuni*, which was set to 100 units +/- standard deviations. (C) Organization of the *E. coli* (*Ec*), *Salmonella enterica* serovar Typhimurium (*St*), and *C. jejuni* (*Cj*) FliG proteins. The FliG proteins possess an N-terminal, middle, and C-terminal domain shown in various shades of pink. In the N-terminal domain of *C. jejuni* FliG, there is an additional domain of 11 amino acids at the beginning of the N-terminal domain (red). Shown below the wild-type *C. jejuni* FliG proteins are mutant FliG proteins with in-frame deletions of specific regions analyzed in this study. (D) Immunoblot analysis and arylsulfatase assay examining *flaB*::*astA* expression of wild-type *C. jejuni* and isogenic *fliG* mutants. For immunoblotting analysis, whole-cell lysates of wild-type *C. jejuni* or isogenic mutants lacking different flagellar genes were analyzed with α-FliF, α-FliG, or α-RpoA antisera. The arylsulfatase assay was performed in triplicate with the level of expression of *flaB*::*astA* in each mutant is relative to wild-type *C. jejuni*, which was set to 100 units +/- standard deviations.

In comparing FliF sequences among motile bacteria, we noticed that the C-terminus of FliF of *C. jejuni* and *H. pylori* (a closely related ε-proteobacterium) is extended by six to seven amino acids relative to FliF of *E. coli* or *Salmonella* species (Figure 9A and data not shown). The importance of these C-terminal six amino acids of *C. jejuni* FliF were revealed when production of $FliF_{\Delta C'6AA}$ which lacked these amino acids failed to restore FliF and FliG protein levels or *flaB*:*astA* expression (Figure 9A and 9B). Alanine-scanning mutagenesis of these C-terminal six amino acids revealed that only K558 was essential for the stability of FliF and FliG, *flaB*::*astA* expression, and motility; the remaining five C-terminal amino acids of *C. jejuni* FliF could be altered and not affect production of FliF or FliG or gene expression (as an example $FliF_{E557A}$ is shown in Figure 9A, 9B, and data not shown).

Based on the organization of FliG in other flagellated organisms (120), we predict that *C. jejuni* FliG is organized into three domains: an N-terminal domain of ~110 amino acids that interacts with FliF; a middle domain that interacts with the stator proteins of the motor; and a C-terminal domain that interacts with the FliM motor switch protein (Figure 9C). However, we

found that *C. jejuni* and *H. pylori* FliG proteins contain an N-terminal extension of 11-12 amino acids (Figure 9C and data not shown). We constructed in-frame *fliG* chromosomal mutations lacking various domains of the protein and then examined the resultant mutants for production of FliF and FliG and *flaB*::*astA* expression. We found that removal of either the middle or C-terminal domain resulted in stable production of FliF and truncated FliG proteins with approximately a 60% reduction in *flaB*::*astA* expression (Figure 9D). However, removal of both the middle and C-terminal domains so that only the N-terminal domain was produced allowed for the production of FliF and a stable truncated FliG with only a 20% reduction in *flaB*::*astA* expression. These data largely suggest that the middle and C-terminal domains of FliG are not required to activate the FlgSR TCS and σ^{54}-dependent flagellar gene expression. When the N-terminal domain of FliG was deleted, a stable, truncated FliG protein was produced, but full-length FliF was not observed. Instead, a protein corresponding to a truncated FliF protein of 25-kDa was produced (Figure 9D). Furthermore, *flaB*::*astA* expression was reduced 32-fold relative to wild-type *C. jejuni*. These data indicate that the N-terminal domain of FliG is essential for stability of FliF. $FliG_{\Delta I2\text{-}Y12}$, which lacks the N-terminal extension unique to *C. jejuni* and *H. pylori* FliG proteins did not produce a stable protein and resulted in production of a truncated FliF protein of 25 kDa (Figure 9D). Additionally, *flaB::astA* expression was reduced about 32-fold relative to wild-type *C. jejuni*. These results indicate that the C-terminal extension to FliF and the N-terminal extension to FliG are required for the stability of these proteins in *C. jejuni* to promote flagellar gene expression and motility. Furthermore, it is possible that these two

proteins may interact through these atypical extensions, with K558 of FliF being essential to this process.

FliF and FliG Interactions with FlgS

Based on our genetic data that the cytoplasmic domain of FliF and the N-terminal domain of the FliG rotor are required for expression of σ^{54}-dependent flagellar genes, we hypothesized that these proteins may form a cytoplasmic signal upon multimerization into the respective MS and C rings that can be detected by FlgS to initiate signal transduction. If so, we may be able to detect an *in vivo* interaction between FlgS and FliF and/or FliG in *C. jejuni*. Additionally, we predicted that we could also monitor interactions between FliF and FliG as these are known to interact in the flagellum.

To analyze potential *in vivo* interactions between FlgS, FliF, and FliG, we expressed FliF with an N-terminal FLAG tag (FLAG-FliF) in *C. jejuni* Δ*fliF* and FliG with a C-terminal FLAG tag in *C. jejuni* Δ*fliG*. In the mutants harboring the empty FLAG-tag vector, FliF and FliG production was greatly reduced in either of the mutants due to both proteins being required for the stability of each other (Fig 10A). However, expression of the FLAG-tagged proteins in the mutants resulted in the stable production of both proteins (Fig 10A). For monitoring interactions between proteins, these *C. jejuni* strains were grown and either treated with a crosslinking agent to trap transient protein interactions in intact cells or processed in the absence of the crosslinker. After lysis of bacterial cells, FLAG-FliF and FliG-FLAG proteins were immunoprecipitated with

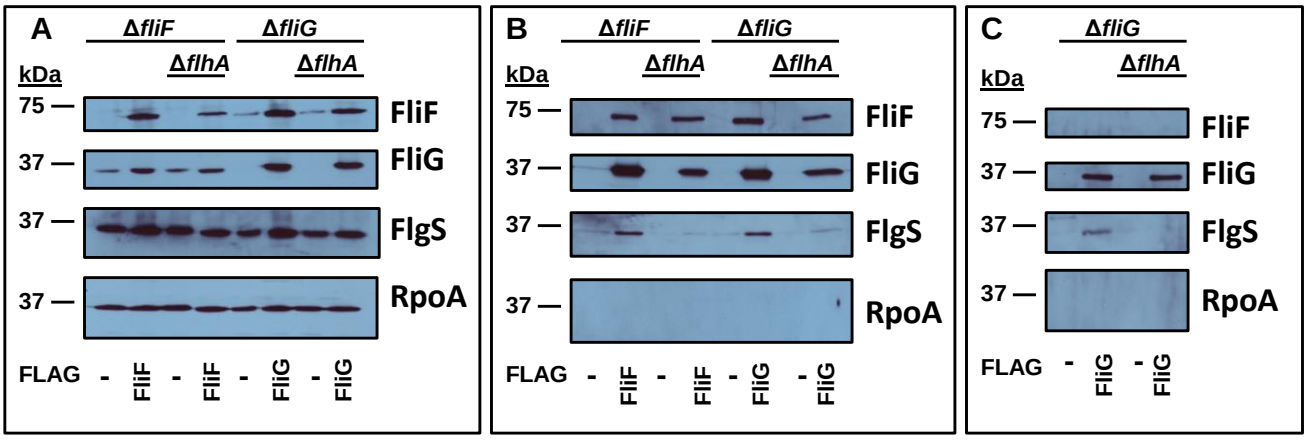

Figure 10: Analysis of FlgS Interactions with FliF and FliG *in vivo*. (A) Whole cell lysates of *C. jejuni* mutants lacking flagellar genes were analyzed by immunoblot analysis with specific α-FliF, α-FliG, α-FlgS, or α-RpoA antisera. Each Δ*fliF*, Δ*fliF* Δ*flhA*, Δ*fliG*, and Δ*fliG* Δ*flhA* mutant was complemented *in trans* with empty vector (-) or a plasmid to express FLAG-FliF (FliF) or FliG-FLAG (FliG). (B) Immunoblot analysis of coimmunoprecipitated *C. jejuni* proteins after crosslinking to analyze interacting proteins using specific antisera. Either Flag-FliF (FliF) or FliG-FLAG (FliG) were immunoprecipitated using an α-FLAG antibody. (C) Immunoblot analysis of coimmunoprecipitated *C. jejuni* proteins without crosslinking after α-FLAG immunoprecipitation of FliG-FLAG (FliG) to analyze interacting proteins using specific antisera.

α-FLAG resin. We found that in crosslinked cells, native FliG coimmunoprecipitated with FLAG-FliF and native FliF coimmunoprecipitated with FliG-FLAG (Figure 10B). However, interactions between these proteins were not detected without the crosslinking agent (Figure 10C and data not shown), possibly due to difficulties in recovering native FliF-FliG complexes due to FliF being an inner membrane protein and FliG being a soluble protein that interacts with FliF at the cytoplasmic face of the MS ring.

Immunoblot analysis of FlgS in the coimmunoprecipitated fractions of these lysates revealed that FlgS interacted with FLAG-FliF only in crosslinked samples (Figure 10B and data not shown). Furthermore, FlgS coimmunoprecipitated with FliG-FLAG in both crosslinked and

non-crosslinked cells (Figure 10B and 10C). As a negative control, the FLAG-tagged FliF or FliG proteins did not coimmunoprecipitate RpoA, the α subunit of RNA polymerase, regardless of the presence or absence of the crosslinking agent (Figure 10B and 10C). These results suggest that FlgS specifically and directly interacts *in vivo* with FliG and likely interacts with FliF *in vivo* either through a direct interaction or via a complex with FliG.

Our data above suggested that FliF and FliG can only form a signal to be detected by FlgS upon interaction with the T3SS. Therefore, we determined if *in vivo* FliF and FliG interactions with FlgS were dependent on the flagellar T3SS. Therefore, we expressed FLAG-tagged FliF or FliG proteins in a *C. jejuni* Δ*flhA* background that also lacked native *fliF* or *fliG*. In these mutants, FliF and FliG co-immunoprecipitated each other, indicating that FliF and FliG interacted independently of the T3SS (Figure 10B). However, FlgS did not coimmunoprecipitate with FLAG-FliF or FliG-FLAG in the Δ*flhA* mutant, either with or without a crosslinking agent (Figure 10B,10C, and data not shown). These results indicate that a FliF and FliG require the T3SS to form a domain to interact with FlgS. We conclude that FliF and FliG interactions with the T3SS likely facilitate multimerization of these proteins into the MS and C rings, which forms a multisubunit binding domain for FlgS to initiate signal transduction.

Identification of a FliF cytoplasmic subdomain required for signal transduction

As described above, the N-terminal cytoplasmic domain of FliF and the middle and C-terminal domains of FliG are not essential for signal transduction through FlgSR and expression of σ^{54}-dependent flagellar genes. We next investigated whether a subdomain of the C-terminal

100-amino acid cytoplasmic domain of FliF was essential for activating FlgSR and σ^{54}-dependent flagellar gene expression.

To this end, we constructed a series of *fliF* mutants that encoded mutant proteins that lacked an increasing number of residues from the C-terminus of FliF. Due to the importance of K558 for the stability of FliF and FliG, all constructs contained the C-terminal six amino acids of FliF (which includes K558). These *fliF* mutants were examined for their ability to restore flagellar gene expression to *C. jejuni* Δ*fliF* when expressed *in trans* from a plasmid. Unfortunately, the only stable FliF mutant proteins we obtained were $FliF_{\Delta K507\text{-}E554}$, $FliF_{\Delta K519\text{-}E554}$, and $FliF_{\Delta L510\text{-}I521}$; FliF mutants lacking greater or smaller amino acid deletions in increments of 12 amino acids were unstable and could not be studied further (Figure 11A and 11B). $FliF_{\Delta K507\text{-}E554}$ lacks the 48 C-terminal amino acids of FliF (excluding the extreme six C-terminal amino acids). This protein supported wild-type levels of FliG production but did not restore *flaB::astA* expression (Figure 11A and 11B), indicating that this 48-amino acid subdomain is essential for signal transduction through FlgSR and σ^{54}-dependent flagellar gene expression. This region of FliF can be subdivided into two subdomains: a 36-amino acid region ($FliF_{K519\text{-}E554}$) with some similarity to other FliF proteins and a 12-amino acid region ($FliF_{L510\text{-}I521}$) that is unique to the FliF proteins of *C. jejuni* and *H. pylori*, but absent in FliF from *E. coli* or *Salmonella* species. Like $FliF_{\Delta K507\text{-}E554}$, $FliF_{\Delta K519\text{-}E554}$ restored production of FliG, but failed to

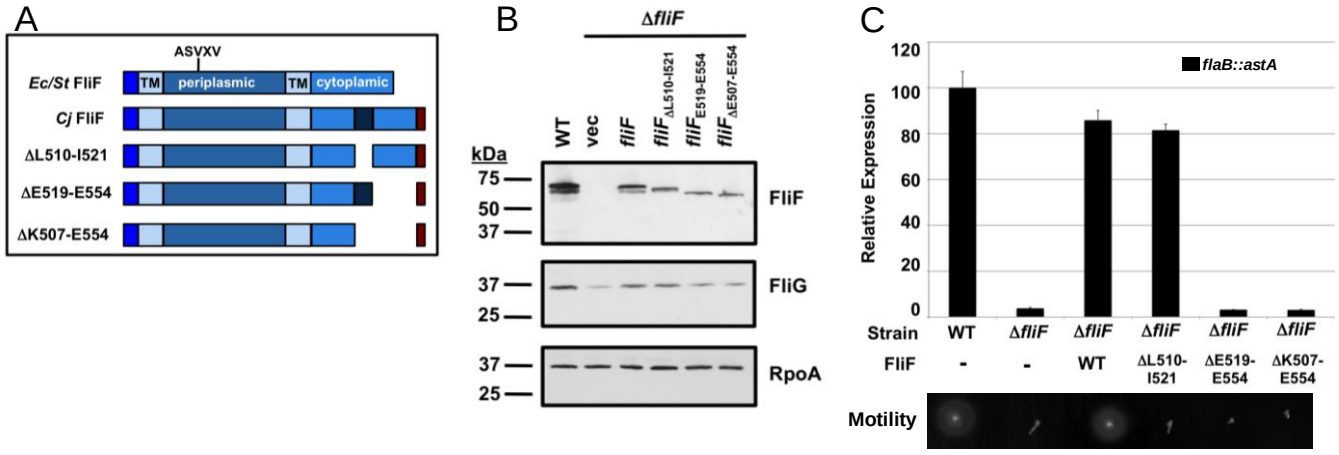

Figure 11. Analysis of FliF Mutant Proteins with Specific Cytoplasmic Domain Deletions. (A) *C. jejuni* FliF proteins with in-frame deletions of specific subdomains of the C-terminal cytoplasmic domain. The proteins are organized into domains as described in Figure 9. (B) Immunoblot analysis and arylsulfatase assay examining *flaB*::*astA* expression, and motility phenotypes of wild-type *C. jejuni* and isogenic *fliF* mutants. For immunoblotting analysis, whole-cell lysates of wild-type *C. jejuni* or isogenic mutants lacking different flagellar genes were analyzed with α-FliF, α-FliG, or α-RpoA antisera. The arylsulfatase assay was performed in triplicate with the level of expression of *flaB*::*astA* in each mutant is relative to wild-type *C. jejuni*, which was set to 100 units +/- standard deviations. Motility was examined by inoculating motility agar with strains and examining motility after 24 h.

restore *flaB*::*astA* expression. In contrast, the $FliF_{\Delta K510\text{-}I521}$ mutant protein restored both production of FliG and expression of *flaB::astA*, indicating that this domain of FliF is not required for activation of FlgS (Figure 11B). Unexpectedly, we discovered expression of $FliF_{\Delta K507\text{-}E554}$ in the Δ*fliF* mutant did not restore motility (Figure 11C), suggesting that this subdomain may assist in flagellar motor function. These data indicate that the cytoplasmic region required for FlgS activation and expression of σ^{54}-depedent flagellar gene expression is limited to a 36-amino acid region within K519-E554 of FliF.

A potential FliF-FlhA interaction domain is required for σ^{54}-dependent flagellar gene expression.

A previous study in *Salmonella enterica* serovar Typhimurium analyzed suppressor mutants that restored motility to a *fliF*$_{\Delta A174\text{-}S175}$ mutant (189). A174 and S175 are located within a conserved ASVXV motif of many FliF proteins and are predicted to reside in the periplasmic domain of FliF (Figure 11A). Suppressor mutations were all localized to different locations in FlhA, a component of the T3SS. It was postulated from this study that FliF may interact with the T3SS via FlhA, with FlhA suppressor mutant proteins compensating for a potential displacement caused by FliF$_{\Delta A175\text{-}S176}$.

Data from a subsequent study suggested that FlhA assists in the recruitment of FliF to the flagellar T3SS and multimerization of FliF into the MS ring surrounding the T3SS (15). Therefore, we hypothesized that the ASVXV motif of FliF may be essential for direct or indirect interactions with FlhA, which may facilitate multimerization into the MS ring. If true, the implications of this hypothesis for *C. jejuni* is that mutation of the ASVXV motif may ultimately disrupt the formation of a direct signal composed by the cytoplasmic domains of FliF in the fully-formed MS ring that is directly sensed by FlgS to promote σ^{54}-dependent flagellar gene expression. To test this hypothesis, we deleted A175 and S176 from the ASVXV motif within *C. jejuni* FliF and tested whether the mutant proteins supported σ^{54}-dependent flagellar

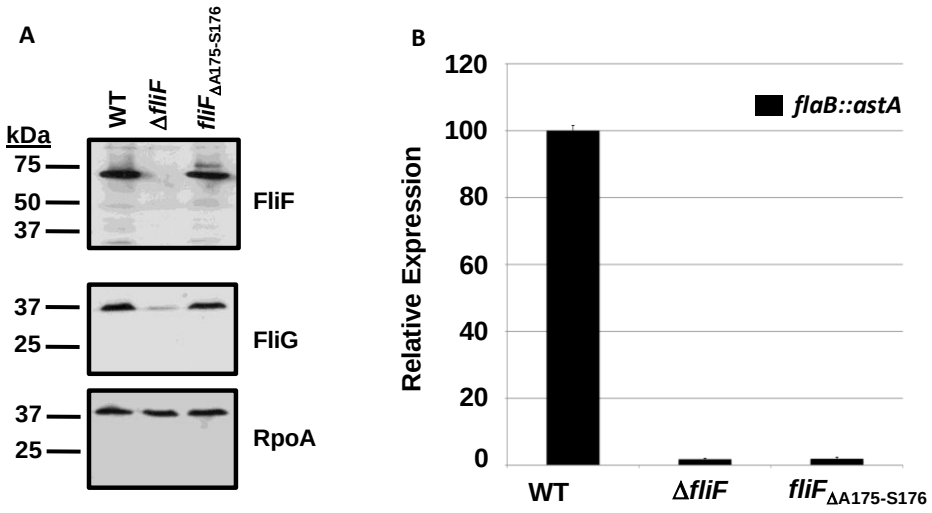

Figure 12. Analysis of FliF Mutant Proteins with Specific Periplasmic Domain Deletions. (A) *C. jejuni* FliF proteins with in-frame deletions of A175 and S176 of the ASVXV motif or specific subdomains of the C-terminal cytoplasmic domain. The proteins are organized into domains as described in Figure 3. (B) Immunoblot analysis, arylsulfatase assay examining *flaB*::*astA* expression, and motility phenotypes of wild-type *C. jejuni* and isogenic *fliF* mutants. For immunoblotting analysis, whole-cell lysates of wild-type *C. jejuni* or isogenic mutants lacking different flagellar genes were analyzed with α-FliF, α-FliG, or α-RpoA antisera. The arylsulfatase assay was performed in triplicate with the level of expression of *flaB*::*astA* in each mutant is relative to wild-type *C. jejuni*, which was set to 100 units +/- standard deviations. Motility was examined by inoculating motility agar with strains and examining motility after 24 h.

gene expression. We found that $FliF_{\Delta A174\text{-}S175}$ was stable in *C. jejuni* and allowed for WT levels of FliG production (Figure 12A). However, $FliF_{\Delta A175\text{-}S176}$ did not support σ^{54}-dependent flagellar gene expression (Figure 12B). Assuming that the ASVXV motif of FliF is essential for recruitment to the flagellar T3SS, these results indicate that FliF interactions with the T3SS are essential for MS ring formation and generation of a specific signal within the cytoplasmic domains of the MS ring activate the FlgSR TCS and σ^{54}-dependent flagellar gene expression.

Discussion

Despite their prevalence in numerous bacterial species, the actual direct signal that is sensed by various TCSs is only known for an extraordinary small number of sensor kinases. In polarly-flagellated bacteria such as species of *Vibrio*, *Pseudomonas*, *Campylobacter*, and *Helicobacter*, a similar TCS is required to activate expression of σ^{54}-dependent flagellar genes. Based on results from previous studies with the *C. jejuni* FlgSR TCS, we proposed that the direct signal sensed by FlgS to activate expression of σ^{54}-dependent rod and hook genes likely emanates from the flagellar T3SS as all components of the T3SS were required for transcription of these sets of genes.

In this study, we explored other flagellar proteins that may be essential for activating FlgSR and expression of σ^{54}-dependent flagellar genes. We discovered that FliF, which forms the MS ring housing the T3SS, and FliG, the rotor component of the flagellar C ring, are both required to activate FlgSR and σ^{54}-dependent flagellar gene expression. Furthermore, we detected a direct *in vivo* FlgS-FliG interaction and also a FlgS-FliF interaction, although the latter required a crosslinking agent. Additionally, these interactions of FliF or FliG with FlgS were dependent on the presence of a wild-type flagellar T3SS. These data strongly suggest a model whereby FliF and FliG require the T3SS to form a direct signal for FlgS to initiate signal transduction that facilitates σ^{54}-dependent flagellar gene expression. We suspect the requirement of the T3SS for FliF and FliG forming such a signal is due to these proteins requiring the T3SS to multimerize efficiently into mature MS and C ring structures with specific domains that can be directly sensed by FlgS. This study illustrates that the MS and C rings of flagella can function as

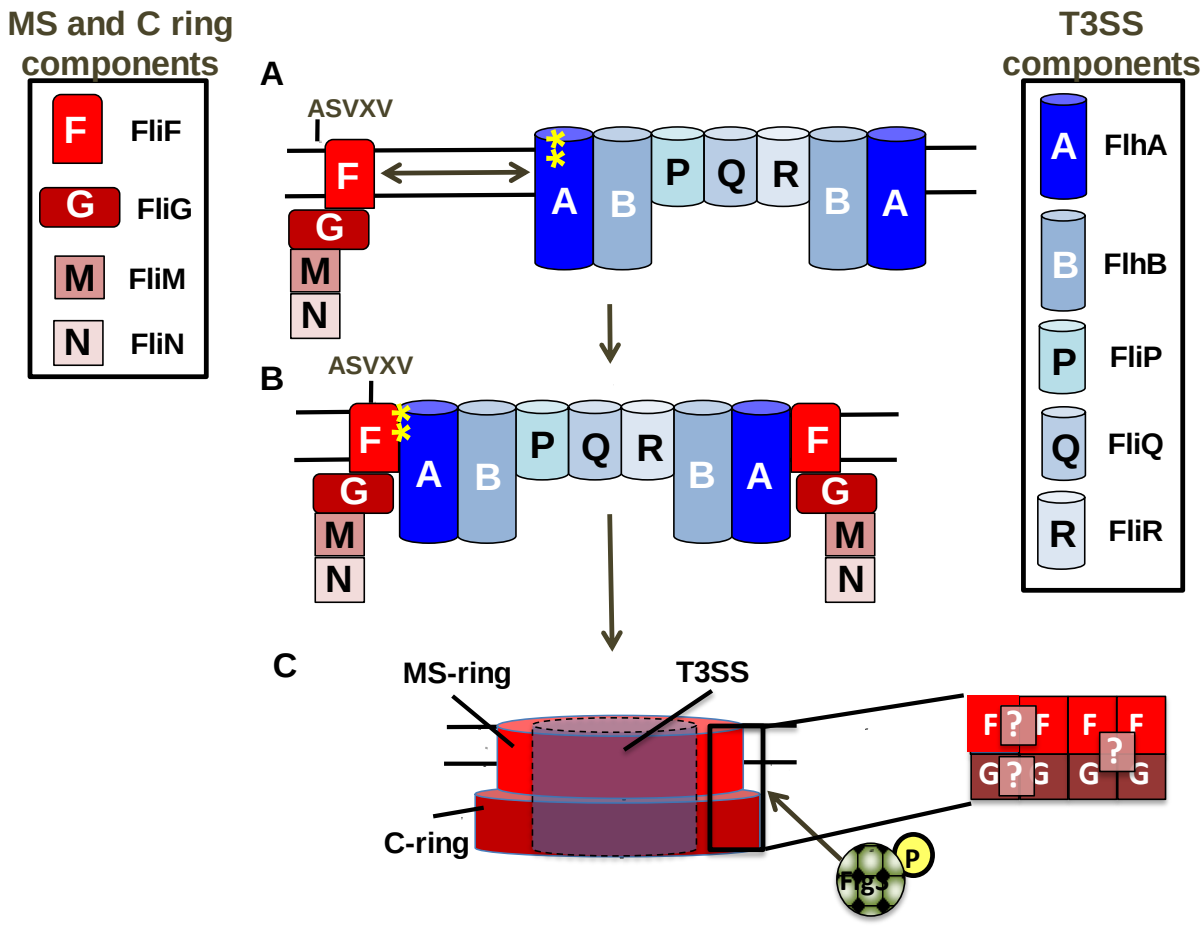

Figure 13. A Model for the Activation of FlgS. A model to illustrate the components required for FlgS activation. (A) FliF and FliG proteins interact via cytoplasmic domains unique to *C. jejuni* and *H. pylori*. FliM and FliN likely also interact with FliG. FliF contains a periplasmic ASVXV motif. Assembly of the flagellar T3SS components occurs in the IM of *C. jejuni*. (B) Once the flagellar T3SS is assembled, FlhA likely recruits FliF to the site of flagellar formation via unknown residues (yellow stars) that interact with the ASVXV motif in FliF. (C) After proper localization, FliF multimerizes into the MS ring and FliG, FliM, and FliN form the C ring. We hypothesize that FlgS detects some cytoplasmic epitope of FliF and/or FliG multimers to activate FlgS autophosphorylation.

signaling modules in bacteria and extends their function beyond being only a structural or functional component of the flagellar basal body and motor.

Based on our experimental evidence, we propose a model where the flagellar T3SS is critical for FliF-FliG complexes to multimerize into mature MS and C rings, which possess domains essential for signaling to FlgS (Figure 13). If a single FliF-FliG complex was sufficient for signal transduction, then we would have expected to observe FliF and FliG to activate gene expression in the absence of the T3SS. Instead, FliF and FliG interacted with each other in a *C. jejuni* T3SS mutant, yet were deficient for activating FlgS. FliG is a soluble rotor component of the C ring, and in well-characterized flagellar systems, does not appear to directly interact with flagellar T3SS components. However, FliF is an inner membrane protein with periplasmic and cytoplasmic domains that forms the MS ring, which houses the T3SS. Thus FliF is expected to directly interact with the T3SS. In studies analyzing the biogenesis of the *E. coli* flagellar T3SS, the T3SS protein FlhA and FliF colocalized, supporting a model whereby FlhA and other T3SS components assembly first into a complex and then recruit FliF, which subsequently multimerizes into the MS ring (15). In another study analyzing a *S. enterica* mutant producing FliF lacking A174 and S175 in a conserved periplasmic ASVXV motif, suppressor mutations that restored motility to this mutant were all localized to different regions in FlhA (189). These results of these studies suggest that FlhA is a leading candidate to interact with and promote the recruitment of FliF to the flagellar T3SS, which likely promotes MS ring formation. In this study, we made a similar mutation in *C. jejuni fliF* that deleted the alanine and serine residues of the ASVXV motif and found that this mutant did not support σ^{54}-dependent flagellar gene expression. Furthermore, *in vivo* interactions between FlgS and FliF or FliG were abolished in a *C. jejuni* Δ*flhA* mutant. Therefore, these results strongly suggest that FliF and FliG multimerize

into the MS and C ring in a T3SS-dependent manner to subsequently initiate signal transduction via the FlgSR TCS (Figure 13).

Currently, it is unclear if the signaling domain within the MS and/or C rings of the *C. jejuni* flagellum is exclusive to a subdomain of FliF or includes subdomains of both FliF and FliG. We have limited the requirements of FliF and FliG for signaling to a 36-amino acid cytoplasmic domain of FliF and the N-terminal 100 amino acids of FliG. The first ten amino acids of FliG are required for the stability of *C. jejuni* FliF and FliG. Thus, we currently do not know if the N-terminus of FliG may form a direct signal for FlgS or is required indirectly for signal transduction by being essential for the stability of FliF. However, FliG and FlgS interacted directly without crosslinking cells, suggesting that FliG may indeed form part of a direct signaling domain for FlgS. In contrast, FliF-FlgS interactions were only detected *in vivo* in the presence a crosslinking agent. However, we only detected an interaction between FliF and FliG, a known interacting partner, in the presence of a crosslinker as well. Furthermore, a specific 36-amino acid cytoplasmic region of FliF was required for signaling. Therefore, our leading hypothesis is that both FliF and FliG likely possess signaling domains and neighboring FliF-FliG complexes in a fully-assembled MS and C ring of a *C. jejuni* flagellum form a specific domain directly sensed by FlgS to initiate signal transduction and σ^{54}-dependent flagellar gene expression (Figure 13). However, we cannot fully exclude the possibility that a signaling domain could be composed solely by FliF-FliF subunits in a MS ring or FliG-FliG subunits in a C ring.

A surprising finding in our study is that *C. jejuni* FliF and FliG require each other for stability, an observation that has not been observed before in motile bacteria. Requirements for the stability of these proteins were localized to K558 at the C-terminus of FliF and the N-terminal ten amino acids of FliG, both of which are not present in FliF and FliG proteins of *E. coli* and *S. enterica*. It is curious that *C. jejuni* has evolved FliF and FliG with these residues to promote stability of the proteins when FliF and FliG proteins of other bacteria do not have these domains and are stable without each other (185). However, we envision that the purpose of these domains in FliF and FliG is to create a checkpoint for *C. jejuni* to monitor the correct formation of the initial flagellar structures (i.e., the MS and C rings and the T3SS) before initiating σ^{54}-dependent flagellar gene expression. If FliF and FliG cannot properly interact in a *C. jejuni* cell, then these proteins are likely targeted for degradation, which eliminates any possibility of part of a signal to initiate signal transduction via the FlgSR TCS from forming. This potential checkpoint mechanism may ensure that a *C. jejuni* cell lacking *fliF* or *fliG* expression or FliF-FliG interactions does not proceed with unnecessary flagellar gene transcription and protein production if the cell cannot construct a correct nascent flagellar structure.

The results of our study in *C. jejuni* have potentially broad implications for understanding signals that stimulate σ^{54}-dependent flagellar gene expression in a variety of polar flagellates. In other well-studied polarly-flagellated bacterial pathogens, such as *Vibrio*, *Pseudomonas*, and *Helicobacter* species, a FlgSR-like TCS is required for expression of σ^{54}-dependent flagellar genes, yet the specific signals that stimulate these TCSs are currently unknown. In the flagellar transcriptional regulatory cascades of these bacteria, *fliF*, *fliG*, and

genes for the flagellar T3SS are expressed before the genes that are dependent on their respective FlgSR-like TCSs are expressed. Thus, it is very possible that multimerization of FliF and FliG into the MS and C rings in a T3SS-dependent manner in these other bacteria, results in the formation of a similar direct signal that can be specifically detected by the respective FlgS homologs. If so, then this mode of signaling may be interpreted as being important for some aspect in the ability of these bacteria to construct flagella specifically at the polar regions of their cell bodies. Thus, our study may have revealed a common signaling domain present in the flagellar MS and C ring structures of flagella that stimulates flagellar gene expression and promotes flagellar biosynthesis in a wide range of bacterial species.

CHAPTER FIVE

A Specificity Determinant for Phosphorylation in a Response Regulator Prevents *in vivo* Cross-talk and Modification by Acetyl Phosphate

Introduction

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 employ 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 (159). 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 (160). *In vivo* cross-phosphorylation, or crosstalk, between non-cognate HK and RRs is usually maintained at a minimum. If crosstalk between two TCSs occurs, correct responses to specific signals may be diminished or inhibited.

Mechanisms exist to insulate a TCS from crosstalk and ensure that intrasystem signal transduction fidelity is preserved. In many TCSs, specific amino acids mediate molecular recognition between cognate HK and RR pairs (162-164, 190). 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, non-cognate HKs, or low molecular weight phosphodonors such as acetyl phosphate (AcP) (159, 166, 174, 191, 192). 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 (3, 11). Initiation of signal transduction through FlgSR is dependent upon components of the flagellar type III secretion system (T3SS) (13, 19). 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 (13).

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 (18, 140, 141). In contrast, FlgR lacking its CTD ($FlgR_{\Delta CTD}$) activated WT levels of σ^{54}-dependent gene expression in the presence of FlgS (19). 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 (19), $FlgR_{\Delta CTD}$ 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. Since $FlgR_{\Delta CTD}$ in the Δ*flgS* mutant required

the phosphorylated D51 residue to activate gene expression, $FlgR_{\Delta CTD}$ must have autophosphorylated via a non-cognate 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 crosstalk 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 expression hindered flagellation. As a result of the CTD limiting crosstalk 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 crosstalk mostly involved activities of HKs, our work identified a domain within a RR that specifically prevents crosstalk 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. 14A). 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 (18, 140). 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 to 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 1) (19). 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 electrophoretic mobility shift assays (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. 14B) (9). Binding by WT FlgR was specific as excess unlabeled P_{flgDE2} DNA, but not P_{gyrA} DNA (a FlgR-independent promoter) competed for binding (Fig. 14C). In contrast, $FlgR_{\Delta CTD}$ only bound P_{flgDE2} at the highest protein concentration examined, but binding was non-specific as both unlabeled P_{flgDE2} and P_{gyrA} DNA reduced residual binding of $FlgR_{\Delta CTD}$ to labeled P_{flgDE2} DNA (Fig. 14C).

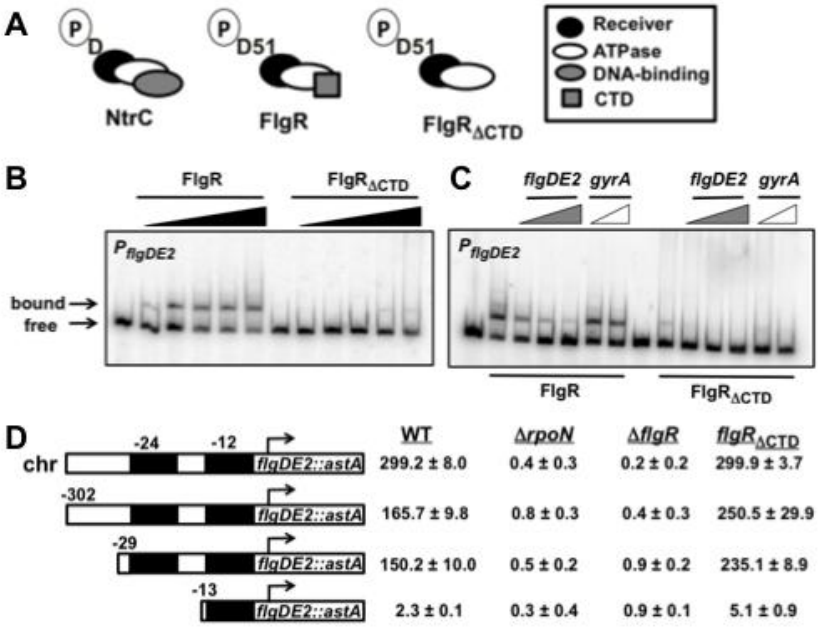

Figure 14. Analysis of the Requirements of FlgR/DNA Interactions for σ^{54}-dependent Flagellar Gene Expression. (A) Domain organization of *E. coli* NtrC, *C. jejuni* WT FlgR, and *C. jejuni* FlgR$_{\Delta CTD}$. The phosphorylated aspartic acid (D or D51; P denotes phosphoryl group) in receiver domains 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 1μM. (C) Increasing ratios of unlabeled P_{flgDE2} DNA (1:1, 5:1, or 10:1) or P_{gyr} DNA (1:1 or 10:1) to labeled P_{flgDE2} DNA were incubated with 1μM of FlgR proteins. (D) Expression of *flgDE2::astA* with 5' promoter truncations in WT C. jejuni or *flgR*$_{\Delta CTD}$, Δ*rpoN* (σ^{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 the 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* (193), we analyzed expression of *flgDE2*::*astA* with 5' promoter truncations on plasmids in *C. jejuni* strains.

Since WT FlgR but not $FlgR_{\Delta CTD}$ specifically bound P_{flgDE2} DNA from -302 to +79 (Fig. 14B and 14C), 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. 14D, 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. 14D). 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. 14D). 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 (Fig. 14D, Fig. 15, and Table 1). In a Δ*flgS* mutant, WT FlgR did not activate σ^{54}-dependent flagellar gene expression (Fig. 15) (3). In contrast, $FlgR_{\Delta CTD}$ activated expression of σ^{54}-dependent genes without FlgS (Fig. 15) (19). 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. 15). Furthermore, $FlgR_{\Delta CTD}$ required phosphorylation of D51 in the receiver domain to activate gene expression in a Δ*flgS* mutant (19). These results indicated that the FlgR CTD may limit *in vivo* phosphotransfer to FlgR by non-cognate HKs or other phosphodonors, in addition to a non-essential 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 out of ~3750 Tn mutants with 7- to 180-fold reductions in expression of the σ^{54}-dependent *flaB*::*astA* transcriptional reporter (Fig. 15). All mutants contained a Tn in genes likely affecting the acetogenesis pathway, a multi-step process that converts pyruvate to ATP and acetate (reviewed in 168). An intermediate in this pathway is AcP, a low molecular weight phosphodonor often used to autophosphorylate many RRs *in vitro*, and a few RRs *in vivo* (172, 173, 191, 194). Six independent Tn insertions were within *pta*,

Table 1. Transcriptional Analysis of Many σ^{54}-dependent Flagellar Genes in Wild-type *C. jejuni* and *flgR* Mutant Strains

	Strain		
Gene*	WT	Δ*flgR*	$flgR_{\Delta CTD}$
flaG	1.00 ± 0.30[†]	0.13 ± 0.05	2.09 ± 0.66
flgB	1.00 ± 0.05	0.14 ± 0.03	2.19 ± 0.51
flgD	1.00 ± 0.26	0.03 ± 0.01	1.26 ± 0.16
flgE	1.00 ± 0.05	0.02 ± 0.01	1.54 ± 0.15
flgF	1.00 ± 0.10	0.55 ± 0.05	1.88 ± 0.16
flgH	1.00 ± 0.54	0.13 ± 0.06	0.74 ± 0.07
flgI	1.00 ± 0.23	0.24 ± 0.03	2.36 ± 1.05
flgK	1.00 ± 0.27	0.24 ± 0.08	0.96 ± 0.45
fliK	1.00 ± 0.07	0.07 ± 0.04	1.20 ± 0.26

* For genes that are part of flagellar operons, the first gene of the operon shown was analyzed for expression in *C. jejuni* strains

[†] The levels of expression of each gene in each strain is relative to wild-type *C. jejuni*, which was set at 1.0. All analysis was performed in triplicate and the standard deviations were calculated.

encoding phosphotransacetylase, which reversibly converts acetyl-Coenzyme A (Ac-CoA) and inorganic phosphate to CoA and AcP (Fig. 15). 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

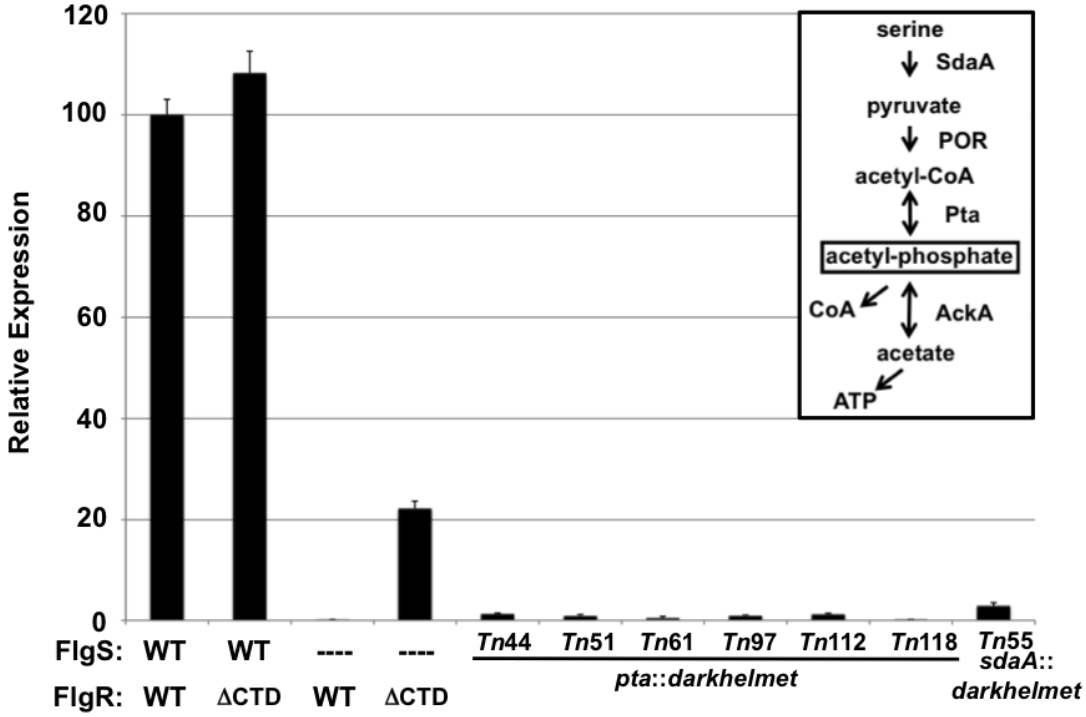

Figure 15. Identification of *darkhelmet* Tn Mutants with Reduced FlgR$_{\Delta CTD}$ Activity in Δ*flgS* Mutants. Arylsulfatase assay examining expression of the σ^{54}-dependent *flaB::astA* transcriptional fusion in *C. jejuni* strains grown on MH agar. The level of *flaB::astA* expression in each 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 (168), and predicted to be intact in *C. jejuni* (169, 170).

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* (168, 169, 195). To analyze possible *in vivo* AcP-mediated activation of

Table 2. 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 WT FlgSR TCS, which was set to 100 units.
†Not Determined

$FlgR_{\Delta CTD}$, *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 (171, 172, 196). 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 2). 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 2). 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 2). 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 2). We next examined if the

acetogenesis pathway could influenced 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 2). However, $FlgR_{\Delta CTD}$-stimulated *flaB*::*astA* expression in the presence of FlgS increased 18% when *ackA* was deleted (Table 2). 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 three-fold greater level of autophosphorylation of $FlgR_{\Delta CTD}$ compared to WT FlgR (Fig. 16A). 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 as phosphotransfer from FlgS to $FlgR_{\Delta CTD}$ was approximately 25-70% less than WT FlgR over time (Fig. 16B). Therefore, the CTD specifically limited crosstalk and phosphotransfer from AcP to FlgR.

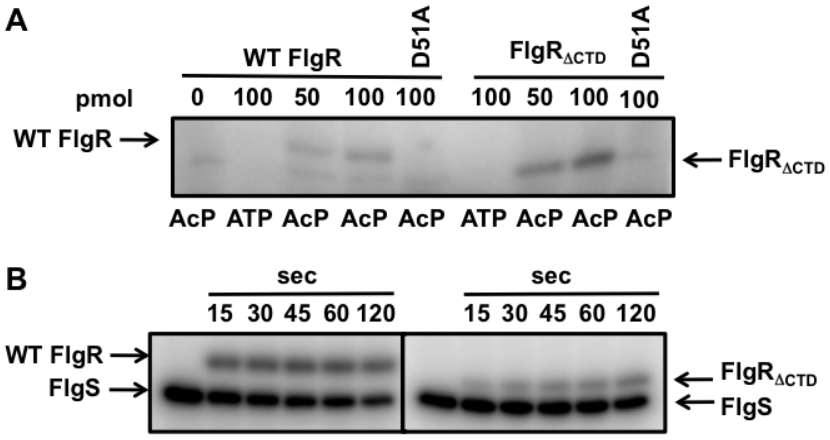

Figure 16. ***In Vitro*** **Phosphorylation of FlgR Proteins.** (A) Autophosphorylation of FlgR proteins with Ac[^{32}P] or [γ^{32}P]ATP. WT FlgR or FlgR$_{\Delta CTD}$ (50 and 100 pmol) or respective D51A mutants (100pmol) are indicated. (B) Phospotransfer from FlgS to FlgR proteins. Six picomoles of WT FlgR or FlgR$_{\Delta CTD}$ were mixed with autophosphorylated ^{32}P-FlgS and removed after 15-120 s.

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 if 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* 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. 17). 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. 17). These results suggested that even in the presence of WT FlgS, $FlgR_{\Delta CTD}$, but not WT FlgR, is responsive to AcP to result in augmented levels of gene expression.

We next examined *flaB*::*astA* expression upon modulating the physiology of *C. jejuni* strains lacking FlgS. Due to 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. 17). However, upon increasing pyruvate concentrations, *flaB*::*astA* expression increased ~14-fold (Fig. 17). 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. 17). 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. 17). 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. 18).

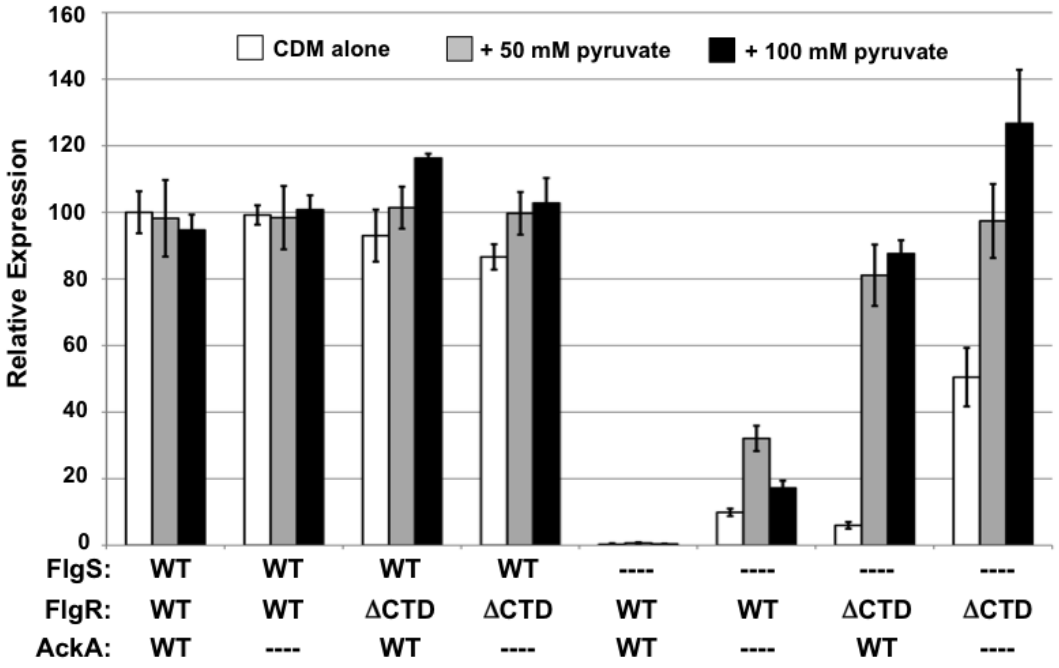

Figure 17. FlgS-independent Activation of $FlgR_{\Delta CTD}$ by Altering *C. jejuni* Physiology. Arylsulfatase assay examining *flaB::astA* expression after growth on CDM alone (white bars), CDM with 50 mM (gray bars), or 100 mM (black bars) excess sodium pyruvate. The level of *flaB::astA* expression 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. Δ CTD indicates $FlgR_{\Delta CTD}$. Dashes indicate deletions of respective genes. Error bars indicate SDs.

In contrast, *C. jejuni* producing WT FlgR without FlgS was not responsive to increasing pyruvate levels as measured by *flaB*::*astA* expression (Fig. 17). 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 three-fold by increasing pyruvate levels (Fig. 17). However, *flaB*::*astA* expression levels were dramatically lower with AcP-dependent activation of WT FlgR than $FlgR_{\Delta CTD}$.

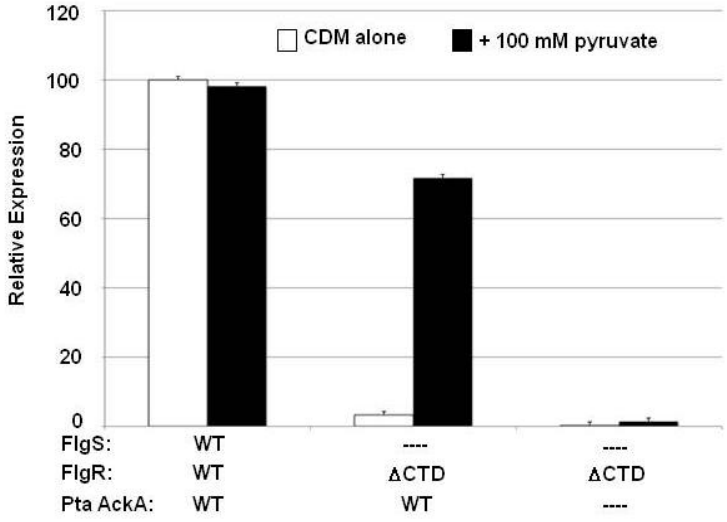

Figure 18. FlgS-independent Activation of $FlgR_{\Delta CTD}$ Requires the Acetogenesis Pathway. Arylsulfatase assay examining *flaB::astA* expression after growth on CDM alone (white bars) or CDM with 100 mM sodium pyruvate (black bars). The level of *flaB::astA* expression in each strain is relative to WT C. jejuni producing the WT FlgSR TCS grown on CDM alone, which was set to 100 units. The FlgS, FlgR, Pta, and AckA proteins produced in each strain are indicated. ΔCTD indicates $FlgR_{\Delta CTD}$. Dashes indicate deletion of respective genes. Error bars indicate SDs.

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 3 and Table 4). 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 4). Considering that pyruvate augmented $FlgR_{\Delta CTD}$-dependent flagellar gene expression but not flagellation in the

Table 3. Flagellation Due to AcP-dependent Activation of FlgR Proteins by Altering *C. jejuni* Physiology

		Number of Flagella per Bacterium*		
Strain	Media	2	1	0
WT	CDM	64.4 ± 7.3	29.4 ± 7.2	6.1 ± 0.1
WT	CDM + 100 mM pyruvate	67.2 ± 1.2	26.7 ± 6.1	6.0 ± 4.9
Δ*flgS flgR*$_{\Delta CTD}$	CDM	0 ± 0	0.9 ± 0	99.2 ± 0
Δ*flgS flgR*$_{\Delta CTD}$	CDM + 100 mM pyruvate	3.9 ± 0.7	25.6 ± 4.0	70.5 ± 3.3
Δ*flgS flgR*$_{\Delta CTD}$ Δ*ackA*	CDM	0 ± 0	5.4 ± 2.7	94.6 ± 2.7
Δ*flgS flgR*$_{\Delta CTD}$ Δ*ackA*	CDM + 100 mM pyruvate	8.5 ± 3.2	48.7 ± 6.8	45.1 ± 13.1

*The percentage of the population producing a songle 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.

presence of FlgS (Fig. 17 and Table 4), these findings suggested 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. 17), 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 3). Upon deletion of *ackA*, the population of flagellated Δ*flgS flgR*$_{\Delta CTD}$ cells increased to 56% with pyruvate supplementation (Table 3). Despite increases in flagellation, the number of Δ*flgS flgR*$_{\Delta CTD}$ cells producing two flagella (the predominant WT phenotype) was always lower than

Table 4. Flagellation of *C. jejuni* Mutant Strains Producing WT FlgS Due to AcP-dependent Activation of FlgR Proteins by Altering Physiology.

		Number of Flagella per Bacterium*		
Strain	Media	2	1	0
Δ*ackA*	CDM	75 ± 1	22 ± 0	3 ± 1
Δ*ackA*	CDM + pyruvate	75 ± 3	21 ± 4	4 ± 1
$flgR_{\Delta CTD}$	CDM	72 ± 1	24 ± 1	4 ± 0
$flgR_{\Delta CTD}$	CDM + pyruvate	74 ± 9	23 ± 10	3 ± 1
$flgR_{\Delta CTD}$ Δ*ackA*	CDM	74 ± 1	23 ± 1	3 ± 0
$flgR_{\Delta CTD}$ Δ*ackA*	CDM + pyruvate	69 ± 1	26 ± 1	5 ± 1

* The percentage of the population producing a single flagellum at both poles (2 flagella), a single flagellum at one pole (1 flagellum), or no flagella (0) after growth on CDM or CDM with 100 mM excess sodium pyruvate are indicated. Data are from the average of two experiments ± standard deviation. Analysis of WT *C. jejuni* is indicated in Table 2.

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. 17). However, coupling FlgR activation 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

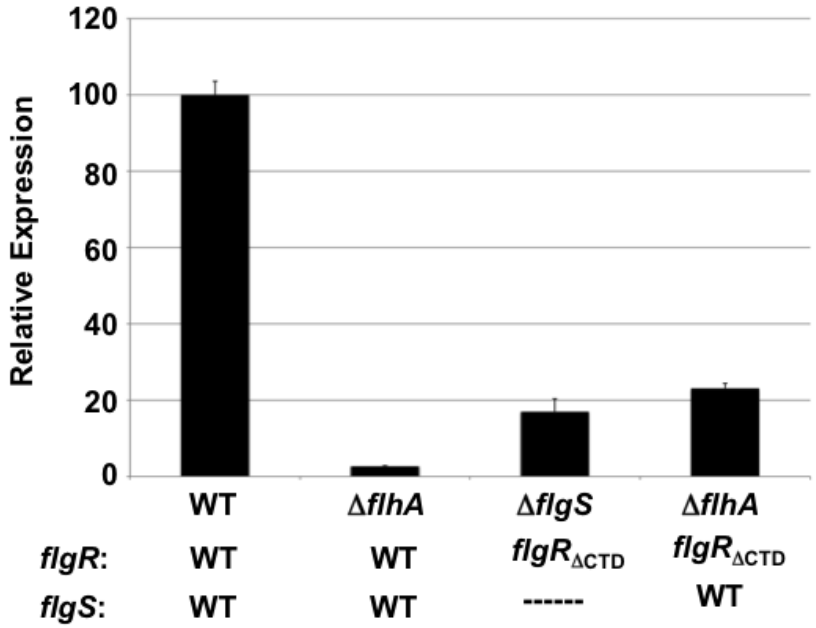

Figure 19. Examination of and *in vivo* FlgS phosphatase activity for AcP-activated $FlgR_{\Delta CTD}$. Arylsulfatase assay examining *flaB::astA* expression after growth on MH agar. The level of *flaB::astA* expression in each strain is relative to WT C. jejuni producing WT FlgSR TCS, which was set 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.

FlgS phosphatase activity has not directly been examined. If one exists, then *in vivo* AcP-mediated activation of FlgR may prevented by both 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 (3, 13). 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. 19) (13). 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 crosstalk with AcP and reduce gene expression. Furthermore, these findings demonstrated the importance of the CTD in eliminating crosstalk of FlgR with AcP and influences from metabolism. As such, the CTD prevented 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 crosstalk 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 crosstalk between two TCSs may cause signaling interference and obstruct responses to activating stimuli. Mechanisms identified to date that promote intrasystem signaling fidelity and limit intersystem crosstalk include molecular recognition between cognate HK and RR pairs and phosphatase activities of some HKs that control levels of phosphorylation of cognate RRs (159, 162-164, 190).

We identified a domain in a RR that limits *in vivo* crosstalk specifically from the metabolite AcP, rather than from a non-cognate 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 (18, 140, 141). Without the 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 (174), 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 (172, 173). While 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 non-cognate 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* crosstalk between AcP and FlgR. Barbieri *et al*. (197) 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 (198). 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 to result 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. Since 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 RNAP 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 *H. pylori*, which naturally lacks a CTD (199). These features of FlgR proteins in different bacteria may be significant in expanding mechanisms of transcriptional initiation by the NtrC family of RRs as many of these proteins promote only limited transcription in the absence of DNA-binding under physiological conditions (18, 141).

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 crosstalk

between FlgR and AcP when conditions are not conducive for flagellation. Since 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 crosstalk with small phosphodonor metabolites.

CHAPTER SIX

Conclusions and Recommendations

My work has focused on the discovery and characterization of molecular mechanisms that regulate σ^{54}-dependent flagellar gene expression in *C. jejuni*. The collection of experimental data presented herein describes the identification and characterization of proteins required to initiate signaling through the FlgSR TCS to result in expression of σ^{54}-dependent rod, hook, and minor flagellin genes. Additionally, this work described the identification of a determinant in the FlgR RR that maintains signaling specificity within the FlgSR TCS to ensure activation of expression of flagellar genes in the presence of the proper activating signal. While this work characterized components of the flagellar signaling pathway in *C. jejuni*, the regulatory mechanisms elucidated are likely applicable to a broad range of bacterial regulatory systems.

Previous work identified components required for expression of σ^{54}-dependent flagellar genes including the flagellar T3SS components (FlhA, FlhB, FliP, and FliR), the FlgSR TCS, and FlhF, a GTPase that functions to localize flagella to polar regions of the cell (3, 152). While the requirement for FlhF in activation of the flagellar gene expression is not understood, studies demonstrated that activation of the FlgSR TCS requires formation of the flagellar T3SS. Since FlgS is a cytoplasmic HK, previous reports hypothesized that a cytoplasmic domain of the flagellar T3SS is likely the activating signal (13). However, the specific domain sensed by FlgS to activate the signal transduction pathway was not identified.

The studies presented in Chapter Four identified additional flagellar components required for the activation of σ^{54}-dependent gene expression. These components include a protein of the flagellar T3SS (FliQ), the rod protein (FliE), the MS ring (FliF) that houses the flagellar T3SS, and the C ring rotor component (FliG) that is localized on the cytoplasmic side of the MS ring. While the contributions of FliQ and FliE to σ^{54}-dependent flagellar gene expression was not analyzed in this work, domain analysis of FliF and FliG suggested that cytoplasmic domains are important for the activation of the FlgSR TCS to result in σ^{54}-dependent gene expression. My findings strongly suggest that one or both of the cytoplasmic domains of FliF and FliG could be the direct signal sensed by FlgS. Additionally, my analysis of FliG and the cytoplasmic domain of FliF revealed that these proteins require each other for stability, unlike observations of FliF and FliG homologs in other motility systems.

With this work and new data regarding formation of T3SSs, I had to rethink our previous model for what the activating signal for FlgS is and how it is generated. My work showed that the flagellar T3SS, the MS ring, and rotor component of the C ring are required for activation, but none of these factors were solely sufficient for activation. Previous studies performed to analyze the order of assembly of the T3SSs in injectisome and flagellar systems have demonstrated nucleation of the T3SS components occurs first followed by localization of FliF and FliG (or respective homologs) to the T3SS where they form the multimeric MS and C rings, respectively (14-16). Based on this evidence and my observations that the flagellar T3SS, FliF, and FliG are required to activate flagellar gene expression, I hypothesized that activation of the FlgSR TCS is likely dependent on some cytoplasmic epitope formed by the MS ring or C ring

structures. Because a FliF mutant **(*fliF*$_{\Delta A175\text{-}S176}$)** that is predicted not to interact with the flagellar T3SS did not activate gene expression, assembly of the flagellar T3SS is likely an indirect event required to activate the FlgSR TCS. Based on our genetic and biochemical evidence, I propose a model for the activation of σ^{54}-dependent gene expression (Figure 13). As illustrated, the flagellar T3SS components likely form first at the bacterial pole in the IM. My data suggest that FliF and FliG must interact for their stability and this interaction is independent of the flagellar T3SS. Once the T3SS is formed, an interaction between FliF and presumably at least the FlhA component of the T3SS and perhaps other components of the flagellar T3SS occurs. This interaction may be dependent on A175 and S176 of FliF (189). Once these components interact, polymerization of the FliF and FliG subunits into the homopolymeric MS ring and C ring occurs (Figure 13). My data suggest that this multimerization facilitates the formation of a cytoplasmic epitope sensed by FlgS to initiate FlgSR and σ^{54}-dependent gene expression. My data suggest that the signal is not a FliF-FliG heterodimer because these two proteins interact in a flagellar T3SS mutant but do not activate σ^{54}-dependent gene expression. The activating signal could consist of FliF-FliF homomultimers, FliG-FliG homomutimers, or another $FliF_2$-$FliG_2$ complex within formed MS and C ring structures. This requirement for multiple components and their formation of a higher-ordered structure indicates a level of commitment by the bacterium where FliF and FliG form a regulatory checkpoint so that flagellar biosynthesis can only progress if the appropriate ring structures are formed to generate signal to activate FlgS.

Like *C. jejuni*, many clinically-relevant pathogens regulate flagellar gene expression using σ^{54} and homologous TCSs (e.g. *Pseudomonas*, *Vibrio*, and *Helicobacter spp.*) (6, 133, 134,

137). All of these bacteria build polar flagella and express the flagellar T3SS components, FliF, FliG, FliE, and their relevant TCSs before expression of rod and hook gene are activated in a σ^{54}-dependent manner (6, 133, 134). Thus, all of the potential activating proteins appear to be present before the respective TCS is stimulated to initiate gene expression. Therefore, our findings of an activating signal for *C. jejuni* FlgSR may have broad implications for understanding regulatory mechanisms that are required for building flagella in polar flagellates.

Preliminary studies to analyze protein-protein interactions between flagellar proteins and FlgS involved expression of FLAG-FliF and FliG-FLAG proteins in *C. jejuni*. FlgS coimmunoprecipitated with these FLAG-tagged proteins suggesting a direct interaction between the flagellar components and the HK. However, expression of FlgS-FLAG did not result in co-immunoprecipitation with FliF or FliG. Because expression resulted in severe over-expression of FlgS, interpretation of these data is difficult. Future studies should be focused on expression of FlgS-FLAG from its native chromosomal locus because normal levels of expression may reveal biologically-relevant interactions with FliF and/or FliG. Additionally, expression of FLAG-tagged cytoplasmic domains of FliF and FliG could be analyzed to investigate the minimal requirements for the FlgS interaction.

A salient feature of my proposed model is that the MS and C ring only form with the flagellar T3SS and formation of these structures creates a physical signal to be detected by FlgS. While FlgS coimmunoprecipitated with FliF and/or FliG in the presence of a WT T3SS, it did not coimmunoprecipitate with these proteins in a Δ*flhA* mutant. These findings indicate that FliF

and FliG require a T3SS to form a multisubunit binding domain that FlgS can interact with to initiate signal transduction.

Like the FliF, FliG, and T3SS mutants, expression of flagellar genes was dramatically reduced in a *C. jejuni* Δ*fliE* mutant. Of all the flagellar proteins in flagellar motility systems, perhaps the least is known about FliE. FliE is predicted to interact with the periplasmic side of the flagellar T3SS and also possibly the periplasmic face of FliF in the MS ring (200, 201). Due to its periplasmic location, it is difficult to propose that FliE is a direct signal to be detected by a cytoplasmic FlgS protein. However, it is possible that FliE assists in the formation of an activating signal for FlgS. One possibility is that FliE could assist in periplasmic interactions between FliF and the T3SS or stabilize polymerization of the MS ring. Therefore, future analysis of FliE in *C. jejuni* should be focused on determining if FliE promotes these activities. Furthermore, if either of these hypotheses are true, I predict that FlgS would not coimmunoprecipitate with either FliF or FliG in a Δ*fliE* mutant.

In other studies from Chapter Four, I found proteins that form the basal body are required for wild-type σ^{54}-dependent gene expression. One problem with this observation is that three of these genes (*flgB*, *flgC*, and *fliE*) that were required for WT levels of σ^{54} gene expression appear to be grouped together in an operon (*flgBCfliE*) with a conserved σ^{54}-binding site upstream of *flgB*. If these genes are required for σ^{54}-dependent gene expression, then how can they stimulate gene expression if they are dependent on σ^{54} for expression? In preliminary studies, I found WT expression of *flgC* and *fliE* in a Δ*rpoN* (σ^{54}) mutant suggesting that these proteins are not dependent on σ^{54} for their expression in *C. jejuni*. Analysis of *flgB* expression revealed that it is

expressed from both σ^{54}-dependent and independent promoters. Together, these data showed that *flgB, flgC,* and *fliE* are expressed independently of σ^{54} and thus their production does not require activation of the FlgSR TCS. Based on the genetic analysis in Chapter Four, rod proteins are likely present during assembly of the flagellar T3SS, the MS ring, and C ring and are required for full activation of the FlgSR TCS.

Parallel studies identified components of the MS ring (FliF), C ring (FliM and FliN), FlhF, and FlhG are required for proper cell division of *C. jejuni (*158). Removal or inactivation of any one of these components caused mislocalization of the division site to the poles and a resultant minicell phenotype. This suggested that FliF may assist in polar formation of the MS and C rings and FlhG may recognize these components at the pole to function in a mechanism to inhibit polar cell division. This finding highlights the importance that *C. jejuni* has placed on its flagellar system because flagellar substrates are not only important for motility and signal transduction, but also for division of the bacterium to generate viable daughter cells. Future studies to identify the components of the flagellar base that interact with FlhF and/or FlhG will be important for understanding how *C. jejuni* cells divide and could identify novel targets to inhibit cell growth.

As demonstrated in Chapter 5, FlgR in *C. jejuni* is an unusual member of the NtrC-family of transcriptional activators because it activated σ^{54}-RNAP independent of DNA-binding, unlike the majority of NtrC-like proteins. Future studies of FlgR in *C. jejuni* should identify the minimal requirements to activate σ^{54}-dependent gene expression using *in vitro* transcription with purified minimal transcriptional machinery. Interestingly, a small group of RRs in other

bacterial systems such as CtcC in *C. trachomatis,* FlgR in *H. pylori,* and Rrp2 in *B. Burgdorferi* also activated σ^{54}-dependent gene expression through unusual mechanisms (202, reviewed in 203). CtcC and FlgR altogether lack a CTD containing the DNA-binding motif whereas Rrp2 binds DNA indiscriminately to activate σ^{54}-dependent gene expression (199, 202, 203). Therefore these RR may form a new subfamily of NtrC transcriptional activators that stimulate target gene expression in the absence of specific DNA-binding. These proteins may instead activate σ^{54}-RNAP from solution without a need to be tethered to DNA. Additional studies with these proteins will likely reveal new mechanism for transcriptional activation of the NtrC family of proteins.

Instead of functioning as a DNA-binding domain, my data showed that the CTD of FlgR is important to maintain the specificity of the phosphodonor for FlgR. In the absence of the CTD, the small-molecule AcP functions as a direct phosphodonor to activate $FlgR_{\Delta CTD}$ in the absence of the cognate HK, FlgS. While the bifunctional kinase and phosphatase activities of many HKs in TCSs tightly regulate the phosphorylation state of their cognate RR, I showed that FlgS does not have phosphatase activity *in vivo*. Therefore, I hypothesized that the FlgSR TCS evolved the CTD of FlgR to maintain specificity and limit autophosphorylation using non-cognate phosphodonors in the absence of FlgS phosphatase activity. This study was the first to show that a RR can specifically limit AcP from serving as a phosphodonor and suggested the mechanism could function in other bacteria that have TCSs with a HK lacking phosphatase activity or in orphan RRs.

In light of this work, the question of how the CTD of FlgR is limiting AcP-dependent phosphorylation is important. While structural studies on OmpR/PhoB RR proteins suggests interdomain contacts between the receiver and effector domain are responsible for determining how susceptible a RR is to AcP-dependent phosphorylation, the molecular mechanism to prevent this mode of phosphorylation in NtrC family proteins has not yet been elucidated. Structural studies to identify conformational differences between FlgR and $FlgR_{\Delta CTD}$ in both the phosphorylated and inactive state may offer insight into the molecular mechanism to discriminate against phosphorylation of small molecules such as AcP.

In addition, examination of RRs that lack a CTD but still activate σ^{54}-dependent gene expression could offer insight into how signaling proteins discriminate against small molecule phosphodonors such as AcP. A sequence alignment of FlgR from *H. pylori* strain G27 show that it lacks a CTD altogether but still activates σ^{54}-dependent gene expression. Preliminary analysis revealed minimal expression of a σ^{54}-dependent gene in a Δ*flgS* background relative to WT. Presuming that *H. pylori* G27 generates AcP, these data illustrate that while *H. pylori* FlgR does not contain a CTD, some alternative mechanism restricts FlgR from AcP-mediated phosphorylation in the absence of phosphatase activity by *H. pylori* FlgS to maintain signaling specificity.

The results of my studies further elucidated the requirements to specifically activate signal transduction via the FlgSR TCS. These results revealed a new function for MS and C rings as signaling proteins and this mechanism may be broadly applicable to other motile pathogens. Additionally, this work determined how signaling specificity is maintained through

the FlgSR TCS in which FlgS lacks phosphatase activity and FlgR contains a novel domain that limits phosphodonors to only FlgS. In conclusion, my work has resulted in the identification of signal transduction and specificity mechanisms that not only are not only relevant to *C. jejuni* biology, but are likely applicable to other bacterial species.

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