

INFLUENCE OF THE FLAGELLAR BASAL BODY ON TRANSCRIPTION OF THE RpoN
REGULON IN *HELICOBACTER PYLORI*

by

JENNIFER WING-WAH TSANG

(Under the Direction of Timothy R. Hoover)

ABSTRACT

Helicobacter pylori is an Epsilonproteobacteria capable of causing gastritis, ulcers, and gastric cancer when it colonizes the human stomach epithelium. Functional flagella are required for the migration of the bacterium through the gastric mucus lining to the gastric epithelial cells. Flagellar biogenesis requires over fifty genes that are transcribed as their products are needed. The sequential expression of flagellar genes is regulated by all three sigma factors found in *H. pylori* (RpoD, RpoN, and FliA). Genes needed early in assembly are dependent on RpoD, genes needed in the middle stage of assembly are dependent on RpoN, and genes needed late in assembly are dependent on FliA. Transcription of the RpoN-dependent genes is activated by a two-component system consisting of the sensor kinase FlgS and the response regulator FlgR. The cellular cue sensed by the FlgS/FlgR two-component system is currently unknown though several studies have linked the expression of RpoN-dependent genes to the presence of the flagellar protein export apparatus. FliO is a membrane-bound component of the export apparatus and is required for optimal expression of the RpoN regulon. The effect of FliO on transcription of the RpoN regulon may be indirect as FliO is required to support wild-type levels of FlhA, another membrane-bound component of the export apparatus. A deletion analysis revealed that

the first 24 residues of FlhA (FlhA_{NT}) are needed for the transcription of RpoN-dependent genes. Interaction between FlhA_{NT} and FlgS was demonstrated using biolayer interferometry, although FlhA_{NT} alone was unable to stimulate autophosphorylation of FlgS. Loss of other structures such as the C ring and FliH, both which facilitate the shuttling of flagellar substrates to the export apparatus, inhibited expression of RpoN-dependent flagellar genes suggesting that these proteins may be important for regulating FlgS activity. Loss of FliE and FlgBC, the first substrates secreted by the export apparatus, increased expression of RpoN-dependent genes. These results support a model where FlgS is brought to the export apparatus via interactions with FliH and the C ring where it can sense the status of flagellar assembly to initiate a signal transduction response resulting in the expression of the RpoN regulon.

INDEX WORDS: *Helicobacter pylori*, flagella, transcription, gene regulation, sigma factor, flagellar export apparatus, two-component system, RpoN, FlhA, FliO, FlgS

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DEDICATION

This work is dedicated to my Mom and Dad who taught me to aim high and to do the best that I can, to my high school biology teacher David Journey whose classes sparked my interest in microbiology, to my undergraduate research advisor Dr. Michelle Dziejman who gave me my first opportunity to experience “real research”, and to my graduate advisor Dr. Timothy Hoover who provided patient guidance through the past several years.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Clinical Significance

Helicobacter pylori is the causative agent for a variety of gastric diseases such as gastritis, peptic ulcers, gastric cancer, and B cell MALT (mucosa-associated lymphoid tissue) lymphoma. Because of its link to gastric cancer and lymphoma, *H. pylori* was classified as a Class I carcinogen by the World Health Organization in 1994 (1). *H. pylori* is found in a large proportion of the worldwide population though most individuals infected with *H. pylori* remain asymptomatic. About 10-20% of individuals infected will develop gastric or duodenal ulcers in their lifetime and about 1-2% of infected individuals will develop gastric cancer or B cell MALT lymphoma (2, 3). Stress was once thought to be the major cause of peptic ulcer disease and treatment by inhibiting gastric acid production were often unsuccessful resulting in relapse of symptoms. In 1982, Barry Marshall and Robin Warren first cultured *H. pylori* from human stomach biopsies and found that most stomach biopsies from patients with gastric inflammation or ulcers were positive for *H. pylori* (4). Approximately 90-95% of duodenal ulcers and 70-75% of gastric ulcers are linked with *H. pylori* infection (2). Because of their discovery, Marshall and Warren received the Nobel Prize in Physiology or Medicine in 2005.

The mode of transmission of *H. pylori* is currently unknown though it has been suspected that infections are acquired through a fecal-oral route and/or an oral-oral route as *H. pylori* has been detected in saliva, feces, and vomitus (reviewed in (3)). It is also possible that *H. pylori*

may reside in the environment in a viable but non-culturable state until it enters a suitable host (5). Most individuals infected with *H. pylori* seem to acquire the bacterium early in childhood, most likely from family members, and infections can persist for the lifetime if left untreated (6, 7).

Treatment of *H. pylori* infections is often difficult and the standard triple therapy treatment consists of two antibiotics and a proton pump inhibitor. Though this triple therapy regimen is generally successful in treating *H. pylori* infections, it may be difficult for patients to take up to 20 pills a day for two weeks and the medications can cause many side effects. Because gastric acid can degrade certain drugs, such as amoxicillin and clarithromycin, treatment may require higher doses for the antibiotics to be effective (8). Additionally, antibiotic resistance is becoming more prevalent resulting in some cases requiring more than one round of antibiotics.

H. pylori is traditionally considered a pathogen, but its role in human health is more nuanced. *Helicobacter* species inhabit the gastrointestinal tract of many mammals and birds, and most *Helicobacter* species are host specific, implying a co-evolution of the bacterium with its host. *H. pylori* has been estimated to have co-evolved with humans for the past 60,000 years (9). Some researchers have suggested that *H. pylori* may play a protective role from some diseases. For instance, Martin Blaser hypothesized that *H. pylori* may be part of the normal flora of the stomach and the decline of *H. pylori* colonization in some areas correspond to an increase in esophageal adenocarcinoma cases (10). Healthy individuals carrying strains of *H. pylori* that produce CagA were less likely to develop acid reflux disease, Barrett's esophagus (11) and asthma (12). However, it is currently debated whether these trends indicate a causal relationship between *H. pylori* and disease.

Microbiological Characteristics of *H. pylori*

H. pylori, formerly called *Campylobacter pylori*, belongs to the subphylum of Proteobacteria called the Epsilonproteobacteria. Many of the Epsilonproteobacteria thrive in the digestive tract including the intestinal pathogen, *Campylobacter jejuni* and the cattle gut symbiont, *Wolinella succinogenes*. Aside from the well-known pathogenic members of the Epsilonproteobacteria, other members of the Epsilonproteobacteria have been isolated from hydrothermal vents, cold vents and terrestrial systems (13, 14).

Typically, *H. pylori* holds a spiral shape about two to four microns in length and about half a micron in width. It possesses two to six polar, sheathed flagella. After prolonged passage in culture or exposure to antibiotics, the morphology changes to a coccoid form, that can represent dying cells, viable cells with culturability or viable but non-culturable (VBNC) cells (15). VBNC coccoid cells can evade detection by traditional culture methods but can be identified by PCR. Because VBNC cells arise under stress conditions, these forms of *H. pylori* reside in biofilms (16). Coccoid forms of *H. pylori* have been found in about 80% of stomach tissue colonized with *H. pylori* (17).

H. pylori is a fastidious organism and requires stringent growth conditions. Because it is a microaerophile, it thrives only in low concentrations of oxygen, and in the laboratory, it is typically grown under an atmosphere of 2-4% O₂, 5% CO₂ and 91-93% N₂. The media used for growing *H. pylori* are complex and require the addition of serum or blood. A chemically defined medium has been created for the growth of *H. pylori* (18, 19) but is not routinely used due to low growth yields. Growth media for *H. pylori* at a neutral pH as *H. pylori* grows only between pH 5.0 to 8.0.

The size of the *H. pylori* genome is approximately 1.7 Mbp, which is considerably smaller than that of *Escherichia coli* (approximately 4.6 Mbp) (20). The *H. pylori* genome also has a G+C content of about 39% (20). *H. pylori* is naturally transformable which contributes to the vast genetic diversity in this species. Some strains of *H. pylori* carry cryptic plasmids whose functions are unknown and do not encode antibiotic resistance markers or virulence factors (21). These cryptic plasmids have been adapted to create the shuttle vectors, pHel2 and pHel3, for genetic manipulation in the laboratory (22).

Colonization and Virulence Factors

H. pylori possesses several factors that promote colonization of gastric epithelial cells. The stomach is a harsh environment and *H. pylori* has several ways to combat the stressors of stomach life. Surprisingly, *H. pylori* is not an acidophile though it lives in the stomach. Rather, the bacterium buffers the area around itself using copious amounts of urease. Urease is one of the most abundant proteins in *H. pylori* totally about 6% of soluble proteins found in crude extracts (23). It catalyzes the hydrolysis of urea to ammonia and carbamate. The carbamate produced is then degraded by spontaneous hydrolysis to ammonia and carbonic acid. The production of ammonia, a basic molecule, increases the local pH around the bacterium making it hospitable for survival in the stomach (24). In order to persist in the stomach, *H. pylori* must also synthesize flagella. The flagellum is needed for the bacterium to swim away from the acidic conditions of the stomach lumen and through the gastric mucin lining to reach the more neutral conditions at the gastric epithelium. Non-motile mutants of *H. pylori* are unable to colonize the gastric lining in animal models (25, 26). *H. pylori* must also be able to combat reactive oxygen species produced from the host immune response and from the microaerophilic environment of

the stomach. Enzymes such as catalase, superoxide dismutase and methionine sulphoxide reductase combat oxidative stress and are important for colonization (27-29). Superoxide dismutase catalyzes the breakdown of the reactive superoxide (O_2^-) into oxygen and hydrogen peroxide which is then further broken down into water and oxygen by catalase. Methionine sulphoxides can arise in proteins exposed to reactive oxygen species and methionine sulphoxide reductase reduces methionine sulfoxide to methionine to restore biological function to these damaged proteins.

Aside from factors that are required for stable colonization and survival in the stomach, *H. pylori* produces proteins that are immunogenic and induce a host response. Individuals that carry strains possessing the highly immunogenic protein CagA (cytotoxin-associated gene A) have a greater chance of developing stomach cancer or peptic ulcers compared to individuals carrying strains that lack CagA (30). CagA is encoded on the *cag* pathogenicity island (*cag* PAI) which is thought to have been introduced in *H. pylori* by horizontal gene transfer from an unknown organism. Many of the genes encoded on the ~37 kb *cag* PAI encode components of a Type IV secretion apparatus, a syringe-like structure that facilitates the translocation of CagA and peptidoglycan into the host cell (31). Once inside the host cell, CagA can interfere with host signal transduction pathways leading to variety of effects such as disruption of tight junctions and polarity, inflammation, altered apoptosis and proliferation of epithelial cells (reviewed in (32)).

VacA (vacuolating cytotoxin A) is another virulence factor that also targets a range of host cell pathways. It induces the formation of large endo-lysosomal compartment that is toxic to the host cell upon accumulation (33). Once VacA is secreted by *H. pylori*, it undergoes proteolytic cleavage to yield two smaller products, p33 and p55. p33 is responsible for the

vacuolating activity of VacA and p55 plays a role in binding to host cells. VacA has also been shown to induce apoptosis in the human gastric epithelial cell line AGS (34) and appears to increase the epithelial permeability of Fe^{3+} and Ni^{2+} ions which may serve as nutrient sources for *H. pylori* in vivo (35).

Flagellar Biogenesis

The *Helicobacter pylori* flagellum consists of three main structures: the basal body, the hook, and the filament. The basal body is a multifaceted structure consisting of several structures with unique functions such as the export apparatus which secretes flagellar protein substrates across the cell membrane, the C ring which controls the rotational direction of the flagellum, the motor, rings that anchor the flagellum to the membranes, and the rod which traverses the membranes. Distal to the rod is the hook, which forms a curved structure that translates the torque generated from the motor to the movement of the flagellum, and the filaments which forms the long tail-like structure of the flagellum. The export apparatus consists of six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ, and FliR) and three soluble components (FliH, FliI, and FliJ) which bring flagellar protein substrates to the membrane-bound portion of the export apparatus for secretion (reviewed in (36)).

Biogenesis of the flagellum involves all three sigma factors found in *H. pylori* (RpoD, RpoN, and FliA). In *H. pylori* and other bacteria that possess flagella, the transcription of flagellar genes is organized into a cascade where genes encoding components needed earlier in flagellar biogenesis are expressed before genes that encode components required later in flagellar biogenesis. Genes that encode the early structures involved in flagellar biogenesis are regulated by RpoD, genes encoding structures in the middle stages of flagellar biogenesis are regulated by

RpoN, and genes encoding structures needed at the last stage of flagellar biogenesis are regulated by FliA (37). Transcription of each set of flagellar genes is also regulated by the activity of transcriptional factors. For instance, expression of the RpoN-dependent genes occurs only when the components of the FlgS/FlgR two-component system are phosphorylated (38, 39) and transcription of FliA-dependent genes occurs when the anti sigma factor, FlgM, is sequestered away from FliA (40, 41). Details of flagellar gene regulation in *H. pylori* and other bacteria are discussed in Chapter 2.

Research Summary

The overall aim of my research is to identify the signals needed to stimulate activation of the sensor kinase FlgS and the response regulator FlgR and thereby initiate transcription of the RpoN-dependent genes. Several studies have indicated a role of the flagellar export apparatus in stimulating expression of the RpoN-dependent genes (42, 43). FliO is one of the most diverse flagellar export apparatus proteins among different bacteria. In *H. pylori*, FliO has a large N-terminal periplasmic domain, followed by a transmembrane region, and a C-terminal cytoplasmic domain. The N terminus of FliO is much larger in *H. pylori* than that in *Salmonella* or *E. coli*, and I wished to determine whether this portion of FliO had a unique function to the *H. pylori* RpoN flagellar regulon. This hypothesis seemed reasonable since flagellar gene regulation in *Salmonella* and *E. coli* does not involve RpoN. To investigate the role of FliO in flagellar biogenesis, I replaced the *fliO* locus with the chloramphenicol acetyltransferase (*cat*) gene and found that these cells were severely defective in motility and expression of the RpoN-dependent genes (44). Truncated versions of FliO in which the N terminus or the C terminus were deleted (FliO_{ΔN} and FliO_{ΔC}, respectively) were able to support motility and expression of

RpoN-dependent genes (44). Wild-type levels of FlhA were found in the membrane of cells containing FliO_{ΔN} and FliO_{ΔC} but not in the $\Delta fliO$ mutant (44) indicating that the transmembrane region of FliO is important for the stability or insertion of proteins to form the flagellar export apparatus.

To investigate the role of FlhA in the regulation of RpoN-dependent flagellar genes, I made a $\Delta flhA$ mutant and several truncated version of FlhA. All versions were non-motile and unable to export flagellar substrates (45). Depending on the strain used, some truncation mutants, such as the *flhA77* mutant, in which only the first 77 residues of FlhA were expressed, were able to support expression of RpoN-dependent genes (45). This suggests that there is a region in the first 77 residues of FlhA that is required for expression of RpoN-dependent genes. The first 24 residues of FlhA seem to be the most likely candidate because this region is cytoplasmic and can potentially stimulate FlgS activity. I introduced plasmids containing either the full-length *flhA* (*pflhA*) or the N-terminal truncation of *flhA* (*pflhA_{ΔNT}*) into the $\Delta flhA$ and noticed that *pflhA* complemented motility and expression of RpoN-dependent genes where as *pflhA_{ΔNT}* did not. Additionally we demonstrated a strong interaction between FlgS and FlhA_{NT} suggesting that FlhA_{NT} may be required to stimulate FlgS activity. However, it does not appear that FlhA_{NT} stimulates FlgS autokinase activity suggesting that other components of the flagellar basal body may be needed to initiate the activity of FlgS.

Lastly, I investigated the role of flagellar structures outside the export apparatus have on expression of RpoN-dependent flagellar genes. I constructed mutants in which C ring components or soluble export apparatus components were deleted and found that in these mutants, expression of RpoN-dependent flagellar genes was decreased. The soluble components of the export apparatus and the C ring have been shown to facility the transport of flagellar

substrates to the export apparatus for secretion (46). It is possible that these components also shuttle FlgS to the export pore where it can recognize the state of the export apparatus to regulate transcription of RpoN-dependent genes. I am currently exploring this hypothesis by examining potential interactions between FlgS and C ring proteins or soluble components of the export apparatus using biolayer interferometry. I have also found that exported flagellar substrates such as FliE (MS-rod linker) and FlgBC (rod substrates) may influence the expression of RpoN-dependent genes. Deletions of *fliE* and *flgBC* both resulted in elevated levels of RpoN-dependent gene expression with *fliE* deletion having a greater effect than *flgBC* deletion.

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CHAPTER 2

THEMES AND VARIATIONS: REGULATION OF RpoN-DEPENDENT FLAGELLAR GENES ACROSS DIVERSE BACTERIAL SPECIES¹

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Abstract

Flagellar biogenesis in bacteria is a complex process in which the transcription of dozens of structural and regulatory genes is coordinated with the assembly of the flagellum. Although the overall process of flagellar biogenesis is conserved among bacteria, the mechanisms used to regulate flagellar gene expression vary greatly among different bacterial species. Many bacteria use the alternative sigma factor σ^{54} (also known as RpoN) to transcribe specific sets of flagellar genes. These bacteria include members of the Epsilonproteobacteria (e.g., *Helicobacter pylori* and *Campylobacter jejuni*), Gammaproteobacteria (e.g., *Vibrio* and *Pseudomonas* species), and Alphaproteobacteria (e.g., *Caulobacter crescentus*). This review characterizes the flagellar transcriptional hierarchies in these bacteria and examines what is known about how flagellar gene regulation is linked with other processes including growth phase, quorum sensing and host colonization.

Introduction

The flagellum is an exquisitely complex nanomachine that is the primary means for motility in many bacteria. Given that motility plays a vital role in important microbial processes such as chemotaxis, host colonization and biofilm formation, understanding how bacteria regulate flagellar biogenesis is critical for developing new strategies for the control of harmful microbes and manipulation of useful ones. Flagellar biogenesis is a highly ordered process that involves the coordinated regulation of dozens of structural and regulatory genes with the assembly of the flagellum. As expected for choreographing such an intricate process, flagellar biogenesis involves some of the most sophisticated regulatory mechanisms found in microbiology.

Although the structure of the flagellum differs slightly between Gram-negative type and Gram-positive type bacteria, in all cases the bacterial flagellum is comprised of three main parts: the basal body, hook and filament (1). Components of the basal body are located within or are closely associated with the cell envelope. The basal body consists of several distinct structures, including the C ring, a type III secretion system known as the flagellar protein export apparatus, the flagellar motor, the rod, and rings that anchor the flagellum to the membrane (Fig. 2.1A). Both Gram-negative and Gram-positive type bacteria possess MS and P rings, located in the inner membrane and peptidoglycan layer, respectively. Gram-negative type bacteria possess another ring known as the L ring located in the outer membrane. These rings provide support for the rod as it goes through the cell envelope. Depending on the bacterial species, the C ring consists of a complex of three or four different types of protein subunits assembled around the export pore of the flagellar protein export apparatus and extending into the cytoplasm. The C ring has a role in switching the rotational direction of the flagellum, but has an additional role in directing protein substrates to the flagellar protein export apparatus for transport (2). The flagellar protein export apparatus (Fig. 2.1B) transports axial components of the flagellum (i.e., proteins that constitute the rod, hook and filament) across the cell membrane where they are incorporated at the distal end of the nascent flagellum (1, 3, 4).

Despite the commonality in flagellar architecture, the ways in which bacteria regulate expression of their flagellar genes vary remarkably (reviewed by Smith and Hoover (5) and Anderson et al. (6)). This review focuses on systems which utilize the alternative sigma factor σ^{54} (also known as RpoN) for regulating transcription of specific sets of flagellar genes. Sigma factors bind core RNA polymerase and allow the resulting RNA polymerase holoenzyme to recognize specific promoter sequences (7). All bacteria possess a primary sigma factor (RpoD;

referred to as σ^{70} in *E. coli*), and most also utilize one or more alternative sigma factors for transcription of specific sets of genes. A unique feature of RpoN-dependent transcription is the requirement of an activator to stimulate the transition of a closed complex between σ^{54} -RNA polymerase holoenzyme (σ^{54} -holoenzyme) and the promoter to an open promoter complex that is able to initiate transcription (8, 9). *Escherichia coli* and *Salmonella* are the archetypes for flagellar biogenesis and gene regulation, but these bacteria do not use RpoN for transcription of flagellar genes. Nevertheless, we refer to the *E. coli/Salmonella* systems throughout the review to make inferences for other systems that are not as well characterized. The bacteria that utilize RpoN in flagellar gene expression constitute a diverse group of microorganisms, some of which are human or animal pathogens. We discuss aspects of flagellar gene regulation in response to host and environmental stimuli and potential ramifications of these responses for survival and persistence of the bacteria.

Transcriptional Hierarchies Governing Flagellar Gene Expression

Though microorganisms utilize various mechanisms to control flagellar gene expression, in most (if not all) bacteria the flagellar genes are transcribed in an organized fashion where genes that encode components needed early in flagellar biogenesis are transcribed before genes encoding proteins required later in the assembly process (Fig. 2.2). To a certain extent these transcriptional hierarchies are governed by the organization of the flagellar genes into different regulons based on the sigma factors required for their transcription. These transcriptional hierarchies are further coordinated by regulatory proteins that control expression of various sets or classes of genes. However, the mechanism by which such a hierarchy is regulated in a given bacterial species is not always understood and varies among organisms. A master regulator(s)

initiates the flagellar gene transcription cascade and is generally thought to couple flagellar biogenesis to the cell cycle. For some bacteria, however, master regulators have yet to be identified. The master regulator stimulates transcription of the early genes which generally encode components of the basal body as well as an array of regulatory proteins which, depending on the bacterium, includes RpoN, FliA (σ^{28}), FlgM (an anti- σ^{28} factor) or other proteins. Following completion of the basal body and hook, late genes encoding components of the filament are expressed.

***H. pylori* and *C. jejuni*.** The best studied members of the Epsilonproteobacteria are *Campylobacter jejuni* and *Helicobacter pylori*. *C. jejuni* is a food-borne pathogen that colonizes the intestinal tract where it causes severe diarrhea, while *H. pylori* colonizes the stomach where it can cause peptic ulcer disease which can progress into gastric cancer if left untreated. Both organisms synthesize polar flagella that are required for colonization of the host (10-13). Moreover, the degree of motility appears to be important for *H. pylori* virulence as more motile strains are able to maintain higher bacterial density and inflammation in the stomach cardia than those that are less motile (14).

In *H. pylori* and *C. jejuni*, flagellar gene regulation involves the primary sigma factor RpoD, as well as the alternative sigma factors RpoN and FliA (Fig. 2.2). A master regulator which activates transcription of the early flagellar genes has not been identified for *H. pylori* or *C. jejuni*. It is possible that these bacteria lack a true master regulator of flagellar biogenesis, a possibility that was suggested by Niehus and co-workers since motility is required for the obligate parasitic lifestyle of *H. pylori* (15). Alternatively, *H. pylori* or *C. jejuni* could possess a master regulator for flagellar biogenesis that has other essential roles which might explain why it has not been identified from mutagenesis screens.

The organization of flagellar genes into regulons based on the sigma factor required for transcription is very similar, though not identical, in *H. pylori* and *C. jejuni*. RpoD is required for transcription of genes encoding components of the basal body as well as regulatory proteins that control transcription of genes required at later stages of flagellar biogenesis (15). Other genes regulated by RpoD include *flhF* and *flhG* whose products influence the localization of flagella to the cell pole and number of flagella per cell, respectively (16-18). It is not known if transcription of the early flagellar genes is temporally regulated and intimately associated with the cell cycle as occurs in *E. coli* and *Salmonella* (19, 20) or if these genes are transcribed constitutively. Distinguishing between these two options would require one to follow temporal changes of early flagellar genes transcription in synchronous cultures, which has not been reported.

Transcription of genes needed midway through flagellar biogenesis is dependent on RpoN. These genes encode the proximal rod proteins, the hook protein, hook-associated proteins, the hook length control protein (FliK), a minor flagellin (FlaB), and enzymes required for glycosylation of the flagellins (15, 21, 22). The transcription of the RpoN-dependent genes is activated by a two-component regulatory system consisting of the sensor kinase FlgS and the response regulator FlgR (21, 23-25). FlgS differs from most sensor kinases in that it is not membrane bound. The signal or cellular cue to which FlgS responds has yet to be identified, but several studies (see below) have implicated the flagellar protein export apparatus in this process. FlgS uses ATP to autophosphorylate a specific histidine residue in response to the cellular cue, and this phosphate is subsequently transferred to a specific aspartate residue in FlgR to activate it. Based on studies with other RpoN-dependent activators (reviewed by Bush and Dixon (26)), phosphorylation of FlgR likely stimulates oligomerization of the protein from a dimer to a

hexamer capable of activating transcription. RpoN-dependent activators typically possess an N-terminal regulatory domain (often a response regulator domain as is the case in FlgR), an AAA+ ATPase domain that engages σ^{54} and couples the hydrolysis of ATP with open complex formation, and a C-terminal DNA-binding domain that binds an enhancer sequence (reviewed by Schumacher et al. (27)). *H. pylori* FlgR is unusual in that it lacks a C-terminal DNA-binding domain and activates transcription by contacting σ^{54} -holoenzyme in the closed promoter complex without binding DNA (24). Although *C. jejuni* FlgR possesses a C-terminal domain, it is not needed for activation of the RpoN regulon suggesting that like its counterpart in *H. pylori*, *C. jejuni* FlgR engages the closed promoter complex from solution (28). While the C-terminal domain of *C. jejuni* FlgR does not appear to play a role in DNA binding, it does prevent phosphorylation of the protein by acetyl phosphate which can interfere with normal flagellation (29).

The flagellar protein export apparatus has a major role in regulating expression of the RpoN regulon of *H. pylori* and *C. jejuni* as evidenced by the observation that mutations which interfere with formation of the export apparatus inhibit expression of RpoN-dependent flagellar genes. Porwollik and co-workers showed that inactivation of *fliI* or *fliQ* (encode a cytosolic ATPase component and integral membrane component of the export apparatus, respectively) in *H. pylori* results in reduced levels of the minor flagellin, FlaB, and the hook protein, FlgE, both of which depend on RpoN for their expression (30). Allan and colleagues showed that mutations in *flhB* (encodes an integral membrane component of the export apparatus) result in reduced levels of FlaB and FlgE in *H. pylori* (31); and Smith and co-workers subsequently demonstrated that disrupting *flhB* inhibited expression of RpoN-dependent reporter genes (32). Using DNA microarrays, Niehus and co-workers (15) demonstrated that FlhA (an integral membrane

component of the export apparatus) is required for transcription of the entire RpoN regulon. Hendrixson and DiRita (33) reported that deleting any one of several integral membrane components of the export apparatus (FlhA, FlhB, FliP or FliR) inhibited expression of an RpoN-dependent flagellar reporter gene in *C. jejuni*. Studies by Tsang and co-workers revealed that *H. pylori* strains which stably express truncated forms of FlhA are able to transcribe RpoN-dependent genes at levels that are close to wild-type but are unable to export flagellar protein substrates (34). Thus, any model for how the export apparatus influences transcription of the RpoN regulon must take into account that the export apparatus need not transport protein substrates to stimulate the RpoN regulon. Boll and Hendrixson recently showed that loss of the MS ring protein FliF or the C ring protein FliG inhibits transcription of RpoN-dependent flagellar genes in *C. jejuni* (35). The MS ring is the first flagellar structure assembled and in its absence, the export apparatus presumably is not formed which would account for the inhibition of the RpoN regulon in the *fliF* mutant. FliG may facilitate autophosphorylation of FlgS either by directly stimulating FlgS or by promoting assembly of the export apparatus into a conformation that stimulates FlgS activity (35).

The export apparatus is an ideal indicator for the status of flagellar assembly as it undergoes a major conformational change upon completion of the hook-basal body structure. In the early stages of flagellar biogenesis the export apparatus is in a conformation that transports rod- and hook-type substrates. Upon completion of the mature hook-basal body structure, the export apparatus undergoes a conformational change which is accompanied by a switch in substrate specificity to filament-type substrates (36). This conformational change involves an autocleavage event in the C-terminal, cytoplasmic domain of the export apparatus protein FlhB (referred to as FlhB_C) as well as interactions between the C-terminal domain of the hook-length

control protein FliK and FlhB_C (37). The change in conformation and substrate specificity of the export apparatus may serve as a cellular cue for the temporal regulation of flagellar genes. Indeed, in *E. coli* and *Salmonella* the anti- σ^{28} factor FlgM is a filament-type substrate and is secreted by the export apparatus upon completion of the mature hook-basal body structure which allows transcription of the σ^{28} -dependent flagellar genes to occur (38).

Transcription of the RpoN-dependent flagellar genes in *H. pylori* also requires the putative RpoN chaperone FlgZ (HP0958) (39, 40). FlgZ was first implicated in having a role in flagellar biogenesis in *H. pylori* when it was identified as interacting with RpoN in a high-throughput screen of a yeast two-hybrid system (<https://pim.hybrigenics.com/>) (41). In the absence of FlgZ, RpoN is turned over rapidly in *H. pylori* with a half-life of about 30 minutes compared to a half-life greater than 4 hours in wild type (40). The molecular basis for how FlgZ protects RpoN from rapid turnover is not known, but it is possible that FlgZ binds RpoN to protect it from proteolysis or that FlgZ facilitates the association of RpoN with core RNA polymerase which serves to protect RpoN from degradation. Overexpression of RpoN restores motility in a *H. pylori flgZ* mutant indicating that FlgZ is not essential for flagellar biogenesis (40). In addition to its potential role as an RpoN chaperone, FlgZ may have additional roles in flagellar biogenesis as it was also shown to interact with the flagellar export apparatus protein FliH in the yeast two-hybrid system (<https://pim.hybrigenics.com/>) (41). In addition, Douillard and co-workers demonstrated that FlgZ binds the *flaA* transcript and is needed for optimal expression of FlaA (42). These researchers proposed that FlgZ functions with FliH to direct *flaA* transcripts to the flagellar protein export apparatus where translation of the transcripts could be coupled with secretion of the nascent FlaA (42).

Transcription of genes needed late in flagellar biogenesis in *H. pylori* and *C. jejuni*, which includes genes encoding the major flagellin, filament cap and associated chaperones, is dependent on σ^{28} (FliA) (15). Similar to *E. coli* and *Salmonella*, the *H. pylori* FliA regulon is negatively regulated by FlgM (43). Unlike *E. coli* and *Salmonella*, the inhibitory effect of FlgM on FliA in *H. pylori* is thought to be alleviated via interactions between FlgM and the C-terminal, cytoplasmic domain of FlhA (FlhA_C) rather than by secretion of FlgM via the export apparatus (44). *C. jejuni* also possesses a FlgM homolog (45), but unlike *H. pylori*, *C. jejuni* FlgM appears to be secreted from the cell via the flagellar export apparatus (46). Interestingly, interaction of *C. jejuni* FlgM with FliA is temperature-dependent, occurring at 42°C (the optimal growth temperature for *C. jejuni*) but not 37°C (46). The primary function of *C. jejuni* FlgM is not to inhibit FliA activity during formation of the hook-basal body structure, but rather to limit the length of the flagellar filament by suppressing expression of the FliA-dependent flagellin (FlaA) and the RpoN-dependent flagellin (FlaB) (46). The mechanism by which FlgM represses transcription of RpoN-dependent genes in *C. jejuni* is unknown, but Wösten and co-workers have speculated that accumulation of the FliA/FlgM complex may inhibit phosphorylation of FlgS and/or FlgR (46). FlgM also appears to inhibit transcription of RpoN-dependent genes in *H. pylori*, but so far this has only been demonstrated in an *flhA* mutant background (15).

***Vibrio* species.** The vibrios are typically saltwater microorganisms and several species cause food-borne illnesses. *Vibrio* species vary greatly in flagella morphology. *Vibrio cholerae* has a single polar sheathed flagellum, while *Vibrio parahaemolyticus* and *Vibrio alginolyticus* produce a single polar sheathed flagellum and lateral, peritrichous flagella, and *Vibrio fischeri* has a tuft of polar sheathed flagella. Lateral flagella promote swarming and colonization of surfaces and thereby enhance biofilm formation upon encountering highly viscous media or solid surfaces.

The flagellar gene transcriptional hierarchy in *Vibrio cholerae* is governed by RpoN and FliA (47). *flrA* encodes an RpoN-dependent activator and is the master regulator for the flagellar gene transcriptional hierarchy (47). FlrA is regulated by cyclic di-GMP, a secondary messenger with roles in biofilm formation and virulence, which binds to FlrA and prevents it from binding the *flrBC* promoter (48). Early and middle genes are RpoN-dependent but they rely on different transcriptional activators for their transcription. FlrA, along with RpoN, is required for transcription of the early genes which encode the components of the MS ring, C ring and export apparatus. Other early genes encode the regulatory proteins FlrB, FlrC and FliA. FlrB and FlrC constitute the sensor kinase and response regulator, respectively, of a two-component system that regulates transcription of the middle genes in conjunction with RpoN (49). The middle genes encode the basal body-hook structures and the core flagellin, FlaA. The late genes are dependent on FliA for their transcription and encode alternative flagellins, FlgM and components of the flagellar motor. As in *Salmonella*, FlgM is secreted via the flagellar protein export apparatus to relieve the inhibition on FliA activity in *V. cholerae* (50). *flgA* is located upstream of *flgM* and its promoter (RpoN- and FliA-independent) may drive transcription of *flgM* such that FliA is repressed until formation of the mature hook-basal body structure (47).

The signal(s) that stimulate the FlrB/FlrC two-component system is not known. FlrD is necessary for transcription of middle and late flagellar genes, making it a candidate for regulating the FlrB/FlrC two-component system (51). Additionally, FlrD contains HAMP domains (domain present in histidine kinases, adenyl cyclases, methyl-accepting proteins, and phosphatases) that are usually found in integral membrane proteins that are part of signal transduction pathways (51, 52). Moisi and co-workers showed that FlrD is inserted into the

inner membrane and proposed that it senses the completion of the MS ring-switch-export apparatus structure and communicates this information to FlrB (51).

The regulation of flagellar genes in *V. parahaemolyticus* is more complex since the bacterium is capable of producing lateral flagella in addition to polar flagella (reviewed by Merino et al. (53) and McCarter (54)). The polar flagellar system (Fla) is constitutively expressed while the lateral flagellar system (Laf) is expressed upon impedance of the polar flagella. The lateral flagella are used for swarming motility and enhances biofilm formation and host colonization by the bacterium (55, 56). When grown planktonically, the bacterium produces polar flagella but when grown on highly viscous or solid medium (53) or during iron-limiting conditions (57), the bacterium produces lateral flagella. Thus the polar flagellum acts as a mechanosensor to regulate expression of the lateral flagella genes through a mechanism that has yet to be defined. The dual flagellar system suited for locomotion under different conditions allows *V. parahaemolyticus* to be highly adaptive to changing habitats, including planktonic environments, surfaces and biofilms.

The polar and lateral flagellar systems do not share any structural or regulatory components (aside from RpoN), but the regulatory networks that control polar and lateral flagellar systems in *V. parahaemolyticus* are similar to that of *V. cholerae*. The lateral flagellar genes are activated by LafK and, like the polar flagellar system, lateral flagellar genes are both RpoN- and FliA-dependent (58). Although LafK is homologous to master regulators of other flagellar systems, LafK does not appear to be a master regulator for the lateral flagellar system as the *fliM_L* operon (contains genes encoding components of the C ring and export apparatus) is not LafK-dependent (59). This suggests that there is another level of regulation preceding expression of these genes.

***Pseudomonas* species.** *Pseudomonas aeruginosa* is an aerobic Gram-negative Gammaproteobacterium that causes opportunistic infections, particularly in patients with cystic fibrosis where it results in inflammation and sepsis (reviewed by Veessenmeyer et al. (60) and Balasubramanian et al. (61)). *P. aeruginosa*, which thrives on surfaces and forms biofilms, is a prevalent agent of nosocomial infections. Wild-type *P. aeruginosa* cells form biofilms which can cause chronic infections. *P. aeruginosa* possesses a single polar flagellum that is required for virulence (62). Motile strains of *P. aeruginosa* induce activation of the inflammasome (a multiprotein oligomer responsible for the activation of the inflammatory response) whereas non-motile strains have a markedly reduced ability to induce inflammasome activation (63). Non-motile strains of *P. aeruginosa* may have an advantage during chronic infection by evading stimulation of the innate immune responses. However, a non-motile *flgK* mutant is defective in surface attachment suggesting a role for flagella and/or motility in the initial cell-to-surface interaction (64). Mutants in *fliM* and *cheY* are also non-motile and are unable to form biofilm cap structures on initial biofilm colonies (65). Other *Pseudomonas* species include *Pseudomonas fluorescens* which possess multiple flagella and is found in the soil and water. Some *P. fluorescens* strains have been shown to protect plant roots against fungal infections (66). *P. fluorescens* does not typically cause disease in humans, but it is an opportunistic pathogen in immunocompromised individuals (67, 68).

Like the Epsilonproteobacteria and *Vibrio*, *P. aeruginosa* possesses a four-tiered transcriptional hierarchy for regulating flagellar gene expression. FleQ is the master regulator for flagellar synthesis and *fleQ* is regulated by global factors outside the flagellar regulons, such as cyclic di-GMP, a signaling molecule important in modulating the transition between planktonic and biofilm lifestyles (69). The early flagellar genes are dependent on FleQ and

RpoN for their transcription and encode components of the basal body as well as the filament cap, which is somewhat surprising since it is not needed until late in flagellar biogenesis (70). The early genes also encode regulatory proteins which include *flhF*, *fleN*, *fleS* and *fleR* (70, 71). FleN is an anti-activator of FleQ that maintains the normal flagellum copy number of one per cell by down-regulating genes encoding early flagellar components via a negative feedback mechanism (72, 73). FleS and FleR form a two-component system that is required for the transcription of the middle RpoN-dependent genes (70). The signal(s) required for FleS activation is unknown. The middle genes encode components of the basal body, including rod, L ring, hook, hook cap and hook-filament junction proteins (70). The late genes are FliA-dependent and encode FliC (flagellin), FleL (filament length control protein), FlgM, FlgN (a protein required for initiation of filament assembly) and some chemotaxis proteins (70). Transcription of *fliA* is constitutive (70), and FlgM (whose transcription is also FleQ dependent, but not RpoN-dependent) repress the activity of FliA until completion of the hook basal body structure, at which point it is secreted from the cytoplasm via the export apparatus which alleviates its inhibition of FliA.

Caulobacter crescentus. *Caulobacter crescentus* is an Alphaproteobacterium found in freshwater that is a model organism for cell cycle studies. *C. crescentus* divides asymmetrically to produce two cells with distinct morphologies: a swarmer cell and a stalked cell. The non-motile stalked cell contains a polar stalk which secretes an exopolysaccharide that facilitates adhesion to surfaces. In addition, the stalked cell initiates DNA replication upon the start of cell division. In contrast, the swarmer cell is motile via a polar flagellum and DNA replication is repressed for a defined length of time until the cell differentiates into a stalked cell. Flagellar

biogenesis is coordinated with the cell division cycle such that all progeny swarmer cells possess a functional flagellum.

Flagellar genes in *C. crescentus* are regulated by two sigma factors. The initiation of DNA replication in the swarmer cell results in expression and activation of the global transcription factor CtrA. CtrA also acts as the master regulator for flagellar gene expression by activating transcription of the early genes (encode MS ring, C ring, and export apparatus, FlbD, FliX). Additionally, CtrA controls expression of *rpoD* (74) and is needed for transcription of the early flagellar genes (75). FlbD and FliX are transcriptional regulators that regulate expression of the early and middle genes. The middle and late genes (encode hook and flagellin proteins) are transcribed only after the components encoded by the early genes have been assembled into the nascent flagellum. FlbD is an RpoN-dependent transcriptional activator which binds *ftr* (flagella transcriptional regulation) sequence motifs to regulate gene expression. Once the early genes are expressed, FlbD binds the *ftr* sequence motifs in the promoter regions of early flagellar genes to inhibit transcription of these genes. For example, FlbD binds the *ftr4* site located upstream of the early gene *fliF* to repress its transcription once the early genes have been expressed (76). In contrast, FlbD stimulates transcription of the middle genes by binding to the *ftr* sites of the middle and late genes (76, 77). FlbD activity is regulated by FliX, which acts to either inhibit or stimulate FlbD. In the absence of early flagellar structures, FliX inhibits FlbD activity by binding FlbD preventing it from binding *ftr* sites (78). Once the early flagellar structures are formed FliX stimulates FlbD. The mechanism for this stimulation is unknown, but it may involve another factor such as an assembled component of the flagellum which converts FliX into a positive regulator, or it may involve covalent modifications of FliX or FlbD (78). Although FlbD possesses an N-terminal response regulator domain, FlbD activity does not

appear to be regulated by phosphorylation via a sensor kinase. The exact cellular cues that regulate FlbD are not known, but FlbD activity is linked to cell division (79).

Unlike the bacteria discussed thus far, flagellar biogenesis in *C. crescentus* does not involve FliA. Rather, the six flagellin genes found in *C. crescentus* are RpoN-dependent. The expression of the flagellin genes is regulated by FlbD and FlbT which binds the 5' UTR of flagellin transcripts and represses translation until the hook-basal body structure is assembled (80). Though the mechanism for this temporal regulation is unknown, one possibility is that FlbT represses translation of flagellin genes until a positive factor binds the 5' UTR region of flagellins to promote translation upon assembly of the mature hook-basal body structure (80).

Coupling Flagellar Biosynthesis with Other Cellular Activities

Though flagellar biogenesis is a complex process in itself, it is further complicated by its coordination with other cellular processes. Flagellar biosynthesis is timed with other cellular activities such as cell replication and quorum sensing. Environmental stimuli also play a role in regulating flagellar biogenesis as bacteria respond to changes in the environment by altering behavior needed for survival. Many of these responses involve altering the state of flagellar biogenesis within these different contexts.

Modulating flagellar gene expression during the cell cycle. Flagellar biosynthesis is linked to the cell cycle through various mechanisms and involves coordinating flagellar gene expression with growth phase and cell division. In *V. cholerae*, chemotaxis and motility genes are up-regulated as the population enters stationary phase. Of the 114 genes identified with roles in chemotaxis and flagellar biogenesis, 72 genes were up-regulated more than 2-fold during stationary phase compared to mid-exponential phase (81). The up-regulation of 60 of the 72

chemotaxis and flagellar genes is dependent on RpoS, indicating a correlation between flagellar biogenesis and entry into stationary phase (81). The coordination between entry into stationary phase and flagellar biogenesis may aid the bacterium in exiting the host when nutrients become depleted and prepare the bacterium for colonization of other niches or survival in environmental reservoirs.

C. crescentus is a particularly good organism for studying how flagellar biogenesis is integrated with the cell cycle because of its two distinct cell morphologies. The flagellar filament is assembled just prior to cell division and all progeny swarmer cells possess a fully functional flagellum. The synthesis of the flagellum is intimately linked to the cell cycle through regulatory proteins such as CtrA, FlbD and FliX that are involved in both the cell cycle and flagellar biogenesis. This dual functionality may exist to ensure that each swarmer cell is flagellated. The master regulator for flagellar biogenesis, CtrA, regulates at least 95 genes (82), including the cell-division genes *ftsZ* (83) and *ftsQA* (84). Cells of a *fliX* mutant become filamentous as they enter late log phase (85) demonstrating a role for this flagellar regulatory protein in cell division. Flagellar biogenesis is also linked to the cell cycle via FlbT and FlaF. FlaF is required for flagellar biogenesis and motility (86). FlbT protein levels are constant throughout the cell cycle, while FlaF levels correlate to flagellin levels, both of which peak just prior to cell division (86). Since FlbT is a negative regulator for flagellin translation and peaks in flagellin levels correlate with FlaF levels, this observation suggests that FlaF may temporally modulate FlbT activity (86).

Expression of *Vibrio vulnificus* FlhF, which is needed for polar localization of the flagellum, is regulated by the quorum sensing master regulator SmcR. *flhF* transcript levels in a *smcR* mutant are higher than wild-type levels indicating that SmcR represses *flhF* expression

(87). In wild-type cells, *flhF* transcript levels are highest during exponential phase and decrease upon entry into stationary phase (87). In contrast, transcript levels of *smcR* increase as cultures enter stationary phase suggesting that SmcR plays a major role for growth phase-dependent variation of *flhF* expression (87). This growth phase-dependent gene expression may be important in host colonization as during the initial infection bacterial cell densities are low but at later stages of colonization when bacterial cell densities are higher motility may not be necessary.

Expression of flagellar genes is regulated in *P. fluorescens* throughout the growth phases by the Gac (GacA/GasS) two-component system which limits flagellar biosynthesis during exponential growth by down-regulating transcription of *fleQ* (encodes the master regulator) (88). The Gac system positively regulates production of virulence factors and quorum-sensing molecules and is required for full virulence in animal and plant hosts (89). Levels of the Gac system components are regulated in response to growth phase as *gacA* and *gacS* transcript levels are highest at mid-exponential growth phase (90). The concomitant up-regulation of virulence factors and the down-regulation of flagellar genes by the Gac system may be a way to coordinate virulence and motility with growth phase so that virulence factors are expressed at high cell densities during host colonization and flagellar genes are turned off to facilitate attachment to the host.

Connections between quorum sensing, host colonization and flagellar gene expression. In many pathogenic bacteria, flagella aid in surface attachment and colonization. In such cases, successful colonization may require cooperation between quorum sensing and flagellar synthesis to integrate surface attachment and virulence gene expression at high cell densities in pathogens. Surface colonization can lead to biofilm formation which is generally more resistant to host

defenses and antimicrobials. Pathogens use different mechanisms to couple expression of virulence genes with flagellar gene expression in the context of the host. This includes modulating quorum sensing and host colonization based on the status of the flagellum or modulating flagellar biogenesis based on quorum sensing signals.

Quorum sensing is used to regulate gene expression in response to cell density. This is achieved through the secretion of a signaling molecule called an autoinducer (AI). AIs are produced by bacteria and travel across the cell membrane and accumulate in the environment. Upon reaching a critical concentration, the AIs stimulate transcription of genes required for group behavior. *luxS* is responsible for the production of AI-2 (91) and an *H. pylori* mutant in *luxS* exhibits decreased motility compared to wild type (92). *flhA* transcript levels are decreased in the *luxS* mutant, but addition of 4, 5-dihydroxy-2,3-pentanedione (functions as AI-2) into the growth medium restores *flhA* transcript levels to wild-type levels (92). Like the *H. pylori luxS* mutant, a *C. jejuni luxS* mutant is less motile than wild type (93) and the mutant displays reduced transcript levels of *flaA* (94). Using DNA microarrays, He and co-workers found that a majority of the flagellar genes are down-regulated in a *C. jejuni luxS* mutant (93). Expression of chemotaxis genes, however, is unchanged in the *luxS* mutant indicating that the motility defect of the *luxS* mutant is due to defects in flagellar biogenesis and not chemotaxis (93). A rationale for the link between cell density and flagellar biogenesis is that it may aid in colonization of host tissue by ensuring that a sufficient number of flagellated bacteria are present to establish a successful infection.

Regulatory proteins involved in flagellar biogenesis can regulate quorum sensing and colonization, or they themselves can be regulated by quorum sensing and colonization factors. For example, the RpoN-dependent activator FlrC is required not only for flagellar biogenesis but

also appears to play a role in host colonization by *V. cholerae*. Mutants expressing a FlrC variant incapable of being phosphorylated fail to produce flagella and are therefore defective in colonization (49). Interestingly, a mutant expressing a constitutively active form of FlrC is also deficient in colonization even though the cells are motile and produce flagella (49). This observation demonstrates the need for coordinating flagellar biogenesis and pathogenesis for successful colonization. Conversely, Vfr, a major quorum sensing and virulence regulator in *P. aeruginosa*, down-regulates expression of flagellar genes by repressing transcription from the *flgQ* promoter (95). Inhibition of flagellar biogenesis by Vfr may aid *P. aeruginosa* in persisting at the site of infection to achieve high cell densities and virulence gene expression.

HapR is a transcriptional regulator involved in quorum sensing in *V. cholerae* which also regulates expression of virulence genes. HapR represses expression of several virulence genes (96) and *hapR* expression is regulated by an unknown mechanism involving FliA (97). *hapR* expression is depressed in *flgM* and *flgD* mutants (both of these mutations alleviate FlgM repression on FliA activity), while deletion of *fliA* results in elevated *hapR* expression (97). The authors of this study speculated that expression of HapR is regulated by a regulator or small, non-coding RNA (sRNA) that is part of the FliA regulon. FliA-mediated regulation of *hapR* appears to be linked to the shearing of flagella from bacteria that penetrate the mucin layer to infect the host. This shearing of the flagella leads to secretion of FlgM via the flagellar protein export apparatus and results in increased expression of FliA-dependent genes and repression of *hapR* expression (97). The outcome of such a mechanism is that virulence genes which are negatively regulated by HapR are expressed specifically when *V. cholerae* penetrates the intestinal mucous layer. *hapR* production is also regulated by the quorum sensing protein LuxO, further linking virulence to cell density. LuxO is an RpoN-dependent activator (98) which along

with HapR, regulates motility, protease production and biofilm formation (96). High cell densities are common during late stages of infection and the HapR-mediated repression of virulence genes may aid *V. cholerae* in detaching and finding new colonization sites (81, 96).

V. parahaemolyticus uses its polar flagellum to detect surfaces by sensing impedance to the polar flagellum rotation rate (99). Once a surface is encountered, expression of the lateral flagellar genes is induced. Lateral flagella are important for host colonization as mutants that do not produce lateral flagella are deficient in adherence to HeLa cells and biofilm formation (56). Gode-Potratz and co-workers identified ~70 genes that are surface-responsive, most of which are positively regulated (59). Some of the surface-regulated genes that are up-regulated are virulence genes, such as a putative GbpA homolog and components of a type III secretion system (59). GbpA from *V. cholerae* promotes adherence to chitinous surfaces of zooplankton and human epithelia cells (100) which is consistent with the observed up-regulation of *gbpA* in *V. parahaemolyticus* when it encounters a surface.

The *P. aeruginosa* flagellum is an important determinant in the susceptibility of the bacterium to host defenses. *P. aeruginosa fliC* mutants are unable to up-regulate transcription of *lasI* and *rhII* which encode enzymes that synthesize quorum sensing homoserine lactones (101). These quorum sensing molecules regulate production of exoproteases, which degrade surfactant protein-A (SP-A), an antimicrobial that opsonizes and permeabilizes membranes of lung pathogens (101). In the absence of these exoproteases, the *P. aeruginosa fliC* mutants are cleared from the lung (101). Though it is not known why *fliC* mutants are unable to up-regulate transcription of *lasI* and *rhII*, it is not due to the inability to sense the environment (101). *P. aeruginosa fliE*, *flgE*, *fliC*, and *fliD* mutants are also attenuated in LPS biosynthesis which compromises the integrity of their outer membranes making them more susceptible to SP-A

(102). Additionally, *flgE* and *fliC* mutants make less pyocyanin, a redox-active toxic secondary metabolite that is crucial for lung infection (103, 104).

Reacting to pH changes by gastrointestinal pathogens. Gastrointestinal pathogens encounter varying environmental conditions as they travel from the mouth to the stomach and then to the intestines. The gastric pathogen *H. pylori* has the added burden of long-term survival in the acidic environment of the stomach. Pathogens that colonize the intestinal tract must also survive passage through the stomach in high enough numbers to be able to colonize their target organs. Thus, it is not surprising that gastrointestinal pathogens often modulate gene expression in response to acidic conditions.

Merrell and co-workers found that expression of approximately 7% of the *H. pylori* genes are altered by a shift to low pH (105). Many of these genes are involved in flagellar synthesis with the majority of those being RpoN-dependent (105). FliA-dependent genes, such as *flgM* in *H. pylori* (105) and *flaA* in *C. jejuni* (106) are also up-regulated at low pH conditions. The up-regulation of flagellar genes is critical for survival of *H. pylori* as the bacterium is not able to survive at low pH but must swim across the mucous layer to the underlying gastric epithelium to establish a successful infection. In addition to enhancing transcription of flagellar genes, acidic environments also stimulate motility and swimming speeds, potentially in response to increased concentration of protons in the stomach (105).

The basis for up-regulation of the RpoN-dependent flagellar genes in *H. pylori* likely involves a stimulation of FlgS activity at low pH. Wen and co-workers reported that *H. pylori* requires FlgS to survive acid stress (30 minute exposure to pH 2.5) (107). These researchers found that besides the known RpoN-dependent flagellar genes, FlgS was required for the up-regulation of 86 genes in response to acid stress, many of which were previously known to be

required for acid stress survival (107). It is not known if FlgS responds directly to acid stress or if the stress signal is mediated through another factor.

Altering flagellar biogenesis by phase variation. In some bacteria flagellar biogenesis is subject to phase variation events that affect expression of specific flagellar genes. Phase variation causes changes in phenotypes at frequencies that are much higher than random mutations and contribute to heterogeneity within a population. The ability of *H. pylori* to persist in the host may involve the phase variable dependent expression of flagella that can serve as an advantage for adaptation to the host and for evading the host immune response. *C. jejuni* utilizes phase variation to turn on and off production of flagella, a strategy that increases commensal colonization in poultry (108). Many phase variation events occur by random reversible changes in the length of short DNA sequence repeats resulting from slipped strand mispairing. These events can take place within the coding sequence or in the promoter of a gene. *C. jejuni* phase variants in which expression of *flgR* is in the OFF state result from the addition or removal of a nucleotide within one of two homopolymeric nucleotide tracts consisting of adenine and thymine. These nucleotide tracts are located within the coding sequence of *flgR* causing the sequence to shift out-of-frame (108). Revertants to the ON state arise upon the addition or removal of a nucleotide within the original mutated homopolymeric nucleotide tract to restore the wild-type sequence. Alternatively, pseudo-revertants can arise in which nucleotides are removed or added close to the original mutated homopolymeric nucleotide tract to restore the correct reading frame but which result in changes in the amino acid sequence of the protein (108). *flgS* is also regulated by phase variation in *C. jejuni*, providing multiple levels of phase variable control for the RpoN regulon (109, 110). Similar mechanisms of phase variation also

exist for *flhA* in *C. coli* (111) and *fliP* in *H. pylori* (112) which leads to the formation of truncated proteins that prevent flagellar biogenesis.

A second type of phase variation in flagellar biogenesis is mediated through alterations in DNA methylation which impact the expression of specific flagellar genes. Host-adapted bacterial pathogens, such as *H. pylori*, often possess DNA methyltransferases and type III restriction-modification (R-M) systems that contain simple tandem DNA repeats which undergo slipped-strand mispairing during DNA replication that causes frameshift mutations and phase variation (113). R-M systems, widespread among many bacteria, confer protection from invasion by foreign DNA. Type III R-M systems are composed of a methyltransferase (*mod*) and an endonuclease (*res*) gene, whose gene products typically function together. The switching between ON and OFF states of a DNA methyltransferase can regulate expression of a “phasevarion,” via differential methylation of the genome in ON and OFF states (114). Many of these phase-variable methyltransferase genes are associated with an inactive *res* gene resulting from frameshift mutation or are orphans that are not associated with a *res* gene, suggesting that DNA restriction is not the primary role of these methyltransferases (reviewed by Fox et al. (115)). For example, *H. pylori modH* encodes a variable DNA methyltransferase that depending on the strain, the cognate *res* gene contains a nonsense or frameshift mutation or is apparently absent (114). *modH* OFF strains have decreased expression of *flaA* and *fliK* compared to the *modH* ON strain (114). FlaA has a low ability to activate innate immunity via the Toll-like receptor 5 (116) and modulating expression of the flagellin may be advantageous in evading the host response. Another *H. pylori* methyltransferase with an inactive *res* gene is *hpyAVIBM* (117, 118). *hpyAVIBM* encodes a C⁵ cytosine methyltransferase and contains AG repeats in its open reading frame, making it susceptible to phase variation via frame shift mutations (119). For

instance, *hpyAVIBM* from strain 26695 and strain HPAG1 have five AG repeats whereas the four AG repeats in strain San 74 results in the translation of a truncated protein (119). Deletion of *hpyAVIBM* results in both the up-regulation and down-regulation of *rpoN*, *fliR*, *fliD*, *fliS*, *motA*, *fliK*, and *flgK* depending on the strain used (119) indicating that phase variation of *hpyAVIBM* can alter flagellar biogenesis.

P. aeruginosa populations can possess two phenotypic variants, one which forms small, rough colonies (S) and another which forms large, flat (L) colonies. S variants form biofilms in non-agitated liquid cultures whereas the L variants do not (120). S variants possess defects in flagellum-mediated swimming, flagellum-mediated swarming, and type IV-pilus-mediated twitching, but are able to revert back to the L phenotype at relatively high frequencies suggesting that the shift between phenotypes is regulated by phase variation (120). Phase variation also occurs in *P. fluorescens* which colonizes the alfalfa rhizosphere. Phenotypic variants arise during rhizosphere colonization and are dependent on the activity of a site-specific recombinase (121). C variants have wild-type colony morphology while F and S variants have a translucent and diffuse colony morphology (121). F and S variants, which colonize distal parts of the roots and swim faster than the C variants, overproduce *fliC* (flagellin) transcripts and synthesize flagellin filaments that are ~3 times longer than those of the C variant (121).

Conclusions and Future Directions

Flagellar biogenesis is a carefully choreographed process in which many different components of the flagellum are made as they are needed for assembly into the nascent flagellum. Bacteria have evolved elaborate and sundry regulatory networks to couple flagellar gene expression with assembly. A driving force for the evolution of such regulatory networks is

undoubtedly the environmental and ecological constraints imposed by a particular bacterium's niche. In addition, the degree to which a given bacterial species needs to couple flagellar biogenesis to other cellular processes, such as cell division, has also likely shaped the evolution of these regulatory networks. We focused here on bacteria which utilize RpoN for flagellar biogenesis, yet even among these bacteria the mechanisms used to regulate expression of the RpoN-dependent flagellar genes vary greatly. For most of the systems described in this review we have only scratched the surface in our understanding of how they operate. For each of these systems, fundamental questions remain to be answered such as: are all of the flagellar genes of a given system expressed as part of a transcription hierarchy or are some genes expressed constitutively? Are there regulatory mechanisms within RpoN flagellar regulons that fine tune gene expression so that components of the flagellum are made precisely when needed? What are the cellular cues that are sensed by the transcriptional machinery that regulate expression of RpoN-dependent flagellar genes? How does the flagellar export apparatus control expression of RpoN-dependent flagellar genes in various bacteria? Does FlgZ have a regulatory role in flagellar biogenesis in *H. pylori* and other bacteria? As our understanding of the molecular genetics of diverse bacterial species expands, novel regulatory mechanisms that control expression of flagellar genes within these bacteria will undoubtedly come to light. For example, it was only recently that sRNAs were identified in *H. pylori*, some of which are potentially FliA-dependent (122) and may have roles in flagellar biogenesis. Finally, with increasingly sophisticated methods in bioinformatics, genomics and proteomics, further characterization of flagellar regulatory networks will provide important insights into the mechanisms governing gene expression as well as identify new regulatory proteins and mechanisms.

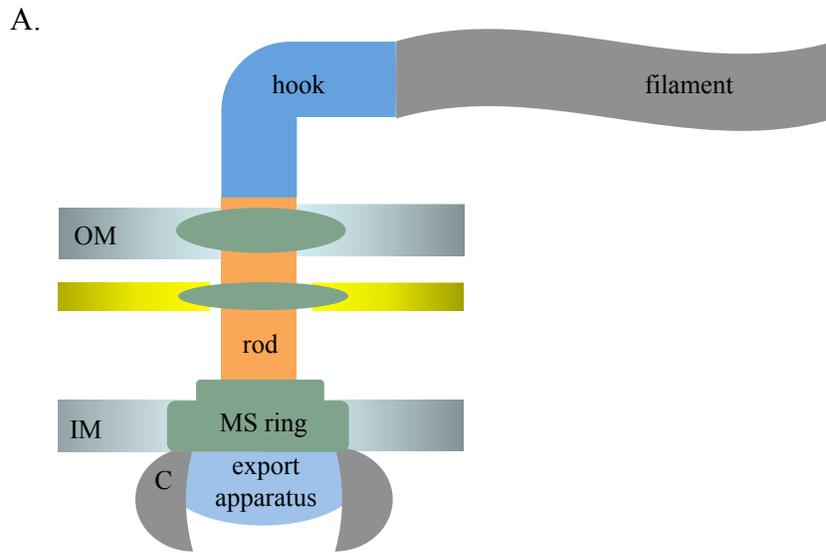
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Author Contributions

JT and TRH wrote the paper.



B.

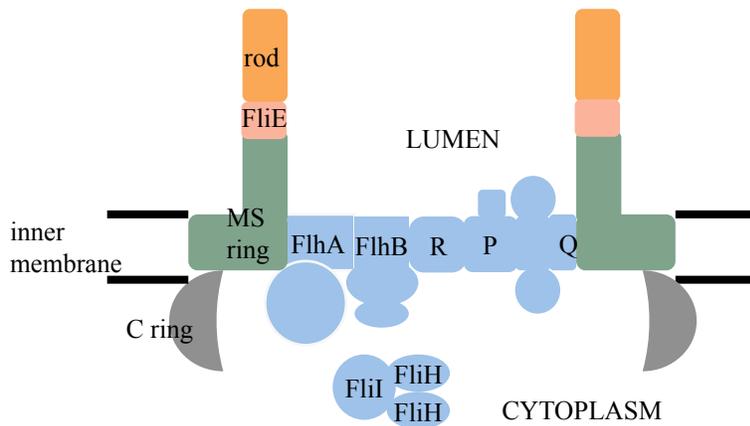


Figure 2.1: Structure of the flagellum. (A) An overview of the structure of the flagellum in Gram-negative bacteria. Abbreviations are as follows: L ring (L), P ring (P), outer membrane (OM), inner membrane (IM), and C ring (C). (B) Components of the flagellar basal body. Export apparatus proteins are shown in light blue. Abbreviations of the export apparatus proteins are as follows: FliR (R), FliP (P), FliO (O), and FliQ (Q). Details of the organization of the export apparatus proteins are not known, although results from genetic studies suggest

associations between FlhA and the MS ring (123), FlhB and FliR (124), and FliO and FliP (125). FliI is an ATPase which forms a heterotrimer with FliH. These proteins function together with other chaperones (not shown) to shuttle protein substrates to the export pore. Upon docking with a platform formed by the large cytoplasmic domains of FlhA and FlhB, a larger FliI₆FliH₁₂ complex is formed. In most bacteria the C ring is composed of three different types of protein subunits (FliG, FliM and FliN). The C ring in *Salmonella* contains an estimated 26 copies of FliG, 34 copies of FliM and ~136 copies of FliN. FliG is closest to the membrane and interacts with the MS ring, while FliN is the most distal to the membrane and FliM is situated between FliG and FliN. The *H. pylori* C ring contains FliG, FliM and FliN plus an additional protein subunit (FliY) that shares homology with FliN (126). Additional information on the bacterial flagellar protein export apparatus can be found in reviews by Minamino et al. (1) and Macnab (127).

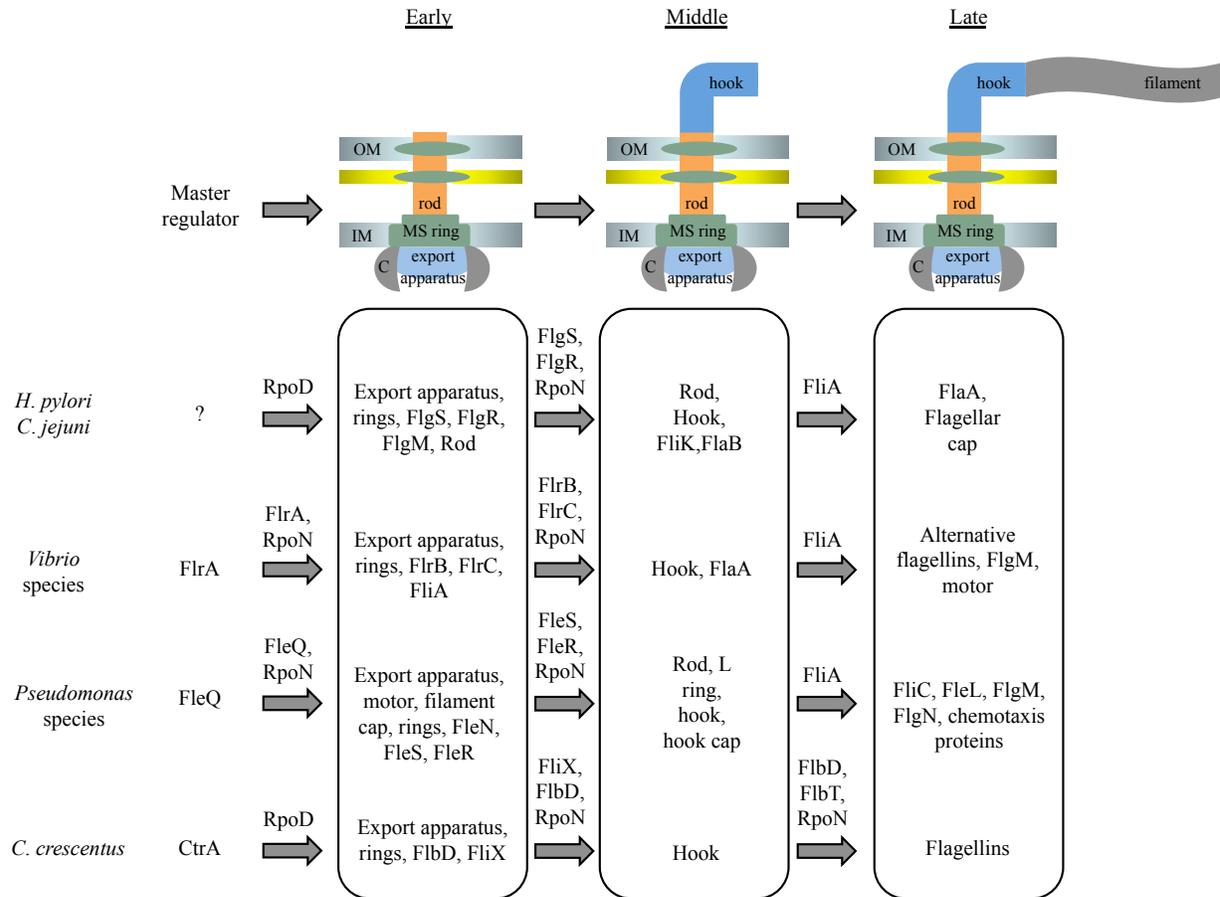


Figure 2.2: Diversity in flagellar gene transcription hierarchies. Transcriptional hierarchies are compared between the Epsilonproteobacteria, *Vibrio*, *Pseudomonas* and *C. crescentus*. Regulatory proteins and sigma factors involved in controlling the transcriptional hierarchies are indicated above the arrows. Early, middle, and late genes are indicated between the arrows.

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CHAPTER 3

REQUIREMENT OF THE FLAGELLAR PROTEIN EXPORT APPARATUS COMPONENT FliO FOR OPTIMAL EXPRESSION OF FLAGELLAR GENES IN *HELICOBACTER PYLORI*¹

¹ Tsang, J., and T. R. Hoover. 2014. *Journal of Bacteriology*. **196**: 15, 2709-2717.
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Abstract

Flagellar biogenesis in *Helicobacter pylori* involves the coordinated expression of flagellar genes with assembly of the flagellum. The *H. pylori* flagellar genes are organized into three regulons based on the sigma factor needed for their transcription (RpoD (σ^{80}), RpoN (σ^{54}) or FliA (σ^{28})). Transcription of RpoN-dependent genes is activated by a two-component system consisting of the sensor kinase FlgS and the response regulator FlgR. While the cellular cues sensed by the FlgS/FlgR two-component system remain to be elucidated, previous studies revealed that disrupting certain components of the flagellar export apparatus inhibited transcription of the RpoN regulon. FliO is the least conserved of the membrane bound components of the export apparatus and has not been annotated for any of the *H. pylori* genomes sequenced to date. A PSI-BLAST analysis identified a potential *H. pylori* FliO which membrane topology algorithms predict to possess a large N-terminal periplasmic domain that is absent in FliO of *Escherichia coli* and *Salmonella*, the paradigms for flagella structure/function studies. FliO was necessary for flagellar biogenesis as well as wild-type levels of motility and transcription of RpoN-dependent and FliA-dependent flagellar genes in *H. pylori* strain B128. FliO also appears to be required for wild-type levels of the export apparatus protein FlhA in the membrane. Interestingly, the periplasmic and cytoplasmic domains were somewhat dispensable for flagellar gene regulation and assembly, suggesting that these domains have relatively minor roles in flagellar synthesis.

Introduction

Helicobacter pylori is a member of the Epsilonproteobacteria that colonizes the human gastric mucosa where it can cause a variety of diseases, including chronic gastritis, peptic and duodenal ulcers, B cell MALT lymphoma and gastric adenocarcinoma (1-3). *H. pylori* is highly

motile via a cluster of sheathed polar flagella. Motility of *H. pylori* is required for virulence as non-motile mutants are unable to colonize the gastric mucosa of model animal systems (4, 5).

The bacterial flagellum is a helical propeller driven by a rotary motor and consists of three basic structures referred to as the basal body, hook and filament (6). The basal body is a complex, multifaceted structure that contains the flagellar rod, rings, motor, switch complex, and a specialized type III secretion system that transports axial components of the flagellum (e.g., rod, hook and filament proteins) across the cell membrane (6-8). In *Salmonella*, a model organism for bacterial flagellum studies, the flagellar protein export apparatus consists of integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR) which form an export pore and cytoplasmic components (FliI, FliH and FliJ) which deliver protein substrates to the export pore (9). With the exception of FliO, orthologs for all of the export apparatus proteins have been tentatively identified or annotated in at least some of the over 200 *H. pylori* genomes that have been sequenced to date.

Where it has been examined, expression of flagellar genes is controlled by a transcriptional hierarchy that coordinates the synthesis of flagellar proteins with assembly of the nascent flagellum (10, 11). Transcription of flagellar genes in *H. pylori* is governed by all three sigma factors found in the bacterium (Fig. 3.1). Sigma (σ) factors bind core RNA polymerase and allow the resulting RNA polymerase holoenzymes to recognize specific promoters (12). Transcription of genes required early in flagellar assembly in *H. pylori* is dependent on the primary σ factor RpoD (σ^{80}), while transcription of genes needed later in flagellar biogenesis is dependent on RpoN (σ^{54}) and transcription of genes needed near the end of the assembly process is dependent on FliA (σ^{28}) (13). The organization of *H. pylori* flagellar genes into three regulons based on the σ factor needed for transcription suggests a framework for a transcriptional

hierarchy operating in conjunction with flagellar assembly. The molecular mechanisms by which such a hierarchy might be regulated, however, have yet to be elucidated.

Products of the RpoN-dependent genes in *H. pylori* include rod proteins, hook protein, hook-associated proteins, the hook length control protein (FliK), a minor flagellin (FlaB), and enzymes required for flagellin glycosylation (13-15). A two-component regulatory system composed of the sensor kinase FlgS and the response regulator FlgR activates transcription of the *H. pylori* RpoN regulon (13, 14, 16, 17). The *H. pylori* RpoN regulon is linked with the export apparatus as mutations in genes encoding various components of the export apparatus generally inhibit expression of RpoN-dependent flagellar genes (13, 18-21). While the mechanism by which the export apparatus influences expression of the *H. pylori* RpoN regulon is not known, it is likely mediated through the FlgS/FlgR two-component system. In support of this hypothesis, a constitutively active form of FlgR partially restored expression of an RpoN-dependent reporter gene in export apparatus mutants of *Campylobacter jejuni* (a member of the Epsilonproteobacteria that is closely related to *H. pylori*) (22).

To further examine the role of the export apparatus in expression of the *H. pylori* RpoN regulon, we characterized a potential FliO (HP0583 in *H. pylori* 26695) in *H. pylori* strain B128. FliO shows the least conservation among export apparatus proteins, and even appears to be absent in some systems (23). Its role in the export apparatus is poorly defined, but deletion of *fliO* in *Salmonella* results in a drastic decrease in motility (24). The *H. pylori* FliO homolog differs from *Salmonella* FliO in that it is predicted to possess a large N-terminal periplasmic domain that is absent in the *Salmonella* protein (Fig. 3.2B). FliO was required for wild-type motility and flagellation, as well as transcription of both RpoN-dependent and FliA-dependent flagellar genes in *H. pylori*. Interestingly, FliO variants that lacked either the periplasmic or

cytoplasmic domains partially restored flagellation as well as transcription of RpoN-dependent and FliA-dependent flagellar genes suggesting the transmembrane region of FliO is the most important determinant of the protein for flagellar biogenesis. Deletion of *fliO* resulted in reduced FlhA levels in the membrane suggesting that FliO is required for the assembly or stability of other components of the export apparatus.

Materials and Methods

Bacterial strains and growth conditions. *Escherichia coli* DH5 α was used for cloning and plasmid construction. *E. coli* strains were grown at 37°C in Luria-Bertani broth or agar supplemented with ampicillin (100 μ g/ml), kanamycin (30 μ g/ml), or chloramphenicol (30 μ g/ml) when appropriate. *H. pylori* B128 (kindly provided by Dr. Richard Peek, Jr.) was grown at 37°C microaerobically (2% O₂, 5% CO₂, and 93% N₂) on tryptic soy agar (TSA) supplemented with 10% horse serum or under an atmosphere consisting of 8.3% O₂, 4.6% CO₂, 9.2% H₂, and 77.9% N₂ in brain-heart infusion (BHI) broth supplemented with 0.4% β -cyclodextrin. Kanamycin (30 μ g/ml) or chloramphenicol (30 μ g/ml) was added to the medium used to culture *H. pylori* when appropriate.

DNA sequencing. The region surrounding the putative *fliO* in *H. pylori* B128 was amplified using primers B128 *fliO* forward and B128 *fliO* reverse with iProof high-fidelity DNA polymerase (Bio-Rad) and cloned into pGEM-T Easy (Promega). Primers used are listed in Table 3.1. Both strands of the cloned DNA within the resulting plasmid were sequenced. All DNA sequencing was done using a commercial service (Genewiz, Inc., South Plainfield, NJ).

Construction of *fliO* mutation in *H. pylori* B128. The *fliO* homolog in *H. pylori* B128 (locus tag HPB128_25g10) was deleted as follows. Overlapping PCR was used to generate an

amplicon that contained a chloramphenicol acetyltransferase (*cat*) gene flanked by ~500 bp regions located upstream and downstream of the *fliO* homolog. iProof DNA polymerase was used for all PCR procedures for strain construction. Genomic DNA from *H. pylori* 26695 was prepared using the Wizard Genomic DNA purification kit (Promega) and was used as template to amplify the regions flanking the *fliO* homolog, and the *cat* cassette was amplified from pUC20cat (25) using the *cat* forward and *cat* reverse primers. Overlapping PCR via the complementary regions of the amplified *cat* cassette and the amplicons of the regions flanking *fliO* generated a product with the *cat* cassette between the flanking regions. The final amplicon was introduced into *H. pylori* B128 by natural transformation and chloramphenicol resistant mutants were selected on TSA medium supplemented with the antibiotic. The *fliO* mutation in *H. pylori* B128 was confirmed by amplifying the regions around the targeted genes and sequencing the resulting amplicons.

Complementation of the Δ *fliO* mutant. The *fliO* deletion mutant was complemented by expressing wild-type *fliO* or truncated versions of *fliO* on the shuttle vector pHel3 (26). These *fliO* alleles were placed under the native promoter located upstream of *fliN* (27). The *fliN* promoter region was amplified by the primers, P*fliN* forward and P*fliN* reverse. The P*fliN* reverse primer contains a sequence complementary to the primer, *fliO* forward. The wild-type *fliO* was amplified with primers, *fliO* forward and *fliO* reverse. The *fliN* promoter and *fliO* were fused by overlapping PCR. The resulting amplicon was cloned into pGEM-T Easy, sequenced and subcloned into pHel3 (26) to create p*fliO*, which was introduced into *H. pylori* by natural transformation.

Truncated *fliO* alleles were created by overlapping PCR to express either the N terminus of FliO or the C terminus of FliO along with the transmembrane regions. For the N-terminal

deletion (*fliO_{ΔN}*), amino acids 26 to 174 were replaced with the FLAG tag (DYKDDDDK). The *fliN* promoter and the first 75 bp of *fliO* were amplified with primers P*fliN* forward and *fliO_{ΔN}* reverse and the last 360 bp of *fliO* was amplified with primers *fliO_{ΔN}* forward and *fliO* reverse from *pfliO*. The primers *fliO_{ΔN}* reverse and *fliO_{ΔN}* forward have reverse and complementary sequences that code for the FLAG tag at their 5' end, which were used in overlapping PCR to create the *fliO_{ΔN}* allele. For the C-terminal deletion (*fliO_{ΔC}*), amino acids 217 to 283 were replaced with the FLAG tag. The *fliN* promoter and the first 648 bp of *fliO* were amplified with primers P*fliN* forward and *fliO_{ΔC}* reverse and the last 33 bp of *fliO* were amplified with *fliO_{ΔC}* forward and *fliO* reverse 2 from *pfliO*. The primers *fliO_{ΔC}* reverse and *fliO_{ΔC}* forward also have reverse complementary sequences that encode the FLAG tag at their 5' end, which are used in overlapping PCR to create the *fliO_{ΔC}* allele. For the N- and C-terminal deletion, plasmid *pfliO_{ΔN}* was used as the template. Primers P*fliN* forward and *fliO_{ΔC}* 2 reverse were used to amplify the *fliN* promoter and *fliO_{TM}* to introduce a C-terminal deletion within the *fliO_{ΔN}* allele. Amplicons bearing the three *fliO* alleles were cloned into pGEM-T Easy (Promega), sequenced and subcloned into pHel3. The resulting plasmids bearing the truncated *fliO* alleles (*pfliO_{ΔN}*, *pfliO_{ΔC}* and *pfliO_{TM}*) were introduced into the Δ *fliO* mutant by natural transformation.

Construction of *rpoN* mutant. A disruption in *rpoN* was constructed in *H. pylori* B128 by allelic exchange. The suicide vector used to generate the *rpoN* mutant was created by amplifying *rpoN* from *H. pylori* 26695 genomic DNA and cloning the resulting amplicon into the NdeI and HindIII sites of pET28a. A unique EcoRI site was introduced ~370 bp downstream of the start codon of *rpoN* as described (28) and a *cat* cassette was inserted into the EcoRI site. This plasmid was introduced into *H. pylori* B128 by natural transformation. The *rpoN* mutation in *H.*

pylori was confirmed by amplifying the region surrounding *rpoN* and sequencing of the resulting amplicon.

Motility assay. Motility was assayed using semisolid medium consisting of Mueller-Hinton broth and 0.4% Noble agar. After autoclaving, the medium was supplemented with sterile 10% heat inactivated horse serum, 10 μ M FeSO₄, and 20 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.0). Kanamycin (30 μ g/ml) and chloramphenicol (30 μ g/ml) were supplemented when appropriate. A sterile toothpick was used to inoculate the cells into the agar. Plates were incubated at 37°C under an atmosphere consisting of 2% O₂, 5% CO₂, and 93% N₂. Diameters of the spreading *H. pylori* cells were measured after 7 days. For each strain, four stabs were analyzed. Statistical significance was determined using the two-sample *t*-test.

Electron microscopy. Strains were grown to mid- to late-log phase in BHI supplemented with 0.4% β -cyclodextrin to an OD₆₀₀ of 0.5 to 1.0. Kanamycin (30 μ g/ml) was included in the medium for cultures of cells carrying derivatives of pHel3. Cells were pelleted by centrifugation for 1 min at 16,000 x g and then resuspended in half strength Karnovsky's fixative (2.5% gluteraldehyde, 2% paraformaldehyde, 0.1M cacodylate buffer). Cells were fixed for 5 min and then transferred to 300-mesh Formvar-coated copper grids. After 5 minutes, grids were washed with 0.1 M cacodylate buffer, followed by a wash with deionized water. Excess liquid was wicked off with filter paper between washes. One drop of 1% uranyl acetate was applied to the grids for 30 seconds and then wicked off with filter paper. Grids were washed in deionized water and dried at room temperature overnight. Cells were visualized using the FEI Tecnai20 transmission electron microscope. For each strain, at least 115 cells were included for determining the proportion of flagellated cells and the number of flagella per cell. Statistical

analyses were performed using the Mann-Whitney U test to determine whether strains were significantly different from one another with regard to the number of flagella per cell.

Motility enrichment. Semisolid agar medium was inoculated by the pre-enrichment *ΔfliO* stock as described in Materials and Methods. After growth for seven days, cells from the edge of the halo were used to inoculate a fresh semisolid agar plate and to make a frozen glycerol stock.

This motility enrichment process was repeated three times in the subsequent weeks to obtain the post-enrichment stock. After all stocks were made, the level of motility from each stock was determined on semisolid agar plates. Cells from the pre-motility enrichment stock and from the post-motility enrichment stock were analyzed by electron microscopy as described above.

Statistical significance between the pre-motility enrichment and post-motility enrichment samples was analyzed using the Mann-Whitney U test and the Fisher exact test.

RNA extraction and cDNA synthesis. *H. pylori* cells were grown on TSA supplemented with 10% horse serum for 18 hours before harvesting and resuspending into 1 ml of nuclease-free water. Cells were pelleted by centrifugation for 1 min at 16,000 x g and then resuspended into 100 μl of nuclease-free deionized water. The Aurum Total RNA Mini Kit (Bio-Rad) was used to isolate RNA, and the RNA solution was treated with the TURBO DNA-free Kit (Ambion) to remove any contaminating DNA. RNA was quantified using a BioPhotometer (Eppendorf) and RNA quality was confirmed on a 1.2% agarose gel. Single-strand cDNA was synthesized from 200 ng of RNA using the iScript cDNA Synthesis Kit (Bio-Rad). The efficiency of cDNA synthesis was assessed by analyzing a series of serial dilutions of each cDNA preparation using quantitative reverse-transcription polymerase chain reaction. If cDNA synthesis occurred at 100% efficiency, a theoretical -3.4 change in cycle threshold is expected for each 10-fold dilution of cDNA.

Quantitative reverse-transcription PCR. Transcript levels of *flaA*, *flaB*, *flgE*, *flgS*, *flgR*, *fliA* and *flhA* were monitored by qRT-PCR using the Bio-Rad iCycler iQ System. Primers used are listed in Table 3.2. *gyrA* transcript levels were measured as a reference gene as *gyrA* levels remain constant during exponential phase (29). Specificity and efficiency of each primer pair was confirmed by PCR using genomic DNA and by qRT-PCR on a serial dilution of wild-type cDNA. Each qRT-PCR reaction, totaling 20 μ l, consisted of 10 μ l of iQ SYBR Green Supermix (Bio-Rad), 5 μ l of 100- fold diluted cDNA from the cDNA synthesis reaction, and 200 nM of each primer. In separate reactions, 5 μ l of dH₂O was used in place of diluted cDNA as a no template control and 5 μ l of 100-fold diluted RNA was used in place of cDNA as a control to ensure that cDNA samples did not contain contaminating DNA from the RNA extractions. A melt curve analysis was performed at the end of each experiment. Experiments were performed in technical triplicate for three biological replicates of each strain. Gene expression levels were quantified by the $2^{-\Delta\Delta Ct}$ method (30). Statistical significance was determined using the two-sample *t*-test.

Detection of FlhA. For detection of FlhA, *H. pylori* membrane fractions were collected as described previously (31). Cells were grown for three days on TSA agar plates supplemented with 10% horse serum and antibiotics where appropriate and resuspended into a buffer containing 10% sucrose, 20 mM HEPES, and 1 mM EDTA, pH 7.4 (Buffer A). Cells were lysed by three passages through a French pressure cell at 10,000 kPa. Unlysed cells and cellular debris were removed by centrifugation for 15 min at 6,000 x g. Membranes were separated from cytoplasmic proteins by centrifugation for 60 min at 100,000 x g and collected into a 0.25 ml pad of 30% sucrose, 20 mM HEPES, and 1 mM EDTA, pH 7.4. The membrane fraction was diluted in Buffer A and the centrifugation was repeated. The cytoplasmic fraction was precipitated

using trichloroacetic acid as describe previously (32). Protein concentrations of the fractions were determined using the bicinchoninic acid protein assay. Membrane fractions were analyzed by western blot using affinity-purified FlhA antibodies (31) or KatA antiserum (kindly provided by Stéphane Benoit). Goat anti-rabbit horseradish peroxidase-conjugated antibody (Bio-Rad) was used as a secondary antibody. Antigen-antibody complexes were detected by chemiluminescence using the SuperSignal West Pico Luminol/Enhancer Solution and SuperSignal West Stable Peroxide Solution (Thermo Scientific). Blots were visualized using the FluoroChem E imager (ProteinSimple). Cross-reacting proteins in the western blots were quantified by densitometry using ImageJ (<http://rsbweb.nih.gov/ij/>) for three biological replicates and statistical significance was determined using the two-sample *t*-test.

Detection of RpoN. For detection of *H. pylori* RpoN, antibodies directed against MBP-RpoN were affinity-purified using the AminoLink Plus Immobilization Kit (Thermo Scientific) as follows. MBP-RpoN was purified as previously described (28) and immobilized to the AminoLink Plus Resin following the manufacturer's protocol. Two milliliters of antiserum was buffer-exchanged into phosphate-buffered saline (PBS) by diluting with 13 ml PBS, reducing volume to 0.5 ml using an Amicon Ultra-15 centrifugal filter two times. The buffer-exchanged antiserum was incubated with the AminoLink Plus Resin in the column to allow for binding to MBP-RpoN. The column was washed with 10 ml PBS before eluting the antibody with 8 ml of 0.1M glycine, pH 2.5. The eluted antibody was dialyzed against 4 L citric acid-phosphate buffer (55 mM citric acid, 50 mM K₂HPO₄, pH 5.5) for 18 hours, followed by 4 L Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6) two times for 3 hours each and one time for 18 hours. The dialyzed antiserum was stored at -20°C.

H. pylori cytoplasmic fractions were prepared for western blot analysis as follows. Cells were grown on agar medium for 24 hours before resuspending into 3 ml PBS. Cells were lysed with three passages through a French press at 10,000 kPa. Cellular debris was removed by centrifugation for 15 min at 6000 x g. Membranes were separated from cytoplasmic proteins by centrifugation for 60 min at 100,000 x g. The supernatant containing the cytoplasmic proteins was concentrated by trichloroacetic acid precipitation as described previously (32). Protein concentrations were determined using the bicinchoninic acid protein assay (Thermo Scientific) following the manufacturer's instructions. 20 µg protein was analyzed by western blotting using the affinity-purified RpoN antibody. Western blots were analyzed using the secondary antibody and imaged as described above.

Results

Identification of a FliO homolog in *H. pylori*. Of the six integral membrane proteins that comprise the flagellar protein export apparatus, FliO is the least conserved and may even be absent in some bacteria. FliO homologs have not been identified in any of the more than 200 *H. pylori* strains whose genomes have been sequenced to date. However, a gene downstream of *fliN* is annotated as a member of the FliO protein family (pfam04347) in *Helicobacter bilis*, *Helicobacter cinaedi* and *Helicobacter hepaticus*. In addition, Pallen and co-workers tentatively identified a *fliO* homolog located immediately downstream of *fliN* (encodes a flagellar motor switch protein) in *C. jejuni* by analyzing the open reading frame using PSI-BLAST (23). The annotation of the *C. jejuni* *fliO* homolog is consistent with the synteny of *fliN* and *fliO* in other bacteria, including *E. coli* and *Salmonella*. Immediately downstream of *fliN* in *H. pylori* 26695 is an open reading frame (HP0583) which we identified as encoding a potential FliO homolog

from PSI-BLAST analysis (Fig. 3.2A). Of the FliO homologs found in the 247 sequenced *H. pylori* genomes in Joint Genome Institute's Integrated Microbial Genomes (IMG) database, FliO homologs among *H. pylori* strains shared 84-100% identity and 88-100% similarity with the corrected FliO sequence from *H. pylori* B128 (see below).

Potential *fliO* homologs are similarly located immediately downstream of *fliN* in other *Helicobacter* species. An examination of sequence information for the 18 *Helicobacter* species in the National Center for Biotechnology Information (NCBI) and the IMG databases, however, revealed that the putative FliO proteins in these bacteria vary markedly in their sequence. Only about half of these FliO proteins (8 out of 17) had significant homology with *H. pylori* FliO over their entire length (expected values $<1e^{-10}$ from a BLAST analysis for the entire 293 amino acid sequence of *H. pylori* FliO). These FliO homologs share 25-79% identity and 45-86% similarity with *H. pylori* FliO. Expected values for comparison of *H. pylori* FliO with the FliO homologs from the remaining *Helicobacter* species were $>1e^{-3}$ in the BLAST analysis. A BLAST analysis of the N-terminal region and the C-terminal region of FliO (see below) indicated that neither domain was more conserved than the other. Out of the 8 species with significant homology to the *H. pylori* FliO protein, 5 species contained homology with the N-terminal region of FliO with an e-value $< 2 e^{-4}$ and 6 species contained homology with the C-terminal region of FliO with an e-value $< 2 e^{-5}$. In contrast to the FliO homologs, sequences of the other integral membrane components of the flagellar protein export apparatus (FlhA, FlhB, FliP, FliQ and FliR) and FliN were highly conserved among the various *Helicobacter* species. Comparing sequences of these proteins from *H. pylori* with their counterparts from the 17 other *Helicobacter* species revealed robust sequence conservation over the entire lengths of the proteins, with FliR displaying the

least conservation (46-96% identity and 67-98% similarity) and FliQ displaying the highest conservation (71-98% identity and 85-98% similarity).

Because *H. pylori* 26695 is non-motile due to a frameshift mutation in *fliP* (33), *H. pylori* B128 was chosen for our studies. The predicted open reading frame located downstream of *fliN* in *H. pylori* B128 (locus tag HPB128_25g10) is substantially shorter than its counterpart in *H. pylori* 26695, corresponding to amino acid residues Met-13 through Pro-142 of the *H. pylori* 26695 FliO homolog. To determine if the *H. pylori* B128 protein is indeed truncated, the region surrounding the predicted reading frame was amplified using a high fidelity DNA polymerase and sequenced. The sequence of the amplified DNA differed from that of the corresponding region in the draft sequence of the *H. pylori* B128 genome by two nucleotides out of 918 base pairs of DNA sequence. The draft genome sequence contains a TA base pair at position 391 which was not present in the sequence of the amplicon. This additional base pair results in a TAA stop codon immediately following codon 130 and corresponds to Pro-142 of the *H. pylori* 26695 FliO homolog. The genome sequence also contains an AT base pair located 192 nucleotides downstream of the other insertion and was likewise not present in the amplicon sequence. Some flagellar genes, such as *flgR* from *C. jejuni*, are known to be subject to phase variation resulting from the loss or gain of a nucleotide in homopolymeric tract (34). Neither of the differences between the *fliO* sequences from the draft genome sequence and that which we determined, however, was within a homopolymeric tract. Thus, the differences in the sequences do not appear to be due to phase variation, but instead may be due to sequencing errors in the draft sequence of the *H. pylori* B128 genome.

The revised sequence for the region surrounding the *H. pylori* B128 *fliO* homolog revealed an open reading frame encoding a protein of 293 amino acid residues which shared 92%

amino acid identify with the *H. pylori* 26695 FliO homolog over its entire length. *H. pylori* FliO is significantly larger than that of *E. coli* or *Salmonella* which is only 125 amino acid residues in length. There are two potential AUG start codons 36 nucleotides apart in *fliO* from *H. pylori* strains B128 and 26695 as well as most of the other 240 *H. pylori* strains whose genome sequences are in the IMG database. It is unclear which of the AUG sequences is the actual start codon. For many of the *H. pylori* FliO proteins in the IMG database the first AUG is indicated as the start codon (51 out of 246), while the second AUG is indicated as the start codon for the remaining *H. pylori* FliO proteins. For *H. pylori* 26695 and B128, the upstream AUG overlaps *fliN* by four nucleotides. There is not a well-defined ribosome binding site upstream of either of the potential start codons for *H. pylori* B128 *fliO* (sequences upstream of the first and second AUG codons are 5'-TTATCTCGCTAAAAATTC-3' and 5'-TTACTGAGCGCTACTTTG-3', respectively). Given the ambiguity in assigning the start codon of *fliO*, we included the first AUG codon in all plasmid constructs used for expression of full-length or truncated versions of *fliO*.

The topology of the *H. pylori* B128 FliO was analyzed using two topology prediction programs – TopPred (<http://mobylipe.pasteur.fr/cgi-bin/portal.py?#forms::toppred>) and TMPred (http://www.ch.embnet.org/software/TMPRED_form.html). The programs predicted two transmembrane regions corresponding to amino acid residues 1-21 and 187-207 or 1-18 and 188-206, respectively. TMPred predicted the C-terminal region of the *H. pylori* B128 FliO homolog to be located on the cytoplasmic side of the membrane and the N-terminal region of the protein to be located on the periplasmic side of the membrane (Fig. 3.2B). This is consistent with the topology of the *Salmonella* FliO determined by Barker and co-workers using membrane topology prediction programs and chimeric proteins in which FliO was fused to alkaline

phosphatase or green fluorescent protein (24). The predicted periplasmic domain of the *H. pylori* FliO homolog (~160 amino acid residues) is much larger than that in FliO from *E. coli* and *Salmonella* (22 amino acid residues).

The *H. pylori* B128 FliO homolog is required for normal motility and flagellar biogenesis.

The *H. pylori* B128 *fliO* homolog was disrupted with the *cat* cassette and the phenotype of the resulting mutant was analyzed. The motility of the $\Delta fliO$ mutant was significantly reduced (Fig. 3.3B) but at least some of the cells appeared to be motile as evidenced by the small halo around the point of inoculation which was not observed with the non-motile $\Delta flhA$ mutant (Fig. 3.3G) (31). Transmission electron microscopy revealed that most of the $\Delta fliO$ mutant cells were aflagellated (93.5%), although some cells possessed either a single flagellum (6%) or two flagella (0.5%) (Fig. 3.4B). In contrast, the majority of wild-type cells were flagellated (93%) with most of the cells possessing two to four flagella (Fig. 3.4A). These results suggested that FliO is required for optimal flagellar biogenesis but is not absolutely essential since some $\Delta fliO$ mutant cells were flagellated.

We attempted to complement the $\Delta fliO$ mutation by expressing *fliO* from the *hp0405* locus in the *H. pylori* chromosome, but we were unable to achieve significant restoration of motility (data not shown). We postulate that the failure to complement the $\Delta fliO$ mutation with a copy of *fliO* introduced into the *hp0405* locus is due contextual issues which resulted in poor expression of the gene. Introducing a wild-type copy of *fliO* on the shuttle vector pHel3 (*pfliO*) into the $\Delta fliO$ mutant ($\Delta fliO$ / *pfliO*) resulted in a partial restoration of motility in the soft agar (Fig. 3.3D). Introduction of *pfliO* into the wild-type strain (WT / *pfliO*) reduced the motility of the strain in soft agar to that of $\Delta fliO$ / *pfliO* (Fig. 3.3C), whereas introduction of just pHel3 into the wild-type strain had no effect on motility (data not shown). The copy number of pHel3 is

~10 (26) and the additional copies of *fliO* may result in elevated levels of FliO which could interfere with assembly of the export apparatus or regulation of the transcriptional machinery controlling flagellar gene expression. Alternatively, the plasmid-borne *fliO* may interfere with motility by titrating transcription factors needed for the normal regulation of other flagellar genes. The $\Delta fliO$ / *pfliO* and WT / *pfliO* strains were flagellated to similar extents with ~70% of the cells possessing from one to five flagella (Fig. 3.4C-D). Taken together, these results verify that deletion of the *H. pylori fliO* homolog was responsible for decreased flagellar biogenesis.

Barker and co-workers showed that incubation of a *Salmonella* $\Delta fliO$ mutant in soft agar gives rise to motile pseudorevertants that contain extragenic bypass mutations in *fliP* (24). To determine if the flagellated *H. pylori* $\Delta fliO$ cells similarly possessed extragenic bypass mutations we tried to enrich for cells in which a higher proportion of the population was flagellated. To do so, the $\Delta fliO$ mutant was subjected to repeated rounds of inoculation in soft agar, allowing the cultures to grow for several days, and then picking bacteria from the periphery of the halo and re-inoculating these cells into fresh soft agar. The $\Delta fliO$ mutant displayed a slight increase in motility during the enrichment (Fig. 3.5) but there was not a concomitant increase in the proportion of flagellated cells (Fig. 3.6). These observations suggest that the flagellated $\Delta fliO$ cells do not have stable extragenic bypass mutations that allow them to produce flagella, but rather that the export apparatus stochastically assembles with low efficacy into a fully functional form in the absence of FliO.

FliO is required for wild-type levels of flagellar gene expression. To determine if disruption of *fliO* affected flagellar gene expression, transcript levels of two RpoN-dependent flagellar genes, *flaB* and *flgE*, and one FliA-dependent flagellar gene, *flaA*, were examined in wild-type and $\Delta fliO$ mutant strains. Compared to wild type, the $\Delta fliO$ mutant displayed a ~24-fold

reduction in *flaB* and *flgE* transcript levels (Figs. 3.4A-B), and a ~7-fold reduction in *flaA* transcript levels (Fig. 3.7C). The *fliO* mutant displayed wild-type levels of *flgR*, *flgS* and *fliA* transcripts (Fig. 3.7D) and RpoN protein (Fig. 3.7E), indicating that the lower levels of *flaB*, *flgE* and *flaA* transcripts in the mutant were not due to reduced expression of regulatory genes known to be required for the transcription of the RpoN and FliA regulons. Introduction of *pfliO* into the Δ *fliO* mutant resulted in a partial restoration of *flaB*, *flgE* and *flaA* transcript levels (Figs. 3.4A-C). Introducing *pfliO* into the wild-type strain depressed *flaB* and *flgE* transcript levels such that they were similar to those for the complemented Δ *fliO* mutants (Figs. 3.4A-B).

The C-terminal and N-terminal domains of FliO are not individually essential for flagellar biogenesis. Barker and co-workers reported that overexpression of either the C-terminal domain of FliO or the first 95 amino acid residues of FliO restored motility in a *Salmonella fliO* deletion mutant (24). We wished to determine if truncated versions of FliO could similarly restore motility in the *H. pylori* Δ *fliO* mutant. Therefore, we constructed plasmids containing alleles encoding truncated versions of FliO in which the N-terminal domain (residues 26 to 174; *pfliO_{ΔN}*) or the C-terminal domain (residues 217 to 283; *pfliO_{ΔC}*) was deleted and examined the ability of the FliO variants to restore flagellar biogenesis in the Δ *fliO* mutant. A FLAG tag was introduced into the truncated FliO proteins encoded by the *fliO_{ΔN}* and *fliO_{ΔC}* alleles to verify that the proteins were expressed and incorporated into the cell membrane. However, we were unable to identify the truncated FliO proteins in immunoblots of membrane fractions prepared from strains bearing the *pfliO_{ΔN}* or *pfliO_{ΔC}* alleles (data not shown). Our failure to detect the truncated FliO proteins was likely due to the low level at which these proteins were expressed and the presence other cross-reacting proteins in the region of the gel where we expected the FliO variants to migrate. Despite our failure to detect the truncated FliO proteins, introduction of the plasmid-borne copies

of either the *fliO_{ΔN}* or the *fliO_{ΔC}* allele into the Δ *fliO* mutant partially restored motility (Fig. 3.3E-F) and increased the proportion of flagellated cells from 6.5% to ~40% (Fig. 3.4E-F). Despite being indistinguishable in the degree to which they were flagellated, the Δ *fliO* strain complemented with *pfliO_{ΔC}* appeared to be more motile than the Δ *fliO* strain complemented with *pfliO_{ΔN}* (Figs. 3.3E and 3.3F). The reason for this difference in motility is not known, but it may indicate a role for the predicted periplasmic domain of FliO in chemotaxis or flagellar function. Introduction of the plasmid-borne *fliO_{TM}* allele in which both the N- and C- terminal regions were deleted did not restore motility (data not shown). It is not known if the FliOTM variant was stably expressed and inserted into the membrane so studies with the Δ *fliO* mutant bearing the *fliO_{TM}* allele were not pursued further. Transcription of *flaB*, *flgE* and *flaA* was restored in the Δ *fliO* mutant complemented with either of the *fliO_{ΔN}* or *fliO_{ΔC}* allele to levels that were comparable to that observed with *pfliO* (Figs. 3.4A-C). Taken together, these data indicate that the C-terminal and the N-terminal regions of FliO are individually dispensable for regulating transcription of the RpoN and FliA regulons.

FliO is needed for the assembly or stability of FlhA in the flagellar protein export

apparatus. We wished to determine if loss of FliO affected the expression and/or localization of other integral membrane components of the flagellar protein export apparatus which could account for the defects in flagellar biogenesis and gene expression seen in the Δ *fliO* mutant. To test this hypothesis, incorporation of FlhA into the membrane of the wild-type and Δ *fliO* strains, as well as the strains expressing the *fliO_{ΔC}* or *fliO_{ΔN}* allele was analyzed by western blotting. FlhA was detected in membrane fractions from wild-type cells but not membrane fractions from the Δ *flhA* mutant (Fig. 3.8, lanes 1 and 2). FlhA levels in the Δ *fliO* mutant were reduced ~2-fold compared to wild type (Fig. 3.8, lane 3). FlhA levels in the WT / *pfliO* strain were similar to that

in the wild type (Fig. 3.8, lane 4) and FlhA levels were restored to close to wild-type levels in the $\Delta fliO$ strain that expressed either the full-length *fliO* or the *fliO*_{ΔN} or *fliO*_{ΔC} allele (Fig. 3.8, lane 5-7). FlhA was not detected in the soluble fraction from any of the strains (data not shown). To confirm that comparable amounts of membrane proteins were analyzed for each of the samples, we examined catalase (KatA) levels in each sample by western blotting. KatA associates with the cell membrane through interaction with KapA, a twin-arginine target protein (35, 36). KatA was not detected in the soluble fractions of the cell extracts (Fig. 3.9) and so its presence in the membrane is a good control for protein load. KatA levels within the various membrane samples did not differ from each other significantly (p -value >0.05) indicating that the amounts of total membrane protein analyzed for the samples were comparable (Fig. 3.8). *flhA* transcript levels in the $\Delta fliO$ mutant were similar to wild-type levels suggesting that the changes in FlhA levels in the $\Delta fliO$ mutant membrane were not due to decreased transcription of *flhA* (data not shown). Taken together, these data indicate that FliO is needed for wild-type accumulation of FlhA in the export apparatus, perhaps by influencing the stability of FlhA or its assembly into the export apparatus. The transmembrane portion of FliO appears to be primarily responsible for the stability of FlhA since FliO variants which lack either the N-terminal or C-terminal domain support near normal accumulation of FlhA in the membrane. It is possible that levels of other components of the export apparatus are similarly reduced in the $\Delta fliO$ mutant.

Discussion

FliO exhibits the lowest conservation among the integral membrane components of the flagellar protein export apparatus. FliO is apparently absent in some systems such as *Aquifex aeolicus* or the lateral flagellar systems of *Chromobacterium violaceum* and *Vibrio*

parahaemolyticus (23). Given the low degree of conservation of FliO sequences even within the genera *Helicobacter*, it is possible that these systems that are thought to lack FliO have highly divergent FliO homologs which have been overlooked. FliO homologs in some bacteria, including *Bacillus* species and *Borrelia* species, are annotated as FliZ and are substantially larger than FliO from *E. coli* or *Salmonella*. FliO homologs in *H. pylori* and *C. jejuni* are similarly larger than their counterparts in *Salmonella* and *E. coli*, and are predicted to possess large periplasmic domains that are absent in *E. coli* or *Salmonella* FliO.

Loss of FliO in *H. pylori* appears to affect the organization or stability of other components within the export apparatus as levels of FlhA were reduced ~2-fold in the Δ *fliO* mutant (Fig. 3.8). *Salmonella* FlhA and FliO have been shown to interact with each other in affinity blots (37) and FliO may be necessary to stabilize FlhA in the membrane. The ability of mutations in *fliP* to suppress the Δ *fliO* mutation in *Salmonella* (24) suggests that FliO and FliP interact within the export apparatus. Further support for the interaction between these proteins comes from the existence of a *fliOP* gene fusion in *Buchnera aphidicola* (38). Though *B. aphidicola* does not produce flagella because it lacks the genes needed for filament formation, it does form hook-basal body complexes which may be used for export of other proteins (39, 40).

Although the mechanisms *H. pylori* and related bacteria use to couple transcription of the RpoN-dependent flagellar genes with assembly of the flagellum remain to be defined, a considerable amount of evidence has linked expression of the RpoN regulon in *H. pylori* and *C. jejuni* with the flagellar protein export apparatus. Inactivation of *fliQ* or *flhB* in *H. pylori* results in reduced levels of the minor flagellin FlaB and the hook protein FlgE, both of which are dependent on RpoN for their expression (19, 41). Using DNA microarray assays, Niehus and co-workers (13) demonstrated that FlhA is required for transcription of the RpoN regulon, and

deletion of *flhB* similarly inhibits expression of RpoN-dependent reporter genes (32).

Hendrixson and DiRita reported that deleting any one of several export apparatus components (FlhA, FlhB, FliP and FliR) inhibited expression of an RpoN-dependent flagellar reporter gene in *C. jejuni* (42).

We postulate that the export apparatus influences expression of the *H. pylori* RpoN-dependent flagellar genes through interactions with one or more of the regulatory proteins that control transcription of the RpoN regulon. A likely candidate for such interactions is the sensor kinase FlgS which, upon stimulation, would support the phosphorylation of FlgR allowing it to activate transcription of the RpoN regulon. Boll and Hendrixson proposed similar models for regulation of the RpoN regulon in *C. jejuni* and further showed that FlgS immunoprecipitates with FliF (flagellar MS ring protein) and FliG (a component of the C ring) (43). They proposed two models for how FliF, FliG and the export apparatus may activate FlgS autokinase activity. In the first model, the formation of the MS and C rings creates a cytoplasmic platform that is sensed by FlgS. In this model, other proteins such as FlhA, may contribute to the interaction between FlgS, FliG and FliF (43). Alternatively, formation of the MS and C rings may be required for assembly of a conformation of the export apparatus capable of interacting productively with FlgS (43). Our observation that loss of FliO negatively impacts the level of at least one other export apparatus component (i.e., FlhA), the phenotype of the *H. pylori* Δ *fliO* mutant is consistent with either of the models proposed by Boll and Hendrixson.

The results presented here show that FliO is unique among the export apparatus components in that while it is required for wild-type transcription of RpoN-dependent flagellar genes, it is not as essential as other export apparatus components such as FlhA or FlhB (22, 31, 32) where their loss abolishes flagellar biogenesis and motility. The molecular basis by which

the flagellar protein export apparatus influences transcription of the RpoN-dependent and FliA-dependent genes in *H. pylori* is complex. Characterizing the roles of FliO and other components of the export apparatus is critical for understanding how *H. pylori* coordinates flagellar gene expression with assembly of the flagellum.

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Author Contributions

JT and TRH designed the experiments and wrote the paper. JT performed the experiments and analyzed the data.

Table 3.1: Oligonucleotides used for this study.

<u>Primer</u>	<u>Sequence</u>
B128 fliO forward	GAT TCT AAC GCC ATT GTG AA
B128 fliO reverse	TCC TGT TAG AAT GAT CCA CA
cat forward	GAT ATA GAT TGA AAA GTG GAT
cat reverse	TTA TCA GTG CGA CAA ACT GGG
fliO upstream forward	GTC AGA AAC AGA AGC TAA TAA
fliO upstream reverse	ATC CAC TTT TCA ATC TAT ATC TCT TCA GCC AGT AAC ATA AAA
fliO downstream forward	CCC AGT TTG TCG CAC TGA TAA TAC GGC CTG CTT TTA GAC A
fliO downstream reverse	AGA AAG CTG GTT AGG GTC AA
PfliN forward	CTC GAG ACT CAG CGA TCT GTT TAA AGA
PfliN reverse	AGA TAA TAT ACA ATG GCG TTA GTT TCT TTT TCG GCT ACT TTT AA
fliO forward	CTA ACG CCA TTG TAT ATT ATC T
fliO reverse	ATT TTT CCC TAA CTT AGC AGG
fliO _N reverse	TTT ATC ATC ATC ATC TTT ATA ATC GTC ATC GTT TAA AGA TAT TT
fliO _N forward	GAT TAT AAA GAT GAT GAT GAT AAA AAC ACT CTA GAG TTA AAA C
fliO _C reverse	TTT ATC ATC ATC ATC TTT ATA ATC AGA CCC TTG TGT GGG
fliO _C forward	GAT TAT AAA GAT GAT GAT GAT AAA AAA TTA GGA AAT
fliO _C 2 reverse	GGA TCC TTA AGA CCT TTG TGT GGG

Table 3.2: qRT-PCR oligonucleotide primers used for this study.

<u>Primer</u>	<u>Sequence</u>
gyrA qPCR forward	GCT AGG ATC GTG GGT GAT GT
gyrA qPCR reverse	TGG CTT CAG TGT AAC GCA TC
flaA qPCR forward	GGC AAG CGT TAT TGT CTG GT
flaA qPCR reverse	CGA TAC GAA CCT GAC CGA TT
flgE qPCR forward	TGC GAA CGT GAA TAC CAC TG
flgE qPCR reverse	GTC ATT CTG CCC TGC TAA CC
flaB qPCR forward	ATC GCC GCT TTA ACT TCT CA
flaB qPCR reverse	CGC CAT CCC ACT AGA ATC AT
flgS qPCR forward	AGC CCT TTA TGA GTG GGT CA
flgS qPCR reverse	TTG AAC ACC TCA TGG CTT TG
flgR qPCR forward	GCC ATT GAT TCC ATT CGT TT
flgR qPCR reverse	GCC TGT CGT TTG CTC TCT TC
fliA qPCR forward	GCC TAT GAT GAG CAG CAA CA
fliA qPCR reverse	GAA TCG TTT AAC GCG CTC TC
flhA qPCR forward	GCG ATA TTA TCA CGG CGT TT
flhA qPCR reverse	CGC GCC CTA ACT TCA GTA AC

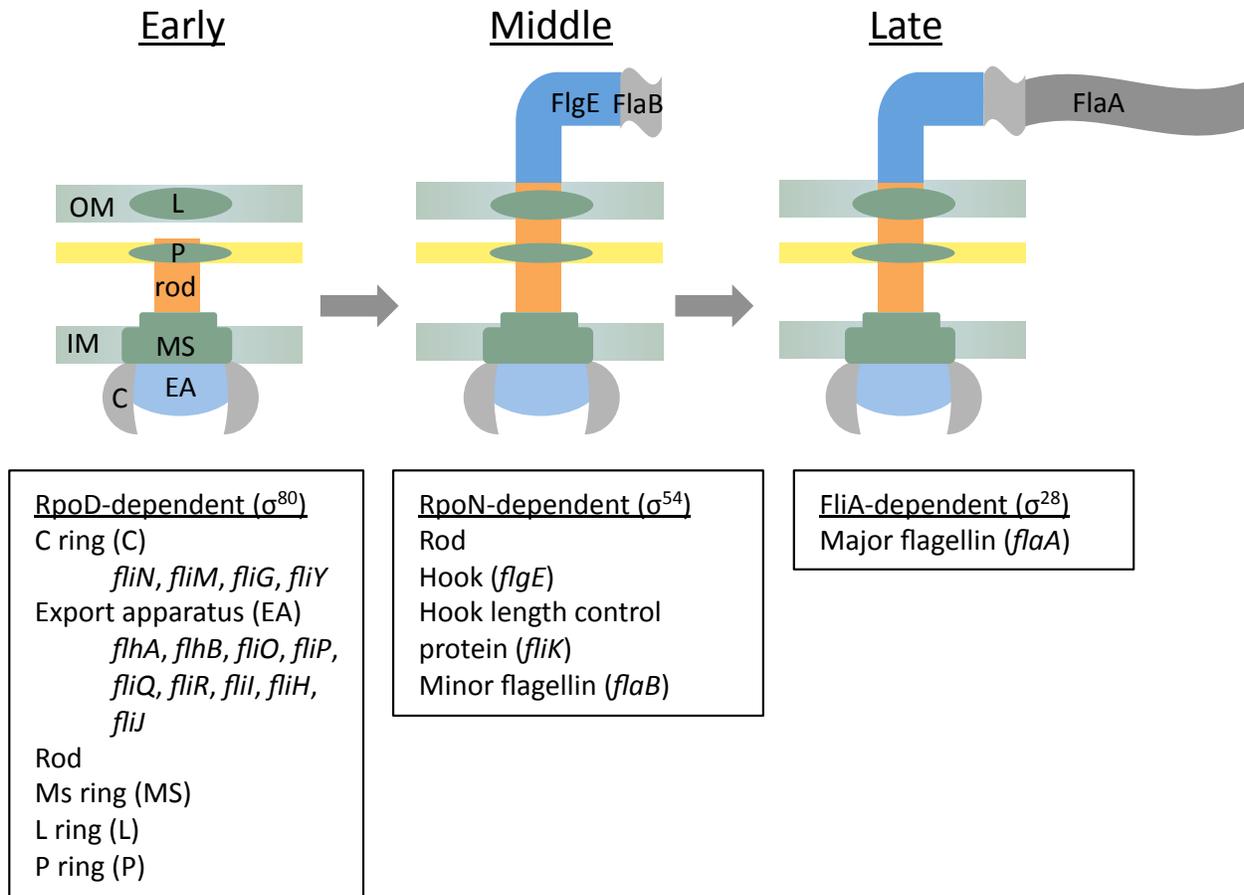
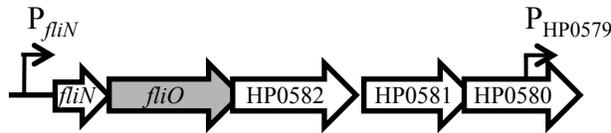
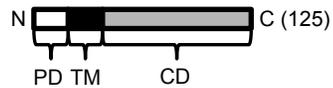


Figure 3.1: A transcriptional hierarchy for expression of flagellar genes. Only proteins and genes mentioned in this study are included in this figure.



B

Salmonella FliO



H. pylori FliO



Figure 3.2: Genome organization around the *hp0583* locus and predicted FliO structural

features. (A) *fliO* (*hp0583*) is predicted to be in an operon with *fliN*, *hp0582*, *hp0581*, and *hp0580*. The promoter for this operon (P_{fliN}) is located upstream of *fliN* (27) which encodes a flagellar motor switch protein. *hp0582* encodes a member of the TonB family C-terminal domain, *hp0581* encodes a possible dihydroorotase (*pyrC*) involved in pyrimidine biosynthesis, and *hp0580* encodes a possible neuraminidase/sialidase). (B) The FliO protein in *H. pylori* has an N-terminal domain not present in *Salmonella* or *E. coli* FliO proteins. The *Salmonella* FliO protein (top) is much shorter than the *H. pylori* FliO homolog (bottom). Transmembrane regions (TM) are shown in black, while N-terminal periplasmic domains (PD) are shown in white and C-terminal cytoplasmic domains (CD) are shown in grey. The predicted lengths of the proteins are indicated in parentheses.

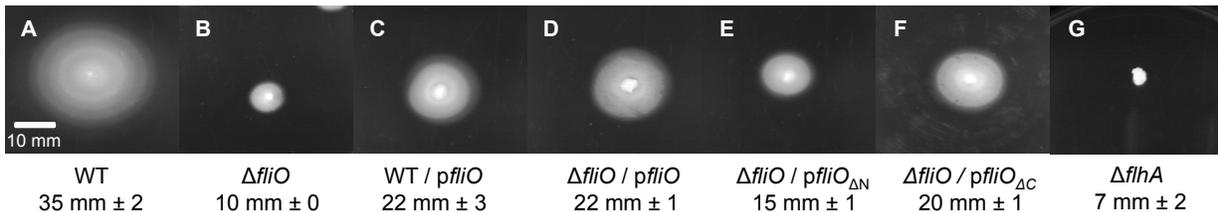


Figure 3.3: Effects of *fliO* alleles on motility. Motility of the $\Delta fliO$ mutant and *fliO* variants were assessed by stab inoculation on 0.4% agar plates. Measurements indicate diameter of halo around site of inoculation after 7 days of incubation in microaerobic conditions. For each strain, four different inoculations were analyzed. The motility of the wild-type strain (WT) was significantly different from that of the other strains (p -value < 0.002). Similarly, the motility of the $\Delta fliO$ mutant differed significantly from each of the complemented strains shown (p -value < 0.002), but not the $\Delta fliO$ / *pfliO*_{TM} (data not shown). A $\Delta flhA$ mutant was used as a non-motile control. Although the $\Delta fliO$ mutant clearly shows a halo around the point of inoculation that is not seen for the $\Delta flhA$, the size of the growth rings for the two strains did not differ significantly (p -value = 0.14).

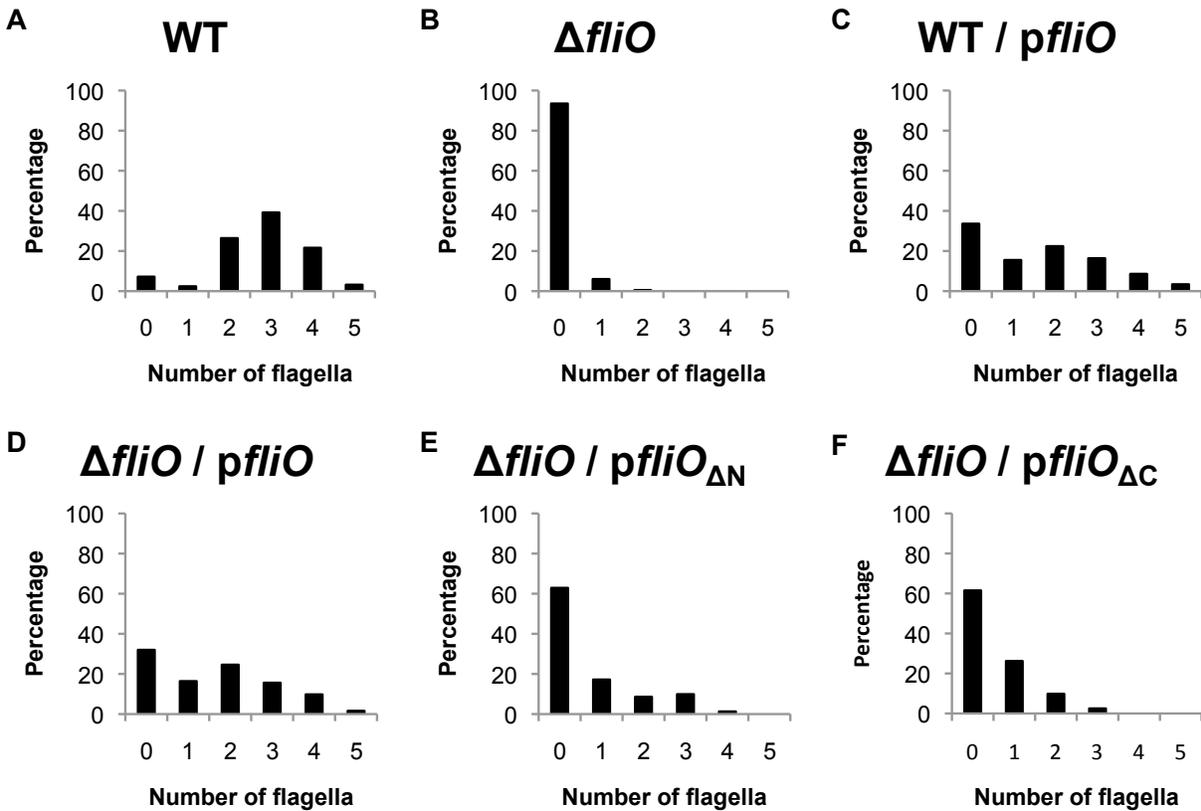


Figure 3.4: Effect of *fliO* mutations on flagellation in *H. pylori*. *H. pylori* strains bearing various *fliO* alleles were examined by transmission electron microscopy after negative staining to determine the number of flagella per cell. At least 115 cells were counted for each strain. Bars indicate percentage of cells that possessed the specified number of flagella. All strains were significantly different from one another (p -value < 0.0001) with the exception of WT / *pfliO* and $\Delta fliO$ / *pfliO* (p -value = 0.98) and $\Delta fliO$ / *pfliO* $_{\Delta N}$ and $\Delta fliO$ / *pfliO* $_{\Delta C}$ (p -value = 0.71).

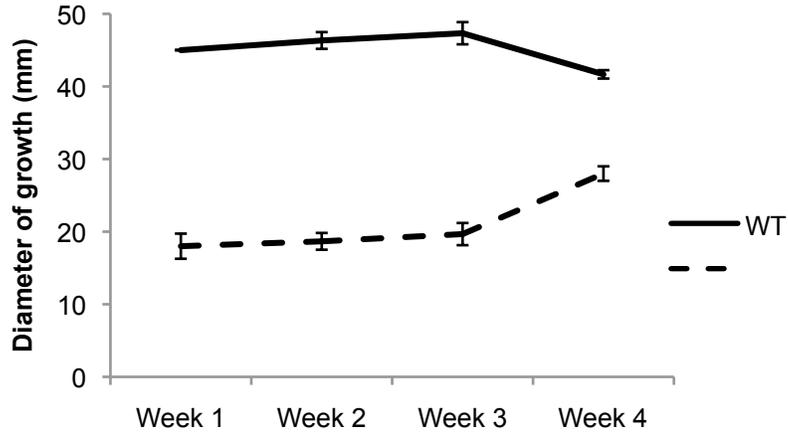


Figure 3.5. Motility enrichment of wild type and $\Delta fliO$. Motility from each enrichment stock was analyzed on semisolid agar medium. Plates were incubated for seven days before halo diameters were measured.

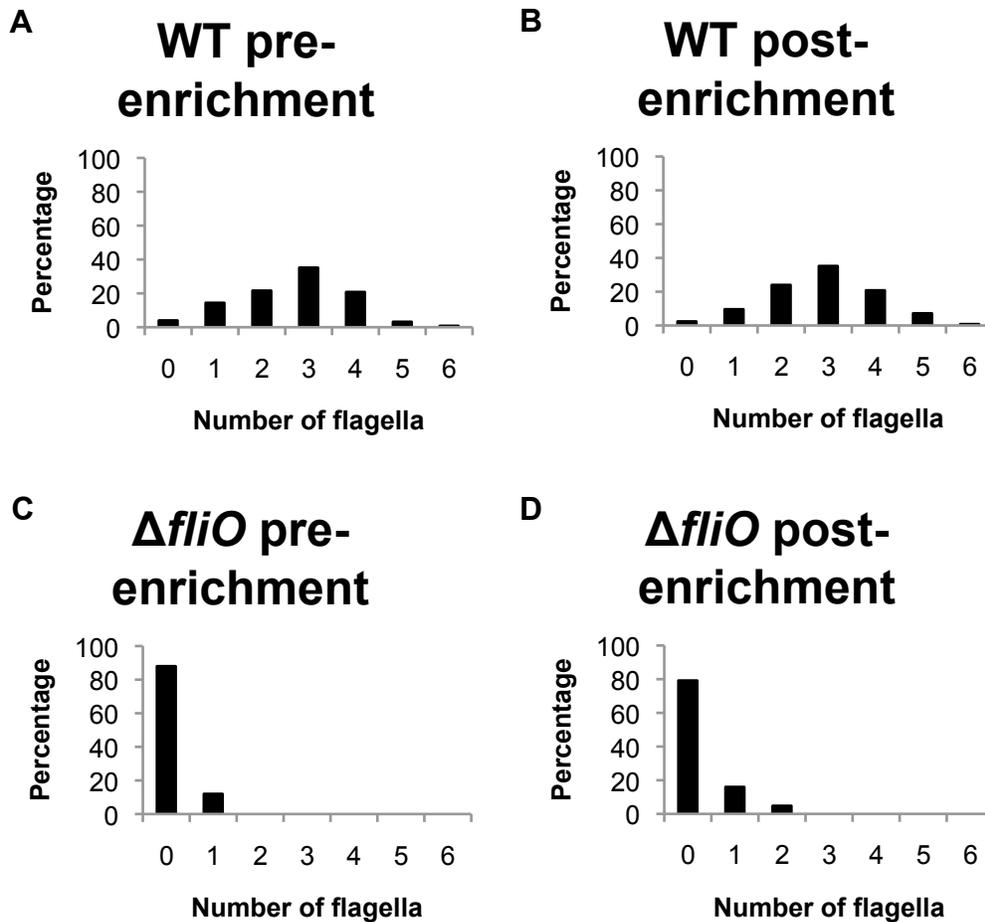


Figure 3.6. Effects of motility enrichment on flagellar numbers. The number of flagella per cell was analyzed by electron microscopy after negative staining of cells. For each strain, 125 cells were visualized. Bars represent the percentage of cells possessing the same number of flagella. Pre- and post-enrichment populations were not significantly different from one another (WT p -value=0.27, $\Delta fliO$ p -value = 0.20). In the wild type, there was no significant difference in the number of cells with 0-3 flagella versus 4-6 flagella (p -value = 0.57) and in the $\Delta fliO$ mutant, there was no significant difference in the number of aflagellated versus flagellated cells (p -value = 0.09) pre- and post-enrichment.

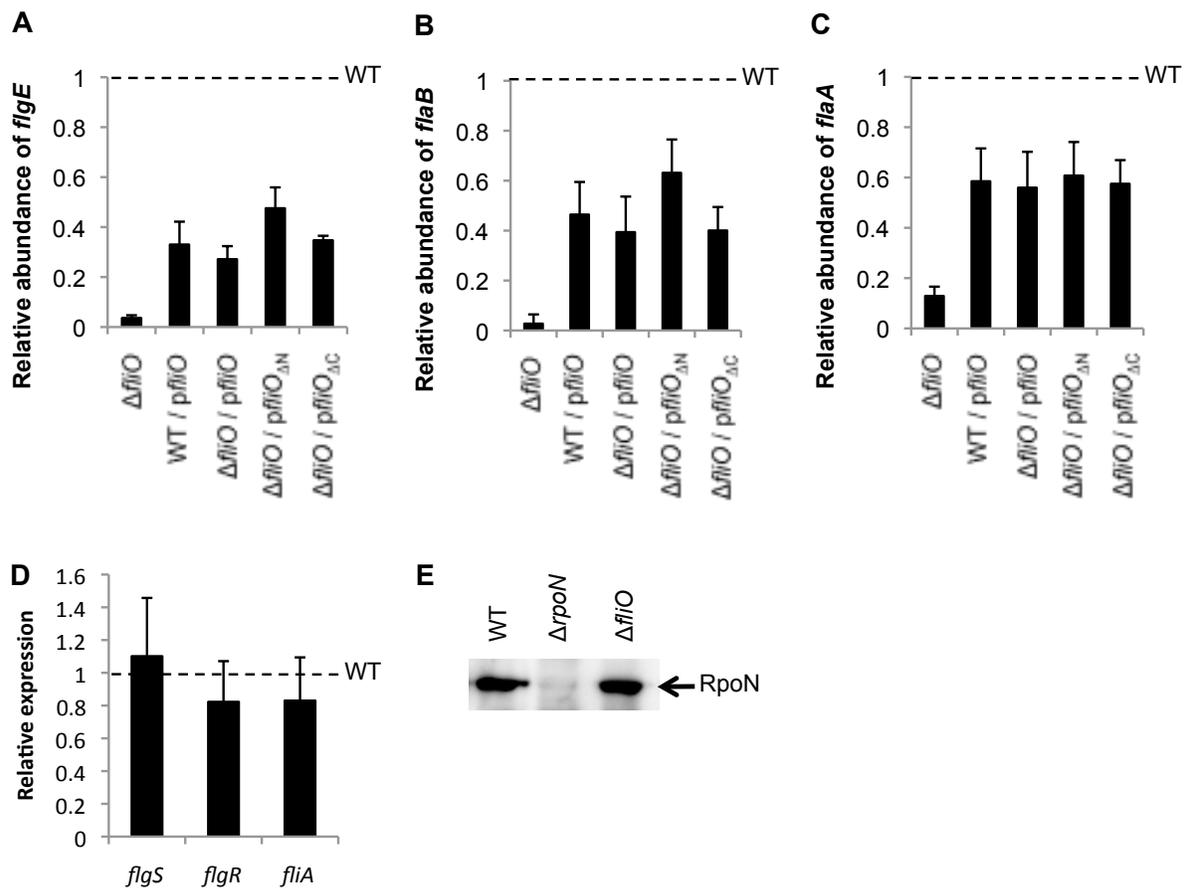


Figure 3.7: Effects of *fliO* alleles on transcription of specific flagellar genes. Transcript levels of *flgE* (A) and *flaB* (B) and *flaA* (C) were assessed by qRT-PCR and normalized to transcript levels of *gyrA*. Relative expression of each strain relative to wild type is plotted on the y-axis with wild type levels indicated by the dashed line at 1.0. All transcript levels measured in the $\Delta fliO$ mutant were significantly different from wild type (p -value <0.05). All transcript levels measured in the WT / *pfliO*, $\Delta fliO$ / *pfliO*, $\Delta fliO$ / *pfliO*_{ΔN}, and $\Delta fliO$ / *pfliO*_{ΔC} strains are significantly different from the $\Delta fliO$:*cat* mutant (p -value <0.05). (D) Transcript levels of regulatory genes, *flgS*, *flgR* and *fliA* in the $\Delta fliO$ were determined by qRT-PCR and normalized to transcript levels of *gyrA*. Relative expression of each gene compared to wild type (dashed line) is plotted on the y-axis and were not significantly different from wild-type levels (p -value

>0.05). (E) RpoN levels in the WT, $\Delta rpoN$, and $\Delta fliO$ mutants were accessed by western blotting using the soluble fraction. RpoN levels in the $\Delta fliO$ mutant were not significantly different than in the wild type (p -value > 0.05).

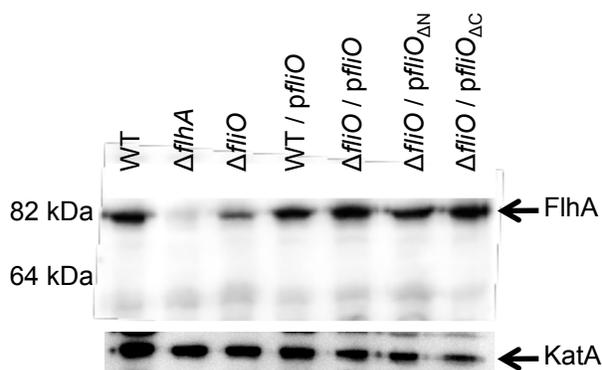


Figure 3.8: Detection of FlhA in wild-type and mutant strains. Membrane fractions prepared from various *H. pylori* strains were analyzed by western blotting using affinity purified antibodies directed against a peptide corresponding to the N-terminal 25 amino acid residues of FlhA (top). 20 μg of protein were loaded into each lane. FlhA from each lane was quantified using ImageJ. FlhA levels in the $\Delta fliO$ mutants were 2- to 3-fold lower than in wild type levels (p -value < 0.03) whereas FlhA levels in the complemented strains were not significantly different from those in wild type (p -value > 0.4). The same membrane fractions were analyzed by western blotting against KatA as a loading control (bottom). 4 μg of protein were loaded into each lane and KatA levels were quantified using ImageJ. KatA levels in the membrane fractions of the $\Delta fliO$ mutant were not significantly different from those in wild type (p -value > 0.1).



Figure 3.9: Detection of KatA in cytoplasmic fractions. 4 μ g of each fraction was loaded into a 10% SDS-PAGE gel. Lane one contains WT membrane and is a positive control for the western blot. All subsequent lanes contain the soluble fraction.

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CHAPTER 4

INSERTION MUTATIONS IN *HELICOBACTER PYLORI flhA* REVEAL STRAIN DIFFERENCES IN RpoN-DEPENDENT GENE EXPRESSION¹

¹ Tsang, J., T. G. Smith, L. E. Pereira, and T. R. Hoover. 2013. *Microbiology*. **159**: 58-67.
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Summary

Flagellar biogenesis in the gastric pathogen *Helicobacter pylori* involves a transcriptional hierarchy that utilizes all three sigma factors found in this bacterium (RpoD, RpoN, and FliA). Transcription of the RpoN-dependent genes requires the sensor kinase FlgS and response regulator FlgR. It is thought that FlgS senses some cellular cue to regulate transcription of the RpoN-dependent flagellar genes, but this signal has yet to be identified. Previous studies showed transcription of the RpoN-dependent genes is inhibited by mutations in *flhA*, which encodes a membrane-bound component of the flagellar protein export apparatus. We found that depending on the *H. pylori* strain used, insertion mutations in *flhA* had different effects on expression of RpoN-dependent genes. Mutations in *flhA* in *H. pylori* strains B128 and ATCC 434504 were generated by inserting a chloramphenicol resistance cassette so as to effectively eliminate expression of the gene ($\Delta flhA$); or within the gene following codon 77 (designated *flhA77*) or codon 454 (designated *flhA454*) which could allow expression of truncated FlhA proteins. All three *flhA* mutations severely inhibited transcription of the RpoN-dependent genes *flaB* and *flgE* in *H. pylori* B128. In contrast, levels of *flaB* and *flgE* transcripts in *H. pylori* ATCC 43504 bearing either *flhA77* or *flhA454*, but not $\Delta flhA$, were ~60% of wild-type levels. The FlhA⁴⁵⁴ variant was detected in membrane fractions prepared from *H. pylori* ATCC 43504, but not *H. pylori* B128, which may account for the phenotypic differences in the *flhA* mutations of the two strains. Taken together, these findings suggest that only the N-terminal region of FlhA is needed for transcription of the RpoN regulon. Interestingly, expression of a *flaB*'-'*xylE* reporter gene in *H. pylori* ATCC 43504 bearing the *flhA77* allele was ~8-fold higher than that of a strain with the wild-type allele, suggesting that expression of *flaB* is not only regulated at the level of transcription but is also regulated post-transcriptionally.

Introduction

Helicobacter pylori is a member of the ϵ -Proteobacteria that colonizes the human gastric mucosa where it can cause a variety of diseases, including peptic and duodenal ulcers, B cell MALT lymphoma and gastric adenocarcinoma (1-3). *H. pylori* must be motile to colonize the gastric mucosa (4, 5), which it achieves via a cluster of sheathed polar flagella (5-7).

The bacterial flagellum is a complex structure consisting of a basal body, hook and filament. The basal body is embedded in the bacterial cell envelope and consists of a rotary motor, a switch complex that regulates the direction of rotation, flagellar rod and rings, and a type III secretion system that transports flagellar subunits across the cell membrane (8-10). The flagellar protein export apparatus consists of six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR) which form an export pore, and three cytoplasmic components, FliI, FliH and FliJ, which deliver protein substrates to the export pore (11).

Assembly of the bacterial flagellum is a highly ordered process in which the basal body is assembled first, followed by the hook and then the filament. Where it has been examined, flagellar gene expression is controlled by a transcriptional hierarchy that coordinates synthesis of flagellar proteins with assembly of the flagellum (12, 13). The best studied example of such coordinated gene control is transcription of the *Salmonella* filament subunit (flagellin) gene, which is dependent on FliA (σ^{28}) and is negatively regulated by the anti- σ^{28} factor FlgM (14). Upon formation of the mature hook, the export apparatus undergoes a conformational change that results in a switch in substrate specificity from rod-/hook-type to filament-type substrates (15). FlgM is exported as a filament-type substrate from the cell at this point, which results in transcription of the FliA regulon (14).

H. pylori flagellar genes are organized in multiple regulons which are based on the sigma factor required for their transcription. In general, genes needed early in flagellar assembly are dependent on RpoD (σ^{80} ; primary σ factor in *H. pylori*) for their transcription, while genes required later in assembly are dependent on RpoN (σ^{54}), and genes required at the end of the pathway are FliA (σ^{28})-dependent (16-19). A two-component regulatory system consisting of the sensor kinase FlgS and the response regulator FlgR is required for transcription of the *H. pylori* RpoN-dependent flagellar genes (19, 20). Expression of the *H. pylori* RpoN regulon is linked with the flagellar protein export apparatus as mutations that disrupt the *H. pylori* export apparatus inhibits transcription of RpoN-dependent flagellar genes (18, 21-24). The export apparatus similarly affects expression of RpoN-dependent flagellar genes in *Campylobacter jejuni*, another member of the ϵ -Proteobacteria (25).

Though not part of the export apparatus, the hook length control protein, FliK, plays a pivotal role in the switch in substrate specificity of the export apparatus (26). Disrupting *fliK* in *H. pylori* or *C. jejuni* stimulates expression of RpoN-dependent genes, further suggesting a link between the export apparatus and RpoN regulons in these bacteria (27-29).

The mechanism by which the export apparatus affects the RpoN regulon in *H. pylori* and other members of the ϵ -Proteobacteria is not known, but it may do so by modulating FlgS activity. In support of this hypothesis, a variant of FlgR that functions independently of FlgS partially restores expression of RpoN-dependent reporter genes in *C. jejuni* mutants in which *flhA*, *flhB* or *fliP* are disrupted (30). It is possible that FlgS responds to conformational changes in the export apparatus during flagellar assembly.

We report here that *flhA* insertion mutations in two *H. pylori* strains manifest differences in expression of RpoN-dependent genes. In *H. pylori* B128, all disruptions in *flhA* that we

examined inhibited transcription of *flaB* and *flgE*, two RpoN-dependent flagellar genes. In *H. pylori* ATCC 43504, however, disruptions within *flhA* that had the potential to produce truncated FlhA proteins allowed transcription of *flaB* and *flgE*. Interestingly, expression of a *flaB*'-'*xylE* reporter gene in one of the *flhA* insertion mutants in *H. pylori* ATCC 43504 was ~8-fold higher than that of the wild-type strain, suggesting that *flaB* is regulated post-transcriptionally, possibly at the level of translation.

Methods

Bacterial strains and culture conditions. *H. pylori* strains ATCC 43504, 26695 and B128 were cultured routinely at 37°C in a microaerobic atmosphere (2% O₂/5% CO₂/93% N₂) on tryptic soy agar (TSA) supplemented with 5% horse serum as described previously (29). Where indicated, the medium for culturing *H. pylori* was supplemented with 30 µg/ml chloramphenicol or 30 µg/ml kanamycin. *Escherichia coli* DH5α was used for replicating plasmids for routine cloning procedures and cultured in Luria-Bertani medium supplemented with 100 µg/ml ampicillin, 30 mg/ml kanamycin or 30 µg/ml chloramphenicol. *H. pylori* motility was assessed using semisolid Mueller-Hinton medium supplemented with 10% horse serum and 10 µM FeSO₄ as described previously (29).

Construction of *H. pylori* mutants. Genomic DNA from *H. pylori* 26695 was prepared using the Wizard Genomic DNA purification kit (Promega). DNA was amplified by polymerase chain reaction (PCR) using *H. pylori* 26695 genomic DNA as a template and Taq DNA polymerase (Promega), Pfu Turbo Hotstart DNA polymerase (Stratagene), or iProof DNA polymerase (Bio-Rad). When Pfu Turbo Hotstart or iProof DNA polymerase were used for PCR, the resulting amplicons were incubated with Taq DNA polymerase at 72°C for 10 min to add single, 3'-A

overhangs to the ends to facilitate T/A cloning into the vector pGEM-T (Promega). DNA primers used for PCR are indicated in Table 4.1.

To construct the $\Delta flhA$ allele, a ~690 bp DNA fragment which included ~90 bp of *rps15* (encodes ribosomal protein S15; gene is upstream and divergent to *flhA*), 140 bp of intergenic region upstream of *flhA*, and 460 bp of the 5'-end of *flhA*, was amplified and cloned into pGEM-T to create plasmid pLP120. A suicide vector for introducing the $\Delta flhA$ mutant into the chromosome was created by digesting pLP120 with HindIII and Eco47III, filling-in the ends of the cut DNA, and then inserting a 1.3 kb SmaI fragment containing a chloramphenicol transacetylase (*cat*) gene from pSKAT4 (31) into the plasmid by blunt-end ligation. The replacement deleted the region corresponding to 90 nucleotides upstream of the predicted start codon of *flhA* through codon 76 of *flhA*. To construct the *flhA77* allele, a DNA fragment corresponding to the first 993 bp of *flhA* was amplified and cloned into pGEM-T to create plasmid pLP78. A 500 bp Eco47III fragment in pLP78 was replaced with the SmaI fragment from pSKAT4 containing the *cat* cassette by blunt-end ligation, which replaced codons 77 to 242 of *flhA* with the *cat* cassette and generated a suicide vector for introducing the *flhA77* allele into the chromosome. To construct the *flhA454* allele, a 1068 bp DNA fragment corresponding to 968 bp from the 5'-end to 165 bp from the 3'-end of *flhA* was amplified and cloned into pGEM-T to create plasmid pLP113. The resulting plasmid was used as a template for site-directed mutagenesis using the QuickChange II site-directed mutagenesis kit (Stratagene) and the primers indicated in Table 4.1. The mutagenic primers introduced a unique EcoRI site that overlapped codons 454 and 455 of *flhA*. A 1.3 kb EcoRI fragment from pSKAT4 containing the *cat* cassette was cloned into the EcoRI site that had been added into pLP113 to create a suicide vector for introducing the *flhA454* allele into the chromosome. The resulting suicide plasmids were

introduced into *H. pylori* ATCC 43504 or B128 by natural transformation, and transformants were selected as described previously (32). Replacement of the wild-type *flhA* allele with the mutant alleles was confirmed by PCR.

To construct the $\Delta hp1042$ mutant, *hp1042* along with 500 bp DNA upstream and 540 bp DNA downstream of the gene were amplified using the *hp1042* forward and *hp1042* reverse primers indicated in Table 4.1. The amplicon was cloned into pGEM-T, and the resulting plasmid was used as a template for inverse PCR using the *hp1042* inverse forward and *hp1042* inverse reverse primers (Table 4.1). These primers annealed to sequences immediately downstream and upstream of *hp1042*, respectively, and introduced EcoRV sites. Amplicons were circularized using T4 DNA ligase, and a 1.3 kb EcoRI fragment from pSKAT4 containing the *cat* cassette that was filled-in was ligated into the EcoRV sites of the plasmid. The resulting suicide plasmid was introduced into *H. pylori* ATCC 43504 by natural transformation, and transformants were selected as described previously (32). Insertion of the *cat* cassette into *hp1042* locus in the *H. pylori* ATCC 43504 chromosome was confirmed by PCR.

XylE assays. A reporter plasmid bearing a *flaB*'-*xylE* reporter gene on the shuttle vector pHel3 (33) was described previously (32). *H. pylori* strains containing the reporter plasmid were cultured for 36 h on agar medium supplemented with kanamycin to maintain the plasmid. Cells were resuspended in 50 mM potassium phosphate buffer, pH 7.0, to a cell density of 1 optical density at 600 nm (OD_{600}), which corresponded to $\sim 1 \times 10^9$ cfu/ml. Reactions were initiated by adding 0.1 ml cell suspension to 0.9 ml of 3 mM catechol in 50 mM potassium phosphate buffer, pH 7.0, which had been warmed for 5 min at 37°C. Reactions were incubated for 2 min at 37°C, stopped by adding 0.5 ml of 1 M sodium carbonate, and clarified by centrifugation at $\sim 16,000 \times g$ for 2 min. The absorbance of each reaction was measured at 375 nm using a Shimadzu UV-

160U spectrophotometer to determine the amount of product (2-hydroxymuconic semialdehyde) that had been formed. One unit of XylE activity corresponds to 1 μ mole of 2-hydroxymuconic semialdehyde produced/min, and was calculated by multiplying the final A_{375} value for each sample by 22.7. XylE activity levels were expressed as units of XylE activity per 10^8 cells. XylE activity for each strain was determined from at least 6 statistical replicates for two or more biological replicates. Student's t-test was used to determine the standard deviation with 95% confidence intervals ($P = 0.05$).

Detection of FlhA proteins. A multiple antigen peptide (MAP) was synthesized in which the first 25 amino acid residues from the N-terminus of *H. pylori* 26695 FlhA (MANERSKLAFKKTFFVFKRFLQSKD) were linked to a peptidyl core of seven lysine residues (Bio-Synthesis, Inc.). The MAP was used to immunize a New Zealand white rabbit (Cocalico Biologicals, Inc.), and antibodies that recognized the FlhA peptide were affinity purified from the resulting antiserum as follows. A peptide consisting of the first 25 residues of FlhA plus a cysteine residue at the C-terminus was synthesized (GenScript, Inc.) and covalently attached to SulfoLink Coupling Resin (Thermo Scientific) following the manufacturer's protocol that accompanied the SulfoLink Immobilization Kit. Two ml of antiserum was prepared by diluting with 13 ml of phosphate-buffered saline (PBS), reducing the volume to 0.5 ml using an Amicon Ultra-15 centrifugal filter, adding another 13 ml PBS, and then reducing the volume to 0.5 ml again. The buffered-exchanged antiserum was applied to the SulfoLink column; the column was capped and gently rocked for 1 h at room temperature to allow the antibodies to bind to the FlhA peptide immobilized on the resin. The resin was washed five times with 2 ml of PBS, and then antibodies were eluted in three 2-ml washes of 0.1 M glycine, pH 2.5. The eluted antibodies were dialyzed against citric acid-phosphate buffer (55 mM citric acid, 50 mM K-

$_2\text{HPO}_4$, pH 5.5), followed by Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6), then stored at -20°C .

H. pylori membranes were prepared for western blot analysis as follows. *H. pylori* strains were grown for ~72 h on agar medium and then resuspended in a buffer containing 10% sucrose, 20 mM HEPES, and 1 mM EDTA pH 7.4. Cells were lysed by three passages through a French press at 10,000 kPa. Cellular debris was removed by centrifugation for 15 min at 6,000 x g. Membranes were separated from cytoplasmic proteins by centrifugation for 60 min at 100,000 x g, and collected in 0.25 ml of 30% sucrose, 20 mM HEPES, and 1 mM EDTA, pH 7.4. The membrane fraction was diluted in 20 mM HEPES, and 1 mM EDTA, pH 7.4, and the centrifugation (60 min at 100,000 x g) was repeated. Protein concentrations of the membrane fractions were measured using the bicinchoninic acid protein assay (Thermo Scientific) following the manufacturer's instructions. The purified *H. pylori* membranes were analyzed by western blotting using the affinity purified FlhA antibodies as the primary antibody and goat-anti-rabbit-HRP conjugated secondary antibody (Bio-Rad). Antigen-antibody complexes on the blot were detected by chemiluminescence using SuperSignal West Pico Luminol/Enhancer Solution and SuperSignal West Stable Peroxide Solution (Thermo Scientific). Blots were visualized with a FluoroChem E imager (ProteinSimple).

Detection of exported flagellar proteins. Plasmid pTS14 is a derivative of pHel3 that carries *H. pylori flgE* under control of its native promoter and a sequence encoding the FLAG tag (DYKDDDDK) fused to the 3'-end of *flgE* (29). *H. pylori* strains containing pTS14 were cultured in brain-heart infusion medium supplemented with 0.4% β -cyclodextrin and 30 $\mu\text{g/ml}$ kanamycin in sealed serum bottles at 37°C under a microaerobic atmosphere with gentle shaking for 24 h as described (29). *H. pylori* extracellular and cytoplasmic proteins were prepared and

concentrated using trichloroacetic acid as described previously (29). Protein concentrations were measured using the bicinchoninic acid protein assay. For each sample analyzed, approximately the same amount of total protein was separated by SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. A monoclonal peroxidase conjugated antibody directed against the FLAG epitope (Sigma) and enhanced chemiluminescence were used to detect FlgE-FLAG. Antiserum directed against *H. pylori* FlaB (34), a peroxidase-conjugated goat anti-rabbit antibody (MP Biomedicals) and enhanced chemiluminescence were used to detect *H. pylori* flagellins. *H. pylori* FlaB and FlaA share 58% amino acid identity and the antiserum directed against FlaB cross-reacts with FlaA (32, 34).

RNA extraction and cDNA synthesis. *H. pylori* cells were grown on TSA for 18 hours before harvesting and resuspended into 1 ml of nuclease-free deionized water. Cells were pelleted by centrifugation and then resuspended in 100 μ l of nuclease-free deionized water. RNA was isolated from the cell pellets using the Aurum Total RNA Mini Kit (Bio-Rad). The resulting RNA solutions were treated with the TURBO DNA-free Kit (Ambion) to remove contaminating DNA. Single-strand cDNA was synthesized from 200 ng of RNA using the iScript cDNA Synthesis Kit (Bio-Rad).

Quantitative RT-PCR. Transcript levels of *flaA*, *flaB*, and *flgE* were monitored by quantitative reverse-transcription PCR (qRT-PCR) using the Bio-Rad iCycler iQ System. *gyrA* transcript levels were used as an internal control for the qRT-PCR assays. Primer specificity was confirmed by PCR using genomic DNA. Each qRT-PCR assay, totaling 20 μ l, consisted of 10 μ l of iQ SYBR Green Supermix, 5 μ l of 100-fold diluted cDNA from the cDNA synthesis reaction, and 200 nM primer. Experiments were performed in technical triplicate from three

different RNA isolations of each strain. Gene expression levels were quantified by the $2^{-\Delta\Delta ct}$ method (35).

DNA Sequencing. A region of the *H. pylori* 43504 chromosome that included *flgM*, the gene upstream of *flgM* (*slyD*), and three genes downstream of *flgM* (a gene encoding a predicted cytosine specific DNA methyltransferase, a potential *flgN* homolog, and *flgK*, respectively) was amplified using iProof DNA polymerase. After treating the resulting amplicon with Taq DNA polymerase to add A residues to the 3'-ends the amplified DNA fragment was cloned into pGEMT-Easy and both strands of the cloned DNA were sequenced by Genewiz, Inc. (South Plainfield, NJ) using the primers indicated in Table 4.1. The DNA sequence from *H. pylori* ATCC 43504 was analyzed using the Basic Local Alignment Search Tool (BLAST). Sequences of the ORFS and intergenic regions from the corresponding regions of the sequenced genomes for *H. pylori* 26695 and B128 were obtained from the Integrated Microbial Genomes database (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>). Nucleotide and predicted protein sequences were aligned using the ClustalW2 multiple sequence alignment program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>). DNA sequences and predicted amino sequences of *H. pylori* ATCC 43504 *flgM* and the surrounding genes were deposited in GenBank (accession numbers JQ31766, JQ31767, JQ31768, JQ317769 and JQ31770).

Results

Insertions in *flhA* affect transcription of RpoN-dependent flagellar genes differently in *H. pylori* strains B128 and ATCC 43504. *Salmonella* FlhA is 692 amino acid residues in length and consists of an N-terminal transmembrane domain (FlhA_{TM}) predicted to contain six to eight membrane-spanning helices and a large C-terminal cytoplasmic domain referred to as FlhA_C

(36). The National Center for Biotechnology Information database contains complete sequences for FlhA proteins from over 20 *H. pylori* strains. These FlhA sequences are >98% identical over their entire length of 733 amino acid residues. The sequences of FlhA from *H. pylori* strains 26695 and B128 were analyzed using three different programs for predicting the membrane topology of proteins: TMHMM Server v 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), Tmpred (http://www.ch.embnet.org/software/TMPRED_form.html), and HMMTOP (<http://www.enzim.hu/hmmtop/>). The results of this analysis indicated that the predicted membrane topology of *H. pylori* FlhA was similar to that of *Salmonella* FlhA (Fig. 4.1).

Previous studies by Niehus and co-workers (18) showed that disrupting *flhA* at codon 456 (located within the FlhA_C domain) in *H. pylori* N6 or 88-3887 (a motile isolate of strain 26695) with a kanamycin-resistance cassette resulted in reduced transcript levels of both RpoN-dependent and FliA-dependent flagellar genes, but not RpoD-dependent flagellar genes. To examine further the role of FlhA in flagellar gene regulation, we generated three *flhA* mutant alleles in *H. pylori* ATCC 43504 and B128 by disrupting *flhA* at various positions (Fig. 4.1) and analyzed the phenotypes of the resulting mutants. For one of the *flhA* alleles the region corresponding to 90 nucleotides upstream of the predicted start codon of *flhA* through codon 77 of *flhA* was replaced with a *cat* cassette. Since this mutation should abolish expression of the entire *flhA* gene it was referred to as a $\Delta flhA$ mutation. For the second allele codons 77 to 242 of *flhA* were replaced with the *cat* cassette, and this allele was referred to as *flhA77*. The *flhA77* allele has the potential to produce a truncated FlhA which would include the first two membrane-spanning helices of the protein (Fig. 4.1). A third *flhA* allele was generated by introducing the *cat* cassette between codons 454 and 455, and the resulting allele was designated as *flhA454*.

The *flhA454* allele could potentially produce a truncated FlhA that would include all of FlhA_{TM} plus part of FlhA_C.

As expected, *H. pylori* strains bearing any of the mutant *flhA* alleles were deficient in motility as assessed using soft agar plates (data not shown). To assess how the *flhA* alleles affected transcription of selected flagellar genes in the *H. pylori* strains, transcript levels of two RpoN-dependent flagellar genes (*flgE*, encodes the hook protein; and *flaB*, encodes a minor flagellin), and one FliA-dependent flagellar gene (*flaA*, encodes the major flagellin) were determined by qRT-PCR. In agreement with the previous report by Niehus and co-workers (18), levels of *flaB*, *flgE* and *flaA* transcripts were reduced significantly in *H. pylori* B128 derivatives bearing any of the three *flhA* mutant alleles (Fig. 4.2A). Levels of *flaB* and *flgE* transcripts in the three *flhA* mutants were <1% of those in the parental strain. Transcription of *flaA* in the *H. pylori* B128 *flhA* mutants appeared to be affected to a lesser degree as *flaA* transcript levels in the *flhA* mutants were ~15% of wild-type levels.

Surprisingly, the flagellar gene transcript profiles for the *flhA* mutants in *H. pylori* ATCC 43504 were strikingly different from those in *H. pylori* B128 (Fig. 4.2B). Relative to wild type, the amount of *flaA* transcripts in the *H. pylori* ATCC 43504 Δ *flhA* mutant was reduced to a level (~12%) that was similar to those in the *H. pylori* B128 *flhA* mutants. In the *H. pylori* ATCC 43504 strains bearing the *flhA77* or *flhA454* alleles, however, *flaA* transcript levels were reduced further to ~5% of wild-type levels. Differences between the *flhA* mutants in *H. pylori* B128 and 43504 were more dramatic with regard to the levels of *flaB* and *flgE* transcripts. As observed in *H. pylori* B128, *flaB* and *flgE* transcript levels in the *H. pylori* ATCC 43504 Δ *flhA* mutant were severely reduced. In contrast to *H. pylori* B128, levels of *flaB* and *flgE* transcripts in *H. pylori* ATCC 43504 strains that carried the *flhA77* or *flhA454* alleles were similar (60 to 80%) to those

in the parental strain. The *flhA* mutants in *H. pylori* ATCC 43504 were reconstructed and three clones for each mutation were analyzed. The phenotypes of the reconstructed strains were like those of the original strains, arguing that the phenotypes were not due to variants within the parental population.

Niehus and co-workers reported that disrupting *flgM* (encodes the anti- σ^{28} factor) restored transcription of RpoN-dependent genes in an *flhA* mutant (18). The molecular basis for this restoration is not known. To determine if the unexpected phenotype of the *H. pylori* ATCC 43504 strains bearing the *flhA77* or *flhA454* alleles might be accounted for by a mutation in *flgM*, we sequenced the gene and surrounding region in *H. pylori* ATCC 43504. The predicted amino acid sequences of FlgM proteins from *H. pylori* ATCC 43504 and *H. pylori* B128 were identical with the exception of two conservative changes – *H. pylori* ATCC 43504 FlgM has isoleucine at positions 2 and 4, while *H. pylori* B128 FlgM has valine at these positions. The ~300 bp intergenic region upstream of *flgM* in the two strains was also the same with the exception of three 1-bp insertions and one 2-bp insertion in the *H. pylori* B128 sequence. Taken together, these findings argue against the idea that the phenotype of the *H. pylori* ATCC 43504 strains bearing the *flhA77* or *flhA454* alleles is due to a mutation in *flgM*.

***H. pylori flhA* mutants are defective in export of flagellar protein substrates.** Given that *H. pylori* ATCC 43504 bearing the *flhA77* or *flhA454* alleles were competent to transcribe RpoN-dependent flagellar genes, we wished to verify that these strains were deficient in secreting protein substrates. To test for deficiencies in export of rod-/hook-type substrates, we monitored secretion of a FlgE-FLAG protein in which the FLAG epitope was fused to the C-terminus of the hook protein FlgE (29). The FlgE-FLAG protein was expressed from the native, RpoN-dependent *flgE* promoter and was carried on the shuttle vector pHel3. A monoclonal antibody

directed against the FLAG tag was used to monitor expression and export of the FlgE-FLAG protein. *H. pylori* strains harboring the plasmid for expression of the FlgE-FLAG protein retained the native *flgE* allele in the chromosome. To test for export deficiencies of filament-type substrates, antiserum directed against *H. pylori* FlaB was used to monitor the expression and secretion of flagellins in the *H. pylori* strains.

Cultures of *H. pylori* ATCC 43504 and mutant derivatives were grown in serum-free liquid medium and the extracellular and soluble cytoplasmic proteins from the cultures were analyzed by western blotting. FlgE-FLAG protein was detected in the cytoplasmic fractions of strains bearing the *flhA77* and *flhA454* mutant strains, but not the Δ *flhA* mutant (Fig. 4.3A). These data were consistent with the results of the qRT-PCR assays showing that *flgE* is transcribed poorly in the *H. pylori* ATCC 43504 Δ *flhA* mutant but is transcribed close to wild-type levels in strains bearing the *flhA77* or *flhA454* alleles (Fig. 4.2B). The FlgE-FLAG protein was detected readily in the wild-type extracellular fraction (i.e., TCA-precipitated material from growth medium that had been clarified by centrifugation – this material included flagellar protein subunits as well as sheared flagella), but not those of the *flhA77* and *flhA454* mutants even though the protein was present in the cytoplasmic fractions of these mutant strains. A small amount of FlgE-FLAG protein was detected in the extracellular fraction of the *flhA77* mutant which may have been due to cell lysis. Alternatively, the extracellular FlgE-FLAG may have been due to a low level of export activity by the mutant flagellar protein export apparatus. In support of this latter suggestion, Schmitz and co-workers reported that cells of a *H. pylori flhA* mutant displayed an occasional assembled hook structure indicating that export of rod-/hook-type substrates was possible to a limited extent in the absence of the full-length FlhA (21).

Likewise consistent with the results of the qRT-PCR assays, *H. pylori* ATCC 43504 bearing the *flhA77* or *flhA454* alleles, but not the Δ *flhA* allele, expressed flagellin proteins (Fig. 4.3B). FlaA and FlaB are difficult to resolve by SDS-polyacrylamide gel electrophoresis since they are very similar in size (53,284 Dal and 53,882 Dal, respectively), and the antiserum directed against FlaB cross-reacts with both FlaB and FlaA (32, 34). Thus, the protein band indicated as ‘flagellin’ in figure 3B may be a mixture of FlaA and FlaB in samples prepared from the wild-type strain. Given the very low levels of *flaA* transcript in the *flhA77* and *flhA454* mutants, we infer that that most of the flagellin detected in the cytoplasmic fractions of these mutants is FlaB. Flagellin was detected in the extracellular fraction of the wild-type strain, but not in that of the *flhA77* and *flhA454* mutants. The extracellular fraction of the wild-type strain contained a cross-reacting protein that migrated slightly higher than the cytoplasmic flagellin. This protein was not consistently observed in the all of the samples which were analyzed (data not shown). *H. pylori* flagellins are glycosylated (37), and this slower migrating species could be flagellin that is glycosylated to a greater degree. The results presented here clearly demonstrate that *H. pylori* ATCC 43504 strains with the *flhA77* or *flhA454* alleles are capable of expressing RpoN-dependent flagellar genes, but are deficient in exporting the products of these genes.

The product of the *flhA454* allele is stably expressed in *H. pylori* ATCC 43504 but not *H. pylori* B128. We wished to address the issue of why the *flhA77* and *flhA454* alleles enabled transcription of RpoN-dependent genes in *H. pylori* ATCC 43504 but not *H. pylori* B128. We postulated that the truncated FlhA proteins encoded by these *flhA* alleles were expressed and incorporated into the export apparatus in *H. pylori* ATCC 43504, but not *H. pylori* B128. To test this hypothesis, membrane fractions from *H. pylori* strains were analyzed by western blotting using antibodies that recognized epitopes located within the first 25 amino acid residues at the N-

terminus of FlhA. Full-length FlhA was detected in membrane fractions prepared from *H. pylori* ATCC 43504 and *H. pylori* B128 in samples that contained as little as 5 µg total protein (Fig. 4.4A; data shown for *H. pylori* B128 only). The predicted size of the FlhA⁴⁵⁴ variant is ~53 kDal, which was determined by sequencing the *flhA454* allele (predicted amino acid sequence included FlhA sequence up to Glu-454 plus an additional 27 residues resulting from the cassette inserted between codons 454 and 455 of *flhA*). A protein that cross-reacted with the purified FlhA antibodies and was ~53 kDal in size was present in membrane fractions prepared from *H. pylori* ATCC 43504 bearing the *flhA454* allele, but not in the *H. pylori* ATCC 43504 Δ *flhA* mutant (Fig. 4.4B). We infer that this protein is the FlhA⁴⁵⁴ variant. In contrast, we were unable to detect the FlhA⁴⁵⁴ variant in membrane fractions prepared from *H. pylori* B128 (Fig. 4.4B) even when the amount of sample analyzed was increased to 80 µg total protein (data not shown). These results were consistent with the hypothesis that FlhA⁴⁵⁴ variant is expressed and incorporated into the export apparatus in *H. pylori* ATCC 43504, but not in *H. pylori* B128.

The predicted size of the FlhA⁷⁷ variant is ~18 kDal which was determined from the sequence the *flhA77* allele. Samples from *H. pylori* B128 bearing either the *flhA77* or Δ *flhA* alleles contained two cross-reacting bands in the region where a protein of this size would be expected to migrate (Fig. 4.4C). Two faint bands of similar size were observed for samples from *H. pylori* ATCC 43504 bearing the *flhA77* allele, but not in the Δ *flhA* mutant (Fig. 4.4C). Given the low intensities of these bands in *H. pylori* ATCC 43504 bearing the *flhA77* allele together with the observation that bands of similar sizes are present in the membrane fractions prepared from *H. pylori* B128 bearing either the *flhA77* or Δ *flhA* alleles, it was not possible to ascertain from the data if the FlhA⁷⁷ variant is stably expressed in *H. pylori* ATCC 43504 but not *H. pylori* B128.

Expression of a *flaB*'-*xylE* reporter gene is enhanced in *H. pylori* ATCC 43504 harboring the *flhA77* or *flhA454* alleles. The results of the western blot analysis demonstrated that the RpoN-dependent flagellar genes *flaB* and FLAG-tagged *flgE* were expressed in the *H. pylori* ATCC 43504 harboring the *flhA77* or *flhA454* alleles. Since the western blot assays were not quantitative, we compared expression of a *flaB*'-*xylE* reporter gene in the *H. pylori* ATCC 43504 *flhA* mutants and parental strain. As expected based on the results of the qRT-PCR assays, expression of the *flaB*'-*xylE* reporter gene was significantly reduced in the Δ *flhA* mutant compared to the wild-type strain (Fig. 4.5). Surprisingly, expression of the *flaB*'-*xylE* reporter gene was up-regulated ~8-fold in the *flhA77* mutant compared to the wild-type strain. Given that *flaB* transcript levels in the *flhA77* mutant were ~60% of those in the wild-type strain (Fig. 4.2B), these data strongly suggest that transcripts from the *flaB*'-*xylE* reporter gene are translated more efficiently in the *flhA77* mutant than they are in the wild-type background. Expression of the *flaB*'-*xylE* reporter gene was also significantly higher in the *H. pylori* strain bearing the *flhA454* allele compared to wild type, though to a lesser extent than for the *flhA77* mutant. We considered the possibility that the enhanced expression of the *flaB*'-*xylE* reporter gene in the *flhA77* mutant compared to the wild-type strain was due to polar effects on the downstream gene, *hp1042*. This does not appear to be the case though, as disrupting *hp1042* in *H. pylori* ATCC 43504 did not result in the up-regulation of the *flaB*'-*xylE* reporter gene (XylE activities for the wild-type and Δ *hp1042:cat* mutant were 0.65 ± 0.26 and 0.72 ± 0.30 , respectively).

Discussion

Assembly of the bacterial flagellum is a highly ordered and sophisticated process that involves the coordinated expression and regulation of dozens of structural, accessory and

regulatory genes. Elucidating the mechanism by which the flagellar protein export apparatus controls transcription of RpoN-dependent flagellar genes in *H. pylori* is critical for dissecting the regulatory circuitries that coordinate gene expression with assembly of the flagellum in *H. pylori* and related bacteria.

An unexpected finding from the studies reported here is that some mutations in *flhA* allow expression of RpoN-dependent flagellar genes in *H. pylori* ATCC 43504, but not in *H. pylori* B128. The results with *H. pylori* B128 are consistent with those reported previously for insertion mutations in *flhA* for *H. pylori* strains N6 and 88-3887 (18, 21). We found that the FlhA⁴⁵⁴ variant is stably expressed in *H. pylori* ATCC 43504 but not *H. pylori* B128 (Fig. 4.4B), which we believe accounts for the phenotypic differences of the *flhA* mutants in these strains. We were unable to obtain conclusive evidence that the FlhA⁷⁷ variant was stably expressed in *H. pylori* ATCC 43504 (Fig. 4.4C). It is possible that the FlhA⁷⁷ variant can interact with other export apparatus components in *H. pylori* ATCC 43504 without being incorporated into the cell membrane. There is precedence for such conjecture as Barker et al. (38) showed in *Salmonella* that expression of the cytoplasmic domain of the export apparatus protein FliO was sufficient to restore motility in a Δ *fliO* mutant.

The phenotypes of the *flhA* mutants in *H. pylori* ATCC 43504 indicate that only the first 77 amino acids (or less) of FlhA are needed for transcription of RpoN-dependent flagellar genes. This portion of FlhA is predicted to contain a stretch of 25 amino acid residues at the N-terminus of the protein that extends into the cytoplasm, as well as the first two transmembrane helices (Fig. 4.1). The N-terminus of *Salmonella* FlhA, which is similarly predicted to be located on the cytoplasmic side of the membrane, is required for export function and is believed to interact with the soluble export apparatus protein FliI and other export apparatus components or protein

substrates (36, 39). We speculate that the N-terminus of FlhA may interact with FlgS to modulate its activity and thereby affect transcription of the RpoN regulon. Alternatively, the N-terminus of FlhA may influence the assembly or conformation of other components of the export apparatus which, in turn, could modulate transcription of the RpoN regulon.

Not only does FlhA have a role in regulation of RpoN-dependent flagellar genes, but it also functions in regulating FliA (σ^{28})-dependent genes in *H. pylori*. Rust *et al.* (40) showed that the anti- σ^{28} factor, FlgM, interacts with the C-terminal domain of FlhA (FlhA_C), and suggested that FlgM function is modulated through these interactions rather than FlgM secretion. The absence of all or part of FlhA_C may have accounted for the down-regulation of the FliA-dependent *flaA* gene in the *H. pylori flhA* mutants examined in this study as well as previous studies (18).

In addition to the transcriptional control of flagellar genes, flagellar biogenesis in *H. pylori* involves other levels of regulation. Douillard and co-workers (41) reported that *H. pylori* protein HP0958 (also referred to as FlgZ) binds the *flaA* mRNA, resulting in decreased stability but enhanced translation of the transcript. Further possible evidence for post-transcriptional control of flagellar genes in *H. pylori* comes from studies by Xiao and co-workers (42) in which they identified a natural antisense transcript complementary to a partial sequence of *fliM* (encodes a flagellar motor switch protein in the C ring) in *H. pylori* 26695. Although the authors of this study did not determine if the antisense RNA affected translation or stability of *fliM* transcripts, it seems reasonable to postulate such a regulatory role. Sharma and co-workers (43) identified three small non-coding RNAs (sRNAs) in *H. pylori* 26695 which they predicted to be transcribed from FliA-dependent promoters. Since the only known role for FliA in *H. pylori* is

flagellar biogenesis, it is reasonable to postulate that these sRNAs have roles in regulating the expression of specific flagellar genes.

Despite having a slightly lower level of *flaB* transcript than the wild-type strain, the *H. pylori* ATCC 43504 *flhA77* mutant expressed the *flaB*'-'*xylE* reporter gene at a significantly higher level than the wild-type strain (Figs. 4.2B and 4.5). These data suggest the *flaB*'-'*xylE* transcripts were translated more efficiently in the *flhA77* mutant background, and point to a possible regulatory mechanism which controls translation of *flaB* mRNA. Since *flaA* mRNA levels were significantly reduced in *H. pylori* ATCC 43504 bearing the *flhA77* allele, transcription of other FliA-dependent genes in this strain is also likely to be depressed. Thus, if any of the potential FliA-dependent sRNAs inhibit translation of the *flaB* mRNA, reduced levels of these sRNAs in the *H. pylori* ATCC 43504 *flhA77* mutant may account for the enhanced expression of the *flaB*'-'*xylE* reporter gene in this strain.

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Author Contributions

JT, TGS, LP, and TRH designed the experiments. JT, TGS, and LP performed the experiments and analyzed the data. JT and TRH wrote the paper.

Table 4.1: Oligonucleotide primers used in the study.

<i>ΔflhA</i> forward	5'-CACTTGCACCTCACAAGAAC-3'
<i>ΔflhA</i> reverse	5'-CTTCAGTAACCCTAGTAGAGC-3'
<i>flhA77</i> forward	5'-ATGGCAAACGAACGCTCCAAATTAG-3'
<i>flhA77</i> reverse	5'-GCCATGCGATGAATAAAAAGAGAC-3'
<i>flhA454</i> forward	5'-GACTCTCTTTTTATTCATCGCATG-3'
<i>flhA454</i> reverse	5'-CAAGACTTTCATGGCCTCTTC-3'
<i>flhA454</i> EcoRI forward	5'-CCCCAACGCATTATGAATTCAAACCTAAAGGCATTGTG-3'
<i>flhA454</i> EcoRI reverse	5'-CACAATGCCTTTAAGTTTGAATTCATAATGCGTTGGGGG-3'
<i>hp1042</i> forward	5'-GTTACATGAAAAAATCCCCATTAA-3'
<i>hp1042</i> reverse	5'-GTGTTTGGTTGGGTGGTATGA-3'
<i>hp1042</i> inverse forward	5'-GGATATCAACTAAGCTGTTTAGAAAAAACTAA-3'
<i>hp1042</i> inverse reverse	5'-GGATATCTCTCCTTTTTTATCAATTATTTATCC-3'
Seq1 forward	5'-ATGCAAACCATGATTTAGAGT-3'
Seq1 reverse	5'-TTATTGTTTAATCCCCAATAAAG-3'
Seq2 forward	5'-AAGCAGCTATTTGCAAGAAG-3'
Seq2 reverse	5'-CAGGCTCGCCTTCAATATG-3'
Seq3 forward	5'-GCTCTTGATAGGGTAGCTG-3'
Seq3 reverse	5'-GCTCGCTTCATCAATGTCC-3'
Seq4 forward	5'-TATCAAAGCCTTGCAAGAAGC-3'
Seq4 reverse	5'-GTCTCTTATATCGCGCTCG-3'
Seq5 forward	5'-TAGTATTTGCTTGAATACGGC-3'
Seq5 reverse	5'-CCTAAAGGGAAATGAAATGG-3'
Seq6 forward	5'-GGGTGCATGATGAGTTTG-3'
Seq6 reverse	5'-GAGAAGTCTCATGCAAGTTG-3'
Seq7 forward	5'-ACGCTCAAAGTGCGAGTC-3'

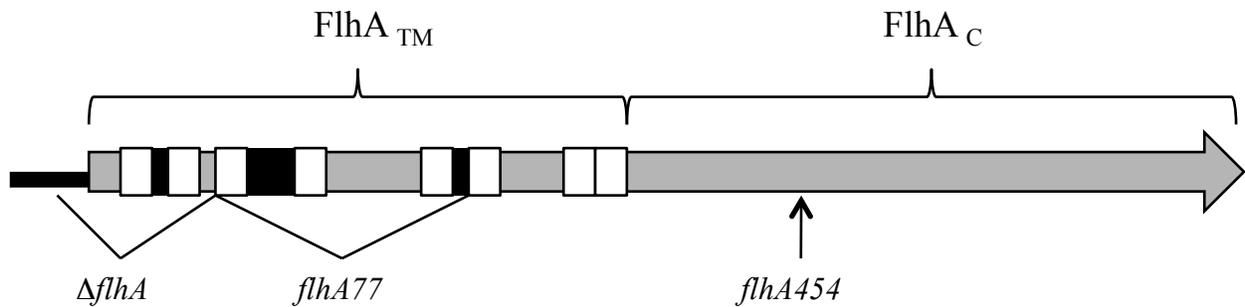


Figure 4.1: Diagram of *H. pylori* FlhA. The FlhA_{TM} and FlhA_C domains of the protein are indicated. White boxes denote the approximate position of predicted transmembrane spanning helices, black boxes indicate putative periplasmic regions of the protein, and gray regions indicate predicted cytoplasmic regions of the protein. The predicted topology of *H. pylori* FlhA is based on a comparison with the predicted topology of *Salmonella* FlhA done by McMurry and co-workers (36) along with membrane topology prediction programs as indicated in Materials and Methods. The heavy black line corresponds to the intergenic region upstream of *flhA*. The corresponding regions of the gene which were replaced with the *cat* cassette in the $\Delta flhA$ and *flhA77* mutants are indicated, as is the approximate location of the *cat* cassette in the *flhA454* mutant.

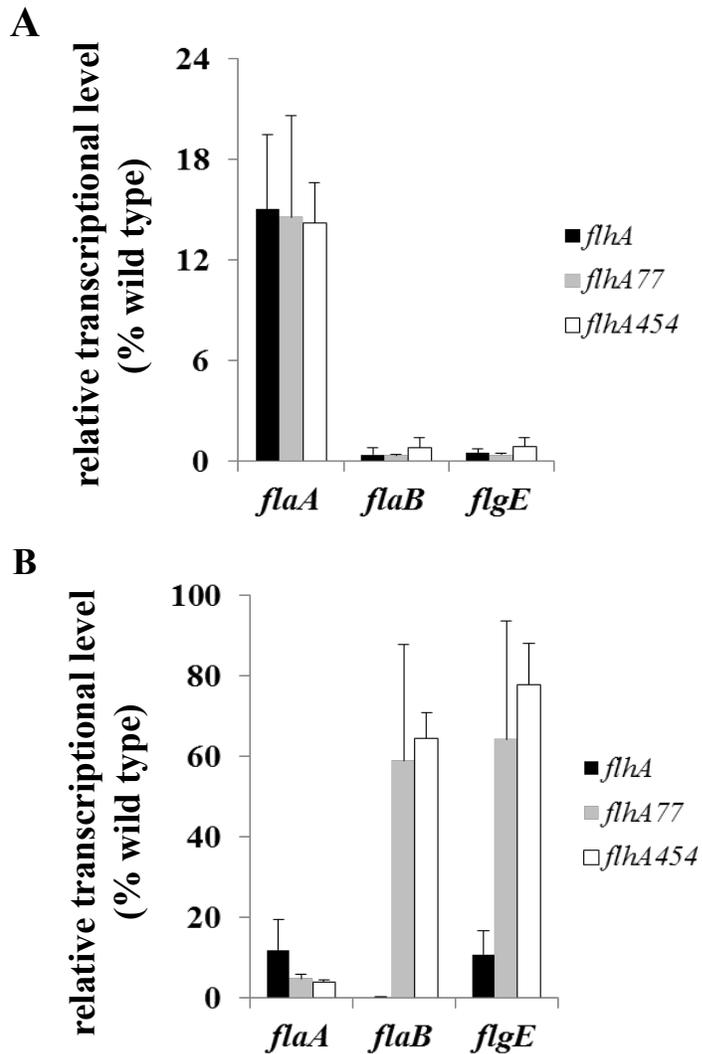


Figure 4.2: Relative levels of *flaA*, *flaB* and *flgE* transcripts in *H. pylori* strains containing various *flhA* alleles. Transcript levels were determined by qRT-PCR and compared relative to wild-type levels. (A) Relative levels of *flaA*, *flaB* and *flgE* transcripts in *H. pylori* B128 harboring the $\Delta flhA$ (*flhA*, black bars), *flhA77* (gray bars) or *flhA454* (open bars) allele. (B) Relative levels of *flaA*, *flaB* and *flgE* transcripts in *H. pylori* ATCC 43504 harboring the $\Delta flhA$

(*flhA*, black bars), *flhA77* (gray bars) or *flhA454* (open bars) allele. Note the differences in scales for the y-axes of the two graphs. Error bars correspond to one standard deviation.

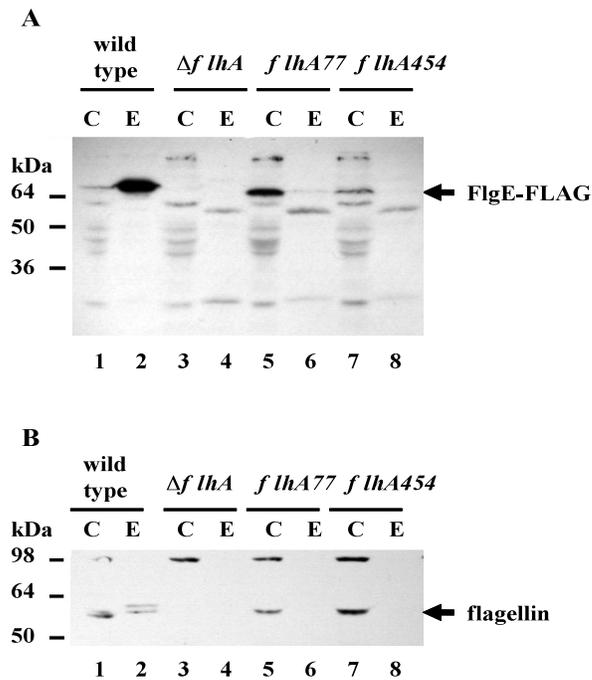


Figure 4.3: Western blot analyses of hook and flagellin proteins exported from *H. pylori* ATCC 43504 bearing various *flhA* alleles. (A) Western blot analysis of hook FlgE-FLAG fusion protein in cytoplasmic (C) and extracellular (E) protein fractions. The indicated strains containing the FlgE-FLAG expression vector pTS14 were analyzed using 15 μ g total protein for each cytoplasmic sample and 20 μ g for each extracellular sample. (B) Western blot analysis of flagellins in soluble cytoplasmic and extracellular protein fractions. The same protein fractions and amounts were analyzed as in part A.

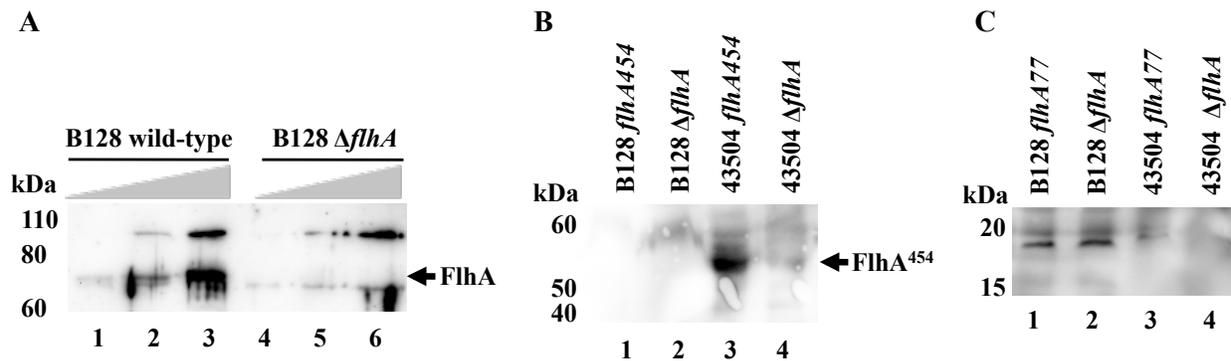


Figure 4.4: Western blot analysis of wild-type and truncated forms of FlhA. (A) Membrane fractions prepared from *H. pylori* B128 wild-type and $\Delta flhA$ mutant were analyzed by western blotting using affinity-purified antibodies. Lanes 1 and 4 contain 5 μ g protein, lanes 2 and 5 contain 10 μ g protein, and lanes 3 and 6 contain 20 μ g protein. FlhA, which is immediately above a faint band seen in samples from both the wild-type and $\Delta flhA$ mutant, is indicated by the arrow. Approximate positions of molecular weight markers are indicated at the left of the figure. (B) Membrane fractions prepared from *H. pylori* B128 bearing either the *flhA454* or $\Delta flhA$ allele, or *H. pylori* ATCC 43504 bearing either the *flhA454* or $\Delta flhA$ allele were analyzed by western blotting. Each lane was loaded with 20 μ g protein. Approximate positions of molecular weight markers are indicated at the left of the figure. (C) Membrane fractions prepared from *H. pylori* B128 bearing either the *flhA77* or $\Delta flhA$ allele, or *H. pylori* ATCC 43504 bearing either the *flhA77* or $\Delta flhA$ allele were analyzed by western blotting. Each lane was loaded with 20 μ g protein. Approximate positions of molecular weight markers are indicated at the left of the figure.

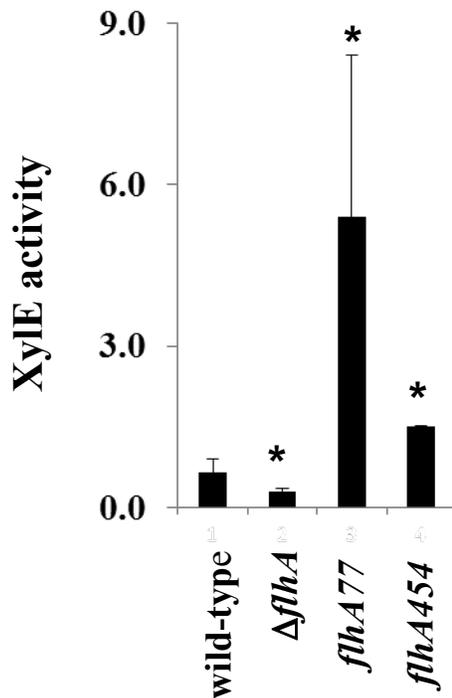


Figure 4.5: Expression of a *flaB*'-*xylE* reporter gene in *H. pylori* ATCC 43504 bearing various *flhA* alleles. Whole cell Xyle assays were carried out for *H. pylori* ATCC 43504 bearing the wild-type, $\Delta flhA$, *flhA77* or *flhA454* allele. Xyle activity indicated for the y-axis is reported in units of Xyle activity per 10^8 cells. One unit of Xyle activity corresponds to 1 μ mole of 2-hydroxymuconic semialdehyde produced/min. Enzyme activities for each strain were determined from at least 6 statistical replicates for two or more biological replicates. Error bars correspond to one standard deviation. Asterisks indicate values that differed significantly from wild-type Xyle activity ($P = 0.05$).

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CHAPTER 5

BINDING OF *HELICOBACTER PYLORI* FlgS TO THE N-TERMINUS OF FlhA AND ITS
IMPLICATIONS IN SIGNAL TRANSDUCTION

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Abstract

Flagellar biogenesis is a complex process that involves multiple checkpoints to coordinate transcription of flagellar genes with assembly of the flagellum. Transcription in flagellar genes in *Helicobacter pylori* is regulated by the three sigma factors found in this bacterium (RpoD, RpoN and FliA). Previous studies have shown that components of the flagellar Type III Secretion System are required for expression of the RpoN-dependent genes. Transcription of the RpoN regulon is activated by a two-component system consisting of the sensor kinase FlgS and the response regulator FlgR. However, it is unclear what initiates signal transduction which results in activation of the RpoN regulon by the FlgS/FlgR two-component system. We showed previously that expression of the first 77 amino acid residues of FlhA was sufficient to support transcription of the RpoN regulon. Using the optical biosensing technique biolayer interferometry, we report here that a peptide corresponding to the first 24 amino acid residues of FlhA (FlhA_{NT}) binds FlgS with nanomolar affinity via the C-terminal half of FlgS. FlhA_{NT} did not stimulate FlgS autophosphorylation in vitro, suggesting that FlhA_{NT} may facilitate interactions with FlgS and other structures needed to stimulate phosphorylation.

Introduction

Helicobacter pylori is member of the subphylum ϵ -Proteobacterium and a causative agent of significant pathologies in the stomach. Approximately 50% of the world population is infected with *H. pylori* though only a small fraction of infected individuals exhibit disease symptoms. *Helicobacter pylori* possesses 2-6 polar flagella that are used to burrow through the mucus layer lining the stomach epithelium. Colonization of the gastric mucosa is dependent on motility as non-flagellated mutants are unable to colonize (1).

The flagellum is a complex structure comprised of ~30 different types of protein subunits organized into three basic parts, namely the basal body, hook, and filament. The basal body is located at the cell membrane and contains a specialized Type III secretion system (referred to as the flagellar protein export apparatus), rings (MS, P, L and C), rod, and motor components. The export apparatus is responsible for the secretion of the axial components of the flagellum (i.e., rod, hook and filament proteins) across the inner membrane and consists of six integral membrane proteins (FliO, FliP, FliQ, FliR, FlhA, and FlhB) and three cytoplasmic proteins (FliH, FliI, and FliJ) that shuttle flagellar substrates to the integral membrane component of the export apparatus (2, 3). The C ring (or switch complex) is located at the cytoplasmic side of the inner membrane. The C ring in *H. pylori* consists of four proteins, FliG, FliM, FliN, and FliY (4, 5). In addition to controlling the rotational direction of the flagellum, the C ring interacts with the soluble components of the export apparatus to shuttle flagellar substrates to the export apparatus for secretion (3, 6, 7). Flagellar substrates are exported in the order of assembly with components located at the base of the flagellum being assembled first.

The synthesis of the flagellum is a complex process involving over 50 genes that code for structural and regulatory components and related chemotaxis components. Synthesis of the flagellum involves tight coordination between expression of flagellar genes and assembly of the nascent flagellum. The temporal expression of flagellar genes is controlled by the three sigma factors found in *H. pylori*: RpoD (σ^{80}), RpoN (σ^{54}) and FliA (σ^{28}). Binding of sigma factors to core RNA polymerase allows polymerase to recognize specific promoters (8). Different sigma factors recognize different promoter sequences allowing transcription of a specific set of genes.

Transcription of genes required early in flagellar assembly in *H. pylori* is regulated by RpoD (9). In *Salmonella enterica* and *Caulobacter crescentus*, master regulators that initiate

flagellar gene transcription cascade have been identified (10, 11). The master regulator coordinates assembly of the flagellum with the cell cycle and initiates a transcriptional hierarchy by stimulating transcription of genes required early in assembly of the nascent flagellum. In *H. pylori* a master regulator has not been identified, possibly because motility is required for its obligate parasitic lifestyle (9) or because it has other essential roles which prevent identification through mutagenesis screens.

Transcription of genes needed midway through flagellar biogenesis which encode components of the hook, hook-associated proteins and a minor flagellin (FlaB), is regulated by RpoN (9). Transcription of the RpoN-dependent genes is regulated by a two-component system consisting of the cytoplasmic sensor kinase FlgS and the response regulator FlgR (12, 13). The cellular cue sensed by FlgS to initiate signal transduction resulting in transcriptional activation of the RpoN regulon has been linked to the flagellar protein export apparatus, although the export apparatus does not need to be secretion-competent (9, 14-16).

Transcription of genes whose products are required late in flagellar assembly (major flagellin FlaA, filament cap protein and chaperones proteins) is regulated by FliA. The activity of FliA is regulated by the anti-sigma factor FlgM for which when bound to FliA represses its ability to interact with core RNA polymerase (17). In *Salmonella*, the inhibition of FliA activity is relieved when FlgM is secreted by the flagellar protein export apparatus as a filament-type substrate (18). FlgM is not exported efficiently in *H. pylori*; instead its inhibition on FliA is alleviated through an unknown mechanism which may involve binding to FlhA (19).

H. pylori FlhA is 733 amino acid residues in length and consists of an N-terminal transmembrane domain and a C-terminal cytoplasmic domain (Fig. 5.1A). We showed previously that a truncated FlhA protein consisting of the first 77 residues is sufficient to support

transcription of RpoN-dependent genes in *H. pylori* (16). We extend the analysis of FlhA and its potential role in signal transduction by examining a region of FlhA corresponding to the first 24 amino acid residues of the protein (FlhA_{NT}) and is predicted to be exposed on the cytoplasmic side of the membrane. We show that a FlhA variant that lacks FlhA_{NT} is unable to support transcription of RpoN-dependent genes in *H. pylori*. Expression of the FlhA variant from the shuttle vector pHel3 inhibited motility in wild-type *H. pylori* strain ATCC 43504 suggesting that the protein was stably expressed and incorporated into the export apparatus. Taken together, these findings suggest that FlhA_{NT} is required for transcription of the RpoN regulon. Using biolayer interferometry and microscale thermophoresis, we showed that a peptide corresponding to FlhA_{NT} binds FlgS with nanomolar affinity via the C-terminal half of FlgS. The FlhA_{NT} peptide did not stimulate autophosphorylation of FlgS, although this does not preclude a role for FlhA_{NT} in signal transduction. The export apparatus is predicted to contain nine FlhA subunits, each of which could bind FlgS. We postulate that binding of FlgS to FlhA_{NT} facilitates interaction of FlgS with itself to promote dimerization and stimulate the autokinase activity of the protein. Alternatively, binding of FlgS to FlhA_{NT} may facilitate interactions of FlgS with other components of the flagellar basal body to initiate signal transduction.

Materials and Methods

Bacterial strains and growth conditions. *Escherichia coli* strains were grown at 37°C in Luria-Bertani broth or agar. Kanamycin (30 µg/ml) or ampicillin (100 µg/ml) was added to the medium when appropriate. *H. pylori* strains were grown at 37°C on tryptic soy agar supplemented with 10% horse serum under an atmosphere consisting of 2% O₂, 5% CO₂ and

93% N₂. Kanamycin (30 µg/ml) or chloramphenicol (30 µg/ml) was added to the medium when appropriate.

Strain construction. $\Delta flhA$ mutants were constructed in *H. pylori* B128 and ATCC 43504 as previously described (16). Briefly, the region corresponding to 90 nucleotides upstream of the start codon through codon 77 of *flhA* was replaced with a chloramphenicol transacetylase (*cat*) cassette. Genomic DNA from *H. pylori* 26695 was extracted using the Wizard Genomic DNA purification kit (Promega) and used as the template for creating *flhA* alleles. The $\Delta flhA$ mutant was complemented with plasmids expressing various forms of *flhA* with its native promoter. Primers SphI PflhA F2 and KpnI flhA R2 were used to amplify *flhA* and its native promoter. This region was cloned into pCR2.1 and subcloned into pHel3 (20, 21) via the SphI and KpnI sites. The resulting plasmid containing the *flhA* region was called *pflhA*. Another plasmid was generated to create an in-frame deletion of codons 2-24 (*pflhA_{ΔNT}*). Primers SphI PflhA F2 and flhA24 R were used to amplify the *flhA* promoter and the start codon of *flhA*. Primers flhA24 F and KpnI flhA R2 were used to amplify codons 25 through 56 nucleotides downstream of the *flhA* stop codon. Primer flhA24 F contains sequences reverse and complementary to flhA24 R. This region was used to perform overlapping PCR to create an in-frame deletion of codons 2 through 24. The resulting amplicon was cloned into pCR2.1 (Life Technologies) and subcloned into pHel3 and the new plasmid was named *pflhA_{ΔNT}*. Plasmids *pflhA* and *pflhA_{ΔNT}* were introduced into the $\Delta flhA$ mutant by natural transformation. All constructs made were confirmed by PCR and sequencing of the resulting amplicons. Primers used in for strain construction are listed in Table 5.1.

Motility assay. Motility of *H. pylori* cells was assayed on soft agar plates consisting of Mueller Hinton Broth and 0.4% noble agar. After autoclaving, sterile heat-inactivated horse serum (10%

final), FeSO₄ (10 μM final concentration), and 2-(N-morpholino)ethanesulfonic acid (20 mM final concentration) were added to the medium. Sterile toothpicks were used to inoculate cells into the center of the agar plate. Plates were incubated at 37°C under an atmosphere of 2% O₂, 5% CO₂ and 93% N₂. Diameters of the spreading cells were measured after one week. Motility assays were done in triplicate. Diameter sizes were averaged and analyzed statistically using Student's t-test.

Quantitative reverse-transcription PCR. RNA was extracted as previously described (16). Briefly, *H. pylori* cells were grown on TSA supplemented with 10% horse serum for 18 hours. Cells were harvested and resuspended in 100 μl of water. RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad) and contaminating DNA was removed using the TURBO DNA-free kit (Ambion). Single stranded cDNA was synthesized from 200 ng RNA using the iScript cDNA synthesis kit (Bio-Rad). Relative transcript levels of *flaB*, *flgE*, and *flaA* were determined using quantitative reverse-transcription PCR (qRT-PCR) as described in (15). Primers used for this study are listed in Table 5.1.

FlgS purification. *flgS* was amplified from *H. pylori* ATCC 43504 using primers FlgS F and FlgS R with *Pfu* polymerase. The amplicon was cloned into the NdeI and the BamHI sites of pET19b. The resulting construct (*pflgS*) was verified by PCR and sequencing. For expression and purification of FlgS, *pflgS* was transformed into *E. coli* KRX cells. Cultures were grown at 37°C in Terrific Broth to an OD₆₀₀ of 0.4. Rhamnose (0.1% final) and isopropyl-β-D-thiogalactopyranoside (IPTG) (1 mM final) were added to the culture to induce expression of His-FlgS. After overnight growth at room temperature, cells were harvested by centrifugation and resuspended into Buffer A (50 mM phosphate buffer pH 8.0, 500 mM NaCl, 10 mM imidazole, 10% glycerol, 0.05% Tween-20).

Plasmids encoding fragments of FlgS were synthesized by DNA 2.0. The parent vector pJ414 (DNA 2.0) contains a T7 promoter and a terminator sequence flanking a multicloning site. DNA sequences encoding residues 1-169 and residues 170-230 along with a N-terminal His-tag were cloned into the NdeI and BamHI site of this vector to create the plasmids *pflgS_N* and *pflgS_C*, respectively. For expression and purification, *pflgS_N* and *pflgS_C* were transformed into *E. coli* BL21 (DE3) pLysS. Cells were induced with IPTG (1 mM final).

Purification of all forms of FlgS was performed as follows. All purification steps were carried out at 4° C. Cells were lysed by three passages through a French pressure cell at 10,000 kPa. Unlysed cells were removed by centrifugation for 15 minutes at 6,000 x g. His-FlgS was purified using the His-Pur Ni-NTA resin (Thermo Scientific). The His-Pur Ni-NTA resin was equilibrated in Buffer A and then incubated with the lysate for 1 hr on an end-over-end tube rotator. The resin containing any bound proteins was separated from unbound lysates by centrifugation and washed with Buffer B (50 mM phosphate buffer pH 8.0, 500 mM NaCl, 25 mM imidazole, 10% glycerol, 0.05% Tween-20). The resin mixture was then transferred to a column to further wash with Buffer B. His-FlgS was eluted from the resin using Buffer C (50 mM phosphate buffer pH 8.0, 500 mM NaCl, 250 mM imidazole, 10% glycerol, 0.05% Tween-20). For phosphorylation studies, eluted proteins were dialyzed in 10 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM dithiothreitol (DTT), 0.05% Tween-20, 100 mM potassiumthiocyanate, and 0.1 mM EDTA). For biosensing experiments, FlgS was exchanged into binding buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% Tween-20, 10% glycerol, 1 mM DTT). Protein concentration was determined by a bicinchoninic acid protein assay (Pierce) or Bradford assay.

FlhA_{NT} peptide. A peptide corresponding to FlhA_{NT} was synthesized by GenScript. The N-terminal sequence from FlhA (FlhA_{NT}) was MANERSKLAFKKTFFVFKRFLQSKDC. A randomized peptide (FFKLSQKKSFERKDQSDLKFRKLRK) was also generated from a rearrangement of the FlhA_{NT} sequence. Peptides corresponding to the residues 1-14 (FlhA_{NTn}) and 15-25 (FlhA_{NTc}) were also synthesized by Biomatik.

Optical biosensing. All biolayer interferometry (BLI) measurements were made on a FortéBio (Menlo Park, CA) Octet QK biosensor using streptavidin (“SA”) sensors. Assays were performed in 96-well microplates at 25 °C. All volumes were 200 µL. Ligand peptides used in this study were synthesized with an N-terminal biotin group (Biomatik, Cambridge, Canada). After loading ligands onto SA sensors, a baseline was established in binding buffer prior to association at varying analyte concentrations. Dissociation was subsequently measured in buffer only. Raw data were analyzed with GraphPad Prism.

Microscale thermophoresis. Microscale thermophoresis measurements were performed on a NanoTemper Technologies Monolith NT.115 instrument. FlgS was labeled with the amine reactive NT-647 dye. Labeled protein was separated from unreacted dye by passage over a gel filtration column. Sixteen serial dilutions of FlhA_{NT} were equilibrated with a constant concentration of FlgS. Thermophoresis was measured at steady state as Hot/Cold ratio. Ratios were fit to a single-state binding model to generate K_D.

In vitro phosphorylation assay. The phosphorylation state of FlgS was monitored using a phosphate affinity polyacrylamide (Phos-tag) gel electrophoresis system (Wako Chemistry USA, Inc) as previously described (22). A buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, and 20 µM MgCl₂ was used for all dilutions. Reactions contained 2 µg FlgS, 200 nM FlhA_{NT} and were initiated with 1 mM ATP in a total volume of 25 µL. Reaction were incubated at room

temperature for 2 minutes, 5 minutes, and 10 minutes before stopping the reaction with the addition of 6X SDS-PAGE loading dye (168 mM Tris base, pH 6.8, 7% SDS, 0.3% Bromphenol Blue, 34% glycerol, 4% 2-mercaptoethanol). Phos-tag gels were made according to manufacturer protocols. 15 μ L of the reaction was loaded onto the gel. Gels were stained with a Commassie brilliant blue solution to visualize unphosphorylated and phosphorylated proteins.

Results

The amino terminus of FlhA is required for motility in *H. pylori*. *H. pylori* FlhA is 733 amino acid residues in length and the largest component of the membrane-integrated portion of the flagellar protein export apparatus. Like its counterpart in *Salmonella*, hydropathy analysis predicts the membrane topology of *H. pylori* FlhA to consist of a small N terminal cytoplasmic region followed by eight membrane-spanning helices and a large cytoplasmic region (Fig. 5.1A). Previous studies showed that a *flhA* null mutant (Δ *flhA*) in *H. pylori* ATCC 43504 does not support transcription of the RpoN regulon, while a strain expressing only the first 77 residues of FlhA, which consists of the N-terminal cytoplasmic region and the first two transmembrane helices, is able to support wild-type levels of RpoN-dependent gene transcription (16). Based on these results, we hypothesized that the N-terminal cytoplasmic region of FlhA (FlhA_{NT}) may play a role in regulation of the RpoN regulon perhaps via interaction with the histidine kinase FlgS.

To test this hypothesis, we monitored the ability of a plasmid expressing the full-length FlhA (*pflhA*) and a plasmid expressing an N-terminal deletion of FlhA (*pflhA*_{NT}) to complement motility defects of the Δ *flhA* mutant in the *H. pylori* ATCC 43504 background. Plasmids contained *flhA* alleles downstream of their native *flhA* promoter. Transformation with *pflhA*

partially restored motility in the $\Delta flhA$ background ($\Delta flhA/pflhA$) (Fig. 5.1B). Introduction of *pflhA* in the wild-type background (WT/*pflhA*) reduced motility, possibly due to elevated levels of FlhA resulting from the increased copy number of *flhA* (pHel3 has a copy number of ~ 10 (20)). We observed similar results where overexpression of an export apparatus protein in otherwise wild-type cells resulted in decreased motility (15, 23). In contrast to *pflhA*, introduction of *pflhA_{ΔNT}* in the $\Delta flhA$ mutant ($\Delta flhA/pflhA_{\Delta NT}$) did not restore motility (Fig. 5.1B), suggesting that the N-terminal portion of FlhA is required for motility. Introduction of *pflhA_{ΔNT}* in the wild-type background (WT/*pflhA_{ΔNT}*) decreased motility indicating that the truncated FlhA_{ΔNT} allele interferes with the function of full-length FlhA proteins in *H. pylori* ATCC 43504. We infer from this observation that FlhA_{ΔNT} variant is stably expressed and incorporated into the export apparatus.

We observed previously that *H. pylori* strains ATCC 43504 and B128 differed with regard to their ability to stably express truncated FlhA variants, although we do not understand the reason for these differences (16). In this study, we observed differences in motility between these two strains when transformed with *pflhA_{ΔNT}*. As observed for the *H. pylori* ATCC 43504 $\Delta flhA$ mutant, transformation of the *H. pylori* B128 $\Delta flhA$ mutant with *pflhA* restored motility whereas *pflhA_{ΔNT}* did not (Fig. 5.1B). However, significant differences in inhibition of motility upon transformation of the wild type with *pflhA_{ΔNT}* were observed. Transformation of *H. pylori* ATCC 43504 with *pflhA_{ΔNT}* resulted in about a 2-fold decrease in halo diameter in soft agar assays as compared to the wild type whereas transformation of *H. pylori* B128 with *pflhA_{ΔNT}* did not have any effect on motility (Fig. 5.1C). Differences in membrane localization of truncated FlhA alleles between the two strains may account for these observations.

N terminus of FlhA is required for RpoN-dependent flagellar gene expression. To determine whether the FlhA_{NT} allele affects transcription of the RpoN regulon quantitative reverse transcription PCR (qRT-PCR) was used to monitor expression of two RpoN-dependent genes, *flaB* and *flgE*. Transcript levels of *flaB* and *flgE* were normalized to transcript levels of *gyrA* which appears to be expressed at a relatively constant level (24). Transcript levels of *flaB* and *flgE* in the $\Delta flhA$ mutant were consistent with our previous results (16) where there was no transcription of *flaB* and transcription of *flgE* was about 6-fold lower than wild-type levels (Fig. 5.2). Introduction of *pflhA* into the $\Delta flhA$ mutant partially restored transcript levels, whereas introduction of *pflhA*_{NT} failed to restore transcript levels in the $\Delta flhA$ mutant suggesting that FlhA_{NT} is required for transcription of the RpoN regulon.

The N-terminus of FlhA binds FlgS via its C-terminal kinase domain. Biolayer interferometry (BLI) is an optical biosensing technique similar to the better known surface plasmon resonance (SPR) as both methods allow for measurements of intermolecular interactions between an analyte to an immobilized ligand in real-time (25-27). Ligand molecules are tethered to a fiber optic sensor and then exposed to an analyte for association. Binding is measured by a shift in the interference pattern of white light reflected from the end of the sensor. After the association phase, sensors are moved to a buffer-only solution and dissociation is monitored. By examining several different analyte concentrations and fitting raw data to global binding models rate and affinity constants can be determined (25).

A synthetic peptide consisting of the first 25 residues of FlhA (FlhA_{NT}) was used as ligand to measure interactions with FlgS. As shown in Fig. 5.3, binding at five different FlgS concentrations ranging from 31-500 nM readily fit a one-state global model where interactions between FlhA_{NT} and FlgS exist in one conformation. The estimated K_D was 21 nM with

subsidiary k_{on} of $2.9 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ and k_{off} $6.2 \times 10^{-4} \text{ s}^{-1}$. Goodness of fit parameters indicated excellent fits as the standard error of the K_{D} is $4.4 \times 10^{-10} \text{ M}^{-1}\text{s}^{-1}$. Controls of analyte screened against sensors without ligands resulted in negligible nonspecific binding (Fig. 5.4). Microscale thermophoresis (reviewed in (28, 29)), a solution method not requiring tethering of one of the binding partners to a surface yielded a K_{D} of 62 nM, which was in reasonable agreement with the BLI results (Fig. 5.3B). An initial attempt to discern critical residues for binding was performed with synthetic peptides corresponding to residues 1-14 (FlhA_{NTn}) and 15-25 (FlhA_{NTc}). Neither evinced binding to FlgS as response was indistinguishable from background (Fig. 5.4), suggesting that the binding site is disrupted in the smaller peptides.

Binding was also analyzed for fragments of FlgS. Residues 1-169 (FlgS_N) did not bind FlhA_{NT} (data not shown). However, residues 170-377 (FlgS_C) bound with high affinity, but observed binding was complex and did not fit a one-state model. Association (Fig. 5.5A) and dissociation (Fig. 5.5B) phases for binding of FlhA_{NT} to FlgS_C could both be fit by parallel two-state exponentials for which the majority of the amplitude was accounted for by a fast-on, slow-off state. In this two-state model, FlhA_{NT} and FlgS interact initially in one conformation and then this complex may shift into a second conformation. Plotting observed association rate constant versus analyte concentration yielded a k_{on} for the fast binding of $1.7 \times 10^4 \text{ M}^{-1}\text{s}^{-1}$ (Fig. 5.5C). Pairing that with the slow state ($k_{\text{off}} = 4.0 \times 10^{-4} \text{ s}^{-1}$) gave a K_{D} of 24 nM, which was essentially identical to that of full-length FlgS. Though unlikely to be of biologic significance (see Discussion), the minor components yielded k_{on} of $1.9 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$ and k_{off} of 0.032 s^{-1} for a nominal K_{D} of 17 μM . Neither FlhA_{NTn} nor FlhA_{NTc} exhibited binding to FlgS_C (Fig 5.4D).

FlhA_{NT} does not stimulate autophosphorylation of FlgS. To test the hypothesis that interaction with FlhA stimulates autophosphorylation of FlgS, FlhA_{NT} (200 nM) was incubated

with FlgS (~0.5-1.5 μ M). Phosphorylated proteins were separated from unphosphorylated proteins using the PhoS-tag gel system where phosphorylated proteins migrate slower through the gel than unphosphorylated proteins. Under our assay conditions, we observed a low level of phosphorylation of FlgS in the presence of ATP (Fig. 5.6, lane 2). Addition of FlhA_{NT}, however, did not increase the amount of phosphorylated protein indicating that FlhA_{NT} does not act by itself as a signal to stimulate the autokinase activity of FlgS (Fig. 5.6, lane 3).

Discussion

Like its homologs in *Salmonella* and other species, *H. pylori flhA* is required for motility, presumably to effect flagellar type III secretion (16, 23). Most studies on FlhA have focused on the cytoplasmic C-terminal domain of FlhA. However, McMurry and coworkers showed that a FlhA variant which lacked residues 18 to 22 was able to complement a *flhA* mutant in *Salmonella*, while a FlhA variant that lacked residues 1 to 22 was unable to complement the same *flhA* mutant (23). FlhA_{NT} has also been proposed to interact with FliI as either overproduction of FliI or bypass mutations in FlhA_{NT} improved motility in a Δ *fliH* mutant background, suggesting that FlhA_{NT} has a role in substrate export as well (30).

Our deletion analysis of *H. pylori flhA* suggests that FlhA_{NT} is needed for transcription of the RpoN-dependent genes *flaB* and *flgE* (Fig. 5.3). A caveat of our deletion analysis is that it is unclear whether the truncated FlhA_{NT} is able to localize to the membrane and interact with other components of the export apparatus. In *Salmonella*, FlhA variants that lack most or part of FlhA_{NT} were retained in the membrane fraction (23). Moreover, the protease protection profiles of wild-type FlhA and the FlhA variant which lacked FlhA_{NT} were identical (23). Taken together, these observations suggest that the truncated FlhA proteins properly assemble within

the membrane-embedded portion of the export apparatus (23). Our observation that FlhA_{NT} inhibited motility in *H. pylori* ATCC 43054 (Fig. 5.1) seems consistent with the idea that this FlhA variant is assembled within the export apparatus.

We demonstrated here that FlgS binds FlhA_{NT} with high affinity (Figs. 5.3), and that it is a region within residues 170-377 (FlgS_C) which is responsible for binding FlhA_{NT} (Fig. 5.5). This region of FlgS contains both the Histidine Kinase A (HisKA) domain (residues ~170 – 230) and the Histidine kinase-like ATPase (HATPase_c) domain (residues ~280 – 380) of the protein. Binding of FlhA_{NT} to FlgS_C yielded complex data, acceptable fitting of which required a second component as a parallel state. The fast-on, slow-off state was nearly identical to binding of full-length FlgS, strongly suggesting that the binding site for FlhA_{NT} on FlgS lies completely within the C-terminal half of the protein. The second state, relatively slow on and fast off, was of low affinity and accounted for no more than about 10% of the amplitude. While it could represent some biologically relevant event such as a conformational change, we consider it far more likely a result of expressing the C-terminal fragment of FlgS alone, perhaps due to alterations of the binding site.

The high affinity of FlgS for FlhA_{NT} suggests the interactions between these proteins are biologically relevant. Our attempts to show that FlhA_{NT} stimulates the autokinase activity of FlgS, however, proved unsuccessful. There are a couple possible reasons to explain this. Firstly, FlhA_{NT} may not be the signal that stimulates FlgS autokinase activity. Secondly, FlhA_{NT} may be the signal but is unable to stimulate FlgS activity outside its native context within the export apparatus. Thirdly, FlhA_{NT} may be part of a complex that functions as the signal for stimulating FlgS autokinase activity. Such a complex could simply include other copies of FlhA_{NT}. The export apparatus is predicted to contain nine FlhA subunits (31), and it is possible that binding of

FlgS monomers to the N-termini of these FlhA subunits facilitates dimerization of FlgS and thereby stimulate the autokinase activity of the protein. Alternatively, FlhA_{NT} may need to work in conjunction with other proteins to stimulate FlgS autophosphorylation. Good candidates for such auxiliary proteins are the MS ring protein FliF and the C ring protein FliG as Boll and Hendrixson showed that FlgS could be cross-linked to these proteins in vivo in *C. jejuni* (32). In support of this supposition, FlhA_{NT}, the C-terminal domain of FliF (FliF_C) and FliG are likely to be closely associated with each other as FlhA has been shown to interact with FliF (33) and FliF_C has been shown to interact with FliG (34, 35).

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Author Contributions

JT, TH, TRH, and JLM designed the experiments. JT, TH, and JLM performed the experiments and analyzed the data. JT, TRH, and JLM wrote the paper.

Table 5.1: Primers used in this study

Primer name	Sequence
SphI flhA F2	ATG TCA GCA TGC ATA ACG ACC TCC TAA TTG GTA A
KpnI flhA R2	CAT GGT ACC CAA TGT GTG AAA GGT GGT AAA C
flhA24 R	CAT AAT CTA AAA TCA ATG CCT
flhA24 F	AGG CAT TGA TTT TAG ATT ATG GAC TTA GCC CTT GTG GTC
gyrA F	GCT AGG ATC GTG GGT GAT GT
gyrA R	TGG CTT CAG TGT AAC GCA TC
flgE F	TGC GAA CGT GAA TAC CAC TG
flgE R	GTC ATT CTG CCC TGC TAA CC
flaB F	ATC GCC GCT TTA ACT TCT CA
flaB R	CGC CAT CCC ACT AGA ATC AT
FlgS F	GTC TCG CAT ATG AAA AAA TCC AAG CAC TTA AAA C
FlgS R	CCT ATC GGA TCC AAA TTA AGA AGC GTT AAG AAT

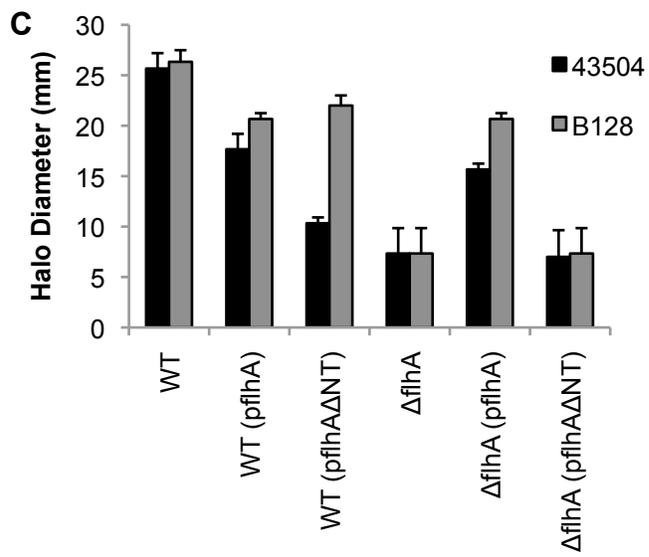
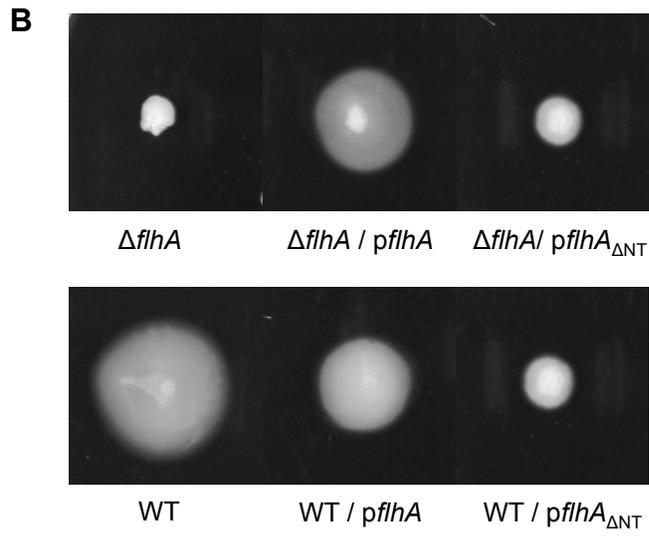
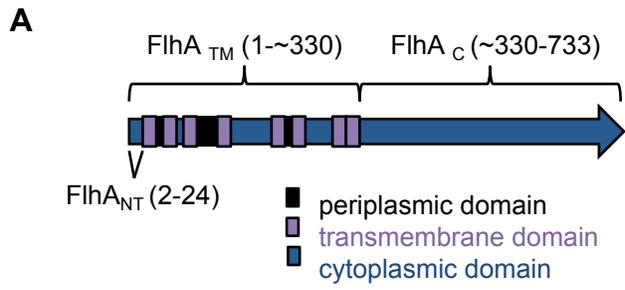


Figure 5.1: FlhA_{NT} is required for motility. (A) Transmembrane topography as inferred by hydropathy analysis. Abbreviations as follows: FlhA_{NT} (N-terminal cytoplasmic domain), FlhA_{TM} (the transmembrane domain), FlhA_C (cytoplasmic domain). The sequence deleted (residues 2-24) for construct *pflhA_{ΔNT}* is indicated at left. (B) Motility assessed on soft agar plates. Top row, $\Delta flhA$ from parent strain 43504 transformed with empty vector, *pflhA* and *pflhA_{ΔNT}*. Bottom row, 43504 parental strain transformed with the same plasmids as the top row. (C) Strain-specific motility differences. The parent strain for *flhA* deletion is indicated by color with motility results quantified for each transformation. Error bars indicate standard error from three separate trials.

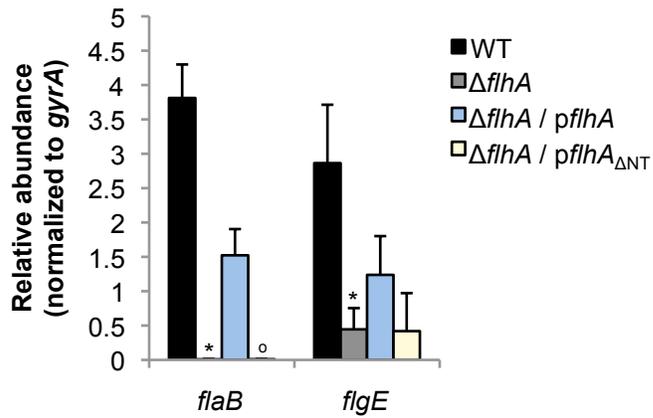


Figure 5.2: Flh_{ΔNT} is required for transcription of RpoN-dependent genes. Transcript levels of *flaB* and *flgE* are dramatically reduced in $\Delta flhA$ (grey) compared to wild-type (black). Transcript levels of *flaB* and *flgE* are partially restored in the $\Delta flhA/pflhA$ strain (blue) but not the $\Delta flhA/pflhA_{\Delta NT}$ strain (tan).

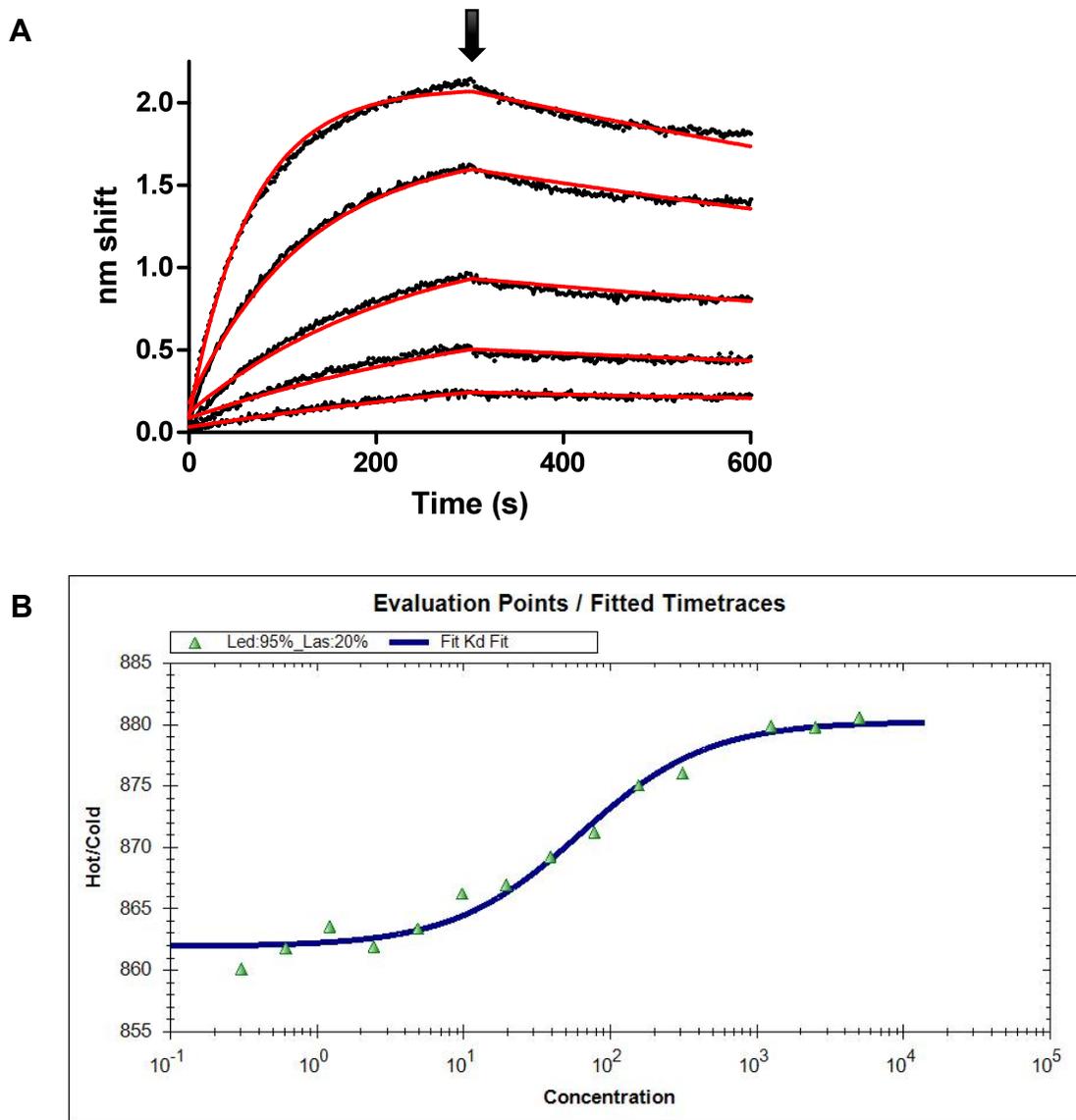


Figure 5.3: Analysis of FlhA_{NT} binding to FlgS. (A) BLI analysis of FlhA_{NT} binding to FlgS. Raw data are shown in black with fits to a global one-state model as red lines. Analyte concentrations were 31, 62, 125, 250 and 500 nM. Arrow indicates the start of the dissociation phase when sensors were moved to buffer solution. (B) Microscale thermophoresis of unlabeled FlhA_{NT} peptide binding to FlgS. Data are fit to a binding model in which K_D is 62 nM.

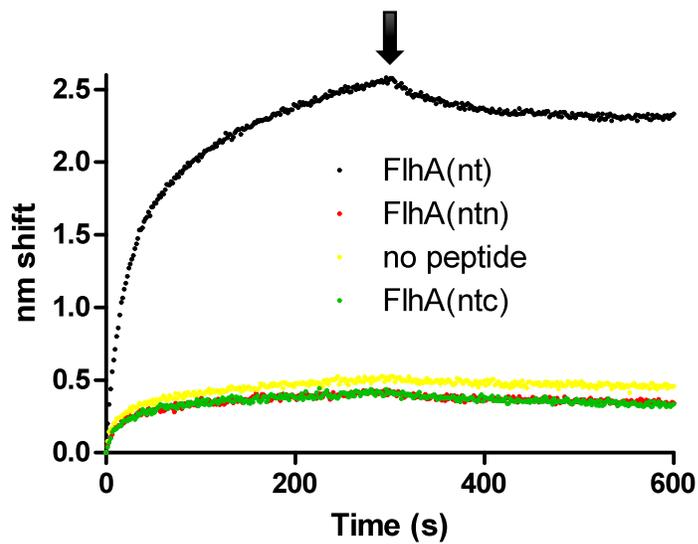


Figure 5.4: Analysis of FlgS binding to truncated forms of FlhA_{NT}. BLI analysis of 1.2 μ M FlgS binding to FlhA_{NT} (black), FlhA_{NTn} (red) and FlhA_{NTc} (green). Nonspecific binding to the sensor only (no ligand) is shown in yellow. Arrow indicates the start of the dissociation phase when sensors were moved to buffer solution.

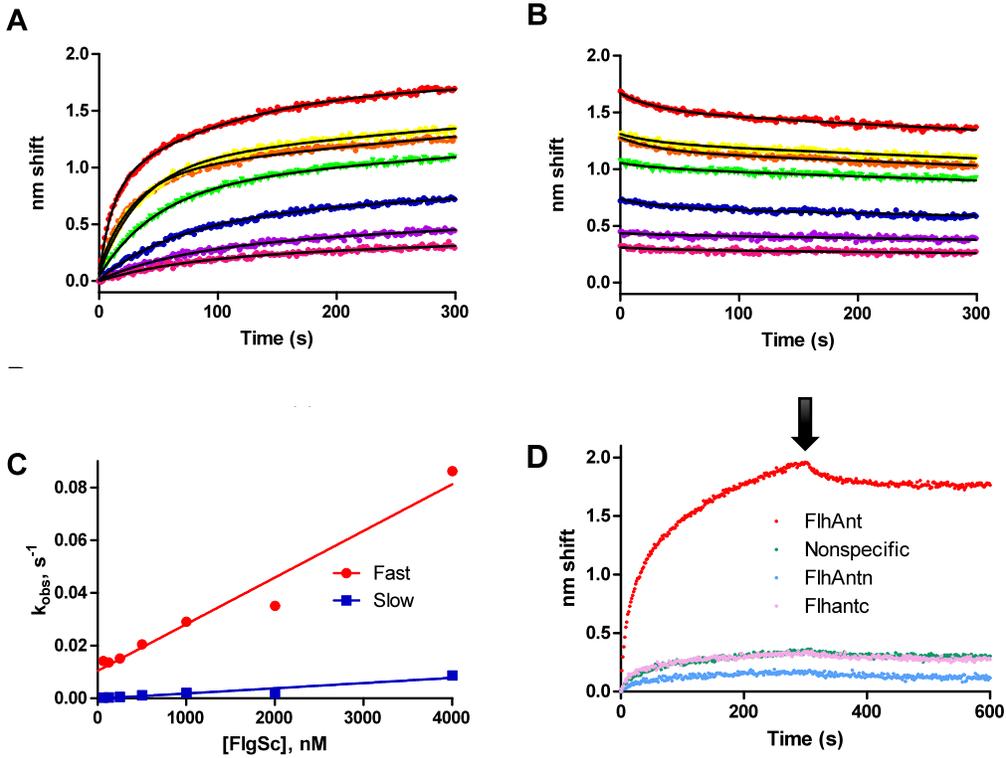


Figure 5.5: BLI analysis of Flh_{ANT} binding to Flg_{SC}. Raw data are shown in colors with fits to a parallel two-state model as black lines during the association phase (A) and dissociation phase (B). (C) k_{obs} determined from the association phase vs. analyte concentration. Each state is plotted separately and k_{on} is equal to the slope. (D) 1 μ M Flg_{SC} binding to peptides Flh_{ANT} (red), Flh_{ANTn} (blue) and Flh_{ANTc} (pink). Nonspecific binding to the sensor only (no ligand) is shown in green. Arrow indicates the start of the dissociation phase when sensors were moved to buffer solution.

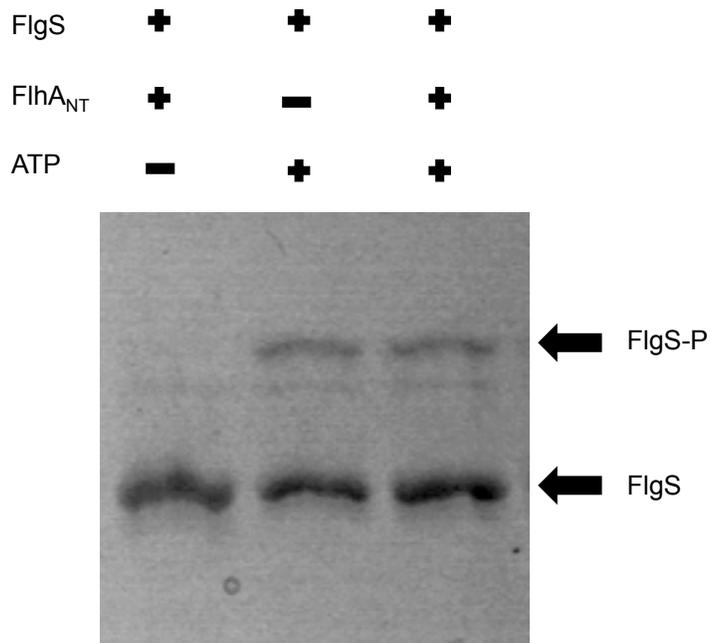


Figure 5.6: Phosphorylation of FlgS monitored by an *in vitro* phosphorylation assay.

Phosphorylated proteins were separated from non-phosphorylated proteins using the Phos-tag gel system. Addition of FlgS, FlhA_{NT} and ATP are indicated above the image.

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CHAPTER 6

BASAL BODY STRUCTURES DIFFERENTIALLY AFFECT TRANSCRIPTION OF RpoN-
AND FliA- DEPENDENT FLAGELLAR GENES IN *HELICOBACTER PYLORI*¹

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Abstract

Flagellar biogenesis in *Helicobacter pylori* requires the coordinated expression of dozens of genes with assembly of the flagellum via a transcriptional hierarchy governed by three sigma factors, RpoD (σ^{80}), RpoN (σ^{54}), and FliA (σ^{28}). Previous studies showed that loss of flagellar protein export apparatus components inhibits transcription of flagellar genes. The FlgS/FlgR two-component system activates transcription of RpoN-dependent genes though it has been unclear how this activation occurs. We report here on how disrupting flagellar biogenesis at various points affects transcription of RpoN-dependent (*flaB* and *flgE*) and FliA-dependent genes (*flaA*). The MS ring (encoded by *fliF*) is one of the earliest flagellar structures assembled. Deletion of *fliF* abolished transcription of the RpoN regulon and inhibited transcription of *flaA* ~4-fold. FliH is a cytoplasmic protein that functions with the C ring protein FliN to shuttle substrates to the export apparatus. Deletions of *fliH*, and genes encoding C ring components (*fliM* and *fliY*) inhibited transcription of *flaB* and *flgE*, but had little or no effect on transcription of *flaA*. Transcription of *flaB* and *flgE* was stimulated in mutants where genes encoding rod proteins (*fliE* and *flgBC*) were deleted, while transcription of *flaA* was reduced ~2-fold in both mutants. We propose that FlgS responds to an assembly checkpoint associated with the export apparatus and that FliH and one or more C ring component may assist FlgS in engaging this flagellar structure.

Introduction

The bacterial flagellum is a complex nanomachine powered by an ion-driven rotary motor that consists of about 30 different types of proteins whose copy numbers range from a few to thousands (Fig. 6.1). The flagellum consists of three basic structures referred to as the basal

body, hook and filament (1). The basal body is an intricate complex that consists of the flagellar rod, rings, motor, switch complex and a specialized Type III Secretion System (T3SS) that transports flagellar proteins across the cell membrane (1-3). The T3SS, also referred to as the flagellar protein export apparatus, consists of integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ and FliR) which form an export pore located within the inner membrane, as well as cytoplasmic components (FliI, FliH and FliJ) that deliver protein substrates to the export pore (4, 5). During flagellar assembly, the export apparatus initially transports rod- and hook-type substrates across the cell membrane into the lumen of the nascent flagellum (6, 7). Upon completion of the mature hook-basal body (HBB) structure, the export apparatus undergoes a conformational change that is accompanied with a switch in substrate specificity to filament-type substrates.

Biogenesis of the bacterial flagellum involves a transcriptional hierarchy that is responsive to checkpoints in flagellar assembly so that expression of specific flagellar genes occurs as their products are needed for formation of the nascent flagellum. In *Salmonella enterica* serovar Typhimurium (the model organism for flagellar biogenesis studies), flagellar genes needed early in assembly require the primary sigma (σ) factor RpoD (σ^{70}) for their transcription, while the late flagellar genes (e.g., flagellin gene) require the alternative σ factor FliA (σ^{28}) for their transcription (reviewed in (8)). Flagellar biogenesis in *H. pylori* and *Campylobacter jejuni* (both of which are members of the subphylum ϵ -Proteobacteria) similarly involves RpoD and FliA, but also involves the alternative σ factor RpoN (σ^{54}). Transcription of *H. pylori* and *C. jejuni* genes required early in flagellar assembly is dependent on RpoD (σ^{80}), while transcription of genes needed midway through flagellar biogenesis is dependent on RpoN and transcription of genes needed late in the assembly process is dependent on FliA (9-11). The

organization of *H. pylori* flagellar genes into regulons based on the σ factor needed for transcription suggests a framework for a transcriptional hierarchy that operates in conjunction with assembly of the flagellum. The assembly checkpoints and mechanisms which regulate such a hierarchy, however, are poorly understood.

In *H. pylori*, the RpoN-dependent genes encode rod proteins (FlgBC), hook protein (FlgE), hook-associated proteins (FlgL and FlgK), hook-length control protein (FliK), a minor flagellin (FlaB), and enzymes required for flagellin glycosylation (9, 12, 13). Transcription of the RpoN regulon is regulated by a two-component system composed of a cytoplasmic sensor kinase, FlgS, and the response regulator FlgR (9, 12, 14, 15). In addition to FlgS and FlgR, several components of the flagellar export apparatus are required for transcription of the RpoN regulons in *H. pylori* and *C. jejuni* (16), although the export apparatus does not need to be competent for secretion to stimulate transcription of the RpoN-dependent genes (17, 18). FlgS is thought to respond to a signal within or near the flagellar T3SS to initiate signal transduction resulting in transcription of the RpoN regulon. Boll and Hendrixson recently proposed a model in which the flagellar export apparatus is required for multimerization of FliF and FliG into the MS ring and rotor component of the C ring, respectively, and formation of this structure is sensed by FlgS to initiate signal transduction in *C. jejuni* (19). The signal sensed by FlgS may involve interactions of the sensor kinase with FliF and FliG as FlgS can be cross-linked to these proteins *in vivo* (19).

Transcription of the FliA-dependent flagellar genes in *Salmonella* and *Escherichia coli* is also intimately linked with the flagellar T3SS through a mechanism which has been well characterized. In the *Salmonella/E. coli* paradigm, the anti- σ^{28} factor, FlgM, inhibits transcription of the FliA-dependent genes by binding FliA and preventing it from engaging the

promoter (20). Upon completion of the HBB complex, FlgM is exported from the cytoplasm by the flagellar T3SS, thereby allowing σ^{28} -RNA polymerase holoenzyme to bind its target promoters to initiate transcription (21). *H. pylori* possesses a FlgM homolog (22), but the inhibitory effect of FlgM on FliA is thought to be alleviated through interactions between FlgM and the cytoplasmic domain of FlhA (FlhA_C) rather than by secretion of FlgM (23).

To understand better the connection between flagellar gene expression and assembly of the flagellum in *H. pylori*, genes encoding various basal body components were disrupted and motility, flagellar biogenesis and transcription of selected flagellar genes were assessed in the resulting mutants. Genes targeted for mutagenesis included the MS ring protein (*fliF*), a soluble component of the flagellar T3SS (*fliH*), C ring elements (*fliM* and *fliY*), and axial components of the flagellum (*fliE* and *flgBC*, which encode the MS ring/rod linker and proximal rod proteins, respectively) (Fig. 6.1). In general, transcription of the FliA-dependent *flaA* was inhibited by mutations that blocked formation of the MS ring or rod, but not by mutations that interfered with assembly of the C ring. Mutations that blocked formation of the MS or C rings inhibited transcription of RpoN-dependent genes, whereas mutations that blocked rod assembly stimulated transcription of RpoN-dependent genes. We postulate that FliH and the C ring are required to facilitate interactions between FlgS and a target associated with the flagellar T3SS to initiate signal transduction in *H. pylori*.

Materials and Methods

Bacterial strains and growth conditions. *Escherichia coli* DH5 α was used for cloning and plasmid construction. *E. coli* strains were grown in Luria-Bertani broth or agar medium and were supplemented with ampicillin (100 μ g/ml) and kanamycin (30 μ g/ml) when appropriate.

H. pylori strains were grown microaerobically at 37°C under an atmosphere consisting of 2% O₂, 5% CO₂, and 93% N₂ on tryptic soy agar (TSA) supplemented with 10% horse serum or shaking under an atmosphere consisting of 8.3% O₂, 4.6% CO₂, 9.2% H₂, and 77.9% N₂ in brain-heart infusion (BHI) broth supplemented with 0.4% β-cyclodextrin. Kanamycin (30 μg/ml) or chloramphenicol (30 μg/ml) was supplemented to the medium used to culture *H. pylori* when appropriate.

Mutant construction. Mutations in *fliF*, *fliM*, *fliH*, *fliY*, *fliE* and *flgBC* were generated in *H. pylori* B128 by the following general procedure. For each mutation, overlapping PCR was used to generate an amplicon that contained a chloramphenicol acetyltransferase (*cat*) gene flanked by ~500 bp regions located upstream and downstream of the target gene. iProof high-fidelity DNA polymerase (Bio-Rad) was used for all PCR procedures. Primers used for PCR are listed in Table 6.1. The region upstream of the target gene was amplified using the upstream forward and the upstream reverse primers. The 5'-end of the upstream reverse primer contained sequence corresponding to one end of the *cat* cassette. The region downstream of the target gene was amplified using the downstream forward and the downstream reverse primers. The 5'-end of the downstream forward primer contained sequence corresponding to the other end of the *cat* cassette. Genomic DNA from *H. pylori* B128 was prepared using the Wizard Genomic DNA purification kit (Promega) and was used as the template to amplify regions upstream and downstream of the target gene. The *cat* cassette was amplified from pUC20cat (24) using the *cat* forward and *cat* reverse primers. Overlapping PCR via the complementary regions of the amplified *cat* cassette and the amplicons of the regions flanking the target gene generated a PCR product with the *cat* cassette between the flanking regions. The resulting amplicons were introduced into *H. pylori* B128 by natural transformation using the following protocol. *H. pylori*

cells were spotted onto a TSA plate and grown for 6 hours. The amplicon was mixed with the cells and incubated for 18 hours before transferring cells onto TSA plates containing the appropriate antibiotics for selection. Plates were incubated for 5 days to allow for colonies to appear. Replacement of the target gene with the *cat* cassette was confirmed by PCR using genomic DNA from chloramphenicol-resistant transformants as a template. The resulting amplicons were sequenced to verify that the mutant alleles were correct.

Mutants in *flgR* and *rpoN* were constructed by transformation and allelic exchange of plasmids containing the deletion alleles. The plasmid used to create the *flgR* insertion mutant was described in Brahmachary *et al.* (15) and contains the *cat* cassette inserted in a unique restriction site within *flgR*. This plasmid was transformed into *H. pylori* B128 for allelic exchange with the wild-type *flgR* allele. The *rpoN* mutant was created using a plasmid containing *rpoN* interrupted with a *cat* cassette. The *rpoN* gene was amplified from *H. pylori* 26695 and cloned into the NdeI and HindIII sites of pET28a. A unique EcoRI site was introduced into *rpoN* as described in Pereira *et al.* (25). The *cat* cassette was inserted into this site and the resulting plasmid was transformed into *H. pylori* B128. Mutants were confirmed by PCR and sequencing of the resulting amplicons.

Complementation of mutants. *fliM*, *fliMY*, *fliE*, and *flgBC* were amplified from genomic DNA isolated from *H. pylori* B128 using their respective forward and reverse primers indicated in Table 1. The *fliE* and *flgBC* regions amplified contained their native promoters. For the *fliM* and *fliMY* complementation plasmids, overlapping PCR was used to create a fusion which introduced the *fliN* promoter region upstream of these genes. The forward primer for each of the final PCR products contained a XhoI site at the 5'-end and the reverse primer for each of the final PCR product contained a BamHI site at the 5'-end. All resulting PCR products were cloned

into pGEM-T Easy, sequenced and introduced into pHel3 by cloning the DNA into the unique XhoI and BamHI restriction sites in pHel3. The resulting plasmids were introduced into the deletion mutants by natural transformation.

Motility assay. Motility was assessed using a semisolid medium consisting of Mueller-Hinton broth and 0.4% Noble agar. After autoclaving, the medium was supplemented with sterile 10% heat inactivated horse serum, 10 μ M FeSO₄, and 20 mM 2-(N-morpholino)ethanesulfonic acid (pH 6.0). Kanamycin (30 μ g/ml) or chloramphenicol (30 μ g/ml) were supplemented when appropriate. Sterile toothpicks were used to stab inoculate cells into the agar. Plates were incubated at 37 °C under an atmosphere consisting of 2% O₂, 5% CO₂, and 93% N₂. Diameters of the spreading *H. pylori* cells were measured after 7 days.

RNA extraction and cDNA synthesis. *H. pylori* cells were grown on TSA plates supplemented with 10% horse serum for 18 hours before harvesting and resuspended into 1 ml of nuclease-free water. Alternatively, *H. pylori* strains were grown in BHI liquid medium supplemented with 0.4% β -cyclodextrin to mid- to late-log phase and 1 ml of cells were harvested. Cells were pelleted and resuspended into 100 μ l of nuclease-free deionized water. The Aurum Total RNA Mini Kit (Bio-Rad) was used to isolate RNA, and the RNA solution was treated with the TURBO DNA-free Kit (Ambion) to remove any contaminating DNA. RNA was quantified using a BioPhotometer (Eppendorf) and RNA quality was confirmed on a 1.2% agarose gel. Single-strand cDNA was synthesized from 200 ng of RNA using the iScript cDNA Synthesis Kit (Bio-Rad).

Quantitative reverse-transcription PCR. Transcript levels of *flaA*, *flaB*, *flgE*, *flgS*, *flgR* and *fliA* were monitored by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) as described previously (26). Primers used are listed in Table 6.2. *gyrA* transcript levels were

measured as a reference gene. Specificity and efficiency of each primer pair was confirmed by PCR using genomic DNA and by qRT-PCR on a serial dilution of wild-type RNA. Each qRT-PCR reaction, totaling 20 μ l, consisted of 10 μ l of iQ SYBR Green Supermix (Bio-Rad), 5 μ l of 100-fold diluted cDNA from the cDNA synthesis reaction, and 200 nM of each primer. A melt curve analysis was performed at the end of each experiment. Experiments were performed on the Bio-Rad iCycler iQ System in technical triplicate for three biological replicates of each strain. Gene expression levels were quantified by the $2^{-\Delta\Delta ct}$ method (27). Statistical significance was determined using the two-sample *t*-test.

Electron microscopy. Strains were grown to mid- to late-log phase in BHI supplemented with 0.4% β -cyclodextrin to an OD₆₀₀ of 0.5 to 1.0. Kanamycin (30 μ g/ml) and chloramphenicol (μ g/ml) were included in the growth medium as necessary. Cells were spun down and resuspended in half strength Karnovsky's fixative (2.5% gluteraldehyde, 2% paraformaldehyde, 0.1M cacodylate buffer). Cells were fixed for 5 minutes and then incubated for 5 minutes on 300-mesh Formvar/carbon-coated copper grids. Grids were washed with 0.1 M cacodylate buffer, followed by a wash with deionized water. Excess liquid was wicked off with filter paper between washes. One drop of 1% uranyl acetate was applied to the grids for 30 seconds and then wicked off with filter paper. Grids were washed in deionized water and dried at room temperature overnight. Cells were visualized using the FEI Tecnai20 transmission electron microscope. For each strain, at least 115 cells were included for quantifying flagellated cells and determining the number of flagella per cell. Statistical analyses were performed using the Mann-Whitney U test to determine whether strains differed significantly from one another with regard to the distribution of the number of flagella per cell.

Detection of RpoN proteins. Antiserum generated against MBP-RpoN was affinity purified prior to use using the AminoLink Plus Immobilization Kit (Thermo Scientific). MBP-RpoN was purified as previously described (25) and immobilized to the AminoLink Plus Resin following the manufacturer's protocol. Antiserum generated against MBP-RpoN was buffer-exchanged two times into phosphate-buffered saline (PBS) by diluting with 13 ml PBS and reducing the volume to 0.5 ml using an Amicon Ultra-15 centrifugal filter. The buffer-exchanged antiserum was incubated in the column to allow for binding to MBP-RpoN. The column was washed with 10 ml PBS before eluting the antibody with 8 ml of 0.1M glycine, pH 2.5. The eluted antibody was dialyzed into citric acid-phosphate buffer (55 mM citric acid, 50 mM K₂HPO₄, pH 5.5), followed by Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.6) and stored at -20°C.

H. pylori cytoplasmic fractions were prepared for Western blot analysis. Cells were grown on agar medium for 24 hours before resuspending into 3 ml PBS. Cells were lysed with three passages through a French press at 10,000 kPa. Unlysed cells were removed by centrifugation for 15 min at 6000 x g. Membranes were separated from cytoplasmic proteins by two centrifugations for 60 min at 100,000 x g. The supernatant containing the cytoplasmic proteins was concentrated by trichloroacetic acid precipitation as described previously (17). Protein concentrations were determined using the bicinchoninic acid protein assay (Thermo Scientific) following the manufacturer's instructions. 20 µg protein was analyzed by Western blotting using the affinity-purified RpoN antibody and goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Antigen-antibody complexes were detected by chemiluminescence using SuperSignal West Pico Luminol/Enhancer Solution and SuperSignal West Stable Peroxide Solution (Thermo Scientific). Blots were visualized on the FluoroChem E imager (ProteinSimple).

Results and Discussion

The MS ring is required for transcription of the RpoN regulon. The MS ring consists of a complex of FliF proteins (28) and acts as a mounting platform for the rotor, switch complex (29) and the rod of the flagellum (30). The assembly of the MS ring is thought to be coordinated with the assembly of the flagellar T3SS in *Salmonella* (31), and FlhA may even promote oligomerization of the MS ring in *E. coli* (32). Since the MS ring is one of the earliest flagellar structure assembled and is a converging point for assembly of many of the basal body proteins, we wished to learn how the MS ring impacts flagellar gene expression in *H. pylori*.

A $\Delta fliF$ mutant was constructed in *H. pylori* B128 by replacement of *fliF* with the *cat* cassette and the phenotype of the resulting mutant was analyzed. As expected, the resulting strain was non-motile on soft agar plates and all cells analyzed by transmission electron microscopy were aflagellated (Fig. 6.2A and 6.2B). Transcript levels of *flaB* and *flgE* (both RpoN-dependent) and *flaA* (FliA-dependent) were analyzed in the $\Delta fliF$ mutant and parental strain by qRT-PCR and normalized to the transcript levels of *gyrA*. Cells for the qRT-PCR assays were obtained from cells grown on agar medium and in broth. The trends in transcript levels were similar for cells grown under the two conditions, but there tended to be less variation between biological replicates for cells grown on agar medium (data not shown), and so for further qRT-PCR assays we used cells grown on agar medium. Transcript levels of *flaB* and *flgE* in the $\Delta fliF$ mutant were similar to those in a $\Delta rpoN$ or $\Delta flgR$ mutant (Fig. 6.2C), indicating that the RpoN regulon is completely inactivated in the $\Delta fliF$ mutant. Boll and Hendrixson reported that deletion of *fliF* similarly results in the complete failure to express an RpoN-dependent reporter gene in *C. jejuni* (19).

Levels of *flaA* transcript in the Δ *fliF* mutant were ~4 fold lower than wild-type levels (Fig. 6.2C). The *H. pylori* FliA regulon is negatively regulated by the binding of the anti- σ^{28} factor FlgM to FliA. Colland and co-workers demonstrated that *flaA* transcript levels were increased ~2.5-fold in a *flgM* deletion compared to wild type and decreased only ~2-fold in a *flgM* overexpression strain (22), suggesting that FlgM does not completely abrogate expression of *flaA*. The inhibitory effect of FlgM on the *H. pylori* FliA regulon is believed to be alleviated by interactions between FlgM and components of the export apparatus (23). The level of inhibition in the Δ *fliF* mutant was similar to that observed for *H. pylori* B128 strains bearing deletions of the export apparatus genes *flhA* (18) or *fliO* (26) indicating that the phenotypes seen in the Δ *fliF* mutant may be due to a failure of the export apparatus to assemble properly.

C ring proteins are required for transcription of RpoN-dependent genes. The C ring is a cup-like structure which extends into the cytoplasm and is closely associated with the MS ring and flagellar T3SS. The C ring in *Salmonella* is comprised of three proteins, FliG, FliM and FliN, whereas the C ring in *H. pylori* consists of these same proteins but also contains an additional protein, FliY. The C ring has roles in rotor function, switching the rotational direction of the flagellum, and flagellar protein export (reviewed in (4)). FliG is located closest to the cell membrane and interacts with FliF (33) and the flagellar stator protein MotA (34) to generate torque for rotation of the rotor. FliN is the outermost protein in the C ring, and FliM is situated between FliG and FliN where it interacts with both proteins (35). FliN is believed to have a role in flagellar protein export as it interacts with FliH (5). Some bacteria, such as *Bacillus subtilis* have FliY in place of FliN. *B. subtilis* FliY consists of a domain that is homologous to FliN and a second domain that shares homology with the chemotaxis protein CheC, which is a phosphatase of CheY-phosphate (36).

Lowenthal and co-workers demonstrated that all four C ring proteins are required for flagellar biogenesis in *H. pylori* (37), but did not report on whether the C ring proteins were required for transcription of the RpoN or FliA regulons. Allan and co-workers reported that FliG is required for flagellar biogenesis in *H. pylori* and showed that *flaA* transcript levels are reduced in the *fliG* mutant (38). To determine if other C ring proteins are required for flagellar gene expression, we individually replaced *fliM* and *fliY* with the *cat* cassette and analyzed the phenotypes of the resulting mutants. The $\Delta fliM$ mutant was non-motile (Fig. 6.3A) and transmission electron microscopy revealed that most of the cells (77%) lacked flagella, while approximately 90% of the wild-type cells were flagellated (Fig. 6.3B). Of the $\Delta fliM$ mutant cells that were flagellated, most possessed one or two flagella, while most of the flagellated wild-type cells possessed two to four flagella (Fig. 6.3B). Transcript levels of *flaB* and *flgE* were about 3-fold lower in the $\Delta fliM$ mutant compared to wild type, whereas *flaA* transcript levels in the mutant were comparable to wild type (Fig. 6.3C) indicate that the C ring is needed for transcription of RpoN-dependent flagellar genes but not FliA-dependent genes.

The effect of the $\Delta fliY$ mutation on flagellar biogenesis was more severe than that of $\Delta fliM$ mutation. About 96% of the $\Delta fliY$ mutant cells lacked flagella, and cells that were flagellated possessed only a single flagellum (Fig 6.3B). Transcript levels of *flaB* and *flgE* in the $\Delta fliY$ mutant were ~18-fold lower than wild-type levels, whereas *flaA* transcript levels in the $\Delta fliY$ mutant did not differ significantly from wild type (Fig. 6.3C). The phenotypic differences between the $\Delta fliY$ and $\Delta fliM$ mutants were unexpected since *fliM* and *fliY* are in the same operon and the $\Delta fliM$ mutation was expected to have a polar effect on *fliY*. Complementation assays confirmed the polarity of the $\Delta fliM$ mutation on *fliY* as introduction of a plasmid-borne copy of

fliMY into the Δ *fliM* mutant restored motility, but the introduction of a plasmid-borne *fliM* did not (Fig. 6.3A). Additionally, the same plasmid restored motility in the Δ *fliY* mutant (Fig. 6.3A).

The inhibited expression of the RpoN-dependent genes observed for the *fliM* and *fliY* mutants suggests the C ring is needed for signal transduction via the FlgS/FlgR two-component system. It is somewhat puzzling why the loss of FliY had a more profound effect on flagellar biogenesis than did loss of FliM and FliY together. It is not known where *H. pylori* FliY localizes within the C ring, but it seems reasonable to suspect it is situated in the outer part of the C ring since some bacteria possess FliY in lieu of FliN. If FliN and FliY bind to similar regions of FliM, it is possible that in the absence of FliY additional copies of FliN are incorporated into the C ring and the additional copies of FliN interfere with signal transduction. In contrast to our results, Boll and Hendrixson observed that deletion of *fliM* or *fliY* did not inhibit expression of an RpoN-dependent *flaB::astA* transcriptional reporter in *C. jejuni* (19). In fact, expression of the *flaB::astA* reporter gene was stimulated ~40% in the *C. jejuni* *fliM* mutant compared to wild type (19). The phenotypic differences between *H. pylori* and *C. jejuni* C ring protein mutants were unexpected since the organization of the flagellar transcriptional hierarchies and the repertoire of regulatory proteins used by these two bacteria are quite similar. The results with the *C. jejuni* *fliM* and *fliY* mutants indicate that, in contrast to *H. pylori*, the C ring in *C. jejuni* does not facilitate (and may actually impede) signal transduction leading to transcriptional activation of the RpoN regulon. It is not obvious why *H. pylori* and *C. jejuni* differ with regard to the role of the C ring in transcriptional regulation of the RpoN regulon. One possible explanation is that there is a greater demand for synthesis of flagellar proteins in *H. pylori* since it produces multiple flagella (two to six) per cell while *C. jejuni* produces only a single flagellum per cell. Since the RpoN-dependent genes are expressed in a temporal fashion, *H. pylori* may require a very robust

signal transduction response by the FlgS/FlgR two-component system to ensure that enough flagellar subunits are made for the coordinated and simultaneous assembly of multiple flagella. It is possible that the C ring in *H. pylori* may facilitate the interaction of FlgS with the basal body whereas in *C. jejuni* the C ring may not interact with FlgS.

FliH is required for transcription of RpoN-dependent genes. FliI is an ATPase that functions with FliH to facilitate the initial entry of protein substrates to the export gate of the flagellar protein export apparatus (39). FliH prevents the ATPase activity of FliI when it is not engaged in secreting proteins (40, 41). FliI forms a heterotrimer with FliH (FliH₂FliI) which forms a hexameric ring (42) around FliJ (FliH₁₂FliI₆FliJ complex) (43). The FliH₁₂FliI₆FliJ complex is believed to function as a substrate loader that couples energy from the proton motive force with protein transport across the cell membrane (44). FliH₂FliI is proposed to deliver chaperone-substrate complexes from the cytoplasm to the export gate and is thought to be facilitated through interactions between FliH and FliN (5). FliH is also thought to interact with FlhA to anchor the FliH₁₂FliI₆FliJ complex to the export platform of the flagellar T3SS (45). Bai and co-workers examined the assembly dynamics of a FliI-YFP fusion protein (44) and proposed a model in which the FliH₁₂FliI₆FliJ complex remains associated with the export gate and FliH₂FliI complexes carrying flagellar substrates are interchangeable to deliver new substrates to the export gate. Given the presence of the FliH₁₂FliI₆FliJ complex within the cavity of the C ring, we wished to determine if FliH influenced transcription of the RpoN-dependent genes.

A Δ *fliH* mutant was constructed and the phenotype of the resulting mutant was analyzed. The Δ *fliH* mutant exhibited slight motility on the soft agar plate (Fig. 6.3A). Approximately 16% of the Δ *fliH* mutant cells were flagellated, most of which possessed either one or two flagella (Fig. 6.3B). The C ring is needed for chemotaxis (46), which likely explains why the

$\Delta fliH$ mutant displayed some motility in the soft agar assay while the $\Delta fliM$ mutant did not, even though a higher percentage of the $\Delta fliM$ mutant cells were flagellated. Deletion of *fliH* resulted in a ~6-fold decrease in transcription of *flaB* and *flgE* compared to wild type but no change in transcription of *flaA* (Fig. 6.3C). These findings suggest that FliH, like the C ring, facilitates signal transduction by the FlgS/FlgR two-component system. We attempted to complement the $\Delta fliH$ mutation by introducing *fliH* carried on pHel3 into the mutant but were unable to obtain transformants for unknown reasons.

Early flagellar export substrates influence RpoN- and FliA-dependent gene transcription.

FliE, FlgB, and FlgC are the earliest substrates transported by the export apparatus, and we wished to determine how loss of these proteins might impact transcription of the RpoN and FliA regulons. FliE is a linker protein between the MS ring and rod (30), while FlgB and FlgC form the proximal rod (47). The *fliE* and *flgBC* genes were replaced with the *cat* cassette and the phenotypes of the resulting mutants were analyzed. As expected, the $\Delta fliE$ and $\Delta flgBC$ mutant strains were non-motile and aflagellated (Figs. 6.4A and 6.4B). Motility was partially restored in the $\Delta fliE$ and $\Delta flgBC$ mutants by introducing *fliE* and *flgBC*, respectively, into the mutants via the shuttle vector pHel3 (Fig. 6.4A), verifying that deletion of the genes was responsible for loss of motility.

The effects on flagellar gene transcription in the $\Delta fliE$ mutant were more pronounced than in the $\Delta flgBC$ mutant. The $\Delta fliE$ mutant showed ~9-fold increase in the levels of *flaB* and *flgE* transcripts, whereas the $\Delta flgBC$ mutant displayed a ~4-fold increase in *flaB* and *flgE* transcript levels compared to wild-type levels (Fig. 6.4C). Levels of *flaA* transcript in both the $\Delta fliE$ and the $\Delta flgBC$ mutants were ~2-fold lower than that in wild type (Fig. 6.4C).

In contrast to what we observed for the *H. pylori* *fliE* and *flgBC* mutants, Boll and Hendrixson reported that disruption of *fliE*, *flgB* or *flgC* in *C. jejuni* inhibits expression of the RpoN-dependent *flaB::astA* reporter gene (19). Lack of FliE in *C. jejuni* resulted in a 50-fold reduction in expression of the reporter, while lack of FlgB or FlgC resulted in a 3- to 5-fold reduction in expression of the reporter (19). It is not obvious why disrupting *fliE* or *flgBC* has such radically different effects on expression of the RpoN-dependent genes in *H. pylori* and *C. jejuni*. Given that FliE and the rod proteins are external to the cell membrane, they likely exert their effects on expression of the RpoN regulon through interactions with the MS ring or membrane components of the export apparatus. The observation that disruption of *fliE* had the most profound effect on expression of the RpoN regulon in both *H. pylori* and *C. jejuni* is consistent with this hypothesis since FliE has a closer association with the MS ring and export apparatus.

Mutations in basal body genes minimally affect expression of genes encoding regulatory proteins for the RpoN and FliA regulons. To examine the possibility that the changes in flagellar gene expression observed for the various mutant strains were due to altered levels of regulatory proteins, transcript levels of *flgS*, *flgR*, and *fliA* and protein levels of RpoN were quantified in all mutants analyzed (Fig. 6.5A and 6.5B). With few exceptions, levels of *flgS*, *flgR* and *fliA* transcripts in the mutants were similar to those in the wild-type strain (Fig. 6.5A). Transcript levels of *flgS* in the Δ *fliE* and Δ *flgBC* mutants were ~2-fold higher compared to wild type (Fig. 6.5A) which may have, at least partially, accounted for the up-regulation of the RpoN-dependent genes in these mutants (Fig. 6.4C). Transcript levels of *flgS* were also slightly elevated (~1.5-fold) in the Δ *fliF* mutant (Fig. 6.5A). *H. pylori* *flgS* is in an operon with other two class I flagellar genes (i.e., early flagellar genes transcribed by σ^{80} -RNA polymerase

holoenzyme), *flgI* (encodes P ring protein) and HP0245, the product of which shares homology with the flagellar rod assembly protein/muramidase FlgJ. Since the *fliF*, *fliE* and *flgBC* mutants are all defective in assembly of the proximal rod, the up-regulation of *flgS* in these mutants may indicate that expression of at least some of the class I flagellar genes in *H. pylori* is coupled with rod assembly. This would be an interesting line of inquiry to pursue as we are unaware of any reports of regulatory proteins that control expression of the class I flagellar genes in *H. pylori*. Transcript levels of *flgR* were slightly elevated (~1.5-fold) in the Δ *fliF* mutant compared to wild type (Fig. 6.5A), but not in any of the other mutants. Unlike *flgS*, *flgR* does not appear to be in an operon with other flagellar genes. Levels of *fliA* transcript were ~2-fold lower in the Δ *fliM* compared to wild type (Fig. 6.5A), but this decrease in the level of *fliA* transcript did not affect transcription of *flaA* (Fig. 6.3C). Western blot analysis of the *H. pylori* mutants indicated that levels of RpoN were unaffected by the mutations (Fig. 6.5B). Taken together, the qRT-PCR and western blot analysis suggest the regulatory proteins that are known to control transcription of the RpoN-dependent and FliA-dependent genes are present at normal levels in the flagellar basal body mutants. These findings indicate the down-regulation of the RpoN-dependent in the *fliF*, *fliM*, *fliY* and *fliH* mutants likely results from either the failure to create the flagellar structure which acts as the regulatory checkpoint for expression the RpoN regulon or the inability of the FlgS/FlgR two-component system to respond efficiently to the regulatory checkpoint.

Conclusions

In summary, we show here that in addition to the export apparatus, proteins that comprise other flagellar basal body substructures have potential roles in regulating transcription of the RpoN and FliA regulons. These basal body proteins may affect the structure and/or activity of

the export apparatus to influence regulation of the RpoN and FliA regulons. Given the conformational changes that occur within the export gate during flagellar biogenesis, it is reasonable to speculate that such conformational changes communicate the status of flagellar assembly to the transcription machinery that controls the RpoN and FliA regulons.

We propose a model for how *H. pylori* couples the expression of the RpoN regulon with assembly of the flagellum via the FlgS/FlgR two-component system (Fig. 6.6). Prior to assembly of the MS ring and flagellar T3SS FlgS is in an inactive state (Fig. 6.6A). Formation of a nascent basal body structure consisting of the MS ring, flagellar T3SS and C ring is a regulatory checkpoint that is sensed by FlgS, resulting in initiating of signal transduction and culminating in transcriptional activation of the RpoN regulon (Fig. 6.6B). Sensing of this assembly checkpoint by FlgS may stimulate the autokinase activity of FlgS by promoting dimerization of the protein. Although the exact nature of the cellular cue that is sensed by FlgS remains to be defined, FliF, FliG and FlhA are likely candidates for FlgS interaction partners that have roles in stimulating FlgS activity. Boll and Hendrixson showed that FlgS immunoprecipitated with FliF and FliG following *in vivo* cross-linking with formaldehyde in *C. jejuni* (19), suggesting an intimate association of FlgS with FliG and the C-terminal, cytoplasmic domain of FliF (FliF_C). In collaboration with Jonathan McMurry, we found that FlgS binds the N-terminus of FlhA (FlhA_{NT}) with high affinity *in vitro* using biolayer interferometry (Tsang *et al.*, manuscript in preparation). FlhA_{NT} is predicted to be exposed on the cytoplasmic side of the membrane (18) and is likely in close proximity to FliF_C and FliG, so it is possible that all three of these proteins interact with FlgS to communicate the status of flagellar assembly to the transcriptional machinery that controls expression of the RpoN regulon. Morimoto and co-workers estimated the flagellar T3SS to contain nine FlhA subunits (31), indicating that multiple

FlgS subunits may bind simultaneously near the flagellar T3SS exit port. Binding of multiple subunits of FlgS in this region could facilitate dimerization of the protein or its interactions with other flagellar components, such as FliG or FliF_C. We postulate that FliH and the C ring proteins facilitate signal transduction by the FlgS/FlgR two-component system in *H. pylori* by assisting FlgS in binding to the flagellar substructure which serves as the regulatory checkpoint.

Alternatively, FlgS may remain stably bound to the regulatory checkpoint structure and FlgR must diffuse into the cavity of the C ring to engage FlgS, in which case, FliH and the C ring may assist FlgR in reaching FlgS. At some later point in flagellar biogenesis, signal transduction via the FlgS/FlgR two-component system is presumably switched off once the flagellar subunits encoded by genes within the RpoN regulon are no longer required. One possibility is that competition by rod and hook substrates for interaction with the export apparatus may lead to the downregulation of the RpoN regulon that either prevents FlgS from binding regulatory signals in the basal body region or FlgR from interacting with phosphorylated FlgS (Fig. 6.6C). In addition, the formation of the HBB is a good candidate for a regulatory checkpoint that turns off expression of the RpoN regulon as the proteins encoded by RpoN-dependent genes are either incorporated into the nascent flagellum or presumably synthesized and awaiting transport following the switch in substrate specificity of the export apparatus (Fig. 6.6D).

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Author Contributions

JT and TRH designed the experiments and wrote the paper. JT performed the experiments and analyzed the data.

Table 6.1: Oligonucleotides used for this study.

Primer	Sequence
cat forward	GAT ATA GAT TGA AAA GTG GAT
cat reverse	TTA TCA GTG CGA CAA ACT GGG
fliF upstream forward	ATC GCT AAT TTA GGC TCT TCT
fliF upstream reverse	ATC CAC TTT TCA ATC TAT ATC CAA GGC GGT GAT TAA AAC CC
fliF downstream forward	CCC AGT TTG TCG CAC TGA TAA CGC CCT GAT GAA ATC GCC A
fliF downstream reverse	ATG TGG GCT AAA ATC AAG GC
fliH upstream forward	GAA AGC TGA GAT CTC TAT TAG
fliH upstream reverse	ATC CAC TTT TCA ATC TAT ATC GTA AAT TTG CCA TGC TCT TA
fliH downstream forward	CCC AGT TTG TCG CAC TGA TAA AGG AGG CGT TAT GAT CAC TA
fliH downstream reverse	AAA TCC CTG CGC TAA TGC T
fliY upstream forward	CCC TAT GAT TGA CAG ACT ATT A
fliY upstream reverse	ATC CAC TTT TCA ATC TAT ATC TGG AGC CTT ACC CAC TAA CC
fliY downstream forward	CCC AGT TTG TCG CAC TGA TAA GAC AAG GTG ATC GCT AAG G
fliY downstream reverse	CCA GCA TGC CCG CAT GCA
fliE upstream forward	CGC TCA AAA CAA TCA AAT TAC C
fliE upstream reverse	ATC CAC TTT TCA ATC TAT ATC TTG TCC GTG TTA AGC TCA GAG
fliE downstream forward	CCC AGT TTG TCG CAC TGA TAA TGC TTG AAG TGC GCA ACA AAG
fliE downstream reverse	TTG GGC GTG AGA TTA TAG GCT
flgBC upstream forward	AAC ATG CCA ATA ATG AGT AAT G
flgBC upstream reverse	ATC CAC TTT TCA ATC TAT ATC ACC GAT AAT CCA ACG CTT TAT
flgBC downstream forward	CCC AGT TTG TCG CAC TGA TAA ATG TCG CAG CTT TCC AAA GC
flgBC downstream reverse	TTG ATG GTG AAA TCT GTT AAA AG
PfliN forward	ACT CAG CGA TCT GTT TAA AGA
PfliN reverse	AGA TAA TAT ACA ATG GCG TTA GTT TCT TTT TCG GCT ACT TTT AA
fliM upstream forward	AAT GGG GCG ATG TTA GAT TAT
fliM upstream reverse	ATC CAC TTT TCA ATC TAT ATC ATC ATA GAG GGT TAC GCT G
fliM downstream forward	CCC AGT TTG TCG CAC TGA TAA GAA GTG ATC TAT AGC GAA AAA
fliM downstream reverse	CTC AAA AGC CGC CGT AGT
fliM comp forward	TTA AAA GTA GCC GAA AAA GAA AGG TGC GTA AAT CCT TAG G
BamHI fliM comp reverse	GGA TCC TTG AAT AAA AAT CTT AAT AAA ATC
XhoI PfliN forward	CTC GAG ACT CAG CGA TCT GTT TAA AGA
PfliN 2 reverse	TTT CTT TTT CGG CTA CTT TTA A
XhoI FliE comp F	CTC GAG ACG ATT TGT AAC AAT TTA TGG
BamHI FliE comp R	GGA TCC GGC GAT CAT TTA AAG AGA AGC
XhoI FlgBC comp F	CTC GAG TAG GAT TTC CTT GAT CTT AGT
BamHI FlgBC comp R	GGA TCC CAT AGA ACC TCC TTA AGC TCT
FliY comp F	TTA AAA GTA GCC GAA AAA GAA AAT GAA AAT AGA AGA AGA GTG AGA A
BamHI fliY comp reverse	GGA TCC CAT AAT TCC TTC CTT CTT TAC

Table 6.2: qRT-PCR oligonucleotide primers used

Primer	Sequence
gyrA qPCR forward	GCT AGG ATC GTG GGT GAT GT
gyrA qPCR reverse	TGG CTT CAG TGT AAC GCA TC
flaA qPCR forward	GGC AAG CGT TAT TGT CTG GT
flaA qPCR reverse	CGA TAC GAA CCT GAC CGA TT
flgE qPCR forward	TGC GAA CGT GAA TAC CAC TG
flgE qPCR reverse	GTC ATT CTG CCC TGC TAA CC
flaB qPCR forward	ATC GCC GCT TTA ACT TCT CA
flaB qPCR reverse	CGC CAT CCC ACT AGA ATC AT
flgS qPCR forward	AGC CCT TTA TGA GTG GGT CA
flgS qPCR reverse	TTG AAC ACC TCA TGG CTT TG
flgR qPCR forward	GCC ATT GAT TCC ATT CGT TT
flgR qPCR reverse	GCC TGT CGT TTG CTC TCT TC
fliA qPCR forward	GCC TAT GAT GAG CAG CAA CA
fliA qPCR reverse	GAA TCG TTT AAC GCG CTC TC

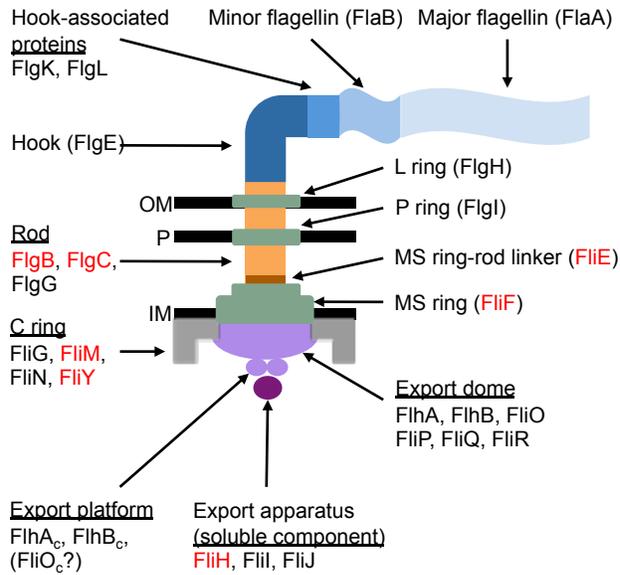


Figure 6.1: Structure of the *H. pylori* flagellum. A brief overview of the major components of the flagellum in *H. pylori*. Components targeted for mutagenesis are labeled in red.

Abbreviations are as follows: outer membrane (OM), inner membrane (IM), and peptidoglycan (P).

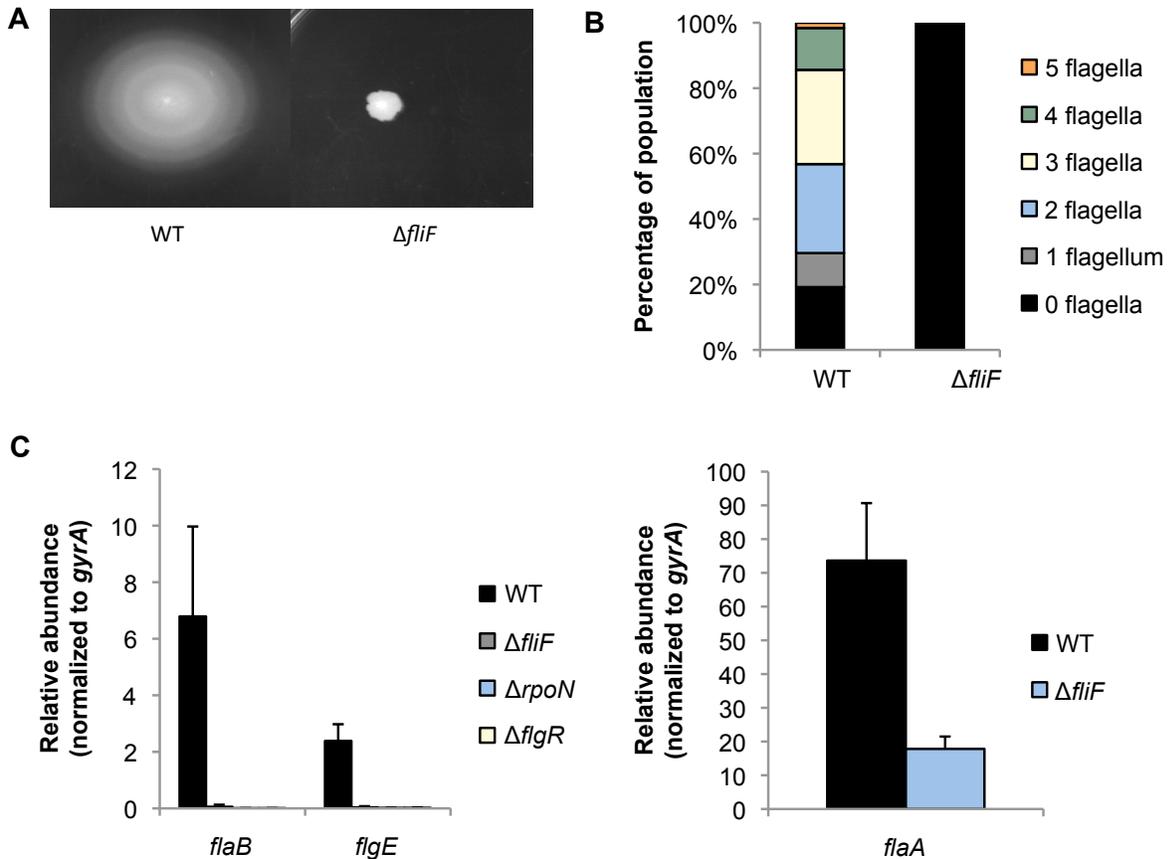


Figure 6.2: Effects of *fliF* deletion on flagellar gene transcription and motility. (A) Motility of $\Delta fliF$ mutant was assessed on 0.4% agar plates after 7 days of incubation in microaerobic conditions. Measurements indicate the diameter of the halo around the site of inoculation. Halo diameter for the wild type was 36 mm \pm 2 and halo diameter for the $\Delta fliF$ mutant was 7 mm \pm 1. (B) The number of flagella per cell was analyzed by electron microscopy after negative staining of cells. At least 115 cells were visualized per strain. (C) Transcript levels of *flaB* and *flgE* (left) and *flaA* (right) were detected by qRT-PCR. All results are significantly different from wild type (P-value <0.03).

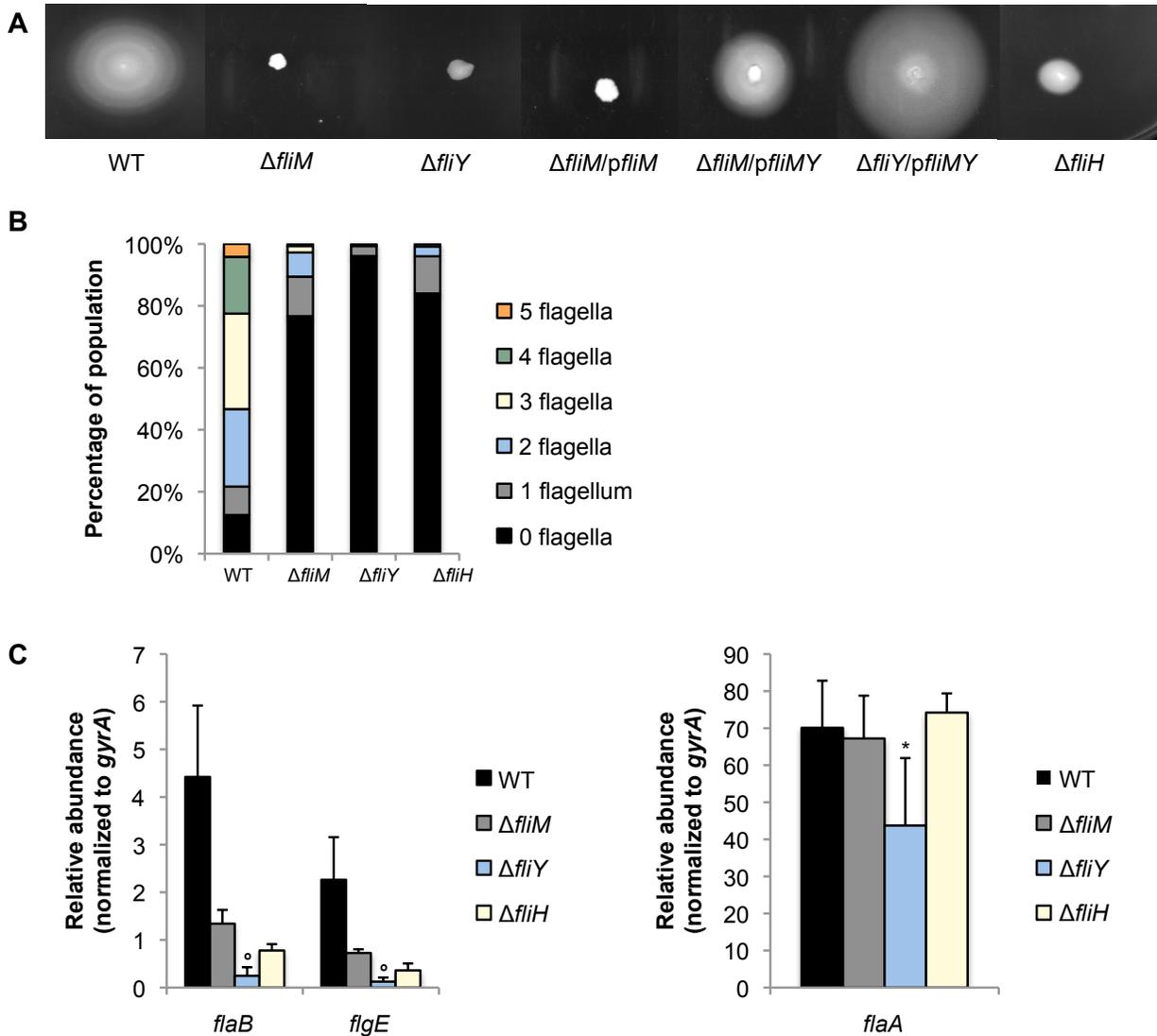


Figure 6.3: Effects of *fliM*, *fliY*, and *fliH* deletion on flagellar gene transcription and motility. (A) Motility of $\Delta fliM$, $\Delta fliY$ and $\Delta fliH$ mutants was determined by stab inoculation on 0.4% agar plates. The $\Delta fliM$ mutant was complemented with a plasmid containing either *fliM* or *fliMY* ($\Delta fliM/pfliM$ or $\Delta fliM/pfliMY$, respectively) and the $\Delta fliY$ mutant was complemented with a plasmid containing *fliMY* ($\Delta fliY/pfliMY$). Measurements indicate diameter of halo around site of inoculation after 7 days of incubation in microaerobic conditions. Halo diameter are as follows: WT 35 mm \pm 2, $\Delta fliM$ 5 mm \pm 1, $\Delta fliY$ 7 mm \pm 1, $\Delta fliM/pfliM$ 5 mm \pm 1, $\Delta fliM/pfliMY$

24 mm \pm 2, Δ *fliY*/*pfliMY* 43 mm \pm 3 and Δ *fliH* 12 mm \pm 1 (B) The number of flagella per cell was analyzed by electron microscopy after negative staining of cells. At least 115 cells were visualized per strain. (C) Transcript levels of *flaB* and *flgE* (left) and *flaA* (right) were assessed by qRT-PCR. All transcript levels of *flaB* and *flgE* are significantly different than wild type (P-value <0.006). Transcript levels in Δ *fliY* that are significantly different from that in Δ *fliM* are marked with a circle (P-value < 0.05). The asterisk indicates significance from wild-type transcript levels of *flaA* (P-value <0.05).

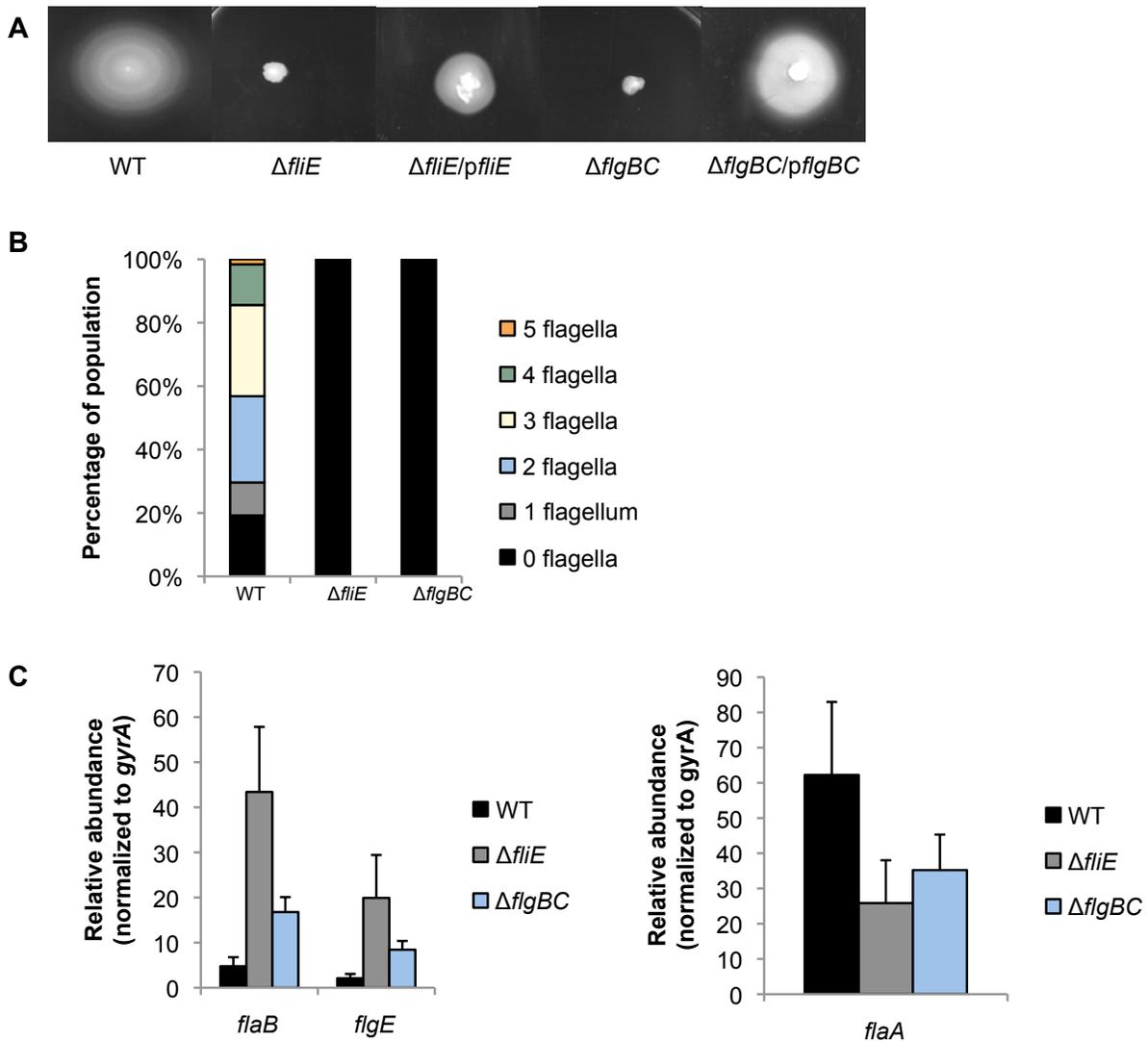


Figure 6.4: Effects of *fliE* and *flgBC* deletion on flagellar gene transcription and motility.

(A) Motility of $\Delta fliE$ and $\Delta flgBC$ mutants was assessed on 0.4% agar plates after 7 days of incubation in microaerobic conditions. The $\Delta fliE$ and $\Delta flgBC$ mutants were complemented with their respective genes on a plasmid. Measurements indicate the diameter of the halo around the site of inoculation. Halo diameters are as follows: WT 35 mm \pm 2, $\Delta fliE$ 7 mm \pm 1, $\Delta fliE/pfliE$ 15 mm \pm 3, $\Delta flgBC$ 7 mm \pm 1, and $\Delta flgBC/pflgBC$ 26 mm \pm 1. (B) The number of flagella per

cell was analyzed by electron microscopy after negative staining of cells. At least 115 cells were visualized per strain. (C) Transcript levels of *flaB* and *flgE* (left) and *flaA* (right) were detected by qRT-PCR. All results are significantly different from wild type (P-value <0.05) with the exception of *flaA* levels in the $\Delta flgBC$ (P-value = 0.08).

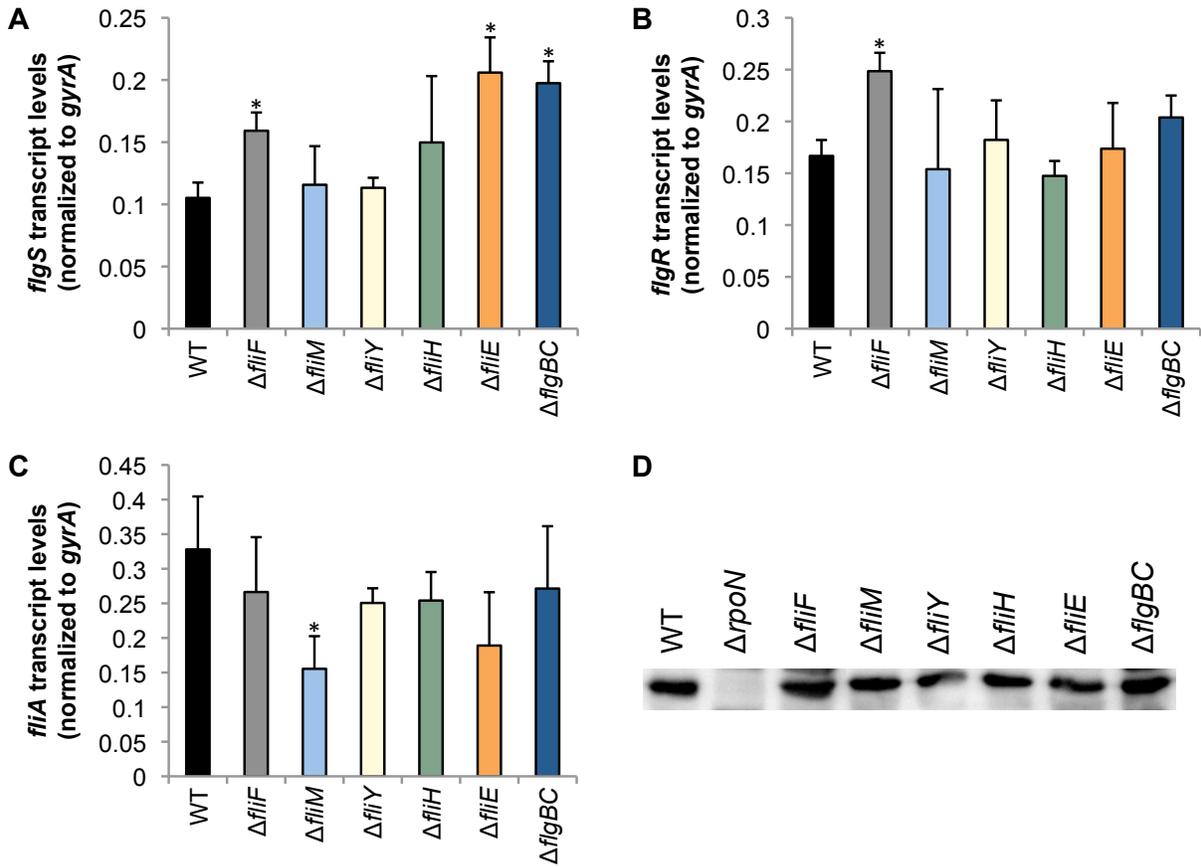


Figure 6.5: Transcript and protein levels of *flgS*, *flgR*, *fliA*, and RpoN among mutants. (A-C) Transcript levels of *flgS* (A), *flgR* (B), and *fliA* (C) were quantified by qRT-PCR. Asterisks indicate significant difference from wild type (P-value < 0.05) (D) Immunoblot of RpoN from *H. pylori* wild-type and mutant cytoplasmic protein fractions. Each lane contains 20 μ g protein. The immunoblot was probed with affinity-purified antibody directed against the MBP-tagged RpoN.

Assembly process

State of the RpoN regulon during assembly

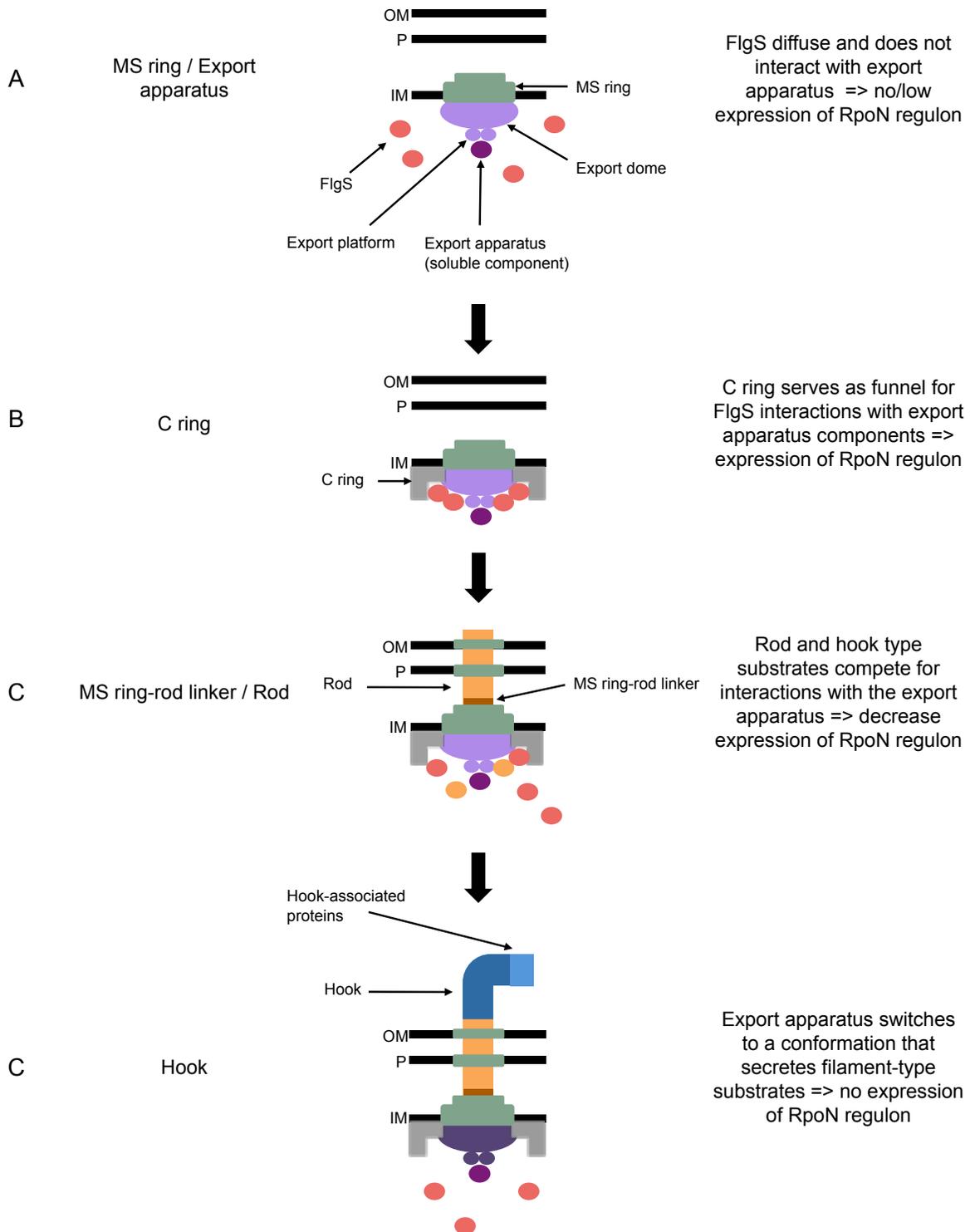


Figure 6.6: Model for the activation of FlgS by interactions with basal body components.

(A) The assembly of the flagellum begins with the assembly of the MS ring and the export apparatus. During this time, FlgS is diffuse throughout the cytoplasm and does not interact productively with the export apparatus. (B) Upon completion of the C ring, it may transiently interact with FlgS thereby facilitating interactions of FlgS and the export apparatus resulting in the expression of the RpoN regulon. (C) Export of flagellar substrates such as the MS ring-rod linker and the rod may compete with FlgS for interaction with the export apparatus resulting in a decrease in the expression of the RpoN regulon. (D) Upon completion of the HBB complex, the export apparatus switches to a conformation that exports filament-type substrates. This conformational change may be a regulatory checkpoint that turns off expression of the RpoN-dependent genes.

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CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions and Future Directions Related to this Work

Helicobacter pylori is a remarkable pathogen in that it is a chronic colonizer of the stomach, an acidic environment that is inhospitable to many bacteria. *H. pylori* is not an acidophile, however, and must swim through the layer lining the gastric epithelial cells to maintain a long-term association with the host. This is also important as the contents of the lumen are emptied within a few hours and the mucus layer is also sloughed off quickly. Studies on flagellar biogenesis in *H. pylori* may aid in the detection or treatment of this significant human pathogen in light of increasing antibiotic resistance. Additionally, these studies may also be applicable for other closely related bacteria such as *Campylobacter jejuni*, especially when the model organisms for flagellar biogenesis studies, *E. coli* and *Salmonella*, have several aspects of flagellar biogenesis which differ significantly from those in *H. pylori* and *C. jejuni*.

The work here provides new insight into how bacteria couple flagellar gene expression with assembly of the flagellum and illustrates the complexity of flagellar biogenesis. Over fifty structural and regulatory genes are involved in the synthesis of the flagellum in *H. pylori*. The assembly of the flagellum is a highly ordered process in that substrates that are needed in the early stages of flagellar biogenesis are made before substrates that are needed in the later stages of assembly. The transcription of different classes of flagellar genes is controlled by the three sigma factors found in *H. pylori*. The goal of my research is to elucidate the cellular cues

involved in activating expression of the RpoN-dependent genes which encode elements needed in the middle stage of flagellar biogenesis. A central player in this regulation is a two-component system consisting of the sensor kinase FlgS and the response regulator FlgR. Our working hypothesis is that FlgS senses the state of flagellar assembly via interactions with flagellar protein export apparatus. Upon sensing a regulatory checkpoint in flagellar assembly, FlgS undergoes autophosphorylation and then transfers the phosphate to FlgR which can then work with RpoN-RNA polymerase holoenzyme to stimulate transcription of the RpoN regulon.

Many studies have shown the importance of specific components of the export apparatus in the expression of the RpoN regulon. I continued these studies by focusing on two export apparatus proteins FliO and FlhA. FliO exhibits the most diversity between species among the export apparatus proteins and deletion of *fliO* in *H. pylori* exhibited low levels of expression of the RpoN regulon. Compared to *Salmonella* and *E. coli*, the FliO in *H. pylori* has an extended N-terminal periplasmic region and I had hoped that this portion had unique function in the regulation of the RpoN regulon which is absent in *Salmonella* and *E. coli*. However, this was not the case and it appeared that only the transmembrane portion of FliO was necessary for expression of the RpoN regulon. The requirement of FliO for expression of the RpoN regulon may be indirect as in its absence the level of another component of the export apparatus, FlhA, was reduced. The expression of specific domains in the $\Delta fliO$ background will further clarify my results as the truncated FliO proteins from my studies included sequence corresponding to the predicted transmembrane region of FliO. Barker and coworkers demonstrated in *Salmonella* that the full length FliO enhanced expression of a tagged FliP in a $\Delta fliO$ -*fliP* background but the cytoplasmic portion of FliO by itself was unable to do so, suggesting that the stability of FliP in the export apparatus is dependent on FliO (1). By constructing FliO alleles that lack sequence

corresponding to the transmembrane region in *H. pylori* Δ *fliO*, the effect of the transmembrane region of FliO on the stability of other export apparatus components can be determined.

In contrast to FliO, I have found that the N-terminus of FlhA (FlhA_{NT}) is essential for the expression of RpoN-dependent genes. In addition, we found that FlgS binds FlhA_{NT} with high affinity ($K_d \sim 20$ nM). However, I have not been able to show that this interaction stimulates autophosphorylation of FlgS. It is possible that the other export apparatus components are necessary to stimulate autophosphorylation of FlgS. The ability to isolate and enrich for intact export apparatus complexes from membrane fractions of wild type and various export apparatus mutants should aid in the identification of structural components needed to stimulate FlgS activity. Further characterization of the interactions between FlgS and various export apparatus components along with studies on whether these interactions lead to FlgS autophosphorylation may distinguish whether specific export apparatus components facilitates interaction of FlgS with its stimulus or whether it is the stimulus itself.

My work has also shown the importance of other basal body components in the regulation of RpoN-dependent flagellar genes. Loss of C ring components (FliM and FliY) or FliH resulted in decreased expression of the RpoN regulon. Our working hypothesis is that the binding of FlgS to a regulatory checkpoint associated with the export apparatus is facilitated by interactions of FlgS with components of the soluble portion of the export apparatus (FliH and perhaps FliI) and the C ring. Alternatively, FlgS may remain stably bound to the regulatory checkpoint and FliH and the C ring proteins assist FlgR in interacting with FlgS. We plan to continue these studies by determining whether FlgS or FlgR interacts with the C ring or soluble components of the export apparatus by biolayer interferometry.

Deletion of other structures, such as the MS ring-rod linker (FliE) and proximal rod proteins (FlgB and FlgC) resulted in an up-regulation of the RpoN regulon. This suggests that the failure to assemble the flagellar rod results in a prolonged signaling via the regulatory checkpoint that is sensed by FlgS. The hook-length control protein, FliK, is also likely involved in modulating the signal that is sensed by FlgS as disruption of *fliK* similarly results in enhanced expression of RpoN-dependent genes (2, 3). FliK senses the length of the hook and initiates a conformational change in the export apparatus upon formation of the mature hook which results in a change in substrate specificity of the export apparatus from rod-/hook-type substrates to filament-type substrates. This conformation change may switch off the signal sensed by FlgS and thereby turn off expression of the RpoN regulon.

General Future Directions for Flagellar Studies in *H. pylori*

In addition to characterizing the cellular cue that is sensed by FlgS to initiate signal transduction and the subsequent activation of the RpoN regulon, a fruitful line of questioning is whether FlgS remains stably associated with the flagellar basal body once it is activated or does it dissociate from the basal body to engage FlgR. If FlgS binds stably to the export apparatus once it is activated, then presumably FlgR would need to be recruited to the export apparatus to interact with FlgS. These questions could be addressed using fluorescence microscopy to monitor the cellular localization of FlgS and FlgR proteins that are fused to fluorescent proteins (e.g., yellow fluorescent protein or cyan fluorescent protein).

Another area of flagellar gene regulation in *H. pylori* which has been overlooked is expression of the flagellar genes which are required at the earliest steps of flagellar biogenesis. A master regulator that initiates flagellar biogenesis in *H. pylori* has yet to be discovered. It has

been suggested that these early flagellar genes are constitutively expressed (4). However, it seems likely that expression of at least some of the early flagellar genes is regulated.

On a more global scale, the identification of genes whose expression is dependent on RpoN or FliA by RNA-seq may elucidate the mechanisms of coordination of flagellar assembly with other cellular processes. It was relatively recently that small RNAs (sRNAs) were discovered in *H. pylori* by differential RNA-seq (5). Three of these sRNAs were predicted to have FliA-dependent promoters and thus may serve roles in regulating flagellar components for early and middle stages of flagellar biogenesis. I have begun a mutational analysis of these sRNAs. One of these sRNAs, sRNA_T, is predicted to interact with *flhF* transcript and we plan to determine the role of sRNA_T on regulating *flhF* transcript levels and FlhF protein levels. Additionally, RNA-seq experiments that compare transcript levels between the wild type and the Δ sRNA_T mutant will demonstrate whether sRNA_T affects stability of other transcripts.

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