

DETERMINANTS INFLUENCING POLAR FLAGELLAR BIOSYNTHESIS AND  
CELL DIVISION IN *CAMPYLOBACTER JEJUNI*

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## DEDICATION

To my parents, Ibrahim and Hadiye Balaban.

For their love and support.

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DETERMINANTS INFLUENCING POLAR FLAGELLAR BIOSYNTHESIS AND  
CELL DIVISION IN *CAMPYLOBACTER JEJUNI*

by

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CELL DIVISION IN *CAMPYLOBACTER JEJUNI*

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The University of Texas Southwestern Medical Center at Dallas, 2011

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*Campylobacter jejuni* is a worldwide leading cause of bacterial gastrointestinal disease. The natural habitat of this organism is the gastrointestinal tracts of warm-blooded animals, especially poultry, where the bacterium promotes a harmless commensal colonization. The abundance of *C. jejuni* in poultry creates a risk for food-borne infections to human populations. Flagellar motility by *C. jejuni* is required to colonize both human and animal hosts. For motility, *C. jejuni* produces amphitrichous flagella, resulting in the formation of a single flagellum at both poles. This work explored factors that regulate numerical and spatial parameters for amphitrichous flagellation. Two factors that have been identified to control flagellar placement and

numbers in polarly-flagellated bacteria are the FlhF GTPase and the FlhG ATPase. FlhF has been shown to be required for regulation of flagellar gene expression and flagellar placement in some *Pseudomonas* and *Vibrio* species. Characterization of FlhF in *C. jejuni* was accomplished by creating point mutants in C-terminal GTPase domain of FlhF to decrease its GTPase activity. GTPase mutants, unlike mutants that lack FlhF, did not have a significant reduction in  $\sigma^{54}$ -dependent flagellar gene expression. Instead, a significant proportion of the population produced flagella at lateral sites or produced multiple flagella at a pole, whereas wild-type bacteria produced single polar flagella. Further experiments suggested that FlhF functions downstream of the FlgSR-flagellar export apparatus (FEA) pathway to activate  $\sigma^{54}$ -dependent flagellar gene expression. Thus, our data suggested that FlhF and its GTPase activity are required for distinct processes in flagellar gene regulation. FlhG has been shown to control flagellar numbers in *Pseudomonas* and *Vibrio* species. We examined *flhG* mutants and confirmed that FlhG regulates flagellar numbers. *C. jejuni flhG* mutants also demonstrated a minicell phenotype, which is the result of division erroneously occurring at polar regions. Further examination revealed that FlhG and the flagellar base components compose a novel division inhibition system to spatially prevent polar division and encourage septation at the cellular midpoint for symmetrical division. This work greatly extends our understanding of factors that govern spatial and numerical patterns of polar flagellation and has identified an unprecedented system to spatially regulate division in bacteria.

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## PRIOR PUBLICATIONS

**Balaban, M.**, Joslin, S. N. and D. R. Hendrixson. 2009. FlhF and Its GTPase Activity Are Required for Distinct Processes in Flagellar Gene Regulation and Biosynthesis in *Campylobacter jejuni*. *J Bacteriol* **191**:6602-6611

**Balaban, M.** and D. R. Hendrixson. 2011. Flagellar Base Structure Is Required for Division Site Determination Through the Flagellar Number Regulator FlhG. *Submitted*

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**LIST OF ABBREVIATIONS**

Bs	<i>Bacillus subtilis</i>
cat	Chloramphenicol acetyl transferase
CDT	Cytolethal distending toxin
Cj	<i>Campylobacter jejuni</i>
DAPI	4',6-diamidino-2-phenylindole
Ec	<i>Escherichia coli</i>
FEA	Flagellar export apparatus
GAP	GTPase activating protein
GBS	Guillian-Barré syndrome
GEF	GTP exchange factor
GFP	Green fluorescent protein
GST	Glutathione S-Transferase
Hp	<i>Helicobacter pylori</i>
IVIG	Intravenous immunoglobulin
LB	Luria-Bertani
LOS	Lipooligosaccharide
µm	Micrometer
OD	Optical Density
Pa	<i>Pseudomonas aeruginosa</i>
Pp	<i>Pseudomonas putida</i>
RNAP	RNA polymerase

RT-PCR	Real-time reverse transcription polymerase chain reaction
Sec	General secretory pathway
SIMIBI	Signal recognition particle, MinD and BioD
SRP	Signal recognition particle
T3SS	Type three secretion system
TCA	Tricarboxyl acid
TCS	Two-component system
Va	<i>Vibrio alginolyticus</i>
Vc	<i>Vibrio cholerae</i>
WT	Wild-type
WCL	Whole cell lysate

## CHAPTER ONE

### INTRODUCTION

*Campylobacter jejuni* is a Gram-negative, helical-shaped organism in the  $\epsilon$ -proteobacterial class. *C. jejuni* is a leading cause of bacterial diarrheal disease in developed countries and a major cause of gastroenteritis throughout the world (128). In contrast, *C. jejuni* lives as a commensal organism in warm-blooded animals, including poultry and livestock, making the bacterium an important agricultural and food safety concern (135). The prevalence of *C. jejuni* in avian species, particularly chickens, is very high and results in over 50% of retail chicken meat being contaminated with the bacterium (189). Both its prevalence in the human food supply and a low infectious dose contribute to the high incidence of *C. jejuni* gastroenteritis, termed Campylobacteriosis (147).

The importance of *C. jejuni* in human health and food safety was first recognized during the 1980s (100). In addition to acute gastroenteritis, *C. jejuni* is linked to a post-infection syndrome called Guillian-Barré syndrome (GBS), which is an autoimmune disorder that can result in temporary paralysis of the peripheral nervous system (147). Lipooligosaccharide structures found on the surface of *C. jejuni* mimic the gangliosides found on nerve cells in the human body, which is thought to cause an autoimmune reaction detrimental to the nervous system that leads to paralysis (97).

Factors that are important in *C. jejuni* pathogenesis of disease are the polysaccharide capsule, cytolethal distending toxin, adhesion factors and flagellar

motility (147). Flagellar motility is the only determinant that has been proven to be required for both infection of humans to cause disease and infection of poultry to promote commensal colonization (13).

*C. jejuni* usually produces a single flagellum at both poles, resulting in amphitrichous flagellation. The other flagellation patterns observed in bacteria are: monotrichous, a single flagellum at a single pole; lophotrichous, multiple flagella at a single pole; and peritrichous, multiple flagella distributed over the bacterial surface. To maintain amphitrichous flagellation, *C. jejuni* must have a mechanism to control spatial and numerical patterns of flagellar biosynthesis. For instance, a daughter cell lacks a flagellum at the new pole after division. Thus, the bacterium must have a mechanism to ensure that a flagellum is constructed at the new pole and only one flagellum is produced at this site.

Flagellar biosynthesis pathways are well studied in peritrichous bacteria, which do not demonstrate strict restrictions in the location and numbers of flagella. In general, factors required for numerical and spatial control of flagellar biosynthesis in a broad range of polarly-flagellated bacteria are not well understood. Some research has been performed with *Pseudomonas* and *Vibrio* species to identify mechanisms facilitating monotrichous flagellation. In these studies, it was shown that the FlhF GTPase is required for polar placement of flagella in addition to some requirements for flagellar gene expression (33, 132, 140). Another protein, the putative FlhG ATPase, was shown to influence numbers of flagella by either controlling the expression or the activity of the master transcriptional regulator in these polarly-flagellated bacteria (33, 34).

My study focused on identifying and understanding factors that control flagellar placement and number for amphitrichous flagellation in *C. jejuni*. In Chapter III, I described work demonstrating that the FlhF GTPase is involved in two distinct steps for flagellar biosynthesis in *C. jejuni*. The GTPase activity of FlhF is required for correct polar placement of flagella, whereas this activity is not required for flagellar gene expression. Instead, FlhF, in a GTPase-independent manner, is required for  $\sigma^{54}$ -dependent flagellar gene expression. In the following chapter, I describe work identifying FlhG as a factor that controls flagellar number in *C. jejuni*. Strains lacking *flhG* frequently produce more than one flagellum at a single pole. In addition to a role in numerical control of flagella, I show that FlhG is required for division site determination. In many bacterial systems, the MinD ATPase, along with other proteins, regulate where division sites form (16). However, *C. jejuni* lacks the canonical Min system. I found that FlhG, which is homologous to MinD, compensates for the lack of MinD in *C. jejuni* and influences the placement of the division site to initiate division. Further investigations revealed that polar flagellar biosynthesis, and more specifically components of the flagellar MS ring and switch complex, are required for FlhG to mediate its role in division site determination. These findings indicate that flagellar biosynthesis and division are linked in *C. jejuni*. Furthermore, these findings reveal a new paradigm for regulating placement of bacterial division sites that may occur in a broad range of polarly-flagellated bacteria.

## CHAPTER TWO Review of the Literature

### GENERAL INFORMATION ABOUT CAMPYLOBACTERS

#### History and Taxonomy of Campylobacters

*Campylobacter jejuni* is a Gram-negative,  $\epsilon$ -proteobacteria and a very prominent member the bacterial genus *Campylobacter*. “Campylobacter” means “curved bacteria”, which refers to the helical shape of the organism (Figure 1). *Campylobacters* was first observed in the stool samples of infants with diarrhea in 1886 by the German scientist Theodor Escherich, but they were described as vibrio-like bacteria (95). Therefore, *Campylobacter* species were thought to belong to the *Vibrio* genus until they were recognized as an independent taxonomic entity in 1963 (40, 156). The first *Campylobacter* species discovered was *Campylobacter fetus*, which was observed in 1906 from sheep that had abortive pregnancies (161). *Campylobacter jejuni* was first successfully isolated from cattle that showed symptoms of diarrhea (85). The first isolation of *C. jejuni* from humans with diarrhea was in 1971 (144). The development of 16S rRNA sequencing techniques in the 1980’s and whole genome sequencing in the following decades have provided a more accurate classification of Campylobacters (40).



**Figure 1. Electron Micrograph of *Campylobacter jejuni*.** *C. jejuni* 81-176 is a clinical isolate that has been isolated from a patient with gastroenteritis (100). Bar represents 1  $\mu\text{m}$ .

The genus *Campylobacter*, includes some important pathogens. The most important pathogens of humans that are frequent causes of diarrheal disease are *C. jejuni* and *Campylobacter coli*. The other important pathogenic species include *C. fetus*, which is responsible for abortions in many agriculturally-important animals (123). Other species in this genus are known to colonize both humans and warm-blooded animals, and in some cases promote disease, but the most well-studied organism in this genus is *C. jejuni* (40).

### **General Properties of Campylobacters**

As the name implies, *Campylobacter* species have a helical shape, with cell bodies between 0.5 to 5  $\mu\text{m}$  in length (40). Most species demonstrate amphitrichous flagellation, with a single flagellum at both poles, but some species produce either no flagella or multiple polar flagella (145). Unlike another prominent  $\epsilon$ -proteobacteria,

*Helicobacter pylori*, which has a lipid membrane around its flagella to result in sheathed flagella, the flagella of *Campylobacter* species are not sheathed (54).

Swimming motility of *C. jejuni* is conferred by the flagella, which are whip-like structures that extend from the bacteria that function like a propeller (58). When the flagella turn counterclockwise, they push the bacterium to promote smooth swimming in a specific direction. When they turn clockwise, it causes tumbling to change directions. These patterns of movements are tightly regulated so that the bacterium moves toward favorable environments for growth (41). The rotating action of the flagellum and the helical shape of the bacterium allow *Campylobacter* species to turn like a corkscrew with a high velocity of motility, especially in viscous environments such as the mucus layer covering the gut epithelia (44). Having a flagellum at each pole also promotes a characteristic darting motility for *C. jejuni*. When observed under microscope, the bacteria move back and forth rapidly along a short and straight path (139, 159). This darting motility is thought to assist in increasing interactions with intestinal epithelial cells and persistence in intestinal mucosal surfaces in hosts (168).

*Campylobacter* species have fairly unusual properties of growth when compared to many other bacterial pathogens. Campylobacters are thermophilic organisms, with optimal growth temperatures from 37 °C to 42 °C. The thermophilic nature of *C. jejuni* provides an advantage for colonizing the natural avian host, which has a body temperature of 42 °C (5).

Many species of *Campylobacter* require a microaerobic environment for growth, which means lower O<sub>2</sub> concentrations and higher CO<sub>2</sub> concentrations compared to atmospheric conditions. The standard microaerobic environment used to grow *C. jejuni*

typically consists of 85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% O<sub>2</sub> (5). Some species require extra hydrogen sources in the environment for growth in microaerobic conditions. In addition, some species can be grown under anaerobic conditions (40).

Another interesting growth characteristic of Campylobacters is their preference of carbon source. The members of this genus are assachrolytic, and unable to use sugars to generate energy, which makes the bacteria dependent on amino acids and metabolites of tricarboxyl acid cycle (TCA) for energy production. The amino acids that are preferentially metabolized by *C. jejuni* are serine, glutamate, aspartate and proline (93). In addition to these amino acids, TCA metabolites like pyruvate are used by *C. jejuni* to generate energy (93). Despite not using sugars for energy production, Campylobacters devote much energy to synthesize sugars to glycosylate outer membrane components and proteins (174). The glycosylated structures have important functions in Campylobacters, such as evasion of host immune response and other biological activities (186).

Campylobacters are usually found associated with warm-blooded animals. They colonize avian and mammalian species as commensals and pathogens depending on the host and bacterial species. For example, *C. jejuni* lives as a commensal in the intestinal tracts of wild and domesticated mammals and birds (5). In chickens, the bacteria colonize the lower intestinal tract including the ceca and the large intestine by occupying the luminal mucosal surfaces without invading the epithelia. However, *C. jejuni* causes disease when it colonizes humans. In humans, *C. jejuni* colonizes the lower intestines and colon and is able to invade epithelial cells to result in inflammation and diarrheal disease (186). An *ex vivo* reservoir for this species is not well examined. Since outbreak cases of *C. jejuni* disease are due to consumption of contaminated water and milk, a

reservoir likely exists for an important *ex vivo* lifestyle (138). Future studies are required to clearly identify and analyze the viability of *C. jejuni* in these reservoirs.

### **Diseases of Campylobacters**

*Campylobacter* species cause an array of diseases, ranging from enteric to abortive diseases. Many *Campylobacter* species have weak correlations with other diseases and it remains unclear if these bacteria are indeed pathogens at this time. For example, *Campylobacter hyointestinalis* can be found in pigs with porcine proliferative enteritis and diarrhea, but it is unclear if this bacterium is a cause of this disease (179). *C. sputorum* has been associated with abortive diseases, bronchitis and diarrhea, but several subspecies of the bacterium are found in both healthy and diseased animals. In contrast, the most well-defined pathogens of this genus are *C. fetus*, *C. coli* and *C. jejuni*.

*C. fetus* causes abortions and infertility in cattle and sheep due to its ability to colonize the genital tracts of these animals. *C. fetus* can also cause sepsis in humans, especially in immunodeficient patients and neonates (14). *C. coli* and *C. jejuni* cause campylobacteriosis, an acute diarrheal disease in humans that can become an inflammatory enteritis resulting in bloody diarrhea or dysentery (30). Symptoms of campylobacteriosis include fever, abdominal pain and cramping. The disease usually resolves in a week, but can be shortened by a few days if antibiotic therapy is given. It is estimated that each year approximately 2.5 million people develop diarrheal diseases due to *C. jejuni* infection (21).

In about one in every 1000 cases, *Campylobacter* infection in humans is followed by an autoimmune peripheral neuropathy called Guillian-Barré syndrome (GBS) (5).

This syndrome manifests itself as weakness of limbs, starting from legs, spreading to arms and face, and ending with the complete loss of tendonal reflexes. In severe cases, artificial ventilation support is required. Importantly, approximately 40% of the GBS patients reported a preceding *Campylobacter* infection.

The most common manifestation of GBS is acute inflammatory demyelinating polyneuropathy in which the immune system attacks the myelin sheaths that covers the axons of the motor neurons of the peripheral nervous system. Miller-Fisher syndrome, another form of GBS, is characterized by a descending paralysis. The antibody response that is responsible for GBS is thought to be elicited against LOS structures that are found on the surface of *C. jejuni* (187). As such, almost 90% of patients with Miller-Fisher syndrome have antibodies against lipooligosaccharide (LOS) moieties found on the outer surface of *C. jejuni* (55). LOS structures are composed of a liposaccharide base and a variable glycosylated epitope on this base structure. Some glycosylation epitopes that are added to the LOS molecules mimic the molecular structures of gangliosides found on the peripheral neurons of the human nervous system. *C. jejuni* elicits an antibody response against LOS that can cross react with gangliosides on neurons, causing neuropathy of the peripheral nervous system. The structure of LOS and the mimicry will be discussed in detail below.

Treatment for GBS includes plasma exchange or plasmapheresis, which removes the autoimmune antibodies from the patients, or introduction of intravenous immunoglobulins (IVIG). A possible mechanism of action for IVIG is that the immunoglobulins block the Fc receptors on macrophages to stop the destructive actions of these immune cells on the myelinated sheath on nerves (80). The patients usually

fully recover after many weeks. Mortality may occur when pulmonary or autonomic neurological disorders are affected.

## EPIDEMIOLOGY AND PATHOGENESIS OF *C. jejuni*

### Pathology of Campylobacteriosis

*C. jejuni* promotes two different associations with hosts, commensalism in poultry and other animals and pathogenesis in humans. Unlike in human infection, *C. jejuni* does not elicit inflammation of the intestine or diarrhea as a commensal in birds. Differences in interactions of *C. jejuni* with host tissues and host responses are likely responsible for these different outcomes of infection. However, factors that trigger these differences in host responses have not yet been identified and more research is required to uncover the mechanism of pathogenesis of disease.

Symptoms common to campylobacteriosis include diarrhea, inflammation of the colon, infiltration of neutrophils into enteric mucosa, blood in stool, fever and abdominal pain (17, 186). High IL-8 levels, which contribute to inflammation, is another characteristics of *C. jejuni* disease (186).

Mice normally are normally not colonized by *C. jejuni* for prolonged periods and do not exhibit symptoms of disease (176). Some immunodeficient mice are more susceptible to disease and colonization. Mice that lack IL-10 and NF- $\kappa$ B (49, 115), components of innate immunity are more susceptible to infection, suggesting that innate immunity is a major factor in preventing *C. jejuni* pathogenesis.

**Epidemiology of *C. jejuni***

*C. jejuni* is the most common cause of bacterial diarrheal disease in the developed world. Although *C. jejuni* causes a self-limiting disease, around 120 people die each year due to *C. jejuni* infection in the USA. It is also a very common cause of disease in developing countries, mainly among children. Recurrent infections during childhood in developing countries are thought to confer protective immunity. As such, campylobacteriosis is not commonly reported in adults in developing countries. According to the FoodNet surveillance program, 13 cases of *C. jejuni* disease were reported per 100,000 individuals in the USA in 2009 (48), making *C. jejuni* only second to *Salmonella* species. Because of the relatively mild nature of *C. jejuni* enteritis or failure to use techniques to properly isolate *C. jejuni* from infected individuals, the actual number of cases of *C. jejuni* disease in the USA is thought to be much higher, possibly reaching 2.5 million cases per year (20). *C. jejuni* disease in developed countries is mostly seen in all ages, but with higher incidence in children under 5 years, young adults and elderly. The disease shows a seasonal distribution, which increases in March, peaks during the summer months of June and July, and decreases through the fall months. The seasonal pattern is thought to be related to lack of adequate refrigeration or freezing of meat products.

Campylobacter infections in the USA showed a steep decrease in 1999 and stabilized afterwards, which is likely due to food-safety regulations that were imposed by the government on the food industry (138). These regulations include increased chlorination of the meat products and some other contamination-reducing procedures.

### *Transmission of Campylobacter jejuni*

Most of the infections due to *C. jejuni* are associated with consumption or handling of contaminated food or water. Because the organism lives as a commensal in avian species, the occurrence of *C. jejuni* in poultry meat is very high. Sources that are most often identified as causes of *C. jejuni* transmission to humans include chicken meat, barbeque meat, pork, wild-game meat, raw or contaminated milk and water (5). In several studies conducted in industrialized countries, retail chicken meats are highly contaminated with *C. jejuni* (5). The contamination of meat products usually occurs during slaughtering and processing when intestinal contents are released and contaminate the meat.

A logical approach to reduce the incidence of *C. jejuni* disease is to eliminate or decrease *C. jejuni* colonization in poultry and animals. Various approaches have attempted to use vaccines or antimicrobials to reduce colonization rates of *C. jejuni* in agriculture. The widespread use of antibiotics has proven to be unsuccessful, as exemplified with the case of ciprofloxacin. *C. jejuni* has developed very rapid resistance to this antibiotic, eliminating its use in medicine to treat *C. jejuni* infections (119, 188). Also, accumulating resistance to fluoroquinolones actually increased the fitness of *C. jejuni* for colonization of poultry and augmented the colonization burden of poultry with *C. jejuni* (188).

### **Treatment of Campylobacteriosis**

Campylobacteriosis is usually self-limiting disease that is relatively mild in nature. Often, rehydration therapy is the only medical intervention recommended. In

some cases, the disease can manifest itself as bloody diarrhea or dysentery. The administration of antibiotics might be employed in these cases. For infected immunocompromised patients, antibiotic therapy is recommended as the infection can lead to bacteremia if untreated. If antibiotics are used, the antibiotics of choice include erythromycin and azithromycin. Recently, ciproflaxin resistance in *C. jejuni* increased to levels where this antibiotic is no more suitable to treat *C. jejuni* infections.

### **Virulence Factors of *C. jejuni***

The proposed virulence or colonization factors of *C. jejuni* to infect humans and poultry include adhesins, polysaccharide capsule, LOS, flagellar motility and cytolethal distending toxin (CDT).

#### *Adhesion Factors*

In *in vitro* models of infection, *C. jejuni* attaches to and invades colonic and intestinal epithelial cells, but at a rate lower than other commonly studied intestinal pathogens (50). The most well-studied factors required for adhesion of *C. jejuni* to host cells are JlpA and CadF, which bind to heat-shock protein Hsp90 $\alpha$  and fibronectin, respectively (83, 98). LOS structures, flagellins and the major outer membrane protein have been implicated as potential adhesins for eukaryotic cells as well (50).

After adherence, *C. jejuni* is able to penetrate epithelial cells (175). While *C. jejuni* can invade non-polarized epithelial cells, *C. jejuni* was also observed to invade from the basolateral surface of polarized intestinal epithelial cells (127). If this system is relevant *in vivo*, *C. jejuni* may be trafficked to the basolateral surface of the intestinal

epithelial barrier via M cells of the intestinal epithelia. Some evidence suggest that internalization is likely facilitated by reorganizing microtubules of host cells and also dependent on caveolin-1 (162, 180). Two factors that may facilitate invasion of eukaryotic cells include flagellar motility and possibly the flagellar secreted proteins CiaB and FlaC (99, 162). However, the functions of these proteins in invasion or intracellular survival are not known. The FspA proteins are also secreted in a flagellum-dependent manner. FspA1 and FspA2 are encoded by two different alleles, with FspA1 inducing apoptosis of eukaryotic cells (146). However, the relevance of these proteins for virulence is not known.

#### *Polysaccharide Capsule*

*C. jejuni* strains produce a polysaccharide capsule that can vary between strains. As such, the capsular polysaccharide is the basis for a hemaagglutination-based Penner serotyping procedure for classification of *C. jejuni* strains (90). Capsular polysaccharides are extracellular structures that often protect bacteria from adverse environmental conditions and immune responses (60). Until several years ago, the capsular polysaccharides of *C. jejuni* were originally thought to be high molecular weight LOS structures (90). The first evidence that *C. jejuni* produces a capsule was shown in 1996 by Chart et al, and the genes for capsule production later confirmed after the complete sequencing of the genome (23). The general structure of the capsule includes repeating oligosaccharide units with variable modifications attached to a dipalmitoyl-glycerophosphate lipid anchor (24, 31). The capsular structure shows significant variation amongst strains as the *kps* gene cluster, which encodes the enzymes required to

synthesize capsular polysaccharide, is variable among strains (141). In addition to protection against the actions of serum, the capsular polysaccharide is required by *C. jejuni* for invasion of epithelial cells, colonization of avian host and virulence in ferret model of disease (8).

#### *Lipooligosaccharide (LOS)*

Another proposed virulence factor is the LOS structure that is found in the outer membrane of the *C. jejuni*. Instead of having a traditional lipopolysaccharide (LPS), *C. jejuni* has a unique LOS, consisting of a lipid A structure, which is similar to those found in other Gram-negative organisms, and an oligosaccharide structure decorated with a variable outer core (89). The lipid A structure is an endotoxic molecule, although *C. jejuni* lipid A is slightly less active than its the well-studied *E. coli* counterpart (155). The oligosaccharide inner core structures are fairly conserved amongst *C. jejuni* strains, containing the trisaccharide  $L,D\text{-Hep-}\alpha(1,3)\text{-}L,D\text{-Hep}(1,5)\text{-Kdo}$  with only some minor strain variations to this structure. The outer core of the oligosaccharide structure of *C. jejuni* LOS often contains N-acetylneuraminic acid (Neu5Ac), also known as sialic acid (89). Addition of sialic acid residues to the oligosaccharide structures creates epitopes that mimic gangliosides found on human neuronal cells (7). Antibodies that are generated against *C. jejuni* LOS structures can also bind to gangliosides on neuronal cells and damage these cells (134). This autoimmune reaction is thought to be the predominant cause of GBS.

In *C. jejuni*, interstrain variation of LOS outer core structure is achieved through different combination of genes that encode enzymes responsible for synthesizing the

glycosylated epitopes (51). In addition, intrastrain variation can occur through phase variation of genes encoding these enzymes (51). The homopolymeric tracts within the genes cause phase-variable production of certain enzymes, resulting in different decoration of LOS outer core structures.

#### *Cytolethal Distending Toxin*

A well-described toxin produced by *C. jejuni* is the cytolethal distending toxin (CDT), although there is still debate concerning how it functions to cause cell death or its relevance in promoting pathogenesis of *C. jejuni* disease. The toxin is composed of three subunits, CdtA, CdtB and CdtC (104). The CdtA and CdtC subunits are responsible for binding to target cells whereas CdtB performs the effector functions of the toxin (105). After internalization, CdtB is localized to the nucleus of the target cell (120). The protein has homology to DNase I and has been shown to cause double-strand DNA breaks to result in cell cycle arrest and eventually cytotoxicity (104). In addition, *in vitro* studies showed that CDT induces IL-8 secretion in intestinal epithelial cells (69). While the cytotoxicity is observed *in vitro* with human intestinal cells, it is not observed during commensal colonization of chickens. Even though the *cdt* genes are expressed in chicken ceca, CDT does not cause cytotoxicity or inflammation during commensalism (1).

#### *Flagellar Motility*

The most well established virulence and colonization factor for *C. jejuni* is flagellar motility. As mentioned above, the flagellar organelle is both used in swimming motility and as a machinery to secrete proteins that may be involved in virulence and

colonization processes. In human volunteer studies, flagellar motility is so far the only factor known to be required for establishment of infection (13). *C. jejuni* also requires flagellar motility for colonization of chicken intestine to promote commensalism (129). The colonization levels of non-motile mutants are significantly lower than wild-type bacteria. In addition, chemotactic motility to properly direct the orientation of flagellar motility is required for optimal colonization of chickens (66). These findings suggest that flagellar motility is required by the bacteria to find the correct niche in the gut of hosts to initiate and maintain infection.

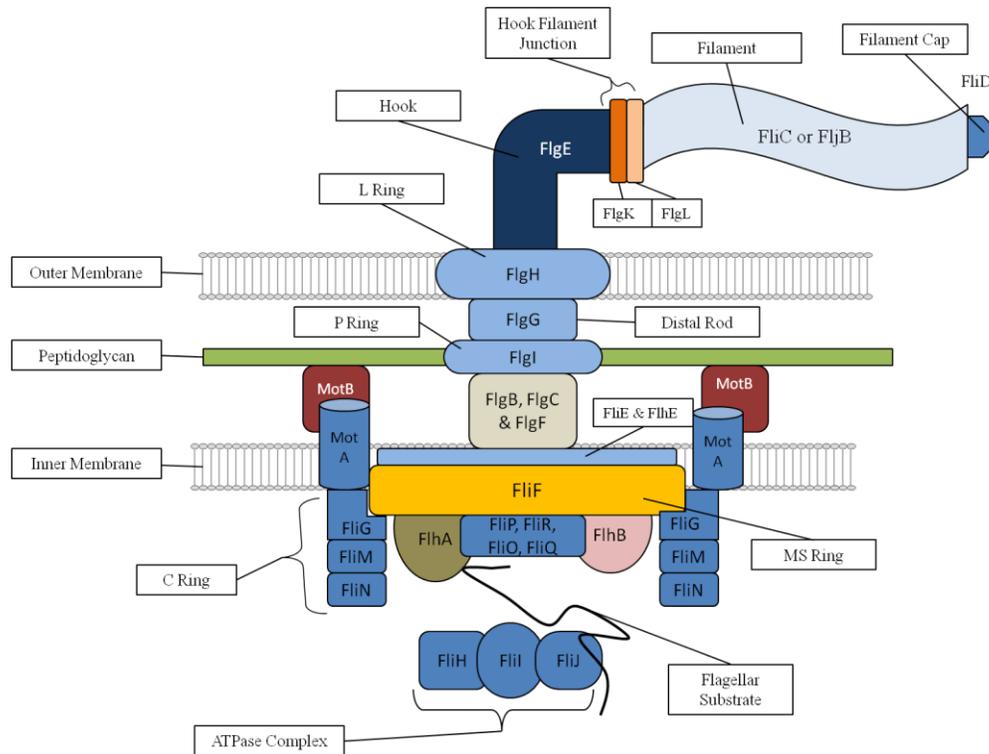
### **FLAGELLAR MOTILITY**

Living organisms change their surroundings for several reasons, including avoiding unfavorable conditions, searching for nutrients and reproduction. Every domain of life has a means of mobility. Animals have muscle and skeletal systems that promote movement. Plants move towards the sun by changing the turgor pressure in their specific tissues while their seeds have become adapted to be efficiently dispersed by natural factors such as wind. Unicellular organisms use subcellular structures to move within their habitat. Many bacterial species use flagella or pili for locomotion. Flagella are also used in unicellular eukaryotic organisms and in some specialized cells of higher animals and plants, such as the sperm cells. However, eukaryotic flagella are structurally and functionally different than bacterial flagella. Eukaryotic flagella are composed of tubulin-based structures, which provide the force for locomotion by generating waves. Bacterial flagella are composed of flagellin proteins and the force is generated as the

whip-like structure rotates in a helical manner like a propeller. In this section, the structure and biosynthesis of a canonical flagellar organelle will be explained.

### **Bacterial Flagellar Biosynthesis**

Historically, the most well-studied organisms in terms of flagellar biosynthesis are *Salmonella typhimurium* and *Escherichia coli*. In the first parts of this section, genetic and biosynthetic processes that regulate flagellar organelle development in *E. coli* and *S. typhimurium* will be explained. Because these model organisms produce peritrichous flagella, a discussion of different requirements for biosynthesis of flagella in polarly-flagellated bacteria, using *C. jejuni* as a model, will follow.



**Figure 2. Schematic diagram of the bacterial flagellum.** The bacterial flagellum consists of three main substructural complexes, basal body, hook and filament. (Adapted from Chevance et. al. 2008 (25))

### Structure of A Flagellum

The flagellum is composed of three substructures: an extracellular whip-like filament, an outer membrane-associated hook, and basal body which is located in the periplasm, inner membrane and cytoplasm (Figure 2). This general structural composition is conserved throughout almost all bacterial species.

## **Basal Body**

The basal body is responsible for a variety of functions. These functions include connecting the flagellar filament and hook to the bacterial cell body, secreting flagellar proteins, rotating the filament, and controlling the direction of flagellar rotation. The basal body is composed of a few different substructures including the periplasmic rod and rings, the inner membrane MS ring, and the cytoplasmic C ring which are described in further detail below.

### *MS Ring*

The inner membrane MS ring is thought to be the first structure that is assembled to begin flagellar biosynthesis (113). The MS ring is a homomultimeric membrane protein complex, consisting of 26 FliF monomers (84). The MS ring connects the rod to the motor/switch complex (C ring) and also houses the flagellar type III secretion system (T3SS), which is also known as flagellar export apparatus (FEA).

### *Flagellar Export Apparatus (FEA)*

The FEA is a multimeric inner membrane-bound protein complex that is required to secrete most flagellar proteins across the inner membrane for construction of the rod, hook and the filament. The elements of this complex are the FlhA, FlhB, FliP, FliR, FliQ and FliO proteins (113). The FEA machinery shows a significant resemblance and homology to T3SS, and it is thought to be a specialized T3SS for the flagellum. Almost all extracytoplasmic flagellar proteins are secreted through the FEA (112). The formation of the FEA can also initiate some regulatory signals in flagellar biosynthesis

pathway (68, 130), which will be discussed below. FlhB and FlhA have large cytoplasmic domains and are thought to have regulatory functions in secretion. FlhA is involved in secretion of flagellar proteins, working as a docking station for the substrates (152). FlhB is responsible for controlling substrate specificity of FEA-mediated secretion throughout steps in flagellar biosynthesis (125). For instance, after completion of the rod and hook, the FEA must specifically secrete flagellin subunits for filament synthesis. After the hook reaches a certain length, an autocleavage event occurs in FlhB that modifies the FEA so that it specifically secretes flagellins (45). The control of substrate switching is discussed below.

The other FEA elements FliP, FliR, FliO and FliQ are also required for secretion through FEA. Together, they likely form a channel that allows proteins to pass through the inner membrane in a partially-unfolded stage (113). In addition to these membrane-related FEA elements, there are cytoplasmic components, such as the FliI ATPase and its regulator/chaperone FliH, which facilitate efficient FEA-dependent secretion (126).

#### *Motor/Switch Complex*

The motor/switch complex forms a large component of the C ring. The C ring is located at the cytoplasmic face of the MS ring and is the most proximal component of the flagellum found at the flagellar base in the cytoplasm. Parts of the C ring functions as the flagellar rotor to turn the flagellum and the switch to control direction of rotation. In addition, the C ring assists in secretion of flagellar proteins.

The C ring component found at the base of the MS ring is the rotor formed by FliG. FliG has a similar symmetrical organization as the MS ring, 26 subunits aligned

with FliF of the MS ring and additional eight subunits are tilted to fit the structure (6). Protein-protein interactions between FliG and the MS ring facilitate its function of the rotor. The rotor rotates through a proton motive force generated by the movement of protons through MotA and MotB stator complexes, which are located in the inner membrane and interact with the rotor (11). As the protons flow through MotAB, they cause the rotor to move, which results in rotation of the flagellum.

FliM and FliN form a heteromeric complex attached to the base of the FliG rotor to complete the C ring structure (6). FliM interacts with regulators of the chemotaxis system to cause reversible rotational switching of the flagellum between clockwise and counterclockwise movements (170). When flagella are rotating counterclockwise, the bacteria swim in a smooth, straight direction. When the rotor moves clockwise, a tumbling motion occurs that changes the orientation of bacteria. The rate of this switching accounts for tactic movement of bacteria. Long stretches of smooth swimming and less frequent tumbling will occur when travelling towards a favorable environment, which is sensed by the chemotaxis system.

In addition to their motor/switch functions, the C ring elements also create an inverted cup structure at the cytoplasmic surface of the basal body (42). This cup structure is thought to act as a molecular funnel that concentrates flagellar proteins that are to be secreted by FEA. The role of FliN is to interact with chaperones and secretion substrates to position them in close proximity the FEA for secretion (53). Absence of the C ring creates defects in flagellar biosynthesis.

### *Rod*

The rod proteins include FlgB, FlgC, FlgF, FlgG, and FliE. FliE is thought to be the most proximal rod protein and FlgG has been shown to be the most distal (133) relative to inner membrane (72, 131). However, the order of the remaining subunits at the rod is not precisely known. The rod structure spans the periplasm and transmits rotational force from the rotor and MS ring to the hook.

Another rod protein, FlgJ assists in construction of this structure. FlgJ has two domains with two distinct functions. The N-terminal domain of FlgJ is responsible for scaffolding of the polymerizing rod structures, and the C-terminal domain has muramidase activity, which is required for efficient penetration through the rigid peptidoglycan layer by cleaving the glycosidic bonds (70).

### *P and L Rings*

The basal body has specific ring structures that are designed to facilitate the passage of the rod through the peptidoglycan layer and the outer membrane. In addition, these rings stabilize the rod structure. The ring in the peptidoglycan layer is called the P ring and the outer membrane ring is the L ring.

P ring formation requires two proteins, FlgI and FlgA (133). The ring is composed of of FlgI monomers, which are linked by disulfide bonds (71). FlgA is the periplasmic chaperone for FlgI (133). Both of these proteins are secreted via the Sec system rather than the FEA. After formation of the P ring, the rod penetrates the peptidoglycan layer.

The L ring forms a pore structure that allows penetration of rod through the outer membrane. The L ring is built from FlgH, which is an outer membrane lipoprotein (154). The C-terminus of FlgH interacts with FlgI, which suggests that the L ring interacts with P ring. Another requirement for L ring formation is the ceasing of polymerization of distal rod protein FlgG (26). This creates an additional step of regulation in the building of the flagellar structure by preventing an immature pore forming in the outer membrane when the rod structure has yet to be made.

## **Hook**

The hook is an extracellular structure that connects the flagellar basal body to the filament. The hook is a flexible structure and serves as a fulcrum to orient flagella properly during rotation. The hook is structurally composed of the FlgE protein (6) . Hook assembly begins after rod assembly is completed. The N-terminus of FlgJ is thought to be the capping protein for the rod structure. After rod polymerization is completed, FlgJ is displaced by the FlgD hook-capping protein to facilitate FlgE polymerization (113). The hook is made of 120 monomers of FlgE, which makes a helical structure of 55 nm in length.

The length of the hook is finely controlled by the protein FliK. FliK serves as a molecular ruler to tightly control the length of the hook (158). FliK is secreted in an unfolded stage through the FEA and the growing hook. When the hook is of a desired length, FliK is in an extended, unfolded state with the N-terminus of FliK positioned at the FEA and the C-terminus at the tip of the hook. At this unfolded state, FliK initiates an auto cleavage event in FlhB of the FEA that causes the FEA to then switch substrates

of secretion from FlgE to flagellins. The completed hook is then connected to the filament by two hook-associated proteins, FlgK and FlgL.

## **Filament**

The filament is a rigid helical structure, which gives it an ability to create a force to push bacteria forward when it is rotating. The filament is composed of protein subunits called flagellins. Flagellins have conserved N- and C-termini throughout different bacterial species with their central region showing variation. The N- and C-termini are involved in correct folding of the flagellins to promote polymerization (177). The central portion of the protein is exposed upon polymerization in the filament and usually has antigenic properties. Polymerization of flagellins into the filament requires the filament-capping protein FliD (81). FliD facilitates the correct folding and polymerization of flagellins into the filament structure at the tip of the filament. The flagellin subunits are translocated to the tip of the flagellum in a partially unfolded state, and with the help of FliD, they are assembled into a polymeric structure.

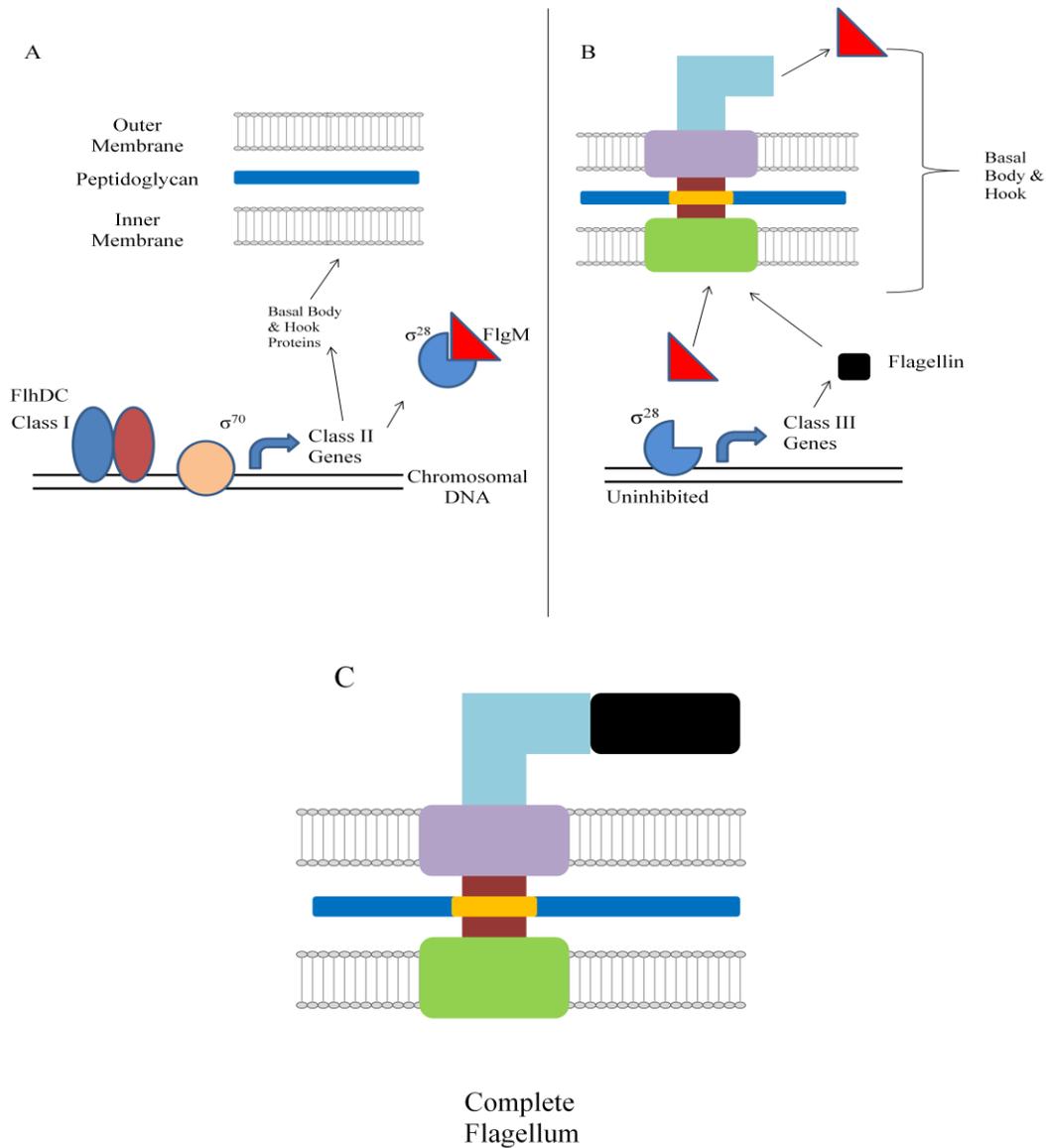
Depending on the bacterial species, more than one flagellin may be encoded in the genome, and these different flagellins may be incorporated into the filament structure in different ratios. In the case of *C. jejuni* there are two flagellins. FlaA is the major flagellin required for motility, whereas FlaB is a minor component of the filament that is dispensable for motility (59).

## **CONTROL OF FLAGELLAR GENE EXPRESSION AND BIOSYNTHESIS**

To construct a functional flagellum, more than 50 genes must be expressed in the correct order and at correct levels to produce flagellar proteins, which then must interact in the proper order for flagellar biosynthesis. In addition, the expression of flagellar genes is tightly controlled because construction of a flagellum and motility is expensive in terms of energy and protein resources. These factors combined create a necessity to tightly control flagellar organelle development.

### **Control of Flagellar Biosynthesis in Peritrichous *E. coli* and *S. typhimurium***

As *E. coli* and *S. typhimurium* are the most well-studied models of flagellar gene regulation, regulatory mechanisms in these bacteria will be examined first (25, 27). Flagellar genes are organized into three classes based on their temporal order of expression. The first class consists of genes encoding the master transcriptional regulator that initiates flagellar gene expression (Figure 3). The second class consists of genes encoding basal body proteins, hook proteins, the alternative sigma factor  $\sigma^{28}$  and the repressor of  $\sigma^{28}$ , FlgM. The third class consists of genes encoding flagellins, chaperones, motor proteins and chemotaxis system.



**Figure 3. Flagellar biosynthesis cascade in *E.coli* and *Salmonella*.** (A) Expression of class II genes by the action of the class I-encoded master regulator results in expression of basal body and hook genes. (B) The completed basal body-hook structure secretes the anti- $\sigma$  factor FlgM, relieving  $\sigma^{28}$  and resulting in expression of class III flagellar genes. (C) With the expression of all three classes in an ordered manner, the flagellar organelle is constructed properly.

### *The FlhDC Master Transcriptional Regulator*

*E. coli* and *Salmonella* induce expression of flagellar genes when required. The factors that affect flagellar biosynthesis include temperature, osmotic conditions, pH of the environment, planktonic lifestyle, location in host and cell cycle (163). All these inputs affect transcriptional and post-transcriptional regulation of genes encoding the master regulator, FlhDC. FlhDC acts as an activator of expression of the class II flagellar genes with  $\sigma^{70}$ . As a complex, FlhDC binds specific DNA sequences in target promoters (109). The detailed analysis of *E.coli* and *Salmonella* promoters revealed a consensus sequence composed of a 17-18 bp imperfect palindromic sequences with 11-12 bp of spacer region (29). FlhDC interacts with C-terminal domain of RNA polymerase  $\alpha$ -subunit to promote positive expression of class II flagellar genes (108).

### *Class II Flagellar Genes*

FlhDC-dependent class II genes include those encoding the MS ring, the FEA, P and L rings, rod, hook and C ring structures (25). In addition to these structural elements, two regulatory genes are classified as class II flagellar genes. These genes are *fliA*, encoding  $\sigma^{28}$  and *flgM* encoding the anti- $\sigma$  factor for  $\sigma^{28}$  (163). Upon expression of class II genes, the MS ring, C ring and FEA are constructed to facilitate secretion of rod and hook proteins to complete the basal body structure.

### *Class III Flagellar Genes*

The major flagellin gene in *E. coli* and *Salmonella* is *fliC*, a class III gene that requires  $\sigma^{28}$  for expression (27). Expression of *fliC* is inhibited until the hook is

synthesized, which completes the basal body structure. Inhibition of *fliC* and class III gene expression is mediated through FlgM suppressing  $\sigma^{28}$  by a direct interaction that prevents  $\sigma^{28}$  from interacting with RNA polymerase (137). Upon completion of the hook structure, FlgM is secreted through the hook-basal body structure via the FEA (88). The decrease of FlgM concentrations in the cytoplasm leads to release of  $\sigma^{28}$  from suppression of FlgM so that  $\sigma^{28}$  can interact with RNA polymerase (103).

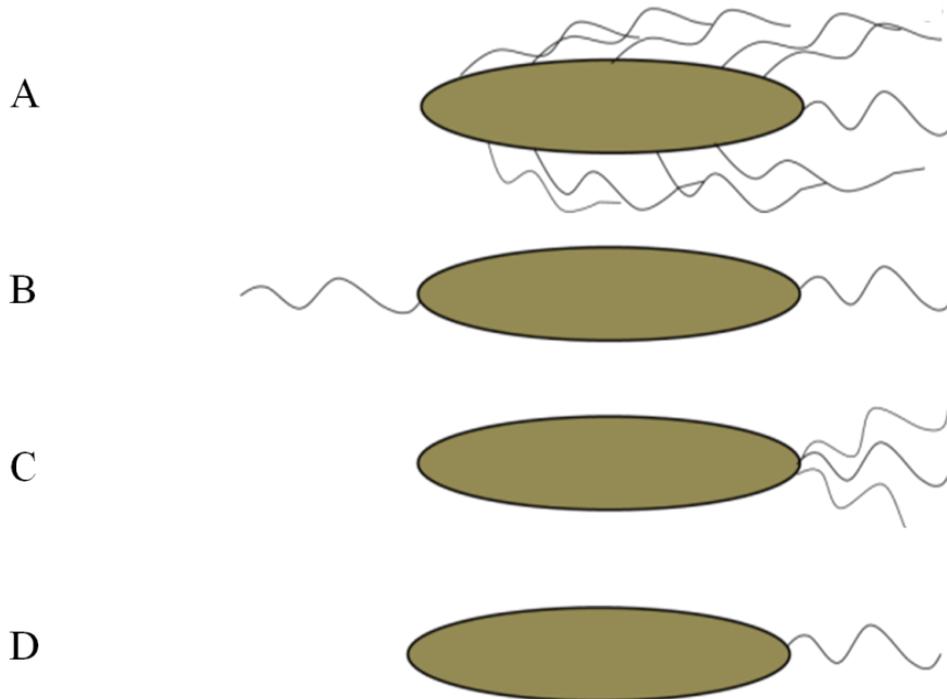
In addition to *fliC*,  $\sigma^{28}$  drives the transcription of genes encoding the MotA and MotB stator proteins, chemotaxis system, flagellin chaperones and the FlgM anti- $\sigma^{28}$  factor (27). Expression of *flgM* creates a negative feedback loop to prevent expression of unnecessary amounts of class III genes (27). The expression of class III genes and their incorporation into the flagellar structure completes the formation of a fully-functional flagellum.

## **CONTROL OF FLAGELLAR GENE EXPRESSION AND BIOSYNTHESIS IN POLARLY-FLAGELLATED BACTERIA**

### **Flagellation Patterns in Bacteria**

The number and positioning of flagella can be different between bacterial species. *E. coli* and *Salmonella* are peritrichous organisms, with multiple flagella distributed on the surface of the bacteria (Figure 4A). *C. jejuni* is amphitrichous producing a single flagellum at both poles of the bacterium (Figure 4B). Another  $\epsilon$ -proteobacterium *Helicobacter pylori* is lophotrichous, producing multiple flagella only at one pole (Figure 4C). Other major pathogens such as *Vibrio cholerae* and *Pseudomonas*

*aeruginosa* are monotrichous by having a single polar flagellum only at one pole (Figure 4D). There are some examples of bacterial species, like *Vibrio parahaemolyticus*, which have two separate flagellar systems, monotrichous flagella and multiple lateral flagella, which are under control of different regulatory mechanisms (121). As apparent in these multiple organisms, the numbers and placement of flagellar organelles varies greatly, which requires different control mechanisms in each bacterium to obtain its characteristic pattern of flagellar biosynthesis.



**Figure 4. Different flagellar patterns in bacteria.** Each diagram shows a different pattern of flagellar organization including (A) peritrichous, (B) amphitrichous, (C) lophotrichous and (D) monotrichous flagellation.

### **Flagellar Gene Expression in Polarity-flagellated Bacteria**

The regulatory mechanism governing flagellar gene expression in *E. coli* and *Salmonella* species are not strictly conserved in polarity-flagellated bacteria. The major difference observed in polarity-flagellated systems is employment of an additional sigma factor,  $\sigma^{54}$ , to regulate flagellar gene expression.

Early work established *Vibrio* and *Pseudomonas* species as model organisms to understand flagellar gene expression and biosynthesis in polarity-flagellated bacteria. In these systems, there is a four-tiered regulatory cascade, the first class consisting of the master transcriptional regulator (36, 149). *C. jejuni* flagellar biosynthesis cascade shows similarity to these systems, but a master transcriptional regulator has not been identified in *C. jejuni*. In the following section, I will describe current understanding of flagellar biosynthesis cascade in *C. jejuni*, after first describing master regulators in other polarity-flagellated bacteria.

#### *The Master Regulator in Polarity-flagellated Bacteria*

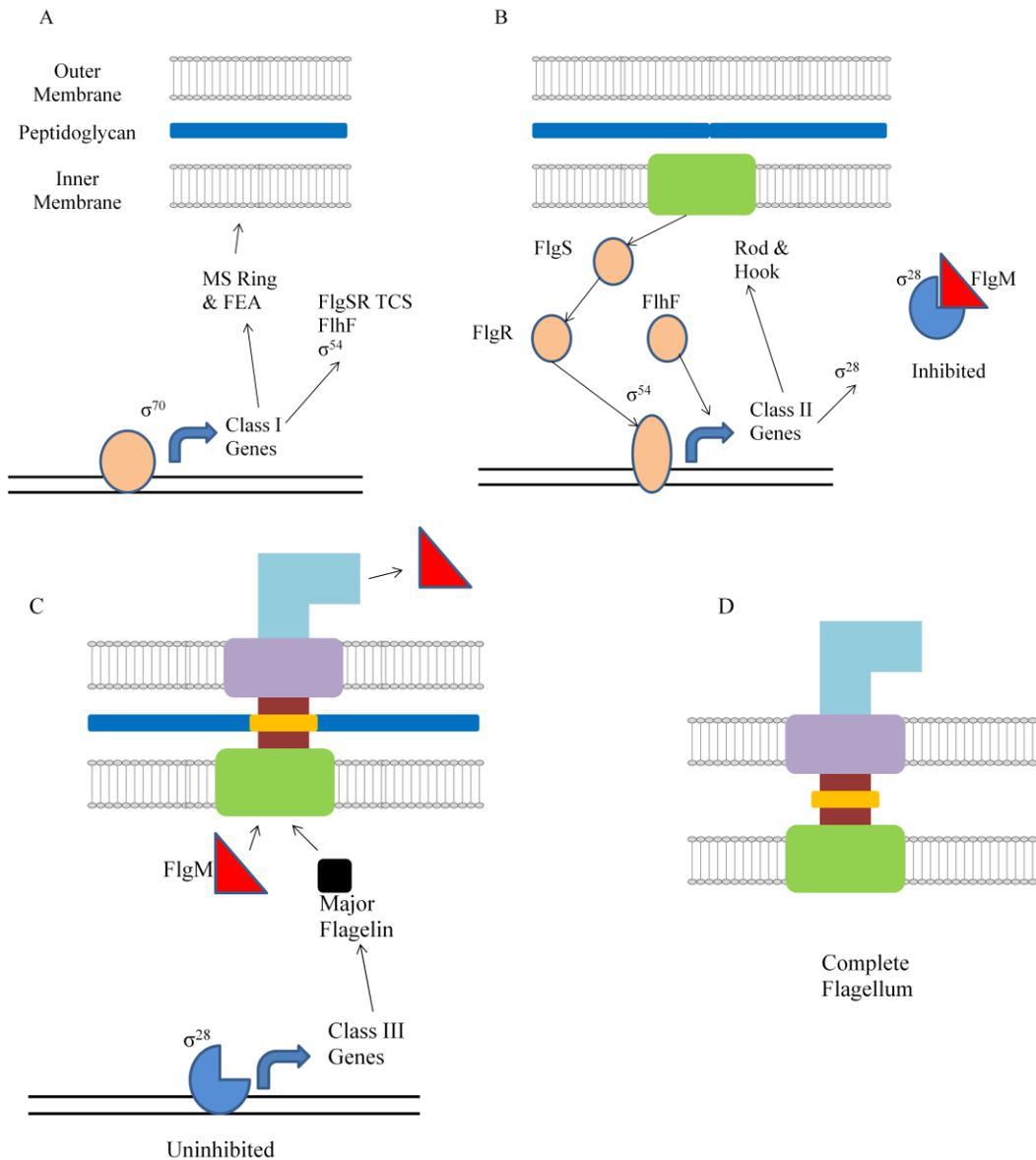
Flagellar gene expression in *V. cholera* and *P. aeruginosa* is ultimately controlled by a master transcriptional regulator. In *V. cholerae* and *P. aeruginosa*, FlrA and FleQ are the master regulators, respectively (118). Expression or the activity of the master transcriptional regulators in these organisms is mediated by FlhG/FleN (33, 35), whose functions will be discussed in sections below and in the results section of this work.

These master transcriptional regulators control expression of class II genes, which are required for expression of later classes of flagellar genes. Tight control of

master transcriptional regulators in *V. cholerae* and *P. aeruginosa* are required for precise expression of most flagellar genes and flagellar biosynthesis.

### **Control of Flagellar Biosynthesis in *C. jejuni***

As with other motile bacteria, flagellar biosynthesis is tightly regulated in *C. jejuni*. Also, *C. jejuni* produces amphitrichous flagella, suggesting that spatial and numerical regulation of flagellar biosynthesis exists in this organism. Research has been performed to investigate genetic regulation of flagellar gene expression, and spatial and numerical control of flagellar biosynthesis in *C. jejuni*. While the scopes of these investigations are not as extensive as in *E.coli* or *Salmonella*, a significant amount of knowledge has been collected that is described below.



**Figure 5. The Flagellar biosynthesis cascade in *C. jejuni*.** *C. jejuni* flagellar genes are composed of three classes. (A) Class I genes encode the FEA, MS ring, and regulatory elements such as FlgSR and FlhF. (B) Formation of the FEA relays a signal to FlgSR two-component system and activates  $\sigma^{54}$ -dependent gene expression with FlhF, resulting in expression of class II genes. (C) Class II genes encode rod and hook proteins. The formation of rod and hook structures allow the secretion of anti- $\sigma^{28}$  factor FlgM, resulting in  $\sigma^{28}$ -dependent expression of class III genes. (D) Expression of class III genes and secretion of flagellins conclude flagellar biosynthesis to result in a completed flagellar organelle.

### **Organization of Flagellar Genes in *C. jejuni***

In *C. jejuni*, three classes of flagellar gene expression have been observed based on their ordered expression (Figure 5). A different  $\sigma$  factor is required to drive the expression of each class, including:  $\sigma^{70}$  for class I genes (Figure 5A),  $\sigma^{54}$  for class II genes (Figure 5B), and  $\sigma^{28}$  for class III genes (Figure 5C).

#### *Class I Genes*

A significant difference between the *C. jejuni* flagellar transcriptional cascades compared to other bacteria is the lack of a master transcriptional regulator atop the cascade. Thus, different genes compose the class I genes in *C. jejuni*. Class I genes in *C. jejuni* are located in operons with housekeeping genes, suggesting that expression of these genes might not be controlled via a master regulator and may be constitutively expressed.

*C. jejuni* class I flagellar genes consists of genes encoding the MS ring and the FEA. In addition, genes encoding the FlgSR two-component system (TCS) and the FlhF GTPase are also class I genes. Although further analysis is necessary, almost all of these genes are thought to be required for expression of class II flagellar genes.

A regulatory system has been proposed for how the FEA, FlgSR and  $\sigma^{54}$  may function together to activate expression of  $\sigma^{54}$ -dependent class II genes (68). According to this model, formation of a functional FEA creates a signal that is sensed by the FlgS sensor histidine kinase (86). FlgS then autophosphorylates and transfers the phosphate to the FlgR response regulator, which in turn activates  $\sigma^{54}$ -dependent flagellar gene

expression (87). Supporting this hypothesis, FEA mutants that are able to form but are defective in secretion were able to promote expression of  $\sigma^{54}$ -dependent genes, even though they are incapable of producing flagella (86).

### *Class II Genes*

The class II flagellar genes of *C. jejuni* encode the rod, hook and minor flagellin proteins (52). Expression of these genes facilitates biosynthesis of the rod and hook structures. A recent finding showed that *fliA*, encoding  $\sigma^{28}$ , has two transcriptional start sites, one  $\sigma^{70}$ -dependent and the other  $\sigma^{54}$ -dependent which makes *fliA* a class I and II flagellar gene (A. Barrero-Tobon, unpublished data). In addition, *flgM* encoding the anti- $\sigma$  factor for  $\sigma^{28}$  is a  $\sigma^{54}$ -dependent class II gene (181). Like in other systems, FlgM represses the activity of  $\sigma^{28}$  until hook biosynthesis is complete (68). Completion of the hook structure results in secretion of FlgM, thus allowing class III gene expression to proceed.

### *Class III Genes*

The last gene class expressed during flagellar biosynthesis are class III genes, which are expressed under the control of  $\sigma^{28}$  transcriptional factor (19, 52). The class III genes encode the major flagellin FlaA and other minor filament proteins (19, 68).

## **Control of Flagellar Placement and Number in Polarly-Flagellated Bacteria**

Polarly-flagellated bacteria are restricted in producing flagella only at poles and producing only a limited number of flagella. Thus, unlike peritrichous bacteria, polarly-

flagellated bacteria need mechanisms to govern numerical and spatial parameters of flagellar biosynthesis. Two proteins that have been implicated in these roles in polar flagellar biosynthesis include the FlhF GTPase and FlhG (also called FleN).

#### *FlhF as a Regulator of Flagellar Biosynthesis and Placement*

The FlhF GTPase was identified to have roles in flagellar gene expression in *V. cholerae* and *H. pylori* (33, 136). *V. cholerae*  $\Delta$ *flhF* mutants are mostly aflagellated due to reduced flagellar gene expression (33). However, the small subset of the population that produce flagella mislocalize these organelle to the lateral sites instead of polar sites (56). In addition, in *P. aeruginosa* and *Pseudomonas putida*, the FlhF GTPase is required for correct placement of flagella to the polar sites (132, 140). The mislocalized flagella were still competent for rotation in *P. aeruginosa*, but gave the bacteria uncoordinated motility (132). In *P. putida*, overexpression of *flhF* caused an increase in flagellar numbers (140). These findings suggest that the FlhF GTPase is a positive factor for flagellar biosynthesis and is required for polar placement of flagella in polarly-flagellated bacteria. This idea is further suggested by observing co-localization of FlhF with flagella in *P. aeruginosa* (132). Thus, a flagellum is present at the pole where FlhF is present.

As described above, flagellar biosynthesis is initiated at the inner membrane and extends extracellularly from the bacterium. The first flagellar structural elements that are thought to form are the MS ring, the FEA and the C ring, which together form the cytoplasmic base of flagella. Sites of flagellar formation are likely determined by the localization of these elements. Evidence suggests that FlhF may initiate the polar formation of the initial base components of a flagellum (56). FliF, which forms the MS

ring, was shown to localize to a single pole of *V. cholerae* in the presence of FlhF, whereas FliF was homogeneously distributed over the membrane in the absence of FlhF (56). This data suggests that FlhF is required for polar localization, but not membrane insertion of the MS ring in *V. cholerae*.

In *C. jejuni*, FlhF is required for flagellar gene expression and biosynthesis. In Chapter IV, I describe work identifying GTPase-dependent and GTPase-independent roles of FlhF in flagellar gene expression and spatial regulation of flagellar biosynthesis.

#### *FlhG as a Regulator of Flagellar Number*

*flhG* is usually found downstream of *flhF* in the genomes of polarly-flagellated bacteria. In *P. aeruginosa*, the ortholog of FlhG is named FleN, but for ease of reading I will refer to these proteins only as FlhG.

In monotrichous *V. cholera*, deletion of *flhG* causes the mutant to produce multiple, but polarly-localized flagella (33). Expression of all classes of flagellar genes in  $\Delta flhG$  mutant of *V. cholerae* was increased, including the gene for the FlrA master transcriptional regulator (33). Thus, FlhG is likely involved in a mechanism to appropriately control *flrA* expression so that levels of flagellar genes are tightly controlled to only allow the production of one flagellum.

In monotrichous *P. aeruginosa*, FlhG is also required to limit production of a single flagellum to one pole (34). In a *P. aeruginosa flhG* mutant, multiple polar flagella and an increase in expression of all flagellar genes except for the class I master transcriptional regulator *fleQ* were observed. This finding suggests that *P. aeruginosa* FlhG controls the activity of master regulator FleQ to limit the expression of downstream

flagellar genes (34, 35). Further analysis indicated that FlhG and FleQ interact, suggesting that FlhG directly reduces the activity of FleQ as a master transcriptional regulator (35).

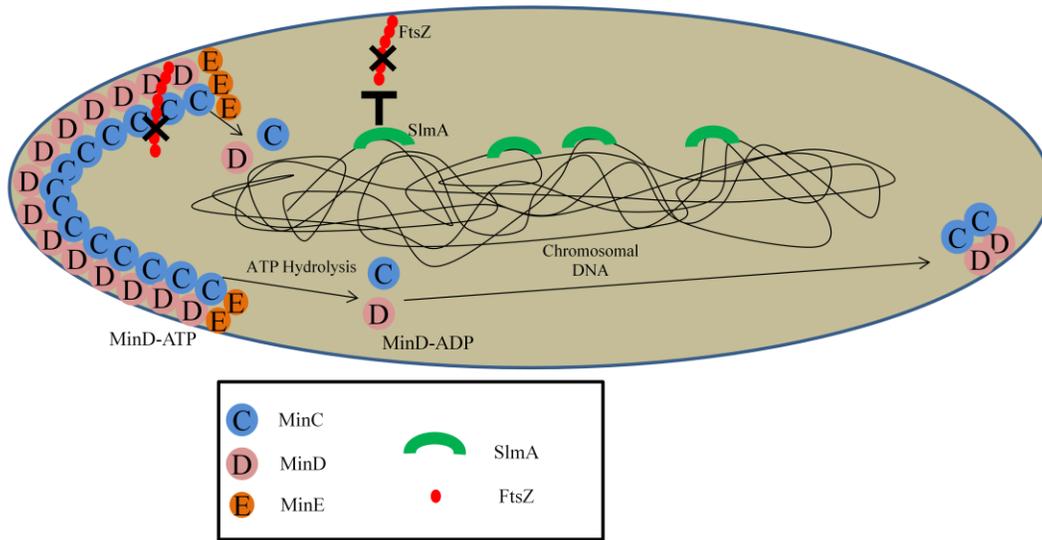
FlhG is also present in bacteria that do not seem to possess a master transcriptional regulator. Examples for such bacterial species include *H. pylori* and *C. jejuni*. In *H. pylori*, an aflagellated phenotype for a  $\Delta flhG$  mutant was observed (171). In chapter V, I describe work analyzing the role of FlhG in *C. jejuni*. Like its role in other polarly-flagellated organisms, I found that FlhG controls flagellar number with a mutant producing extra flagella at the poles. However, we also found an unexpected second role for FlhG in control of placement of division sites.

## BACTERIAL CELL DIVISION MECHANISMS

Bacteria divide by binary fission, which most often results in symmetrical division to generate two identical daughter cells. Division is tightly controlled by complex mechanisms. Division begins with DNA replication and segregation of the two chromosomal copies to opposite poles of bacteria to equally distribute the genetic material to the two eventual daughter cells. Septation then occurs after DNA segregation by precisely placing the divisome to the cellular midpoint. In this section, I will briefly explain the mechanisms that bacteria use to influence where the division machinery is placed in a cell to divide. Two major proteins that will be discussed in this section are MinD and MipZ, which are homologs of FlhG. Reviewing these systems will be helpful in understanding a role for FlhG in division of *C. jejuni*, which is described in chapter V.

**Bacterial Cytokinesis**

Bacterial cell division is a fairly conserved process among different bacterial species. The first protein that initiates bacterial cytokinesis is FtsZ (12). FtsZ is a GTPase homologous to tubulin that is capable of polymerizing into protofilaments to form a ring structure (Z-ring) necessary to initiate cell division (37). After Z-ring formation at the cytoplasmic surface of the inner membrane at the midpoint of a bacterial cell, other factors are recruited to the site of division. These proteins include FtsA and ZipA, which tether the Z-ring to the cytoplasmic membrane and recruit additional factors needed to complete septation and cell division (2, 110). At the last step, enzymes such as FtsI are recruited to the divisome to generate peptidoglycan to separate two daughter bacteria.



**Figure 6. The Min System and Nucleoid Occlusion in *E. coli*.** *E. coli* employs two systems for division site determination: The Min system prevents polar divisome formation by inhibiting FtsZ ring formation through the action of MinC. MinC is recruited to the membrane by the carrier protein MinD. MinCD complexes are kept away from midpoint of the bacterium by the ATPase-activating topological specificity factor MinE. Nucleoid occlusion prevents formation of divisomes over the chromosome and DNA fragmentation through the FtsZ polymerization inhibitor SlmA. SlmA binds DNA and directly interact with FtsZ. The presence of SlmA-bound DNA at midregion prevents Z ring formation at the midpoint until DNA replication and segregation to polar regions.

### Regulation of Z-ring Formation

An important step in initiating symmetrical division is the precise placement of the Z-ring to the cellular midpoint. Spatial regulation of the Z-ring may be accomplished by nucleoid occlusion, which prevents Z-ring formation over chromosomes, and by the Min system, which works to inhibit polar division (Figure 6).

#### *Nucleoid Occlusion*

Division at sites where condensed chromosomal DNA is present is inhibited by a mechanism known as nucleoid occlusion (Figure 6). The details of this mechanism are

not fully understood, but this mechanism is thought to be present in a wide variety of bacterial species. In *E. coli*, the factor responsible for nucleoid occlusion is SlmA.

SlmA is associated with chromosomal DNA and inhibits Z-ring formation by binding to FtsZ directly (28). This mechanism is thought to prevent division over DNA which may cause fragmentation of the genetic material. *E. coli* cells with intact nucleoid occlusion machinery precisely places the division site to the midpoint more often than *slmA* mutants, to result in identical daughter cells. A similar mechanism is present in Gram-positive *Bacillus subtilis*, which is mediated by the Noc protein (183). Although these proteins do not share sequence homology, they have similar functions. Both SlmA and Noc bind to chromosomal DNA and inhibit FtsZ polymerization (28, 183). SlmA can directly inhibit FtsZ polymerization, but no such observation was made for Noc *in vitro*.

### *Min System*

In a dividing bacterium, polar sites are protected from division largely due to the Min system. In *min* mutants isolated several decades ago, division was found to occur at polar sites to result in small bacterial particles called minicells (4). The mechanisms protecting polar sites from division have been extensively studied in *E. coli*, *B. subtilis* and *Caulobacter crescentus*, which will be discussed below.

### *The Min System in E. coli*

Minicells were first observed in *E. coli* by Adler et. al., in 1961 (3). Initial studies revealed that minicells were the result of mutations at the *min* locus, which was

later shown to encode three important proteins that control of division site determination. These proteins include MinC, MinD and MinE (Figure 6).

MinC is an actual inhibitor of FtsZ polymerization into the Z-ring (77). MinC was shown to directly interact with FtsZ to inhibit its polymerization (77). When the N-terminal domain that inhibits FtsZ polymerization is mutated, minicells are produced, suggesting that Z-ring formation was not strictly localized to midpoint (74). To inhibit Z-ring formation at poles, MinC must be located at polar sites. MinC achieves this specific localization by directly interacting with the MinD ATPase (78).

MinD is an ATPase that binds MinC and interacts with membranes in an ATP-dependent manner (78). Upon ATP binding, a conformational change occurs in MinD that results in its C-terminal amphipathic helix interacting with the cytoplasmic face of the inner membrane (166, 167). In addition, ATP-binding also causes MinD dimerization and binding to MinC (78, 190), which results in targeting MinC to the membrane where it inhibits FtsZ polymerization into the Z-ring (78). Mutations that disrupt MinC-MinD binding or the absence of MinD also cause the minicell phenotype, suggesting that MinD is an essential factor for the Min system.

ATP-bound MinCD complexes can bind to any region of the bacterial inner membrane. Thus, MinCD complexes require the MinE as a topological specificity factor to inhibit division only at polar sites (76). MinE is a small membrane protein with two domains, an N-terminal domain that interacts with MinD and a C-terminal domain that is required for interacting with other MinE proteins for polymerization into a ring structure (94, 111). MinE binding to MinD has two consequences: it displaces MinC from MinD and it induces the ATPase activity of MinD, which causes release of MinD from the

membrane (73, 78, 151). With the action of MinE, MinCD complexes are cleared from the membrane.

In *E. coli*, MinCD complexes first accumulate at one pole and begin extending towards the midpoint (151). MinE forms a ring at the midpoint and moves towards the pole with MinCD, resulting in dissociation of these complexes at this pole (79). This mechanism leads to oscillation of MinCD complexes between poles in *E. coli*, while keeping the cellular midpoint relatively free from MinCD so that the Z-ring can form at this region to promote symmetrical division.

#### *The Non-Oscillating Min System in B. subtilis*

*B. subtilis* has also been extensively studied for mechanisms that govern cytokinesis and division site determination. *B. subtilis* employs nucleoid occlusion and Min systems to regulate symmetrical division. However, the *B. subtilis* Min system lacks the MinE topological specificity factor, suggesting it uses another mechanism to ensure that MinCD complexes only inhibit division at discrete locations. Unlike *E. coli*, the MinCD complexes of *B. subtilis* do not oscillate between poles.

In *B. subtilis*, MinCD complexes are recruited to the forming septum. DivIVA is the topological specificity factor for MinCD and is recruited to the septum after the Z-ring has initially formed (57). DivIVA then recruits MinCD to the divisome when the Z-ring is no longer sensitive to depolymerization by MinCD so that a normal division can still proceed (57). Placing MinCD at a developing septum is thought to be beneficial by inhibiting a second division event once the new pole is formed after the first division event (57, 172).

### *Division Site Determination in C. crescentus*

The Min system and nucleoid occlusion are commonly used in different bacterial species to spatially regulate division. However, some species of bacteria lack one or both of these systems. *C. crescentus* is one bacterium which lacks both of these systems. Instead, *C. crescentus* utilizes a MinD homolog, MipZ to achieve division site determination by an alternative mechanism.

Unlike *E. coli* or *B. subtilis* MinDs, MipZ itself inhibits FtsZ polymerization into the Z-ring formation (169). Topological specificity of MipZ is achieved via MipZ interacting with ParB bound to the origin of newly replicated chromosomal DNA. In *C. crescentus*, a non-replicating cell has the chromosomal origin is located at the pole. After a round of DNA replication, the chromosomes are segregated to two different poles of the bacterium. By MipZ binding to ParB, which is bound to DNA origin, ParB carries MipZ to the opposite pole of the bacterium. MipZ causes depolymerization of FtsZ present at the opposite pole from the last round of division, which encourages FtsZ to reform the Z-ring at an off-center point to promote asymmetrical division for stalked and swarmer cell formation.

By analyzing division in different bacteria, it is clear that bacteria can utilize different systems to influence where division sites form. As I describe in chapter V, I have identified a new mechanism for spatially regulating where division occurs in bacteria. I found that *C. jejuni* lacks the canonical Min system and instead employs FlhG and components of polar flagella to inhibit polar division so that FtsZ forms the Z-ring at the midpoint of the bacterial cell for symmetrical division.

## CHAPTER THREE

### MATERIALS AND METHODS

**Bacterial strains.** The *C. jejuni* strain that is used in this study is *C. jejuni subsp. jejuni* strain 81-176. This strain is a clinical isolate from a human with gastroenteritis (100). *C. jejuni* 81-176 is widely used in laboratories for research purposes and has been shown to promote disease in humans and colonization of gastrointestinal tract in chickens (13, 66). Routine growth of *C. jejuni* was performed on Mueller-Hinton (MH) agar containing 10 µg/ml of trimethoprim (TMP) at 37 °C under microaerobic conditions (85% N<sub>2</sub>, 10% CO<sub>2</sub> and 5% O<sub>2</sub>). Strains were grown on MH agar with 50 µg/ml kanamycin, 10 µg/ml chloramphenicol, 0.5, 1, 2 or 5 mg/ml streptomycin when necessary. In certain experiments, strains were grown in MH broth for 6 hours with 12.5 or 15 µg/ml cephalexin. All strains were stored at -80 °C in MH broth with 15% glycerol. *E. coli* DH5α and XL-1 Blue were grown on Luria-Bertani agar or broth containing 100 µg/ml ampicillin, 50 µg/ml kanamycin or 15 µg/ml chloramphenicol when necessary. All *E. coli* strains were stored at -80 °C in LB broth with 20% glycerol.

**Construction of strains.** Mutants of *C. jejuni* were constructed by previously described methods (65). *C. jejuni* 81-176 *rpsL*<sup>Sm</sup> mutant strains previously constructed include:  $\Delta flhF$  (DRH1054; (67));  $\Delta flhA$  (DRH946; (67));  $\Delta flhB$  (SNJ471; (86));  $\DeltafliP$  (DRH1065; (67)); and  $\DeltafliR$  (DRH755; (67)),  $\Delta rpoN$  (DRH321; (65)) and  $\Delta astA$  (DRH461; (67)); (9). *E. coli* DH5α was used for all cloning procedures.

pDRH416 was used in PCR-mediated mutagenesis to provide plasmids containing various mutations and deletions of *flhF* including (67, 114): *flhF* <sub>$\Delta$ G</sub> (which lacks amino acids 274 – 476, removing the GTPase domain); (pSNJ132), *flhF*<sub>K295A</sub> (pDRH1275), *flhF*<sub>D321A</sub> (pMB620), and *flhF*<sub>R324A</sub> (pMB619). These plasmids were electroporated into DRH1054 (81-176 Sm<sup>R</sup> *flhF*::*cat-rpsL*; (67)) to replace *flhF*::*cat-rpsL* with the *flhF* mutant alleles on the chromosome. To delete *astA* in these genetic backgrounds and in DRH1056 (81-176 Sm<sup>R</sup>  $\Delta$ *flhF*; (67)), the strains were first electroporated with pDRH424 to create *cat*::*rpsL* insertions in *astA* and then pDRH449 to remove the *astA*::*cat-rpsL* mutation (and thus *astA*) from the chromosome (67).

Replacement of *flgR* with *flgR* alleles encoding deletions of the N-terminal receiver domain (FlgR <sub>$\Delta$ receiver</sub>) or the C-terminal domain (FlgR <sub>$\Delta$ CTD</sub>) was achieved by first electroporating DRH1438 (81-176 Sm<sup>R</sup>  $\Delta$ *astA*  $\Delta$ *flhF*) with pDRH443 to create *kan*::*rpsL* insertion in *flgR* (SNJ170; (67)). This strain was then electroporated with pDRH1855 or pDRH1856 to replace *flgR*::*kan-rpsL* with the *flgR* alleles encoding the domain deletions (87).

To create a  $\Delta$ *astA*  $\Delta$ *flhF*  $\Delta$ *flhB* mutant of *C. jejuni*, DRH1438 (81-176 Sm<sup>R</sup>  $\Delta$ *astA*  $\Delta$ *flhF*) was electroporated with pDRH781 to create a *cat*::*rpsL* insertion in *flhB* (MB511; (67)). This strain was then electroporated with pDRH742 to remove *flhB*::*cat-rpsL* (and thus *flhB*) from the chromosome of *C. jejuni* to generate MB532 (81-176 Sm<sup>R</sup>  $\Delta$ *astA*  $\Delta$ *flhF*  $\Delta$ *flhB*; (67)).

For construction of a  $\Delta$ *astA*  $\Delta$ *flgG* mutant, the *flgFG* locus of *C. jejuni* 81-176 was amplified by PCR with primers containing 5' BamHI sites. After cloning the DNA into BamHI-digested pUC19 to generate pDRH1349, a *cat-rpsL* cassette from pDRH265

was inserted into the BglII site within *flgG* to create pDRH2567 (65). DRH461 (81-176 Sm<sup>R</sup>  $\Delta astA$ ; (67)) was electroporated with pDRH2567 to create DRH2623 (81-176 Sm<sup>R</sup>  $\Delta astA flgG::cat-rpsL$ ). pDRH1349 was subjected to PCR-mediated deletion mutagenesis to delete the entire coding sequence of *flgG* from the plasmid (creating pDRH2425). This plasmid was electroporated into DRH2623 to remove *flgG::cat-rpsL* (and thus *flgG*) from the chromosome to generate SNJ925 (81-176 Sm<sup>R</sup>  $\Delta astA \Delta flgG$ ).

Deletion of *fliF* from *C. jejuni* was performed by first amplifying the *fliF* locus from *C. jejuni* 81-176 by PCR with primers containing 5' BamHI sites. After cloning the DNA into BamHI-digested pUC19 to create pDRH1777, a *cat-rpsL* cassette from pDRH265 was inserted into the MfeI site within *fliF* to create pDRH1814 (65). This plasmid was electroporated into DRH212 (81-176 Sm<sup>R</sup>; (65)) to create DRH2067 (81-176 Sm<sup>R</sup> *fliF::cat-rpsL*). pDRH1777 was used in PCR-mediated deletion mutagenesis to delete codons 2 through 548 of the coding sequence of *fliF*, fusing the start codon to the last 13 codons of *fliF*. This plasmid was electroporated into DRH2067 to remove *fliF::cat-rpsL* (and thus *fliF*) from the chromosome to create DRH2074 (81-176 Sm<sup>R</sup>  $\Delta fliF$ ).

To create *C. jejuni*  $\Delta flhG$  mutant, *flhG* was amplified with 700 bp of flanking sequence by PCR from the *C. jejuni* 81-176 chromosomal DNA with primers containing 5' BamHI sites. After cloning into BamHI-digested pUC19 (creating pSMS248), an MscI restriction site was introduced into the coding sequence of *flhG* using PCR-mediated mutagenesis (creating pSMS259). In addition, PCR-mediated mutagenesis was performed with pSMS248 to change the codon for D61 to a codon for alanine (creating pMB951 containing the *flhG<sub>D61A</sub>* allele) and to delete in-frame codons 14 - 241 (creating

pMB752 containing the  $\Delta flhG$  allele). A *Sma*I-digested *cat-rspL* cassette (from pDRH265; (65)) was inserted into the *Msc*I site within *flhG* to create pSMS275 and pSMS279. *C. jejuni* 81-176 Sm<sup>R</sup> (DRH212; (65)) was electroporated with pSMS275 and transformants were recovered on MH agar containing 10  $\mu$ g/ml chloramphenicol. Two *C. jejuni* 81-176 Sm<sup>R</sup> *flhG::cat-rspL* transformants (SMS368 and SMS370) were verified by PCR.

*C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta flhG$  was created by electroporating SMS370 with pMB752. Transformants were recovered on MH agar containing 0.5 – 2 mg/ml streptomycin. Deletion of a major portion of *flhG* in one transformant (MB770) was verified by PCR. *C. jejuni* 81-176 Sm<sup>R</sup> *flhG*<sub>D61A</sub> was created by electroporating SMS368 with pMB951. Transformants were recovered on MH agar containing 0.5 – 2 mg/ml streptomycin. The presence of *flhG*<sub>D61A</sub> at the native location on the chromosome in two transformants (MB1040 and MB1054) was verified by PCR and sequencing.

*C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta flhG \Delta astA$  was created by electroporating 81-176 Sm<sup>R</sup>  $\Delta astA$  (DRH461; (67)) with pSMS279 and transformants were recovered on MH agar containing 10  $\mu$ g/ml chloramphenicol. One *C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta astA flhG::cat-rpsL$  transformant (DRH2133) was verified by PCR. To replace *flhG::cat-rpsL* with the  $\Delta flhG$  allele, DRH2133 was electroporated with pMB752. Transformants were recovered on MH agar containing 0.5 – 2 mg/ml streptomycin. Deletion of a major portion of *flhG* in one transformant (MB771) was verified by PCR.

Construction of *C. jejuni fliG* mutant was initiated by amplifying *fliG* with 700 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primers

containing 5' BamHI sites. After cloning into BamHI-digested pUC19 (creating pDRH2407), a SmaI-digested *cat-rspL* cassette (from pDRH265) was inserted into the ClaI restriction site within *fliG* to create pALU115. *C. jejuni* 81-176 Sm<sup>R</sup> (DRH212) was electroporated with pALU115 and transformants were recovered on MH agar containing 10 µg/ml chloramphenicol. One *C. jejuni* 81-176 Sm<sup>R</sup> *fliG::cat-rspL* transformant (DRH2469) was verified by PCR.

Creation of *C. jejuni fliM* mutant was achieved by first amplifying *fliM* with 700 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primers containing 5' BamHI sites. After cloning into BamHI-digested pUC19 (creating pJMB532), a SmaI-digested *cat-rspL* cassette (from pDRH265) was inserted into the EcoRV restriction site within *fliM* to create pJMB572. *C. jejuni* 81-176 Sm<sup>R</sup> (DRH212) was electroporated with pJMB572 and transformants were recovered on MH agar containing 10 µg/ml chloramphenicol. One *C. jejuni* 81-176 Sm<sup>R</sup> *fliM::cat-rspL* transformant (DRH3304) was verified by PCR.

*C. jejuni fliN* mutant was constructed by amplifying *fliN* with 1 kb of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primers containing 5' BamHI sites. After cloning into BamHI-digested pUC19 (creating pDRH1350), a SmaI-digested *cat-rspL* cassette (from pDRH265) was inserted into the EcoRV restriction site within *fliN* to create pDRH1367. *C. jejuni* 81-176 Sm<sup>R</sup> (DRH212) was electroporated with pDRH1367 and transformants were recovered on MH agar containing 10 µg/ml chloramphenicol. One *C. jejuni* 81-176 Sm<sup>R</sup> *fliN::cat-rspL* transformant (DRH1407) was verified by PCR.

To construct a *C. jejuni fliQ* deletion, *fliQ* was amplified with 700 bp of flanking sequence by PCR from *C. jejuni* 81-176 chromosomal DNA with primers containing 5' BamHI sites. After cloning into BamHI-digested pUC19 (creating pDRH1454), a MscI restriction site was introduced into the coding sequence of *fliQ* using PCR-mediated mutagenesis (creating pSMS462). In addition, PCR-mediated mutagenesis was performed with pDRH1454 to delete in-frame codons 10-72 (creating pSMS443 containing the  $\Delta fliQ$  allele). A SmaI-digested *cat-rspL* cassette (from pDRH265) was inserted into the MscI site within *fliQ* to create pSMS469. *C. jejuni* 81-176 Sm<sup>R</sup> (DRH212) was electroporated with pSMS469 and transformants were recovered on MH agar containing 10 µg/ml chloramphenicol. One *C. jejuni* 81-176 Sm<sup>R</sup> *fliQ::cat-rspL* transformant (SMS508) was verified by PCR. *C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta fliQ$  was created by electroporating SMS508 with pSMS443. Transformants were recovered on MH agar containing 0.5 – 2 mg/ml streptomycin. Deletion of a major portion of *fliQ* in one transformant (DAR101) was verified by PCR.

To create a *C. jejuni*  $\Delta fliE$  mutant, the *flgBCfliE* locus was amplified with 600 to 700 bp of flanking sequence by PCR from the *C. jejuni* 81-176 chromosomal DNA with primers containing 5' BamHI sites. After cloning into BamHI-digested pUC19 (creating pDRH2428), a StuI restriction site was introduced into the coding sequence of *fliE* using PCR-mediated mutagenesis (creating pSNJ822). In addition, PCR-mediated mutagenesis was performed with pDRH2428 to fuse in-frame the start codon to the last 20 codons of *fliE* (which results in deletion of codons 2 through 78) to create pSNJ918 containing the  $\Delta fliE$  allele. A SmaI-digested *cat-rspL* cassette (from pDRH265) was inserted into the

StuI site within *fliE* to create pSNJ878. *C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta$ *astA* (DRH461) was electroporated with pSNJ878 and transformants were recovered on MH agar containing 10  $\mu$ g/ml chloramphenicol. One *C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta$ *astA* *fliE*::*cat-rspL* transformant (SNJ907) was verified by PCR. *C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta$ *astA*  $\Delta$ *fliE* was created by electroporating SNJ907 with pSNJ918. Transformants were recovered on MH agar containing 0.5 – 2 mg/ml streptomycin. Deletion of a major portion of *fliE* in one transformant (SNJ915) was verified by PCR.

#### **Construction of plasmids for complementation and overexpression of genes.**

Complementation of the *C. jejuni* 81-176 Sm<sup>R</sup>  $\Delta$ *flhG* mutant with *flhG* or *minD* alleles was accomplished by amplifying the alleles with primers containing 5' BamHI restriction sites immediately upstream of the start and stop codons of the respective genes. *flhG* alleles were amplified from the chromosomal DNA of *C. jejuni* 81-176, *H. pylori* J99, and *V. cholerae* O395. *minD* alleles were amplified from the chromosomal DNA of *H. pylori* J99, *V. cholerae* O395, and *E. coli* MG1655. The alleles were cloned into BamHI-digested pCE107, an *E. coli*-*C. jejuni* shuttle vector containing the  $\sigma^{28}$ -dependent *flaA* promoter of *C. jejuni* followed by a BamHI site fused in-frame to a gene for the *Zoanthus* species green-fluorescent protein (ZsGreen). Insertion of *flhG* or *minD* alleles in the correct orientation placed a stop codon between the allele and the gene for GFP, preventing the formation of a fusion protein. All plasmids were sequenced and then transformed into DH5 $\alpha$ /RK212.1 (46). The plasmids were then conjugated into the appropriate *C. jejuni* 81-176 by a previously published method (61).

For overexpression of *ftsZ* in the *C. jejuni* Sm<sup>R</sup> *flhG*<sub>D61A</sub> mutant (MB1054), *ftsZ* was amplified from chromosomal DNA of *C. jejuni* 81-176 using primers containing 5' BamHI restriction sites immediately upstream of the start and stop codons of the gene. *ftsZ* was cloned into BamHI-digested pCE107 and then conjugated into the *flhG*<sub>D61A</sub> as described above.

To overexpress *flhG* in the *C. jejuni*  $\Delta$ *flhF* (DRH1056), *fliF* (DRH2074), *fliM* (DRH3304), and *fliN* (DRH1407) mutants, the coding sequence of *flhG* was amplified from chromosomal DNA from *C. jejuni* 81-176 with primers containing BamHI restriction sites in-frame to codon 2 and the stop codon. *flhG* was then cloned into BamHI-digested pECO101, an *E. coli*-*C. jejuni* shuttle vector containing the promoter of the chloramphenicol-acetyltransferase (*cat*) gene (178). After screening for correct orientation of *flhG* and sequencing, one plasmid (pMB1230) was transformed into DH5 $\alpha$ /RK212.1 for conjugation into *C. jejuni* mutants as described above. Constitutive expression of *flhG* from the *cat* promoter on the plasmid, along with constitutive expression of *flhG* from the native chromosomal locus allowed for expression of *flhG* at increased levels relative to wild-type *C. jejuni*.

**Bioinformatic analysis.** *In silico* analysis of protein homologies were performed by BlastP, Blast2 and ClustalW2 programs (<http://www.ncbi.nlm.nih.gov/BLAST>; <http://www.ebi.ac.uk/Tools/clustalW2/index.html>). Secondary structure analysis was done by Jpred3 (<http://www.compbio.dundee.ac.uk/www-jpred/index.html>).

**Purification of FlhF and FlhG proteins.** Wild-type *flhF*, *flhF*<sub>K295A</sub>, *flhF*<sub>D321A</sub>, *flhF*<sub>R324A</sub>, wild-type *flhG* and *flhG*<sub>D61A</sub> from codon 2 to the stop codon and *flhF*<sub>ΔG</sub> from codons 2 through 273 were PCR amplified with primers containing in-frame 5' BamHI sites. The DNA fragments were digested with BamHI and ligated into BamHI-digested pQE30 to create the following plasmids that carry N-terminal His<sub>6</sub>-tags: pDRH2270 (wild-type FlhF), pMB174 (FlhF<sub>K295A</sub>), pMB640 (FlhF<sub>D321A</sub>), pMB681 (FlhF<sub>R324A</sub>), pMB176 (FlhF<sub>ΔG</sub>), pMB180 (wild-type FlhG) and pMB1067 (FlhG<sub>D61A</sub>). These constructs were then transformed into *E. coli* XL-1 Blue. Bacteria were grown in 500 ml LB broth (for purification of wild-type FlhF, FlhF<sub>D321A</sub>, FlhF<sub>R324A</sub>, FlhF<sub>ΔG</sub>, FlhG and FlhG<sub>D61A</sub>) or 2 liters of LB broth (for purification of FlhF<sub>K295A</sub>) at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of 0.8 and induced with 1 mM (final concentration) of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37°C for 1 h. The bacteria were washed once with 350 mM NaCl, 10 mM MgCl<sub>2</sub>, and 10 mM KCl (pH 8.0) and then resuspended in 40 ml of the same buffer containing 1% Nonidet P-40 and 1 tablet of Complete protease inhibitor cocktail (Roche). For FlhG and FlhG<sub>D61A</sub> proteins, resuspension buffer did not contain Nonidet P-40. The resuspended bacteria were passaged four times through an EmulsiFlex-C5 cell disrupter (Avesin) at 15,000 to 20,000 lb/in<sup>2</sup>. Nickel-nitrilotriacetic acid agarose beads (Qiagen) were used for purification of proteins under native conditions according to the manufacturer's instructions. Eluted proteins after chromatography were analyzed by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Fractions with the highest purity of FlhF and FlhG proteins were combined, and glycerol was added to a final concentration of 10%. Protein preparations

were frozen at  $-80^{\circ}\text{C}$ . The concentrations of protein samples were measured by Bradford assay prior to use.

**Generation of polyclonal antiserum against *C. jejuni* proteins.** For purification of wild- type FlhF for generation of antisera, XL1-Blue/pDRH2270 was grown in 500 ml LB containing 100  $\mu\text{g/ml}$  ampicillin to an  $\text{OD}_{600}$  of 0.6 at  $37^{\circ}\text{C}$ . IPTG was added to a final concentration of 0.1 mM, and the culture was further incubated for 4 h. The bacteria were lysed as described above, and His<sub>6</sub>-FlhF was purified from the insoluble material after solubilization in 8 M urea with nickel-nitrilotriacetic acid agarose according to the manufacturer's instructions. The coding sequence of *flgG* and *fliF* was cloned into pGEX-4T-2 and transformed into BL21(DE3) to create pJMB515 (encoding the glutathione *S*-transferase [GST]-FlgG fusion protein) and pDRH2266 (encoding the GST-FliF fusion protein). For induction of protein production, 500 ml and 3 liters of LB broth with 100  $\mu\text{g/ml}$  of ampicillin was inoculated with overnight cultures of BL21(DE3)/pJMB515 or BL21(DE3)/pDRH2266, respectively, and grown to an  $\text{OD}_{600}$  of 0.6. The bacteria were induced for 4 h with 0.1 mM IPTG at  $37^{\circ}\text{C}$ , and the bacteria were lysed as described above. After recovery of the soluble fraction after centrifugation, the fusion proteins were purified by standard procedures using glutathione Sepharose 4B.

The purified His<sub>6</sub>-FlhF, GST-FlgG, and GST-FliF proteins were used to immunize mice by standard procedures for antiserum generation by a commercial vendor (Cocalico Biologicals).

**Fluorescent microscopy analysis.** For each strain, growth from freezer stocks was restreaked onto MH agar containing appropriate antibiotics and grown for 16 h under microaerobic conditions at 37 °C. For untreated wild-type *C. jejuni* and *flhG<sub>D61A</sub>* mutant strains, bacteria were resuspended from agar plates in MH broth and then diluted to OD<sub>600</sub> 1.0. For cephalixin-treated strains, bacteria were resuspended from agar plates and grown in MH broth containing 15 µg/ml cephalixin for 6 hours. Approximately 1.5 ml of bacterial culture was pelleted in a microcentrifuge and then resuspended in 4% formalin for fixation. Then, 350 µl of fixed cells were stained with 20 µl of FM4-64 at a 1 mg/ml concentration for 15 min. Samples were then added to poly-L-lysine-coated chamber slides. After 5 min, excess liquid was removed with a vacuum. ProLong Gold antifade reagent containing DAPI (Invitrogen) was applied to the chamber slides. After 24 h, fluorescent images were obtained with an Applied Precision PersonalDV deconvolution microscope with an Olympus 100x objective lens and a CoolSNAP\_HQ2 camera. Images were processed using the ImageJ program.

**Arylsulfatase transcriptional reporter assays.** Transcriptional reporter gene fusions were constructed by inserting the SmaI *astA-kan* cassette from pDRH580 into the NcoI site of *flhA* in pDRH664 (to generate pDRH867 and creating *flhA::astA-kan*), the MscI site of *fliP* in pSNJ128 (to generate pMB109 and creating *fliP::astA-kan*), and the StuI site of *flhB* of pDRH742 (to generate pMB144 and creating *flhB::astA-kan*). *C. jejuni* strains in the  $\Delta astA$  background were electroporated with pDRH532, pDRH608, pDRH610, pDRH867, pMB109, and pMB144 to create promoterless *astA* transcriptional fusions to *flgDE2*, *flaA*, *flaB*, *flhA*, *fliP*, and *flhB* respectively. Arylsulfatase production

from the transcriptional fusions in these strains was measured by previously published methods (63, 67, 185).

**Motility assays.** Motility phenotypes of *C. jejuni* wild-type and mutant strains were assessed as previously described (23). Briefly, strains from 16 h growth plates were suspended in MH broth to an OD<sub>600</sub> of 0.8 and stabbed into semisolid MH motility agar by using a sterilized inoculating needle. The plates were incubated for 24 h at 37°C in microaerobic conditions and then visualized for motility.

**Transmission electron microscopy.** For each strain, growth from freezer stocks were restreaked onto MH agar containing appropriate antibiotics and grown for 16 h under microaerobic conditions at 37 °C. Strains were resuspended in PBS to an OD<sub>600</sub> between 0.6 – 1.0, pelleted for 3 min at full speed in a microcentrifuge, and then resuspended in 2% gluteraldehyde and 0.1 M cacodylate solution. For cephalixin-treated strains, bacteria were resuspended from agar plates and grown in MH broth containing 15 µg/ml or 12.5 µg/ml cephalixin for 6 hours and then fixed with glyteraldehyde. After 1 h of fixation on ice, samples were stained with 2% uranyl acetate and visualized with a FEI Technai G2 Spirit BioTWIN transmission electron microscope.

**Quantification of bacterial populations according to bacterial cell length and flagellar pattern.** Data from two separate experiments were combined and averaged to determine the proportion of bacterial populations producing different numbers of polar flagella or cell bodies of different lengths. In total, over 210 individual bacteria were

analyzed for each strain. For analysis of flagellar numbers, each bacterium was divided into one of three categories: > 2 flagella, bacteria that produced at least two flagella at one pole; WT flagella, bacteria that produced a single flagellum at one or both poles; or 0 flagella, bacteria that were aflagellated. For analysis of the lengths of cell bodies, each bacterium was divided into one of four categories: < 0.5  $\mu\text{m}$ , minicells; 0.5 – 1  $\mu\text{m}$ ; 1 – 2  $\mu\text{m}$ ; and > 2  $\mu\text{m}$ . After averaging, the standard error for each population was calculated.

***In vitro* GTP and ATP Hydrolysis Assays.** The nucleotide hydrolysis activity of purified proteins over time was performed by using a modified version of the protocol published by Weiss et al (48). Reactions consisted of 500 nM purified wild-type and mutant FlhF proteins, 6  $\mu\text{Ci}$  [ $\gamma$ -<sup>32</sup>P]GTP or [ $\gamma$ -<sup>32</sup>P]ATP (100 nM, final concentration), 50 mM sodium acetate, 40 mM KCl, 5.4 mM MgCl<sub>2</sub>, 0.1 M EDTA, 3% glycerol, 1 mM dithiothreitol, and 0.1 mg/ml acetylated bovine serum albumin in a final volume of 55  $\mu\text{l}$ . At time points of 0, 5, 10, 20, and 30 min, 10  $\mu\text{l}$  was removed and added to 990  $\mu\text{l}$  of 1 N formic acid to stop the reaction. Released phosphate in 5  $\mu\text{l}$  of each stopped reaction was separated from the nucleoside by thin-layer chromatography using Cellulose PEI plates (J.T. Baker) in a buffer containing 0.4 M K<sub>2</sub>HPO<sub>4</sub> and 0.7 M boric acid. The plates were air dried and autoradiographed by using a Storm 820 phosphorimager (Amersham Biosciences). The data were analyzed by the manufacturer's software.

**Fractionation of *C. jejuni* strains for protein localization analysis.** Total proteins from whole-cell lysates (WCL) and total membrane of *C. jejuni* strains were fractionated by a protocol that was described previously (3). For whole-cell lysates, protein samples were

loaded to represent the proteins recovered from 200- $\mu$ l aliquots of bacterial cultures which had been equilibrated to the same density. For fractions representing total membrane, the amounts loaded represented protein samples obtained from 500  $\mu$ l of bacterial culture (for detection of FliF, AtpF, or RpoA) or 5 ml of bacterial culture (for detection of FlhB).

**Immunoblotting analysis of FlhF, FlgS, FlgR, FlgG, FliF and FlhB proteins.**

Immunoblotting analysis was performed with specific primary antiserum at the following dilutions:  $\alpha$ -FlhF M1 (1:3000),  $\alpha$ -RpoA M59 (1:3500; (86)),  $\alpha$ -FlhB Rab476 (1:1000; (86)),  $\alpha$ -FliF M1 (1:1000),  $\alpha$ -FlgS Rab11 (1:3500; (64)),  $\alpha$ -FlgR Rab13 (1:5000; (64)),  $\alpha$ -AtpF M3 (1:1000), and  $\alpha$ -FlgG M69 (1:1000). Secondary antibodies were used at 1:5000 to 1:10,000 dilutions.

**Real-time reverse transcription-PCR (RT-PCR) analyses.** *C. jejuni* strains DRH212 (81-176 Sm<sup>r</sup>), DRH1056 (81-176 Sm<sup>r</sup>  $\Delta$ *flhF*), MB630 [81-176 Sm<sup>r</sup> *flhF*<sub>D321A</sub>], and MB628 [81-176 Sm<sup>r</sup> *flhF*<sub>R324A</sub>] were grown from frozen stocks on MH agar containing appropriate antibiotics at 37°C for 48 h and restreaked 16 h prior to use. 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/ $\mu$ l of RNA was used in a Sybr green PCR master mix. Real-time reverse transcription-PCR (RT-PCR) was performed using a 7500 real-time PCR system (Applied Biosystems). Detection of mRNA for 16S rRNA served as an endogenous control, and the transcript levels of *rpoN*, *flgG*, *flhA*, *flhB*, and *fliF* in *flhF*

mutants were compared relative to those of the wild-type strain (DRH212). The following primer pairs were used for real-time RT-PCR analysis:

*rpoN* RT F, 5'-TTAGCACTTGATTTAGAACGCAATG-3', and

*rpoN* RT R, 5'-GGGATAAGTCCTCTTTCACAACCTTAAATA-3';

*flgG* RT F, 5'-AATTCTGCGCGACTTTTCTTAAA-3', and

*flgG* RT R, 5'-AGCGCAGCAAACACAAATTG-3';

*flhA* RT R, 5'-AATACAATCACGCCAATGACCAT-3', and

*flhA* RT F, 5'-GCCCTGAAGCTGTGAGTGAGA-3';

*flhB* RT F, 5'-CGACGCATTATGCCGTAGCTA-3', and

*flhB* RT R, 5'-TGTTTTATGCGAAGAGCGAGAAA-3';

*fliF* RT F2, 5'-TTTAAACGAGGAAAGAACAGGTAGAAA-3\_, and

*fliF* RT R2, 5'-GTCAAGTCCTTCAACAGGACCTATATTA-3'; and

16S rRNA F, 5'-CCGGAATCGCTAGTAATCGTAGA-3', and

16S rRNA R, 5'-ACGGGCGGTGAGTACAAGAC-3'.

## CHAPTER FOUR

### FLHF AND ITS GTPASE ACTIVITY ARE REQUIRED FOR DISTINCT PROCESSES IN FLAGELLAR GENE REGULATION AND BIOSYNTHESIS IN *CAMPYLOBACTER JEJUNI*\*

#### Introduction

*C. jejuni* belongs to a subset of motile bacteria that produce polarly-localized flagella. These bacteria produce the putative FlhF GTPase, which is required in each bacterium for at least one of the following: expression of a subset of flagellar genes, biosynthesis of flagella, or the polar placement of the flagella. Bioinformatic analysis indicates that the FlhF proteins belong to the SIMIBI class of NTP-binding proteins (106). More specifically, the GTPase domains of FlhF proteins are most similar to those of the signal recognition particle (SRP)-pathway GTPases, such as Ffh and FtsY. Because of the homology of the GTPase domains, these three proteins may form a unique subset within the SIMIBI proteins. Whereas the GTPase activity of the interacting Ffh and FtsY proteins have been extensively characterized (122, 143, 148, 157), little is known about the GTP-hydrolysis activity of FlhF. However, no biochemical analysis has been performed to verify or characterize the ability of an FlhF protein to hydrolyze GTP. As such, no studies have correlated the biochemical activity of FlhF in relation to GTP hydrolysis with the role that FlhF performs in flagellar gene expression or biosynthesis.

Previous work revealed the details of the regulatory cascades governing flagellar gene expression in *C. jejuni*. Formation of the flagellar export apparatus (FEA) that

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secretes most of the flagellar proteins out of the cytoplasm to form the flagellum, is required to activate the FlgS sensor kinase to begin a phosphorelay to the cognate FlgR response regulator (86, 87). Once activated by phosphorylation, FlgR likely interacts with  $\sigma^{54}$  in RNA polymerase to initiate expression of many flagellar genes encoding components of the flagellar basal body, rod, and hook (67, 87). After formation of the hook, *flaA* encoding the major flagellin is expressed via  $\sigma^{28}$  and RNA polymerase to generate the flagellar filament and complete flagellar biosynthesis (19, 65, 67, 82, 182).

Previously, in two separate genetic analyses, it was found that *flhF* mutants of *C. jejuni* are non-motile and reduced over 10-fold for expression of  $\sigma^{54}$ -dependent flagellar genes, indicating that FlhF is required for both flagellar gene expression and biosynthesis (67). However, it is unclear how FlhF influences expression of  $\sigma^{54}$ -dependent flagellar genes. Furthermore, it is unknown if the GTPase activity of FlhF is required for flagellar gene expression or biosynthesis in *C. jejuni*.

In this chapter, I show that *C. jejuni* FlhF specifically hydrolyzes GTP, confirming that FlhF is a GTPase, and this GTPase function of FlhF influences spatial control of flagellar biosynthesis. Whereas the FlhF protein is required for motility, flagellar biosynthesis, and expression of  $\sigma^{54}$ -dependent flagellar genes, the GTPase activity of the protein only significantly influences proper biosynthesis of flagella. These results suggest that multiple biochemical activities of FlhF (including GTPase activity and likely other as yet uncharacterized activities mediated by other domains) are required at distinct steps in flagellar gene expression and biosynthesis. In addition, this chapter provides biochemical and genetic evidence that FlhF likely functions in a separate

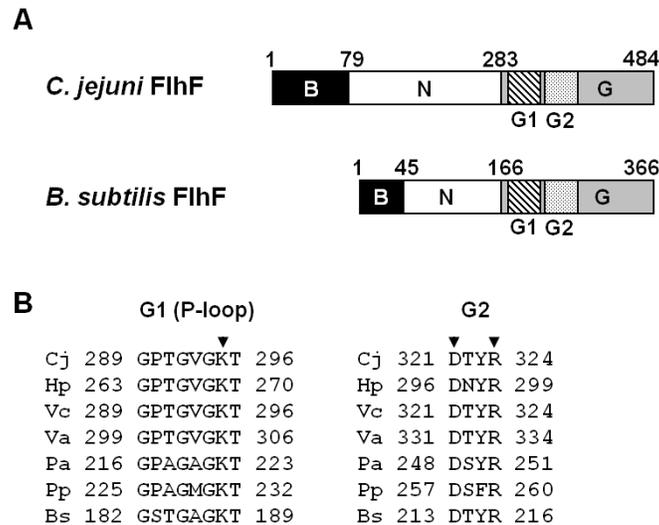
pathway from the FEA-FlgSR pathway in *C. jejuni* to influence expression of  $\sigma^{54}$ -dependent flagellar genes. In summary, in this chapter I conclude that FlhF have two distinct functions, GTPase-dependent role in polar placement of flagella and GTPase-independent role in  $\sigma^{54}$ -dependent flagellar gene expression in *C. jejuni*.

## Results

### *FlhF is a GTPase*

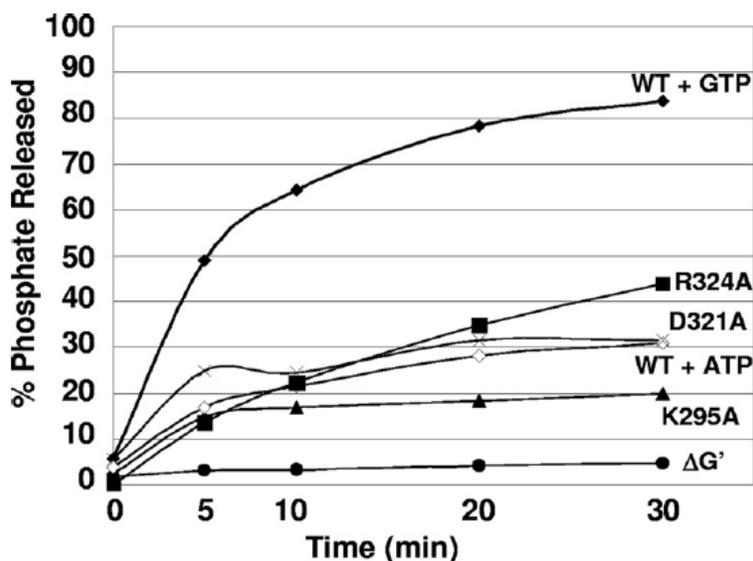
Based on previous annotation of domains of the FlhF protein of *B. subtilis* (10), *C. jejuni* FlhF can be divided into three domains: an N-terminal basic (B) domain (amino acids 1-78), a middle N domain (amino acids 79-272), and a C-terminal G domain containing the GTPase domain (amino acids 283-484) (Figure 7A). Bioinformatic analysis revealed that the B and N domains of the proteins share some homology (21 to 27.9% identity, 46.5 to 51.1% similarity), but FlhF of *C. jejuni* contains an additional 107 amino acids in the putative B and N domains not found in *B. subtilis* FlhF. Similarly, the B and N domains of other FlhF proteins from *P. putida*, *P. aeruginosa*, *H. pylori*, *V. cholerae*, and *V. alginolyticus* also contain additional amino acids, suggesting that these domains may confer functional and structural differences to their respective FlhF proteins compared to that of *B. subtilis*. More homology was noted between the G domain of the *C. jejuni* and *B. subtilis* proteins (35.6% identity and 56.5% similarity), with most of the homology occurring within the putative GTPase domain. Two subdomains of the G domain, G1 (containing the P-loop of GTPases) and G2 demonstrate conservation of residues predicted to be required for GTPase activity of the FlhF proteins (41) (Figure

7B). Despite conservation of GTPase domains, no studies have analyzed if the proteins are able to hydrolyze GTP. We performed *in vitro* and *in vivo* biochemical and genetic analysis to determine if FlhF of *C. jejuni* is a GTPase and if this biochemical activity of FlhF is required for distinct steps in flagellar gene regulation or flagellar biosynthesis.



**Figure 7. Domain organization and sequence alignments of FlhF proteins.** (A) The FlhF proteins of *C. jejuni* 81-176 (GenBank accession number YP\_999790) and *B. subtilis* strain 168 (GenBank accession number CAA47062) can be divided into three domains: an N-terminal basic domain (“B”, black rectangles), a central N domain (“N”, white rectangles) and a C-terminal GTPase domain (“G”, grey rectangles). Within the GTPase domain, multiple conserved subdomains are evident including G1 containing the P-loop domain (striped box) and G2 containing the DXXR motif (dotted box). Numbers above proteins indicate boundaries of domains. (B) ClustalW alignment of the G1 and G2 domains of FlhF proteins from *C. jejuni* 81-176 (Cj), *H. pylori* 26695 (Hp, GenBank accession number NP\_207825), *V. cholerae* O395 (Vc, GenBank accession number YP\_001217595), *V. alginolyticus* 12G01 (Va, GenBank accession number ZP\_01258825), *P. aeruginosa* PAO1 (Pa, GenBank accession number AAG04842), *P. putida* MK1 (Pp, GenBank accession number AF67042), and *B. subtilis* (Bs). Numbers flanking each sequence indicate position of amino acids in the respective proteins. Arrowheads indicate conserved amino acids changed to alanine residues in the *C. jejuni* FlhF<sub>K295A</sub>, FlhF<sub>D321A</sub>, and FlhF<sub>R324A</sub> mutant proteins analyzed in this study.

Wild-type FlhF and FlhF mutant proteins predicted to be defective in GTP hydrolysis were purified. FlhF<sub>ΔG</sub> lacks amino acids 274 - 476, which removes most of the GTPase domain and presumably eliminates the hydrolysis activity of the protein. FlhF<sub>K295A</sub> contains an alanine substitution at a conserved lysine in the G1 (P-loop) region of the GTPase domain that is usually essential for GTP hydrolysis (153) (Figure 7B). FlhF<sub>D321A</sub> and FlhF<sub>R324A</sub> contain alanine instead of an aspartic acid or arginine at a conserved DXXR motif of the G2 region of the GTPase domain, which has been proposed to assist in GTP hydrolysis in the SIMIBI class of GTPases (106) (Figure 7B). However, verification of the requirement of the DXXR motif for the GTPase activity of an FlhF protein has yet to be performed.

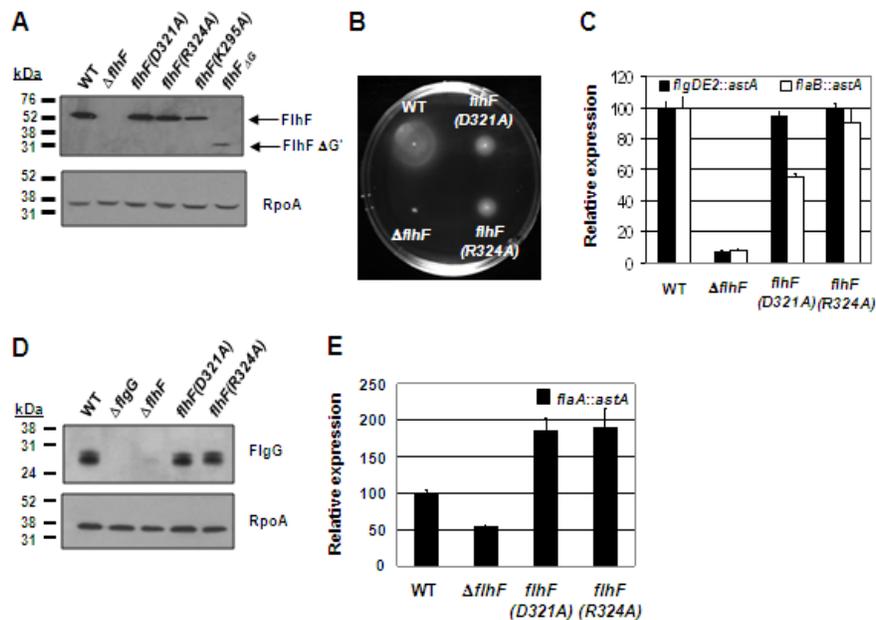


**Figure 8. Nucleotide-hydrolysis activity of wild-type and mutant FlhF proteins.** Released phosphate from  $[\gamma\text{-}^{32}\text{P}]$  GTP or ATP was monitored in reactions with purified wild-type and FlhF mutant proteins over 30 minutes. FlhF proteins and nucleotides were used at a final concentration of 0.5  $\mu\text{M}$  and 0.1  $\mu\text{M}$ , respectively. Hydrolysis activity is expressed as the percentage of labeled phosphate released compared to the amount of labeled phosphate in non-hydrolyzed nucleotides remaining at each time point. Data presented are from a representative assay. Closed symbols indicate phosphate released from GTP with addition of: WT FlhF (◆), FlhF<sub>K295A</sub> (▲), FlhF<sub>D321A</sub> (×), FlhF<sub>R324A</sub> (■), and FlhF<sub>ΔG'</sub> (●). Open symbols indicate phosphate released from ATP with addition of WT FlhF (◇).

The FlhF proteins were analyzed for GTPase activity by monitoring release of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{GTP}$  over time. Wild-type FlhF hydrolyzed GTP, with over 80% of the  $^{32}\text{P}$  detected as free phosphate by the end of the assay (Figure 8). In contrast, FlhF<sub>R324A</sub>, FlhF<sub>D321A</sub> and FlhF<sub>K295A</sub> were reduced over 60 – 75% for GTP hydrolysis (Figure 8). As expected, FlhF<sub>ΔG'</sub> was the most defective for GTP hydrolysis, with an over 90% reduction in activity. In comparison to GTP, wild-type FlhF only demonstrated limited hydrolysis of ATP, suggesting that FlhF is more efficient at hydrolyzing GTP compared to other nucleotides.

*The GTPase activity of FlhF is specifically required for proper biosynthesis of flagella.*

Wild-type *flhF* of *C. jejuni* was replaced with *flhF* alleles encoding the FlhF mutant proteins described above to determine if defects in GTPase activity impact flagellar gene expression, biosynthesis, or motility. FlhF<sub>D321A</sub> and FlhF<sub>R324A</sub> were detected as stable proteins in *C. jejuni*, produced at similar levels as wild-type FlhF (Figure 9A). However, FlhF<sub>K295A</sub> and FlhF<sub>ΔG</sub> were produced at less than 25% of the levels of wild-type FlhF. Because the reduced levels or stability of FlhF<sub>K295A</sub> and FlhF<sub>ΔG</sub> may have caused difficulty in interpreting results of potential defects in flagellar gene expression and biosynthesis that may be unrelated to defects in GTP hydrolysis, these respective *C. jejuni* mutants were not further characterized in vivo.



**Figure 9. Analysis of *flhF* mutants for flagellar gene expression, motility, and protein production.** A. Immunoblot analysis of production of wild-type and FlhF mutant proteins in *C. jejuni*. FlhF proteins from WCL were detected with murine  $\alpha$ -FlhF M1 antiserum (top panel) and RpoA from WCL was detected with murine  $\alpha$ -RpoA M59 antiserum to verify equal loading of samples (bottom panel). Arrows indicate FlhF or FlhF $\Delta$ G'. Strains include DRH212 (WT 81-176 Sm<sup>R</sup>), DRH1056 (81-176 Sm<sup>R</sup>  $\Delta flhF$ ), MB630 (81-176 Sm<sup>R</sup> *flhF*<sub>D321A</sub>), MB628 (81-176 Sm<sup>R</sup> *flhF*<sub>R324A</sub>), DRH1302 (81-176 Sm<sup>R</sup> *flhF*<sub>K295A</sub>), and SNJ206 (81-176 Sm<sup>R</sup> *flhF* <sub>$\Delta$ G'</sub>). B. Motility phenotypes of wild-type and mutant *C. jejuni* strains in MH semi-solid agar. Strains include DRH212 (wild-type 81-176 Sm<sup>R</sup>), DRH1056 (81-176 Sm<sup>R</sup>  $\Delta flhF$ ), MB630 (81-176 Sm<sup>R</sup> *flhF*<sub>D321A</sub>), and MB628 (81-176 Sm<sup>R</sup> *flhF*<sub>R324A</sub>). C. Arylsulfatase assays measuring expression of the  $\sigma^{54}$ -dependent transcriptional fusions, *flgDE2::astA* and *flaB::astA*, in *C. jejuni* strains. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity  $\pm$  standard deviation relative to the amount of expression of each transcriptional fusion in wild-type 81-176 Sm<sup>R</sup>  $\Delta astA$ , which was set to 100 arylsulfatase units. For expression of *flgDE2::astA* (black bars), strains include wild-type DRH533, SNJ150, MB657, and MB662. For expression of *flaB::astA* (white bars), strains include wild-type DRH665, SNJ155, MB659, and MB666. D. Immunoblot analysis of production of FlgG in *C. jejuni* strains.  $\alpha$ -FlgG M69 murine antiserum was used to detect FlgG in WCL in the top panel. Lower panel is an immunoblot for RpoA detected with  $\alpha$ -RpoA M59 murine antiserum to verify equal loading of samples. Strains include DRH212 (WT 81-176 Sm<sup>R</sup>), SNJ925 (81-176 Sm<sup>R</sup>  $\Delta astA$   $\Delta flgG$ ), DRH1056 (81-176 Sm<sup>R</sup>  $\Delta flhF$ ), MB630 (81-176 Sm<sup>R</sup> *flhF*<sub>D321A</sub>), and MB628 (81-176 Sm<sup>R</sup> *flhF*<sub>R324A</sub>). E. Arylsulfatase assays measuring expression of *flaA::astA* in *C. jejuni* strains. Results are

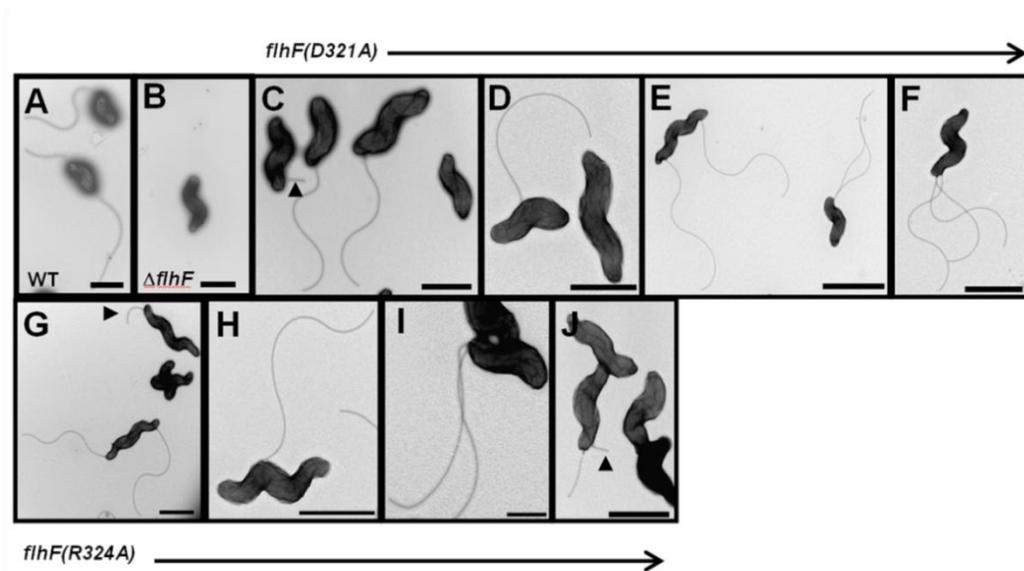
from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity  $\pm$  standard deviation relative to the amount of expression of each transcriptional fusion in wild-type 81-176 Sm<sup>R</sup>  $\Delta astA$  which was set to 100 arylsulfatase units. Strains include DRH655, SNJ154, MB704, and MB706.

Unlike wild-type *C. jejuni* which was fully motile, a  $\Delta flhF$  mutant (generated by removing codons 2 – 476 of *flhF* from the chromosome; (67)) was non-motile (Figure 9B). The *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants displayed an intermediate phenotype with a level of motility sharply reduced compared to the wild-type strain (Figure 9B). These data suggest that complete lack of FlhF is more detrimental to motility than producing an FlhF mutant protein hindered for GTPase activity. Attempts to recover transconjugants with wild-type *flhF* expressed from a constitutive promoter *in trans* to complement any *flhF* mutant were unsuccessful, suggesting that this method of complementation of *flhF* mutants may be lethal to the bacterium (data not shown). However, interruption of the two genes downstream of *flhF* did not result in non-motile phenotypes like that caused by deletion of *flhF* (data not shown). These results confirm that any motility defects associated with the  $\Delta flhF$  mutant or *flhF* point mutations were directly due to mutation of *flhF* and not due to any possible polar defects in expression of downstream genes that could be essential for flagellar motility.

We next analyzed if reduced motility of the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants was due to reduced expression of  $\sigma^{54}$ -dependent flagellar genes. Expression of two  $\sigma^{54}$ -dependent flagellar genes was measured by examining the levels of arylsulfatase produced from a transcriptional fusion of *flgDE2* and *flaB* to a promoterless *astA* construct. Compared to the wild-type strain, the  $\Delta flhF$  mutant of *C. jejuni* demonstrated

a 12- to 15-fold reduction in expression of  $\sigma^{54}$ -dependent flagellar genes (Figure 9C). In contrast, expression of *flgDE2* was not affected in the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants. We did detect a minor reduction (less than two-fold) in expression of *flaB* in the *flhF*<sub>D321A</sub> mutant, but not in the *flhF*<sub>R324A</sub> mutant. These results suggest that impairing the GTPase activity of FlhF does not significantly influence the ability of *C. jejuni* to express flagellar genes. We also assessed the ability of the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants to produce proteins encoded by  $\sigma^{54}$ -dependent transcripts. For this analysis, we analyzed production of FlgG, which forms the periplasmic distal rod structure of the flagellum. Like *flgDE2* and *flaB*, *flgG* is predicted to be dependent on  $\sigma^{54}$  and FlhF for expression, but this analysis has never been performed. By real-time RT-PCR analysis, we confirmed that the  $\Delta$ *flhF* mutant demonstrated a five-fold reduction in expression of *flgG* relative to the wild-type strain, which resulted in a very minimal level of FlgG production (Figure 9D and data not shown). Similar to our analysis of *flgDE2* and *flaB* expression, we only detected a slight reduction of expression of *flgG* in the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants of about 25% (data not shown). In addition, the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants produced wild-type levels of FlgG (Figure 9D), indicating that production of proteins from  $\sigma^{54}$ -dependent transcripts was likely not affected in FlhF mutants hindered for GTPase activity. Analysis of expression of the  $\sigma^{28}$ -dependent flagellar gene *flaA* revealed that the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants expressed approximately 70% more *flaA* than the wild-type strain, whereas the  $\Delta$ *flhF* mutant expressed about 50% less. Whereas the significance of increased expression of *flaA* in these mutants is unknown regarding if this increase would negatively impact flagellar motility, the fact that these mutants do not

have decreases in *flaA* expression indicate that these mutants are not defective in regulating pathways for controlling activity of  $\sigma^{28}$  for expression of essential flagellar genes (Figure 9E).



**Figure 10. Flagellar biosynthesis phenotypes of *C. jejuni* wild-type and *flhF* mutant strains.** Transmission electron microscopy of negatively stained bacteria was performed. All micrographs are between 11,500X and 20,500X magnification. Bars equal 1  $\mu\text{m}$ . A. DRH212 (WT 81-176 Sm<sup>R</sup>). B. DRH1056 (81-176 Sm<sup>R</sup>  $\Delta flhF$ ). C-F. MB630 (81-176 Sm<sup>R</sup> *flhF*<sub>D321A</sub>). G-J. MB628 (81-176 Sm<sup>R</sup> *flhF*<sub>R324A</sub>). Arrowheads indicate truncated flagella produced by some bacteria in panels C, G, and J.

By transmission electron microscopy, we found that the reduced motility phenotypes of the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants were due to defects in proper flagellar biosynthesis. The production of a single flagellum at one or both poles of the bacterium was the normal flagellar biosynthesis phenotype produced by over 92% of individual wild-type *C. jejuni* (Figure 10A and Table 1). In contrast, the  $\Delta flhF$  mutant rarely produced a single flagellum at one pole (Figure 10B and Table 1). The *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants produced a variety of flagellar biosynthesis phenotypes, with only

approximately one-third producing a single flagellum at one or both bacterial poles (part of the normal phenotype; Figure 10C and Table 1). Another one-third of the mutants produced no flagella, compared to only 7.2% of wild-type bacteria with no flagella (Figure 10C, 10D, 10G, and 10J and Table 1). The remaining 30 – 37% of the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants produced a variety of improper flagellar biosynthesis phenotypes not detected in wild-type *C. jejuni*, which included the production of two or more flagella at one pole (Figure 10E, 10F, 10I, and 10J), a flagellum at a non-polar site (flagella either produced slightly off the polar ends or at a more lateral site on the bacterium; Figures 10D, 10E, 10G, and 10H), or a significantly shorter flagellum (Figures 10C, 10G, and 10J). The increase in the aflagellated or improper flagellar phenotypes likely contributed to the reduction in flagellar motility observed in these mutants (Figure 9B). These results indicate that the GTPase activity of FlhF is specifically required for proper flagellar biosynthesis, and may perform only a minor role, if any, in flagellar gene expression or protein production. Since the  $\Delta$ *flhF* mutant is defective for  $\sigma^{54}$ -dependent flagellar gene expression, we hypothesize that some other as yet uncharacterized activity of FlhF is required by *C. jejuni* for expression of wild-type levels of  $\sigma^{54}$ -dependent flagellar genes. Thus, inherent biochemical activities are required for distinct steps in flagellar gene expression and biosynthesis.

Relevant Genotype	Percentage of population producing:			
	2 flagella	1 flagellum	0 flagella	other forms
Wild-type	73.9	18.9	7.2	0
$\Delta flhF$	0	1.6	98.4	0
$flhF_{D321A}$	1.6	29.0	32.3	37.1
$flhF_{R324A}$	4.1	29.3	35.8	30.9

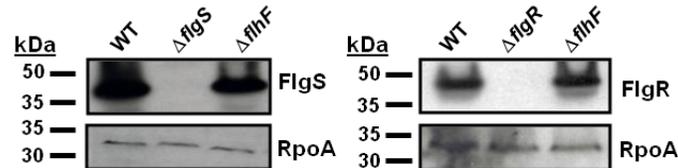
**Table 1. Analysis of proper flagellar number and placement of wild-type and *C. jejuni* mutant strains.** Strains used include DRH212 (wild-type 81-176 Sm<sup>R</sup>), DRH1056 (81-176 Sm<sup>R</sup>  $\Delta flhF$ ), MB630 (81-176 Sm<sup>R</sup>  $flhF_{D321A}$ ), and MB628 (81-176 Sm<sup>R</sup>  $flhF_{R324A}$ ). Over 110 individual bacteria for each strain were examined to determine the percentage of bacteria producing each phenotype. Phenotypes included: bacteria that produced a single flagellum at both poles (2 flagella), a single flagellum at only one pole (1 flagellum), or no flagella (0 flagella). Bacteria producing other forms included those that produced more than one flagellum at a single pole, a flagellum at a non-polar or lateral site, or a significantly shortened flagellum.

*FlhF likely functions in a separate pathway from the fea-flgsr pathway to activate expression of  $\sigma^{54}$ -dependent flagellar genes.*

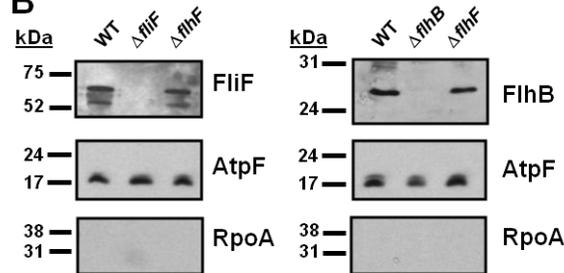
Previous work has established that the formation of the FEA, rather than its secretory activity, contributes to a signal that is sensed by the cytoplasmic FlgS sensor kinase to result in autophosphorylation and phosphorelay to the cognate response regulator FlgR (86, 87). Activation of FlgR stimulates  $\sigma^{54}$  in RNA polymerase for expression of flagellar genes. Elimination of any component of the FEA, FlgS, or FlgR results in 12- to 800-fold decreases in  $\sigma^{54}$ -dependent flagellar gene expression (20). To provide a better understanding where FlhF may function in regulatory pathways for

expression of  $\sigma^{54}$ -dependent flagellar genes, we analyzed if FlhF is required for production of the components of the FEA-FlgSR pathway.

**A**



**B**

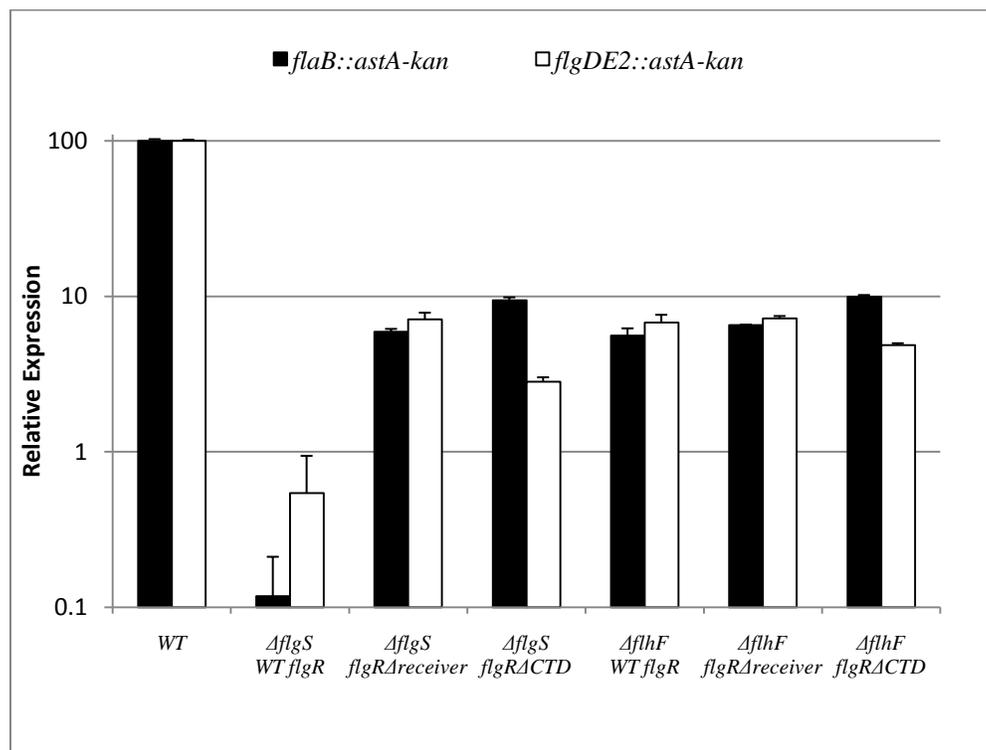


**Figure 11. Formation of the FEA-FlgSR signaling pathway components and activity of the FEA in *C. jejuni* wild-type and *flhF* mutant strains.** *A.* Immunoblot analysis of FlgS and FlgR production in WCL of *C. jejuni* strains. Immunoblots were performed with  $\alpha$ -FlgS Rab11 rabbit antiserum and  $\alpha$ -FlgR Rab13 rabbit antiserum. Control immunoblots were performed with  $\alpha$ -RpoA M59 antisera to verify equal loading of protein samples. Strains include DRH212 (WT 81-176 Sm<sup>R</sup>), DRH460 (81-176 Sm<sup>R</sup>  $\Delta$ *flgS*; (67)), DRH737 (81-176 Sm<sup>R</sup>  $\Delta$ *flgR*; (67)), DRH1056 (81-176 Sm<sup>R</sup>  $\Delta$ *flhF*). *B.* Immunoblot analysis of FliF and FlhB production and localization to the membrane fraction in *C. jejuni* strains. Total membranes were isolated from wild-type and mutant *C. jejuni* strains and then analyzed by immunoblot analysis. Immunoblots were performed with  $\alpha$ -FliF M1 murine antiserum and  $\alpha$ -FlhB Rab476 rabbit antiserum. Control immunoblots were performed with  $\alpha$ -AtpF M3 and  $\alpha$ -RpoA M59 murine antisera to verify presence of AtpF and absence of RpoA in the membrane preparations. The membrane proteins from 500  $\mu$ l of bacterial culture at equivalent densities were analyzed for FliF, AtpF, and RpoA and from 5 ml of bacterial culture at equivalent densities was analyzed for FlhB. Strains include DRH212 (wild-type 81-176 Sm<sup>R</sup>), DRH2074 (81-176 Sm<sup>R</sup>  $\Delta$ *fliF*), SNJ471 (81-176 Sm<sup>R</sup>  $\Delta$ *flhB*), DRH1056 (81-176 Sm<sup>R</sup>  $\Delta$ *flhF*).

As shown in Figure 11A, no differences in the levels of the FlgS and FlgR proteins were observed in whole cell lysates (WCL) of wild-type and  $\Delta flhF$  mutant strains. Comparing wild-type and  $\Delta flhF$  mutant strains by real-time RT-PCR revealed no differences in the levels of expression of *fliF*, *flhA*, and *flhB*, encoding three components of the FEA (data not shown). In addition, we did not detect significant differences in the production or membrane-localization of two components of the flagellar export apparatus, FlhB and FliF, between wild-type and  $\Delta flhF$  mutant strains (Figure 11B). Purity of membrane preparations from *C. jejuni* strains was verified by monitoring the presence of the inner membrane protein AtpF, a component of ATP synthase, and the absence of the cytoplasmic RpoA protein, a component of RNA polymerase (Figure 11B). Whereas we could detect FlhB and FliF localized to the membrane of *C. jejuni*, generation of antisera specific for the other FEA components was not successful. Thus, the immunoblotting analysis was incomplete to ensure that all components of the FEA were produced and localized to the membrane properly. The observation that a small minority (less than 2%) of individual  $\Delta flhF$  bacteria produce a single flagellum is indirect and secondary evidence that FlhF is not absolutely required for FEA formation since the FEA is required to secrete proteins to build the flagellum (Table 1). Because of these combined results, we conclude that FlhF is likely not required for production of components of the FEA-FlgSR signaling pathway.

We conducted additional experiments to determine if FlhF functions within the FEA-FlgSR signaling pathway or in a separate pathway that converges with the FEA-FlgSR pathway to activate expression of  $\sigma^{54}$ -dependent flagellar genes. We first ensured

that *flhF* was not required for expression of *rpoN* encoding  $\sigma^{54}$ . Quantitative real-time RT-PCR did not reveal any differences in expression of *rpoN* between wild-type and the  $\Delta flhF$  mutant strains (data not shown). We were unable to monitor the levels of  $\sigma^{54}$  as antisera generated against a portion of the protein were not able to detect native  $\sigma^{54}$  in *C. jejuni*.



**Figure 12. Relative flagellar expression levels  $\Delta flhF$  in partially constitutively active *flgR* mutants.** Arylsulfatase assays measuring expression of *flaB::astA* and *flgDE2::astA* in *C. jejuni* strains. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity  $\pm$  standard deviation relative to the amount of expression of each transcriptional fusion in wild-type 81-176 Sm<sup>R</sup>  $\Delta astA$  which was set to 100 arylsulfatase units. Strains include DRH533, DRH665, DRH936, DRH939, SNJ123, SNJ136, SNJ150, SNJ155, SNJ252, SNJ255, SNJ258, SNJ264, MB553 and MB555.

In previous work, partially constitutively active forms of FlgR lacking the N-terminal receiver domain (FlgR<sub>Δreceiver</sub>) or the C-terminal domain (FlgR<sub>ΔCTD</sub>) of the protein were shown to suppress the phenotypes of an FEA or *flgS* mutant for expression of  $\sigma^{54}$ -dependent flagellar genes (86, 87). These results indicated that FlgR functioned downstream of the FEA and FlgS in the signaling cascade for expression of  $\sigma^{54}$ -dependent flagellar genes. A similar suppressor analysis was performed to determine if FlgR<sub>Δreceiver</sub> or FlgR<sub>ΔCTD</sub> could restore flagellar gene expression to a  $\Delta$ *flhF* mutant. However, these partially-constitutively active FlgR mutant proteins did not suppress the  $\Delta$ *flhF* mutant for expression of  $\sigma^{54}$ -dependent flagellar genes (Figure 12). These results suggest that FlhF does not function upstream of the FlgSR in the FEA-FlgSR signaling cascade.

Relevant Genotype	Relative Expression of <i>flaB::astA</i> reporter
WT	100 ± 8.12
$\Delta$ <i>flhF</i>	5.37 ± 0.44
$\Delta$ <i>flhB</i>	3.41 ± 0.01
$\Delta$ <i>flhB</i> $\Delta$ <i>flhF</i>	1.54 ± 0.24

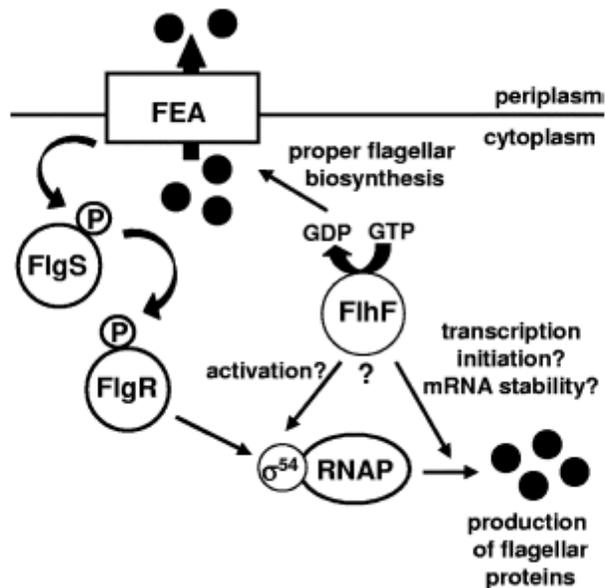
**Table 2. Relative expression level of *flaB::astA* reporter in a *flhB flhF* double mutant.** Arylsulfatase assays measuring expression of *flaB::astA* in *C. jejuni* strains. Results are from a typical assay with each strain tested in triplicate. Values reported for each strain are average arylsulfatase activity ± standard deviation relative to the amount of expression of each transcriptional fusion in wild-type 81-176 Sm<sup>R</sup>  $\Delta$ *astA* which was set to 100 arylsulfatase units. Strains include DRH665, DRH1830, SNJ155 and MB538.

We then compared the levels of expression of one  $\sigma^{54}$ -dependent flagellar gene, *flaB*, in mutants lacking *flhF* or *flhB* (encoding a component of the FEA), or both *flhF*

and *flhB*. Our analysis revealed that the  $\Delta flhF$  or  $\Delta flhB$  mutant displayed respectively 18- and 29-fold decreases in expression of *flaB::astA*, but the  $\Delta flhF \Delta flhB$  mutant showed a 65-fold decrease in expression of this transcriptional reporter (Table 2). Because the defects of disrupting both the FEA-FlgSR pathway and FlhF were additive, these data provide further evidence that FlhF likely functions in a separate pathway that either converges with or functions downstream of the FEA-FlgSR pathway to activate  $\sigma^{54}$ -dependent flagellar gene expression.

## Discussion

Peritrichous bacteria, which produce multiple flagella over the bacterial surface, have historically served as the model system to analyze regulation of flagellar genes and flagellar biosynthesis. Investigations into polarly-flagellated bacteria have uncovered variations in these pathways, indicating that bacteria have evolved diverse mechanisms to ensure proper expression of flagellar genes and biosynthesis of flagella. One of these differences has been the inclusion of FlhF into flagellar gene regulation and biosynthesis pathways in many polarly-flagellated bacteria. With the studies conducted so far, it is evident that FlhF functions at different steps in flagellar organelle development in these bacteria.



**Figure 13. Model for role of FlhF in activation of expression of  $\sigma^{54}$ -dependent flagellar genes and the GTPase activity of FlhF in proper flagellar biosynthesis.** Construction of the FEA is hypothesized to be required for the formation of a signal sensed by the FlgS sensor kinase for phosphorelay to the cognate FlgR response regulator to activate  $\sigma^{54}$  for expression of a subset of flagellar genes (67, 86, 87). An activity of FlhF independent of GTP hydrolysis is hypothesized to be required for a late step in regulating the expression of  $\sigma^{54}$ -dependent flagellar genes which may include co-activation mechanisms with FlgR to stimulate  $\sigma^{54}$  in RNA polymerase, activation of transcription initiation by  $\sigma^{54}$ -RNA polymerase holoenzyme, or stability of  $\sigma^{54}$ -dependent mRNA transcripts. The GTP-hydrolysis activity of FlhF is hypothesized to be required at an early step in flagellar biosynthesis by possibly influencing the FEA so that a flagellum is constructed and only a single flagellum forms at each pole.

Before this study, no experimental proof had been obtained that FlhF proteins hydrolyze GTP or if the GTPase activity is required for specific steps in production of flagella. By analyzing mutants of *C. jejuni* that either lacked FlhF or produced FlhF proteins with reduced ability to hydrolyze GTP, we identified distinct GTPase-dependent and GTPase-independent steps in flagellar gene regulation and biosynthesis (Figure 13). We discovered that a mutant lacking FlhF expressed approximately 5- to 15-fold lower

levels of three  $\sigma^{54}$ -dependent flagellar genes and over 98% of the bacteria did not produce a flagellum, which contributed to its non-motile phenotype. However, mutants producing FlhF<sub>D321A</sub> and FlhF<sub>R324A</sub>, which in *in vitro* analysis demonstrated only 30-40% of the GTPase activity of wild-type FlhF, were not severely defective for  $\sigma^{54}$ -dependent flagellar gene expression. These mutants were only significantly defective for proper flagellar biosynthesis, which is defined in *C. jejuni* by the production of a single flagellum at one or both bacterial poles. Whereas we feel that our results give credence to our conclusion that the GTPase activity is only required for proper flagellar biosynthesis, the possibility remains that the residual GTPase activity we observed with the FlhF<sub>D321A</sub> and FlhF<sub>R324A</sub> proteins was sufficient for expression of  $\sigma^{54}$ -dependent flagellar genes analyzed in this study. Conclusive analysis that the GTPase activity of FlhF is not required for  $\sigma^{54}$ -dependent flagellar gene expression will require the *in vivo* production of a stable FlhF mutant protein in *C. jejuni* that completely lacks GTPase activity and analysis of multiple  $\sigma^{54}$ -dependent promoters. As was shown by in this study, this approach may be difficult as we were unable to generate such a stable, GTPase-deficient FlhF mutant protein, perhaps due to a requirement of GTP interactions for the proper folding or stability of FlhF. Nonetheless, our results demonstrate that proper flagellar biosynthesis is more sensitive to decreases in GTP hydrolysis by FlhF than  $\sigma^{54}$ -dependent flagellar gene expression.

In our *in vitro* biochemical assays, we identified a requirement of residues K295, D321, and R324 of FlhF for full GTPase activity. K295 is located in the P-loop (G1 region) of the GTPase domain whereas D321 and R324 are in a conserved DXXR motif

of the G2 region that has been postulated to assist in GTP hydrolysis (10). However, these residues in any FlhF protein have not been analyzed to determine if they are required for GTPase activity until this study. Further characterization of these mutant FlhF proteins will be necessary to determine if these proteins are blocked only at the hydrolysis step or have defects in GTP-binding as well. Because evidence from structural studies with FlhF of *B. subtilis* suggest that FlhF may have an unusual mechanism for GTP hydrolysis compared to other SIMIBI family members, these mutant proteins we have created may have the potential to provide insights into the biochemical mechanism of GTP hydrolysis by FlhF (34). In eukaryotic systems, GTPases are often regulated by GTPase activating proteins (GAPs) and GTPase exchange factors (GEFs) (reviewed in (160)). Bacterial versions of these GAPs or GEFs have not been identified for the other SIMIBI members, FtsY or Ffh. Rather, heterodimer formation by FtsY and Ffh has been found to directly stimulate the GTPase activity of each individual protein (47, 148). More extensive biochemical analysis will be required to determine if FlhF has an associated GAP- or GEF-like protein or if the GTPase activity of FlhF is influenced by possible homodimer formation or heterodimer formation with another GTPase.

Because the GTPase domain of FlhF proteins is most similar to those of the bacterial FtsY and Ffh SRP GTPases, some speculation has been made that FlhF may function as a SRP protein specific for flagellar proteins that compose the FEA (18, 101, 106, 140). The bacterial SRP system is required for targeting many proteins to the general secretory (Sec) system (92, 165). These proteins predominantly include those to be inserted in the inner membrane or some proteins that are to be secreted to the periplasm. Considering our analysis of the  $\Delta flhF$  mutant, we do not favor that FlhF

functions exclusively in the targeting of flagellar proteins, such as components of the FEA, to the inner membrane that are essential for flagellar biosynthesis and flagellar gene expression. In the  $\Delta flhF$  mutant, we detected at least two FEA proteins localized to the membrane fraction. Furthermore, in a small minority of individual  $\Delta flhF$  mutant bacteria, a flagellum was detected suggesting that *C. jejuni* does not have an absolute dependence on FlhF for FEA formation and FEA-mediated flagellar protein secretion. Further indirect evidence that FlhF is likely not required to form the FEA is the observation that the  $\Delta flhF$  mutant is reduced 12- to 15-fold for expression of  $\sigma^{54}$ -dependent flagellar genes, but a mutant lacking a component of the FEA (such as a  $\Delta flhB$  mutant) generally demonstrates at least a 25- to 60-fold reduction in expression of these genes (67). Thus, if a  $\Delta flhF$  mutant is lacking one of the FEA components in the inner membrane, we would have expected to see a deficiency in expression of  $\sigma^{54}$ -dependent flagellar genes equivalent to that typically seen in an FEA mutant.

Monitoring formation of the FEA by measuring FEA-dependent secretion of flagellar proteins in the  $\Delta flhF$  mutant would be one method to study if FlhF affects formation of the FEA, but this approach would be difficult since many of the genes that encode the rod, hook, and flagellin proteins that are secreted by the FEA are part of the  $\sigma^{54}$ -dependent regulon, and by consequence, are dependent on FlhF and the FEA for expression. Future studies will focus on characterizing FEA-mediated secretion in  $flhF$  mutants ectopically expressing genes for the rod, hook, and flagellin proteins from non-native, FEA- and FlhF-independent promoters to determine if there is a functional relationship between FlhF and FEA formation.

It is possible that FlhF, and more specifically its GTPase activity, may be required for modulating the activity of the FEA. In support of this hypothesis, the *flhF<sub>D321A</sub>* and *flhF<sub>R324A</sub>* mutants were impaired for proper flagellar biosynthesis as approximately 60 – 65% of these bacteria (compared to ~ 7% of wild-type bacteria) were unable to produce flagella, produced flagella at incorrect positions on the bacterial surface, or produced multiple flagella at a single pole. Since these mutants have a higher propensity for these phenotypes than wild-type bacteria, the GTPase activity of FlhF may be required at early initiation steps with respect to the FEA in flagellar biosynthesis (Figure 10). These steps may include one or more of the following: the proper positioning of the FEA at a pole, ensuring that only one FEA is formed at a pole, monitoring the FEA so that it properly secretes flagellar proteins in the correct order to build a flagellum, or assisting the FEA to more efficiently secrete flagellar proteins. Some evidence for the first hypothesis exists in *V. cholerae* as FlhF appears to assist in properly localizing at least one FEA component, the FliF MS ring, to the old pole in a bacterium (56). If FlhF of *C. jejuni* functions in an analogous process, it will be interesting to study this localization process as *C. jejuni* usually constructs a flagellum at both the old and new pole. Furthermore, it would be interesting to study the localization of the FEA components in the *flhF<sub>D321A</sub>* and *flhF<sub>R324A</sub>* mutants which presumably mislocalize the FEA to produce non-polar flagella or allow for multiple polar FEA formation to contribute to more than one flagellum at a single pole. All of these studies depend on visualization of proteins in *C. jejuni* using fluorescent microscopy with labeled proteins which has been difficult in some strains of *C. jejuni* including the strain used in this study, *C. jejuni* strain 81-176 ((124) and our unpublished observations). To study the

other hypotheses, development of new and better reagents to characterize FEA formation and to monitor flagellar protein secretion in *flhF* mutants is required to provide any insights into how FlhF may influence the activity of the FEA.

We found a more severe defect in flagellar gene regulation and biosynthesis with a mutant lacking *flhF* compared to the GTP-hydrolysis hindered *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> mutants. The severity of the biosynthesis defect in the  $\Delta$ *flhF* mutant is most likely related to the fact that expression of at least three  $\sigma^{54}$ -dependent flagellar genes (and probably all other  $\sigma^{54}$ -dependent flagellar genes) were reduced at least 5- to 15-fold. The decreased expression of multiple genes would greatly reduce the levels of the encoded proteins essential for construction of flagella. Since the *flhF*<sub>D321A</sub> and *flhF*<sub>R324A</sub> were not significantly defective, if at all, for expression of  $\sigma^{54}$ -dependent flagellar genes, these results indicate that other domains or activities of FlhF independent of GTP hydrolysis are required for processes leading to full level of expression of  $\sigma^{54}$ -dependent flagellar genes. These activities may be related to the B or N domains. To determine if the B and N domains of FlhF are specifically required for expression of  $\sigma^{54}$ -dependent flagellar genes, we attempted to create an *flhF* mutant of *C. jejuni* lacking these domains, but this mutant protein was unstable in the bacterium. We continued our analysis of FlhF and revealed that the protein is not required for formation of the FEA or the FlgSR two-component system. Furthermore, we provided data that suggest that the FlgSR system does not function downstream of FlhF for activation of  $\sigma^{54}$  and that the lack of *flhF* and the FEA contribute to greater defects for expression of  $\sigma^{54}$ -dependent flagellar genes than either mutation alone. Thus, our results indicate that FlhF may function in a separate

pathway that converges with or acts downstream of the FEA-FlgSR pathway to stimulate  $\sigma^{54}$  (Figure 13). Future studies will determine if FlhF is required at the step of FlgR-dependent activation of  $\sigma^{54}$  or at a more downstream step such as  $\sigma^{54}$ -RNA polymerase initiation or stability of  $\sigma^{54}$ -dependent mRNAs.

With continued investigation into the FlhF proteins of polarly-flagellated bacteria, it is evident that these proteins are an essential component of regulatory systems for expression of flagellar genes, flagellar biosynthesis, or both. For instance, *flhF* mutants of *P. aeruginosa* are only slightly affected for expression of the major flagellin in *P. aeruginosa*, but this defect is not detrimental to flagellar biosynthesis (132). Instead, FlhF is more specifically required for the polar placement of flagella. Furthermore, expression of *flhF* is dependent on  $\sigma^{54}$ , rather than being required for expression of the  $\sigma^{54}$  flagellar regulon in *P. aeruginosa* (36). *flhF* is also dependent on  $\sigma^{54}$  for expression in *V. cholerae*, but the FlhF protein is required for expression of other  $\sigma^{54}$ -dependent flagellar genes, and consequently, flagellar biosynthesis (32). In *C. jejuni* and *H. pylori*, no evidence exists to indicate that *flhF* expression is  $\sigma^{54}$ -dependent and, therefore, expression may be constitutive or regulated by a yet unknown factor. However, FlhF is required for wild-type levels expression of the  $\sigma^{54}$ -dependent flagellar rod and hook genes in *H. pylori* and *C. jejuni* and flagellar biosynthesis (136). Therefore, polarly-flagellated bacteria have acquired *flhF* and adapted the encoded protein to be involved at different specific steps in flagellar gene regulation and biosynthesis that behoove the individual species. Thus, exploration of the role of FlhF in diverse bacterial species will be required to fully understand the biochemical properties of FlhF and how

these activities function to ensure proper flagellar biosynthesis. Our study has provided a foundation for the molecular characterization of FlhF and how biological properties of FlhF are linked to distinct steps in flagellar gene regulation and biosynthesis.

## CHAPTER FIVE

### FLAGELLAR BASE STRUCTURE IS REQUIRED FOR DIVISION SITE DETERMINATION THROUGH THE FLAGELLAR NUMBER REGULATOR

#### FlhG

##### Introduction

Symmetrical division in bacteria requires spatially regulating the divisome to form specifically at the cellular midpoint. In *Escherichia coli* and *Bacillus subtilis*, the Min system prevents polar division by inhibiting formation of the divisome at poles. The cellular midpoint is kept relatively free of MinCD by topological specificity factors like MinE or DivIVA so that the Z-ring forms at the middle to promote symmetrical division. In *B. subtilis*, the presence of MinCD at the septum is thought to prevent a second division event from occurring at the poles in the newly generated daughter cells immediately after division, while in *E. coli*, oscillating MinCD protects both poles from division. (57).

The MipZ ATPase, a MinD ortholog, spatially regulates Z-ring formation in *Caulobacter crescentus* (169). Unlike MinD, MipZ itself is able to dissociate FtsZ polymers and inhibit Z-ring formation. In *C. crescentus*, MipZ associates with a replicated chromosome as it is segregated to a pole during division. MipZ depolymerizes polar FtsZ polymers present from the last round of division, causing reorganization of FtsZ and formation of the Z-ring near the midpoint. Division in *C. crescentus* is

asymmetrical so that two differentiated daughter cells of different sizes are formed, a larger stalked cell and a smaller swarmer cell.

Unlike MinD or MipZ, which are involved in spatial regulation of the division, another ParA superfamily member FlhG (also called FleN) is involved in numerical regulation of flagellar biosynthesis in polarly-flagellated bacteria (32, 34). Unlike peritrichous organisms, flagellar biosynthesis in polar flagellates is spatially and numerically restricted so that a very limited number of flagella are produced only at one or both poles. In the monotrichous bacterial species, *Vibrio cholerae* and *Pseudomonas aeruginosa*, FlhG/FleN proteins are required to limit flagellar biosynthesis to a single organelle, which forms only at one of the two poles; mutants lacking these proteins produce multiple flagella, but primarily only at one pole (32, 34).

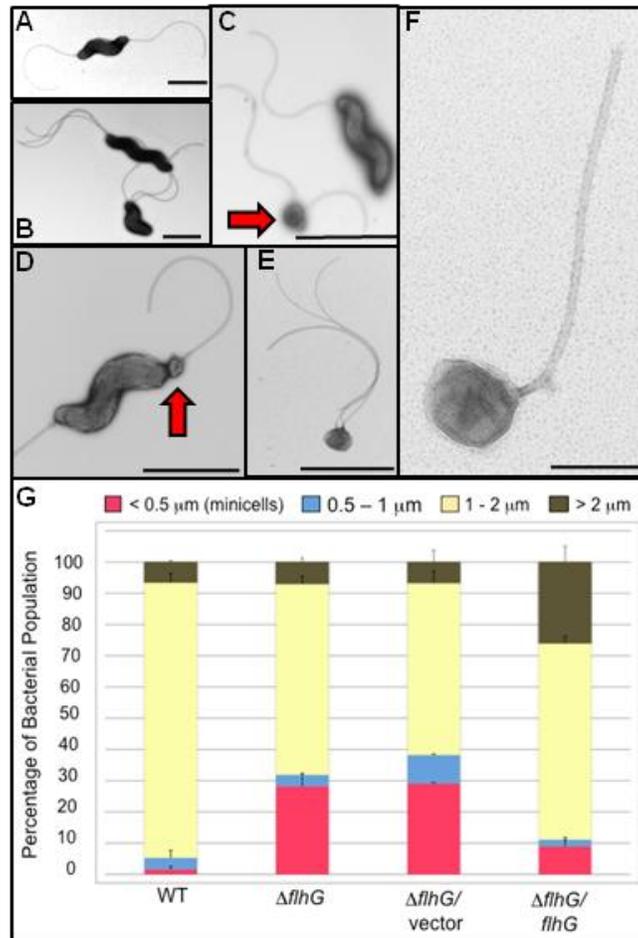
In this chapter, I present work identifying an unprecedented system to spatially regulate symmetrical division that involves FlhG and components of amphitrichous (bipolar) flagella. In addition to limiting poles to a single flagellum, we identified a second function for FlhG in inhibiting polar division in *Campylobacter jejuni*. FlhG activity in division site placement is dependent on polar flagellar biosynthesis, and more specifically, components of the flagellar MS and C rings, which have established motor, switch, and secretory functions for the flagellar organelle. I propose a model whereby the flagellar MS ring and switch complex serve as topological specificity factors for FlhG to inhibit division only at the poles. Furthermore, the results demonstrate that amphitrichous flagellation is not only required for motility in *C. jejuni*, but also to inhibit polar division and promote symmetrical division to generate viable daughter cells. The study presented in this chapter also reveals that FlhG orthologs of other polarly-

flagellated bacteria have the ability to influence placement of division sites, suggesting that polar flagellar biosynthesis may influence division in a broad range of motile bacteria that produce FlhG.

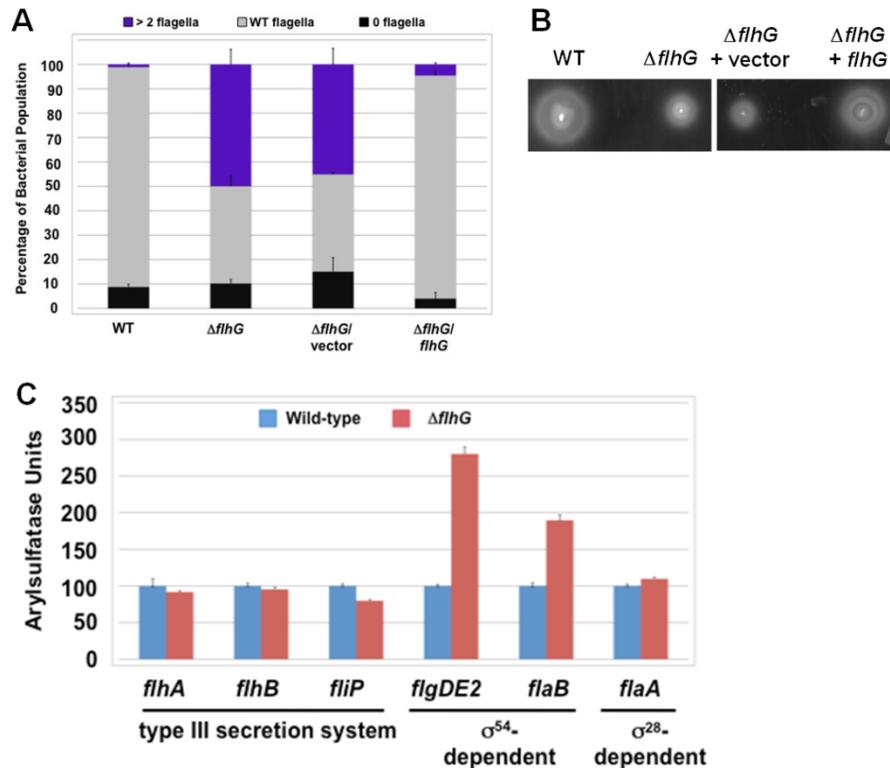
## Results

### *FlhG is Involved in Numerical Control of Flagellar Biosynthesis and Division Site Determination*

Completed genomes of all *Campylobacter* species encode the putative FlhG ATPase, but not MinD, another ParA ATPase superfamily member. Although Min systems have not been analyzed in any polarly-flagellated bacteria, these organisms appear to encode MinD and other Min components presumably to function in division site determination, and FlhG, which numerically regulates flagellar biosynthesis in the monotrichous species, *V. cholerae* and *P. aeruginosa* (32, 34). Therefore, we analyzed *C. jejuni* 81-176 with an in-frame deletion in *flhG* to ascertain a role for FlhG in the biology of the bacterium.



**Figure 14. The polar flagellar number and minicell phenotypes of the *C. jejuni*  $\Delta flhG$  mutant.** (A-F) Electron micrographs of negatively stained (A) WT *C. jejuni* and (B-E)  $\Delta flhG$  mutant strains. Bars = 1  $\mu\text{m}$  (A and E), 2  $\mu\text{m}$  (B-D), and 0.2  $\mu\text{m}$  (F). Red arrows indicated minicells next to normal size bacteria (C) or forming a pole of a bacterium (D). (G) Quantification of lengths of cell bodies of WT *C. jejuni* and  $\Delta flhG$  mutant populations. The long axes of the cell bodies of bacteria were measured. The *C. jejuni*  $\Delta flhG$  mutant was complemented with vector alone, or vector expressing WT *flhG*. Bacteria were classified by the following cell lengths: < 0.5  $\mu\text{m}$ , minicells (red); 0.5 – 1  $\mu\text{m}$  (blue); 1 – 2  $\mu\text{m}$  (yellow); and > 2  $\mu\text{m}$  (brown). The data represent the average of two experiments. Bars represent standard errors.



**Figure 15. Effect on *flhG* mutation on polar flagellar numbers, motility and flagellar gene expression.** (A) Quantification of flagellar numbers of WT *C. jejuni* and  $\Delta flhG$  mutant populations. Individual bacteria were analyzed for the number of flagella produced at each pole. *C. jejuni*  $\Delta flhG$  was complemented with vector alone, or vector expressing WT *flhG*. The data are reported as the percentage of the bacterial population with the following flagellar numerical patterns: >2 flagella, producing two or more flagella at least at one pole (purple); WT flagella, producing a single flagellum at one or both poles (grey); and 0 flagella, aflagellated bacteria (black). Data represent the average of two experiments. Bars represent standard errors. (B) Motility phenotype of WT *C. jejuni* and  $\Delta flhG$  mutant strains in semi-solid agar. Cultures of similar densities were stabbed into motility agar and incubated in microaerobic conditions at 37 °C for 24 h. The *C. jejuni*  $\Delta flhG$  mutant was complemented with empty vector or plasmid expressing *flhG*. (C) Arylsulfatase assays measuring the level of flagellar gene expression in WT *C. jejuni* and  $\Delta flhG$  mutant strains. Transcriptional fusions of flagellar genes linked to a promoterless *astA* gene were used to replace respective WT alleles in *C. jejuni*  $\Delta astA$  (WT *C. jejuni*; blue bars) or *C. jejuni*  $\Delta astA$   $\Delta flhG$  (red bars). Results are from a typical assay with each strain performed in triplicate. Values reported for each strain are average arylsulfatase activity  $\pm$  standard deviation relative to the amount of expression of each transcriptional fusion in WT *C. jejuni*  $\Delta astA$ . Genes analyzed include those for the flagellar type III secretion system (early class of flagellar genes),  $\sigma^{54}$ -dependent middle class of flagellar genes, and  $\sigma^{28}$ -dependent late class of flagella genes.

We first observed that FlhG is required for numerical control of amphitrichous flagellation by examining flagellar biosynthesis of populations of wild-type (WT) *C. jejuni* and  $\Delta flhG$  mutant strains. Over 90% of individual WT cells produced a single flagellum at one or both poles (62% were amphitrichous, 29% were monotrichous), which together were classified as the normal flagellar number phenotype (Fig. 14A and Fig. 15A). Only about 1% of WT *C. jejuni* cells produced more than one flagellum at least at one pole. In contrast, about 40% of *C. jejuni*  $\Delta flhG$  cells produced extra flagella at least at one pole, with a correlative decrease in the population producing WT flagellar numbers (Fig. 14B and Fig. 15A). As a population, the  $\Delta flhG$  mutant was less motile than WT *C. jejuni* (Fig. 15B). Both motility and WT flagellar numbers were restored to the  $\Delta flhG$  mutant by expressing *flhG* *in trans* (Fig. 15A and B).

Unlike *V. cholerae* or *P. aeruginosa* *flhG/fleN* mutants in which increased expression of almost all classes of flagellar genes contributed to extra polar flagellar (32, 34, 35), *C. jejuni*  $\Delta flhG$  did not express increased levels of all flagellar gene classes. Instead, a fairly insignificant increase in expression of less than 2.5-fold was only observed for  $\sigma^{54}$ -dependent flagellar genes (encoding primarily rod and hook proteins), but not for other classes of flagellar genes, such as the early class encoding the flagellar type III secretion system (T3SS) or the late  $\sigma^{28}$ -dependent *flaA* gene encoding the major flagellin (Fig. 15C). These results suggest that FlhG is involved in numerical control of amphitrichous flagellation by a process different from monotrichous bacteria.

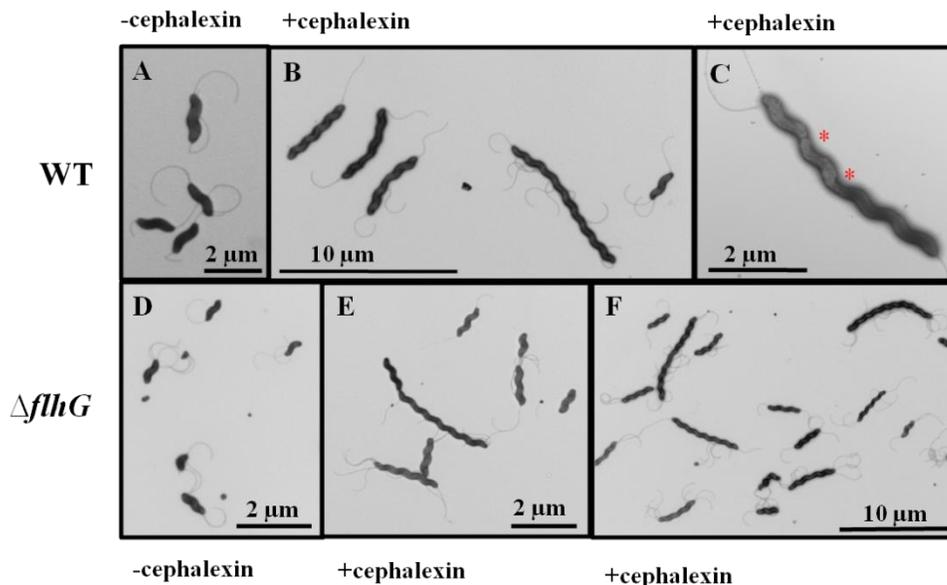
Upon closer examination of the *C. jejuni*  $\Delta flhG$  mutant by electron microscopy, we observed minicells along with bacteria of normal cell lengths (Fig. 14C-F). These

minicells were normally 0.2 - 0.5  $\mu\text{m}$  in diameter and originated from the poles of  $\Delta\text{flhG}$  cells (Fig. 14D). Many minicells were flagellated, and some were multiply flagellated due to the  $\Delta\text{flhG}$  background (Fig. 14C, E, and F). These findings indicate that *C. jejuni* minicells are most likely generated due to division occurring at poles, similar to observations in *E. coli* and *B. subtilis minD* mutants (39, 173).

The minicell phenotype of the *C. jejuni*  $\Delta\text{flhG}$  mutant was pronounced in comparison to WT bacteria. By analyzing the lengths of the long axis of bacterial cells, approximately 88% of WT *C. jejuni* were 1-2  $\mu\text{m}$  in length (Fig. 14G). Only 4% of WT *C. jejuni* were minicells, which we classified as bacterial-derived, spherical particles under 0.5  $\mu\text{m}$  in diameter (Fig. 14G). In contrast, minicells composed 28% of the *C. jejuni*  $\Delta\text{flhG}$  population. Complementation of the  $\Delta\text{flhG}$  mutant *in trans* with WT *flhG* greatly reduced the minicell population to less than 9%, demonstrating that the minicell phenotype of the  $\Delta\text{flhG}$  mutant was due to loss of *flhG*. We also noticed upon complementation that the elongated cell population ( $> 2 \mu\text{m}$ ) increased to 26%, an approximately four-fold increase relative to WT *C. jejuni* (Fig. 14G). This elongated cell phenotype is reminiscent of *E. coli* or *B. subtilis* strains when *minD* is overexpressed (39, 116), further suggesting that FlhG has a functional role similar to MinD homologs in influencing division.

We verified that the minicell phenotype of *C. jejuni*  $\Delta\text{flhG}$  was a result of the process of division. Cephalixin is a late-stage division inhibitor that inactivates FtsI, which produces peptidoglycan at a septum during the final stages of division to separate two daughter cells (15, 164). Treatment of WT *C. jejuni* or the  $\Delta\text{flhG}$  mutant with

cephalexin resulted in cell elongation and growth of *C. jejuni* in chains with septa separating individual cells (Fig. 16A-C). Upon treatment of the  $\Delta flhG$  mutant with cephalexin, we also observed elongation of cells with an additional reduction of the minicell population by half (Fig. 16D-F and Table 3), indicating that minicells were formed in a process that requires division.



**Figure 16. Effect of cephalexin on division in *C. jejuni* wild-type and  $\Delta flhG$  mutant strains.** Wild-type *C. jejuni* or *C. jejuni*  $\Delta flhG$  mutant were grown in liquid culture and then either left untreated (A and D) or exposed to 15  $\mu\text{g}/\text{ml}$  or 12.5  $\mu\text{g}/\text{ml}$  of cephalexin, respectively, for 6 hours (B, C, E, and F). The *C. jejuni*  $\Delta flhG$  mutant demonstrated an increase in sensitivity to cephalexin, which required exposure to a lower concentration of the antibiotic for analysis. Strains were then negatively stained and analyzed by electron microscopy. Observed septa are noted by asterisks in C.

Depending on the type of mutation, mutation of the ATPase domain of *E. coli* or *B. subtilis* MinD or *C. crescentus* MipZ results in minicell production (due to inability to inhibit polar division) or cell elongation (due to increased inhibition of division) (73, 91, 169, 184, 191). We examined a role for the putative ATPase domain of FlhG in

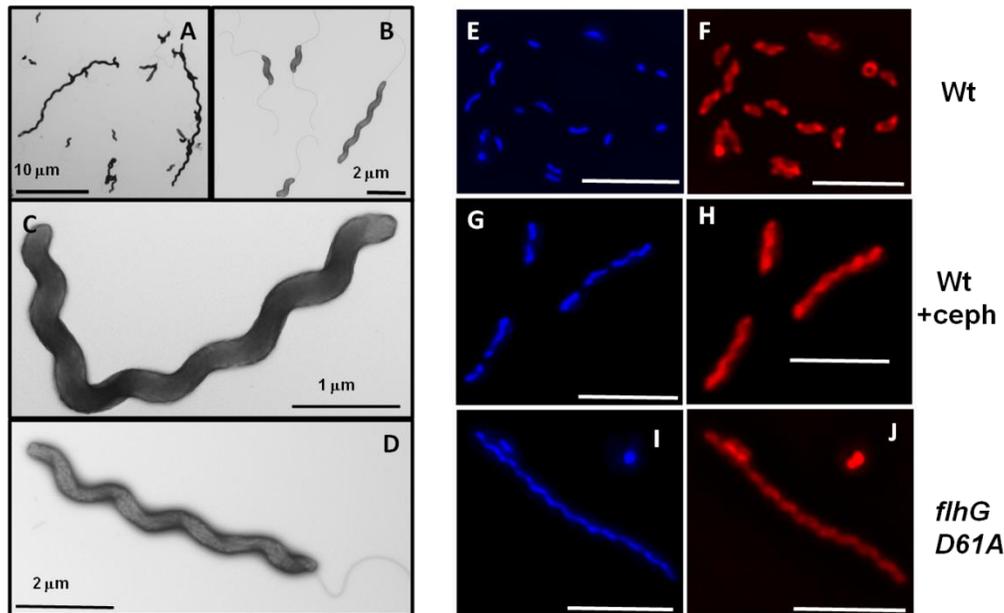
influencing division by mutating a conserved aspartic acid residue that is predicted in MinD and other ParA ATPase family members to be required for ATP hydrolysis (62, 107). To perform these experiments, we replaced chromosomal WT *flhG* with the *flhG<sub>D61A</sub>* allele, which we predicted would encode an FlhG mutant protein locked into an ATP-bound state.

Strain	Treatment	Cell Length			
		< 0.5 $\mu\text{m}$ (minicells)	0.5–1.0 $\mu\text{m}$	1 – 2 $\mu\text{m}$	> 2 $\mu\text{m}$
Wild-type	None	1.5 $\pm$ 0.2	1.9 $\pm$ 1.3	92.4 $\pm$ 2.7	4.3 $\pm$ 1.5
$\Delta$ <i>flhG</i>	None	33.0 $\pm$ 4.3	4.5 $\pm$ 0.2	57.8 $\pm$ 2.8	4.7 $\pm$ 1.4
$\Delta$ <i>flhG</i>	12.5 $\mu\text{g/ml}$ cephalexin	19.2 $\pm$ 2.7	1.9 $\pm$ 0.4	27.8 $\pm$ 6.5	51.1 $\pm$ 3.4
<i>flhG<sub>D61A</sub></i>	None	1.0 $\pm$ 0.34	1.2 $\pm$ 0.2	73.9 $\pm$ 5.8	23.9 $\pm$ 5.5
<i>flhG<sub>D61A</sub></i>	<i>ftsZ</i> overexpression	2.3 $\pm$ 0.2	2.8 $\pm$ 0.9	85.9 $\pm$ 1.4	9.0 $\pm$ 0.8

**Table 3. Minicell production and elongated cell phenotype of the *C. jejuni flhG* mutants upon exposure with cephalexin or *ftsZ* overexpression.** The long axes of the cell bodies of individual bacteria were measured. The population was divided based on proportions with the following cell lengths: minicells, bacteria under 0.5  $\mu\text{m}$ ; 0.5 – 1  $\mu\text{m}$ ; 1 – 2  $\mu\text{m}$ ; and elongated cells, > 2  $\mu\text{m}$ . Values are reported as percentages of the population  $\pm$  standard error. Strains were either exposed to cephalexin for 6 hours or contained a plasmid to overexpress *ftsZ* *in trans* as indicated in the second column. Strains were then fixed and the population of bacterial cells were analyzed for cell lengths.

A greatly elongated cell body phenotype occurring amongst cells of normal length was easily observed in the *flhG<sub>D61A</sub>* mutant (Fig. 17A and B). Approximately 24% of *C. jejuni flhG<sub>D61A</sub>* cells were over 2  $\mu\text{m}$  in length and some were over 10  $\mu\text{m}$ , unlike WT *C. jejuni* in which elongated cells composed only 4% of the population and ranged

from 2 – 3  $\mu\text{m}$  (Table 3, Fig. 17A-D and data not shown). Of note, many *flhG*<sub>D61A</sub> cells appeared to produce WT flagella, with a single flagellum at the poles.



**Figure 17. Elongation of *C. jejuni* cell bodies producing FlhG<sub>D61A</sub>.** (A-D) Electron micrographs of negatively stained *C. jejuni flhG*<sub>D61A</sub>. Bars = 10  $\mu\text{m}$  (A), 2  $\mu\text{m}$  (B and D), and 1  $\mu\text{m}$  (C). (E-J) Fluorescent micrographs of wild-type *C. jejuni*, wild-type *C. jejuni* treated with cephalexin, or *C. jejuni flhG*<sub>D61A</sub>. Bacteria were stained with DAPI (E, G, and I) to stain DNA or FM464 (F, H, and J) to stain the outer membranes. After growth in the liquid culture, wild-type *C. jejuni* was left untreated (E and F) or treated for 6 hours with 15  $\mu\text{g}/\text{ml}$  of cephalexin (G and H). *C. jejuni flhG*<sub>D61A</sub> was grown in the absence of cephalexin (I and J). Bars = 5  $\mu\text{m}$ .

As observed by electron microscopy, many of the *C. jejuni flhG*<sub>D61A</sub> mutant cell bodies appeared to lack septa (Fig 17C and D), suggesting that the structures were predominantly formed by inhibiting an early step in division to result in elongated cells, rather than formed by inhibiting a late step in division to result in a chain of individual, septated bacteria. To further analyze the *C. jejuni flhG*<sub>D61A</sub> cell structures, the cell bodies of WT *C. jejuni*, cephalexin-treated WT *C. jejuni*, and *C. jejuni flhG*<sub>D61A</sub> were compared

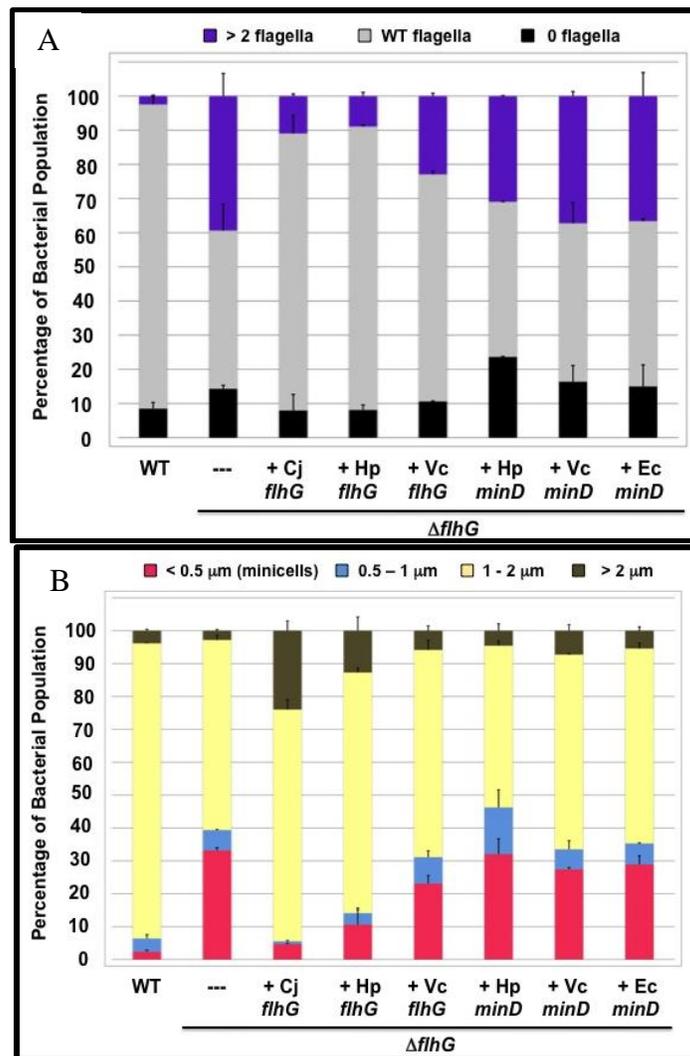
by fluorescent microscopy after staining with DAPI (for DNA) and FM4-64 (for outer membranes). WT *C. jejuni* stained as individual bacteria with DNA throughout the cell (Fig. 17E and F). In contrast, the DNA staining in cephalixin-treated WT *C. jejuni* was segmented, indicating septa were present between regions of stained DNA (Fig. 17G and H), which is consistent with a block in division at a late step mediated by FtsI. In contrast, the DNA staining of *C. jejuni flhG<sub>D61A</sub>* cells was not segmented, suggesting the absence of septa and confirming that *flhG<sub>D61A</sub>* conferred inhibition at an early step of division to result in cell elongation (Fig. 17I and J).

The elongated cell phenotype of *C. jejuni flhG<sub>D61A</sub>* suggests enhanced inhibition of Z-ring formation, which normally initiates division. To determine if FtsZ is a target of inhibition by a mechanism mediated by FlhG, we overexpressed *ftsZ* *in trans* in *C. jejuni flhG<sub>D61A</sub>* and noticed that the elongated cell phenotype was reduced from 24% to 9% (Table 3). These results suggest that FtsZ polymerization into the Z-ring is a target of inhibition for FlhG. We conclude that FlhG is involved in mechanisms to numerically control flagellar biosynthesis and spatially control formation of division sites. We propose that FlhG compensates for the lack of one or more Min components to spatially regulate Z-ring formation and division.

#### *Heterologous FlhG Proteins Function in Division Site Determination in C. jejuni*

We considered if MinD orthologs, either FlhG or MinD proteins from other polarly-flagellated bacteria or *E. coli* MinD, could functionally complement *C. jejuni*  $\Delta$ *flhG* for numerical control of flagellar biosynthesis or division site determination. For these experiments, *C. jejuni*  $\Delta$ *flhG* was complemented *in trans* with a plasmid expressing

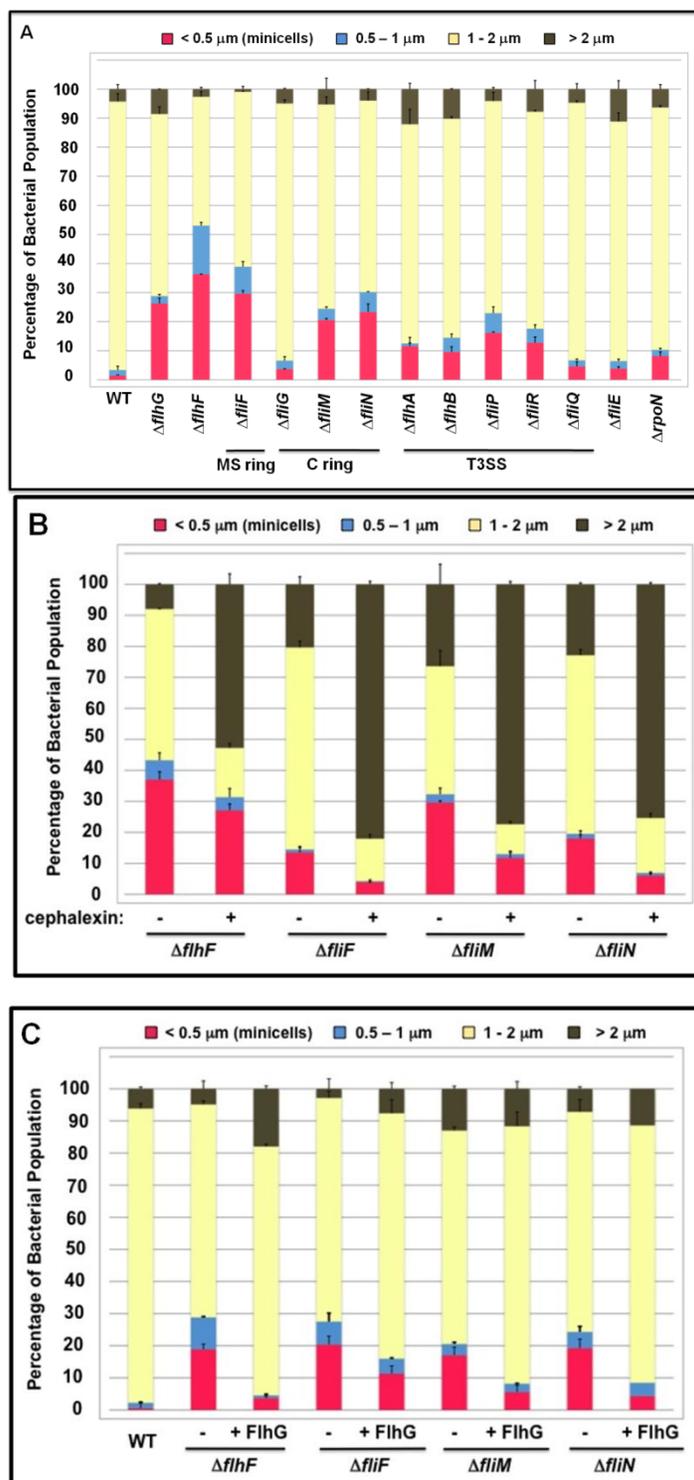
heterologous *flhG* or *minD* genes from the *flaA* promoter, a strong promoter for the major flagellin in *C. jejuni*. We found that *H. pylori* FlhG was just as competent as *C. jejuni* FlhG in reducing extra polar flagella and restoring WT flagellar numbers to *C. jejuni*  $\Delta flhG$  (Fig. 18A). Furthermore, *H. pylori* FlhG dramatically reduced the minicell population in the  $\Delta flhG$  mutant (Fig. 18B). In addition, *V. cholerae* FlhG partially restored both WT flagellar numbers and normal division to *C. jejuni*  $\Delta flhG$  (Fig. 18A and B). In contrast, all MinD proteins failed to complement *C. jejuni*  $\Delta flhG$  for either phenotype (Fig 18A and B). These results indicate that *H. pylori* FlhG, and to a lesser extent *V. cholerae* FlhG, can function in spatial regulation of division site formation and inhibition of polar division. Secondly, these findings suggest that *C. jejuni* has evolved to preferentially use FlhG to determine placement of division sites and numerically control flagellar biosynthesis.



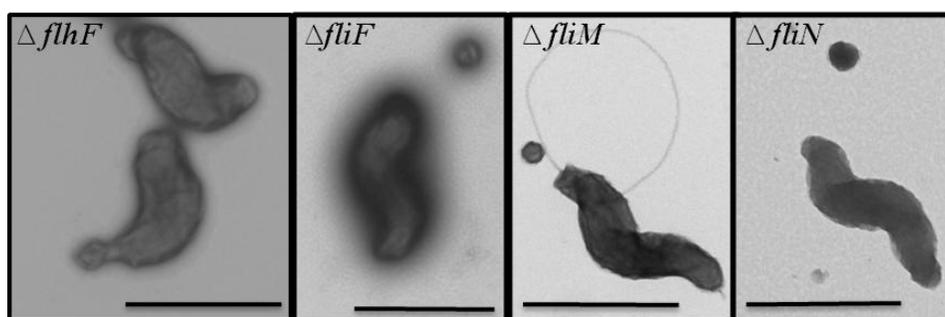
**Figure 18. Complementation of *C. jejuni*  $\Delta flhG$  in trans with heterologous *flhG* or *minD* alleles.** (A and B) The *C. jejuni*  $\Delta flhG$  mutant was complemented *in trans* with vector alone (-), or *flhG* or *minD* from *C. jejuni* (Cj), *H. pylori* (Hp), *V. cholerae* (Vc), or *E. coli* (Ec). (A) Quantification of flagellar numbers of *C. jejuni*  $\Delta flhG$  mutants upon complementation. Individual bacteria were analyzed for the number of flagella produced at each pole. Bacteria were classified as described in Figure 15 A. (B) Quantification of lengths of the cell bodies of *C. jejuni*  $\Delta flhG$  mutants upon complementation. The long axes of the cell bodies of individual bacteria were measured. Bacteria were classified as described in Figure 14 G.

*FlhF and the Flagellar MS Ring and Switch Complex are Required for FlhG to Inhibit Polar Division*

The FlhF GTPase of *C. jejuni* and other polar flagellates has been implicated as a regulatory factor required to express flagellar genes and spatially control polar flagellar biosynthesis (32, 56, 102, 132, 140). Previous studies have provided evidence that the FlhF and FlhG proteins of *C. jejuni* and *Vibrio alginolyticus* potentially interact to mediate their functions in spatial and numerical control of flagellar biosynthesis (102, 142). A current hypothesis for a role of *C. jejuni* FlhF in polar flagellar placement suggest that the GTPase activity of FlhF may influence its positioning to the new pole after division. Of note, polarly localization of FlhF has been observed in *C. jejuni* (43). After polar localization, FlhF may promote organization of the initial flagellar components, such as the motor, switch, and secretory components at the pole (9).



**Figure 19. Characterization of the minicell phenotype of *C. jejuni* flagellar mutants.** (A) Quantification of the lengths of the cell bodies of wild-type *C. jejuni* and mutants lacking a motility gene. (B) Quantification of the lengths of the cell bodies of mutants lacking FlhF, MS ring (FliF), or C ring components (FliM or FliN) after treatment with cephalixin. After growth in liquid broth, strains were incubated in the absence (-) or presence (+) of 15  $\mu\text{g/ml}$  cephalixin for 6 hours. The lengths of the cell bodies were then measured. (C) Quantification of the lengths of the cell bodies of mutants lacking FlhF, MS ring (FliF), and C ring components (FliM and FliN) upon overexpression of *flhG* *in trans*. For (A-C), the long axes of the cell bodies of individual bacteria were measured. Bacteria were classified as described in Figure 14.



**Figure 20. Minicell production in *flhF*, *fliF*, *fliM*, and *fliN* mutants.** Electron micrographs of negative stained *C. jejuni* mutant bacteria and associated minicells. Bars = 1  $\mu\text{m}$

Considering the potential interactions between FlhF and FlhG that may influence the activities of each protein, we examined a *C. jejuni*  $\Delta flhF$  mutant and observed a minicell population that was at least as prevalent as that of *C. jejuni*  $\Delta flhG$  (Fig. 19A and Fig. 20). In addition, the minicell phenotype of the  $\Delta flhF$  mutant was resolved by overexpression of *flhG* *in trans* (Fig. 19B), indicating that minicell production in the mutant is due to inefficient FlhG activity.

We considered two hypotheses for how FlhF may be involved in an FlhG-dependent mechanism to inhibit polar division. First, we considered if either  $\sigma^{54}$ -

dependent flagellar gene expression or flagellar rod biosynthesis, which are both dependent on FlhF (9, 67), are required for FlhG to function in a mechanism to inhibit polar division. If lack of  $\sigma^{54}$ -dependent flagellar gene expression or rod biosynthesis caused the minicell phenotype in the  $\Delta flhF$  mutant, minicell production in a  $\Delta rpoN$  mutant (encoding  $\sigma^{54}$ ) or a  $\Delta fliE$  mutant (encoding a rod protein) would be expected. However, neither mutant demonstrated a significant minicell phenotype compared to the  $\Delta flhG$  or  $\Delta flhF$  mutants (Fig. 19A).

For the second hypothesis, we considered if FlhG may require initial flagellar components, which are likely dependent on FlhF for polar formation, to function in a mechanism to inhibit polar Z-ring formation. The first components of a flagellum that are constructed include: FliF (which forms the inner membrane MS ring); FliG, FliM, and FliN (the motor/switch components of the cytoplasmic C ring); and the flagellar T3SS (which is located within the MS ring) (113). Together, these components mediate motor, switch, and secretory functions for the flagellum. Of note, the MS ring of *V. cholerae* appears to be dependent on FlhF for polar localization (56).

We analyzed a panel of *C. jejuni* mutants lacking these flagellar components for a defect in division that results in minicells. Inactivation of *fliF*, *fliM*, and *fliN* resulted in strong minicell phenotypes comparable to *C. jejuni*  $\Delta flhF$  and  $\Delta flhG$  mutants (Fig. 19A and C). In contrast, a *fliG* mutant or mutants lacking a single component of the flagellar T3SS either did not produce minicells above wild-type levels or only showed a mild minicell phenotype (Fig. 19A). These findings suggest that the MS ring and switch complex (made up of FliM and FliN) are division site determination factors in *C. jejuni*.

To verify that minicells are products of division in the *flhF*, *fliF*, *fliM*, and *fliN* mutants, minicell production was monitored in the mutants after exposure to cephalixin. In each case, minicell production was reduced in cephalixin-treated cells (Fig. 19B). In the case of the *fliF*, *fliM*, and *fliN* mutants, the minicell population was reduced over 50%. We next determined if the minicell phenotypes of the *fliF*, *fliM*, and *fliN* mutants are linked to FlhG, similar to what we observed with *C. jejuni*  $\Delta$ *flhF*. Minicells were reduced in each mutant upon overexpression of *flhG* *in trans* (Fig. 19C), indicating that these mutants presumably have a defect in FlhG activity. These results indicate that in addition to performing motor, switch, and secretory functions for the flagellum, the MS and switch complex influence activities of FlhG necessary to efficiently inhibit polar division and promote correct symmetrical division. Therefore, polar flagellar biosynthesis influences formation of division sites via FlhG.

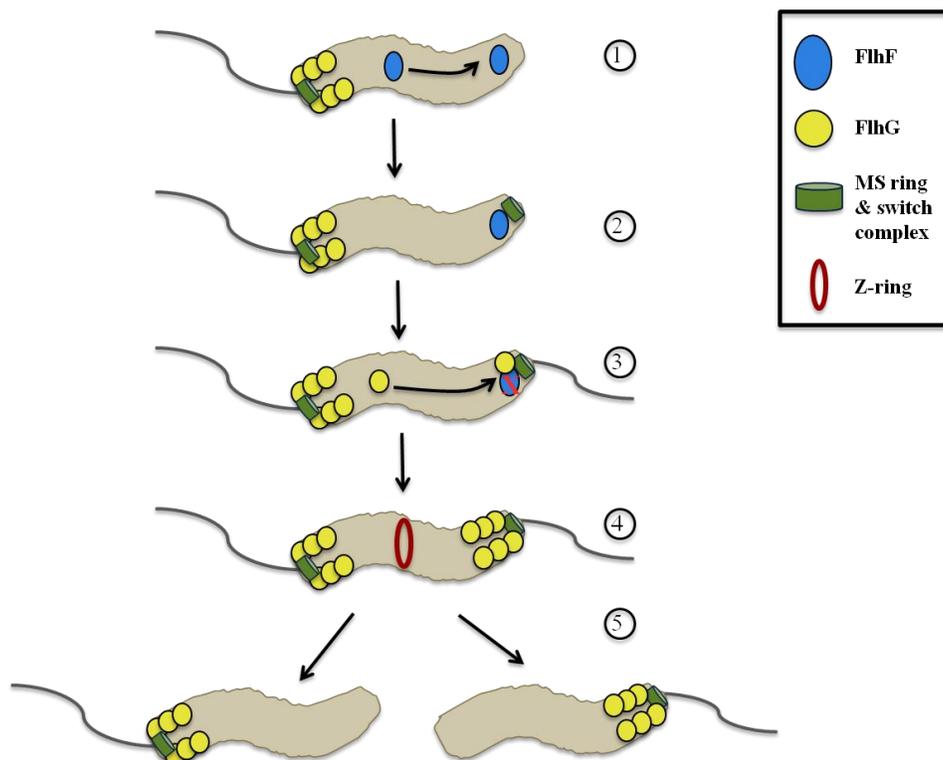
## **Discussion**

In this chapter, I describe a new paradigm for spatially regulating formation of division sites in bacteria. This novel division site determination system is composed of the ParA ATPase family member FlhG and the MS ring and switch complex of polar flagella. In addition to its predicted role in numerical control of polar flagellar biosynthesis, we found that FlhG functions in a mechanism to inhibit polar division, similar to the roles of other ParA family members such as MinD and MipZ. FlhG requires the flagellar MS ring and switch complex to inhibit polar division, which is an unprecedented role for these well established flagellar components. Without these components, FlhG is inefficient in inhibiting polar division, resulting in augmented

production of non-viable minicells. Therefore, synthesis of polar flagella influences formation of division sites at the midpoint of *C. jejuni* so that symmetrical division occurs to produce viable offspring.

Amphitrichous flagellation confers a characteristic darting motility for *C. jejuni* that assists in colonization of intestinal mucosal sites in hosts (159, 168). Our results suggest a previously unrecognized basic biological advantage for amphitrichous flagellation. By having a flagellum-dependent division site determination system, amphitrichous flagellar biosynthesis assists FlhG in inhibiting division at both poles. A strictly monotrichous flagellar pattern in *C. jejuni* may only inhibit division at one pole, whereas peritrichous flagella may inhibit division throughout the cell. As such, amphitrichous flagella efficiently inhibit polar division and encourage symmetrical division to generate the highest number of viable *C. jejuni* daughter cells.

Combining results from previous work with these new findings, a model can be proposed for how biosynthesis of amphitrichous flagella and FlhG influence division in *C. jejuni* (Fig. 21). Our previous findings suggested that the GTPase activity of FlhF is likely required at an early step in flagellar biosynthesis to place flagellar components at a pole (9). Formation of the initial components of a polar flagellum, which include the MS ring and switch complex, by FlhF would provide the necessary structures for FlhG to inhibit polar Z-ring formation and division.



**Figure 21. Proposed model for FlhG-mediated regulation of amphitrichous flagellar biosynthesis and division site formation.** Immediately after division, a daughter cell lacks a flagellum at a new pole. FlhF migrates to the new pole in a GTPase-dependent manner (Step 1). FlhF is likely involved in an early step in flagellar biosynthesis to localize or organize components of the flagellar base, including the MS ring, C ring (including the switch complex), and flagellar T3SS (Step 2). FlhG may migrate to the pole after formation of the MS and switch complex and inhibit FlhF from performing a second round of synthesis of a flagellar base at the pole. Flagellar biosynthesis is completed by construction of the remainder of the flagellum (Step 3). The MS and switch complex may serve as a topological specificity factor for FlhG so that FlhG only inhibits Z-ring formation at the pole. As a consequence, the Z-ring may form at the cellular midpoint due to low FlhG concentrations or activity (Step 4). Symmetrical division results in two daughter cells of normal lengths that lack a flagellum at the new poles (Step 5).

FlhG may be dependent on FlhF or the MS ring and switch complex to localize to the new pole and/or induce an activity of FlhG to inhibit Z-ring formation specifically at the pole. Despite intensive efforts to monitor FlhG localization, we were unable to detect

sufficient fluorescence of a FlhG-GFP fusion protein in *C. jejuni* strains to assess cellular localization (unpublished observations). Furthermore, FlhG has not been sufficiently immunogenic in multiple mammalian species to generate antisera for use in immunofluorescent microscopy procedures to study its localization.

The proteins required for FlhG to inhibit polar division are FliF, FliM and FliN. Curiously, the base of the MS ring (composed by FliF) and the FliM and FliN structures in the C ring are cytoplasmic-accessible portions of the flagellum. In contrast, the flagellar T3SS and FliG, which do not appear to influence division site determination, are internal components of the MS and C rings, respectively. Thus, FlhG may require a superficial domain of the flagellar base composed by the MS and switch complex to inhibit division at a pole. MinE or DivIVA function as topological specificity factors in Min systems to ensure that MinCD complexes only inhibit Z-ring formation at poles (22, 39, 76, 117, 150, 151). These proteins function by ultimately keeping MinCD relatively high at polar regions to inhibit polar division, and relatively low at cellular midpoints to promote Z-ring formation. Without the MS ring and switch complex, FlhG does not inhibit polar division efficiently, but it also appears defective in inhibiting Z-ring formation throughout the cell, as evident by the lack of increased cell elongation in MS ring and switch complex mutants. Thus, the MS ring and switch complex appear to induce an inhibitory mechanism mediated by FlhG primarily evident at the poles. Therefore, the MS ring and switch complex may serve as topological specificity factors for FlhG to inhibit polar division.

MinD orthologs influence division site determination in two ways: 1) by localizing the FtsZ-inhibitor MinC to polar or septal sites (MinD of *E. coli* and

*B. subtilis*); or 2) by directly depolymerizing FtsZ at polar sites (MipZ of *C. crescentus*). *C. jejuni* FlhG is more homologous to MinDs than MipZ, conserving many residues of the putative ATPase domain and a C-terminal amphipathic helix that are functionally required by *E. coli* MinD to influence division (38, 75, 167). However, FlhG lacks residues required for *E. coli* MinD to interact with MinE or MinC (184). It is possible that FlhG may complex with an FtsZ inhibitor in *C. jejuni* that is different than MinC. It is also possible that FlhG itself, perhaps induced by the MS ring and switch complex, is the direct inhibitor of FtsZ polymerization, similar to direct depolymerization of FtsZ by MipZ in *C. crescentus* (169). However, only very limited subdomains of the ATPase domains are conserved between FlhG and MipZ. Hence, no domains outside the MipZ ATPase domain that may inhibit Z-ring formation in *C. crescentus* are conserved in *C. jejuni* FlhG. Thus, FlhG likely functions by a different mechanism than MinD or MipZ in division site determination.

All polarly-flagellated bacteria commonly studied for motility, such as *Vibrio*, *Pseudomonas*, and *Helicobacter* species, encode FlhG and all Min components, except for *Campylobacter* species. A likely hypothesis for most polar flagellates is that FlhG controls numerical parameters of flagellar biosynthesis, whereas the Min system influences division site placement. However, we observed that *H. pylori* FlhG, and to a lesser extent *V. cholerae* FlhG, resolved the minicell phenotype of *C. jejuni*  $\Delta$ *flhG*, indicating that these proteins have the ability to function as division site determination factors. Thus, FlhG may influence division in a broad range of polarly-flagellated bacteria.

In this chapter, I describe a new paradigm that links polar flagellar biosynthesis to division in bacteria. Furthermore, I show how amphitrichous flagellation is beneficial for influencing symmetrical division in *Campylobacter* species so that two viable daughter cells are generated during each round of division. In addition, by this study, we provide a new function for the flagellar MS ring and switch complex in serving as a topological specificity determinant for FlhG so that division is specifically prevented at polar sites. Further exploration of this system will undoubtedly lead to new understandings of division processes that may occur in a broad range of polar flagellates.

## CHAPTER FIVE

### DISCUSSION

#### Summary

The focus of my thesis research is to identify and understand factors that numerically and spatially regulate flagellar biosynthesis. My findings also unexpectedly provided valuable insights into regulation of division in *C. jejuni*. As described in chapter IV, I have characterized FlhF as a positive factor required for  $\sigma^{54}$ -dependent flagellar gene expression, and the GTPase function of FlhF was determined to be a spatial determinant for flagellar placement in *C. jejuni*. Through research described in Chapter V, I found that FlhG has two major and distinct roles, one as a regulator of flagellar number and one as a determinant of division site placement. In addition, I identified a mechanism for how FlhG may execute its function in inhibiting polar division. The evidence presented in Chapter V suggests that FlhG requires the flagellar MS ring and the switch complex of the C ring to inhibit polar division. This latter finding has an additional significance, as it links flagellar biosynthesis to division in *C. jejuni*. Furthermore, my work suggests new functions for the MS ring and C ring, which have well established roles for the flagellar organelle.

#### Characterization of the Role of FlhF in Flagellar Gene Regulation and Biosynthesis

##### *Analysis of the in vitro GTPase activity of FlhF*

Before this study, no functional or biological activity had been associated with the GTPase activity of FlhF. The crystal structure of *B. subtilis* FlhF shows a GTP-bound homodimeric structure (10). This finding suggested that FlhF likely itself was not

sufficient to hydrolyze GTP alone. In addition, the FlhF homologs SRP GTPases demonstrate a heterodimeric structure, where the molecular asymmetry is thought to play a GTPase-activating role. However, FlhF homodimers lack this asymmetry, raising questions if and how the GTPase activity may be regulated. My work showed for the first time that FlhF is a GTPase and identified residues required for full activity. In comparison to other GTPases, I observed a relatively slower GTPase activity. Many GTPases require a GTPase activating protein (GAP) to stimulate the GTP hydrolysis. Although, I demonstrated a GTPase activity for FlhF *in vitro*, it is possible an additional factor in *C. jejuni* may influence FlhF GTPase activity *in vivo* and influence its role in spatial parameters of flagellar biosynthesis.

#### *Role of FlhF in Flagellar Gene Expression in C. jejuni*

In polarly-flagellated bacteria, the FlhF GTPase has been shown to be involved in flagellar gene expression and polar placement of flagella. In *C. jejuni*, we have found that FlhF, but not its GTPase activity, is required for  $\sigma^{54}$ -dependent flagellar gene expression. When FlhF is absent,  $\sigma^{54}$ -dependent flagellar gene expression is decreased. Through research efforts presented in Chapter IV, I provided evidence that FlhF functions either downstream of FlgSR in the FEA-FlgSR pathway or converges with FEA-FlgSR pathway to influence  $\sigma^{54}$ -dependent transcription, mRNA stability or translation.

FlgR, is homologous to the NtrC class of transcriptional regulators (87). However, our laboratory has found that it likely initiates transcription by a mechanism alternate to typical NtrC proteins, including a DNA-binding independent step. It is

possible that FlhF may assist FlgR in interacting or stimulating  $\sigma^{54}$  to initiate expression of flagellar genes. *In vitro* transcription assays with FlgR and  $\sigma^{54}$  may reveal a need for FlhF for transcription. FlhF may alternatively function by stabilizing  $\sigma^{54}$ -dependent mRNAs. FlhF has homology to Ffh and FtsY, which binds to mRNA-ribosome complexes to stall transcription and direct these complexes to the membrane for cotranslational secretion of a polypeptide through the inner membrane. If FlhF functions in stabilization of  $\sigma^{54}$ -dependent mRNAs, we may be able to assess the stability of  $\sigma^{54}$ -dependent mRNAs in wild-type or *flhF* mutant strains over time by Northern blot or RT-PCR after treatment of strains with rifabutin, an RNA polymerase inhibitor. A significant reduction in mRNA stability in the absence of FlhF would suggest that FlhF directly or indirectly binds to mRNAs to promote their stability. Additionally, we may be able to test if FlhF directly binds to  $\sigma^{54}$ -dependent mRNAs *in vitro*.

Due to its homology to SRPs, FlhF might also be involved in translation of flagellar transcripts. Creation of a translational reporter to a  $\sigma^{54}$ -dependent gene can be used to monitor if FlhF is involved in translation of flagellar proteins.

#### *Role of FlhF GTPase Activity in Polar Placement of Flagella in C. jejuni*

In chapter IV, I showed that the FlhF GTPase mutants that have reduced GTP hydrolysis activity, FlhF<sub>D321A</sub> and FlhF<sub>R324A</sub>, cause mislocalization of flagella to lateral sites, multiple flagella at a single pole, and an increase in aflagellated bacterial population. In these GTPase mutants,  $\sigma^{54}$ -dependent flagellar gene expression remained mostly unaffected. These findings suggest that the GTPase activity is involved in a mechanism that precisely controls flagellar placement. Homologs of FlhF, such as the

SRP GTPases, use GTP hydrolysis to coordinate delivery of ribosomes to a membrane channel for cotranslational secretion of proteins. Thus, loss of GTP hydrolysis may lead to dysregulated behavior of FlhF to result in loss of strict localization of flagellar structures, resulting in lateral flagella. Alternatively, the FlhF GTPase mutants may be hyperactive and able to produce multiple base structures at a pole to result in multiple polar flagella.

Flagella are produced in bacteria where the initial basal structures that are required for secretion of flagellar proteins are localized. An experiment supporting this idea comes from *V. cholerae*, where polar localization of FliF-GFP (which composes the MS ring) is dependent on presence of FlhF (56). Thus, FlhF and its GTPase activity are likely required for the localization of base structures that include the MS ring. Preliminary fluorescent studies in *C. jejuni* suggest that while wild-type FlhF localizes to poles, FlhF GTPase mutant proteins localize to polar and lateral sites, suggesting the intrinsic GTPase activity of FlhF might be involved in its polar localization leading to polar formation of initial basal structures of the flagellum (data not shown).

#### *The FlhF GTPase is a Positive Factor for Flagellar Biosynthesis*

A small subset of the FlhF GTPase mutant population produces multiple polar flagella, unlike wild-type bacteria where the number of flagella is restricted to one per pole. The increase in the flagellar number in these mutants may suggest that FlhF mutants are resistant to an inhibitor that maintains flagellar biosynthesis. The potential inhibitor for FlhF might be FlhG. An interaction between these proteins was observed in a yeast two-hybrid study performed in *C. jejuni* (142). In addition, in *V. alginolyticus*,

FlhF and FlhG were found to interact in co-immunoprecipitation studies (102). Finally, *flhG* mutants that produce wild-type FlhF, produce multiple polar flagella, suggesting that FlhF may be uninhibited and able to initiate production of multiple flagella in these cells. These findings together lead to the idea that FlhG inhibits FlhF function in a direct manner.

Previous findings and Figure 9 shows that FlhF (but not its GTPase activity) is required for  $\sigma^{54}$ -dependent flagellar gene expression. While deletion of *flhF* creates an aflagellated phenotype, overexpression of *flhF* in *P. putida*, *V. alginolyticus* and *C. jejuni* caused an increase in the numbers of polar flagella in a subset of the bacterial population ((96, 140) and data not shown). These data indicate that FlhF is a factor that positively influences flagellar biosynthesis, in addition to its role in spatial regulation of flagellar placement.

In the case of *P. putida* and *V. alginolyticus*, FlhF might be the limiting step in flagellar biosynthesis and overexpression of *flhF* might overcome this limit to produce an increased number of flagella (although they possess master transcriptional regulators). A similar rate-limiting phenomenon might be true for *C. jejuni* FlhF with FlhF directly involved in forming the base structures required for biosynthesis of flagellum at polar sites. In addition, a subset of population of FlhF GTPase mutants do not produce flagella, which might be attributed to lack of properly formed flagellar base structures that are required for flagellar biosynthesis.

## **Characterization of FlhG in *C. jejuni***

### *FlhG is Required for Two Distinct Biological Processes in C. jejuni*

FlhG has been identified as a regulator of flagellar number in polarly-flagellated bacteria (33, 34). As such loss of this protein results in an increase in the number of polar flagella (34). Results presented in Chapter V show that FlhG in *C. jejuni* also controls flagellar number. In addition, *flhG* mutants show a pronounced minicell phenotype, implying that FlhG has a role in division site determination by preventing formation of polar division sites.

### *FlhG Controls Numerical Parameters of Flagellar Biosynthesis in C. jejuni*

The loss of FlhG in *C. jejuni* resulted in increased flagellar numbers at the poles of the bacteria. By altering the levels or activity of a master transcriptional regulator, FlhG can influence flagellar gene expression and protein production in other polar flagellates (33, 35). Increased flagellar gene expression and protein production in *flhG* mutants is thought to lead in increased flagellar numbers.

In *V. cholerae* and *P. aeruginosa*, *flhG* is a member of class II flagellar genes, and its expression is driven by the flagellar master transcriptional regulator (33, 36). This type of organization creates a negative feedback loop and results in the downregulation of master transcriptional regulator by FlhG after a brief pulse of activation of class II flagellar genes. As *C. jejuni* lacks a master transcriptional regulator, it is unclear how the *flhG* mutant produces multiple flagella. With our current knowledge, the most plausible target of repression by FlhG may be FlhF. As discussed above, FlhF GTPase mutants

and overexpression of *flhF* resulted in multiple polar flagella phenotypes in the presence of wild-type FlhG, suggesting that modulating FlhF GTPase or levels may overcome a repressive activity of FlhG. One current hypothesis for FlhG function in flagellar biosynthesis of *C. jejuni* that we have proposed suggests that FlhG inhibits the activity of FlhF at the cell poles after one round of flagellar biosynthesis. This inhibition may be bypassed by overexpressing *flhF*, decreasing the GTPase activity of FlhF, or by removing FlhG.

#### *FlhG Influences Division Site Determination in C. jejuni*

In addition to the multiple polar flagellar phenotype, a  $\Delta flhG$  mutant demonstrated a strong minicell phenotype. The minicell phenotype is characteristic of cell division erroneously occurring at the polar sites, which was first observed in *min* mutants of *E. coli* (3, 4). The minicell phenotype has not been reported in *flhG* mutants in other polar flagellates.

Inhibition of Z-ring formation might be directly attributed to FlhG itself, or FlhG might be the carrier protein of an FtsZ-inhibitor analogous to MinC. Similar to *in vitro* experiments with MinC, we may be able to monitor FtsZ polymerization in the presence or absence of FlhG. If the addition of FlhG alone causes depolymerization, this finding would suggest that FlhG may inhibit polar division by directly inhibiting Z-ring formation at the poles. To further characterize FlhG, conserved structures like the C-terminal amphipathic helix can be mutated and resulting mutants can be observed for minicell formation. Also, expression of *C. jejuni* FlhG in a heterologous system like *E. coli* without the Min system is another approach to understand the function of FlhG. If

the minicell phenotype of a *min* mutant of *E. coli* is resolved with overexpression of FlhG, these findings would support that FlhG is the factor that directly inhibits FtsZ polymerization.

The alternative hypothesis for FlhG function is that *C. jejuni* employs a second protein that interacts with FlhG that has the FtsZ-inhibitory activity, like MinC. If the experiments mentioned above fail, a screen can be performed to identify a *C. jejuni* MinC-like protein *in vitro*. A biochemical fractionation of *C. jejuni* extracts can be monitored for FtsZ-depolymerization effects *in vitro*. Fraction with the desired activity can be further purified to identify the proteins that inhibit FtsZ polymerization.

#### *Link Between Flagellar Biosynthesis and Cell Division in C. jejuni*

The main habitat of *C. jejuni* is the intestinal tract of mammalian and avian species, especially poultry animals. Colonization of intestinal tracts of hosts by *C. jejuni* requires flagellar motility. If the commensal lifestyle of *C. jejuni* is the predominant lifestyle, the organism should always have flagella to efficiently colonize and persist in the host. Consistent with this hypothesis, *C. jejuni* does not have a flagellar master transcriptional regulator to control expression of class I genes. Instead, these class I genes are clustered with housekeeping genes and are likely constitutively expressed.

Normally, *C. jejuni* produces one flagellum at each pole. To maintain an amphitrichous flagellar pattern, *C. jejuni* is required to synthesize a new flagellum at new poles of daughter cells after division. This requirement leads to the idea that cell division may be linked to initiation of flagellar biosynthesis. The findings presented in Chapter IV showed that the GTPase function of FlhF is required for efficient polar placement of

flagella, possibly due to FlhF influencing the polar placement of flagellar base structures that are required for secretion of flagellar proteins. Therefore, FlhF may have an intrinsic ability to find new poles to begin flagellar biosynthesis. FlhF may be able to identify new poles, perhaps by interacting with a component of the divisome remaining at new poles.

While it is only a hypothesis that cell division may initiate flagellar biosynthesis, I have provided strong evidence that placement of division sites is strongly dependent on polar flagellar biosynthesis. The absence of FlhF, FliF, FliM and FliN all result in strong minicells phenotypes, suggesting a defect in division site determination. This phenotype can be reversed by overexpression of *flhG in trans*.

These findings suggest some possible mechanisms about the role of FlhF and the flagellar base in the function of FlhG and division site determination. The polar flagellar base structure (composed by FliM and FliN of the C ring and FliF of the MS ring) might be recognized by FlhG to localize to a pole to exert its function. Alternatively, FlhG might require these flagellar base structures to be fully active at the polar regions to inhibit division. The presence of the MS ring is thought to be required for anchoring all flagellar proteins to the inner membrane (27). Therefore, the lack of the MS ring results in lack of all flagellar structures at the pole of the bacterium including the C ring. Thus, the minicell phenotype observed in  $\Delta$ *fliF* mutant might be indirect and due to lack of this mutant to support FliM and FliN biosynthesis into the C ring. Furthermore, the  $\Delta$ *flhF* and *flhF* GTPase mutants show defects in division site determination and this may be due to lack of localizing these flagellar base structures to the polar regions (Figure 19A and data not shown).

The amphitrichous flagellar pattern has historically been believed to give *C. jejuni* its unique darting motility phenotype (159). The results presented in Chapter V showed another important aspect of amphitrichous flagellation. As mentioned in Chapter II, poles of bacteria, if not protected, are vulnerable to improper division events to result in minicell formation. In *C. jejuni*, inhibition of polar division requires that FlhG function at both poles. As described above, FlhG requires flagellar structures to act at the poles. Thus, amphitrichous flagellation is the best flagellation pattern to inhibit polar division and promote symmetrical division.

To clearly determine the dynamics of FlhG localization in *C. jejuni* and which factors are involved in its localization, fluorescently-labeled FlhF and FlhG and other flagellar proteins can be traced over time *in vivo*. Preliminary fluorescent studies suggest that FlhG has a preference for polar regions in wild-type *C. jejuni* (data not shown). The spatial dynamics of FlhF and FlhG will help us understand the inhibitory activity of FlhG for polar division and flagellar biosynthesis. For example, we will be able to determine if FlhF localization to the poles directly recruits FlhG or if another protein like FliF, FliM and FliN localization to the pole is required for proper localization of FlhG.

#### *Differences of in Division Site Determination Between C. jejuni and Other Polarly-flagellated Bacteria*

*In silico* analysis showed that *Campylobacter* species do not encode any Min proteins, other than FlhG which is a ParA ATPase superfamily member like MinD. Although cell division is not well characterized in polarly-flagellated bacteria, the

genomes of major polarly-flagellated bacterial species including *P. aeruginosa*, *V. cholerae* and *H. pylori*, all have genes encoding the Min system in addition to FlhG.

Our studies indicated that FlhG of *C. jejuni* appears to compensate for the lack of MinD and possible other Min proteins to spatially control division site formation. During evolution it is possible that *Campylobacter* species might have lost the Min system, and adapted FlhG, which controls numerical aspects of amphitrichous flagellation, to function in inhibition of polar division.

FlhG proteins from other polar-flagellates resolved the minicell phenotype of the *C. jejuni*  $\Delta flhG$  mutant. In chapter V, we presented results suggesting that *H. pylori* and *V. cholerae* FlhG proteins can compensate for lack of *C. jejuni* FlhG in inhibiting FtsZ function. This finding raises the possibility that FlhF, and perhaps polar flagella, may inhibit polar division in other polarly-flagellates. It is possible that the Min system and FlhG may have partially redundant functions in division site determination in these other polar flagellates. However, these bacteria do not demonstrate amphitrichous flagellation and only produce flagella at one pole. Therefore, FlhG may only be to protect the flagellated pole from Z-ring formation in these bacteria. Thus, the Min system may be required to inhibit polar division sites at both poles.

From my studies, it is obvious that FlhG and the Min system of polarly-flagellated bacteria with a different polar flagellar biosynthesis pattern need to be studied to determine the precise functional roles in division. For example, a lophotrichous species such as *H. pylori* would be interesting to analyze. Creating single and double deletions of *flhG* and *min* genes will reveal if these factors have redundant functions in division site determination, or if they both partially contribute to spatially regulating

division site formation. A possible outcome of this experiment might be that *min* mutants demonstrate division at the pole that lacks flagella, and deletion of *flhG* might further increase the minicell phenotype by making the pole with flagella vulnerable to division.

Also, to further understand spatial and numerical control of flagella in polarly-flagellated bacteria, swapping the *flhF* and *flhG* genes between *H.pylori* and *C.jejuni* will be beneficial, because these closely related bacterial species have significantly different flagellation patterns and flagellar numbers. By observing these types of reconstructed strains, one can have a better understanding about the intrinsic properties of FlhF and FlhG in numerical and spatial control of flagellar biosynthesis.

## **Conclusion**

The studies presented in this thesis suggest that FlhF is a positive factor for flagellar biosynthesis and gene expression. Furthermore, FlhF controls spatial organization of flagella through its GTPase activity. Furthermore, I have provided some evidence that the activity of FlhF may be repressed by the flagellar number regulator FlhG. In addition to control of flagellar number, FlhG is involved in division site determination and requires flagellar substructures such as the MS ring and the C ring to inhibit polar division in *C.jejuni*. In addition to characterizing roles of FlhF and FlhG in *C.jejuni* flagellar biosynthesis, the work presented in this thesis clearly shows a link between determination of division sites and flagellar biosynthesis, and the utilization of an amphitrichous flagellation pattern for control of cell division.

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