CHARACTERIZATION OF THE ACTIVATION OF THE FLGSR TWO-COMPONENT SYSTEM IN *CAMPYLOBACTER JEJUNI*

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Epidemiological studies indicate that *Campylobacter jejuni* is the leading cause of bacterial gastroenteritis worldwide. This organism has the ability to live as a commensal or a pathogen, depending on the host with which it is associated. While colonization of the gastrointestinal tract of many avian and mammalian species results in a harmless commensal relationship, human infection can cause diarrheal disease. In both scenarios flagellar motility is crucial for promoting

optimal host interactions, as non-motile *C. jejuni* colonize the gastrointestinal tracts of commensal hosts at levels significantly lower than motile isolates and are incapable of causing disease in humans. The means by which *C. jejuni* regulates flagellar gene transcription and assembly differ from the well-studied pathways in species of *Salmonella*, *E. coli*, and *Vibrio*. Previous studies found that *C. jejuni* requires the flagellar export apparatus, σ^{54}, and a two-component regulatory system comprised of the FlgS sensor kinase and the FlgR response regulator to activate transcription of the middle and late σ^{54}-dependent flagellar genes. The FlgR response regulator is an NtrC-like protein that can be divided into three domains: an N-terminal domain that is phosphorylated by FlgS, a central σ^{54} interaction domain, and a C-terminal domain of unknown function. Characterization of FlgR was accomplished by generating constructs that lack the N- or C-terminal domains of the protein and the site of phosphorylation. Through genetic and biochemical analyses, we found that both the N- and C-terminal domains have suppressive functions that prevent FlgR activation of σ^{54}-dependent flagellar gene transcription in the absence of FlgS. Our data also indicate that unlike other NtrC-family proteins, the C-terminus of FlgR does not bind DNA and is dispensable for FlgR activity. The FlgS sensor kinase activates FlgR through phosphorylation, but little was known about its activation prior to these studies. We have identified the site of FlgS autophosphorylation and demonstrated that formation of the flagellar export apparatus and the presence of

at least one other flagellum-associated protein is required for autoactivation of this protein. This study provides insight into the unusual regulation of the FlgSR two-component system and its role in activating σ^{54}-dependent flagellar gene transcription.

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PRIOR PUBLICATIONS

Joslin, S. N., and D. R. Hendrixson. 2008. Analysis of the *Campylobacter jejuni* FlgR response regulator suggests integration of diverse mechanisms to activate an NtrC-like protein. *J Bacteriol* **190:**2422-2433.

Joslin, S. N., and D. R. Hendrixson. 2009. Activation of the *Campylobacter jejuni* FlgSR Two-component System is Linked to the Flagellar Export Apparatus. *J Bacteriol* **191:**2656-2667

Portions from these papers have been reproduced in Chapters 2, 3, 4, and 5.

LIST OF FIGURES

LIST OF ABBREVIATIONS

CFU	Colony-forming units
Cj	*Campylobacter jejuni*
cm	centimeters
CTD	C-terminal domain
Ec	*Escherichia coli*
FEA	Flagellar Export Apparatus
g	grams
GBS	Guillain-Barré syndrome
GI	gastrointestinal
h	hour
Hp	*Helicobacter pylori*
HTH	helix-turn-helix
IM	Inner membrane
in	inches
lb	pounds
LB	Luria-Bertaini
LOS	lipooligosaccharide
MCP	methyl-accepting chemotaxis protein

Mem	Membrane fraction
mg	milligram
ml	milliliter
µg	microgram
µl	microliter
MH	Mueller-Hinton
nmol	nanomol
OM	Outer membrane
Pa	*Pseudomonas aeruginosa*
pmol	picomol
PG	peptidoglycan
RNAP	RNA polymerase holoenzyme
Sol	Soluble fraction
St	*Salmonella typhimurium*
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TMP	trimethoprim
UAS	Upstream activating sequence
v	volume
V	volts
Vc	*Vibrio cholerae*
vol	volume

WCL	Whole-cell lysate
wt	weight
WT	wild-type
x g	times gravity

CHAPTER ONE

INTRODUCTION

Campylobacter jejuni is a Gram-negative, microaerobic rod commonly found associated with many animals, including several of agricultural significance. While colonization of the gastrointestinal tract of avian and mammalian species generally results in a harmless commensal relationship, infection of humans often results in gastroenteritis. *C. jejuni* is a leading cause of bacterial gastroenteritis in the United States (173) and has a significant detrimental effect on economic productivity (22).

Flagellar motility is key in allowing *C. jejuni* to establish and maintain both commensal and parasitic relationships with a variety of hosts. Transcription and assembly of the components required to build the flagellum in *C. jejuni* differ substantially from the well-characterized pathways utilized by *Salmonella* species and *E. coli*. Earlier studies indicated that *C. jejuni*, like *Vibrio cholerae* and *Pseudomonas aeruginosa*, utilizes both σ^{28}- and σ^{54}-dependent mechanisms to transcribe flagellar genes (63, 74, 76, 78, 201, 210). However, very little was known about the regulation of the σ^{54}-dependent cascade in this organism. A transposon mutagenesis study revealed that activating the transcription of a middle σ^{54}-dependent flagellar gene in *C. jejuni* requires a two-component

regulatory system comprised of the FlgS sensor kinase and the FlgR response regulator, the FlhF GTPase, and the flagellar export apparatus (FEA) (Figure 1; 78).

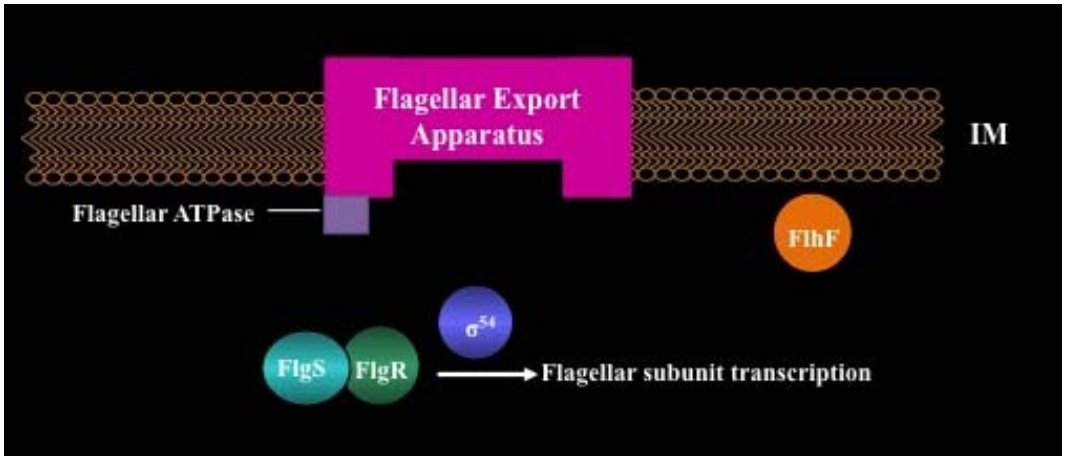

Figure 1: Activation of σ^{54}-dependent flagellar gene expression in *C. jejuni*. A transposon mutagenesis screen (78) revealed that this process requires genes that encode σ^{54}, the FEA (FlhA, FlhB, FliP, FliR), the FlgSR two-component system, and the putative FlhF GTPase. IM: inner membrane.

To more fully understand the regulation of this process, we have worked to characterize mechanisms by which both members of the FlgSR two-component system are activated and function on downstream targets. The first part of this project analyzed the FlgR response regulator protein to understand how it mediates σ^{54}-dependent gene transcription. Bioinformatics analyses indicate that FlgR bears 60% similarity to NtrC, a well-characterized response regulator. NtrC and similar proteins such as DctD and NifA have been studied for over 20 years,

and there is extensive information regarding the biochemistry of these response regulators. We began analyzing FlgR using many of the same approaches used to examine other NtrC-like proteins and found that while FlgR utilizes some of the same mechanisms for activation and mediation of σ^{54}-dependent gene transcription, this protein has many properties that are quite different from the well-characterized members of the NtrC family. For this project we also analyzed the relationship between FlgR and its cognate histidine kinase, FlgS. In addition, the construction of constitutively active forms of the FlgR protein have been useful not only in understanding FlgR function, but also in elucidating the nature of the relationship between the FlgSR two-component system and the FEA. We have found that constitutively active FlgR proteins produced in strains lacking FEA components can partially suppress the phenotype of FEA mutants for expression of σ^{54}-dependent flagellar genes, indicating that FlgSR is linked to and functions downstream of the FEA.

Once we had a better understanding of how FlgR becomes activated and is involved in σ^{54}-dependent gene transcription, we sought to uncover the signal that leads to activation of the FlgSR two-component system. To this end, we have characterized the FlgS sensor kinase and attempted to identify the activating signals that influence its ability to positively regulate flagellar gene expression and motility in *C. jejuni*. Although a previous study established that FlgS is a histidine kinase with the ability to phosphorylate the FlgR protein (210), the site

of autophosphorylation and the signal for FlgS autoactivation were not investigated. We have established that H141 is the likely site of FlgS autophosphorylation and is required for the activation of the kinase and the subsequent activation of FlgR. Preliminary evidence from our lab suggested that the FlgS activating signal could be associated with the FEA, but in an unknown manner. To further investigate this finding, we generated *C. jejuni* mutants in which the FEA assembles in the inner membrane but inefficiently secretes flagellar subunits. The results of these studies suggest that transcription of σ^{54}-dependent flagellar genes is activated following formation of the FEA but likely does not require the secretion of a substrate through this complex. In addition, preliminary studies described in Chapter Six indicate that at least one of the flagellar rod proteins, FliE, is also required for activating the transcription of multiple σ^{54}-dependent flagellar genes. Autoactivation of FlgS by the FEA or FliE could involve direct interactions between FlgS and these proteins, which led us to attempt a number of *in vitro* and *in vivo* techniques to demonstrate whether this occurrs. Although these studies were not successful in providing an interaction partner for FlgS, they did lead our lab to new findings concerning the flagellar gene transcription hierarchy.

The studies performed to complete this project offer new insight into the unusual regulation of *C. jejuni* motility and reveal several atypical mechanisms by which flagellar gene transcription is controlled in this bacterium. This work

expands on previous models for regulating flagellar gene expression in *Campylobacter* by characterizing the FlgSR two-component system and introducing the hypothesis that the FEA and other flagellum-associated proteins may have more complex roles in regulating flagellar gene transcription than previously believed.

CHAPTER TWO

LITERATURE REVIEW

Discovery and classification of *Campylobacter* species

In 1886, Theodor Escherich described small, spiral-shaped bacteria found in the stool samples of children that had died from a diarrheal disease that he termed 'cholera infantum' (reviewed in 198). However, he was unable to culture these organisms using reagents available at the time and concluded that this meant their presence was coincidental and not the cause of illness. Study of the phenotypic and epidemiological evidence presented in Escherich's work has led many scientists to now believe that the organism he described was most likely a species of *Campylobacter* and indeed responsible for the diarrheal disease he observed.

The first half of the 20th century saw the discovery of many pathogenic and commensal *Campylobacters* in humans and a number of other animals, although they were initially classified as species of *Vibrio*. These include a pathogen of livestock that is now known as *Campylobacter fetus*. The name *Vibrio fetus* was initially given to this organism when it was found to be associated with spontaneous abortion in cattle (177) and rare infection of humans (reviewed in 53). Additionally, the species now known as *Campylobacter coli*

and *Campylobacter jejuni* were discovered associated with dysentery of swine (43) and bovine (90), respectively. Human infection with these "Vibrio-like" *Campylobacters* was also described in this era. One of the earliest published cases of a possible human outbreak of *C. jejuni*-associated gastroenteritis occurred in 1938 at two geographically related correctional institutions where more than 300 inmates became ill following the consumption of raw milk products (122). Although investigators did observe "vibrio-like micro-organisms" by microscopy of stool specimens, they were unable to culture the bacteria and, like Escherich, initially disregarded the finding as unrelated to the diarrheal disease in the inmates. However, the preponderance of these organisms in samples from diseased inmates led to the suspicion that they were the causative agent of the outbreak.

The isolation of these "Vibrio-like" organisms from many species of animals, both in the presence and absence of disease, was followed by metabolic and DNA analyses that indicated these organisms were part of a new genus of bacteria, subsequently named *Campylobacter* (175). However, not until new isolation, enrichment, and selective media were developed could the significant contribution of *Campylobacter* to human disease begin to be appreciated. In 1972, Dekeyser and collegues published the first report of *C. jejuni* isolation from the feces of a patient with acute gastroenteritis (38), followed shortly by a similar report of isolation from diarrheic individuals in Africa (21). Since this time,

advanced diagnostic protocols have increased the frequency of *Campylobacter* recovery from clinical samples, leading to the identification of this organism as an important cause of human disease.

The *Campylobacterales*

The order *Campylobacteracea* encompasses not only the genus *Campylobacter*, but also the genetically related and clinically significant *Helicobacter* and *Arcobacter*. Several genera that are likely non-pathogenic are also classified in this order and include *Wollinella* and *Sulfurospirillum*. These bacteria are Gram-negative, usually microaerobic, and have a characteristic S-shaped or spiral morphology (reviewed in 151, 198). Typically they utilize amino acids as a carbon source and do not metabolize sugars, one characteristic that was used to distinguish them from species of *Vibrio* before genomic sequencing technology was available. Some members of this family, including *C. jejuni*, are thermotolerant, capable of growth at 42˚C, while others exhibit optimal growth at temperatures of 37˚C or lower.

Campylobacter jejuni phenotype and genetics

C. jejuni is a fastidious, Gram-negative microaerobe with spiral-shaped morphology (Figure 2). It is highly motile via a single, unsheathed flagellum elaborated from one or both poles of the cell. Although it has morphological

characteristics that led early researchers to classify is as a species of *Vibrio*, the metabolic properties of *C. jejuni* are quite different (reviewed in 99). These include a requirement for low (5%) oxygen concentration and the inability of *C. jejuni* to ferment or oxidize carbohydrates. Additionally, the G+C content of the *C. jejuni* genome is approximately 30% (155), whereas the G+C content of the *V. cholerae* genome is nearly 50% (71).

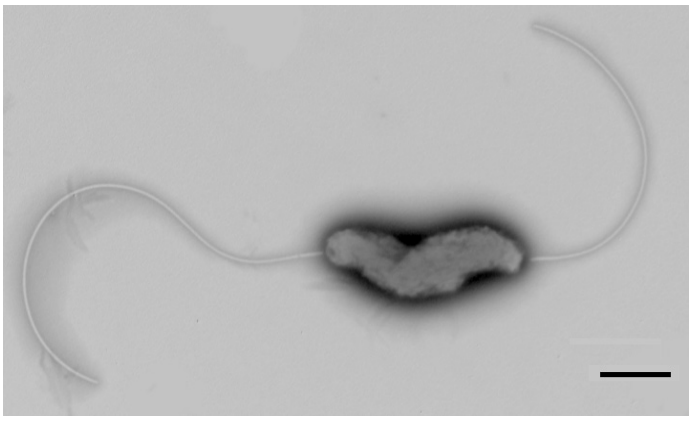

Figure 2: Electron micrograph of *C. jejuni*. 81-176 Sm^R (DRH212) is a spontaneous streptomycin-resistant isolate and is used in our lab as a wild-type strain. Magnification: 16,000X. Bar represents 0.5 μm.

The *C. jejuni* genome is contained on a single circular chromosome and is approximately 1.65 megabases (155). Many isolates also contain extrachromosomal plasmid elements. Comparative genome analysis reveals a large amount of strain diversity among *C. jejuni* isolates (41, 51, 187). Until recently, genetic manipulation of this organism was very difficult and limited.

New technologies have allowed for the generation of in-frame chromosomal deletions, the use of transposon mutagenesis protocols and reporter gene constructs, and extra-chromosomal complementation (36, 76).

Incidence of *Campylobacter jejuni* infection in humans

C. jejuni accounts for the largest number of human infections caused by a *Campylobacter* species in both developing and industrialized nations (reviewed in 58, 176). Additionally, *C. jejuni* is now generally regarded as the most common bacterial cause of gastroenteritis worldwide (200). In the developing world, *C. jejuni*-associated enteritis is most frequently seen in children less than four years of age and is less common in healthy adults (28). Individuals living in industrialized regions are most frequently infected as infants, young adults, and during travel to endemic regions (2).

In the United States, the Foodborne Diseases Active Surveillance Network (FoodNet) estimate of *C. jejuni* incidence in 2006 was approximately 13 cases per 100,000 individuals per year (2). However, this number includes only laboratory-confirmed cases in states that are monitored by FoodNet, and the actual incidence is likely significantly higher, as it is estimated that for every case clinically verified and reported in the United States, there may be 34 cases that are not (173). In Canada, where *C. jejuni* infection is a reportable disease, in 2004 there were approximately 30 documented cases per 100,000 individuals (56). Other

industrialized nations report incidences of *Campylobacter* infection that can range from 400 cases per 100,000 persons in New Zealand in 2003 (11) to 46 cases per 100,000 across 21 European nations in 2007 (1). At least some of the observed differences in incidence are attributable to variability in surveillance, laboratory confirmation, and reporting between countries.

Although *C. jejuni*-associated gastroenteritis is frequently mild and of short duration, the economic cost of *C. jejuni* infections in the United States may be as high as $4 billion per year (22). This includes not only productivity loss for working adults, but also the cost of treating secondary sequelae associated with *C. jejuni* infection.

Clinical aspects of human campylobacteriosis

Human infection usually results from eating improperly prepared or contaminated foods, particularly poultry, and drinking contaminated water or unpasteurized milk. The infectious dose is low, and is estimated to be as few as 500 to 800 organisms (16, 169). Infected individuals generally become symptomatic within 1-7 days following exposure (reviewed in 176). The illness is accompanied primarily by watery-to-bloody diarrhea, abdominal cramping and pain, and fever. Vomiting may be present in children, but is less common in adult patients. Rarely, infection may present as a pseudoappendicitis similar to that

observed in individuals with other gastrointestinal (GI) infection such as *Yersinia enterocolitica*. Illness is self-limiting and generally resolves within a week of the onset of symptoms; however, recovery in some individuals, particularly those with immunodeficiencies, may take significantly longer. Unless treated with antibiotics, patients will frequently continue to shed *C. jejuni* in the feces for several weeks after the resolution of clinical symptoms. Following *C. jejuni*-associated gastritis, approximately 0.1% to 1% of patients may experience autoimmune sequelae, including Guillan-Barré syndrome (GBS), Miller-Fisher syndrome, and reactive arthritis. *C. jejuni* infection is the most common bacterial infection preceding onset of GBS (18).

Campylobacteriosis in immunocompromised individuals

Typically, an immunocompetent individual will resolve symptoms of an acute *Campylobacter* infection within a week of the onset of symptoms. Persons with HIV, hypogammaglobulinemia, and other immunodeficiencies often present with infections that are more severe and prolonged in duration (46, 158). These patients may also become chronic carriers of *C. jejuni* subject to recurrent enteritis and bacteremia, requiring multiple courses of antibiotics to clear the infection. Bacteremic *C. jejuni* infections are observed with greater frequency in the immunocompromised population and can be associated with the formation of skin lesions, cellulitis, and other usual presentations (138, 152, 192).

Additionally, HIV+ patients have an incidence of *Campylobacter* infection that may be up to 40-fold higher than the general population in developed nations (7, 180). In developing countries, HIV+ status contributes to an increased number of adult *C. jejuni* infections and increased mortality in co-infected children (28, 109).

Treatment of Campylobacteriosis

Due to the self-limiting nature of most *C. jejuni* infections, patients are usually given supportive treatment with fluids and electrolytes for rehydration (176). In more severe cases hospitalization with antibiotic treatment may be required. Antibiotic therapy is most effective if it is provided early in disease, or for immunocompromised patients with chronic carriage and recurrent enteritis (206). Duration of symptoms is shortened if antibiotics are given early, and fecal shedding is markedly reduced if antibiotics are given at any point during infection (6, 153, 172). Erythromycin and fluoroquinolones are the most frequently used antibiotics but resistance to the latter is fairly common due to use in agricultural industries (67, 144, 164).

Secondary sequelae following Campylobacteriosis

GBS is an ascending paralysis of the peripheral nervous system that can lead to respiratory failure and death (197). This condition is the most common cause of neuromuscular paralysis in industrialized nations and is widely believed

to develop as an autoimmune condition following infection with certain bacterial or viral pathogens. Although not all cases of GBS are preceded by confirmed clinical infections, diarrheal disease is described as an antecedent to the onset of symptoms in up to 40% of cases and *C. jejuni* is the most commonly associated bacterial species (reviewed in 4, 197). The lipooligosaccharides (LOS) expressed on the surface of different *C. jejuni* strains can mimic the carbohydrate residues on human gangliosides, prompting infected individuals to produce antibodies that are cross-reactive for bacterial LOS and human gangliosides (111). While less than 1 in 1000 individuals with campylobacteriosis develop GBS (137), the morbidity and mortality of this sequelae have significant economic costs (55). Other neuromuscular conditions can follow *C. jejuni* infection, including multifocal motor neuropathy (191, 193) and Miller-Fisher syndrome, a subtype of GBS (104).

Approximately three to six weeks after the onset of diarrheal disease patients may develop pain and swelling in the joints of the knees, fingers, and ankles (194; reviewed in 159). This reactive arthritis is observed following infection with several enteric pathogens and was initially thought to be related to the genetic background of the host (reviewed in 159), although this argument has been discounted by recent population-based studies (68, 161). Reiter's syndrome is also observed in a very small percentage of *C. jejuni* patients and includes the development of arthritis, redness of the eyes, and urethral inflammation.

Studies have also postulated links between *C. jejuni*-associated gastroenteritis and the subsequent development of chronic digestive disorders such as irritable bowel syndrome (195) and Crohn's Disease (13, 116). However, the authors of some of these studies acknowledge confounding psychological factors that might make the link between *C. jejuni* and chronic intestinal pathology less likely (66).

Animal and environmental reservoirs for *C. jejuni*

Campylobacteriosis is most commonly thought of as a food-borne illness. Epidemiological studies have a found a strong link between the consumption of improperly cooked or handled meats, particularly poultry, and the appearance of *C. jejuni*-associated gastritis (39, 70). Poultry animals raised for human consumption frequently have high intestinal burdens of *C. jejuni* that can exceed 10^9 colony-forming units (CFU) per gram of feces (14). While muscle tissue is less frequently contaminated, processing of carcasses can introduce *C. jejuni* to these sites. Unlike *Salmonella*, *C. jejuni* is not thought to be transmitted through raw chicken eggs. Although the outer surface of the eggshell can become contaminated, studies have demonstrated that this bacterium does not survive for more than 48 hours on the shell and is unable to breach this barrier to enter the egg (44).

C. jejuni has also been found associated with cattle raised both for meat and for dairy products (182). This relationship does show seasonal variance, with higher rates of carriage in the summer months, although more than half of a herd can be infected year-round. Increased consumption of raw milk is a significant and growing source of *C. jejuni* infection in industrialized nations (79; reviewed in 108, 205). As dairy cattle are frequently colonized with *C. jejuni*, the spread of fecal material to the udder is thought to be the major source of milk contamination. *Campylobacter* is common in other livestock animals as well, such as swine (207) and lamb (183).

Minor sources of human infection include wild animal reservoirs such as migratory birds, although they can spread *C. jejuni* to uninfected commercial chicken flocks (167). Pets are also thought to be a minor source of *Campylobacter* infection, particularly for patients with AIDS and other immunodeficiencies (59, 72, 209). However, transmission from companion animals to humans is thought to be infrequent and easily managed with proper hygiene.

Campylobacters, including *C. jejuni*, have also been isolated from sewage and freshwater sources and are frequently found in waters that are contaminated with waste from waterfowl or agricultural runoff (150, 199). Under-treated municipal water sources can also contain detectable levels of *C. jejuni* and have been the source of several recent human outbreaks (88, 115, 150). Chlorination of

human water supplies is an effective strategy for reducing the amount of *C.jejuni* present.

Animal models of *C. jejuni* commensalism

C. jejuni is a frequent commensal colonizer of the GI tracts of many animals. The natural relationship that *C. jejuni* has with poultry has provided an excellent natural model for the study of colonization requirements and kinetics: the one-day-old chicken hatchling (12). One day post-hatch, birds can be infected with *C. jejuni* and the bacterial load in the lower GI tract can be quantified at different points following exposure. Some chicks can be experimentally colonized with an innoculum as low as 8 CFU, but ≥900 CFU will successfully colonize all chicks in a study (77). These low innocula are not surprising, as it is has been shown that infected chickens readily transmit *C. jejuni* to naïve flock members and that the bacterium can be transmitted from migratory birds and even insects (196; reviewed in 170).

Other experimental animal models have been developed to study factors that are thought to be involved in colonization of the GI tract, and include athymic and euthymic mice (213), IL-10$^{-/-}$ mice (126), suckling mice (140), *myd88*$^{-/-}$ mice (204) and specific-pathogen free pigs (117). However, none of these models fully recapitulate the relationship between *C. jejuni* and its natural, avian commensal host.

The importance of flagellar motility in commensalism

Non-motile isolates of *C jejuni* are severely impaired for colonization of the one-day-old chick cecum (73, 75, 77, 143, 203). Seven days following infection, animals that received strains with motility defects display *C. jejuni* loads in the lower intestines that are 1,000-10,000 fold reduced as compared to chicks that are inoculated with fully motile strains. Although the precise role for motility in colonization is unknown, some studies have postulated that motility allows attachment to the epithelium or prevents clearance of the bacterium during intestinal peristalsis.

Non-poultry animal models of *C. jejuni* commensalism also address the importance of motility in the establishment of a host-bacteria relationship. Infant mice will clear high doses of non-motile *C. jejuni* within 48 hours of infection but remain colonized with motile bacteria (190). Additionally, mice with limited gut flora can be colonized with wild-type *C. jejuni* but not strains that have mutations in motility-associated genes (26).

Flagellar motility is required for *C. jejuni* to promote human disease

Few studies have been performed analyzing infections in human volunteers with motile and non-motile strains of *C. jejuni*, but the available evidence does support the notion that motility is essential to establish a productive human infection. In one human challenge study, volunteers were given a mixture

containing equal numbers of motile and non-motile *C. jejuni* (16). Stool samples from individuals who developed diarrheal disease contained only the motile strain, suggesting that this trait is necessary to either the establishment or prolongation of disease. Several strains of *C. jejuni* have been shown to invade cultured epithelial cells and multiple groups have utilized this property to analyze putative virulence factors. Because motility appears to be required to promote human disease, loss of motility has been studied in the tissue culture environment. It has been shown that the major flagellin, required for motility, is also required for invasion of INT-407 cells (202) and Caco-2 cells (189). Later studies suggest that while motility is not necessary for the initial attachment of *C. jejuni* to host cells, it is required for the bacteria to cross polarized epithelial cell monolayers (62). Chemical modification of the flagella is also crucial, as mutants impaired for glycosylation of flagellar proteins are deficient for both attachment and penetration of epithelial cells (64).

The role of flagellar motility in animal models of *C. jejuni* pathogenesis

Perhaps due in part to its ability to establish non-pathogenic relationships with many different types of animals, models that have been developed to study *C. jejuni* pathogenesis are limited and often do not replicate salient aspects of human infection (9, 50, 163; reviewed in 42). Weanling ferrets have been developed as a model of *C. jejuni* pathogenesis with some success (52), and have

been used to address the role of motility in infection. Compared to inoculation with wild-type strains, ferrets challenged with *C. jejuni* mutants defective for chemotaxis or glycosylation of flagellar proteins displayed diarrheal symptoms of shorter duration and severity, reiterating the central role flagellar motility has in promoting human pathogenesis (64, 211).

Regulation of flagellar motility in *Escherichia coli* and *Salmonella* species

Flagella are produced by diverse bacterial species to aid in processes including motility and adhesion, allowing bacteria to occupy an environmental niche or maintain a relationship with a host. Flagellar biosynthesis requires coordinating both the expression of over forty flagellar genes and assembly of the encoded flagellar components into the organelle. Several mechanisms of flagellar gene regulation have evolved, with the best understood system exemplified by *Escherichia coli* and *Salmonella* species (reviewed in 27). The process is multi-tiered, with the expression of genes required late in the assembly process dependent on the proper expression of genes and production of proteins required earlier in the process. Flagellar genes of *Salmonella* are grouped into three classes, based on their temporal expression. Class I genes at the top of the cascade encode the master flagellar operon *flhDC*. FlhDC production and activation allows expression of the second class of genes, including an alternative sigma factor, σ^{28} (*fliA*), and a negative regulator of σ^{28}, *flgM*. Genes encoding the

hook, basal body, and a multi-protein complex termed the flagellar export apparatus (FEA) are also included in the class II genes. The FEA consists of several inner membrane-bound proteins and is responsible for the secretion of flagellar components. Additionally, the FlgM protein is secreted through the FEA following its proper assembly. This allows the activation of σ^{28} and expression of the class III genes that encode the flagellar filament subunits. This level of control is necessary for preventing the energetically wasteful expression of class III genes in the absence of a fully-formed, competent FEA.

Regulation of flagellar motility in species of *Vibrio*, *Pseudomonas*, and *Helicobacter*

In species of *Vibrio* and *Pseudomonas*, researchers have found four-tiered transcriptional cascades that utilize both σ^{28} and σ^{54} to express flagellar genes (35, 165). In both of these systems the class I genes encode a master regulator protein: FlrA in *V. cholerae* (93, 102), and FleQ in *P. aeruginosa* (34). Both of these proteins receive global regulatory signals that are currently unknown, and mediate the transcription of σ^{54}-dependent class II genes. Like *Salmonella*, the class II genes in these species include σ^{28}, *flgM*, and the components of the FEA. In species of *Vibrio* and *Pseudomonas* the class II genes also encode a flagellar two-component system not found in pathways utilizing only σ^{28}. These two-component systems are required for the σ^{54}-dependent activation of the class III

genes encoding the hook and basal body. Class III gene expression and export of the encoded proteins leads to the secretion of the FlgM negative regulator and the transcription of σ^{28}-dependent class IV genes, encoding the flagellins and motor proteins.

Like *Vibrio* and *Pseudomonas* species, *Helicobacter pylori* has also been found to utilize both σ^{28} and σ^{54} to transcribe flagellar genes. However, unlike other systems, no master regulator of flagellar biosynthesis has been discovered in *H. pylori* (145). Additionally, while both *Vibrio* and *Pseudomonas* spp. have more than one flagella-associated response regulator protein that functions to activate σ^{54}, *H. pylori* appears to only encode one.

Flagellar motility in *C. jejuni*

Flagellar motility is one of the few traits of *C. jejuni* that has been demonstrated to be associated with both harmless commensal colonization and the establishment of diarrheal disease in humans and other model animals. Much of the work in understanding the regulation of flagellar biosynthesis in *Campylobacter* species has been performed in *C. jejuni* and the closely-related *C. coli*. Genes required for *C. jejuni* motility were initially identified by screening laboratory-cultured populations for non-motile mutants and analyzing the nature of the defect (63, 201). Whole-genome sequencing, the development of transposon mutagenesis protocols, and targeted genetic manipulation of *C. jejuni*

greatly increased the number of postulated motility-associated genes and provided evidence for an unusual means of regulating flagellar biosynthesis in this organism (29, 36, 60, 76, 155).

C. jejuni elaborates a single flagellum at one or both poles, and synthesis of this organelle is accomplished through both σ^{28}- and σ^{54}-dependent transcriptional pathways (63, 74, 76, 78, 201, 210). The use of both σ^{28} and σ^{54} in these pathways indicates that flagellar gene transcription in *C. jejuni* bears more similarity to the regulatory cascades of species of *Vibrio*, *Pseudomonas*, and *Helicobacter* than those of *E. coli* or *Salmonella* species (8, 91, 100, 102, 128, 145, 184). In *C. jejuni*, σ^{28} is involved in transcription of the major flagellin, *flaA*, other minor flagellar components, and *cj0977*, a gene encoding a non-flagellar protein involved in virulence (24, 61, 78, 210). σ^{54}, FlhF (a putative GTPase), the FEA, and the FlgSR two-component system are required for transcription of the middle and late flagellar genes necessary for formation of the hook, basal body, and the minor flagellin, FlaB (24, 76, 78, 210). The FlgS sensor kinase has been hypothesized to sense an unidentified signal, possibly emanating from the FEA and FlhF, which initiates a signal transduction cascade. FlgS can phosphorylate the FlgR response regulator *in vitro* (92, 210), presumably activating FlgR so that it may productively interact with σ^{54} to mediate transcription of target flagellar genes. FlgR is highly homologous to NtrC and the NtrC-like proteins utilized by *Vibrio* (FlrC; 31, 102), *Pseudomonas* (FleR; 168) and *Helicobacter* species (FlgR;

19, 181) to regulate transcription of σ^{54}-dependent flagellar genes, and is likely functioning in a similar manner. The regulation of FlgR activity appears to be two-tiered. First, phase variation of the gene affects whether a full-length, functional version of the protein is even produced in cells (73); and second, the protein is phosphorylated by FlgS to function as a response regulator (92).

Our lab has found that FlgS is also a target of phase variation, making FlgSR the only known two-component regulatory system wherein both proteins are subject to this form of control (73, 75). However, the mechanism by which FlgS is activated and functions as a sensor kinase remains to be characterized. Sequence analyses indicate that this protein appears to contain domains common to many sensor histidine kinases such as the ATP-binding catalytic domain and the histidine-containing phosphotransfer domain (185, 208). Although homology is somewhat weaker in the N-terminal region of the protein, FlgS is similar to the flagella-associated histidine kinases that are required for σ^{54}-dependent flagellar gene expression, and motility in species of *Vibrio*, *Pseudomonas* and *Helicobacter* (31, 102, 145, 168). However, the signals that activate any of these kinases for positively influencing flagellar gene expression are uncharacterized.

In addition to phase-variation of the FlgS and FlgR proteins, production of the flagellin subunits and genes associated with post-translational modification of the flagellum are phase-variable properties in *C. jejuni* that have been shown to affect experimental commensal colonization of poultry, infection of human

volunteers, and invasion in cell culture models (23, 40, 65, 69, 97, 149). Due to the diversity of *C. jejuni* isolates, some of these phase-variable loci are strain-dependent, such as the *maf* genes, which have been shown to phase-vary in strain NCTC 11168 (97), but are partially absent in strain 81-176 (81).

Activation of σ^{54}-dependent gene expression

Many bacteria utilize an alternative sigma factor, σ^{54}, to transcribe genes required for such diverse activities as nitrogen fixation, root nodule formation during plant symbiosis, and flagellar motility (reviewed in 98, 112). Unlike other σ factors, σ^{54}-RNA polymerase (RNAP) holoenzyme alone cannot mediate the opening of DNA at target promoters. Instead, it requires interactions with a σ^{54}-dependent response regulator (also termed "enhancer-binding protein") to mediate this process. While several proteins of this type have been found associated with motility in *Vibrio* (102), *Pseudomonas* (168), *Helicobacter* (181), and *Campylobacter* (87), these proteins have not yet been well-studied. They are, however, homologous to NtrC, a response regulator that is involved in nitrogen metabolism in *E. coli* and has been the focus of extensive study for over 20 years. This σ^{54}-dependent response regulator protein consists of a phosphorylatable N-terminal regulatory (or receiver) domain, a central σ^{54}-interaction domain, and a C-terminal domain (CTD) that contains dimerization determinants and is also indispensable for DNA-binding *in vivo* (45; reviewed in 116). Under nitrogen-

limiting conditions, the NtrB histidine kinase autophosphorylates and donates its phosphate residue to NtrC at residue D54 (103, 147, 174), which activates the protein to promote its oligomerization, DNA binding, and interactions with σ^{54} in the RNAP holoenzyme. In addition, phosphorylation of NtrC stimulates hydrolysis of ATP by the central domain to remodel the closed DNA complex, allowing transcription of the target gene to occur. The CTD allows NtrC to binding to upstream activation sequences (UAS) and directly contact the σ^{54}-RNAP holoenzyme, a requirement for σ^{54}-dependent gene expression to occur. The large volume of work describing the mechanisms of NtrC activation and function provide a starting point for designing experiments to analyze flagellar response regulators.

Bacterial two-component regulatory systems

Responding to changing environmental and intracellular conditions in cells requires efficient communication networks that can rapidly receive and integrate signals. Distributed almost ubiquitously among prokaryotic organisms, two-component regulatory systems allow bacteria to monitor their intracellular and extracellular environments and react by altering the expression of appropriate genes. These systems are typically comprised of a sensor histidine kinase and a response regulator protein (reviewed in 127, 208). The sensor kinase protein contains a domain usually within the N-terminal portion that detects a specific

signal, commonly through an interaction with another protein or a small effector molecule. Activation includes autophosphorylation of the sensor kinase and a conformational change that allows the transmitter domain, usually within the C-terminal portion, to activate a cognate response regulator via phosphotransfer. Some histidine kinases also have the ability to function as a phosphatase to remove a phosphate group from either itself or its cognate response regulator when activity of the regulatory system is not favored. The FlgS sensor kinase described in this study bears low homology to non-FlgS orthologues, but like the well-studied NtrB histidine kinase it is modular, cytoplasmic, and its two-component partner, FlgR, is homologous to the partner of NtrB, NtrC.

Signaling from the histidine kinase to the cognate response regulator protein allows a cell to amplify the signal received and respond quickly to a stimulus. Response regulators have highly diverse functions and in addition to enzymatic activities, many have been shown to bind DNA, RNA, or other proteins (reviewed in 57). In this work, we have focused on a response regulator, FlgR, a protein that bears high sequence homology to the NtrC-like response regulators which function to activate σ^{54}-dependent gene transcription as described above. Approximately 10% of all known bacterial response regulators are within the NtrC family of transcriptional activators (57). Within this class, two means by which phosphorylation of the receiver domains by the histidine kinase ultimately influence the activity of the protein have been described. The

archetypal member of this family, NtrC, is positively regulated by phosphorylation (45), while other members, such as *S. meliloti* DctD and *A. aeolicus* NtrC1 utilize phosphorylation to remove inhibition mediated by the receiver domain (118, 120). These positive and negative regulatory mechanisms both ultimately influence the ability of the regulator to hydrolyze ATP and interact with σ^{54}-RNAP holoenzyme and DNA via the C-terminal helix-turn-helix motif to transcribe target genes under appropriate conditions. Understanding how phosphorylation is utilized to activate FlgR may have broader implications for understanding mechanisms for controlling the activation of NtrC-like response regulators in a number of bacterial species.

Two-component systems related to flagellar motility

Flagellar chemotaxis systems also rely on two-component signaling systems to properly regulate bacterial motility (17, 188). The CheA kinase receives signals from a number of membrane-bound methyl-accepting chemotaxis protein (MCP) receptors (reviewed in 10, 47, 48). Motile bacteria respond via chemotaxis to small molecules that are attractants or repellants, and many of these effectors are bound by the periplasmic domains of MCPs. Through interactions of the cytoplasmic domains of MCP with the CheA kinase, CheA is able to integrate and transmit these signals via phosphorelay to CheY, ultimately

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

Many bacteria have flagellar gene transcription cascades that utilize two-component systems to integrate environmental and internal signals for the proper temporal expression of genes necessary for building the flagellum. As described above, many clinically relevant bacteria including species of *Vibrio*, *Pseudomonas*, *Helicobacter*, and *Campylobacter* require two-component systems for expression of genes encoding the flagellar substructure and flagellins (35, 78, 100, 102, 145). While the intra- or extracellular signals that activate these two-component systems remain largely uncharacterized, this work provides the basis for understanding the activation of FlgS in *C. jejuni* and may be relevant to other flagellar two-component systems.

The flagellar export apparatus and assembly of flagellar substructures

The FEA is comprised of six membrane proteins (FlhA, FlhB, FliP, FliR, FliO, and FliQ) that are believed to form a pore in the inner membrane to allow the secretion of flagellar substrates (132). Surrounding this complex is the MS ring, formed from multiple subunits of the FliF protein (82). Formation of the MS ring may precede FEA formation, although this sequence is not well-understood (110).

In addition to the FEA, there are several flagellar substructures that are required for motility. These include (in order from cell-proximal to cell-distal) the basal body/rod, hook, junction, filament, and filament cap. The proteins that comprise these regions of the flagellum have been studied in *Salmonella* and a likely temporal order of secretion for each of these classes has been described (80). Not surprisingly, the order in which substrates are exported is directly related to their position in the flagellar structure. For example, the proteins that are thought to comprise the rod (FlgB, FlgC, FliE, FlgF, and FlgG) are exported more efficiently before the rod and hook are finished assembling (80). It is known that formation of the FEA, rod, and hook are required for secretion of the FlgM anti-sigma factor, a protein that sequesters σ^{28} activity until the cell is ready to produce the subunits of the flagellar filament and motor proteins (113). However, it is the not the FlgM anti-sigma factor alone that determines which substrates are secreted. One component of the FEA, FlhB, has a key role in determining whether the cell will continue secreting rod and hook substrates or switch to export of filament proteins (49). This substrate switch occurs following autoproteolytic cleavage of FlhB and requires FliK, a protein involved in ensuring that the hook is of the proper length before flagellin export begins (131, 136, 139).

The functions of the FEA may extend beyond mediating the export of flagellar subunits and could include the secretion of proteins involved in virulence

or colonization. It has been observed in *Y. enterocolitica* that the expression and secretion of several non-flagellar proteins requires the FEA (212). Likewise, the *C. jejuni* FEA has also been shown to be required in tissue culture models for the secretion of proteins believed to be virulence factors (105, 179). Transposon mutagenesis in *C. jejuni* revealed that the components of the FEA are required for transcription of middle and late σ^{54}-dependent flagellar genes, revealing that the apparatus could act through the FlgSR two-component system (78). In this work, we have found that not only does the FlgSR two-component system function downstream of the FEA, but the formation of this complex and components of the flagellar rod are required for optimal FlgSR activity. These findings reveal the possibility that the FEA and other flagellum-associated proteins may have functions relating to signaling and motility that have yet to be uncovered.

CHAPTER THREE

MATERIALS AND METHODS

Bacterial strains. *C. jejuni* strain 81-176 is a clinical isolate from a patient presenting with gastroenteritis and has since been shown to promote disease in humans and commensal colonization of the chick gastrointestinal tract (16, 73, 77, 107). *C. jejuni* was routinely grown on Mueller-Hinton (MH) agar containing 10 μg/ml of trimethoprim (TMP) at 37˚C under microaerobic conditions (85% N_2, 10% CO_2, and 5% O_2). As required, strains were grown on MH agar containing 50 μg/ml kanamycin, 20 μg/ml chloramphenicol, or 0.5, 1, 2, or 5 mg/ml streptomycin. All *C. jejuni* strains were stored at –80˚C in a solution of 85% MH broth and 15% glycerol. *E. coli* DH5α, XL1-Blue, and BL21(DE3)pLysE were cultured with Luria-Bertani (LB) agar or broth containing 100 μg/ml ampicillin or 15 μg/ml chloramphenicol as necessary. All *E. coli* strains were stored at –80˚C in a solution of 80% LB broth and 20% glycerol.

Construction of strains. All strains were constructed as previously described (76). To create the *flgR* mutations, pDRH428 (78) was subjected to PCR-mediated mutagenesis (125) to generate in-frame deletions of the receiver and C-terminal domains of FlgR. The $flgR_{\Delta receiver}$ mutation was made by fusing the start

codon to codon 132, removing the intervening 131 codons, to create pDRH1855. pDRH1856 contains *flgR*$_{\Delta CTD}$ which lacks the terminal 51 codons of the gene. This plasmid was subsequently used in PCR-mediated mutagenesis to create pSNJ711 which contains the *flgR*(*D51A*)$_{\Delta CTD}$ mutant. pDRH1865, containing *flgR*$_{central}$, was generated by two steps of PCR mutagenesis to remove the N-terminal 131 codons and the C-terminal 52 codons. PCR-mediated mutagenesis was also performed with pDRH428 to generate plasmid derivatives containing single point-mutations in the coding sequence of *flgR*. These point mutations include D46A (pSNJ511), D51A (pSNJ512), and D58A (pSNJ513). All plasmid constructs were verified by DNA sequence analysis.

All plasmids containing *flgR* mutants were electroporated into strains 81-176 Sm^R *flgR::kan-rpsL* and 81-176 Sm^R Δ*astA flgR::kan-rpsL* (DRH473 and DRH475, respectively; 78), replacing *flgR::kan-rpsL* with the specific *flgR* mutant at the native locus. To introduce the *flgR* mutants into the Δ*flgS* background, 81-176 Sm^R Δ*flgS* (DRH460) and 81-176 Sm^R Δ*astA* Δ*flgS* (DRH911; 78) were electroporated with pDRH443 (containing *flgR::kan-rpsL*; 78). The resulting strains, 81-176 Sm^R Δ*flgS flgR::kan-rpsL* (SNJ767) and 81-176 Sm^R Δ*astA* Δ*flgS flgR::kan-rpsL* (DRH1763), were then electroporated with the plasmids containing specific *flgR* mutations described above. All mutants were recovered on MH agar containing streptomycin and verified by PCR and DNA sequencing.

81-176 Sm^R Δ*astA flgR*(*D51A*) *flgDE2::nemo* (SNJ602) and 81-176 Sm^R Δ*astA flgR*(*D51A*) *flaB::astA* (SNJ605) were complemented *in trans* with wild-type *flgR* on plasmid pDRH818 by conjugation via *E. coli* strain DH5α/pRK212.1 (78). Transconjugants were selected on MH agar containing chloramphenicol and kanamycin and verified by PCR.

flgR from *Helicobacter pylori* strain 26695 was amplified from chromosomal DNA (a kind gift from D. Scott Merrell, Uniformed Services University of the Health Sciences) by PCR. Primers were designed so that in-frame BamHI sites were fused 5' to the second codon and 3' to the stop codon. The PCR product was digested with BamHI and ligated into BamHI-digested pECO102 (77, 78) so that *H. pylori flgR* is expressed from the constitutive chloramphenicol acetyltransferase (*cat*) promoter. Clones were verified by sequencing and used to transform *E. coli* strain DH5α/pRK212.1 for conjugation into 81-176 Sm^R Δ*astA* Δ*flgR flgDE2::nemo* and 81-176 Sm^R Δ*astA* Δ*flgR flaB::astA* (DRH830 and DRH842, respectively; 65) and 81-176 Sm^R Δ*astA* Δ*flgS* Δ*flgR flgDE2::nemo* (SNJ807) and 81-176 Sm^R Δ*astA* Δ*flgS* Δ*flgR flaB*::*astA* (SNJ808). Transconjugants were selected on MH agar containing TMP, kanamycin, and chloramphenicol and were verified by PCR.

To construct *flgS(H141)* mutants, pDRH310 (78) was subjected to PCR-mediated mutagenesis (125) to mutate the histidine codon at position 141 to one for alanine and then verified by DNA sequence analysis. One plasmid,

pDRH1276, was recovered and introduced into 81-176 Sm^R *flgS*::*cat-rpsL* (DRH441; 78) and 81-176 Sm^R Δ*astA* *flgS*::*cat-rpsL* (DRH460; 78) by electroporation. Mutants were recovered on MH agar containing streptomycin and verified by PCR analysis and DNA sequencing. Mutants used for further analysis were designated DRH1323 [81-176 Sm^R *flgS(H141A)*] and SNJ947 [(81-176 Sm^R Δ*astA* *flgS(H141A)*].

We replaced native *flgR* with the $flgR_{\Delta receiver}$ and $flgR_{\Delta CTD}$ alleles in flagellar export apparatus mutants. For Δ*fliP*, Δ*flhA*, and Δ*flhB* mutants, *flgR::kan-rpsL* (pDRH443) was electroporated into strains 81-176 Sm^R Δ*astA* Δ*fliP* (DRH1016), 81-176 Sm^R Δ*astA* Δ*flhA* (DRH979), and 81-176 Sm^R Δ*astA* Δ*flhB* (DRH1734) (65). The resultant strains 81-176 Sm^R Δ*astA* Δ*fliP* *flgR::kan-rpsL* (SNJ158), 81-176 Sm^R Δ*astA* Δ*flhA* *flgR::kan-rpsL* (DRH1765), and 81-176 Sm^R Δ*astA* Δ*flhB* *flgR::kan-rpsL* (DRH1830) were electroporated with pDRH1855 and pDRH1856 containing the $flgR_{\Delta receiver}$ and $flgR_{\Delta CTD}$ alleles (92) respectively. All transformants were selected on MH agar with streptomycin and verified by PCR and DNA sequencing.

C. jejuni Δ*fliI* mutants were constructed by first cloning the *fliI* locus into pUC19 (to generate pDRH1453) and then cloning a SmaI-digested *kan-rpsL* cassette (from pDRH427; 78) into a PmeI site within the *fliI* coding sequence to generate pDRH1506. pDRH1506 was introduced into 81-176 Sm^R Δ*astA* (DRH461; 65) by electroporation, generating 81-176 Sm^R Δ*astA* *fliI*::*kan-rpsL*

(DRH2246), which was recovered on MH agar with kanamycin. pDRH1453 was then used in PCR-mediated mutagenesis (125) to delete a large portion of the coding sequence of the gene by fusing codon 4 to codon 453, creating pDRH1843. DRH2246 was then electroporated with pDRH1843 to replace *fliI*::*kan-rpsL* with the Δ*fliI* allele to create 81-176 Sm^R Δ*astA* Δ*fliI* (DRH2257).

Generation of *flhB* mutants first involved PCR-mediated mutagenesis (125) to create a point mutation, generating a StuI site in the coding sequence of *flhB* in pDRH666 (78) to create pSNJ355. This plasmid was then digested with StuI so that a *cat-rpsL* cassette generated by digestion of pDRH265 (76) with SmaI could be inserted with *flhB*. The resulting plasmid, pSNJ360, was then introduced into DRH461 (81-176 Sm^R Δ*astA*; 78) by electroporation, replacing *flhB* with *flhB*::*cat-rpsL* to generate SNJ404 (81-176 Sm^R Δ*astA flhB*::*cat-rpsL*). PCR-mediated mutagenesis (125) with pDRH666 was used to generate point mutants and in-frame deletions within *flhB*. These mutations and the resulting plasmids include *flhB(N267A)* (pSNJ238), $flhB_{\Delta 214\text{-}218}$ (pSNJ243), $flhB_{\Delta 224\text{-}228}$ (pSNJ236), and $flhB_{\Delta 244\text{-}253}$ (pSNJ237). These plasmids were introduced into SNJ404 by electroporation to replace the *flhB*::*cat-rpsL* allele with the respective *flhB* alleles. Mutants were recovered on MH agar with streptomycin. The resulting strains included SNJ438 [(81-176 Sm^R Δ*astA flhB(N267A)*], SNJ464 (81-176 Sm^R Δ*astA* $flhB_{\Delta 214\text{-}218}$), SNJ428 (81-176 Sm^R Δ*astA* $flhB_{\Delta 224\text{-}228}$), and

SNJ475 (81-176 Sm^R Δ*astA* $flhB_{\Delta 244\text{-}253}$). Mutants were verified by PCR and DNA sequencing.

To generate an in-frame *fliE* deletion, pDRH275, a plasmid containing the *flgBCfliE* locus cloned into the BamHI site of pUC19, was subjected to PCR-mediated mutagenesis to generate a StuI site in the *fliE* gene, creating pSNJ822 (125). The *cat-rpsL* fragment was obtained by digesting pDRH265 (76) with SmaI and ligating the cassette into StuI-digested pSNJ822. The *fliE* allele of DRH461 (81-176 Sm^R Δ*astA*) was replaced with the *fliE::cat-rpsL* construct in pSNJ878 by electroporation. Transformants were selected on MH agar with chloramphenicol and verified by PCR. PCR-mediated mutagenesis was used on pSNJ822 to create a deletion construct of *fliE*, fusing codon 1 to codon 80. Following sequencing of the resulting plasmid, pSNJ918, this construct was used in electroporation of SNJ907 (81-176 Sm^R Δ*astA fliE::cat-rpsL*). 81-176 Sm^R Δ*astA* Δ*fliE* transformants were selected on MH agar with streptomycin. Mutants were verified by PCR and DNA sequencing.

pDRH275 was also used to generate a construct to interrupt *flgB*. The plasmid was digested with StyI and SmaI-digested *cat-rpsL* fragment from pDRH265 (76) was ligated into the site. The resulting plasmid, pDRH2662, was used for electroporation of DRH461 (81-176 Sm^R Δ*astA*). Mutants were selected on MH agar with chloramphenicol and verified by PCR. PCR-mediated mutagenesis was performed to generate in-frame deletions of *flgB* and *flgC* (125).

In *flgB*, codon 1 was fused to codon 133 (generating pSNJ919) and in *flgC*, codon 1 was fused to codon 154 (generating pSNJ1009). SNJ1044 (81-176 Sm^R Δ*astA* *flgB::cat-rpsL*) was electroporated with both of these constructs to create 81-176 Sm^R Δ*astA* Δ*flgB* and 81-176 Sm^R Δ*astA* Δ*flgC*. Mutants were selected on MH agar containing streptomycin and were verified by PCR and DNA sequencing.

The *flgFG* locus was cloned into pDRH1349 and verified by sequencing. To disrupt the *flgF* gene, pUC19::*flgFG* (DRH1349) was subjected to PCR-mediated mutagenesis (125) to create an EcoRV site. This plasmid, pDRH2528, was digested with EcoRV and the *cat-rpsL* fragment from pDRH265 (76) was cloned into this site. The resultant plasmid, pDRH2534, was used in the elecroporation of 81-176 Sm^R (DRH212; 76) and 81-176 Sm^R Δ*astA* (DRH461; 78) to generate 81-176 Sm^R *flgF*::*cat-rpsL* (DRH2550) and 81-176 Sm^R Δ*astA* *flgF*::*cat-rpsL* (SNJ931-932), which were selected on MH agar with chloramphenicol and verified by PCR. PCR-mediated mutagenesis (125) was used to fuse codon 1 to codon 257 and this plasmid, pDRH2504, was used to electroporate DRH2550, creating 81-176 Sm^R Δ*flgF* (SNJ922). Mutants were selected on MH agar with streptomycin and verified by PCR and DNA-sequencing

Disruption of *flgG* also utilized pDRH1349. The plasmid was digested with BglII and the *cat-rpsL* fragment from pDRH265 (76) was cloned into this site. The resulting plasmid, pDRH2566, was used to electroporate 81-176 Sm^R

(DRH212; 76) and 81-176 Sm^R Δ*astA* (DRH461; 78). 81-176 Sm^R *flgG*::*cat-rpsL* (DRH2560) and 81-176 Sm^R Δ*astA flgG*::*cat-rpsL* (DRH2623) were selected on MH agar with chloramphenicol and verified by PCR. The *flgG* deletion plasmid was constructed by PCR-mediated mutagenesis of pDRH1349, and involved removing the entire open reading frame. This plasmid, pDRH2425, was used to electroporate DRH2623. Transformants were selected on MH agar with streptomycin and the 81-176 Sm^R Δ*astA* Δ*flgG* (SNJ925) strain was verified by PCR and DNA sequencing.

flgI was cloned into pUC19 to generate pDRH1348, digested with EcoRV, and the *cat-rpsL* fragment from pDRH265 (76) and the *kan-rpsL* fragment from pDRH437 (78) were cloned into this site. pDRH2536, containing *flgI::cat-rpsL*, was used to generate 81-176 Sm^R *flgI::cat-rpsl* (DRH2701) from 81-176 Sm^R (DRH212; 76). pDRH2658, containing *flgI::kan-rpsL*, was used to generate 81-176 Sm^R Δ*astA flgI::kan-rpsL* (DRH2776) from 81-176 Sm^R Δ*astA* (DRH461; 78)

pUC19::*flgH* (pDRH1878) was digested with PmlI and the *cat-rpsL* fragment from pDRH265 (76) was cloned into this site. The resulting plasmid, pALU101, was used in electroporation of 81-176 Sm^R (DRH212; 76) and 81-176 Sm^R Δ*astA* (DRH461; 78). 81-176 Sm^R *flgH::cat-rpsL* (ALU103) and 81-176 Sm^R Δ*astA flgH::cat-rpsL* (ALU107) were selected on MH agar with chloramphenicol and verified by PCR. PCR-mediated mutagenesis (125) was

used to delete the entire *flgH* open-reading frame, and the plasmid generated, pDRH2422, was electroporated into ALU103 and ALU107 to produce 81-176 Sm^R Δ*flgH* (DRH2449) and 81-176 Sm^R Δ*astA* Δ*flgH* (DRH2468) which were selected on MH agar with streptomycin and verified by PCR and DNA-sequencing.

flgE was cloned into pBR322 (pDRH2464) and digested with SwaI. The *cat-rpsL* fragment from pDRH265 (76) was cloned into this site and the resulting plasmid, pDRH2502, was used in electroporation of 81-176 Sm^R (DRH212; 76) and 81-176 Sm^R Δ*astA* (DRH461; 78). 81-176 Sm^R Δ*astA flgE::cat-rpsL* (DRH2610) and 81-176 Sm^R Δ*astA flgE::cat-rpsL* (SNJ928) were selected on MH agar with chloramphenicol and verified by PCR.

The *flgB::astA* transcriptional reporter was generated by digesting pDRH275, a plasmid containing the *flgBCfliE* locus cloned into the BamHI site of pUC19, with StyI to generate blunt ends. pDRH580 was digested with SmaI to liberate the *astA*-kan cassette (78), which was then ligated into digested pDRH275. DH5a transformants were selected on LB agar containing kanamycin and analyzed by PCR and DNA sequencing.

To construct strains containing transcriptional reporters, plasmids pDRH532 (containing *flgDE2::nemo*), pDRH608 (containing *flaA*::*astA*), pDRH610 (containing *flaB::astA*), pDRH669 (containing *flgD*::*astA*), and pSNJ1051 (*flgB::astA*) were electroporated into *C. jejuni* to replace the native

flgDE2, *flaA*, *flaB*, or *flgD* loci on the chromosome as previously described (78, 178). All mutants were recovered on MH agar containing kanamycin and verified by PCR analysis.

Bioinformatics analyses. Protein homology searches and analyses were performed using BLASTP, BLAST2 and ClustalW2 programs (http://www.ncbi.nlm.nih.gov/BLAST; http://www.ebi.ac.uk/Tools/clustalw2/index.html). HTHs motifs were identified using PBIL-IBCP Gerland (http://pbil.ibcp.fr/htm/index.php).

Generation of polyclonal antiserum against *C. jejuni* proteins. Generation of polyclonal murine antiserum against the RNA polymerase subunit A (RpoA) protein of *C. jejuni* involved first constructing primers with 5' BamHI sites to amplify the coding sequence starting at codon 2 through the stop codon of *rpoA* from *C. jejuni* strain 81-176 (81). Ligation of this DNA fragment into the BamHI site of pQE30 (QIAGEN) and transformation into *E. coli* XL1-Blue allowed for the recovery of pDRH2907, which encodes a 6XHis-RpoA fusion protein. To purify the protein, 1 L of LB was grown to OD_{600} of 0.5 and then the culture was induced for 4 h with 1 mM IPTG. The bacteria were disrupted by passaging twice through an EmulsiFlex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2. The protein was purified under native conditions from the soluble fraction with Ni-

NTA agarose according to the manufacturer's instructions. Polyclonal murine antiserum was generated in mice by standard procedures using a commercial vendor (Cocalico Biologicals).

Detection of FlhB in *C. jejuni* required the generation of rabbit polyclonal antiserum against the cytoplasmic domain of the protein. Because this portion of FlhB in *Salmonella typhimurium* undergoes autoproteolytic processing between the asparagine and proline residues at positions 269 and 270 (54, 133), we attempted to create a soluble, more stable protein to immunize rabbits for antiserum generation. We first used PCR-mediated mutagenesis (125) with pDRH666 (containing the wild-type *flhB* allele; 78) to change the codons for asparagine and proline at positions 267 and 268, respectively, to alanines to generate pDRH2339. After construction of pDRH2339, we then amplified a portion of the *flhB(N267A P268A)* sequence encoding amino acids 209 through 369 that encompasses the predicted entire unprocessed cytoplasmic domain of the protein. Primers were used in PCR so that in-frame BamHI sites were added to the 5' ends of the amplified product. The DNA was then cloned into BamHI-digested pGEX-4T-2 (GE Healthcare) in the correct orientation to produce a GST-$FlhB_{cyto}$ (N267A P268A) fusion protein. The resulting plasmid was designated pDRH2367 and used to transform BL21 (DE3). The resulting strain was grown in 3 L of LB to mid-log phase and then induced with 25 mM IPTG for 3 h at 37 °C. The bacteria were harvested and disrupted by an EmulsiFlex-C5 cell

disrupter (Avesin) at 15,000 to 20,000 lb/in^2. The soluble fraction was obtained by removing the insoluble material by centrifugation at 13,000 rpm for 2 h at 4 °C. The soluble material was rocked with 2.4 ml of glutathione Sepharose 4B (GE Healthcare) for 30 min at room temperature. The protein was then purified according to the manufacturer's instructions. Despite our attempt to create a more stable, unprocessed version of the cytoplasmic domain of FlhB fused to GST, about one third of the recovered purified protein was approximately 29 kDa after SDS-PAGE analysis, which correlates with the size of GST rather than 46 kDa, the predicted size of the full-length fusion protein. Therefore, this protein was only partially stable. These purified products were used to immunize rabbits by standard procedures for antiserum generation using a commercial vendor (Cocalico Biologicals).

Immunoblotting analyses of FlgS, FlgR, FlhB, and FlaA proteins. *C. jejuni* strains were grown from frozen stocks on MH agar containing appropriate antibiotics at 37 °C for 48 h and restreaked 16 h prior to use. SDS-PAGE and immunoblotting of FlgS was performed as previously described with α-FlgS Rab11 or α-FlgR Rab13 rabbit polyclonal antiserum, respectively (73, 75). Briefly, cells were resuspended from 16 h growth plates in MH broth and diluted to OD_{600} of 0.8. One milliliter samples were harvested by centrifugation and washed once with PBS. For whole-cell lysates (WCL), the pellet was

resuspended in 50 ml 1X Laemmli buffer, and 4 ml (for FlgR analysis) or 7 ml (for FlgS analysis) of each resuspended pellet was loaded onto 10% SDS-PAGE gels.

For FlgS localization studies, 5 ml of culture of wild-type and mutant strains at an OD_{600} of 0.8 were prepared as described above, resuspended in 10 mM HEPES, pH 7.4 and broken by sonication. Unbroken cells were removed by centrifugation at 13,000 x g for 5 min at 4 °C, the supernatant was transferred to a new tube and centrifuged at 13,000 x g for 30 min at 4 °C to pellet the total membrane fraction (outer and inner membrane proteins). The supernatant contained soluble proteins (cytoplasmic and periplasmic proteins). Volumes representing equivalent cell numbers of the membrane and soluble proteins were analyzed by 10% SDS-PAGE gel after resuspension and boiling in 1X Laemmli buffer. For detection of FlgS, α-FlgS Rab11 antiserum was used at a dilution of 1:10,000 (73). To detect proteins representative of the cytoplasmic fraction or inner membrane fraction, we analyzed the location of the RpoA cytoplasmic protein and the AtpF inner membrane protein by using α-RpoA M59 antiserum at a dilution of 1:2000 and α-AtpF M3 antiserum at a dilution of 1:1000 (15), followed by a goat a-mouse secondary antibody.

To monitor the stability and location of FlhB proteins, bacteria were grown and 5 ml samples of cultures from wild-type and mutant strains were prepared and sonicated as described above. The total membrane fraction,

containing inner and outer membrane proteins, was recovered by centrifugation at 13,000 rpm for 30 min at 4 °C. The recovered pellet was suspended in 50 ml of 1X Laemmli buffer and loaded onto a 10% SDS-PAGE gel for immunoblot analysis. Primary α-FlhB Rab476 antiserum was used at a concentration of 1:1000 and was rocked with the membrane overnight at 4 ºC. The blot was then washed and incubated with 1:10,000 goat α-rabbit secondary antibody for 4 h at room temperature.

For analysis of FlaA secretion in *C. jejuni* strains, bacteria were grown and resuspended from plates as described above. WCLs from 1 ml of cultures of wild-type and mutant strains were prepared as described above. For recovery of outer membrane proteins, 5 ml cultures of each bacterial strain were prepared and sonicated and the unbroken cells were removed by brief centrifugation at 13,000 rpm for 5 min at 4 °C. The supernatant was recovered and spun at 13,000 rpm for 30 min at 4 °C. The pellet containing insoluble material representing total membrane proteins (inner and outer membrane proteins) was resuspended in 1% *N*-laurylsarcosine sodium salt and incubated for 30 minutes at room temperature to solublize inner membrane proteins. The outer membrane proteins were recovered as the insoluble pellet after centrifugation at 13000 rpm for 30 minutes at 4 °C. Volumes of sample corresponding to 200 ml or 700 ml of bacteria were loaded for analysis of WCLs or outer membrane proteins, respectively.

Immunoblot analysis was performed with 1:10,000 dilution of α-FlaA LLI antiserum (119) and 1:10,000 dilution of goat α-rabbit secondary antibody.

Arylsulfatase reporter assays. Arylsulfatase transcriptional reporter assays were performed as previously described (78). *C. jejuni* strains were grown from frozen stocks on MH agar containing TMP or kanamycin and chloramphenicol as necessary for 48 hours under microaerobic conditions and then restreaked and grown for 16 hours prior to the assay. Strains were resuspended from agar plates and diluted to an OD_{600} of 0.7 to 1.0, washed in arylsulfatase assay buffer and then incubated with 10 mM nitrophenolsulfate and 1 mM tyramine for 1 hour at 37ºC. The assay was terminated with 0.2 N NaOH and the amount of nitrophenol released in each sample was determined by measuring the absorbance of the sample at OD_{410}. The amount of nitrophenol in each sample was determined by comparing the OD_{410} readings of each sample to those from a standard curve of known nitrophenol concentrations to obtain the number of arylsulfatase units produced by each strain. One arylsulfatase unit is defined as the amount of enzyme catalyzing the release of 1 nmol of nitrophenol per hour per OD_{600} 1. Each strain was tested in triplicate and each assay was performed three times.

Motility assays. To compare the respective motility phenotypes of our *C. jejuni* 81-176 derivatives, strains were grown from freezer stocks on MH agar

containing TMP for 48 hours, then restreaked and grown for 16 hours prior to use. Each strain was resuspended in MH broth to an OD_{600} of 1.0, and a sterile needle was used to inoculate semi-solid MH motility media containing TMP. Plates were incubated at 37˚C under microaerobic conditions for 24 to 72 hours prior to analysis.

Transmission electron microscopy. To examine wild-type and *flgR* mutant strains for the production of flagella, strains were grown from freezer stocks onto MH agar containing TMP for 48 hours and then restreaked and grown for 16 hours prior to study. Strains were resuspended from plates in MH broth to an OD_{600} of 1.0 and resuspended in a 2% (v/v) gluteraldehyde solution for fixation. Fixation was completed by incubating strains for 1 hour on ice. All samples were stained with 1% (w/v) uranyl acetate and visualized with a JOEL 1200X transmission electron microscope at 80 kV.

Purification of FlgR, FlgS, FlhA, FlhB, and FliE proteins. Wild-type *flgR* and $flgR_{\Delta receiver}$, $flgR_{\Delta CTD}$, $flgR_{central}$, and *flgR*(*D51A*) were amplified by PCR. Primers for each mutant were designed so that a 5' BamHI site was added in-frame. The other primer to amplify each mutant contained codons for an in-frame 6XHis-tag followed by a stop codon and a BamHI site. After amplification, the DNA fragments were digested with BamHI and ligated into BamHI-digested pT7-7.

For purification, 500 ml LB broth was inoculated with BL21(DE3)pLysE containing each of the expression constructs and grown at 37 ºC to an OD_{600} of 0.4, induced with 1 mM IPTG, and incubated at 30˚C for an additional 4 hours. Bacteria were harvested and passaged twice through an EmusliFlex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in^2. Ni-NTA agarose beads were used to purify the proteins under native conditions, per instruction of the manufacturer (Qiagen). Eluted fractions were analyzed by 10% SDS-PAGE and the eluate fractions containing the highest purity of FlgR proteins were combined for dialysis against a buffer containing 10 mM Tris-HCl, 50 mM KCl, 5% (v/v) glycerol, 0.1 mM EDTA and 1 mM DTT. FlgS was purified as previously described (73) and the eluate fractions were dialyzed against a buffer containing 50 mM Tris-HCl pH 7.5, 100 mM KCl, 25% (v/v) glycerol, and 1 mM DTT. Following dialysis, the concentration of each protein was determined by a Bradford assay and aliquots of each were frozen at –80˚C. To minimize contamination with free phosphates, we used ultra-pure water (Fluka) in all buffers and reagents for purification. *flgS(H141A)* was cloned into pQE30 with the same primers and conditions as *flgS* (73) and was purified in an identical manner.

Purification of the cyoplasmic domains of FlhA and FlhB is described above. These constructs and conditions were used for $FlhA_{cyto}$ and $FlhB_{cyto}$ purification. *fliE* was amplified by PCR and cloned into BamHI-digested pGEX-

4T-2 (GE Healthcare) in the correct orientation to produce a GST-FliE fusion. This protein was purified under the same conditions as GST-$FlhA_{cyto}$.

Autophosphorylation of FlgS. FlgS autophosphorylation assays were performed as described previously using purified 6XHis-FlgS or 6XHis-FlgS(H141A) in the presence of [γ-^{32}P]ATP (92, 210). Briefly, 6 pmol 6xHis-FlgS or 6XHis-FlgS(H141A) was added to a buffer containing 50 mM Tris-HCl, pH 8.0, 75 mM KCl, 2 mM MgCl2, and 1 mM DTT. Ten μCi [γ-^{32}P]ATP was then added. At each time point, a sample was removed and the reaction was stopped by the addition of an equal amount of 2X SDS-PAGE loading buffer. Proteins were resolved on 10% SDS-PAGE and the gels were dried and exposed to a PhosphorImage screen. The screen was read on Storm 820 phosphoimager (Amersham Biosciences) and the data were analyzed using the manufacturer's software.

***In vitro* phosphorylation assays of FlgR by FlgS.** To analyze the ability of FlgS to phosphorylate wild-type and mutant FlgR proteins, we adapted a previously published protocol (210). Six pmol FlgS-6XHis was incubated with 10 μCi [γ-^{32}P]ATP for 15 minutes in a buffer containing 50 mM Tris-HCl pH 8.0, 75 mM KCl, 2 mM $MgCl_2$, and 1 mM DTT. Six pmol of each FlgR-6XHis construct was added to the reaction and incubated for 2 minutes, then stopped by the addition of

2X Laemmli buffer. Samples were analyzed by 10% SDS-PAGE. Following drying of the gels, they were autoradiographed by a Storm 820 Phosphoimager (Amersham Biosciences). Data were analyzed using the manufacturer's software.

DNA-binding assays. Mobility-shift assays were based on the protocols of Porter *et al*. (162) and McIver *et al*. (129). Briefly, DNA fragments corresponding to base pairs 1-250 or 250-500 upstream of the start codon of *C. jejuni* genes *flgDE2* and *flaB* were generated by PCR and 5' end-labeled using T4 polynucleotide kinase (Epicentre). Labeled fragments were purified following electrophoresis in 5% acrylamide gels. DNA probes equivalent to approximately 2000 counts per minute were used in each binding reaction. Either phosphorylated or unphosphorylated wild-type FlgR protein was used in a concentration of up to 10 μg per reaction. Phosphorylation of FlgR was performed with 5 μg FlgS with unlabeled ATP as described above. The phosphorylation reaction was added to a tube containing labeled probe and DNA-binding buffer. Reactions were incubated for 20 minutes at either 4º C or 37º C before electrophoresis on 5% acrylamide gels at 110 V for 3 hours.

Real-time reverse transcription-PCR (RT-PCR) analyses. 81-176 Sm^R (DRH212), 81-176 Δ*flhA* (DRH946), 81-176 Δ*flhB* (SNJ471), 81-176 Δ*fliP* (DRH1065), 81-176 Δ*flgR* (DRH737), 81-176 Δ*astA* Δ*fliE* (SNJ915), 81-176

Δ*astA* Δ*flgB* (SNJ1046), 81-176 Δ*astA* Δ*flgC* (SNJ1048) were grown from frozen stocks on MH agar containing appropriate antibiotics at 37 °C for 48 h and restreaked 16 h prior to use (76, 78). Bacteria were suspended from the agar plates in MH broth and total RNA was extracted from the bacteria with Trizol reagent (Invitrogen). The RNA was then treated with DNase prior to analysis. A final concentration of 50 ng/ml of RNA was used in a Sybr green PCR master mix. Real time RT-PCR was performed using a 7500 real-time PCR system (Applied Biosystems). Detection of mRNA for *gyrA*, encoding DNA gyrase, served as an endogenous control. Analysis of *flgS* and *flgR* transcripts was performed in mutants lacking *flhA*, *flhB*, or *fliP* and compared relative to the wild-type strain (DRH212). Transcript levels of *flgB*, *flgC*, and *fliE* were analyzed in mutants lacking *rpoN* (σ^{54}), *flgR*, *flhA*, *flhB*, *fliP*, *flgB*, *flgC*, and *fliE* and compared to the wild-type strain (DRH212). The following primer pairs were used for real-time RT-PCR analysis:

flgS RT#1, 5'-GCTACAGATATTAGCGATGAAAAACG-3', and

flgS RT#2, 5'-TAGGATTTCTTATCTCATGTGCCAAAT-3';

flgR RT#3, 5'-TCAAGCCAAACTTTTAAGAGCTTTG-3', and

flgR RT#4, 5'-CTATTTTGATGCTTTTCGTACTTCCA-3';

flgB RT #1, 5'-ACAGCTTTGGTAAATCGTGCAA-3', and

flgB RT #2, 5'-CAGGGAATTTCCAAGGTTTTTG-3';

flgC RT #1, 5'-CGTATCCAAATCTAAAGGGTGTAAA-3', and

flgC RT #2, 5'-CTTTGAGCTATAGTTTTTGCACTTGTAAA-3';

fliE RT #1, 5'-GGAGAAGCTGCAATGACAGACA-3', and

fliE RT #2, 5'-CATACTGCTTTCAGCTTTAGTGATAGC-3';

gyrA F, 5'-CGACTTACACGGCCGATTTC-3', and

gyrA R, 5'-ATGCTCTTTGCAGTAACCAAAAAA-3'.

Transposon mutagenesis. Chromosomal DNA from *C. jejuni* 81-176 Δ*astA* Δ*flhA flgDE2*::*nemo* (DRH1021; 78), 81-176 Δ*astA* Δ*flhB flgD*::*astA* (SNJ331) and 81-176 Δ*astA* Δ*fliP flaB*::*astA* (DRH1178; 78) was purified and subjected to *in vitro* random transposon mutagenesis with the *darkhelment* transposon by previously published protocols (75, 76, 78). Twelve *in vitro* transposon mutagenesis reactions were performed with DNA from each strain. Each reaction contained 2 mg of chromosomal DNA, 1 mg of pSpaceball1, and 250 ng of *Himar1* C9 transposase purified from DH5a/pMalC9 (3). After transposition, the mutagenized DNA was repaired and transformed into each respective strain as previously described (76). Transposon mutants were recovered after growth on MH agar containing chloramphenicol and 5-bromo-4-chloro-3-indolyl sulfate (X-S) and then examined for blue or white colony phenotypes.

CHAPTER FOUR

THE *CAMPYLOBACTER JEJUNI* FLGR PROTEIN IS AN UNUSUAL MEMBER OF THE NTRC-FAMILY OF PROTEINS

Introduction

The NtrC-like group of proteins includes a number of response regulators that are involved in regulating various intracellular activities. The members of this class of response regulators mediate σ^{54}-dependent gene expression by utilizing the energy released from ATP hydrolysis to open transcriptional complexes. Within a single bacterium, several response regulators of this class may be required for diverse functions that include nitrogen metabolism, virulence, and flagellar motility. Binding of the response regulator to upstream activation sequences ensures that only the appropriate σ^{54}–dependent genes are transcribed. Sequence analyses indicates that *C. jejuni* and the closely related *H. pylori* each only encode one response regulator thought to function in the activation of σ^{54}, and that is the flagellar response regulator, FlgR.

Results

Comparative analysis of the domain architecture of NtrC and NtrC-like flagellar response regulators

The members of the NtrC family of response regulators are generally modular in structure with each domain contributing to transcriptional activation under the proper conditions. Based on its homology to this class of proteins, FlgR can be divided into three putative domains (Figure 3). The N-terminal domain (amino acids 1-131) resembles a typical response regulator receiver domain, which is often phosphorylated at a specific aspartic acid residue to activate the protein. The central domain of FlgR is homologous to the highly conserved central domain of NtrC family members that hydrolyzes ATP and interacts with the σ^{54} RNAP holoenzyme (reviewed in 186). Most NtrC family members also possess a CTD containing a helix-turn-helix (HTH) motif, necessary for DNA-binding to activate transcription of target genes (30, 84). Bioinformatics analyses comparing the NtrC proteins of *E. coli* and *S. typhimurium* and the FlgR homologues in *P. aeruginosa* (FleR) and *V. cholerae* (FlrC) demonstrate a highly predicted HTH in the CTDs of these proteins (Figure 3). However, analyses of *C. jejuni* FlgR with these proteins suggest an absence of homology between its CTD and those of other NtrC-like proteins. Furthermore, the FlgR CTD does not appear to contain a predicted HTH structure or any other DNA-binding motif, which may imply that this domain of FlgR does not function in DNA interactions. Similar to a previous study, alignment of the FlgR protein of *H. pylori* with these proteins shows that it is prematurely truncated and completely lacks the CTD and presumably a DNA-binding motif (Figure 3; 19).


Receiver Domain
Walker A
Central Domain
Walker B
CTD
HTH

Figure 3. ClustalW alignment of the amino acid sequence of the *C. jejuni* strain 81-176 FlgR protein with those of FlgR homologues and NtrC proteins of other bacteria. The predicted receiver domain (black line), central domain (blue line), and C-terminal domain (CTD; red line) are indicated with a line above the domains. The conserved aspartic acid residue in the receiver domain of each protein that is predicted or has been shown to be phosphorylated by the respective cognate sensor kinase is boxed and indicated with an arrow. The predicted Walker A and Walker B motifs of the ATPase domain in the central domain of each protein are outlined in blue boxes. The helix-turn-helix motifs of the C-terminal domains that have been shown or are predicted to function in DNA binding are boxed in red. Conserved residues are indicated with an asterisk (*); highly conserved residues are indicated by a colon (:); and semi-conserved residues are indicated by a dot (.). Proteins (and GenBank accession numbers) included for analysis are: *C. jejuni* strain 81-176 FlgR (Cj_FlgR; YP_001000703.1); *H. pylori* strain 26695 (Hp_FlgR; NP_207497); *V. cholerae* strain O395 (Vc_FlrC; YP_001217658); *P. aeruginosa* strain PA01 (Pa_FleR; NP_249790); *S. typhimurium* strain LT2 (St_NtrC; NP_003197.1); and *E. coli* strain K-12 (Ec_NtrC; P0AFB8).

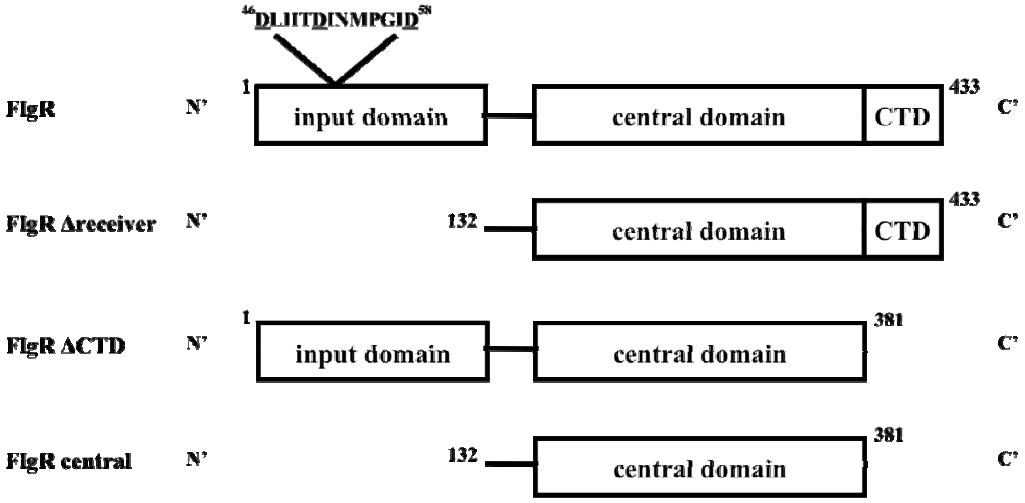

Figure 4. Proposed domains of *C. jejuni* FlgR and diagram of FlgR mutant proteins. Like other well-characterized NtrC family members, FlgR can be divided into three putative modular components: an N-terminal receiver domain, a central domain that has highly conserved domains for ATP hydrolysis and

interactions with σ^{54}, and a C-terminal domain (CTD) that unlike most of other NtrC-like proteins lacks a typical DNA-binding motif. Shown (from top to bottom) are the wild-type *C. jejuni* FlgR protein, $FlgR_{\Delta receiver}$ protein, $FlgR_{\Delta CTD}$ protein, and $FlgR_{central}$ protein that were created and analyzed in this study. Residues 46 through 58 of the receiver domain with the three aspartic acid residues that were altered by site-directed mutagenesis are shown.

FlgR activation is dependent on the phosphorylation of residue D51

Based on the known properties of NtrC-family proteins and the putative features of *C. jejuni* FlgR, we analyzed alterations of the N-terminal receiver domain and the CTD to better understand how activity of the protein is controlled. Alignment of the FlgR receiver with other σ^{54}-dependent response regulators suggested that D51 may be the site of phosphorylation (Figure 4). We performed site-directed mutagenesis to individually change this residue to an alanine. We noticed two other aspartic acids, D46 and D58, present in the same region of the protein that due to their vicinity to D51, could be alternative sites of phosphorylation. Mutants were constructed that changed these residues to alanines to eliminate the possibility that amino acid substitutions in this subregion of the receiver domain could cause non-specific structural changes that result in the inactivation of FlgR. The *flgR* mutants were used to replace wild-type *flgR* on the chromosome of *C. jejuni* 81-176 Sm^R (DRH212; 76) and 81-176 Sm^R Δ*astA* (DRH461; 78). This latter strain is used as the genetic background for analysis of expression of two σ^{54}-dependent flagellar genes, *flaB*, encoding a minor flagellin, and the *flgDE2* operon, encoding flagellar hook-associated proteins. In *C. jejuni*,

these mutant FlgR proteins are stable and produced at levels similar to wild-type FlgR (Figure 5A). Examination of mutants producing FlgR(D46A) or FlgR(D58A) revealed no effect on motility, flagellar biosynthesis, or expression of σ^{54}-dependent flagellar genes (Figures 5B-D). However, alteration of residue D51 of FlgR to an alanine results in a *C. jejuni* mutant that is incapable of promoting motility (Figure 5B), producing flagella (Figure 5C), and activating σ^{54}-dependent flagellar gene expression (Figure 5D). These phenotypes of the *flgR*(*D51A*) mutant are similar to Δ*flgR* mutant with a deletion of codons 17-408 of the gene (Figures 5B-D; 78). We were able to complement all deficiencies of the *flgR*(*D51A*) mutant by introducing wild-type *flgR* on a multicopy plasmid (Figure 5D and data not shown), indicating that FlgR(D51A) was responsible for all observed phenotypes.

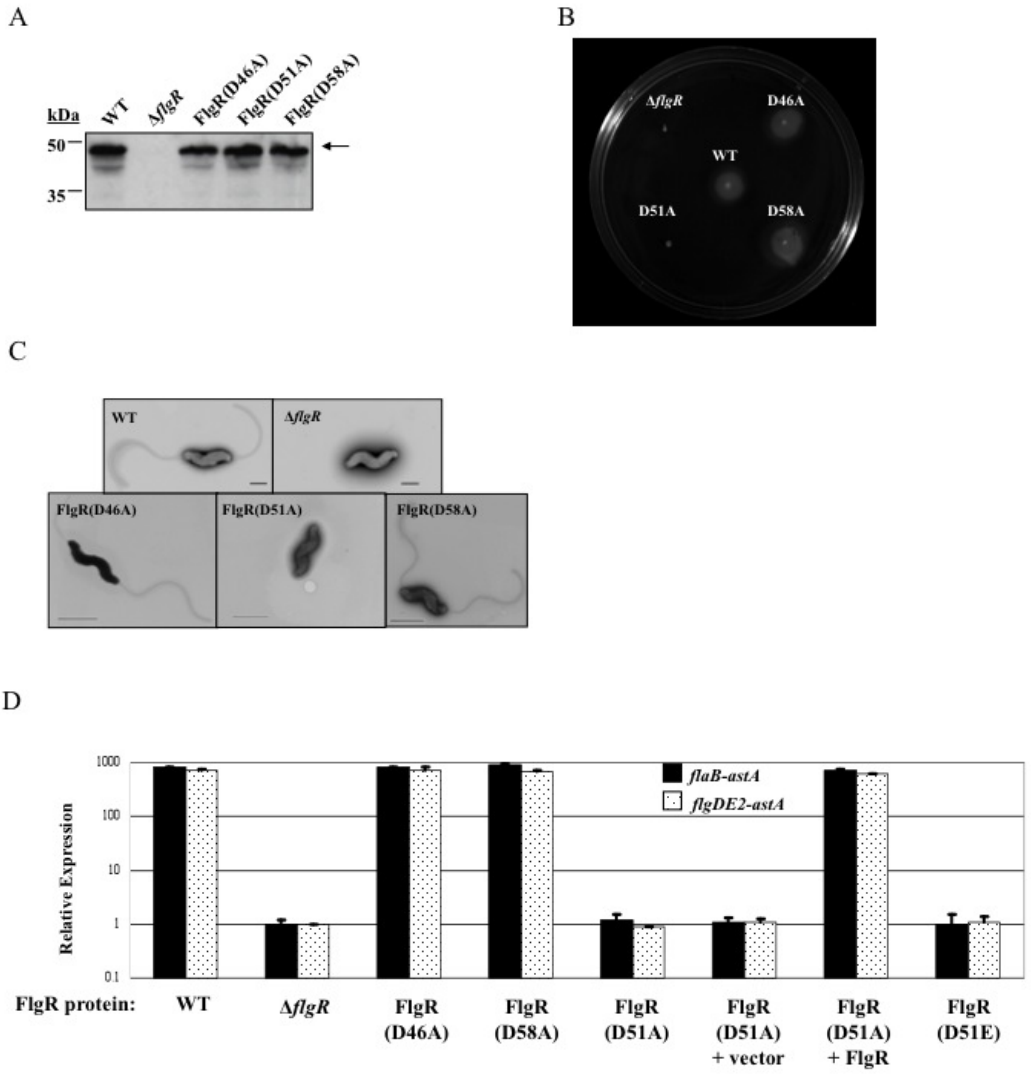

Figure 5. Phenotypic analysis of *C. jejuni* strains producing FlgR mutants. (A) Immunoblot analysis of whole-cell lysates of *C. jejuni* wild-type and mutant strains to detect FlgR proteins. The FlgR protein produced by each strain is listed above the blot. Arrow indicates wild-type and mutant FlgR proteins. (B) Motility phenotypes of *C. jejuni* strains producing wild-type or mutant FlgR proteins in MH semi-solid agar. Strains include DRH212 (81-176 Sm^R), DRH737 (81-176 Sm^R Δ*flgR*), SNJ737 (81-176 Sm^R *flgR(D46A)*), SNJ734 (81-176 Sm^R *flgR*(*D51A*)), SNJ738 (81-176 Sm^R *flgR(D58A)*). (C) Electron micrographs of *C. jejuni* wild-type and mutant strains for analysis of flagellar biosynthesis. All electron micrographs are at 16,000x magnification. The bar in WT and Δ*flgR* represents 0.5 μm and the bar in D46A, D51A, and D58A represents 1 μm.

Strains include DRH212 (81-176 Sm^R), DRH737 (81-176 Sm^R Δ*flgR*), SNJ737 (81-176 Sm^R *flgR D46A*), SNJ734 (81-176 Sm^R *flgR*(*D51A*)), and SNJ738 (81-176 Sm^R *flgR D58A*). (D) Arylsulfatase assays for analysis of expression of *flgDE2::nemo* and *flaB::astA* in *C. jejuni* 81-176 derivatives producing wild-type and FlgR mutant proteins. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in 81-176 Sm^R Δ*astA* Δ*flgR* which was set to 1 arylsulfatase unit. For expression of *flaB*::*astA* (filled bars), strains include DRH665, DRH842, SNJ655, SNJ660, SNJ605, SNJ721, SNJ723, and SNJ341. For expression of *flgDE2*::*nemo* (dotted bars), strains include DRH533, DRH830, SNJ650, SNJ658, SNJ602, SNJ718, SNJ634, and SNJ339. The FlgR protein produced by each strain is listed below the graph.

A previous study has shown that FlgS can mediate the *in vitro* phosphorylation of FlgR, but the phosphorylated residue was not identified (210). Our analyses described above strongly support D51 as the site of phosphorylation. *In vitro* phosphorylation assays were performed and demonstrated that wild-type FlgR but not FlgR(D51A) is phosphorylated in the presence of FlgS (Figure 6). This *in vitro* result confirms our *in vivo* genetic analyses and supports the hypothesis that D51 is the phosphorylated residue of FlgR.

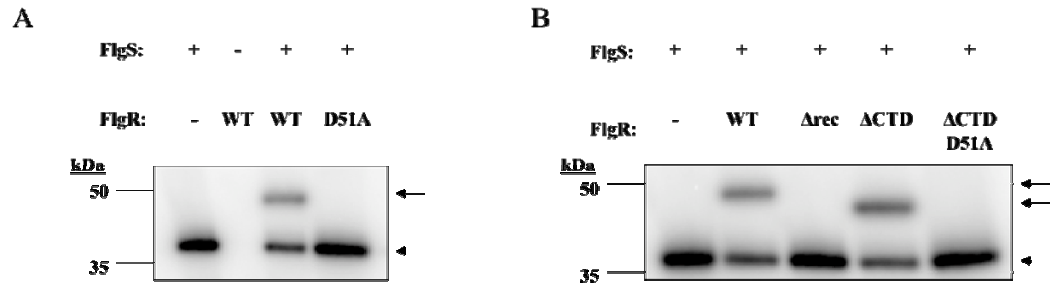

Figure 6. *In vitro* phosphorylation of FlgR proteins by FlgS. FlgR proteins were mixed with FlgS in the presence of [γ-^{32}P]ATP. After incubation, proteins were analyzed by 10% SDS-PAGE followed by autoradiography. (A) Analysis of phosphorylation of wild-type FlgR and FlgR(D51A). (B) Analysis of phosphorylation of wild-type FlgR, FlgR$_{\Delta receiver}$, FlgR$_{\Delta CTD}$, and FlgR(D51A)$_{\Delta CTD}$. The presence or absence of FlgS and the type of FlgR protein used in each reaction is shown above. The arrows indicate phosphorylated FlgR proteins and the arrowhead indicates the phosphorylated FlgS protein.

Glutamic acid substitution at the site of phosphorylation is not an effective strategy for generating a constitutively active FlgR protein.

Upon identification of D51 as the site of FlgR phosphorylation, we attempted to create a constitutively activated FlgR protein by substituting this residue with a glutamic acid as NtrC can be constitutively activated by a similar mutation at its phosphorylated residue, D54 (103). However, this type of substitution does not activate all NtrC-family proteins, as the D55E mutation of *Sinorhizobium meliloti* DctD inactivates the protein (130). We constructed a *flgR(D51E)* allele and used it to replace wild-type *flgR* on the chromosome of *C. jejuni*. The resultant strain produces a stable protein, but σ^{54}-dependent flagellar gene expression and motility are both fully ablated (Figure 5D and data not

shown). Thus, unlike NtrC, FlgR cannot be constitutively activated by glutamic acid substitution at the site of phosphorylation.

Evidence for dual activating and inhibitory functions of the FlgR receiver domain

To further understand how the domains of FlgR influence its activation as a transcriptional regulator, we performed a more global domain analysis of the FlgR protein. An N-terminal receiver domain is often necessary to influence the activity of NtrC-family proteins under appropriate conditions. Within the NtrC subfamily of response regulators the receiver domain can employ either positive or negative modes of regulation which are influenced by the phosphorylation state of the domain (reviewed in 57). For example, the unphosphorylated receiver domains of *S. meliloti* DctD and *Aquifex aeolicus* NtrC1 prevent intermolecular interactions between the central domains that would result in protein activity (118, 120). Thus, removal of the receiver domain of these proteins results in a constitutively active protein in the absence of phosphorylation while the full-length, unphosphorylated protein is held inactive. Phosphorylation of the receiver domain removes this inhibition allowing for oligomerization and ATPase activity, requirements for initiation of target gene transcription. In contrast, the receiver domain of NtrC of *S. typhimurium* positively regulates protein activation upon phosphorylation, as this modification induces conformational changes necessary

for function of the response regulator (37). In NtrC, the receiver domain is essential for activation and removal results in an inactive protein.

As described above, we have shown that phosphorylation of residue D51 is essential for full activation of FlgR to mediate expression of σ^{54}-dependent flagellar genes. Based on the shared homology between the FlgR proteins of *C. jejuni* and *H. pylori* and the domain architecture of the *H. pylori* FlgR protein described in Brahmachary *et al*. (19), we have designated amino acids 1-131 as the receiver domain. To characterize the means by which this domain influences FlgR activity, we generated an in-frame deletion of the N-terminal 131 amino acids of the protein and used this construct (*flgR*$_{\Delta receiver}$) to replace *flgR* at the native locus. As shown in Figure 7A, FlgR$_{\Delta receiver}$ is stable and produced at levels comparable to wild-type FlgR in *C. jejuni*. Analysis of *C. jejuni* producing FlgR$_{\Delta receiver}$ revealed that expression of σ^{54}-dependent reporter genes is approximately 10% of that observed in wild-type bacteria (Figure 5B). However, expression of these genes in the *flgR*$_{\Delta receiver}$ mutant is nearly 100-fold greater than in a Δ*flgR* mutant. Initial observation of the *flgR*$_{\Delta receiver}$ mutant showed a non-motile phenotype (Figure 7C). However, with prolonged incubation for up to three days, slight motility could be observed (data not shown), although this level of motility is of questionable significance. In addition, examination of over 100 bacteria by EM did not reveal any signs of flagellar biosynthesis (Figure 7D). Despite the motility and flagellar biosynthesis phenotypes, our observation that

the level of σ^{54}-dependent flagellar gene expression in 81-176 Sm^R *flgR*$_{\Delta receiver}$ is higher than 81-176 Sm^R Δ*flgR* indicates that the $FlgR_{\Delta receiver}$ is partially active despite lacking the phosphorylated N-terminal receiver domain.

As previously shown, a Δ*flgS* mutant producing wild-type FlgR is non-motile and incapable of activating σ^{54}-dependent flagellar gene expression, demonstrating that wild-type FlgR is inactive in the absence of FlgS and presumably requires phosphorylation to function in the activation of flagellar gene transcription (78, 210). Our data shown above strengthen this hypothesis as FlgR(D51A) is not phosphorylated in the presence of FlgS (Figure 6) and this protein does not support expression of σ^{54}-dependent flagellar genes (Figure 5D). Given that $FlgR_{\Delta receiver}$ lacks the phosphorylated residue, D51, but is still partially active when the cognate sensor kinase is present, we hypothesize that this activity is likely independent of phosphorylation. Indeed, in an *in vitro* phosphorylation assay, $FlgR_{\Delta receiver}$ is not phosphorylated in the presence of FlgS (Figure 6). Thus, the *in vivo* ability of $FlgR_{\Delta receiver}$ to activate flagellar gene expression is likely due to constitutive activity of this protein. If this is the case, then we would expect that unlike wild-type FlgR, $FlgR_{\Delta receiver}$ would activate expression of σ^{54}-dependent flagellar genes in a Δ*flgS* mutant. Therefore, we introduced *flgR*$_{\Delta receiver}$ into a Δ*flgS* background and compared the resulting mutant to a Δ*flgS* mutant (which produces wild-type FlgR). The level of σ^{54}-dependent flagellar gene expression in the Δ*flgS flgR*$_{\Delta receiver}$ mutant is 20- to 80-fold greater than in the

Δ*flgS* mutant (Figure 5E), demonstrating that removal of the N-terminal receiver domain of FlgR increases its activity compared to wild-type FlgR in the absence of FlgS. However, expression of flagellar genes in the Δ*flgS* $flgR_{\Delta receiver}$ mutant is 20 to 30-fold less than wild-type *C. jejuni* (producing both FlgS and FlgR; Figure 7E). In addition, this *flgR* deletion in the Δ*flgS* background does not support motility (data not shown). Considering these results, we propose that one function of the receiver domain is to inhibit FlgR activity in the absence of phosphorylation, and that this inhibition is partially relieved when the receiver domain is artificially removed. Ultimately, we believe the primary function of the receiver domain is to positively regulate the protein upon phosphorylation, since wild-type levels of expression of σ^{54}-dependent flagellar genes are only observed when the receiver domain of FlgR is intact and phosphorylated by FlgS. Thus, the receiver domain appears to have both stimulatory and inhibitory activities based on the state of phosphorylation.

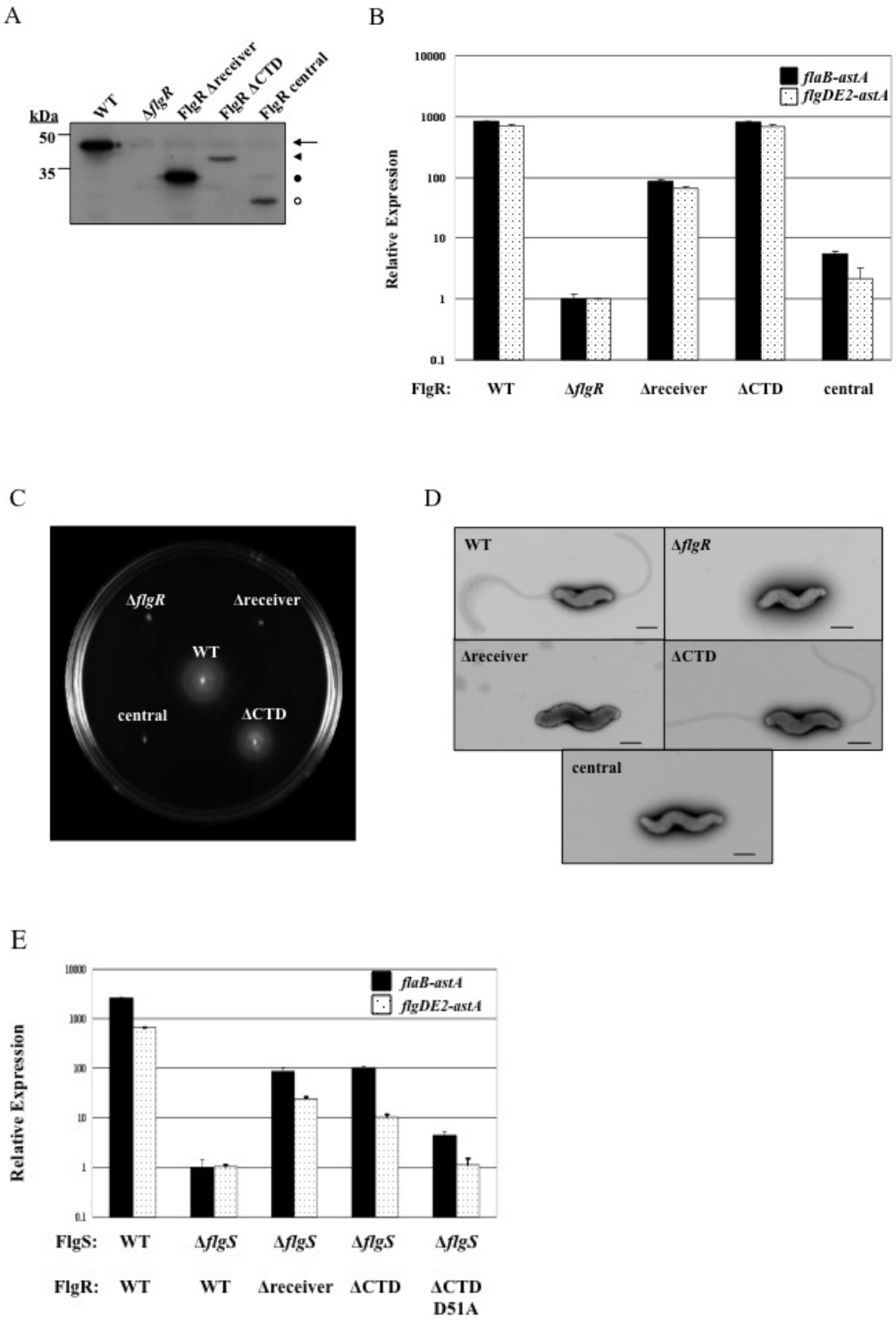
A
kDa
WT
ΔflgR
FlgR Δreceiver
FlgR ΔCTD
FlgR central
50
35
B
Relative Expression
flaB-astA
flgDE2-astA
10000
1000
100
10
1
0.1
FlgR: WT ΔflgR Δreceiver ΔCTD central
C
ΔflgR
Δreceiver
WT
central
ΔCTD
D
WT
ΔflgR
Δreceiver
ΔCTD
central
E
Relative Expression
flaB-astA
flgDE2-astA
FlgS: WT ΔflgS ΔflgS ΔflgS ΔflgS
FlgR: WT WT Δreceiver ΔCTD ΔCTD D51A

Figure 7. Phenotypic analyses of *C. jejuni* strains producing in-frame deletion of domains within FlgR. (A) Immunoblot analysis of whole-cell lysates of *C. jejuni* wild-type and mutant strains to detect FlgR proteins. The FlgR protein produced by each strain is listed above the blot. Arrow indicates full-length FlgR protein, arrowhead indicates $FlgR_{\Delta CTD}$, and filled dot indicates $FlgR_{\Delta receiver}$, and empty dot indicated $FlgR_{central}$. (B) Arylsulfatase assays for analysis of expression of *flgDE2::nemo* and *flaB::astA* in *C. jejuni* 81-176 derivatives producing wild-type and FlgR mutant proteins. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in 81-176 Sm^R Δ*astA* Δ*flgR* which was set to 1 arylsulfatase unit. For expression of *flgDE2*::*astA* (dotted bars), strains include DRH533, DRH830, SNJ118, SNJ120, and SNJ126. For expression of *flaB*::*nemo* (filled boxes), strains include DRH665, DRH842, SNJ145, SNJ138, and SNJ142. The FlgR protein produced by each strain is listed below the graph. (C) Motility phenotypes of *C. jejuni* strains producing wild-type FlgR or FlgR in-frame domain deletions in MH semi-solid agar. Strains include DRH212 (81-176 Sm^R), DRH737 (81-176 Sm^R Δ*flgR*), DRH1901 (81-176 Sm^R $flgR_{\Delta receiver}$), DRH1925 (81-176 Sm^R $flgR_{\Delta CTD}$), and DRH1928 (81-176 Sm^R $flgR_{central}$). Level of motility is shown after 24 hours of incubation. (D) Electron micrographs of *C. jejuni* wild-type and mutant strains for analysis of flagellar biosynthesis. All electron micrographs are at 16,000x magnification. The bar for each micrograph represents 0.5 μm. Strains include DRH212 (81-176 Sm^R), DRH737 (81-176 Sm^R Δ*flgR*), DRH1901 (81-176 Sm^R $flgR_{\Delta receiver}$), DRH1925 (81-176 Sm^R $flgR_{\Delta CTD}$), and DRH1928 (81-176 Sm^R $flgR_{central}$). (E) Arylsulfatase assays for analysis of expression of *flgDE2::nemo* and *flaB::astA* in *C. jejuni* 81-176 Δ*flgS* derivatives producing wild-type and FlgR mutant proteins. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in 81-176 Sm^R Δ*astA* Δ*flgS* which was set to 1 arylsulfatase unit. For expression of *flaB*::*astA* (filled bars), strains include DRH665, DRH939, SNJ136, SNJ268, and SNJ730. For expression of *flgDE2*::*nemo* (dotted bars), strains include DRH533, DRH936, SNJ123, and SNJ264, and SNJ728. The status of *flgS* and *flgR* in each strain is shown below the graph.

Analysis of an alternative role for the CTD in controlling FlgR activity

Bioinformatics analyses using software to identify DNA-binding regions such as HTH motifs suggest that the FlgR CTD lacks the strongly predicted HTH found in other NtrC family response regulators that is required *in vivo* for expression of NtrC target genes (Fig 2; 45). To begin characterizing the putative functions of the CTD, we removed the C-terminal 52 amino acids, generating *flgR*$_{\Delta CTD}$. *H. pylori* FlgR (FlgR$_{Hp}$) naturally lacks these residues, and presumably, DNA-binding activity (19). This construct was used to replace wild-type *flgR* on the chromosome of *C. jejuni*. FlgR$_{\Delta CTD}$ is produced by *C. jejuni* (Figure 7A), but at levels that are substantially lower than FlgR or FlgR$_{\Delta receiver}$, indicating that the CTD may be necessary for FlgR stability.

Despite low observed protein levels, deletion of the CTD had no apparent effects on expression of σ^{54}-dependent flagellar genes (Figure 7B), motility (Figure 7C), or production of flagella (Figure 7D), indicating that in the presence of FlgS, FlgR$_{\Delta CTD}$ behaves similarly to wild-type FlgR. This finding is consistent with FlgR$_{Hp}$, which naturally lacks a CTD but functions to transcribe σ^{54}-dependent flagellar genes (19). We performed DNA-binding assays using the full-length FlgR protein and the promoter regions of *flgDE2* and *flaB*. Although multiple approaches were used, including different phosphorylation states of FlgR and altering incubation conditions, we could not demonstrate any DNA-binding activity (data not shown). This finding was not entirely surprising, however, as

there is evidence that $FlgR_{Hp}$ does not bind DNA to activate transcription (19). We believe our following observations support the hypothesis that *C. jejuni* FlgR lacks a DNA-binding domain: 1) deletion of the CTD does not affect expression of σ^{54}-dependent flagellar genes; 2) a DNA-binding motif cannot be found via bioinformatics analysis of the isolated CTD or full-length FlgR; and 3) the full-length FlgR protein does not appear to bind DNA upstream of σ^{54}-dependent promoters *in vitro*.

Considering our findings and the published reports analyzing *H. pylori* FlgR, we initially suspected that the CTD of *C. jejuni* FlgR is not necessary for DNA-binding and that it may be vestigial. However, by introducing $flgR_{\Delta CTD}$ into a Δ*flgS* background, we discovered that the CTD has an inhibitory function. Similar to our findings with the $FlgR_{\Delta receiver}$ protein, σ^{54}-dependent reporter gene expression mediated by $FlgR_{\Delta CTD}$ in a Δ*flgS* background is 10- to 100-fold greater than the Δ*flgS* mutant that produces wild-type FlgR (Figure 7E). Additionally, production of $FlgR_{\Delta CTD}$ in the Δ*flgS* background restores motility to 20 – 50% of wild-type levels (data not shown). These results indicate that like the receiver domain, the CTD may also inhibit FlgR activity in the absence of phosphorylation. *In vitro* phosphorylation assays show that $FlgR_{\Delta CTD}$ is phosphorylated in the presence of FlgS, and that this modification is dependent on residue D51 like the wild-type protein (Figure 6B). To determine if the *in vivo* activity of $FlgR_{\Delta CTD}$ for expression of flagellar genes in the absence of FlgS is

dependent on phosphorylation of D51, we introduced *flgR*(*D51A*)$_{\Delta CTD}$ into the Δ*flgS* background. In this mutant, expression of σ^{54}-dependent reporter genes is reduced compared to Δ*flgS flgR*$_{\Delta CTD}$ (Figure 7E), indicating that phosphorylation of FlgR$_{\Delta CTD}$ on residue D51 is required in part for its constitutive transcriptional activity. Although FlgR$_{\Delta CTD}$ activity still appears to be largely dependent on phosphorylation of D51 in a mutant lacking FlgS, FlgS cannot be responsible for this modification. Thus, we hypothesize that FlgR$_{\Delta CTD}$ may be phosphorylated by non-FlgS kinases or phosphodonors. This finding stands in contrast to wild-type FlgR that is produced in the Δ*flgS* mutant, as it remains completely inactive without FlgS. In light of these results, we believe it is unlikely that another phosphodonor is capable of phosphorylating full-length, wild-type FlgR *in vivo*.

In light of our findings that both the receiver domain and CTD can inhibit FlgR activity in the absence of phosphorylation by FlgS and that removal of each domain creates a protein with partial constitutive activity, we proposed that deletion of both domains from FlgR may be additive to produce a protein with high constitutive activity. We generated a *C. jejuni* mutant that produced only the central domain of FlgR (*flgR*$_{central}$; amino acids 132-381). Examination of this mutant revealed that the FlgR$_{central}$ protein was produced at very low levels, similar to the FlgR$_{\Delta CTD}$ protein (Figure 7A). Regardless of the presence of FlgS, the FlgR$_{central}$ domain only afforded a very low level of flagellar gene expression (Figure 7B) that did not result in flagellar biosynthesis or motility (Figure 7C-D),

suggesting that this version of the protein is inactive. Currently, we do not understand why removal of both the receiver domain and CTD does not activate the protein. However, a relatively simple explanation is that this severely truncated FlgR protein may have an altered conformation or may not fold properly, resulting in complete inactivation.

Evidence for distinct mechanisms to control the activation of $FlgR_{\Delta CTD}$ and a FlgR orthologue that naturally lacks a CTD

Unlike most members of the NtrC family of transcriptional regulators that contain a CTD to bind DNA, evidence suggests that $FlgR_{Hp}$ naturally lacks this domain and does not bind DNA (19). The work described above also suggests that the CTD of *C. jejuni* FlgR is not required for expression of flagellar genes and inhibits FlgR activity in the absence of phosphorylation. Not using a HTH-containing CTD to interact with DNA *in vivo* distinguishes these FlgR proteins from other NtrC-family members; thus, they may represent a sub-family of NtrC-like proteins that do not require DNA interaction to activate transcription.

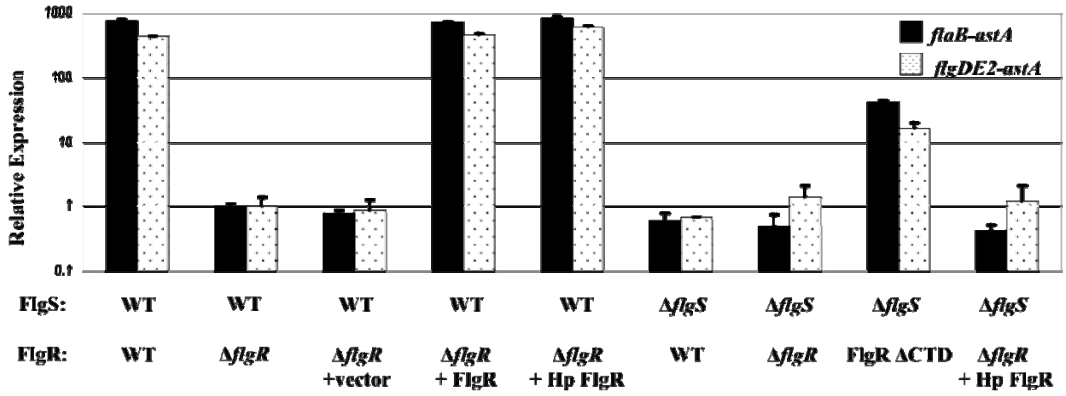

Figure 8. Analysis of the ability of *H. pylori* FlgR to promote expression of σ^{54}-dependent flagellar genes in *C. jejuni* with and without *C. jejuni* FlgS. Arylsulfatase assays for analysis of expression of *flgDE2::nemo* and *flaB::astA* in *C. jejuni* 81-176 derivatives producing wild-type and FlgR mutant proteins. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in 81-176 Sm^R Δ*astA* Δ*flgR* which was set to 1 arylsulfatase unit. For expression of *flaB*::*astA* (filled bars), strains include DRH665, DRH842, DRH 951, DRH966, SNJ759, DRH939, SNJ268, SNJ808, and SNJ821. For expression of *flgDE2*::*nemo* (dotted bars), strains include DRH533, DRH830, SNJ780, DRH954, SNJ756, DRH936, SNJ264, SNJ807, SNJ817. The status of *flgS* and *flgR* in each strain is shown below the graph.

The FlgR proteins of *C. jejuni* strain 81-176 and *H. pylori* strain 26695 are 56% identical and 73% similar, with a major difference being that $FlgR_{Hp}$ lacks the CTD observed in *C. jejuni* FlgR. As *C. jejuni* $FlgR_{\Delta CTD}$ is partially active in the absence of FlgS, we investigated whether the $FlgR_{Hp}$ would be constitutively active without FlgS. First, we examined the ability of $FlgR_{Hp}$ to complement a *C. jejuni* Δ*flgR* mutant in the presence of FlgS. When introduced into 81-176 Sm^R Δ*flgR* on a multicopy plasmid and expressed from the constitutive

chloramphenicol acetyltransferase (*cat*) promoter, $FlgR_{Hp}$ is able to fully restore expression of σ^{54}-dependent flagellar genes (Figure 8), indicating that activation of $FlgR_{Hp}$ and *C. jejuni* FlgR by FlgS is conserved in the two bacteria. However, when $FlgR_{Hp}$ is produced in a Δ*flgS* Δ*flgR* mutant, it is unable to activate expression of σ^{54}-dependent flagellar genes (Figure 8). While $FlgR_{\Delta CTD}$ displays partial constitutive activity in a Δ*flgS* background (Figures 7E and 8), $FlgR_{Hp}$ is entirely inactive in the absence of *C. jejuni* FlgS. These data suggest that a non-FlgS kinase or phosphodonor may exist to phosphorylate $FlgR_{\Delta CTD}$, but not the $FlgR_{Hp}$ protein. Alternatively, $FlgR_{Hp}$ may utilize another means of inhibiting its activity in the absence of phosphorylation. Thus, this subfamily of NtrC-like proteins that may not interact with DNA appear to possess diverse mechanisms to control their activation. This work illustrates that *C. jejuni* FlgR is an unusual member of the well-studied NtrC family of proteins.

Discussion

As with most motile bacteria, controlling flagellar gene expression in *C. jejuni* is important for the proper construction of the flagellum. Combining our studies with previous works, we have revealed that *C. jejuni* has complex and unusual mechanisms for controlling flagellar gene expression that distinguish it from other well-characterized bacteria. Not only is the essential FlgR response regulator subjected to a phase-variable mechanism controlling production of the

protein (73), but it is post-translationally controlled by a signaling cascade through its cognate sensor kinase FlgS (78, 210), this work). Our analysis of FlgR reveals that *C. jejuni* has evolved alternative mechanisms of controlling flagellar gene expression that differ from mechanistically characterized NtrC-like proteins. First, it appears to have a receiver domain that can exert both positive and negative influence over controlling activation of the protein, depending on its phosphorylation state. Second, it has adapted the CTD of FlgR to limit specificity of phosphorylation to the cognate sensor kinase FlgS.

The FlgR receiver domain has activating and inhibitory functions

The well-characterized response regulator NtrC, is positively regulated by phosphorylation (45), while other members of the NtrC family of protein, including *S. meliloti* DctD and *A. aeolicus* NtrC1 utilize phosphorylation to remove inhibition mediated by the receiver domain (118, 120). A few characterized response regulators outside of the NtrC family have receiver domains that utilize both positive and negative regulation, depending on the state of phosphorylation. One of the best-characterized examples of this dual regulation is CheB, a protein involved in chemotaxis in motile bacteria (reviewed in 17). It is comprised of two major domains, an N-terminal receiver that is phosphorylated by the CheA kinase and a C-terminal methylesterase domain that dephosphorylates methyl-accepting chemotaxis proteins. Deletion of the receiver

domain results in a mutant with increased methylesterase activity compared to the unphosphorylated wild-type protein, indicating that the receiver domain inhibits methylesterase activity in an unphosphorylated state (5). However, phosphorylation of the receiver domain enhances methylesterase activity to a greater extent than CheB with a deletion of the entire domain. Thus, the receiver domain has dual roles in inhibition and activation, depending on the state of phosphorylation. The receiver domain of FixJ, a *Sinorhizobium meliloti* protein utilized in nitrogen fixation and microaerobic respiration, is also believed to exert both positive and negative control over the activity of the output domain which is dependent on phosphorylation (33). In this case, phosphorylation removes inhibition by opening the structure of the protein and activates the protein by inducing dimerization.

By analyzing the domains of FlgR, we have developed a model of FlgR activation. We hypothesize that the receiver domain likely activates the protein when phosphorylated and inhibits it in an unphosphorylated state. The highest level of *in vivo* FlgR activity observed occurs when the wild-type protein is phosphorylated in the presence of FlgS. In the Δ*flgR* and *flgR*(*D51A*) mutants as well as the Δ*flgS* mutant (where wild-type FlgR is present but inactive, presumably due to lack of phosphorylation in the absence of FlgS) there is a complete loss of σ^{54}-dependent gene expression. These results suggest that phosphorylation of the receiver domain positively influences FlgR activity. When

we analyzed $FlgR_{\Delta receiver}$ in *C. jejuni*, we noticed an intermediate phenotype between strains with wild-type phosphorylated and unphosphorylated FlgR. While $FlgR_{\Delta receiver}$ activates flagellar gene expression at a level that is 10-fold lower than wild-type FlgR in presence of FlgS, expression is increased 100-fold compared to Δ*flgR*, *flgR*(*D51A*), and Δ*flgS* mutants. Hence, removal of the receiver domain appears to eliminate an inhibitory function mediated by this domain of the protein. Thus, we believe the receiver domain can exert both positive and negative regulatory effects. We hypothesize that wild-type, phosphorylated FlgR and $FlgR_{\Delta receiver}$ compared to wild-type FlgR in an unphosphorylated state will have one or more biochemical activities (e.g., oligomerization, ATP hydrolysis, or interactions with σ^{54}) that are enhanced. Future studies using purified FlgR and $FlgR_{\Delta receiver}$ proteins will focus on characterizing differences in the biochemical properties of these proteins which may elucidate the precise mechanisms by which the receiver domain prevents FlgR activity under non-inducing conditions, and how phosphorylation influences FlgR activation.

The FlgR CTD has alternative functions

Generally, the CTDs of NtrC-like proteins contain a HTH motif necessary for interacting with a specific UAS, mediating transcription from target promoters *in vivo*. The necessity of DNA interactions by these motifs has been studied in

several NtrC-like proteins. The prototypical member, NtrC, requires the CTD for transcriptional activation *in vivo* (45). However, some forms of NtrC with mutations in the CTD are capable of promoting a lower level of transcription *in vitro* (148). While deletion of the CTD of the *R. legumionsarum* DctD regulator results in partial expression from the *dctA* promoter in *E. coli*, the level of expression observed is 100-fold lower than that of DctD containing the CTD (84). Similarly, *R. meliloti* producing NifA lacking its CTD results in a 10-fold reduction in expression of *nifH* relative to a strain containing the wild-type protein (83). The reduced gene expression due to mutation of the CTD in the respective proteins is believed to be largely due to the lack of specific DNA binding to target promoters. Thus, while not always an absolute requirement, DNA binding to appropriate sites is necessary for optimal transcriptional activation by these proteins.

Bioinformatics does not indicate any type of DNA-binding motif located within the CTD of FlgR or in any other portion of the protein. Furthermore, deletion of the FlgR CTD has no observable effect on expression of σ^{54}-dependent genes or motility in *C. jejuni* when the FlgS sensor kinase is present. Thus, FlgR likely does not need to interact with DNA to mediate expression of target genes. DNA-binding assays were performed with full-length FlgR in the presence and absence of FlgS. In both cases, we could not observe any binding to the promoter regions of *flgDE2* or *flaB*. Although this negative result alone cannot be used to

state conclusively that FlgR activates transcription without binding DNA, this data taken in combination with our finding that the CTD is not required for *in vivo* expression of target genes and the lack of any DNA-binding motif suggests that FlgR may not bind DNA to activate transcription. The FlgR orthologue in *H. pylori* may also function without binding DNA, as it naturally lacks the CTD and does not require an upstream activating sequence to initiate transcription (19). In addition, the σ^{54}-dependent CtcC response regulator of *Chlamydia trachomatis* also does not have a CTD, but the means by which its activity is induced and controlled require further characterization (106). Rrp2 of *Borrelia burgdorferi*, another NtrC-like protein, appears to contain a standard CTD but does not need to interact with an enhancer upstream of σ^{54}-dependent promoters to activate expression of target genes (20).

Further exploration into a possible role for the CTD of FlgR allowed us to observe that this portion of the protein may have an alternative function in preventing the receiver domain of FlgR from being modified by non-FlgS kinases or phosphodonors. This hypothesis is supported by the observation that in the absence of FlgS, expression of σ^{54}-dependent flagellar genes mediated by $FlgR_{\Delta CTD}$ is principally dependent on D51, the phosphorylated residue. An increased susceptibility for phosphorylation by such phosphodonors or kinases may account for the ability of $FlgR_{\Delta CTD}$ to promote greater flagellar gene expression in the absence of *flgS*. Further studies will compare the ability of

wild-type FlgR and $FlgR_{\Delta CTD}$ to be phosphorylated by small phosphodonors or non-FlgS kinases. Having identified the CTD as an important domain for putatively controlling phosphorylation of FlgR, future work will more specifically analyze this domain to determine specific amino acid residues or subregions of this domain that are required for its activity.

FlgR and other response regulators with atypical CTDs may represent a new subclass of NtrC-like proteins

Two challenges are created for a bacterium that produces a σ^{54}-dependent response regulator that does not need to bind DNA for transcription of target genes: 1) how the response regulator can interact with σ^{54} at target promoters when not anchored to DNA; and 2) maintenance of response regulator specificity for activating transcription at the correct regulons to accomplish a specific biological function. The most common mechanism for σ^{54}-dependent response regulators to interact with σ^{54} in the RNAP holoenzyme at target promoters involves the regulator binding to the UAS and then bending of the DNA to allow the regulator to directly interact with σ^{54}. The only requirement that these DNA-independent regulators must fulfill is that they interact with σ^{54} bound to the target promoter. While the exact mechanism of interaction of FlgR and other DNA-independent regulators with σ^{54} in RNAP is unknown, these proteins may directly interact with the σ^{54} component of RNAP in solution. Subsequent studies

will focus on how FlgR interacts with σ^{54} in RNAP holoenzyme and if phosphorylation enhances its ability to complex with σ^{54}.

Considering that these DNA-independent regulators could activate transcription at any σ^{54}-dependent promoter, maintaining specificity for expression of only their target genes would seem to present a problem. In most bacteria that have two or more σ^{54}-dependent response regulators, specificity of activation at proper promoters is maintained by UAS-binding. For example, *Pseudomonas putida* must ensure that activation of the NtrC-like protein FleR protein (involved in flagellar motility) does not result in transcription of the genes that are normally regulated by activated XylR (involved in xylene and tolulene catabolism; reviewed in 25). One level of specificity is maintained by the FleR-specific UAS being upstream of flagellar genes whereas the XylR-specific UAS being upstream of the appropriate catabolic genes. Analysis of genome sequences of species of *Campylobacter*, *Helicobacter*, and *Chlamydia* reveals that these bacteria contain only one σ^{54}-dependent response regulator and in each case, the regulator lacks a CTD entirely, or expresses one that does not appear to have a DNA-binding motif (19, 106). Therefore, σ^{54} can only be activated by the sole regulator in these bacteria. In addition, all σ^{54}-dependent promoters in these bacteria are used for expression of genes for a single activity (i.e. flagellar motility in *Campylobacter* and *Helicobacter*). Twelve σ^{54}-dependent promoters are predicted in the *C. jejuni* genome (51, 155) and the gene products of these

promoters are almost all predicted to function in motility. Thus, there is not a need for the extra layer of specificity provided by the regulators binding to specific DNA sequences.

Considering our findings and the work performed by others, we propose that the FlgR proteins of *C. jejuni* and *H. pylori*, CtcC of chlamydial species, and Rrp2 of *B. burgdorferi* may represent a subfamily of NtrC-like proteins that do not interact with DNA at target promoters to activate gene expression. We have demonstrated that within this subfamily, previously undescribed mechanisms to control the activation of the FlgR proteins in *C. jejuni* and *H. pylori* may exist. Both of these wild-type proteins can function with *C. jejuni* FlgS to express σ^{54}-dependent flagellar genes in *C. jejuni*. However, when we compared the activity of *C. jejuni* $FlgR_{\Delta CTD}$ and $FlgR_{Hp}$ (which naturally lacks the CTD) in the *C. jejuni* Δ*flgS* mutant, the *C. jejuni* protein exhibited partial activation but the *H. pylori* protein was completely inactive. Whereas the function of the CTD of *C. jejuni* FlgR has been adapted to prevent inappropriate activity in the absence of FlgS and an activating signal, the lack of a CTD in $FlgR_{Hp}$ does not result in partial constitutive activity. Unlike *C. jejuni* FlgR, $FlgR_{Hp}$ may not interact with an alternative phosphodonor when produced in *C. jejuni*. Alternatively, $FlgR_{Hp}$ may have a different mechanism for keeping the protein inactive under non-inducing conditions that does not depend on a CTD. It remains to be determined how activation of the *H. pylori* FlgR is controlled in the absence of a CTD.

CHAPTER FIVE

FLGS IS A CYTOPLASMIC SENSOR HISTIDINE KINASE AND IS ACTIVATED FOLLOWING FORMATION OF THE FLAGELLAR EXPORT APPARATUS

Introduction

Sensor histidine kinase proteins are utilized to receive signals from the internal or external environment and transduce that signal to a response regulator protein. This allows the amplification of signal and permits the bacterium to undertake the correct response to a multitude of signals. Prior to this work, it was believed that FlgS functioned as a sensor kinase protein for the FlgR response regulator. The following studies allowed us to clarify the role of this protein and understand how it may receive the signals that culminate in σ^{54}-dependent flagellar gene expression.

Results

FlgS is a cytoplasmic protein.

Bioinformatics analyses suggest that the *C. jejuni* FlgS sensor kinase is a cytoplasmic protein. The protein lacks both a predicted signal sequence that would target it for secretion and hypothetical spans of hydrophobic residues that

would be indicative of a protein associated with the inner membrane. To determine if FlgS is localized to the cytoplasm, we fractionated wild-type *C. jejuni* 81-176 Sm^R (DRH212; 76) into a soluble fraction containing cytoplasmic and periplasmic proteins and an insoluble fraction containing proteins associated with the outer and inner membranes. As shown in Figure 9, FlgS was only found in whole-cell lysates (WCLs) and the soluble fraction of wild-type bacteria. In comparison to the location of control proteins, FlgS was present in the same fraction as the soluble cytoplasmic protein RpoA and absent in the fraction containing the insoluble inner membrane protein AtpF. Considering both the bioinformatic and biochemical analyses, we concluded that FlgS is a cytoplasmic protein (Figure 9).

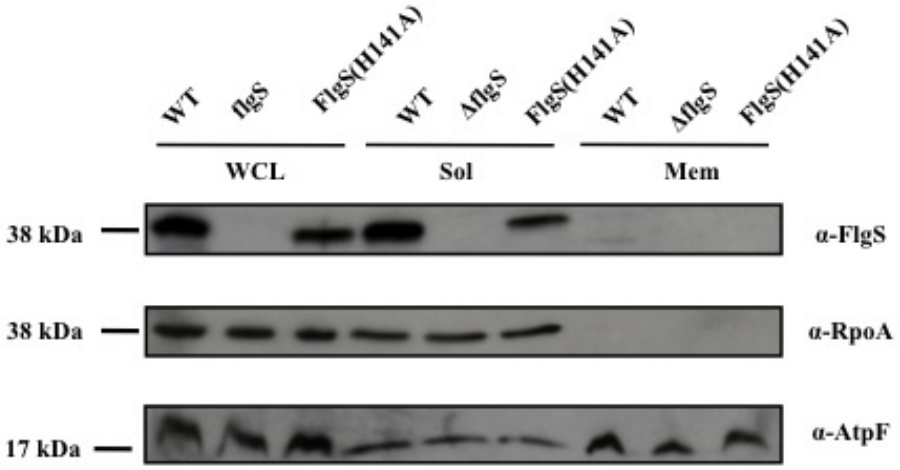

Figure 9: Localization and stability of FlgS proteins in *C. jejuni*. Wild-type *C. jejuni* 81-176 Sm^R (WT; DRH212), 81-176 Sm^R Δ*flgS* (DRH460), and 81-176 Sm^R *flgS(H141A)* (DRH1323) were grown and protein samples were obtained

from whole-cell lysates (WCL), the soluble (Sol) fraction, and the insoluble membrane (Mem) fraction after sonication. α-FlgS Rab11 antiserum was used to detect FlgS proteins (73). α-RpoA M59 antiserum and α-AtpF M3 antiserum was used to detect the soluble cytoplasmic RpoA protein and the insoluble inner membrane protein AtpF (15).

Autophosphorylation of residue H141 is required for FlgS activity.

It has been shown that the autophosphorylation site of the NtrB sensor kinase is residue H139 (146). Alignment of FlgS to NtrB indicated that this phosphorylated residue likely corresponds to H141 of FlgS, an amino acid located within the putative phosphotransfer domain (spanning amino acids 131-195) that receives the phosphate group upon autophosphorylation of other kinases. To determine if H141 is essential for FlgS activity as a kinase and for flagellar gene expression, the wild-type *flgS* allele of *C. jejuni* was replaced with *flgS(H141A)*, which results in production of FlgS with an alanine at position 141 instead of histidine. The resulting mutant was first examined for a potential defect in FlgS stability. We found that while FlgS(H141A) appears to lack any detectable degradation products, the levels of the FlgS(H141A) protein present in WCLs and the soluble fraction was about half the levels of the wild-type FlgS (Figure 9). By comparing the phenotypes of the wild-type and mutant strains, we found that *flgS(H141A)* mutation affected motility, flagellar biosynthesis, and σ^{54}-dependent flagellar gene expression (Figure 10 and data not shown). The non-motile phenotype of the *flgS(H141A)* mutant on semi-solid agar plates at 24 hours after

inoculation was similar to that observed in a Δ*flgS* strain in which *flgS* had been deleted from the chromosome (Figure 10A and not shown; 78). This lack of motility in the *flgS(H141A)* mutant correlated with a complete absence of flagella as analyzed by transmission electron microscopy (data not shown). We then analyzed expression of *flgDE2*- and *flaB-astA* transcriptional fusions in strains producing the FlgS(H141A) protein and found that the level of σ^{54}-dependent flagellar gene expression in a *flgS(H141A)* mutant was equivalent to that of a Δ*flgS* mutant (Figure 10B), indicating that H141 is critical for proper function of FlgS in *C. jejuni*.

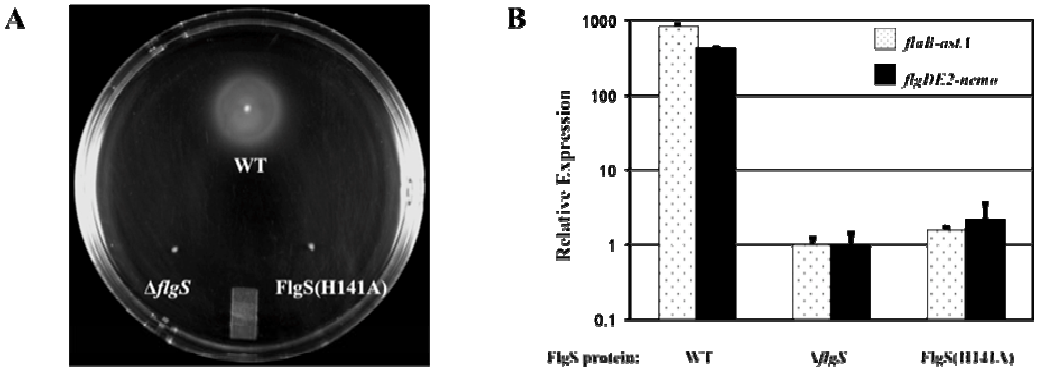

Figure 10. Phenotypic analyses of *C. jejuni* wild-type and *flgS(H141A)* mutant strains. (A) Motility phenotypes of *C. jejuni* strains producing wild-type or mutant FlgS proteins in MH semi-solid agar 24 hours after inoculation. Strains include wild-type 81-176 Sm^R (WT; DRH212), 81-176 Sm^R Δ*flgS* (DRH460), and 81-176 Sm^R *flgS(H141A)* (DRH1323). (B) Arylsulfatase assays for analysis of expression of *flaB*::*astA* and *flgDE2*::*nemo* in *C. jejuni* 81-176 derivatives producing wild-type and FlgS mutant proteins. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in 81-176 Sm^R Δ*astA* Δ*flgS*, which was set to 1

arylsulfatase unit. For expression of *flaB*::*astA* (dotted bars), strains include wild-type DRH665 (81-176 Sm^R Δ*astA flaB*::*astA*; WT), DRH939 (81-176 Sm^R Δ*astA* Δ*flgS flaB*::*astA*), and SNJ958 [81-176 Sm^R Δ*astA flgS(H141A) flaB*::*astA*]. For expression of *flgDE2*::*nemo* (solid bars), strains include wild-type DRH533 (81-176 Sm^R Δ*astA flgDE2*::*nemo*; WT), DRH936 (81-176 Sm^R Δ*astA* Δ*flgS flgDE2*::*nemo*), and SNJ956 [81-176 Sm^R Δ*astA flgS(H141A) flgDE2*::*nemo*]. The FlgS protein produced by each strain is listed below the graph.

Since H141 is the predicted site of phosphorylation, we performed autophosphorylation assays with purified 6XHis-tagged versions of FlgS and FlgS(H141A). Whereas FlgS autophosphorylated and accumulated radiolabeled-phosphate over time, FlgS(H141A) remained unphosphorylated (Figure 11A-B). In previous work, we showed that the FlgR response regulator is modified by phosphorylation in the presence of purified FlgS *in vitro* (92). We performed similar experiments to determine if phosphotransfer to FlgR was abolished in the presence of FlgS(H141A). In these experiments, we observed phosphorelay to FlgR in the presence of wild-type FlgS but not in the presence of the FlgS(H141A) protein (Figure 11C), consistent with the hypothesis that autophosphorylation of FlgS on H141 contributes to phosphotransfer to FlgR. Thus, we believe that H141A is the most likely site of autophosphorylation and is essential for proper function of the protein.

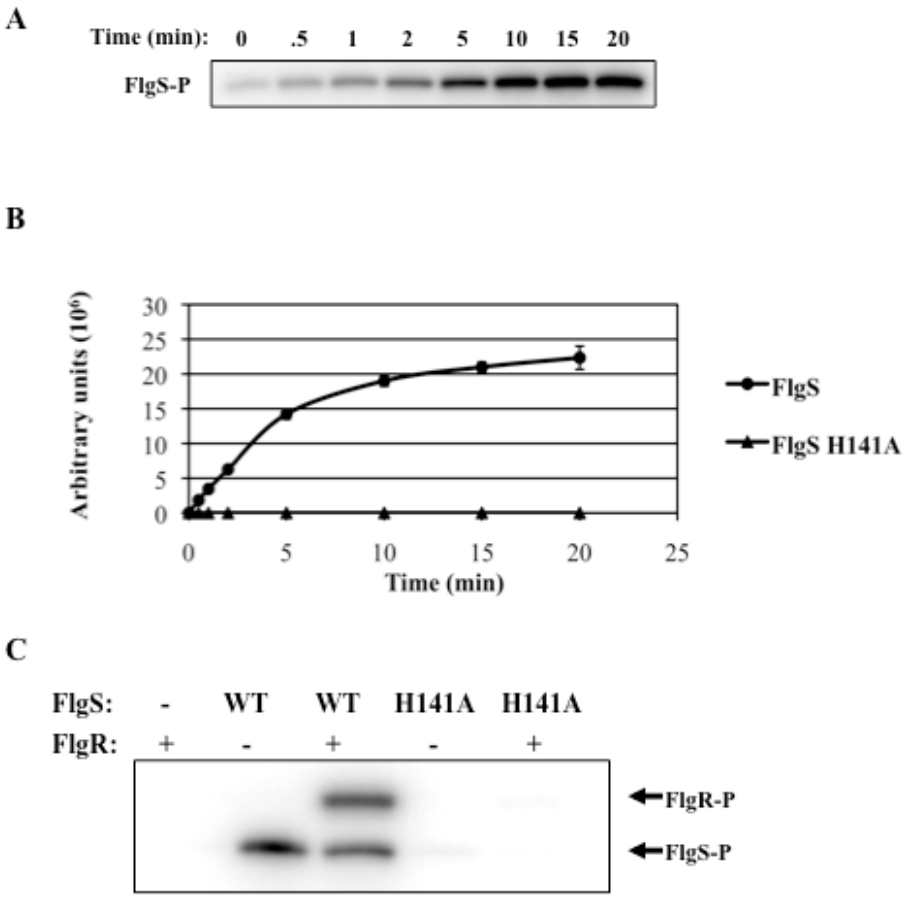

Figure 11. Autophosphorylation of FlgS proteins and phosphorelay to FlgR. (A and B) Analysis of autophosphorylation of 6XHis-FlgS and 6XHis-FlgS(H141A) over time after incubation of proteins with [γ-^{32}P]ATP. (A) A representative gel analyzed by autoradiography from a FlgS autophosphorylation assay is shown. (B) Relative quantification of autophosphorylation of FlgS proteins was determined by densitometry after autoradiography of gels. Three separate FlgS and FlgS(H141A) autophosphorylation assays were performed and the results from these assays were averaged. The amount of incorporation of ^{32}P is reported as arbitrary units from the densitometric analysis. (C) Analysis of phosphorelay to 6XHis-FlgR from 6XHis-tagged FlgS or FlgS(H141A) proteins. FlgS proteins were preincubated with [γ-^{32}P]ATP before addition of 6XHis-FlgR. A representative gel analyzed by autoradiography from a phosphotransfer assay is shown. The presence (+) or absence (-) of FlgR and the FlgS protein used in each reaction are indicated above.

Production of FlgS and FlgR does not depend on the presence of the flagellar export apparatus.

The FEA is a multi-protein complex that translocates flagellar subunits across the inner membrane for incorporation into a functional organelle (reviewed in 124). As mentioned above, many of the FEA components (e.g. FlhA, FlhB, FliP, FliR) in addition to FlgS and FlgR are required for σ^{54}-dependent flagellar gene expression in *C. jejuni* (78). We next performed experiments to determine if the FEA and FlgSR systems are linked together in a regulatory cascade that terminates in activation of expression of σ^{54}-dependent flagellar genes. More specifically, we investigated whether the FEA influences the production or activity of the FlgSR two-component system.

To examine if production of FlgS or FlgR is dependent on the FEA, we performed immunoblot analysis of cell lysates from the wild-type strain or mutant strains lacking *flhA*, *flhB*, or *fliP* that encode some of the proteins composing the FEA. We observed similar levels of FlgS and FlgR present in the wild-type strain and the FEA mutants (Figure 4A), indicating that production of FlgS and FlgR is independent of the FEA. As an additional verification that the FEA does not affect production of FlgS and FlgR, we compared the levels of the *flgS* and *flgR* mRNA transcripts in mutants lacking *flhA*, *flhB*, and *fliP* relative to the wild-type strain by real-time RT-PCR analysis. We did not detect significant fold changes in the levels of the *flgS* or *flgR* mRNAs in the mutant strains compared to wild-

type bacteria (data not shown). Therefore, FEA mutants of *C. jejuni* appear to produce normal levels of FlgS and FlgR transcripts and proteins, but have defects in signaling pathways for stimulation of σ^{54}-dependent flagellar gene expression.

We next analyzed *C. jejuni* to determine if the FlgSR system functions downstream of the FEA in a regulatory cascade to activate expression of σ^{54}-dependent flagellar genes. Previous work from our laboratory generated *flgR* alleles encoding proteins lacking the N-terminal receiver or C-terminal domain (CTD) of the response regulator (92). These proteins were shown to have partial constitutive activity in the absence of the FlgS sensor kinase, indicating that FlgR functions downstream of FlgS (92). We used these *flgR* alleles (*flgR*$_{\Delta receiver}$ and *flgR*$_{\Delta CTD}$) to replace wild-type *flgR* on the chromosome of mutants lacking *flhA*, *flhB*, or *fliP* to determine if these partially constitutively active FlgR proteins suppress the phenotype of the FEA mutants for expression of flagellar genes. As shown previously (78) and in Figure 4B, *flhA*, *flhB*, or *fliP* mutants containing wild-type *flgR* and producing the wild-type protein expressed 40- to 50-fold less of the σ^{54}-dependent *flaB*- and *flgDE2-astA* transcriptional fusions. When *flgR* in these FEA mutants was replaced with the *flgR* alleles encoding FlgR$_{\Delta receiver}$ and FlgR$_{\Delta CTD}$, partial restoration of σ^{54}-dependent flagellar gene expression was observed (Figure 12B). Although the levels of expression were not restored to wild-type levels, they were approximately 5- to 10-fold higher than in the FEA mutants that produced wild-type FlgR. These analyses suggest that FlgSR

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

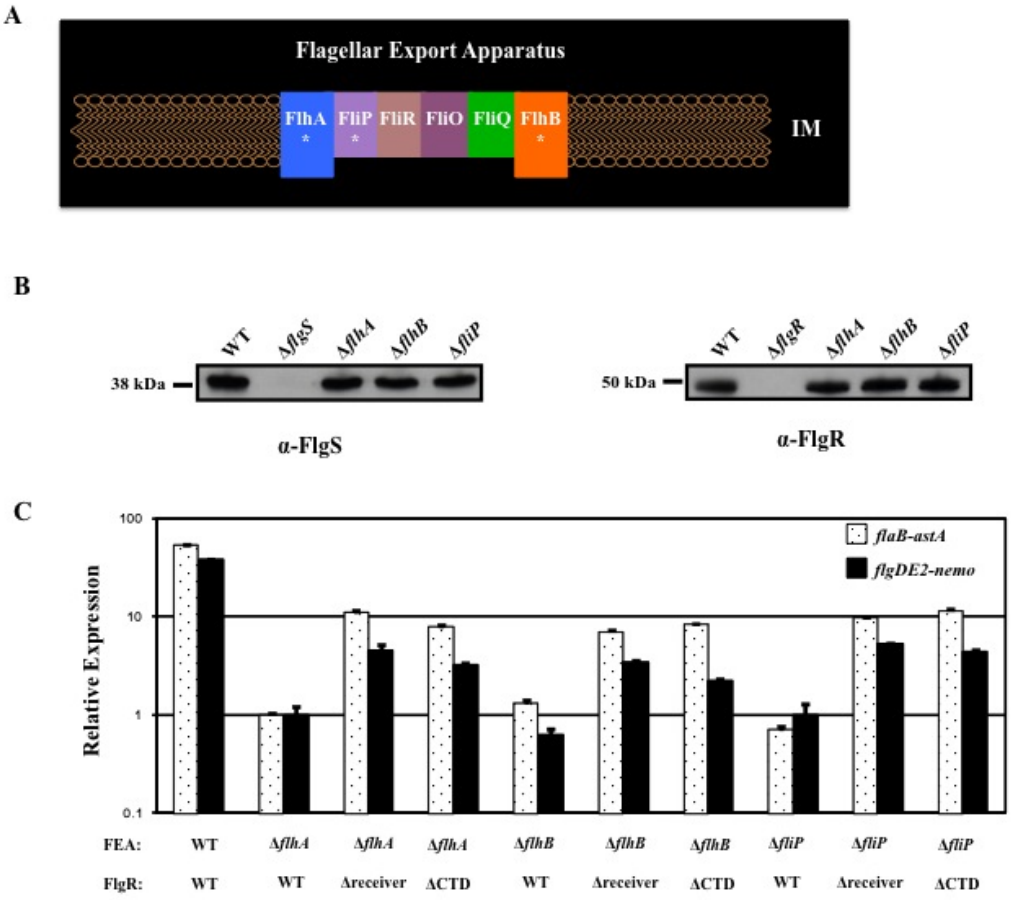

Figure 12. Production of FlgS and FlgR and activity of FlgR proteins in flagellar export apparatus mutants in *C. jejuni*. (A) Model of the FEA. Components that were deleted for this study are indicated by an asterisk. (B). Production of FlgS and FlgR proteins in mutants of *C. jejuni* lacking one component of the FEA. Whole-cell lysates of wild-type and *C. jejuni* mutant strains were prepared for immunoblotting analyses. α-FlgS Rab11 and α-FlgR Rab13 antisera was used to detect the FlgS (left) and FlgR (right) proteins (73). Strains used for analysis include wild-type DRH212 (WT; 81-176 Sm^R), DRH460 (81-176 Sm^R Δ*flgS*), DRH737 (81-176 Sm^R Δ*flgR*), DRH946 (81-176 Sm^R

Δ*flhA*), SNJ471 (81-176 Sm^R Δ*flhB*), and DRH1065 (81-176 Sm^R Δ*fliP*). (C) Arylsulfatase assays for analysis of expression of *flaB::astA* and *flgDE2::nemo* in *C. jejuni* 81-176 Sm^R wild-type or mutant strains lacking a component of the FEA and producing wild-type and FlgR mutant proteins. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in 81-176 Sm^R Δ*astA* Δ*flhA*, which was set to 1 arylsulfatase unit. For expression of *flaB*::*astA* (dotted bars), strains include (from left to right) wild-type DRH665 (WT), DRH1049, SNJ112, SNJ273, DRH1827, SNJ109, SNJ1021, DRH1178, SNJ261, and SNJ1015. For expression of *flgDE2*::*nemo* (solid bars), strains include (from left to right) wild-type DRH533 (WT), DRH1021, SNJ115, SNJ274, DRH1827, SNJ113, SNJ1017, DRH1204, SNJ358, and SNJ1012. The FEA mutation and the type of FlgR protein produced in each strain is shown below the graph.

Formation of the FEA likely initiates activation of the FlgSR system.

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

activity of FlgS may be present in the cell before the FEA is competent for secretion, and the secretory activity of the FEA may be required to inactivate or remove this protein from the cytoplasm, relieving FlgS from repression and allowing for autophosphorylation and phosphorelay to FlgR to ensue.

To distinguish between these possibilities, we generated mutants with FEA complexes that are predicted to assemble in the inner membrane, but are hindered for secretion of flagellar substrates. For this approach, we targeted *fliI* and *flhB* for mutation. FliI functions as an ATPase that dissociates export substrates (e.g., flagellins) from their chaperones in *S. typhimurium* (135, 157). While FliI is not an absolute requirement for secretion of flagellar substrates, its absence substantially reduces the efficiency of this process. Due to significant homology between the FliI proteins of *C. jejuni* and *S. typhimurium* strain LT2 (43% identity and 62% similarity over 424 amino acids), we hypothesize that FliI serves a similar function in *C. jejuni* in increasing the efficiency of secretion of flagellar proteins. Therefore, we deleted *fliI* from the *C. jejuni* genome to create a mutant that possibly impaired the efficiency of FEA-mediated secretion of flagellar proteins.

Previous analysis with *S. typhimurium* revealed that defined mutations can also be made in *flhB* so that the FEA assembles in the inner membrane, but secretion of substrates through the FEA is reduced or blocked (54). These mutations include small, in-frame deletions and point mutations in the FlhB

protein. By aligning the sequences of the *S. typhimurium* and *C. jejuni* strain 81-176 proteins (which are 36% identical and 60% similar across 351 amino acids), we identified regions of the FlhB protein of *C. jejuni* that may be able to be deleted or mutated to result in FEA mutants that form but do not secrete efficiently. To this end, we constructed *flhB* mutant alleles that encoded $FlhB_{\Delta 214-218}$, $FlhB_{\Delta 224-228}$, $FlhB_{\Delta 244-253}$, and FlhB(N267A) mutant proteins. These deletions and mutations in the *C. jejuni* FlhB protein correspond respectively to types of domain deletions and point mutations resulting in the FlhBΔ2, FlhBΔ4, FlhBΔ8-9, and FlhB(N269A) proteins of *S. typhimurium* constructed by Fraser *et al* (54).

After construction of *fliI* and *flhB* mutants of *C. jejuni*, we first analyzed the strains for stability of the respective FlhB protein produced in each by immunoblotting analysis. FlhB is produced as a 42-kDa protein in *S. typhimurium* that is cleaved to a 31-kDa protein by autoproteolysis of the peptide bond between position N269 and P270 (49, 54, 133). Though *flhB* of *C. jejuni* appears to encode a protein of 37 kDa, we predict a similar processing may occur between N267 and P268 to result in an FlhB protein of 30 kDa. Immunoblot analysis of the total membrane fraction of wild-type *C. jejuni* revealed that FlhB appeared as the processed 30-kDa protein (Figure 13A). In three of the four *fliI* and *flhB* mutants, we observed similar levels of processed FlhB proteins, indicating that the mutant FlhB proteins were stable. The *flhB(N267A)* mutant was expected to produce an FlhB protein that is not able to undergo

autoproteolytic processing. Indeed, we observed only the full-length 37-kDa protein in this mutant (Figure 13A). In the *flhB*$_{\Delta 244\text{-}253}$ mutant, we could not detect any mutant FlhB protein. The reason for the lack of detection of this mutant form of FlhB remains unknown, but may be due to the method to generate the α-FlhB antiserum. The antigen that was used to make the α-FlhB antiserum contained amino acids 209-367 of FlhB, which forms the complete cytoplasmic domain of the protein before processing. Due to predicted processing of FlhB at position 267 in *C. jejuni*, ultimately only a maximum of 58 amino acids (amino acids 209-267) in processed FlhB proteins are in common with the antigen that was used to generate the α-FlhB antiserum. Since FlhB$_{\Delta 244\text{-}253}$ lacks 10 of the 58 amino acids of the antigen, we may have destroyed the epitope that the α-FlhB antiserum recognizes may have been destroyed or deleted in this protein to result in its lack of detection. Because the mutant producing FlhB$_{\Delta 244\text{-}253}$ stimulated expression of σ^{54}-dependent flagellar genes (see below), we believe that this protein is being made and is stable, but undetectable with current reagents.

We next determined if these *flhB* and *fliI* mutants were impaired for secretion. To this end, we performed two different analyses. We first determined if motility was reduced since motility is directly dependent on FEA-mediated secretion of flagellar proteins out of the cytoplasm to construct a flagellar organelle. In all the *flhB* and *fliI* mutants, we observed that the level of motility

was ≤ 10% of the wild-type strain, indicating that flagellar motility and presumably secretion through the FEA had been severely impaired (Figure 13A).

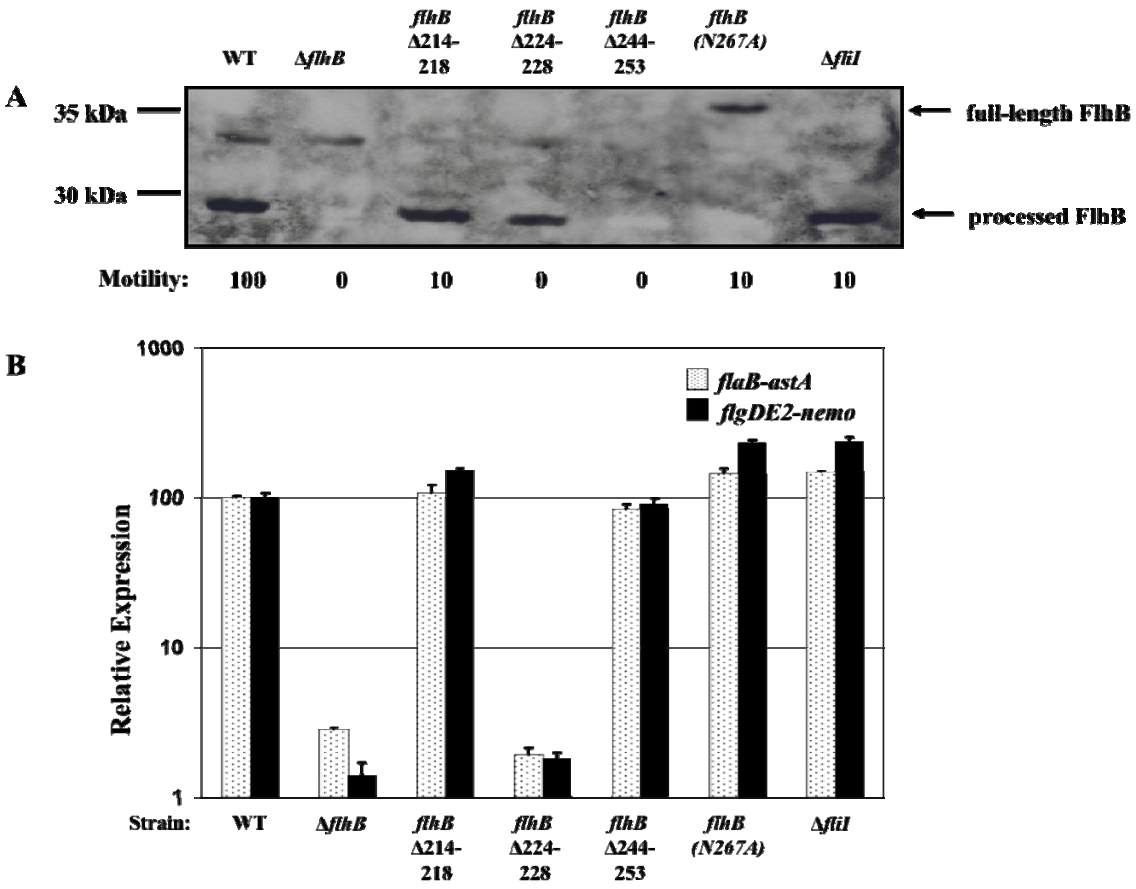

Figure 13. Phenotypic analyses of *C. jejuni* strains with formed but secretion-impaired flagellar export apparatus complexes. (A) Immunoblot analysis of FlhB proteins and motility phenotypes of wild-type *C. jejuni* and *flhB* or *fliI* mutant strains. Total membrane proteins were isolated from wild-type and mutant strains of *C. jejuni*. Equal amounts of proteins from each strain were analyzed. α-FlhB Rab476 antiserum was used to detect the FlhB proteins. Arrows indicate the 37-kDa full-length, unprocessed FlhB protein or the 30-kDa processed FlhB protein. Motility phenotypes of wild-type and mutant strains are indicated below the blot. The diameter of the motile ring around the point of inoculation in MH semi-solid agar was measured after 36 hours of incubation at

37 °C under microaerobic conditions. The level of motility of all mutants is reported relative to the level of motility of the wild-type strain, which was set at 100%. Strains for both analyses include (from left to right) wild-type DRH461 (WT), DRH1734, SNJ464, SNJ428, SNJ475, SNJ438. (B) Arylsulfatase assays for analysis of expression of *flaB*::*astA* and *flgDE2*::*nemo* in *C. jejuni* 81-176 Sm^R wild-type or mutant strains containing a secretion-impaired flagellar export apparatus. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in wild-type 81-176 Sm^R Δ*astA*, which was set to 100 arylsulfatase units. For expression of *flaB*::*astA* (dotted bars), strains include (from left to right) wild-type DRH665 (WT), DRH1830, SNJ467, SNJ434, SNJ508, SNJ442, SNJ422. For expression of *flgDE2*::*nemo* (solid bars), strains include wild-type DRH533 (WT), DRH1827, SNJ466, SNJ433, SNJ504, SNJ439, SNJ457. The type of mutation in the flagellar export apparatus of each strain is shown below the graph.

We next performed a more direct analysis of the secretion competence of the FEA in the derived mutants by monitoring FEA-dependent secretion of the FlaA flagellin protein to the outer membrane of *C. jejuni* strains. Unlike in *S. typhimurium*, the complete regulatory pathways that govern *flaA* expression are not completely understood in *C. jejuni*. In *S. typhimurium*, σ^{28}-dependent expression of *fliC* encoding the major flagellin is repressed in FEA mutants due to cytoplasmic retention of the anti-σ^{28} factor, FlgM (85, 96). In *C. jejuni*, *flaA* is expressed by a σ^{28}-dependent promoter (24, 76, 78, 210). However, evidence for expression of *flaA* and secretion of the encoded protein via the FEA to form a truncated flagellum with partial motility has been observed in a *fliA* (encoding σ^{28}) mutant, indicating that a σ^{28}-independent promoter likely exists (76, 78, 94). Also unlike in *S. typhimurium*, evidence exist that *flaA* expression is only

moderately decreased in certain FEA mutants of *C. jejuni* 81-176, indicating that some expression of *flaA* is independent on the FEA status of the bacterium (78). Furthermore, any existing translation controls for *flaA* mRNAs in *C. jejuni* are uncharacterized. Since evidence that *flaA* expression and FlaA production is not entirely dependent on the status of the FEA in *C. jejuni* as it is in other bacteria has been reported, we analyzed FEA-dependent secretion of FlaA in our defined *flhB* and *fliI* mutants.

We first ensured that *flaA* was expressed in the mutants by monitoring expression of *flaA*::*astA* in these *flhB* and *fliI* mutants. We found that *flaA*::*astA* expression was not defective in three of the mutants [$flhB_{\Delta 214\text{-}218}$, *flhB(N267A)*, and Δ*fliI*]. Rather, expression of *flaA*::*astA* in these mutants was approximately two-fold higher than in the wild-type strain (Figure 14A). Expression of *flaA*::*astA* was slightly reduced in the $flhB_{\Delta 244\text{-}253}$ mutant to approximately 75% of that of the wild-type strain. The remaining mutant, $flhB_{\Delta 224\text{-}228}$, expressed *flaA*::*astA* at a level that was 50% less than the wild-type strain (Figure 14A). The level of expression of *flaA*::*astA* in this mutant was similar to that of Δ*flhB* or Δ*fliA* (lacking σ^{28}) mutants. With the exception of the $flhB_{\Delta 224\text{-}228}$, *flaA*::*astA* expression in the mutants was mostly intact or at a higher level than the wild-type strain.

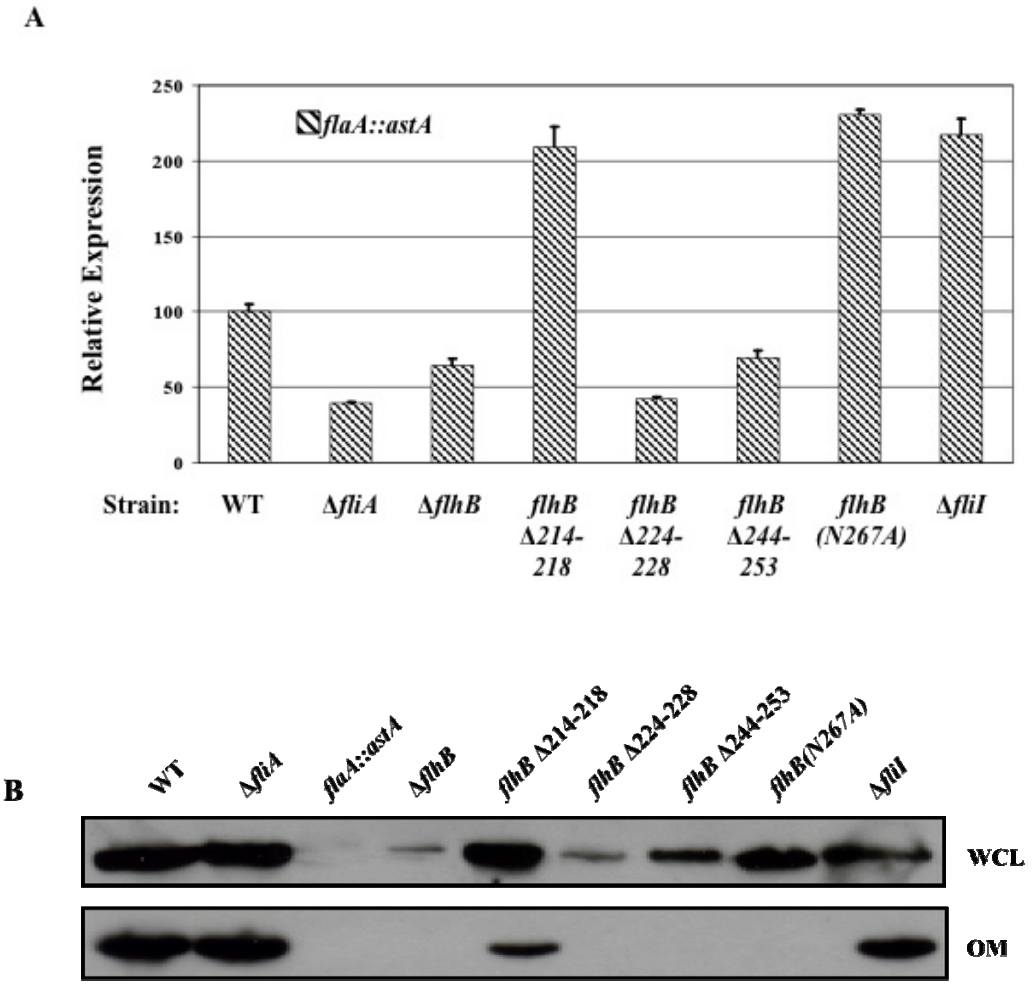

Figure 14. Analysis of *flaA* expression and FlaA secretion mediated by the flagellar export apparatus. (A) Arylsulfatase assays for analysis of expression of *flaA*::*astA* in *C. jejuni* 81-176 Sm^R wild-type or strains with a secretion-impaired FEA. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in wild-type 81-176 Sm^R Δ*astA*, which was set to 100 arylsulfatase units. For expression of *flaA*::*astA*, strains include (from left to right) wild-type DRH655 (WT), DRH1070, SNJ365, SNJ427, SNJ1033, SNJ1034, SNJ1038, SNJ1042. The type of mutation contained in each strain is shown below the graph. (B) Immunoblot analysis of FlaA production in whole-cell lysates and secretion to the outer membrane of wild-type and FEA mutant strains. Whole-cell lysates (WCL) and outer membrane (OM) fractions were isolated from wild-type

and mutant strains of *C. jejuni*. α-FlaA LL-1 antiserum was used to detect the FlaA proteins (119). Strains include (from left to right) wild-type DRH212 (WT), DRH724, DRH655, SNJ471, SNJ464, SNJ428, SNJ475, SNJ438, and DRH2257.

We next monitored FEA-mediated secretion of FlaA by comparing the levels of FlaA associated with outer membranes of wild-type and mutant strains of *C. jejuni*. As shown in Figure 14B, the $flhB_{\Delta 214\text{-}218}$, *flhB(N267A)*, and Δ*fliI* mutants produced comparable levels of FlaA in WCLs but had reduced levels of the protein associated with the outer membrane compared to wild-type bacteria. The most severe mutant was *flhB(N267A)*, which completely lacked FlaA in the outer membrane. The other two mutants, $flhB_{\Delta 214\text{-}218}$ and Δ*fliI* had approximately two- to five-fold reductions of FlaA associated with the outer membrane, suggesting that secretion had been impaired in these mutants. The $flhB_{\Delta 244\text{-}253}$ mutant produced about three-fold less FlaA in WCLs but completely lacked FlaA in the outer membrane. Only one mutant, $flhB_{\Delta 224\text{-}228}$, appeared greatly hindered for FlaA production, similar to a Δ*flhB* mutant.

Considering that four of the five mutants we created appeared to have FEAs with greatly diminished secretion abilities, we then analyzed expression of σ^{54}-dependent flagellar genes in these mutants. In these same four mutants [$flhB_{\Delta 214\text{-}218}$, $flhB_{\Delta 244\text{-}253}$, *flhB(N267A)*, and Δ*fliI*], expression of the *flaB*- and *flgDE2-astA* transcriptional fusions was equal to or slightly higher, than the wild-type strain (Figure 13B). These results indicate that completely blocking or

hindering secretion through the FEA did not affect expression of σ^{54}-dependent flagellar genes. This analysis provided evidence that formation of the FEA, rather than secretory activity of the apparatus, is required and may be the key element to activate the FlgSR system for expression of σ^{54}-dependent flagellar genes.

Only in the mutant that produced the $FlhB_{\Delta 224\text{-}228}$ protein did we observe reduced expression of *flaB*::*astA* and *flgDE2*::*nemo* comparable to that of the Δ*flhB* mutant (Figure 13B). Considering that this mutant also behaved similar to the Δ*flhB* mutant in expression of *flaA*::*astA* and secretion of the FlaA protein, we believe that, like the Δ*flhB* mutant, this mutant may not form a complete FEA. Thus, this mutant may not actually be germane to our goal of creating secretion-incompetent but correctly formed FEAs. However, if a FEA is forming in this mutant, then our alternative hypothesis that a negative regulator may be active and present to inhibit the FlgSR system in a non-secreting bacterium may have some credence. To investigate this hypothesis, we performed transposon mutagenesis with the *darkhelment* transposon (75) in *C. jejuni* 81-176 Δ*astA* Δ*flhA flgDE2*::*nemo*, 81-176 Δ*astA* Δ*flhB flgD*::*astA* and 81-176 Δ*astA* Δ*fliP flaB*::*astA*. These mutants fail to express the σ^{54}-dependent transcriptional *astA* fusions due to the lack of a complete FEA. Disruption of a gene encoding a putative repressor would allow for expression of these transcriptional reporters in the FEA mutants. Such a transposon mutant could be identified by recovering mutants on media containing a chromomeric substrate for arylsulfatase and

observing a switch from a white to blue colony phenotype. Despite screening over 65,000 transposon mutants, we were unable to identify any mutant with a transposon that disrupted a gene for such a negative regulator, suggesting that one may not exist or it is an essential gene. Considering these data as a whole, we propose that the FlgSR activation likely depends on proper assembly of the FEA. While we cannot entirely exclude the possibility that the secretory activity is required for FlgSR activation, our results indicating that four of five *flhB* or *fliI* mutants that were impaired for secretion but did not affect expression of σ^{54}-dependent flagellar genes, coupled with the results from our transposon mutagenesis screen, weaken this hypothesis.

Discussion

Previous studies from our laboratory have found that the proteins of the FEA, the putative FlhF GTPase, and the FlgSR two-component system are required for full expression of σ^{54}-dependent flagellar genes in *C. jejuni* (78, 92). We have also provided evidence that links the FEA to stimulation of the FlgSR two-component regulatory system. We found that activation rather than production of the FlgSR system is dependent on the FEA. Furthermore, we believe that formation of the apparatus rather than the secretory function of the apparatus is key to produce the signal detected by FlgS leading to its activation and subsequent expression of σ^{54}-dependent flagellar genes. Analysis of the

genomic sequences of various *C. jejuni* strains indicates that the consensus σ^{54}-binding site is found in the promoters of most genes that encode the flagellar proteins that are external to the cytoplasm and likely secreted by the FEA (51, 81, 155). Because gene expression and protein production are energetically expensive processes, it is likely that the introduction of a level of transcriptional control imparted by the FEA allows the *C. jejuni* to ensure that σ^{54}-dependent flagellar genes are only expressed and the secreted proteins are only produced after the apparatus has formed.

The FlgS sensor kinase is soluble and phosphorylated on residue H141

In analyzing the sequence of the FlgS sensor kinase, we found that the central and C-terminal portions of the protein contain the histidine-containing phosphotransfer domain and the ATP-catalytic domain (185, 208). These domains are required for accepting a phosphate group on a conserved histidine and for ATP hydrolysis, respectively, for autophosphorylation. Indeed, we found that H141 within the phosphotransfer domain is required for modification by phosphorylation and function of the active FlgS to stimulate expression of σ^{54}-dependent flagellar gene expression. In comparing the amino acid sequence of FlgS to other sensor kinases, the predominant homology with these kinases is almost exclusively localized to these phosphoacceptor and ATP hydrolysis domains. Only a limited homology between the initial 130 amino acids of FlgS

and other sensor kinases is apparent. The sensor kinases that share the most homology to this region of the *C. jejuni* FlgS protein are other FlgS homologues in *Campylobacter* species (almost 100% identity), the FlgS orthologue in *Helicobacter* species (31 -37 % identity and 57 - 66% similarity), and FlrB sensor kinase of *V. cholera* (26% identity and 54% similarity). The N-terminal regions of these proteins bear no obvious motifs to suggest a function or how they may presumably sense a specific factor. Since these N-terminal domains are unique to the group of FlgS orthologues, it is likely that this region of these proteins may function in specifically recognizing the signal necessary to culminate in expression of σ^{54}-dependent flagellar genes. Future studies will focus on further characterizing this domain of the protein.

The FlgSR two-component system is linked to the FEA

In this study, we characterized the relationship between the FlgSR two-component system and the FEA. Previous studies revealed that when FEA components are not produced, *C. jejuni* does not express σ^{54}-dependent flagellar genes (78). To determine the nature of this defect we performed qRT-PCR and immunoblot analysis and found that the transcript and protein levels for both FlgS and FlgR are not affected by the FEA. We then showed that when partially constitutively active forms of FlgR are produced in strains lacking FEA components, partial restoration of reporter gene expression is observed. These

findings led us to believe that FlgSR activity is linked to, and downstream of, the flagellar export apparatus in a regulatory cascade that terminates in expression of σ^{54}-dependent flagellar genes.

FlgSR activation requires formation of the FEA

The analysis presented in this work has allowed us to more precisely clarify the relationship between the FEA and the FlgSR system in σ^{54}-dependent flagellar gene expression. We constructed *C. jejuni* mutants that impaired FEA-mediated secretion to determine if formation of the export apparatus or its secretory activity was required for FlgS activation. Based on our finding that three of four *flhB* mutants and a *fliI* mutant reduced or blocked secretion of the FlaA flagellin but did not negatively affect σ^{54}-dependent gene expression, we concluded that the formation of the FEA in the inner membrane could directly be the signal detected by FlgS to lead to activation of the kinase. Alternatively, formation of the FEA may be indirectly involved by being required for the production of a downstream activating signal. Although these data alone do not define the nature of the communication between the FEA and FlgSR, we have provided a foundation for future studies to understand activation of the system. Characterization of additional FEA proteins and structures such as the inner membrane MS ring and the cytoplasmic C ring that are associated with the FEA (123, 124), along with better reagents to detect complete FEA formation, may

allow us to further define the activating signal emanating from this secretory apparatus.

The FEA may function directly or indirectly in autoactivation of FlgS

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

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

The *C. jejuni* FEA may function as a signaling complex

Our work also suggests a new function in signaling mediated by the FEA in flagellar regulatory cascades. In the well-characterized pathways observed in *E. coli* and *Salmonella*, formation of the FEA ultimately controls activity of the alternative sigma factor σ^{28} involved in expression of genes encoding the major flagellins and some motor proteins (114). The FEA is responsible for secretion of flagellar proteins and the anti-σ factor, FlgM, which represses the activity of σ^{28} until the cell has completed formation of the FEA, basal body, and hook structures required to secrete flagellins to build a filament (85, 96). In this study, we found that the FEA is intimately involved in creating a signal that activates the FlgSR two-component system, leading to activation of σ^{54}-dependent genes expression. Therefore, the FEA performs a different role in influencing signaling for σ^{54}-dependent in a flagellar regulatory transcriptional cascade in *C. jejuni*. This work expands on the known mechanisms of regulating flagellar gene expression and suggests that there are more complex functions associated with the FEA beyond protein secretion.

Applications to flagellar transcription cascades in other bacteria

The flagellar regulatory cascade of *C. jejuni* appears to bear resemblance to the cascades utilized by species of *Helicobacter*, *Vibrio*, and *Pseudomonas* (8, 35, 91, 101, 102, 128, 145, 165, 184). First, all are known to require σ^{54} and a

two-component regulatory system with functional similarity to FlgSR for expression of a subset of flagellar genes. In addition, *Vibrio* and *Pseudomonas* species require the activity of a master regulator protein to initiate transcription of genes encoding FEA proteins and these flagellar two-component regulatory systems (8, 34, 35, 93, 102, 165). However, in *C. jejuni* and *H. pylori*, no master regulator of flagellar biosynthesis has been found, and a current hypothesis is that the expression of genes encoding components of the FEA and FlgSR is largely constitutive (75, 145). In all these bacteria, activation of the flagellar two-component regulatory system leads to the σ^{54}-dependent expression of genes encoding flagellar proteins that are secreted by the FEA (35, 73, 74, 78, 92, 102, 145, 165). Considering the similarity in the composition of these flagellar regulatory cascades, our findings may suggest that the formation of the FEA could influence σ^{54}-dependent flagellar gene expression in number of bacterial species. Further analyses in each of these organisms will be required to determine if this relationship is shared across multiple genera of motile bacteria.

CHAPTER SIX

FORMATION OF FLAGELLAR SUBSTRUCTURES INFLUENCES FLGSR ACTIVATION

Introduction

The work discussed in Chapter Five suggests that the FEA is involved in creating a signal that influences FlgS activation. We have begun studies to further understand the contribution of the FEA to FlgS activation. Two separate reports have been published suggesting possible interactions between the FlgS sensor kinase and the FliE protein in yeast two-hybrid assays (156, 166). FliE is known to be associated with the rod class of exported flagellar substrates, although its precise location and function are still not known (141). This protein may be an adapter between the FliF MS-ring and the proximal rod and is required for the secretion of hook subunits (132, 134). It is predicted that FliE is one of the earliest, if not the first, substrate secreted through a fully-formed FEA, and it has even been suggested that its current classification as a rod-type protein may be incorrect, as it could actually be part of the FEA (80).

If FliE is part of the FEA or exported at a very early point in flagellar biosynthesis, changes in FliE localization could serve as a signal to FlgS that the FEA is fully competent for secretion, especially if FliE does indeed interact with

FlgS as the two-hybrid studies suggest. As described in the previous chapter, attempts were made to demonstrate FlgS interactions with the FEA through chemical crosslinking, affinity chromatography, and affinity blotting. These experiments were also attempted with FliE and were not successful in demonstrating an interaction with FlgS, but did raise questions about the potential contributions of FliE to the σ^{54}-dependent gene expression pathway and the hierarchy of flagellar gene transcription. In this chapter we describe preliminary work in characterizing the genes of the *flgBCfliE* locus and how FliE may contribute to FlgSR activation.

Additionally, we have performed preliminary work in determining the minimal flagellar substructure that must be formed in order to activate transcription of σ^{54}-dependent flagellar genes. To this end, insertional inactivations or in-frame deletions were generated in genes known to encode proteins required to build the proximal and distal rod, hook, and to anchor the flagellum in the periplasm and outer membrane.

Results

The genes of the *flgBCfliE* locus may not be expressed as a single transcript.

The *fliE* gene is located in a putative operon with two other flagellum-associated genes, *flgB* and *flgC*. Genomic arrangement of the *flgBCfliE* locus is

shown below (Figure 15). Our initial hypothesis was that these three genes are co-transcribed, even though the prefixes *flg* and *fli* in the genomic nomenclature indicate that they are expressed on separate transcripts in *Salmonella* and *E. coli* (86). While the *fliE* gene is located near another flagellar operon in *Salmonella*, the *C. jejuni fliE* gene is located adjacent and downstream of *flgC*. Furthermore, in *C. jejuni*, there is very minimal intergenic space between *flgB*, *flgC* and *fliE* and bioinformatic analysis predicts a σ^{54}-dependent promoter upstream of *flgB*. We have not yet found bioinformatic evidence for σ^{28}- or σ^{70}-dependent promoters in front of *flgB*, *flgC* or *fliE*. ClustalW2 analysis indicates that the *C. jejuni* FliE protein shares less than 20% sequence homology with FliE produced by *Salmonella* or *E. coli*. These differences in genomic arrangement and protein sequence could mean that the *C. jejuni* FliE has properties not yet observed in other motile bacteria and could suggest an alternative expression profile in addition to alternative functions.

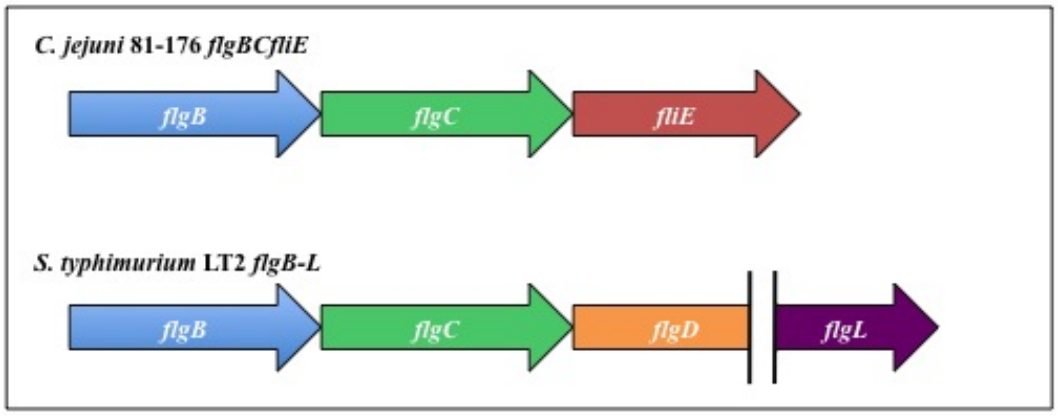

Figure 15. Genomic arrangement of the *flgBCfliE* locus in *C. jejuni* and comparison to the *flgB-L* region in *S. typhimurium* LT2.

Real-time reverse transcriptase PCR was performed to analyze the presence of *flgB*, *flgC*, and *fliE* transcripts in wild-type and several mutant strains. The mutations analyzed included the deletion of σ^{54} (*rpoN*) *flgR*, several members of the FEA, *flgB*, *flgC*, and *fliE*. Microarray data using RNA isolated from a different strain of *C. jejuni* showed that *flgB*, *flgC* and *fliE* transcription were reduced when σ^{54} and *flgR* were insertionally inactivated (95). These data and our prediction that these three genes are co-transcribed led us to believe that we would observe similar changes in transcription among all three genes in the backgrounds tested. We were surprised that our real-time RT-PCR results demonstrated different expression profiles than our predictions and the microarray data suggested. While *flgB* transcription is slightly affected in the FEA mutants, it is more severely affected in the FlgR and σ^{54} mutants (Figure 16). Surprisingly,

it is equally reduced in a *fliE* mutant. This contrasts with *Salmonella*, where a study of the flagellar regulon indicated that there was no change in the expression of a *flgB* reporter construct in a *fliE* mutant (114). Another unusual finding in this study was that neither *flgC* nor *fliE* transcription were significantly affected by the loss of the FEA, FlgR, or σ^{54} (Figure 16), which differs substantially with the microarray data described by Kamal et al (95). *C. jejuni* isolates exhibit a large amount of genomic diversity and there are a number of published differences between the strain utilized by that lab (NCTC11168), the strain utilized in our lab (81-176) and other clinical isolates (41, 121, 160). It is possible that the results described above are another example of the strain-to-strain variations that are common in *C. jejuni*.

Based on these early data, our current hypothesis is that in *C. jejuni* strain 81-176 transcription of *flgB* is at least partially dependent on the σ^{54}-dependent pathway but *flgC* and *fliE* transcription are fully independent of it. Additionally, optimal expression of *flgB* but not *flgC* appears to depend on the presence of FliE. Since expression of *flgB* is most similar in the backgrounds lacking *flgR*, *rpoN* and *fliE*, this could mean that FliE functions as a signaling component rather than part of the FEA. Future studies will address whether the *fliE* deletion has the same phenotype for other σ^{54}-dependent promoters. These data are potentially very exciting, as we may have uncovered a role for FliE that has not been described in any other motile organism. Further studies will be needed to fully

characterize the promoter region(s) of this locus and any potential operons. Primer extension analysis will also allow the identification of alternative transcriptional start sites and advanced bioinformatics analysis should be used determine if σ^{28}- or σ^{70} -promoters exist.

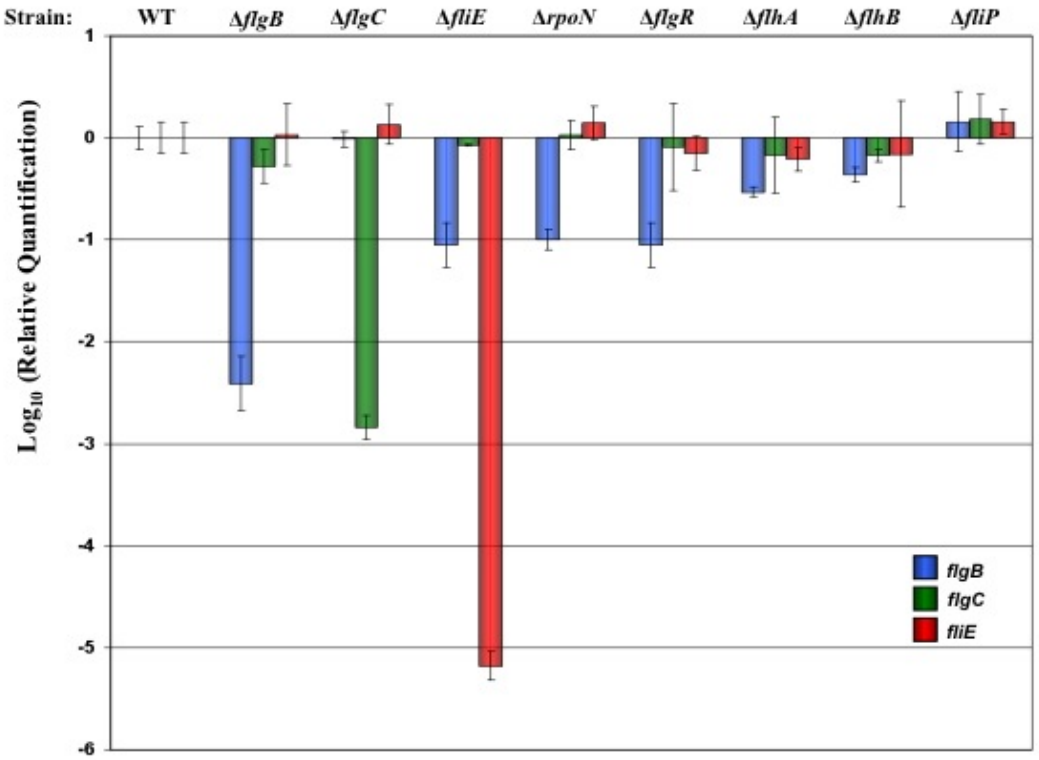

Figure 16: Preliminary real-time RT PCR analysis of *flgB*, *flgC* and *fliE* transcription. All strains were analyzed for the presence of *flgB*, *flgC*, and *fliE* transcripts, and as expected, strains with deletions of the *flgB*, *flgC*, or *fliE* coding sequences have the most severe defects for transcription of these genes. While there is no apparent defect for transcription of *flgC* and *fliE* in the Δ*rpoN*, Δ*flgR*, and FEA mutants, *flgB* transcription appears to be more severely affected in the Δ*rpoN*, Δ*flgR*, and Δ*fliE* mutants.

Linkage of flagellar substructure formation to FlgSR activity and transcriptional hierarchy

The finding that FliE is required for *flgB* transcription led us to wonder whether mutating the components of the flagellar substructures (rod, hook, and the proteins that anchor the flagellum to the periplasm and outer membrane) also affect expression of known or putative σ^{54}-dependent genes. We hypothesized that if only a subset of these proteins is required to activate σ^{54}-dependent gene expression, we could determine the minimum flagellar substructure required for FlgSR activation. This information could also provide clues about potential FlgS interaction partners or proteins with major roles in regulating *C. jejuni* flagellar gene transcription.

The flagellar rod and hook genes (and encoded proteins) analyzed in this portion of the study are *fliE* (putative FEA/rod junction protein), *flgB* (proximal rod), *flgC* (proximal rod) *flgF* (proximal rod), *flgG* (distal rod), *flgE* (hook), *flgI* (periplasmic anchoring protein/P ring), and *flgH* (outer membrane anchoring/L ring) (89, 134, 171) (Figure 17). For each mutant, the respective gene was interrupted with an antibiotic resistance cassette or an in-frame chromosomal deletion was constructed. All mutants demonstrate a complete lack of motility on semi-solid agar (not shown).

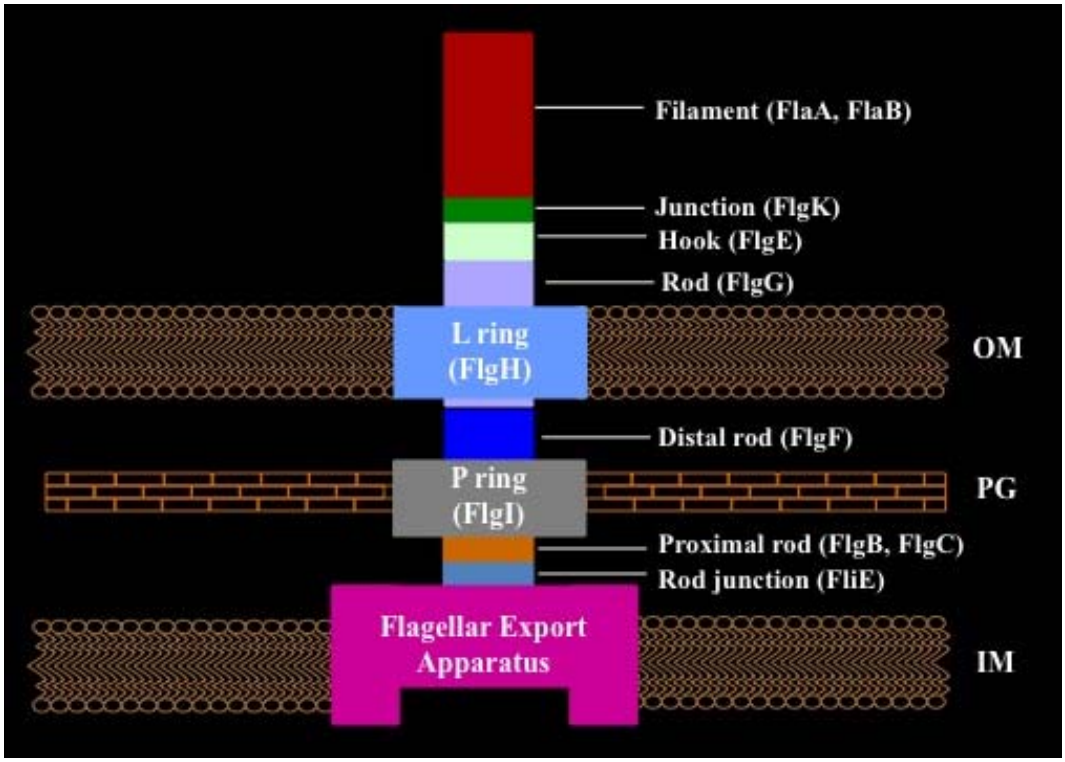

Figure 17. The putative *C. jejuni* flagellar substructure. Arrangement of the proteins is based on work performed with *Salmonella* and *E. coli*

To date, *flgDE2*::*nemo* and *flaB*::*astA* reporter constructs have been introduced into the Δ*flgB*, Δ*flgC*, and Δ*flgG* mutants. Preliminary analysis of the Δ*flgB* and Δ*flgC* mutants has been performed on MH agar with X-S, a chromogenic substrate that turns blue when the reporter constructs are active. Faint blue color was observed when the Δ*flgB* and Δ*flgC* reporter strains were plated on this medium, suggesting that both mutants can support reduced transcription of the reporter constructs. These results are not conclusive, but

suggest that FlgB and FlgC are necessary for the optimal transcription of the σ^{54}-dependent reporter constructs. Full analysis of these reporter strains and the construction of Δ*fliE* reporter strains will allow our lab to more fully determine if, and possibly how, the genes of the *flgBCfliE* locus influence FlgSR activation.

A preliminary arylsulfatase assay with the Δ*flgG* reporter strains indicates that there is no defect for the transcription of the σ^{54}-dependent reporter constructs, suggesting that the distal rod is not required for FlgSR-mediated transcription of middle and late flagellar genes (Figure 18). In fact, this mutant actually displays an increase in the expression of the *flgDE2*::*nemo* reporter construct while *flaB*::*astA* reporter expression was roughly equivalent to *flaB*::*astA* expression in the wild-type strain. This finding is surprising because in *Salmonella* and *E. coli*, if any of the middle genes (e.g. *flgG*) are absent, late genes such as those encoding the flagellins are not expressed (27). In *C. jejuni*, a deletion of the middle gene *flgG* does not appear to reduce transcription from the *flaB* promoter, which may indicate 1) *flaB* is not truly a late gene in *C. jejuni*, or 2) late gene transcription in *C. jejuni* does not require middle gene expression. In addition, the expression of *flgDE2::nemo* is increased 5-fold over wild-type, indicating that there is some modulation of expression of different σ^{54}-dependent genes in the *flgG* mutant. As both constructs are dependent on FlgSR and σ^{54}, it is currently unclear how *C. jejuni* is able to upregulate expression from one promoter without changing expression from the other. To more fully understand

the implications of this finding, our lab must finish construction of the substructure mutants and analyze reporter gene expression in each. When complete, this line of research will provide more insight into the temporal expression of flagellar genes and whether the transcriptional hierarchy observed in *Salmonella* actually exists in this bacterium. A better understanding of the mechanisms and regulation of FlgSR activation may also stem from these studies.

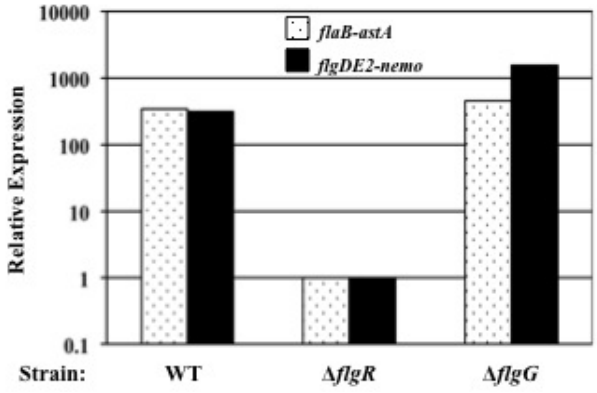

Figure 18: Preliminary analysis of *flaB*::*astA* and *flgDE2*::*nemo* in wild-type, Δ*flgR* and Δ*flgG* backgrounds. Values reported for each strain are arylsulfatase activity relative to the amount of expression of each transcriptional fusion in 81-176 Sm^R Δ*astA* Δ*flgR*, which was set to 1 arylsulfatase unit. For expression of *flaB*::*astA* (dotted bars), strains include wild-type DRH665 (81-176 Sm^R Δ*astA* *flaB*::*astA*; WT), DRH842 (81-176 Sm^R Δ*astA* Δ*flgR* *flaB*::*astA*), and SNJ933 [81-176 Sm^R Δ*astA* Δ*flgG* *flaB*::*astA*]. For expression of *flgDE2*::*nemo* (solid bars), strains include wild-type DRH533 (81-176 Sm^R Δ*astA* *flgDE2*::*nemo*; WT), DRH830 (81-176 Sm^R Δ*astA* Δ*flgR* *flgDE2*::*nemo*), and SNJ934 [81-176 Sm^R Δ*astA* Δ*flgG* *flgDE2*::*nemo*]. The genotype of each strain is listed below the graph.

Discussion

FliE and other flagellar substructure proteins may also influence FlgSR activity

Two independent two-hybrid studies suggest that FlgS interacts with the flagellum-associated protein FliE. Although we were unable to demonstrate direct interactions between FlgS and FliE, genetic analysis revealed that a Δ*fliE* strain is deficient for transcription of *flgB*, a σ^{54}-dependent flagellar gene, and that *fliE* transcription is independent of σ^{54}. In addition, we found that despite a genomic arrangement that suggests co-transcription, *flgB*, *flgC*, and *fliE* might not be expressed as a single transcript. It is possible that the genes are co-transcribed, but that internal terminators or small RNAs may prevent co-translation. Performing RT-PCR analysis will allow us to determine if the transcription of these genes is linked or if they are subject to more complex regulation than previously thought. Regulation of this locus may also vary between strains, as observed by the different expression profile reported by another group (95).

FliE merits further characterization due to its potential interaction with FlgS and influence on the transcription of *flgB*. Studies in *Salmonella* have attempted to characterize this protein, but it still remains poorly understood with respect to function. It is known that FliE interacts with FlgB in *Salmonella*, and it is currently thought that FliE could actually be part of the FEA or function as an adapter between the FEA and the rod proteins (80). If this is also the case in *C.*

jejuni, FliE could have an important role in the activation of σ^{54}-dependent flagellar gene transcription by providing a structural component necessary to build the proximal rod, in addition to influencing the expression of σ^{54}-dependent flagellar genes.

Other components of the flagellar substructure also may contribute to FlgSR activation. Interruption of any of the genes encoding rod (*flgB*, *flgC*, *flgF*, *flgG*), hook (*flgE*), or the periplasmic (*flgI*) and outer membrane (*flgH*) anchoring proteins results in a complete loss of flagellar motility. Completion of this analysis in all flagellar substructure mutants may lead to discoveries about the minimum flagellar substructure that is required for FlgSR activity and could provide indirect evidence for a FlgS binding partner. It could also uncover whether there is a temporal order of σ^{54}-dependent flagellar gene transcription in *C. jejuni*, where the transcription of one set of flagellar genes influences whether another set of flagellar genes can also be transcribed.

CHAPTER SEVEN

SUMMARY AND CONCLUSIONS

My thesis project has focused on understanding how multiple signaling events culminate in the activation of σ^{54}-dependent flagellar gene transcription. We have described work that provides a foundation for understanding the mechanisms for activating both FlgS and FlgR and how this process is linked to formation of the FEA. In Chapter 4, we described studies that demonstrated the FlgR response regulator, while highly homologous to the NtrC class of proteins, utilizes a number of atypical mechanisms to ensure that it is only activated under optimal conditions. These include an input domain that, based on phosphorylation state, has the ability to both positively and negatively regulate FlgR activity. Furthermore, the FlgR CTD appears to function not to bind DNA sequences upstream of target genes, but rather to ensure that FlgR cannot be phosphorylated in the absence of activated FlgS.

Although we were unable to precisely identify the signal that leads to FlgS autophosphorylation, we were able to link this phenomenon to the formation of the FEA in the inner membrane. This finding has led us to hypothesize that the FEA of *C. jejuni* may have functions that extend beyond protein export and could include signaling through a two-component system. Other bacteria that utilize

FlgSR-like two-component systems (e.g. *V. cholerae*, *P. aeruginosa*) to activate flagellar gene expression may also have FEAs that function in the activation of these proteins. Thus, this discovery may have broad applications to many bacteria of clinical importance and could potentially be utilized in the development of novel antimicrobials.

With the information described in Chapters Four, Five, and Six we have developed a model for the activation σ^{54}-dependent flagellar genes in *C. jejuni* (Figure 19). Two findings have led me to show FlgS as potentially interacting with the FEA: 1) a transposon mutagenesis screen did not reveal any potential negative regulator for FlgS that would suppress flagellar gene transcription in a FEA mutant; and more importantly 2) it appears that formation of the FEA, and not secretion of any substrates through this apparatus, stimulates FlgSR activity. It is possible that FlgS is interacting with another flagellum-associated protein for activation, and a current candidate for this hypothesis is the FliE protein, which is believed to be the first substrate secreted through the FEA in *Salmonella* (80) and may have additional functions in *C. jejuni*.

Preliminary studies analyzing mutants that are unable to form substructures of the flagellum (rod, hook, etc.) have also been useful in understanding the requirements for FlgSR activity. These early studies indicate that while all genes encoding the flagellar substructures are required for motility, only a subset appear to be required to activate transcription of the middle and late

reporter constructs *flgDE2*::*nemo* and *flaB*::*astA*. A mutation in the distal rod (*flgG*) does not appear to negatively affect expression of these constructs, while early analysis of mutations in genes encoding the proximal rod (*flgB*, *flgC*) indicate that expression is reduced markedly. That *flgB* and *flgC* may represent a class of "early" flagellar substrates that are required for expression of middle and late flagellar genes should be investigated, as this may provide insight into whether *C. jejuni* has a hierarchy of flagellar gene transcription that is similar to the cascades observed in other motile bacteria.

Studies from another line of investigation in our laboratory have allowed us to begin appreciating the role of FlhF. The *C. jejuni* FlhF protein has been shown to have GTPase activity (Balaban et al, in preparation) and is highly homologous to proteins that are involved in flagellar number and placement in other bacterial species (32, 142, 154). We initially believed that this protein may be required to target the members of the FEA to the inner membrane, thus functioning upstream of FlgR. Immunoblots to analyze the localization of FEA members in a Δ*flhF* background reveal that the proteins are not mislocalized in the absence of FlhF (Balaban et al, in preparation). Furthermore, while a FlhF deletion substantially reduces σ^{54}-dependent gene expression, introducing the *flgR* alleles with partial constitutive activity has no restorative effect in the Δ*flhF* background, indicating that FlgSR may not function downstream of FlhF. Thus, current hypotheses include the intriguing possibilities that FlhF may function in a

related pathway that converges on FlgR-mediated activation of σ^{54}-dependent flagellar genes or it could be involved in aspects of post-transcriptional regulation (Figure 19).

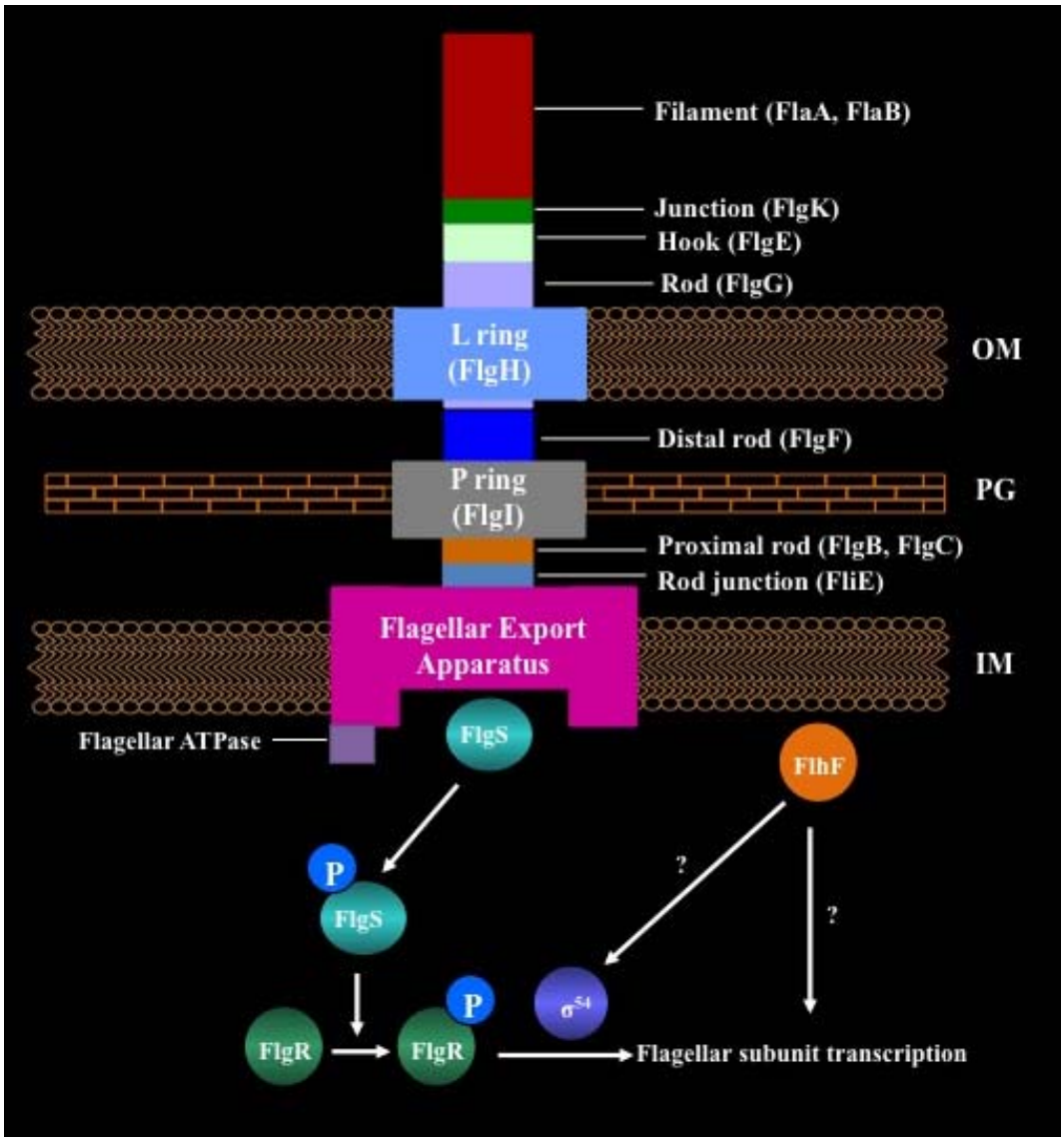

Figure 19: Current model for activation of σ^{54}-dependent flagellar gene expression in *C. jejuni*. FlgS activation is dependent on formation of the FEA and could be due to interactions with members of this protein complex. It is also

possible that FlgS could interact with other proteins that encode the substructures of the rod. FlgR activation is dependent on phosphorylation by FlgS, allowing the response regulator to mediate transcription of σ^{54}-dependent flagellar genes. Our lab is currently in the process of understanding the role of the FlhF GTPase in flagellar gene expression.

Further genetic and biochemical analyses of FlgR must be performed to gain a better understanding of how the protein interacts with σ^{54}, especially if the hypothesis that FlgR does not bind DNA is true. Current studies in our lab have begun examining the minimum promoter required for FlgR to active σ^{54}-dependent flagellar gene transcription. Further confirmation that FlgR activity does not require the binding of DNA is expected from experiments wherein the sequence upstream of the -24/-12 site of a σ^{54}–dependent flagellar gene is deleted. If FlgR is interacting with σ^{54} in solution and not with DNA binding, as we predict, transcription of this gene will be unaffected by the loss of the upstream sequence. Future studies should also include analysis of FlgR ATPase activity and the oligomerization state of the activated protein. Currently we do not know if FlgR acts as a monomer, dimer or multimer. Finally, a crystal structure of FlgR may provide the best clues about the structure and function of each domain, particularly the CTD, and the protein as a whole.

Our lab has recently made progress in identifying the source of $FlgR_{\Delta CTD}$ phosphorylation in the Δ*flgS* background. In a transposon mutagenesis screen of 81-176 Sm^R Δ*flgS* $flgR_{\Delta CTD}$ *flaB*::*astA*, several transposon mutants that had lost

the ability to express the reporter construct were found to have an insertion in the *pta* gene which encodes phosphotransacetylase and is important in aspects of cellular metabolism (Boll and Hendrixson, personal communication). This finding implicates acetyl phosphate as a potential phosphate donor for $FlgR_{\Delta CTD}$ and provides new possibilities for speculation about the true function of the CTD. We previously postulated that the FlgR CTD may have evolved to prevent promiscuous phosphorylation of FlgR by other histidine kinases. This recent discovery may imply a role for the CTD in preventing FlgR activation from by-products of basic cell metabolic activities such as acetyl phosphate, ensuring that FlgR activation is coupled only to flagellar biosynthesis. The *H. pylori* FlgR protein naturally lacks a CTD, but unlike $FlgR_{\Delta CTD}$, it does not function in *C. jejuni* in the absence of FlgS. This suggests that it may have alternative means to prevent non-FlgS phosphorylation. Comparative analyses of $FlgR_{\Delta CTD}$ and $FlgR_{Hp}$ *in vitro* with different phosphodonors may also provide some interesting clues about the evolution of FlgR proteins in related species of bacteria.

The analysis of FlgS presented in this work revealed the protein is a cytoplasmic sensor kinase and that autophosphorylation of residue H141 is crucial for activating σ^{54}-dependent flagellar gene expression. The N-terminal domain of FlgS shows little homology to non-FlgS proteins and is currently of unknown function. We believe that this domain contains the residues that sense the FlgS activating signal, possibly emanating from the FEA or interactions with FliE. The

creation of small amino acid deletions within this domain may allow our group to define which residues are crucial for FlgS activation or protein-protein interactions. We have also found that proper localization of the FEA to the inner membrane, rather than substrate secretion activates FlgS for expression of σ^{54}-dependent flagellar genes. The precise manner in which formation of the FEA influences FlgS activity has not been determined, but future analyses will focus on defining how an activating signal may emanate from the FEA.

Preliminary work indicates that FlgB, FlgC, and FlgG contribute to FlgSR activation, albeit in different manners. Completion of the flagellar substructure studies could describe previously unknown signaling events or FlgS interaction partners. Additionally, identifying all putative σ^{54}-dependent flagellar genes through bioinformatic analysis and confirming the findings through reverse transcriptase-PCR will likely allow our lab to fine-tune the model presented in Figure 19 and could uncover new flagellar genes for future studies. This analysis could also alter the classification of "early," "middle," and "late" *C. jejuni* flagellar genes that is used in our lab. The classification we currently use is based on the transcriptional cascades observed in other bacteria such as species of *Salmonella* (27) and *Pseudomonas* (35), and may not accurately represent the order of gene transcription and proteins secretion in *C. jejuni*.

The work presented here provides a detailed insight into the mechanisms for regulating and activating the FlgSR two-component system. Many of these

findings have pointed toward *C. jejuni* evolving previously undescribed mechanisms for controlling the activities of proteins that are crucial to a regulatory cascade that requires the expression of genes in a precise temporal order. Future analyses will no doubt enhance our understanding of this pathway, but may also show if and how *C. jejuni* can potentially link flagellar motility to metabolism, and the precise role of motility in host interactions for the development of commensalism and disease.

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