SIGNAL TRANSDUCTION PATHWAYS THAT IMPACT POLAR FLAGELLAR BIOGENESIS

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Therefore is the name of it called Babel;

because the LORD did there confound the language of all the earth:

and from thence did the LORD scatter them abroad

upon the face of all the earth.

— Genesis 11:9 KJV

Dedicated to my mother, my first mentor,

Dr. Janet Pinsince Burnham.

Soul vaccination,

All across the nation people been catchin' honkypox (Honkypox)

When you get the notion,

Tower's got the potion - you might look in to set yourself in motion.

— Tower of Power. *Soul Vaccination*. 1973

ACKNOWLEDGEMENTS

Thank you to my brother Robert, because of whom, I have never been alone.

Thank you to my parents for supporting my curiosity from birth to today.

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ABSTRACT

Bacterial flagella are rotating nanomachines required for motility. Flagellar gene expression and protein secretion are coordinated for efficient flagellar biogenesis. Polar flagellates, unlike peritrichous bacteria, commonly order flagellar rod and hook gene transcription as a separate step after production of the MS ring, rotor, and flagellar type III secretion system (fT3SS) core proteins. This thesis describes two different ways MS ring-rotor-fT3SS assembly regulates flagellar gene expression. MS ring-rotor-fT3SS assembly stimulates expression of the next stage of flagellar genes establishing a unique polar flagellar transcriptional program. Conserved regulatory mechanisms in diverse polar flagellates to create this polar flagellar transcriptional program centered on MS ring-rotor-fT3SS assembly have not been thoroughly examined. Using *in silico* and genetic analyses and our previous findings in *Campylobacter jejuni* as a foundation, we observed that a large subset of Gram-negative bacteria with the FlhF/FlhG regulatory system for polar flagellation also possess flagellum-associated two-component signal transduction systems (TCSs). I present data supporting a general theme in polar flagellates where MS ring, rotor, and fT3SS proteins contribute to a regulatory checkpoint during polar flagellar biogenesis. I demonstrated that *Vibrio cholerae* and *Pseudomonas aeruginosa* require the formation of this regulatory checkpoint for the TCSs to directly activate subsequent rod and hook gene transcription, which are hallmarks of the polar flagellar transcriptional program. By reprogramming transcription in *V. cholerae* to more closely follow the peritrichous flagellar transcriptional program, I discovered a link between the polar flagellar transcription program and the activity of FlhF and FlhG flagellar biogenesis regulators in which the transcriptional program allows polar flagellates to continue to produce flagella for motility when FlhF or FlhG activity may be altered. I discovered a second mechanism by which the MS ring-rotor-fT3SS regulates polar flagellar gene expression as *V. cholerae* MS ring-rotor-fT3SS mutants increased expression of *flrB*, the sensor kinase of flagellar FlrBC TCS in *V. cholerae*. This suggested that MS ring-rotor-fT3SS formation may act as a feedback inhibition mechanism to repress the activity of

the master flagellar regulator, FlrA. I examined if this effect was on *flrA* transcription or FlrA activity and found that early flagellar formation appears to impact *V. cholerae* FlrA activity. I hypothesized that early flagellar formation may repress FlrA activity through c-di-GMP in a FlhG-independent or dependent manner. I then examined the effect of DGC and PDE mutants that either 1) increased c-di-GMP levels in a FlhA mutant or 2) were known to affect *V. cholerae* motility to identify DGCs or PDEs that may link early flagellar formation to FlrA activity. I found evidence for two different early flagellar formation feedback inhibition mechanisms: a possibly c-di-GMP-independent mechanism through FlhA, and a c-di-GMP-related mechanism through FlhG, CdgE, and RocS. Although more characterization is needed, our data suggests a complex previously undescribed feedback inhibition mechanism that links completion of the MS ring-rotor-fT3SS complex to both repress FlrA activity and stimulate flagella-associated TCSs.

CONTENTS

PUBLICATIONS

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[1] PM. Burnham and DR. Hendrixson. "*Campylobacter jejuni*: collective components promoting a successful enteric lifestyle." In: *Nat Rev Microbiol.* 16.9 (Sept. 2018), pp. 551–565.

[2] PM. Burnham, WP. Kolar, and DR. Hendrixson. "A Polar Flagellar Transcriptional Program Mediated by Diverse Two-Component Signal Transduction Systems and Basal Flagellar Proteins Is Broadly Conserved in Polar Flagellates." In: *mBio.* 11.2 (Mar. 2020), e03107–19.

I adapted Section 1.1.3.1 and Section 1.1.3.2 from *Burnham et. al. 2018*. Chapter 3 contains the figures and text from *Burnham et. al. 2020*

ACRONYMS

fT3SS Flagellar Type III Secretion System

TCS Two-Component System

HK Sensor Histidine Kinase

RR Response Regulator

c-di-GMP Bis-(3'-5')-cyclic dimeric guanosine monophosphate

EBP Enhancer Binding Protein

WT Wild-Type

DGC Diguanylate Cyclase

PDE Phosphodiesterase

PAS Per-Ant-Sim Domain

CC Coiled-Coil Domain

REC Receiver Domain

MR Master Regulator

NTD Amino-Terminal Domain

MD Middle Domain

CTD Carboxy-Terminal Domain

VEC Vector

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INTRODUCTION TO FLAGELLAR BIOLOGY IN POLAR FLAGELLATES

The bacterial flagellum is a complex structure that is formed by the expression of dozens of genes, and thousands of individual proteins. Not all bacteria produce flagella, but those that do rely on flagella to move away from hazards and to find nutrients. Some pathogenic bacteria use flagella to circumvent the physical and chemical barriers the human body erects to limit bacterial infection. Over the last several decades, bacteriologists have developed a sophisticated understanding of the parts of the bacterial flagellum and how they work together to create a bacterial motility apparatus [1–4]. Increasingly, it has become clear the bacterial flagella is not a machine unto itself, but that stages of flagellar assembly and the completion of the flagella regulates other aspects of bacterial biology [3–5]. This trend seems to be particularly pronounced in polar flagellates, which tightly control flagellar number and express flagella only at their poles [6, 7]. My work in the Hendrixson lab and this thesis describes how early stages of polar flagellar assem-

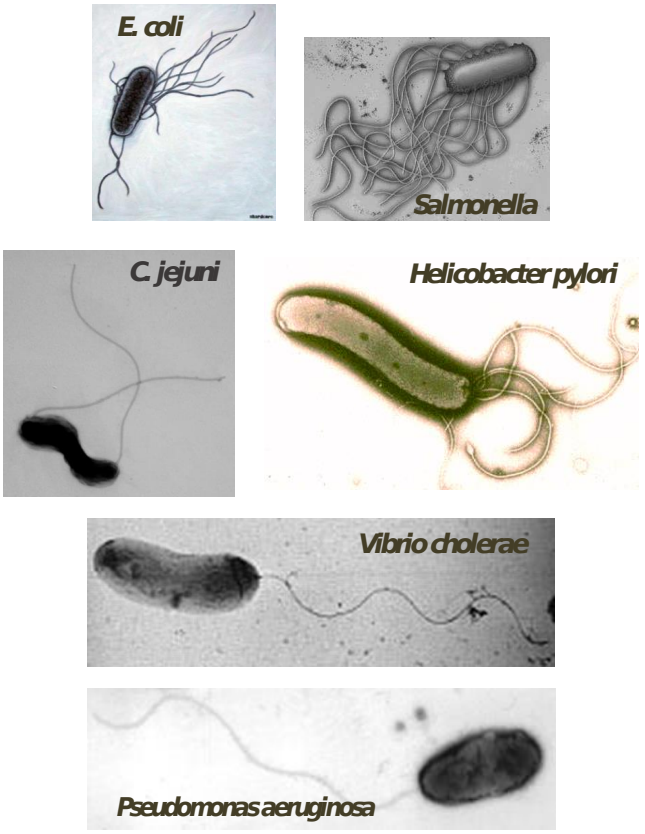

Figure 1: **Flagellation patterns in selected bacteria.** *E. coli* (*top left*) and *Salmonella* (*top right*) express flagella throughout their outer surface in a peritrichous pattern. *C. jejuni* (*middle left*), *H. pylori* (*middle right*), *P. aeruginoa* (*bottom left*), and *V. cholerae* (*bottom right*) express their flagella only at the poles and are considered polar flagellates.

bly both stimulates the transcription of additional flagellar genes and represses the transcription of flagellar components once they are no longer required for flagellar assembly.

This Introduction will delve into the structure of bacterial flagella and the two types of bacterial signaling systems central to my thesis, two-component signal transduction systems (TCS) and c-di-GMP signaling.

1.1 *How to build a flagellum*

Many bacteria produce flagella with unique adaptations for the environments they live in, but many universally conserved components of the bacterial flagellum were initially described in *E. coli* and *Salmonella*, which produce flagella throughout their outer surface in a peritrichous flagellation pattern [1, 3]. The peritrichous flagellum has become representative of a "standard" flagellum in nature.

1.1.1 *How to build a flagellum in E. coli and Salmonella*

Flagellar assembly in *Salmonella* involves the expression of 67 genes [3]. Flagellar gene expression proceeds in two phases: 1) early flagellar gene expression driven by $FlhD_4FlhC_2$ and the RNA polymerase holoenzyme with σ^{70}; and 2) late flagellar gene expression driven by the RNA polymerase holoenzyme with σ^{28}. Flagellar assembly broadly begins with assembly of inner membrane-bound and cytoplasmic flagellar components before extending outward with periplasmic and finally external flagellar components (Figure 2).

1.1.1.1 *FlhDC and the expression of early flagellar genes in E. coli and Salmonella*

Early flagellar gene expression in *E. coli* and *Salmonella* is dependent on a master flagellar regulator, the $FlhD_4FlhC_2$ complex. This complex binds to approximately 48 bp of DNA in the -20 to -80 region of early flagellar gene promoters [8, 9]. $FlhD_4FlhC_2$ bends DNA to promote σ^{70}-dependent flagellar gene expression [8, 9]. $FlhD_4FlhC_2$ and σ^{70} stimulate the ex-

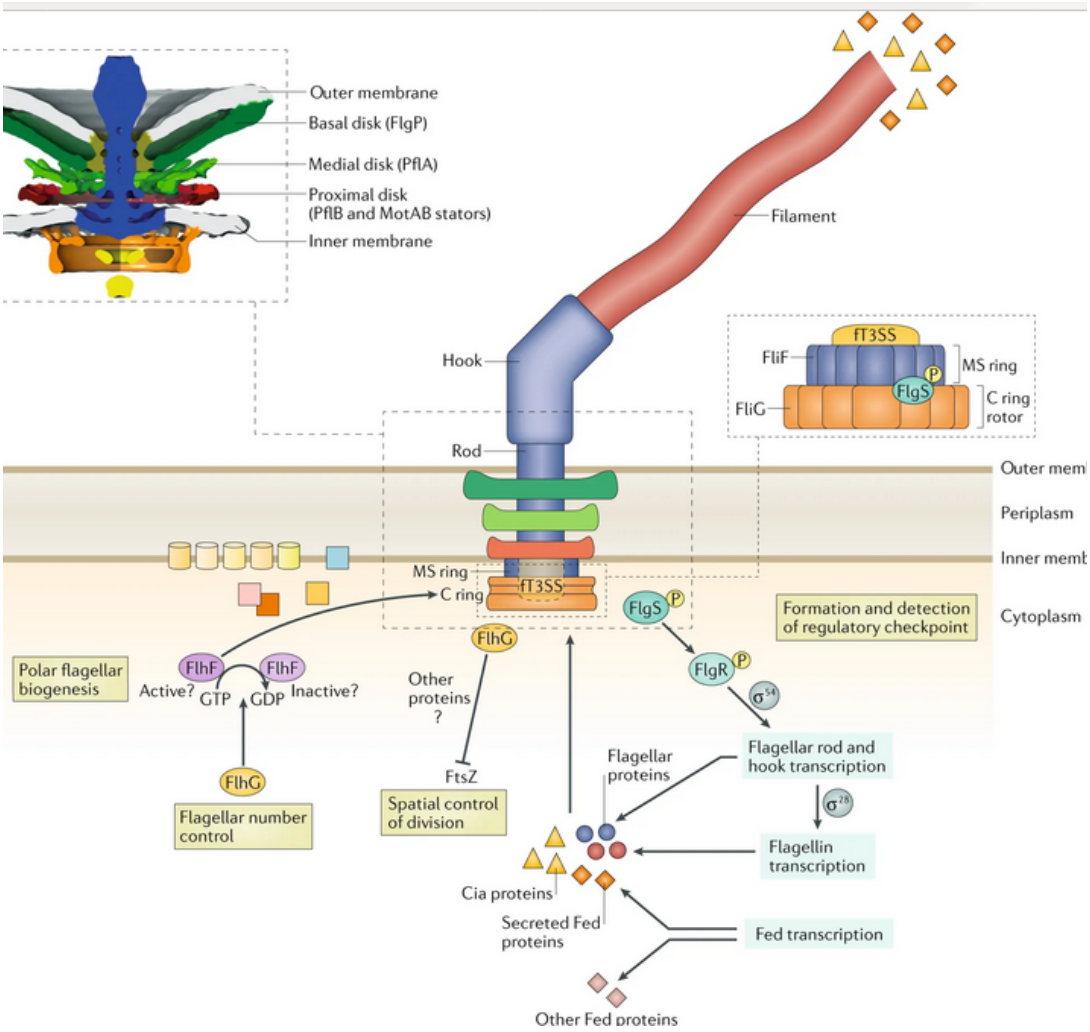

Figure 2: **Multiple biological roles of the *C. jejuni* flagellum.** The flagellum is composed of an MS ring (dark blue) and C ring (orange) that surround the fT3SS core in the inner membrane, a rod and hook structure (dark blue) that transverses the periplasm and outer membrane and an extracellular flagellar filament (red). Three disk structures, the basal disk (dark green), medial disk (bright green) and proximal disk (bright red), surround the flagellar rod in the periplasm. An isosurface rendering of a longitudinal slice of a tomogram of the *C. jejuni* flagellar motor obtained by electron cryotomography that reveals the flagellar motor structure is shown in the upper left panel. The basal disk is composed of FlgP, the medial disk is composed of PflA and the proximal disk is composed of PflB and the MotAB stators. These disk structures incorporate an increased number of stator complexes into the motor and position them at a wider distance from the motor axis to contribute to an increased amount of torque relative to many other bacterial flagellar motors. The flagellar motor switch proteins FliF and FliG multimerize around the fT3SS core proteins in the inner membrane to form the MS ring and rotor of the C ring, respectively, during the initial stage of flagellar biogenesis. The FlgS sensor kinase detects the formation of the MS ring and rotor as a regulatory checkpoint during flagellar biogenesis, probably through direct interaction of multimers of FliF and/or FliG (upper right). After detection, autophosphorylation of FlgS initiates signal transduction to result in phosphorylation of the FlgR response regulator and expression σ^{54}-dependent flagellar rod and hook genes and eventually leads to expression of σ^{28}-dependent flagellins and *fed* gene expression. Flagellar proteins are secreted in a specific order by the fT3SS to build the flagellum. Some Cia and Fed proteins are also secreted by the flagellum to influence host interactions. Polar flagellar biogenesis is regulated in a GTP-dependent manner by the FlhF GTPase and the FlhG ATPase. FlhG seems to control the active and inactive states of FlhF by promoting FlhF GTPase activity, which may influence both flagellar placement and the number of flagella at poles. FlhG also influences a process that prevents the polymerization of the cell division protein FtsZ into the septal Z ring at a pole so that the Z ring forms at the cellular midpoint for symmetrical division. FlhF, fT3SS proteins, FliF, and FliM and FliN C ring proteins influence the ability of FlhG alone or together with other unknown proteins to inhibit Z ring formation at poles. Tomogram of the *C. jejuni* flagellar motor courtesy of Morgan Beeby, Imperial College London, UK.

pression of flagellar genes that encode flagellar components required to build the flagellar type III secretion system (fT3SS), rod, and hook [10]. $FlhD_4FlhC_2$ also represses *flhDC* expression, which links flagellar gene expression to growth phase as $FlhD_4FlhC_2$ accumulates in the cell over time to repress early flagellar gene expression in stationary phase [3].

1.1.1.2 *Flagellar type III secretion system (fT3SS), MS ring, and C ring*

The fT3SS is comprised of several core proteins (FlhA, FlhB, FliO, FliP, FliQ, FliR), an ATPase (FliI), spoke (FliH) and a general fT3SS chaperone (FliJ) (Figure 2) [3, 11]. Completion of the core fT3SS is required for the multimerization of the MS- and C- rings, and FliI ATPase assembly which completes a functional fT3SS for secretion of the flagellar rod and hook (Figure 2) [11].

FliF monomers multimerize to form the MS ring [12]. An interaction between FlhA and FliF is required for an association between the MS ring and fT3SS in *E. coli* and *Salmonella* (Figure 2) [13–15]. The MS ring is embedded within the inner membrane and the cytoplasmic face of the MS ring is required for C ring formation (Figure 2) [12, 16].

The C ring is made up FliG (the rotor component) and FliM and FliN, which together make up the switch complex (Figure 2) [3, 17]. The N-terminus of FliG binds to the cytoplasmic C-terminus of FliF and this interaction is essential for C ring formation [12, 16, 18]. FliG, as the rotor component of the C ring, rotates upon a pushing force created by flagellar stators (Figure 2) [17]. FliM and FliN, as members of the switch complex, are responsible for changing the direction of flagellar rotation (clockwise or counterclockwise, reversibly) in response to chemotactic signals detected by the Che chemosensory systems [17].

1.1.1.3 *Flagellar stators*

Flagellar rotation in *E. coli* and *Salmonella* is powered by up to 11 integral membrane stator units comprised of a $MotA_4MotB_2$ complex [19, 20]. The $MotA_4MotB_2$ stator complex

provides a channel for proton flux to power flagellar rotation through proton motive force, which with steric forces stimulates a "power stroke" to drive rotor rotation [20].

1.1.1.4 *Flagellar rod*

The flagellar rod in *E. coli* and *Salmonella* is comprised of FliE, FlgB, FlgC, FlgF and FlgG, which are secreted through the fT3SS and assemble in the periplasmic space (Figure 2) [1, 21]. An additional rod component, FlgJ, is an autolysin which degrades peptidoglycan for rod assembly through the peptidoglycan layer [22].

1.1.1.5 *Flagellar P and L rings*

FlgI forms the P ring in the peptidoglycan and the FlgH lipoprotein forms the outer-membrane L ring [1]. Both proteins are secreted through the Sec-dependent secretion pathway rather than through the fT3SS and form rings that multimerize around the flagellar rod [1]. The P ring likely acts as a bushing for the flagellar rod as it passes through the peptidoglycan layer [23]. The L ring forms a pore in the outer membrane for the flagellar hook and filament to assemble outside the cell [1].

1.1.1.6 *Flagellar hook, σ^{28}, FlgM, and the expression of late flagellar genes in E. coli and Salmonella*

FlgE binds to the final rod component FlgG and multimerizes into the flagellar hook [3]. Hook completion is measured by a FliK "ruler" protein leads to a substrate specificity change that allows for FlgM secretion out of the cell and the next stage of flagellar gene transcription [24].

FlgM is an anti-σ factor that binds to σ^{28} and represses σ^{28}-dependent gene expression [25–29]. Once, the flagellum secretes FlgM out of the cell, σ^{28}-dependent expression of the FliC or FljB flagellins begins [25–29]. Thus, hook formation acts as a regulatory checkpoint in flagellar assembly that is required for flagellar gene expression to proceed.

Once expressed, flagellins assemble at the end of the hook and underneath the flagellar filament cap FliD (Figure 2) [3]. As the filament grows, FliD remains at the end of flagellar filament to trap flagellins for polymerization, which can grow to incorporate roughly 20,000 flagellin subunits [3].

1.1.2 *How to build a polar flagellum*

There are several perceived advantages for bacteria to produce flagella at the poles. First, polar flagella are more structurally complex and generate more torque than peritrichous flagella, which can allow a bacteria to move in more viscous environments and may allow for better penetration of intestinal mucus [30–34]. Second, polar flagella can substitute for other signaling systems at the pole. For instance, some bacteria such as *C. jejuni* use polar flagellar machinery to prevent cellular division near the poles [35, 36]. Third, polar flagellates have additional stages of flagellar gene expression, which may allow for more signaling events to be linked to flagellar assembly [37–39].

1.1.2.1 *C. jejuni flagella generate more torque than peritrichous flagella.*

As *C. jejuni* primarily exists in association with the host intestinal tract, the *C. jejuni* flagellum has evolved as an ideal motor to power propulsion through viscous milieus, such as intestinal mucus, with a velocity around 40 μm per second in viscosities that normally impede other motile bacteria [4, 31–34]. As such, the *C. jejuni* flagellum is equipped with additional components to generate a higher level of torque for flagellar rotation and motility, which is necessary to move through viscous gut mucus. Electron cryotomography of flagellar motor structures *in situ* revealed three large multimeric disk structures (annotated as basal, medial and proximal disks) that surround the flagellar rod and ring structures between the outer and inner membranes [40] (Figure 2). These disk structures are composed of FlgP, PflA and PflB that act as scaffolds to incorporate MotAB stator complexes into the motor to power rotation via proton transport and impart greater torque on the flagellar rotor [30]. Whereas *E. coli* flagellar motors contain at most 11 stators to generate power [19], the *C. jejuni* flagellar motor incorpo-

rates 17 stators and orients the stators via the disk scaffolds at a greater radial distance from the central motor axis and rotor [30]. This numerical and spatial stator arrangement creates a more powerful motor that generates a higher level of torque for greater propulsion of *C. jejuni* through a range of viscosities [30].

1.1.2.2 *FlhF and FlhG regulate polar flagellar pattern.*

C. jejuni spatially and numerically regulates flagellar biogenesis to create and maintain the amphitrichous flagellation pattern. The *C. jejuni* FlhF GTPase is required for flagellar biogenesis, and mutants with altered GTPase activity produce heterogenous flagellation phenotypes, including normal amphitrichous flagella, lateral flagella, polar hyperflagellation or aflagellation (Figure 2) [35, 36, 38]. The placement of the MS ring-rotor-fT3SS complex on the cell body most likely determines the ultimate position of the nascent flagellum. Although it is unknown how FlhF functions in flagellation, FlhF may regulate polar positioning or organization of fT3SS, MS ring and C ring proteins in a GTP-dependent manner for polar flagellation [35, 36].

The C. jejuni FlhG ATPase likely mediates mediates numerical control of flagellation through FlhF [36, 41]. FlhG stimulates the *in vitro* GTPase activity of FlhF, which likely converts it from an active GTP-bound state that facilitates a step in polar flagellar biogenesis to an inactive GDP-bound state (Figure 2) [36] . It has been hypothesized that accurate control of FlhF activity via FlhG ensures exactly one flagellum is formed at each *C. jejuni* pole for amphitrichous flagellation, which is ideal for motility.

1.1.2.3 *Polar flagellar gene transcription involves extra regulatory steps.*

The cytoplasmic and inner membrane substructures of the flagellum influence other processes in *C. jejuni*. The core of the fT3SS is located in the inner membrane and is surrounded by the MS ring and the cytoplasmic C ring (see Section 1.1.1.2 and Figure 2). These components are required for secretion of most proteins that form the flagellar rod, hook and filament. Formation of the MS ring and rotor around the flagellar fT3SS core by FliF and FliG in *C. jejuni* creates a regulatory checkpoint detected by the FlgSR TCS to activate σ^{54}-dependent

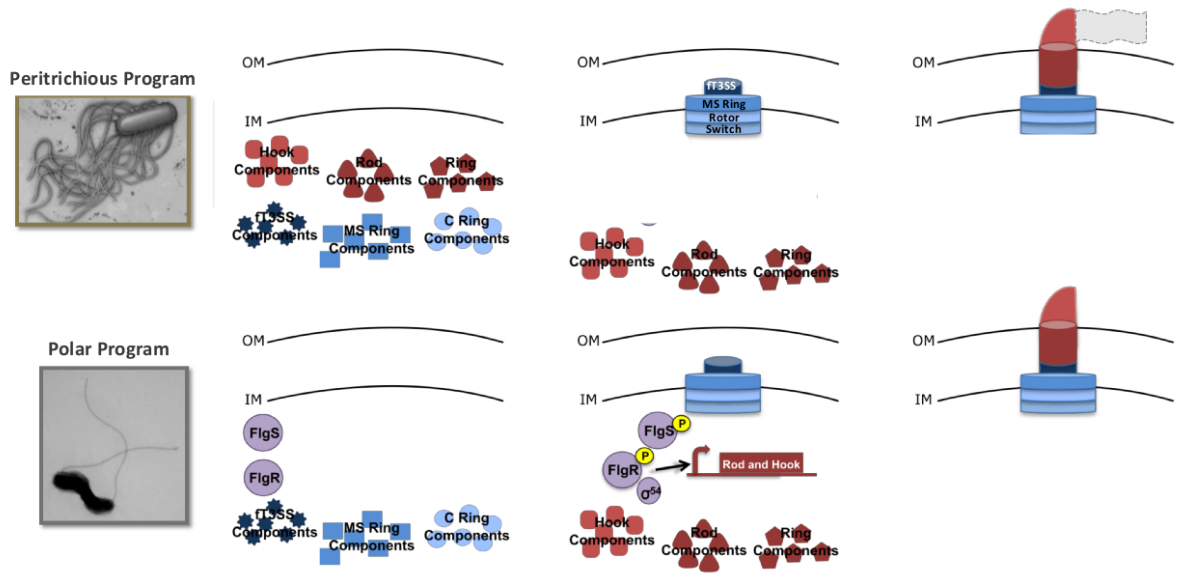

Figure 3: **Summary of early stages of peritrichous and polar flagellar transcriptional cascades.** Peritrichous flagellates such as *Salmonella* generally express flagellar genes including fT3SS, MS ring, C ring, rod, and hook genes using a master flagellar transcriptional regulator such as *Salmonella* FlhDC. The flagellum then self-assembles starting with the fT3SS, MS ring, and C ring, which then allows for the secretion and assembly of the flagellar rod and hook. Completion of the flagellar hook is a conserved regulatory checkpoint (not shown) required for expression of flagellins. Polar flagellates such as *C. jejuni* have an additional regulatory checkpoint in their flagellar transcriptional cascade where multimerization of the MS ring and rotor signals a TCS to stimulate σ^{54}-dependent rod and hook gene expression.

expression of flagellar rod and hook genes (Figure 3) [38, 42–46]. Mutation of any fT3SS protein, FliF or FliG abolishes FlgSR-dependent and σ^{54}-dependent flagellar gene expression [38, 44, 46]. Physical detection of MS ring and rotor formation around the flagellar fT3SS core by two-component system histidine kinase (FlgS) ensures that a competent secretory system has formed before resources are expended to produce substrates for the flagellar fT3SS to build a flagellum (Figure 3) [44]. Thus, the *C. jejuni* flagellum influences signal transduction for its own biogenesis.

1.1.3 *Additional roles for flagella in C. jejuni*

The differences between peritrichous and polarly-flagellated bacteria are not simply that polar flagella produce higher torque and possess machinery to both place them at the poles and control flagellar number, but also that polar flagella in some bacteria have evolved roles outside flagellar-mediated motility.

1.1.3.1 *The polar flagellum as a general virulence secretory apparatus*

Most fT3SSs specifically recognize only flagellar proteins as substrates for secretion to construct flagella. However, some bacterial fT3SSs, including those of *Yersinia enterocolitica* and *C. jejuni*, secrete proteins not involved in flagellar motility [47–54]. The Cia proteins, Fed proteins and flagellin C (FlaC) are secreted by the *C. jejuni* fT3SS [47–53, 55, 56]. The Cia proteins were first discovered as bile-inducible, fT3SS-secreted proteins not involved in motility, but that instead, influence *C. jejuni* interactions with human intestinal cells [47, 55–57]. Some Cia proteins have been reported to localize inside eukaryotic cells to influence *C. jejuni* interactions, although it is unclear the essentiality of secreted Cia proteins for adherence to and invasion of eukaryotic cells across different strains [52, 58–61]. The Fed proteins are co-expressed with many flagellar proteins, and some of these proteins are secreted by the fT3SS [50–52, 60]. These proteins largely influence the commensal colonization capacity of *C. jejuni* for chicks, although individual functions of the Feds are unknown [51, 52]. FlaC is a flagellin-like protein that is secreted but does not influence motility [49]. Instead, FlaC influences invasion of human intestinal cells and recently has been shown to modulate immune responses by promoting cross tolerance to some Toll-like receptors to reduce cytokine production [49, 53].

1.1.3.2 *Polar flagella and bacterial cell division*

C. jejuni flagellar components also influence spatial control of cell division through FlhG [41]. *C. jejuni* lacks a canonical bacterial Min system that spatially regulates septal Z ring formation so that it forms at the midcell for symmetrical division rather than at a pole. *C. jejuni* FlhG shares homology with MinD ATPase of the Min system, but does not encode orthologues of other Min proteins, such as MinC, which inhibits the cell division protein FtsZ from polymerizing into the Z ring. *C. jejuni* Δ*flhG* produces a high level of non-viable minicells, which are products of asymmetrical cell division occruing at poles that often consequently lack chromosomal DNA [41]. *C. jejuni* mutants lacking the FliF MS ring protein, C ring switch proteins, fT3SS proteins or FlhF produce high levels of minicells [41]. Thus, polar flagellar formation, which may begin with FlhF producing the initial MS ring-rotor-fT3SS structure of a single flagellum at a new, unflagellated pole immediately after symmetrical divsion, appears to

influence FlhG (either alone or with other unknown proteins) to inhibit septal Z ring formation at a pole so that a Z ring forms at the cellular midpoint for symmetrical division [36, 41]. These findings suggest a possible additional explanation for an often-pondered question: why does *C. jejuni* produce a fairly rare amphitrichious flagellation pattern? The amphitrichious flagellation pattern appears ideal for the darting motility and efficient migration through viscous milieus encountered naturally, but also has a role in linking polar flagellation to a process that prevents division at a pole for accurate symmetrical division and efficient generation of viable progeny.

1.2 *Two-component systems in flagellar synthesis*

Bacteria are simple single-celled organisms that live in almost every explored environment on Earth. They must respond to environmental cues rapidly in order to produce the proteins that they need to survive environmental challenges. Two-component systems (TCS) are the most abundant signaling system in bacteria and the primary way that bacteria respond to changes in their environment [62, 63]. TCSs have provided a simplistic and flexible two-part framework that through natural selection, allows bacteria to detect and respond to the incredibly diverse environments they live in [62, 63].

TCSs as their name suggests, consist of two parts: a sensor histidine kinase (HK) and a response regulator (RR) [62, 63]. Generally, the HK is responsible for detecting a particular signal in the environment and then transmitting that information via phosphorylation to a RR, which often enable the transcription of bacterial genes important for responding to that particular stimulus [62, 63]. However, this paradigm is an overly simplistic summary of the diversity in how these TCS function and are organized [62, 63]. This section will first describe how TCSs commonly function using two well-characterized TCSs: the *E. coli* EnvZ/OmpR TCS (with a membrane-bound HK) and the *E. coli* NtrB/NtrC TCS (with a cytoplasmic HK).

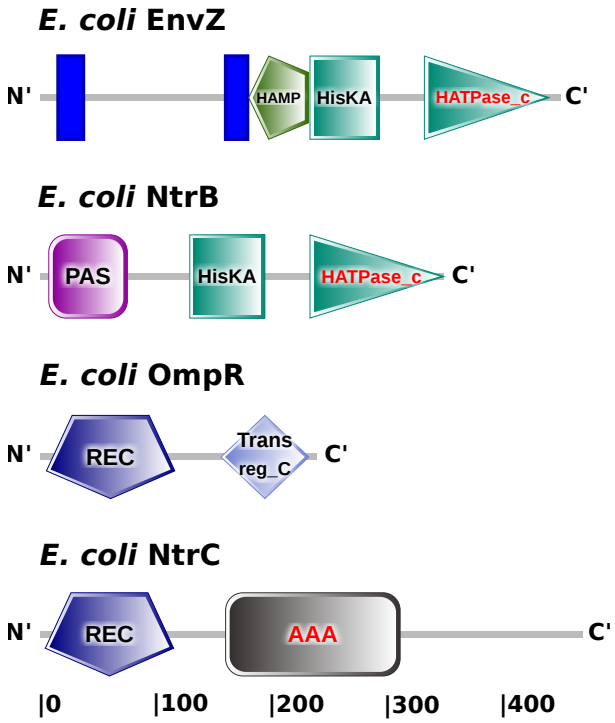

Figure 4: **Domain analysis of *E. coli* EnvZ/OmpR and NtrB/NtrC TCSs.** Protein domains shown are predicted by SMART. EnvZ transmembrane regions are represented as blue rectangles. The EnvZ HAMP linker domain is important for transmitting conformational changes to promote EnvZ kinase activity and is represented as a green pentagon. The NtrB sensor kinase has a predicted PAS domain, which is shown as a purple square. The conserved HisKA and HATPase-C domains of EnvZ and NtrB sensor kinases for histidine autophosphorylation, dimerization, and ATPase activity are shown in turquoise squares and triangles, respectively. The OmpR and NtrC RRs both have N-terminal REC domains which contain the phosphorylation site that enables signal transmission from EnvZ and NtrB respectively. OmpR also has a conserved C-terminal DNA-binding domain, which is represented as a grey diamond. NtrC has a AAA+ ATPase domain, represented as a grey rectangle, which is required for RNAP-σ^{54} holoenzyme turnover at NtrC-dependent promoters.

1.2.1 *The E. coli EnvZ/OmpR two-component system*

The EnvZ/OmpR TCS was the first characterized TCS [64–66]. The EnvZ/OmpR TCS regulates *ompF* and *ompC* expression [64–66]. OmpF and OmpC are major components of the *E. coli* outer membrane and the ratio of OmpF to OmpC is important for resisting osmolalic stress [64–66]. EnvZ acts as an osmolality sensor that activates OmpR to increase *ompC* expression and represses *ompF* expression [67].

Under low osmolality, EnvZ exists as 50-kDa monomer [68, 69]. Starting at the N-terminus, EnvZ begins with a 15 amino acid cytoplasmic domain, then a transmembrane re-

gion, a periplasmic region, a second transmembrane region, a HAMP linker and a C-terminal cytoplasmic region (Figure 4) [70]. The C-terminal cytoplasmic region is important for EnvZ signaling as EnvZ detects high osmolality from the bacterial cytoplasm [70]. The EnvZ cytoplasmic C-terminus possesses a HisKA domain and an ATP-binding domain (Figure 4) [70, 71].

In a single *E. coli* cell there are roughly 100 EnvZ proteins [72, 73]. How *E. coli* EnvZ detects high osmolality as a finely tuned sensor is not entirely clear, but likely occurs through a major and a minor sensing mechanism.

The major sensing mechanism involves sensing osmolytes such as K^+, Na^+ and sucrose [74]. These osmolytes stabilize the His^{243} helical backbone, which causes a conformational shift within the periplasmic domain to promote EnvZ dimerization [69, 75–77]. Several structural changes to EnvZ follow dimerization. First, in general, EnvZ dimerization promotes its kinase activity over its phosphatase activity against OmpR [78]. Second, EnvZ dimerization forms the α-helical bundle in the cytoplasm required for EnvZ kinase and phosphatase activity [79, 80]. Third, EnvZ dimerization places His^{243} of one subunit next to the G2 box of the other subunit, which binds ATP [81, 82]. His^{243} is required for EnvZ autophosphorylation [83–85]. Once in proximity to a neighboring G2 box, His^{243} becomes more nucleophilic, which then attacks the γ-phosphate of ATP to phosphorylate His^{243} [86]. This reaction is stabilized by the glycine-rich G2 box and a Mg^{2+} chelating Asn residue. The resulting negatively charged β-phosphate of ADP product is then countered by the positively charged Arg^{392} [86]. EnvZ is a trans-autophosphorylating HK (a subunit facilitates its neighbor's autophosphorylation), but the general mechanism of autophosphorylation described here is conserved among HKs [86].

The minor sensing mechanism centers on the EnvZ ATP-binding site, which interacts with lipids in the inner membrane [70]. As osmolality increases, the *E. coli* cell shrinks in volume to limit water loss, which increases EnvZ access to lipids and promotes ATP-binding at the site [70].

EnvZ autophosphorylation stimulates the phosphorylation of OmpR. Under low osmolarity, OmpR exists as a 29-kDa monomer [68, 69]. OmpR has two domains, an N-terminal REC domain and a C-terminal DNA-binding domain defined by a winged helix-turned-helix motif [70]. The REC domain has a conserved Asp^{55} residue that is the OmpR phosphorylation site [70].

In a single *E. coli* cell, there are roughly 3500 OmpR proteins in the cytoplasm [72, 73]. EnvZ phosphotransfer from EnvZ His^{243} to OmpR Asp^{55} occurs upon binding [70]. This is not the only way OmpR Asp^{55} phosphorylation occurs as small molecules such as acetylphosphate can serve as phosphodonors (Figure 4) [70].

OmpR phosphorylation stimulates its DNA-binding activity although OmpR, unusually as a DNA-binding RR, has a relatively high degree of DNA-binding activity even when unphosphorylated [70]. Genes within the OmpR regulon, generally have atypical -10 sites that deviate from the *E. coli* consensus sequence and require OmpR to bind near the -35 site of their promoters to recruit the RNA polymerase holoenzyme [70]. OmpR interacts with α subunit of RNAP [70]. OmpR DNA-binding activity when unphosphorylated may allow for some basal expression of the OmpR regulon under typical osmolality [70].

EnvZ His^{243} and a conserved Thr^{247} residue are important for EnvZ phosphatase activity against phosphorylated Asp^{55} on OmpR [85]. However, OmpR dephosphorylation can occur without EnvZ present and the relative importance of this OmpR-P turnover versus EnvZ phosphatase activity against OmpR-P is debated [70].

EnvZ and OmpR function in a 1:1 complex. Given that OmpR exists in the *E. coli* cell at much higher levels than EnvZ (3500 versus 100 molecules), there is always the possibility that an unphosphorylated pool of OmpR could be activated by another HK, which could cause some undesirable cross-talk between bacterial signaling pathways [73]. This is somewhat mitigated by the fact that the OmpR-P half life of 90 min (with Mg^{2+}) is higher than other RR

regulators in *E. coli* (CheY-P: seconds, NtrC-P: 4-minutes) [80]. However, cross-talk between EnvZ/OmpR and other TCSs in *E. coli* does occur, specifically with the CpxA/CpxR TCS [87]. The CpxA HK displays kinase activity towards OmpR even when CpxA is not stimulated, but CpxA kinase activity towards OmpR is barely detectable when CpxR or EnvZ are present [87]. This suggests that the binding affinity of CpxA/CpxR and EnvZ/OmpR outcompetes interactions with non-cognate HKs and RRs and that the resting phosphatase activity of HKs may further reduce cross-talk by dephosphorylating any RR erroneously phosphorylated in the absence of the correct environmental stimuli [87]. Additionally, cross-talk between the CpxA/CpxR and EnvZ/OmpR is further regulated by MzrA [88, 89]. *mzrA* expression is CxpA/CpxR-dependent and when CxpA/CpxR activity increases MrzA levels also increase [88, 89]. This directly links both TCSs as MzrA binds to EnvZ, which increases OmpR phosphorylation and illustrates the complexity underlying seemingly simple bacterial signaling systems [88, 89].

1.2.2 *The E. coli NtrB/NtrC two-component system*

While the EnvZ/OmpR TCS represents many common features in TCSs, the NtrBC TCS is an example of a TCS with a cytoplasmic HK and a RR that acts as a σ^{54} enhancer-binding protein (EBP). These are features of the flagellar TCSs central to my thesis work.

Nitrogen starvation stimulates the expression of nearly 100 genes in *E. coli* to scavenge nitrogen from the environment and shift its internal metabolism to ease nitrogen demand [90–92]. Under nitrogen stress, *E. coli* uses glutamine synthetase to convert ammonia to glutamine for amino acid and nucleotide synthesis. The NtrBC TCS controls this critical response to nitrogen starvation.

E. coli NtrB is a 36-kDa protein expressed from the *glnL* gene [93]. It has a N-terminal Per-Ant-Sim Domain (PAS) domain followed by a conserved HK region that resembles the cytoplasmic domain of EnvZ (Figure 4). The N-terminal PAS domain regulates NtrB kinase and

phosphatase activity (Figure 4) [94].

E. coli NtrB differs from *E. coli* EnvZ because NtrB detects nitrogen starvation indirectly through the activity of an additional protein, PII [95]. PII inhibits NtrB kinase activity and promotes its phosphatase activity except when α-ketoglutarate levels are high and the influence of PII on NtrB activity is weakened [96, 97]. Under these conditions, NtrB kinase activity increases and its phosphatase activity decreases both of which leads to NtrC phosphorylaion [96, 97].

NtrC is 54-kDa protein expressed from the *glnG* gene in *E. coli* [93]. NtrC has three main domains, an N-terminal REC domain, a middle AAA+ ATPase domain, and a C-terminal helix-turn-helix domain (similar to OmpR) (Figure 4) [98]. NtrC, unlike OmpR, does not bind to the α subunit of the RNAP, but rather acts a bacterial enchancer binding protein (EBP). Bacterial RNAP holoenzymes possess different σ units that drive the transcription of different promoters and genes [98]. RNAP with σ^{70} drives expression of housekeeping genes and when σ^{70} binds to the -10 and -35 site in a bacterial promoter, it shifts from an closed to an open conformation to facilitate gene transcription [98]. RNAP with σ^{54} functions by a different mechanism. The σ^{54} subunit binds to the -12 and -24 sites in a bacterial promoter, but is unable to bind to the non-template strand because of the σ^{54} interaction at the -12 site. RNAP with σ^{54} requires an EBP with AAA+ ATPase activity to loosen the inhibitory -12 site interaction and drive gene transcription forward (Figure 4) [98]. Phosphotransfer from NtrB to the N-terminal aspartic acid residue of NtrC promotes NtrC oligomerization [98, 99]. NtrC binds to roughly 80 to 150 bp upstream of bacterial promoters and NtrC phosphorylation allows for the trimerization of NtrC dimers to create a functional NtrC hexamer [98]. NtrC oligomerization promotes NtrC AAA+ ATPase activity, which then enables σ^{54}-dependent gene transcription (Figure 4) [98].

1.2.3 *The conservation and evolution of two-component systems*

E. coli uses a similar phosphotransfer mechanism in the EnvZ/OmpR and NtrBC TCSs to monitor osmolalic stress and nitrogen starvation (See Section 1.2.1 and Section 1.2.2). While phosphotransfer mechanisms between these two systems are similar, the HKs of these systems (EnvZ and NtrB) possess entirely different sensing domains and the RRs of these system (OmpR and NtrC) control gene expression by interacting with different subunits of the RNA polymerase holoenzyme. This work focuses on the three NtrBC-like flagellar TCSs of *C. jejuni*, *V. cholerae*, and *P. aeruginosa* and the differences between these systems is a foundation for a large part of this thesis (See Chapter 3).

TCS are ubiquitous in bacteria and a few can be found in eukaryotes, generally for chloroplast-related functions or as HKs and RRs that perform new activities distinct from the bacterial TCS paradigm (See Section 1.2.1) [63]. The average bacterial genome contains roughly 50 TCSs, while some groups of bacteria such as cyanobacteria and myxobacteria can have over 240 [62]. Most HKs and RRs genes are located near one another on the genome, a survey in *P. aeruginosa* found that of the over 50 *P. aeruginosa* HKs genes, all but 14 HKs had a RR within three open reading frames of the HKs [100]. This proximity between HKs genes and RRs genes likely lead to the evolution of hybrid HKs through gene fusion that possess both HK and RR characteristics and that phosphosphorylate their own conserved aspartic acid residues within RR elements [101].

Genes encoding HKs and RRs are notable among bacterial genes in that horizontal gene transfer does not appear to be the primary way that they are acquired. Instead, many appear to be the product of duplication events [102]. The high degree of similarity in G/C content in HKs and RRs genes relative to the average genomic G/C content and the underrepresentation of phagal or transposon elements neighboring HKs and RRs genes support this hypothesis [100, 102]. That finding does not exclude horizontal gene transfer as a mechanism that generates new TCSs in bacterial species, but only a minority TCSs were likely acquired through horizontal gene

transfer [102].

While TCSs can detect a very diverse group of environmental signals, most TCSs fall within the Che, Ntr, Omp, and Nar families [62]. Duplication of a TCS means that crosstalk between the ancestral TCS and the copied TCS needs to be overcome for a different signaling pathway to emerge. Following duplication of a TCS, only a few mutations are needed to avoid crosstalk between the ancestral TCS and the new TCS [103–105]. Once crosstalk between the ancestral TCS and new TCS is overcome, domain swapping can occur in the new TCS to allow the new TCS to detect different signals from the ancestral TCS [103–105].

1.2.4 *FlgSR and two-component systems in flagellar formation*

Bacteria tightly regulate the assembly of flagella, which are energetically costly to produce and can trigger an immune response within human hosts. Polarly-flagellated bacteria use TCSs to monitor a key step in flagellar assembly and then control expression of flagellar genes required for rod and hook components [106]. The best characterized flagellar TCSs in polar flagellates are *C. jejuni* FlgSR, *V. cholerae* FlrBC, and *P. aeruginosa* FleSR, which are NtrBC-like flagellar TCSs.

1.2.4.1 *The biology of FlgSR in C. jejuni*

Our understanding of the FlgSR system in *C. jejuni* began with early studies into the FlgSR system in another ϵ-proteobacterium, *H. pylori*. FlgR is a NtrC-like RR that is required for σ^{54}-dependent flagellar gene expression in *H. pylori* and *C. jejuni* [107, 108]. Unusually, the gene encoding the cognate HK for FlgR, *flgS*, does not neighbor *flgR* in *H. pylori* or *C. jejuni* [42, 107, 109].

Expression of *flgS* and *flgR* is σ^{70}-dependent and is likely constituitive [42, 107, 109]. Direct transcriptional regulation of *flgS* and *flgR* expression (i.e. through a master flagellar transcriptional regulator as seen in other flagellates) has not been described. Instead of FlgS and

FlgR expression (and as a consequence, flagellation) in *C. jejuni* can be influenced via phase-variation [110, 111]. Homopolymeric tracts (repeats of the same nucleotide) are enriched within coding regions of some genes in the *C. jejuni* genome [112]. A deletion or insertion mutation in these homopolymeric tracts frequently leads to a premature stop codon and a non-functional protein [110, 111]. This phase-variability mechanism means that *C. jejuni* exists within a host as a heterogenous population for several critical phenotypes for virulence including glycosylation and flagellation [112]. *flgS* and *flgR* both have multiple homopolymeric tracts within their coding sequences and non-motile *C. jejuni* mutants often contain deletion mutations in these tracts, which led to a frameshift and premature stop-codon ("phase on" $\rightarrow$ "phase off") [110, 111]. The *flgS* and *flgR* homopolymeric tracts are pronounced in *C. jejuni* relative to other *Campylobacter spp.* and given that non-motile "phase off" *flgS* and *flgR* mutants administered to chickens often revert back to "phase on" motile mutants following infection, phase variability could be a way for *C. jejuni* to regulate flagellation without a master flagellar transcriptional regulator [110, 111].

FlgS is a cytoplamsmic NtrB-like HK that binds to FliF (the MS ring component) and FliG (the rotor component) (See Section 1.2.2) [44, 46]. This interaction appears to be dependent on MS ring and C ring multimerization as fT3SS mutants abolish MS ring multimerization, both disrupting FliF-FlgS and FliG-FlgS interactions and eliminating FlgSR activity [38, 44, 46]. It is unclear if the interactions between FlgS and the MS ring and rotor directly stimulate FlgS autophosphorylation or are required for the N-terminus of FlgS to detect another signal, but MS ring and rotor formation form an important regulatory checkpoint in flagellar gene expression (Figure 3) [38, 44]. However it ultimately occurs, MS ring and rotor formation leads to autophosphorylation of the His^{141} residue of FlgS [46]. FlgS autophosphorylation then leads to phosphotransfer from FlgS to a conserved Asp residue in the N-terminus of FlgR [42, 109].

FlgR is an NtrC-like RR, and FlgR phosphorylation promotes FlgSR- and σ^{54}-dependent flagellar gene expression (See Section 1.2.2) [45]. Unlike NtrC, FlgR lacks a C-terminal helix-turn-helix DNA-binding domain and does not appear to bind to promoters [45]. Instead, the

FlgR C-terminus seems to be important to ensure FlgR specificity toward FlgS as FlgR C-terminal mutants can be phosphorylated from acetyl phosphate or other small molecule phosphodonors [43, 45]. FlgR may stimulate σ^{54}-dependent expression of flagellar genes by binding to RNAP holoenzymes that are already attached to target promoters [43].

1.2.4.2 *The role of FlrBC in Vibrio species*

Shortly after initial studies into FlgSR- and σ^{54}-dependent flagellar gene expression in *C. jejuni*, similar studies were carried out in *V. cholerae*. The FlrBC TCS is required for σ^{54}-dependent flagellar rod and hook gene expression [113]. In contrast to *C. jejuni* FlgSR, *V. cholerae flrB* and *flrC* expression requires a master flagellar transcriptional regulator, FlrA, and σ^{54}, which allows *flrBC* expression to be regulated with the transcriptional machinery that enables shifts from motile and sessile lifestyles in *V. cholerae* (See Section 1.3) [113, 114].

FlrB autophosphorylation is dependent on FliF, which means it may also directly or indirectly detect the formation of a secretion-competent fT3SS as we found with *C. jejuni* FlgSR [115] Upon FlrB autophosphorylation, phosphotransfer from FlrB to FlrC occurs [116]. FlrC is a NtrC-like RR, but it has some key differences from *E. coli* NtrC. FlrC binds to DNA (unlike *C. jejuni* FlgR), but it binds downstream of the *flaA* transcriptional start site (whose expression is the highest among FlrC- and σ^{54}-dependent genes) [117]. This is in contrast to NtrC, where internal NtrC binding sites usually repress gene expression and suggests that FlrC may interact with the RNAP/σ^{54} holoenzyme differently than NtrC [117].

1.2.4.3 *The role of FleSR in Pseudomonas species*

Of the *C. jejuni* FlgSR, *V. cholerae* FlrBC, and *P. aeruginosa* FleSR TCSs, the *P. aeruginosa* FleSR is the most poorly studied in spite of being the first of the three systems to be characterized. *fleS* and *fleR* are expressed in an operon and *fleSR* expression requires a master transcriptional regulator, FleQ, and σ^{54} (like *V. cholerae flrBC*) [37, 118]. Not much is known about what stimulates FleS autophosphorylation or how FleR stimulates σ^{54}-dependent flagellar gene expression.

1.3 *c-di-GMP regulation of flagellar motility*

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (c-di-GMP) is a ubiquitous secondary signaling molecule in bacteria that regulates a wide range of bacterial processes necessary for virulence including biofilm formation, motility, and effector secretion [119–122]. c-di-GMP signal transduction can regulate expression in complex, multifaceted pathways involving multiple signals, sensors, and c-di-GMP-binding proteins or riboswitches can regulate expression at transcriptional or translational levels [123–125]. Broadly speaking, c-di-GMP signaling pathways contain diguanylate cyclases (DGCs) that synthesize c-di-GMP and phosphodiesterases (PDEs) that hydrolyze c-di-GMP, and effectors that bind c-di-GMP to regulate bacterial processes (Figure 5) [123].

1.3.1 *The role of c-di-GMP in bacterial biology*

Intracellular levels of c-di-GMP regulates a wide range of phenotypes in different bacteria. In Gram-negative bacteria, c-di-GMP facilitates the transition from motile to sessile lifestyles and between different modes of virulence among Gram-negative bacterial pathogens [119]. Among bacterial pathogens, *P. aeruginosa* and *V. cholerae* are prominent models for c-di-GMP signaling [126]. c-di-GMP signaling in *P. aeruginosa* influences biofilm formation, twitching motility, chemotaxis, and a small colony variant phenotype found in advanced-stage cystic fibrosis patients [120, 127–129]. c-di-GMP signaling in *V. cholerae* affects cholera toxin transcription and biofilm formation [130, 131]. Additionally, quorum sensing in *V. cholerae* appears to repress expression of proteins containing GGDEF and EAL domains, which are the two most prominent domains in c-di-GMP biochemistry [132].

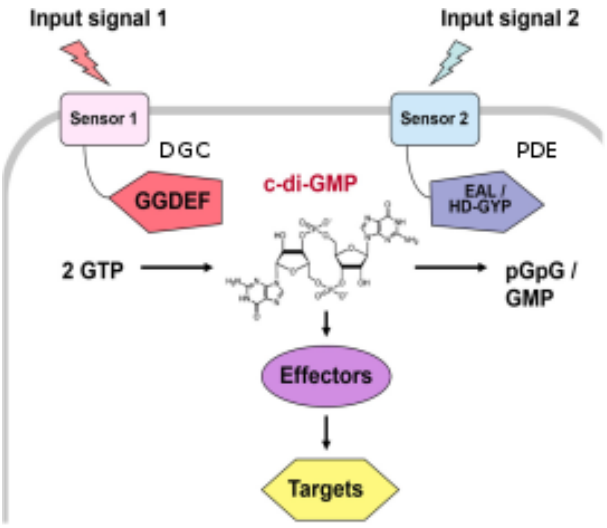

Figure 5: **General c-di-GMP regulatory scheme (adapted from Hengge *et al.*) [123]).** c-di-GMP is a secondary signaling molecule that regulates broad lifestyle changes in some bacteria. c-di-GMP is synthesized by DGCs from GTP in response to extracellular or intracellular stimuli. c-di-GMP binds to several classes of effectors, which can lead to transcriptional, post-transcription, or post-translational regulation. c-di-GMP is hydrolyzed by PDEs through either low-level constituitive PDE activity or in response to stimuli.

1.3.2 *The biochemistry of c-di-GMP signaling*

The primary catalytic domain in DGCs is the GGDEF domain, which contains a highly conserved Gly-Gly-Asp-Glu-Phe motif [119, 133]. The first two glycine residues of GGDEF motif bind GTP specifically [119, 134, 135]. The third and fourth residues of GGDEF motif facilitate Mg^{2+} or Mn^{2+} coordination and are necessary for phosphoester bond formation [119, 134, 135]. DGCs generally function as homodimers and two broad structural mechanisms regulate DGC activity: 1) A signal leads to a conformational change in the DGC homodimer, which brings the GGDEF domains in close proximity to become catalytically active; and 2) c-di-GMP can bind to an inhibitory "RXXD" (I) site five amino acids upstream of the GGDEF motif, preventing DGC activiy [119, 134, 135].

PDEs generally have either EAL domains (Figure 5) [119, 136–138]. EAL domains have a highly conserved Glu-Ala-Leu motif [139]. The glutamic acid residue in the EAL motif directly coordinates one of the metallic cations necessary for c-di-GMP catalysis [140]. PDEs with EAL domains primarily act as homodimers and hydrolyze c-di-GMP in a Mg^{2+} or Mn^{2+} two-metal-ion catalysis mechanism [138, 140]. PDEs with EAL domains can be activated by conformational changes that affect the metal-water coordination at the EAL-EAL dimer interface [119, 140]. PDE activity can be inhibited by acidic conditions and Ca^{2+}, which disrupt metal-water coordination at the EAL motif [119]. HD-GYP domains also have c-di-GMP PDE ac-

tivity, but much less is known about their biochemistry and structure than EAL domains [119]. Many c-di-GMP DGCs and PDEs have GGDEF and EAL or HD-GYP domains in tandem and in most cases, one of these domains is catalytically inactive [119, 141].

c-di-GMP-binding effectors are not well characterized since c-di-GMP can bind to a wide range of proteins with little to no sequence similarity [119]. The best studied c-di-GMP receptor domain, PilZ, is widely conserved among bacterial species, yet many PilZ domains are no longer able to bind c-di-GMP and fulfill different roles [142]. Some proteins with catalytically inactive EAL domains bind to c-di-GMP and act as response regulators [143]. Similarly, some catalytically inactive GGDEF domains retain their upstream inhibition sites to bind c-di-GMP [144]. In addition to protein effectors, there are two known classes of c-di-GMP binding riboswitches, and both are defined by conserved sequence identities [124, 145].

1.3.3 *c-di-GMP regulation of flagellar formation*

In bacteria with c-di-GMP signaling systems, high levels of intracellular c-di-GMP generally represses flagellar gene expression. There are several different kinds of signaling pathways that link c-di-GMP to flagella. For example, *Caulobacter crescentus* flagellar synthesis and holdfast gene expression and *P. aeruginosa* flagellar stator load can increase c-di-GMP levels [146, 147]. For this work, I am primarily interested in how c-di-GMP regulates the activities of the flagellar master regulators in *V. cholerae* (FlrA) and *P. aeruginosa* (FleQ).

Both *V. cholerae* FlrA and *P. aeruginosa* FleQ are NtrC-like σ^{54} EBPs, but they each lack a cognate HK or a conserved aspartic acid residue required for phosphotransfer from a cognate HK [114, 148]. Both FlrA and FleQ drive transciption of the fT3SS and *flrBC*/*fleSR* TCS genes, which are all required for σ^{54}-dependent expression of rod and hook genes (See Sections 1.2.4.3 and 1.2.4.2) [37, 114]. c-di-GMP represses both FlrA and FleQ, with FlhG (or the *P. aeruginosa* FlhG homologue, FleN) appearing to play a role in this repression. However, there have been different findings reported in *V. cholerae* and *P. aeruginosa* and it is unclear how sim-

ilar the three-way relationship between c-di-GMP, FlhG/FleN, and FlrA/FleQ is between both species [149–152].

c-di-GMP inhibits FleQ activity in *P. aeruginosa* by competing with ATP for the FleQ AAA+ ATPase domain and FleN binding to FleQ further enhances this inhibition [149, 150]. FleN deletion mutants have higher *fleSR* expression than WT, but *fleQ* expression is unaffected, which suggests that FleQ activity could be altered by FleN [153, 154]. Additionally, FleN does not affect FleQ DNA-binding activity, and so the mechanism by which FleN influences c-di-GMP inhibition of FleQ-dependent flagellar gene expression is unclear [154].

c-di-GMP binds to FlrA in *V. cholerae*, which prevents FlrA from binding to the promoters of FlrA-dependent flagellar genes [152]. Mutations in the FlrA AAA+ ATPase site removed c-di-GMP inhibition of FlrA activity [152]. Neither of these phenotypes appear to be dependent on FlhG and an interaction between FlhG and FlrA has not been demonstrated to date [152]. However, Correa *et. al.* found that *V. cholerae* Δ*flhG* increased *flrA* expression although this phenotype appeared to be unstable and disappeared after 48 hours [151].

In this thesis, I explore how early polar flagellar formation can both stimulate rod and hook gene expression to drive flagellar assembly forward (Chapter 3) and repress early flagellar gene expression that is no longer required for flagellar assembly (Chapter 4). Both of these projects further our understanding of the role the polar flagellum plays in regulating its own transcription and adds a further layer of complexity to the tightly regulated assembly of flagella.

2

METHODS

2.1 *Growth and storage of C. jejuni strains*

C. jejuni 81-176 strains were stored at −80°C as frozen stocks in a solution of 85% MH broth and 15% glycerol. *C. jejuni* strains were grown from frozen stocks on MH agar for 48 hours under microaerobic conditions: 10% CO_2, 5% O_2, and 85% N_2 at 37°C. *C. jejuni* strains were restreaked on MH agar and incubated for 16 hours under microaerobic conditions at 37°C. Antibiotics were added to MH as needed at the following concentrations: 10 μg/mL trimpethoprim (TMP), 15 μg/mL chloramphenicol (CM), or 0.5, 1, 2, or 5 mg/mL streptomycin (SM).

2.2 *Growth and storage of V. cholerae and P. aeruginosa*

Vibrio cholerae C6706 *lacZ*, a spontaneous *lacZ* mutant of the WT El Tor C6706 strain, and isogenic mutants from this background were used for all analyses involving *V. cholerae* strains [155]. *Pseudomonas aeruginosa* PA14 and isogenic mutants from this background were used for all analyses involving *P. aeruginosa* [156, 157]. *V. cholerae* and *P. aerguniosa* stocks were grown at 30°C or 37°C in LB broth and these strains were stored as frozen stocks at −80°C in a solution of 80% LB and 20% glycerol. Antibiotics or growth inhibitors were added to LB as needed at the following concentrations: 100 μg/mL ampicillin (AMP), 10 μg/mL chloramphenicol (CM), 100 μg/mL kanamycin (KAN), 100 μg/mL streptomycin (SM), 15 μg/mL gentamicin (GM), 12.5 μg/mL tetracycline (TET), and 10% sucrose (SUC).

2.3 *Methods for constructing C. jejuni strains*

C. jejuni 81-176 *rpsL*^{Sm} Δ*astA* Δ*fliM flaB::astA* (PMB979) was generated following previously described procedures to delete *astA* from the chromosome of 81-176 *rpsL*^{Sm} Δ*fliM* (CRG1005) with a two-step electroporation and selection procedure [38, 106]. After recovery of DRH6757 (81-176 *rpsL*^{Sm} Δ*astA* Δ*fliM*), the strain was electroporated with pDRH665 to replace *flaB* with *flaB::astA-kan* on the chromosome. Transformants on MH agar with kanamycin were recovered and screened to isolate PMB979.

2.4 *Methods for constructing V. cholerae strains*

V. cholerae flrA, *flrB*, *flrC*, *fliF*, *flhB*, *fliP*, *fliQ*, *fliR*, *fliA*, and *rpoN* mutants were derived from mutants containing a TnFGL3 insertion (which encodes kanamycin-resistance) in each gene. Each TnFGL3 mutant was prepared for electroporation. Briefly, 100 mL LB containing kanamycin were inoculated with a 1:40 dilution of overnight cultures of each mutant and grown with shaking at 37°C to an optical density at 600 nm (OD_{600}) of 0.8. Bacteria were recovered by centrifugation, washed twice in cold 2 mM $CaCl_2$, washed once in 2 mM $CaCl_2$ with 10% glycerol, and then resuspended in 300 μL 2 mM $CaCl_2$ with 10% glycerol. Approximately 0.4 μg of pFlpE was electroporated into each mutant and the bacteria were grown in LB for 1 hour at 37°C before plating on LB with ampicillin for selection. Ampicillin-resistant colonies were grown overnight at 37°C in 10 mL LB with 0.1% arabinose to induce FLP-mediated recombination to remove a large part of TnFGL3, including the kanamycin-resistance gene, leaving a 192-bp scar within each gene to disrupt the coding sequence. Ten-fold serial dilutions were plated on LB with streptomycin and 40 μg/mL X-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside). White, streptomycin-resistant colonies were then screened for sensitivity to ampicillin and kanamycin and then screened by colony PCR to verify the presence of the 192-bp scar within the coding sequence of each gene.

In-frame deletion of *flhA*, *fliQ*, *fliM*, *fliN*, or *fliG* from the chromosome of *V. cholerae* C6706 lacZ was accomplished by first creating pKAS32-based plasmids that contained DNA for the correct in-frame mutation. Primers with 5' sites for restriction enzymes were designed to amplify from the *V. cholerae* C6706 *lacZ* genome two DNA fragments with 700-1000 nucleotides upstream and downstream of the portion of the gene to be deleted. These fragments were joined together by a second round of PCR and then cloned as a single fragment into the pKAS32 with T4 DNA ligase (New England Biolabs). DNA fragments for in-frame deletion of *flhG* or deletion of domains encoded by *fliG* were generated similarly by PCR and ligated into pGP704sacB28 with Gibson Assembly Mastermix (New England Biolabs).

To delete genes from the chromosome of *V. cholerae* C6706 *lacZ* with pKAS32-based plasmids, the respective plasmids were transformed into *E. coli* SM10λ*pir*. *V. cholerae* C6706 *lacZ* and SM10λ*pir* strains were inoculated from overnight cultures into LB at a 1:20 dilution and grown at 37°C with shaking to OD_{600} 0.6-0.8. *V. cholerae* C6706 *lacZ* was mixed with SM10λ*pir* strains at a ratio of 20:1 and collected onto a filter by vacuum using a manifold. The filters were placed on LB agar for 7 hours at 37°C. Bacteria were collected from filters by washing with 2.5 mL phosphate buffered saline (PBS) and 100 µl was plated on thiosulfate citrate biosalts (TCBS) agar with ampicillin to select for *V. cholerae* C6706 *lacZ* transconjugants. After overnight incubation at 37°C for 24 hours, transconjugants were streaked for individual colonies on LB with ampicillin. Colonies were screened for ampicillin resistance and streptomycin sensitivity and then grown overnight in LB without antibiotics. Ten-fold dilutions of overnight cultures were plated on LB with 1 mg/mL streptomycin. Colonies were screened for ampicillin resistance and by colony PCR to verify generation of the correct in-frame deletion of the specific gene.

For deletion of *flhG*, DGC and PDE genes, and portions of *fliG* and to generate FlrA point mutations on the chromosome of *V. cholerae* C6706 *lacZ*, pGP704sacB28 derivatives containing mutations were first transformed into *E. coli* SM10λ*pir*. *V. cholerae* C6706 *lacZ* and *E. coli* SM10λ*pir* strains were grown overnight at 30°C in LB without NaCl with appro-

priate antibiotics. One mL of *E. coli* SM10λ*pir* strains were collected by centrifugation and then resuspended with 1 mL of the *V. cholerae* C6706 *lacZ* culture. After collection of the bacteria by centrifugation, bacteria were resuspended in 150 μL LB and 50 μL was spotted onto LB agar without NaCl or antibiotics. After overnight incubation at 37°C, each spot was collected in 900 μL LB and vortexed. Ten-fold serial dilutions were plated on LB agar without NaCl, but containing ampicillin and then agar plates were incubated overnight at 30°C. A colony from each transconjugant was restreaked on the same agar. A colony was then grown overnight in LB without NaCl at 30°C. A loopful of culture was streaked on LB agar containing 10% sucrose and lacking NaCl. Agar plates were incubated at room temperature for 36-48 hours. Colonies were screened for streptomycin resistance and ampicillin sensitivity and then screened by colony PCR for generation of the correct in-frame deletion of the specific gene.

Construction of plasmids for creating mutants to express σ^{54}- and FlrBC-dependent flagellar rod and hook operons from the FlrA-dependent promoter of the fliE operon involved a tripartite fusion of three DNA fragments by PCR followed by insertion into the XbaI site of pGP704sacB28. The three DNAs that were fused together in the 5' to 3' orientation included: a region upstream of the promoters for the *flgB*, *flgF*, or *flgK* operons (-998 to -195, -1005 to -158, and -817 to -163, respectively); bases -89 to -1 upstream of the *fliE* coding sequence that contains the FlrA-dependent *fliE* promoter; and the coding sequence of *flgB*, *flgF*, or *flgK*. The plasmids were sequenced and then transformed into *E. coli* SM10λ*pir* for conjugation into *V. cholerae* C6706 lacZ or *V. cholerae* C6706 *lacZ* Δ*flhG* to replace the promoter for a specific operon with the promoter for the *fliE* operon on the chromosome. Conjugation and mutant isolation procedures are described as above. The procedures were repeated with resultant strains to replace two or three promoters for the rod and hook proteins with the *fliE* promoter on the *V. cholerae* chromosome.

Portions of the promoter regions of *flrA*, *flrB*, *flaA*, *flgB*, *flgF*, *flgK*, *fliE*, and *cheV* operons were amplified by PCR with primers SalI or BamHI sites at the 5' ends. These plasmids were then ligated into the SalI and BamHI sites of pTL61T with T4 DNA ligase to create

lacZ transcriptional fusions to the promoters of these operons. These plasmids were first transformed into DH5α and then electroporated into WT *V. cholerae* C6706 *lacZ* or isogenic mutants by procedures described above and recovered on LB agar with ampicillin. Transformants were screened by colony PCR for retention of the *lacZ* transcriptional fusions *in trans* on the pTL61T derivatives.

Plasmids to complement *V. cholerae* C6706 *lacZ* mutants were generated from pACYC184. DNA containing approximately 20 nucleotides upstream and downstream of the coding sequence of each gene was amplified by PCR with primers encoding 5' restriction sites. These fragments were cloned into BamHI- and SalI-digested pACYC184 or EcoRV-digested pACYC184 by T4 DNA ligase or Gibson Assembly Mastermix to disrupt the tetracycline-resistance gene and then transformed into DH5α. Complemented genes were thus expressed from the promoter of the tetracycline-resistance gene. pGP704sacB28-based plasmids carrying $fliF\Delta_{\mathrm{AS200-201}}$ and $fliF\Delta_{\mathrm{AS202-203}}$ were created by amplifying two DNA fragments with approximately 1.5 kb upstream of the two codons to be deleted from *fliF*. These fragments were then joined together by PCR and then cloned into pGP704sacB28 using Gibson Assembly Mastermix (New England Biolabs). These plasmids were then used as template to amplify DNA to clone into pACYC184 for complementation. pACYC184 was also digested with EcoRV and NruI to remove a portion of the tetracycline-resistance gene and religated to create a negative control vector for complementation. Transformants were selected on LB with chloramphenicol. After verification of interruption of the tetracycline-resistance cassette, the plasmids were then electroporated into *V. cholerae* C6706 *lacZ* strains by procedures described above. Transformants were recovered on LB agar with chloramphenicol.

2.5 *Methods for constructing P. aeruginosa strains*

In-frame deletion of genes from the chromosome of PA14 was accomplished by creating pEX18Gm-based plasmids that contained DNA for the correct in-frame mutation. Primers with 5' sites for restriction enzymes were designed to amplify from the PA14 genome DNA

fragments with 700-1000 nucleotides upstream of the portion of the gene to be deleted. These fragments were cloned into pEX18Gm by Gibson Assembly Mastermix or joined together by a second round of PCR and then cloned as a single fragment into pEX18Gm with Gibson Assembly Mastermix or T4 DNA ligase (New England Biolabs). DNA was introduced by electroporation into PA14 to delete genes from the chromosome based on previously described procedures [158, 159]. Briefly, PA14 was grown as overnight cultures in 6 mL LB at 37°C. Bacteria were collected by centrifugation at room temperature and washed three times in room temperature 300 mM sucrose. After the last centrifugation step, bacteria were resuspended in 200 μL of 300 mM sucrose. For electroporation, 100 μL of PA14 resuspension and 300 ng of each pEX18Gm-based plasmid were mixed and electroporated. Bacteria were collected and then grown in 1 mL LB broth without NaCl for 4 hours at 37°C. After growth, 100 μl of culture were plated on Vogel-Bonner Medium E agar (VBEM) with 100 μg/mL gentamicin and then incubated at 30°C for 48 hours. Transformants were patched on VBEM with 100 μg/mL gentamicin and VBEM with 10% sucrose and grown for 24 hours at 30°C. Gentamicin-resistant and sucrose-sensitive colonies were then grown overnight at 37°C in LB without salt. Ten-fold serial dilutions were plated on VBEM with 10% sucrose and grown at 30°C for up to 48 hours. Colonies were patched on LB agar without salt but containing 10% sucrose and LB with 100 μg/mL gentamicin at 37°C to identify sucrose-resistant and gentamicin-sensitive colonies. These colonies were then screened by colony PCR to verify generation of the correct in-frame deletion from the chromosome of PA14.

Chromosomal *flgB-lacZ* and *fliA-lacZ* reporters at the *att* site in *P. aeruginosa* mutants were constructed using mini-CTX-lacZ-based plasmids. Promoter regions of *flgB* and *fliA* operons were amplified by PCR with primers containing restriction sites at the 5' ends. The *flgB* promoter was ligated as a SalI-BamHI fragment into mini-CTX-lacZ and the *fliA* promoter was ligated as a PstI-BamHI fragment into mini-CTX-lacZ with T4 DNA ligase to create *lacZ* transcriptional fusions to the promoters of these operons. These plasmids were transformed into DH5α and transformants were selected for on LB with 12.5 μg/mL tetracycline. Plasmids were then transformed into *E. coli* SY17.1 λ*pir*. WT PA14 and PA14 mutants were then conju-

gated with SY17.1 λ*pir* containing mini-CTX-lacZ harboring *flgB*- or *fliA-lacZ* transcriptional fusions. LB broth with tetracycline was inoculated with a 1:100 dilution of overnight culture of SY17.1 λ*pir* strains and then grown at 37°C with shaking to an OD_{600} 0.3-0.6. Five-hundred μL of overnight cultures of PA14 strains were inoculated into 10 mL LB without antibiotics and incubated without shaking at 42°C during growth of the SY17.1 λ*pir* strains. SY17.1 λ*pir* and PA14 cultures were then combined (0.5 mL of each) and collected by centrifugation. Bacterial pellets were suspended in 40 μL of LB broth and spotted on LB without antibiotics. After overnight incubation at 30°C, the spots were scraped into 1 mL PBS and 10-fold serial dilutions were plated on VBEM containing 100 μg/mL tetracycline. Bacteria were then incubated at 30°C for 48 hours. Colonies were patched onto VBEM with tetracycline and grown at 30°C overnight. Colonies were verified by colony PCR to ensure integration of the mini-CTX-lacZ DNA into the *att* site on the chromosome and then frozen. To remove the backbone of mini-CTX-lacZ, PA14 strains were then conjugated with SY17.1 λ*pir* containing pFLP2, which harbors a plasmid for expression of the FLP recombinase. Conjugation was performed as described above except conjugation mixtures were initially spotted on LB without NaCl and then transconjugants were selected by plating 10-fold serial dilutions on VBEM containing 200 μg/mL carbenicillin. After growth for 48 hours at 30°C, colonies were patched on VBEM containing 200 μg/mL carbenicillin or 100 μg/mL tetracycline. Carbenicillin-resistant and tetracycline-sensitive colonies were then grown in LB without NaCl or antibiotics at 37°C. After overnight growth, 10-fold serial dilutions were plated on VBEM with 10% sucrose and incubated for 2 hours at 30°C. Colonies were then patched on VBEM containing 10% sucrose, 200 μg/mL carbenicillin, or 100 μg/mL tetracycline. Sucrose-resistant, carbenicillin-sensitive, and tetracycline-sensitive colonies were then screened by colony PCR to ensure retention of the *lacZ* transcriptional fusions at the *att* site on the chromosome and removal of the mini-CTX backbone from the genome.

2.6 *Arylsulfatase assays*

Arylsulfatase assays were used to measure the level of expression of the *flaB::astA* transcriptional fusion on the chromosome of *C. jejuni* Δ*astA* strains as previously described [38, 160, 161]. Each strain was analyzed in triplicate, and each assay was performed three times. The level of expression of the transcriptional fusion in each strain was calculated relative to the expression in the WT *C. jejuni* Δ*astA* strain, which was set to 100 units.

2.7 β-*galactosidase assays*

The level of gene expression in *V. cholerae* and *P. aeruginosa* strains was compared by monitoring the β-galactosidase activity of strains harboring *lacZ* transcriptional fusions to specific promoters by standard procedures [162]. Strains were grown in LB at 37°C with shaking to an OD_{600} of approximately 0.8 prior to the start of the assays. Each strain was analyzed in triplicate, and each assay was performed three times. The level of expression of the transcriptional fusion in each strain was calculated relative to the expression in WT *V. cholerae* C6706 *lacZ* or PA14, which was set to 100 units.

2.8 *Immunoblots*

Whole-cell lysates (WCL) of *V. cholerae* and *P. aeruginosa* strains for immunoblot analysis were prepared by first inoculating 5 mL LB with a 1:50 dilution of overnight cultures. Cultures were grown at 37°C with shaking to an OD_{600} of 0.8. One-milliliter aliquots of each culture were recovered by centrifugation in microcentrifuge tubes, washed once with PBS, and then resuspended in 50 μl of 1xSDS-loading buffer. Samples were boiled for 5 minutes prior to separation by SDS-PAGE and transferred to membranes for immunoblotting by standard procedures. For specific detection of proteins in WCL, 10 μl of WCLs was analyzed to detect FliF, FliG, and RpoA, and 25 μl of WCL was analyzed to detect FlrB. Proteins were

detected with specific guinea pig antisera generated as described below. Primary antisera were applied to immunoblots for 1 to 2 hours and used at the following concentrations: *V. cholerae* FliF UTGP151 (1:1,000), *V. cholerae* FliG UTGP198 (1:1,000), *V. cholerae* FlrB UTGP151 (1:2,000), *V. cholerae* RpoA UTGP197 (1:2,000), and *P. aeruginosa* FliG UTGP145 (1:1,000). A 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-guinea pig antibody was then applied for detection of proteins.

2.9 *Antiserum production*

All use of animals in experimentation has been approved by the IACUC at the University of Texas Southwestern Medical Center. Recombinant protein for antiserum production was produced by first cloning the coding sequences from codon 2 to the stop codon of *V. cholerae* *flrB*, *fliG*, and *rpoA* into the SmaI site, the BamHI site, or the BamHI and SalI sites of pGEX4T-2 to create N-terminal fusions of glutathione S-transferase. For recombinant *V. cholerae* FliF, the region of *fliF* encoding the predicted periplasmic domain, from codons 45 to 473, was cloned into the BamHI and SalI sites of pQE30 to create an N-terminal fusion of 6xHis-tag. For recombinant *P. aeruginosa* FliG, the coding sequence from codon 2 to the stop codon was cloned into the BamHI and SmaI sites of pQE30 to create an N-terminal fusion of a 6xHis-tag. Resultant plasmids were transformed into BL21(DE3) or XL1-Blue and then induced in LB broth with 1 mM isopropyl-β- D -thiogalactopyranoside (IPTG). Recombinant protein was purified from the soluble fractions by affinity chromatography according to the manufacturer's instructions. Purified recombinant protein was used to immunize guinea pigs by standard procedures for antiserum generation via a commercial vendor (Cocalico Biologicals).

2.10 *Electron microscopy analysis*

Overnight cultures of *V. cholerae* strains were inoculated into 5 mL LB at a 1:50 dilution and grown at 37°C with shaking to an OD_{600} of approximately 0.8. One milliliter of each culture was pelleted for 3 minutes at 13,200 rpm in a microcentrifuge, resuspended in

2% glutaraldehyde in 0.1 M cacodylate, and then incubated on ice for 1 hours. Copper-coated Formvar grids were negatively glow discharged, and bacterial samples then were applied to the grids. The samples were stained with 2% uranyl acetate and visualized with an FEI Technai G2 Spirit Bio TWIN transmission electron microscope. Flagellar numbers were counted from at least 100 individual cells and averaged from three biological replicates to determine the proportion of bacterial populations producing different flagellation phenotypes: hyperflagellated (producing at two or more flagella at least at one pole), wild-type (producing a single flagellum at one pole), or aflagellated (lacking a flagellum). After averaging, the standard deviations for each population were calculated.

2.11 *Motility assays*

V. cholerae strains were grown from freezer stocks in 5 mL LB overnight at 37°C with shaking. After growth, each strain was inoculated into LB motility agar (containing 0.3% agar) with an inoculation needle. Agar plates then were incubated for 8 hours at 37°C.

2.12 *Bioinformatic methods*

Complete reference bacterial genomes were acquired from `www.ncbi.nlm.nih.gov/assembly` to form a database containing 117 genomes. tBLASTn was run against the genome database to identify the top-scoring hit for each genome in the database for the following protein sequences in FASTA format from the UniProt database: *E. coli* FlgH (P0A6S0), *V. cholerae* FlhF (C3LP19) or *C. jejuni* FlhF (A0A0H3P9N0), and *V. cholerae* FlrB (C3LPE1) or *C. jejuni* FlgS (A0A0H3PDD6). To perform a reciprocal best hit sequence alignment, another tBLASTn search was performed with each top-scoring hit from each genome against the genome containing each protein query and only considered positive hits as those that were able to identify the protein query as the top-scoring hit in the respective genome. 51 sensor histidine kinase sequences were also acquired from the *V. cholerae* N16961 proteome from UniProt. Reciprocal best hit sequence alignments were performed against the reference bacterial genome

database with these 51 *V. cholerae* sensor kinases and calculated the Pearson correlation coefficient between each of the 51 sets of sensor kinase hits and *V. cholerae* FlhF hits in the reference bacterial genomes. For completion of all bioinformatics analysis, the following software was used: tBLASTn (www.ncbi.nlm.nih.gov) for reciprocal best hit sequence alignments, BioPython (www.biopython.org) to read XML files and prepare command lines, Python2.7.6 (www.python.org) to run scripts, and NumPy (www.numpy.org) to calculate Pearson correlation coefficients. Predicted structural domains in proteins were identified by submitting amino acid sequences to SMART (http://smart.embl-heidelberg.de).

2.13 *Statistical test*

Tests for significance in differences in expression of transcriptional reporter assays in Chapter 3 were conducted using the Student's t test (two-tailed distribution with two-sample, equal variance calculations). For analysis of flagellation of *V. cholerae* populations, a Student's t test (two-tailed distribution with two-sample, equal variance calculations) was used to evaluate statistical significance of monotrichous flagellation, aflagellation, or hyperflagellation between WT *V. cholerae* and transcriptional reprogramming mutants in the WT background and between the *V. cholerae* Δ*flhG* strains and transcriptional reprogramming mutants in the Δ*flhG* background. As indicated in the tables, figures, or figure legends, statistically significant differences between relevant strains possessed p values less than 0.05. Dunnett's multiple comparison tests were performed to determine statistical significance of *flrA-lacZ* and *flrB-lacZ* reporter expression in multiple flagellar mutants relative to WT reporter expression in Chapter 4. Dunnett's tests were performed in GraphPadPrism*v*8.3.0.

3

POLAR FLAGELLATES DETECT A COMMON REGULATORY CHECKPOINT

3.1 *The C. jejuni FlgSR TCS and Polar Flagellar TCS.*

Many bacteria synthesize flagella for swimming motility. Each species produces a specific flagellation pattern defined by the spatial arrangement and number of flagella presented on the cell surface. Peritrichous flagellates construct many flagella across the surface, whereas polar flagellates generate flagella only at polar regions. Polar flagellates are further categorized by the number of flagella per cell: monotrichous (one flagellum at one pole), amphitrichous (one flagellum at each pole), and lophotrichous (a tuft of a few flagella at one pole).

Flagellar placement and number in many polar flagellates are controlled by the FlhF GTPase and FlhG/FleN ATPase [6, 163]. FlhF is hypothesized to function in some polar flagellates in a GTP-bound "on" state to perform an unknown essential step for flagellar biogenesis at a pole. Transitioning to a GDP-bound "off" state upon GTP hydrolysis may limit the production of additional polar flagella. In some polar flagellates, FlhG stimulates FlhF GTPase activity *in vitro*, which has been proposed to influence flagellum numbers by controlling *in vivo* FlhF-dependent polar flagellation activities [36, 164–166]. However, FlhG orthologs in other species control polar flagellar number by repressing the activity or expression of a specific master transcriptional regulator so that an ideal level of flagellar genes sufficient to produce the correct number of flagella are expressed [151, 153, 154]. Many molecular details for how FlhF and FlhG control polar flagellation remain elusive. It is anticipated that FlhF and FlhG activities vary among species, resulting in different flagellation patterns in polar flagellates.

Despite different flagellation patterns, many peritrichous and polar flagellates possess some conserved strategies to coordinate flagellar gene transcription with stages of flagellar assembly [3, 10, 167, 168]. These strategies allow for tight regulation of ordered flagellar protein secretion that is conducive to flagellar motor biogenesis. Stages of flagellar assembly can be marked by distinct cues or regulatory checkpoints that are detected by different mechanisms to stimulate gene transcription and protein production to complete the next stage of assembly. Flagellar biogenesis begins by activating the transcription of genes encoding components essential for the initial steps in assembly, which include the flagellar type III secretion system (fT3SS), MS ring, and C ring rotor and switch proteins [8, 9, 37, 113, 114, 148, 169]. MS and C ring formation around the fT3SS core completes biogenesis of a competent fT3SS for export and assembly of rod and hook components [11, 14, 15, 170, 171]. Up to this point, the alternative σ factor σ^{28}, which is required for transcription of flagellins and other motility genes, is inhibited by the anti-σ factor FlgM [25, 26, 28, 29, 167, 168]. Hook biogenesis completes a regulatory checkpoint that facilitates an fT3SS substrate specificity switch to secrete FlgM out of the cell via the fT3SS [27, 167, 168]. Derepression of σ^{28} allows for transcription of genes that complete flagellar filament polymerization and motor assembly.

We previously explored how the amphitrichous polar flagellate *Campylobacter jejuni* coordinates the transcription of flagellar genes with flagellar assembly (Figure 6) [38, 44, 46]. We discovered that fT3SS core proteins (FlhA, FlhB, FliP, FliQ, and FliR), the FliF MS ring, and FliG rotor of the C ring assemble into the MS ring-rotor-fT3SS complex to form a regulatory checkpoint monitored by *C. jejuni*. We found the flagellum-associated FlgSR TCS detects MS ring-rotor-fT3SS formation to directly activate σ^{54}-dependent flagellar rod and hook gene expression (Figure 6) [38, 44, 46]. As a means of signal detection, we observed that the cytoplasmic FlgS sensor kinase of the FlgSR TCS physically interacted with FliF and FliG only after these proteins multimerized into the MS ring and rotor around the fT3SS core (Figure 6) [44]. In mutants defective for MS ring-rotor-fT3SS complex assembly, FlgS did not interact with FliF and FliG. Currently, it is not known whether FlgS interacts with surfaces of adjacent FliF subunits, FliG subunits, or FliF-FliG complexes of the MS ring-rotor structure surrounding the

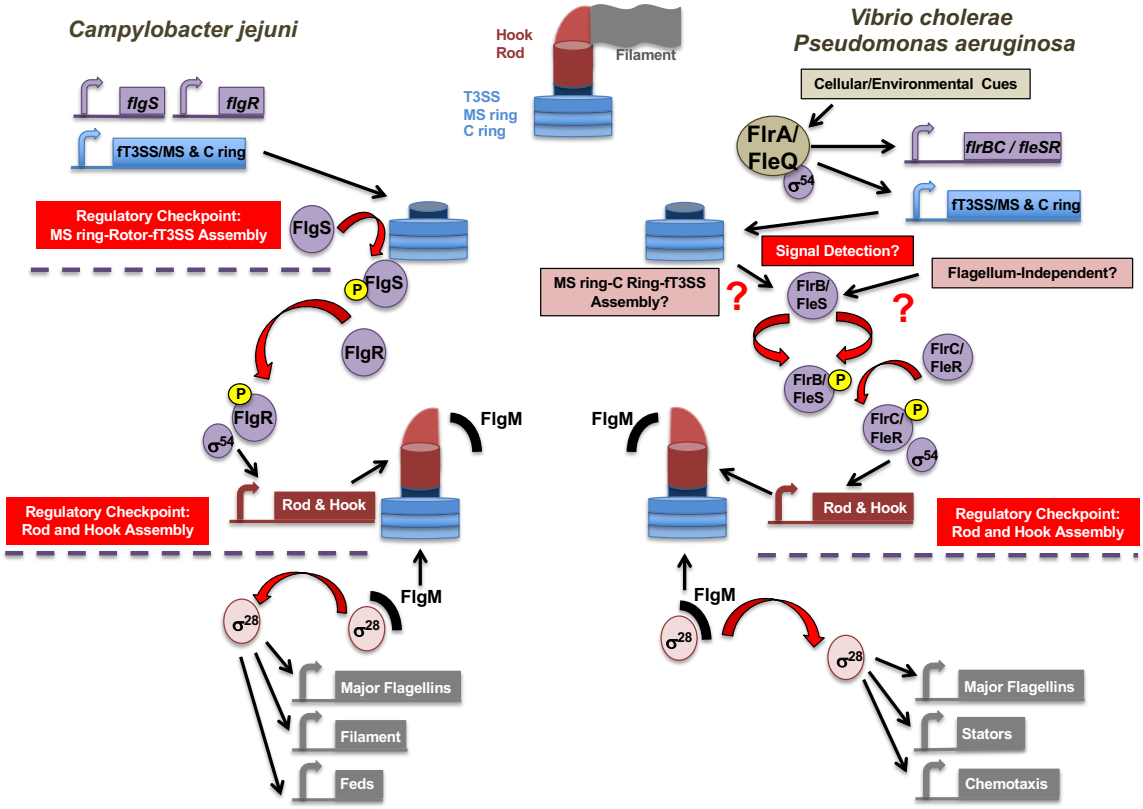

Figure 6: **Defined and unknown regulatory steps of the polar flagellar transcriptional program of *C. jejuni*, *V. cholerae*, and *P. aeruginosa*.** Simplified models of transcriptional regulation of different classes of flagellar genes in the Gram-negative polar flagellates *C. jejuni*, *V. cholerae*, and *P. aeruginosa*. *V. cholerae* and *P. aeruginosa* (right) initiate flagellar gene transcription via master transcriptional regulators (FlrA and FleQ, respectively) to transcribe an initial class of flagellar genes. *C. jejuni* lacks a master transcriptional regulator and expression of this class of flagellar genes appears to be constitutive (left). These initial flagellar genes encode the fT3SS, MS ring, C ring, and a flagellum-associated TCS (FlgSR in *C. jejuni*, FlrBC in *V. cholerae*, and FleSR in *P. aeruginosa*). Expression of the *C. jejuni* flagellar rod, ring, and hook genes is dependent upon σ^{54}, the FlgSR TCS, and the fT3SS, FliF MS ring, and FliG C ring rotor proteins [38, 44, 46]. The formation of the MS ring-rotor-fT3SS complex is an early regulatory checkpoint sensed by the FlgS sensor kinase to initiate phosphotransfer to the FlgR response regulator, which allows FlgR to function with σ^{54} for rod and hook gene expression. (Right) Exploration of factors that may form a similar regulatory checkpoint during flagellar assembly that are required for the activity of the *V. cholerae* FlrBC TCS and the *P. aeruginosa* FleSR TCS addressed in this work. A regulatory checkpoint during late flagellar assembly occurs upon formation of the flagellar rod and hook in all of these organisms and many other peritrichous and polar flagellates. Completion of rod and hook assembly promotes a substrate specificity switch that facilitates secretion of the anti-σ factor FlgM from the cytoplasm and derepression of σ^{28} for transcription of flagellins and other proteins that complete flagellar biogenesis for motor function. *C. jejuni* also contains *fed* genes that are dependent on σ^{28} for expression and are not involved in motility but are required for the colonization of avian species and some virulence processes [51, 52].

fT3SS core. Detection of this regulatory checkpoint by an orthologous FlgSR TCS also may occur in *Helicobacter pylori*, a lophotrichous ϵ-proteobacterium closely related to *C. jejuni*, for transcription of σ^{54}-dependent rod and hook genes [172–175]. Genetic analyses indicate that *H. pylori* FlgSR TCS activity for rod and hook gene transcription is also dependent on fT3SS, MS ring, and C ring proteins, suggesting that this TCS in *H. pylori* also senses the formation of a competent fT3SS [176–181].

Vibrio cholerae and *Pseudomonas aeruginosa* are monotrichous polar flagellates that also produce a flagellum-associated TCS. The *V. cholerae* FlrBC and *P. aeruginosa FleSR* TCSs, like the *C. jejuni* FlgSR TCS, are directly required with σ^{54} for flagellar rod and hook gene expression [34, 182–185]. As with *C. jejuni* FlgSR, signal transduction through *V. cholerae* and *P. aeruginosa* TCSs results in phosphorylation of the cognate FlrC and FleR response regulators to promote their binding to rod and hook gene promoters and directly assist σ^{54} in activating transcription of rod and hook genes [184–187]. Although transcription of the *V. cholerae* and *P. aeruginosa flrBC* and *fleSR* TCSs occurs simultaneously with fT3SS, MS ring, and C ring genes by a master transcriptional regulator, it is not known whether these TCSs sense cues associated with flagellar assembly or flagellum-independent cellular cues to initiate and coordinate flagellar rod and hook gene expression with a stage of flagellar assembly (Figure 6) [182, 183]. Transcription of rod and hook genes after expression of MS ring, C ring, and fT3SS components appears to comprise a polar flagellar transcriptional program not observed in peritrichous flagellates.

Noticeably, many peritrichous flagellates appear to ignore the formation of the MS ring-rotor-fT3SS complex as a regulatory checkpoint that we discovered in *C. jejuni* and is likely present in *H. pylori*. The model peritrichous bacteria *Escherichia coli* and *Salmonella* lack flagellum-associated TCSs and do not employ σ^{54} for flagellar gene transcription. These bacteria also do not require the MS ring, C ring, and fT3SS proteins for rod and hook gene expression [32, 188, 189]. Instead, these bacteria express fT3SS, MS ring, and C ring proteins simultaneously with flagellar rod and hook genes, which we designate for the purposes of this

report “the peritrichous flagellar transcriptional program”. Hence, a competent fT3SS is not required for expression of rod and hook genes in peritrichous flagellates, in contrast to *C. jejuni* and *H. pylori*. These differences raise intriguing questions for flagellar biogenesis in polar flagellates. (i) How common is it for polar flagellates to possess flagellum-associated TCSs? (ii) Do polar flagellates with flagellum-associated TCSs broadly require MS ring, C ring, and fT3SS proteins for activity to result in rod and hook gene expression? (iii) Do polar flagellates employ the respective sensor kinases to detect a regulatory checkpoint formed by the MS ring, C ring, and/or fT3SS? (iv) Do some TCSs detect flagellum-independent cues to activate rod and hook gene expression? (v) Is the polar flagellar transcriptional program that separates production of fT3SS, MS ring, and C ring proteins from rod and hook proteins required for or beneficial to a specific process in polar flagellates to build flagella?

We evaluated the conservation of regulatory systems that order gene expression for a polar flagellar transcriptional program in diverse polar flagellates. Our results, combined with previous findings in α-proteobacteria, indicate a broad, common theme in polar flagellates, whereby different mechanisms are employed to coordinate rod and hook protein production with a stage of flagellar assembly involving the formation of a competent fT3SS. We found that a large subset of polar flagellates with FlhF/FlhG flagellar biogenesis regulatory systems encode orthologous flagellum-associated TCSs. Additional evidence combined with our previous findings suggests that these TCSs have a conserved function in detecting a similar regulatory checkpoint centered around MS ring-rotor-fT3SS complex formation. Our findings suggest that the polar flagellar transcriptional program, rather than a peritrichous one, allows polar flagellates to sustain flagellation and motility if FlhF and/or FlhG activity is altered and provide speculation into the evolution of polar flagellates. Our work provides insight into connections between flagellar transcriptional and biogenesis regulatory systems involved in polar flagellation.

3.2 *Many polar flagellates possess flagellar TCSs*

We followed a bioinformatic strategy to evaluate the prevalence of flagellum-associated TCS in a broad range of Gram-negative polar flagellates. We limited our analysis to Gram-negative bacteria to enable comparisons with previous studies in bacteria that employ flagellum-associated TCSs for direct activation of σ^{54}-dependent flagellar rod and hook gene expression, such as *C. jejuni*, *H. pylori*, *V. cholerae*, and *P. aeruginosa*. For this approach, we performed reciprocal best hit sequence alignments with a set of 117 reference bacterial genomes available at NCBI Assembly to identify Gram-negative flagellates. Genomes included in this reference set were curated by NCBI to represent high-quality, community-standard bacterial genomes or genomes of medically relevant bacteria. Although not every sequenced bacterial genome is included in this reference set, it is sufficient to survey and acquire information regarding features present among phylogenetically diverse bacteria.

We first used *E. coli* FlgH as a marker for Gram-negative bacteria producing flagella (Figure 7A). FlgH forms the L-ring required for external flagella to penetrate the outer membrane barrier in Gram-negative flagellates [190–192]. Since FlhF is involved in polar flagellation, we then used *V. cholerae* FlhF as a marker to predict polar flagellates within the Gram-negative flagellates (Figure 7A) [6, 151, 163]. Our results initially identified 23 of 47 putative Gram-negative flagellates within the reference set as polarly flagellated species (Figure 7B), with two clear false positives: *Bordetella bronchiseptica*, which encodes a predicted FlhF (BN112-0372) but is a known peritrichous organism, and *Burkholderia mallei*, which harbors mutations in multiple flagellar genes and is likely undergoing reductive genome evolution [193, 194]. Twenty of 21 predicted Gram-negative polar flagellates encoded a predicted FlhG ortholog immediately downstream of *flhF*, indicating that an FlhF/FlhG flagellar biogenesis regulatory system was intact; only *Rhodospirillum rubrum* lacked an *flhG* ortholog organized with *flhF*. Of the 24 predicted Gram-negative flagellates lacking FlhF, 18 are known peritrichous organisms. The remaining six are actually polar flagellates with five species from α-proteobacteria, best represented by *Caulobacter crescentus*, which employs factors other

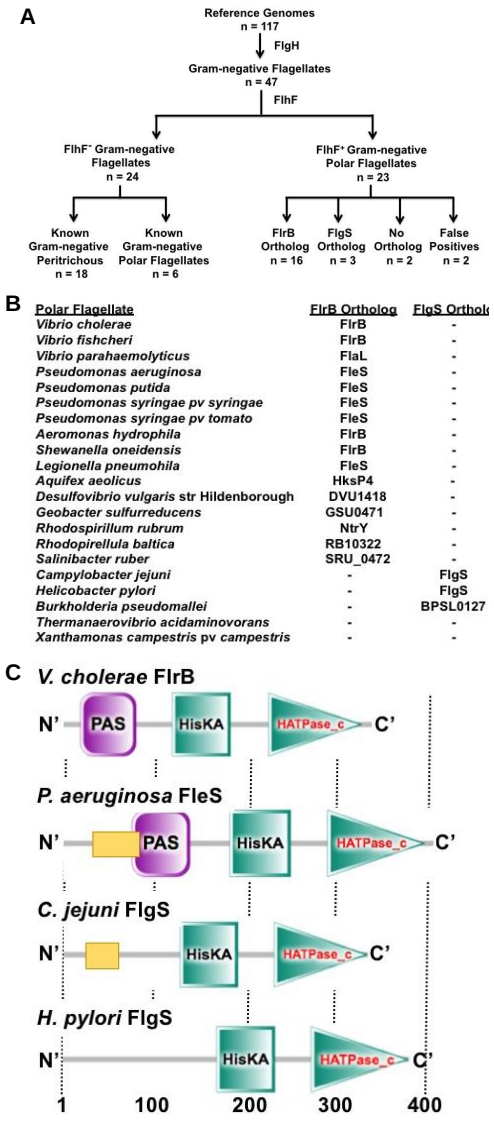

Figure 7: **Bioinformatic analysis of predicted Gram-negative polar flagellates and flagellum-associated TCSs.** (A) Flowchart of the strategy and outcomes of tBLASTn reciprocal best hit sequence alignments of predicted Gram-negative polar flagellates from a reference set of bacterial genomes and those with *V. cholerae* FlrB or *C. jejuni* FlgS orthologs. (B) Sensor kinases of putative flagellum-associated TCS of predicted Gram-negative polar flagellates. For each polar flagellate, the annotated or predicted kinase is indicated as an FlrB or FlgS ortholog depending on the resultant score. (C) Domain analysis of *V. cholerae* FlrB, *P. aeruginosa* FleS, *C. jejuni* FlgS, and *H. pylori* FlgS as predicted by SMART. The conserved HisKA and HATPase-C domains of bacterial sensor kinases for histidine autophosphorylation, dimerization, and ATPase activity are shown in turquoise squares and triangles, respectively. Putative predicted PAS domains are shown as purple squares, and predicted CC domains are shown as gold rectangles. Numbers below indicate approximate positions of amino acids.

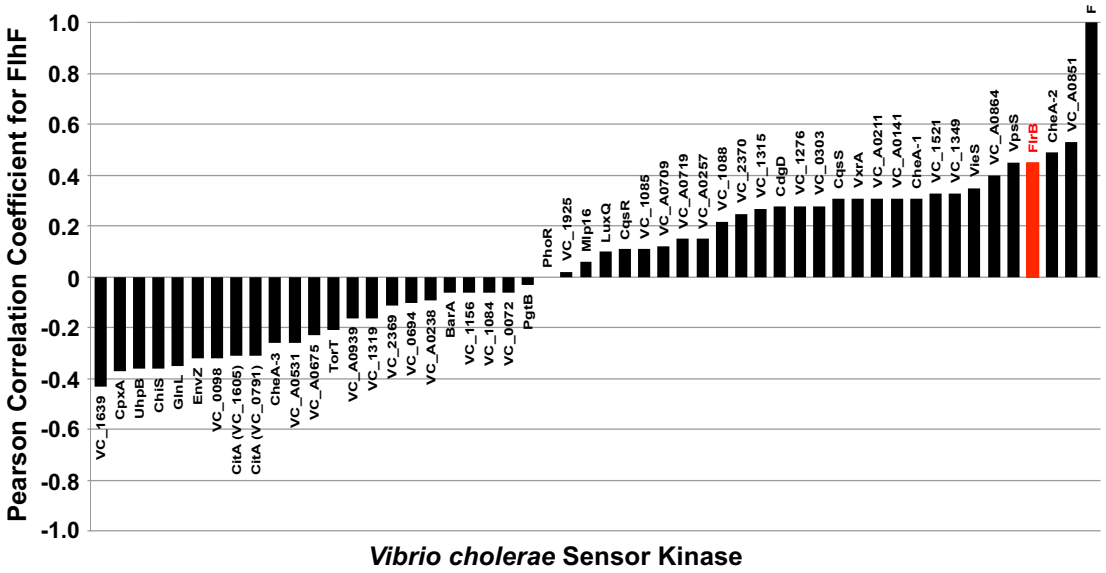

Figure 8: **Correlation of *V. cholerae* sensor kinase orthologs in predicted polar flagellates to *V. cholerae* FlhF orthologs.** The relative correlation of each set of kinase orthologs for the 51 *V. cholerae* sensor kinases taken from each of the reference genomes to FlhF is shown as a Pearson correlation coefficient. Values closer to 1 indicate that the sensor kinase orthologs are more positively correlated with FlhF orthologs. Values greater than 0.4 are considered significant positive correlations. The score for FlrB orthologs is indicated in red.

than FlhF for polar flagellation. Thus, FlgH and FlhF were relatively robust predictors of Gram-negative polar flagellates within the reference set except for α-proteobacterial species.

From the 21 remaining predicted Gram-negative polar flagellates with FlhF orthologs, we performed reciprocal best hit sequence alignments using the *V. cholerae* FlrB sensor kinase of the flagellum-associated FlrBC TCS to identify 16 species with flagellum-associated TCSs (Figure 7A and Figure 7B). Although this approach identified putative flagellum-associated TCS sensor kinases in many polar flagellates (including FleS of the FleSR TCS of different *Pseudomonas* species), *C. jejuni* FlgS was not identified. We repeated our reciprocal best hit sequence alignment analysis using *C. jejuni* FlgS. *C. jejuni* FlgS did not identify FlrB or FleS as orthologs but did identify flagellum-associated sensor kinases in two of the four predicted polar flagellates without FlrB orthologs, *H. pylori* FlgS and *Burkholderia pseudomallei* BPSL0127 (Figure 7A and Figure 7B). These observations suggest that sensor kinases of flagellum-associated TCSs in polar flagellates are divided into distinct FlrB-like or FlgS-like groups. Overall, our bioinformatic analysis indicated that 19 of 21 predicted Gram-negative

polar flagellates from the reference set encode both an FlhF/FlhG regulatory system and a putative flagellum-associated TCS. Thus, our results suggest a high degree of cooccurrence between the two regulatory systems exist in bacterial species that produce polar flagella. The only two Gram-negative FlhF/FlhG-positive species in the reference set that did not encode a flagellum-associated TCS were the known polar flagellate *Xanthamonas campestris pv. campestris*, previously noted to lack a respective TCS, and *Thermanaerovibrio acidaminovorans*, whose prediction as a polar flagellate may be dubious, as it produces lateral flagella on its concave surface (Figure 7B) [195, 196].

To ensure that our FlrB homologs are flagellum-associated sensor kinases rather than conserved unrelated sensor kinases involved in other processes, we examined the correlation of *V. cholerae* sensor kinases with the presence of FlhF across genomes in the reference set (Figure 8). If non-flagellum-associated sensor kinases are within our predicted FlrB orthologs, we would expect a weak to negative correlation of these kinases with FlhF that is indistinguishable from the correlation of a random *V. cholerae* sensor kinase to FlhF. In contrast, if FlrB orthologs are flagellum-associated TCS kinases, we would expect *V. cholerae* FlrB to be one of the kinases most highly correlated with FlhF. We found that predicted FlrB orthologs had a stronger positive correlation to *V. cholerae* FlhF than all but two of 51 *V. cholerae* sensor kinases (Figure 8). One of these kinases is *V. cholerae* CheA, which is the major sensor kinase in the chemotaxis system that influences flagellar rotation and motility in many bacterial species [197, 198]. The other *V. cholerae* kinase is VCA0851, an uncharacterized kinase. These results support our flagellum-associated TCS predictions and further emphasize the correlation between the FlhF/FlhG flagellar biogenesis system and the flagellum-associated TCSs.

Our bioinformatic analysis suggested that the sensor kinases in flagellum-associated TCS systems of Gram-negative polar flagellates belong to two or more unrelated groups and possess different features, since *C. jejuni* FlgS and *V. cholerae* FlrB did not identify each other as orthologs. Many of these sensor kinases are predicted to be cytoplasmic kinases lacking transmembrane domains. Representative kinases such as *C. jejuni* FlgS, *H. pylori* FlgS, *V. cholerae*

FlrB, and *P. aeruginosa* FleS contain similar HisKA and HATPase-c domains that function in histidine autophosphorylation, dimerization, and ATPase activity but have divergent N-terminal sensor domains (Figure 7C) [199, 200]. Both *V. cholerae* FlrB and *P. aeruginosa* FleS contain a predicted PAS domain that is a common sensing domain in sensor kinases; FleS also has a predicted CC domain for potential protein interactions (Figure 7C) [200]. However, the only predicted domain within the *C. jejuni* FlgS sensor region is a CC domain, and no predicted structural domain was identified in *H. pylori* FlgS (Figure 7C). These observations suggest that flagellum-associated sensor kinases within FlhF/FlhG-containing polar flagellates detect different cellular signals or detect similar signals by different mechanisms to initiate signal transduction for flagellar gene expression and polar flagellar biogenesis.

3.3 *The V. cholerae FlrBC TCS requires the fT3SS, MS ring, and rotor for activity.*

As described above, we previously discovered that *C. jejuni* FlgS detects MS ring-rotor-fT3SS assembly by a direct interaction as a regulatory checkpoint to initiate signal transduction for FlgSR- and σ^{54}-dependent transcription of rod and hook genes for the polar flagellar transcriptional program (Figure 6) [44]. The *V. cholerae* and *P. aeruginosa* flagellum-associated FlrBC/FleSR TCSs are expressed simultaneously with the fT3SS, MS ring, and C ring genes. However, investigations to identify what signals are detected by these TCSs to initiate signal transduction for TCS- and σ^{54}-dependent flagellar rod and hook gene expression that occurs in the subsequent tier of the polar flagellar transcriptional program are lacking (Figure 6) [37, 114]. Considering that the sensor domains of *V. cholerae* FlrB and *P. aeruginosa* FleS differ from that of *C. jejuni* FlgS, we hypothesized that the FlrB and FleS kinases detect a similar regulatory checkpoint formed by fT3SS, MS ring, and/or C ring components, but by different means, or they detect a signal independent of flagellar biogenesis (Figure 6). Thus, we first investigated whether disruption of the fT3SS, MS ring, and/or C ring impacted the activity of the *V. cholerae* FlrBC TCS for σ^{54}-dependent rod and hook gene expression. Thus, we first investigated whether disruption of the fT3SS, MS ring, and/or C ring impacted the activity of the

V. cholerae FlrBC TCS for σ^{54}-dependent rod and hook gene expression.

Transcriptional fusions of the promoters of four *V. cholerae* FlrBC- and σ^{54}-dependent operons, *flgBCDE*, *flgFGHIJ*, *flgKL*, and *flaA*, to a promoterless *lacZ* gene were created in WT *V. cholerae* and isogenic flagellar mutants. The former three operons encode rod and hook genes, whereas *flaA* encodes the major flagellin. *V. cholerae* is different from many other flagellates in that the major flagellin is not expressed from a σ^{28}-dependent promoter; instead, minor flagellins are expressed from a σ^{28}-dependent promoter in this bacterium [201]. Of note, the *flgFGHIJ* rod operon was previously proposed to be cotranscribed with the *flgBCDE* rod and hook operon from the *flgB* promoter [114]. However, we identified a putative σ^{54} binding site between *flgE* and *flgF* and constructed an *flgFp-lacZ* transcriptional fusion for analysis. A *cheVp-lacZ* transcriptional fusion served as a control, as *cheV* expression is independent of *FlrBC* TCS and σ^{54} [114].

We verified previous findings that *V. cholerae* Δ*flrA*, Δσ^{54}, Δ*flrB*, and Δ*flrC* (but not Δσ^{28}) mutants were defective for *flaAp-*, *flgBp-*, and *flgKp-lacZ* expression (Figure 9A); expression of these reporters was reduced 6 to 50-fold in these mutants relative to that of WT *V. cholerae*. The defect in Δ*flrA* is due to FlrA functioning as a master transcriptional regulator required for *flrBC* expression (Figure 6) [113, 114, 117]. We also confirmed that an FlrBC- and Δσ^{54}-dependent promoter upstream of *flgF* drives expression of *flgFGHIJ* independently of *flgBp*; *flgFp-lacZ* expression was decreased 50- to 100-fold in FlrBC TCS or Δσ^{54} mutants (Figure 9A).

We next discovered that *V. cholerae* mutants lacking fT3SS core proteins (including FlhA, FlhB, FliP, FliQ, and FliR) and the FliF MS ring protein were defective for FlrBC activity and rod and hook transcription, as expression of the respective transcriptional reporters was reduced 3- to 20-fold (Figure 9A). The C ring FliG rotor protein was also required for FlrBC- and σ^{54}-dependent flagellar gene expression, but the C ring FliM and FliN switch proteins were not to 20-fold (Figure 9A). These results are similar to our findings for requirements

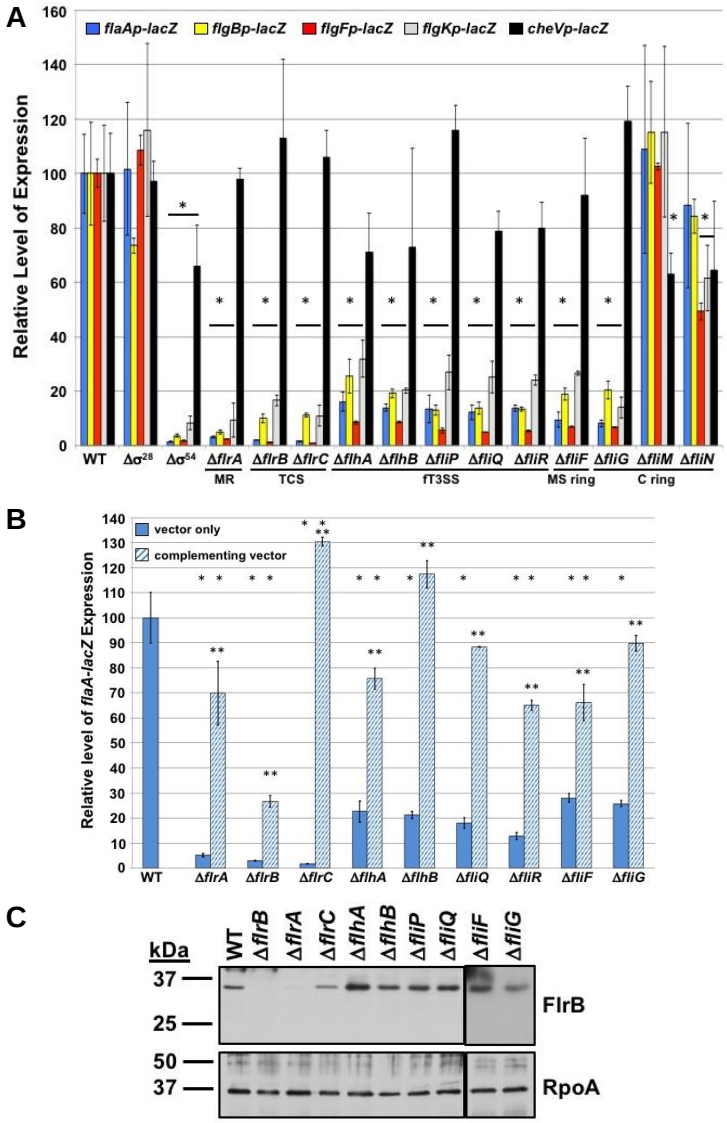

Figure 9: **Analysis of TCS and σ^{54}-dependent flagellar gene expression in flagellar mutants of *V. cholerae*.**(A) Expression of flagellar rod and hook operons in WT *V. cholerae* C6706 and isogenic flagellar mutants. *flgBp*, *flgFp*, *flgKp*, *flaAp*, and *cheVp-lacZ* transcriptional reporters were maintained on plasmids in *V. cholerae* strains. The level of expression of each transcriptional reporter in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. MR, master regulator, TCS, two-component signal transduction system. (B) Expression of *flaAp-lacZ* transcriptional fusion in WT *V. cholerae* and an isogenic mutant containing vector alone (solid blue bars) or vectors to express genes from a constitutive promoter for complementation (hatched blue bars). The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *V. cholerae* with vector alone, which was set to 100 U. For panels A and B, results are from a representative assay with each sample analyzed in triplicate. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates significant difference in expression from the WT (A) or WT containing vector alone (B) ($p<0.05$). Two asterisks indicate significant increase in expression from the respective mutant containing vector only ($p<0.05$). (C) Immunoblot analysis of the FlrB sensor kinase levels in whole-cell lysates of WT *V. cholerae* and isogenic mutants. Specific antiserum to FlrB was used to detect the protein. Detection of RpoA served as a control to ensure equal loading of proteins across strains.

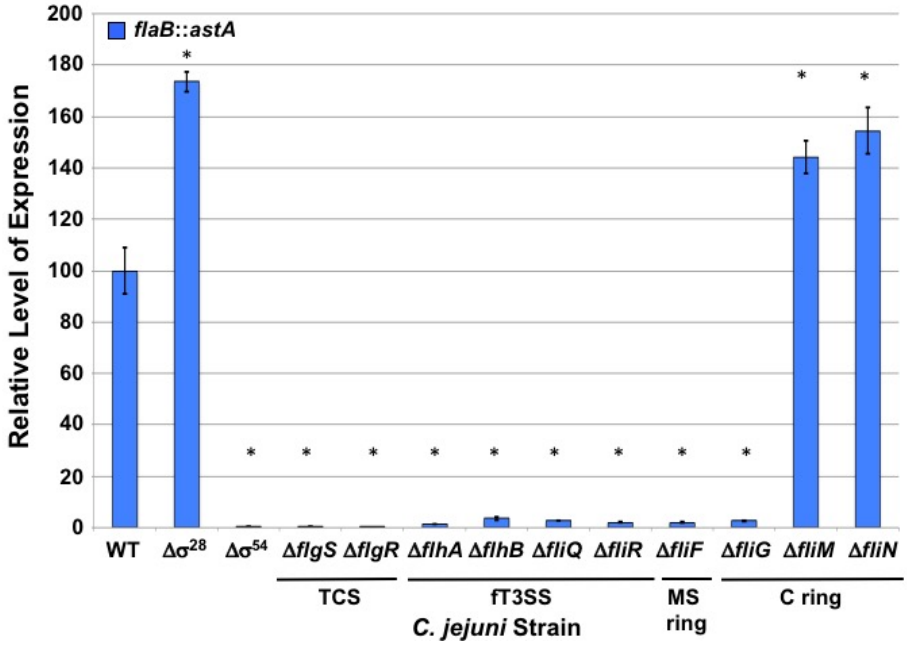

Figure 10: ***C. jejuni* FlgSR TCS activity for the σ^{54}-dependent flagellar gene expression in WT and flagellar mutants.** Expression of the Δ*flaB::astA* transcriptional reporter in the WT *C. jejuni* strain 81-176 and isogenic flagellar mutants is shown and comparable to our previously published analysis [44]. The level of *flaB::astA* expression in each mutant is relative to the expression of WT *C. jejuni*, which was set to 100 U. Results from a representative assay with each sample analyzed in triplicate are shown. Error bars indicate standard deviations of the average levels of expression from three samples. An asterisk indicates the mutant had significantly increased or decreased reporter expression relative to that of the WT strain ($p<0.05$)

for *C. jejuni* FlgSR TCS directly activating σ^{54}-dependent flagellar rod and hook gene expression (Figure 10) [38, 44, 46]. Either no reductions or only modest reductions in *cheVp-lacZ* expression were observed in these flagellar mutants. *In trans* complementation of the *V. cholerae fT3SS*, *fliF*, and *fliG* mutants with the respective genes restored expression of *flaAp-lacZ* to a significantly higher level than that of each mutant with vector alone (and to at least 60% of the level observed in the WT (Figure 9B). We were unable to construct a complementing vector for the *fliP* mutant due to toxicity during attempted construction. *flaAp-lacZ* expression was partially or fully restored in *flrA*, *flrB*, and *flrC* mutants with complementation relative to the mutants with vector alone. Additionally, we verified that FlrB production was unaffected in mutants lacking the fT3SS, FliF, or FliG, which eliminated the possibility that the FlrBC TCS was unstable or not expressed in these mutants as an explanation of their reduction in rod and hook gene expression a 20-fold (Figure 9C). The finding that a lack of individual MS ring, rotor, and fT3SS proteins abolishes FlrBC-dependent gene expression is consistent with our hypothesis that *V. cholerae* flagellar components form a regulatory checkpoint, possibly involving functional fT3SS assembly, required for FlrBC to activate rod and hook gene expression, as we previously demonstrated in *C. jejuni* [38, 44, 46].

3.4 *The P. aeruginosa FleSR TCS requires the fT3SS, MS ring, and rotor for activity.*

We next investigated whether *P. aeruginosa* FleSR-dependent expression of flagellar rod and hook genes was influenced by fT3SS, MS ring, and C ring protein production and possibly assembly into an MS ring-rotor-fT3SS complex. We generated complete or partial in-frame deletion mutants of flagellar genes in *P. aeruginosa* PA14 and then integrated transcriptional fusions of the promoters of the *flgB* rod and hook operon and *fliA* (encoding σ^{28}) to *lacZ* in the *att* site on the chromosome. We verified that transcription of the *P. aeruginosa flgB* rod and hook operon is dependent on σ^{54} and the FleSR TCS, in addition to the FleQ master transcriptional regulator that is required for *fleSR* transcription (Figure 6 and Figure 11) [37, 148]. The *fliAp-lacZ* reporter served as a control, as *fliA* expression is independent of σ^{54} or the FleSR TCS [37]. Deletion of σ^{28} did not affect expression of either reporter. We discovered

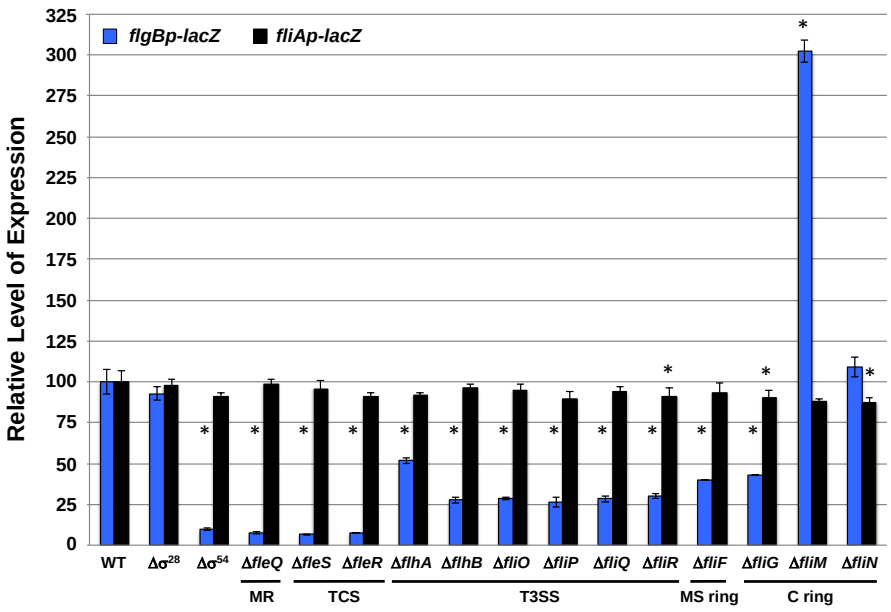

Figure 11: **Analysis of TCS and σ^{54}-dependent flagellar gene expression in flagellar mutants of *P. aeruginosa*.** Expression of the flagellar rod and hook operon in WT *P. aeruginosa* PA14 and isogenic flagellar mutants is shown. *flgBp-lacZ* and *fliAp-lacZ* transcriptional reporters were integrated at the *att* site on the chromosome of *P. aeruginosa* strains. The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *P. aeruginosa*, which was set to 100 U. Results are from a representative assay, with each sample analyzed in triplicate. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates the mutant had a significantly increased or decreased reporter expression relative to that of the WT strain ($p<0.05$). MR, master regulator, TCS, two-component signal transduction system.

that FleSR- and σ^{54}-dependent expression of *flgBp-lacZ* in *P. aeruginosa* required the fT3SS, FliF MS ring, and the FliG C ring proteins, but not FliM or FliN, for full expression, similar to our analysis of the *V. cholerae* FlrBC and *C. jejuni* FlgSR TCSs (Figure 9A, Figure 11, and Figure 10) [38, 44, 46]. Expression of *fliAp-lacZ* was unaffected in these mutants. Thus, our data continue to support that many polar flagellates with the FlhF/FlhG flagellar biogenesis regulatory system contain flagellum-associated TCSs that require flagellar-dependent cues to stimulate expression of σ^{54}-dependent flagellar rod and hook genes as a discrete step for the polar flagellar transcriptional program.

3.5 *Requirements of FliF and FliG for flagellum-associated TCS activity.*

Having established that deletion of individual MS ring, rotor, and fT3SS proteins impeded FlrBC/FleSR TCS activity, we assessed whether these proteins alone or as a part of the MS ring-rotor-fT3SS complex were required for the activity of *V. cholerae* and *P. aeruginosa* TCSs to stimulate flagellar rod and hook gene transcription. Because FlrB and FleS are predicted to be cytoplasmic kinases, we hypothesized that they detect signals within the cytoplasm, similar to *C. jejuni* FlgS sensing formation of the MS ring and rotor by FliF and FliG via a direct interaction with the cytoplasmic domains of these structures [44]. FliF is predicted to contain two transmembrane domains with a large central periplasmic domain and smaller N- and C-terminal cytoplasmic domains. A conserved periplasmic ASVXV motif in FliF is required for flagellation in *Salmonella* [13]. This motif has been hypothesized to promote recruitment of FliF to the fT3SS core via interactions with FlhA and/or FliF multimerization into the MS ring around the fT3SS core. Alteration of the *C. jejuni* FliF ASVXV motif eliminated FlgS interactions with FliF and FliG and abolished FlgSR TCS signal transduction for rod and hook gene expression, supporting the hypothesis that FliF multimerization into the MS ring (and simultaneous rotor formation by FliG) around the complete fT3SS core is required to form a signal directly detected by FlgS [44].

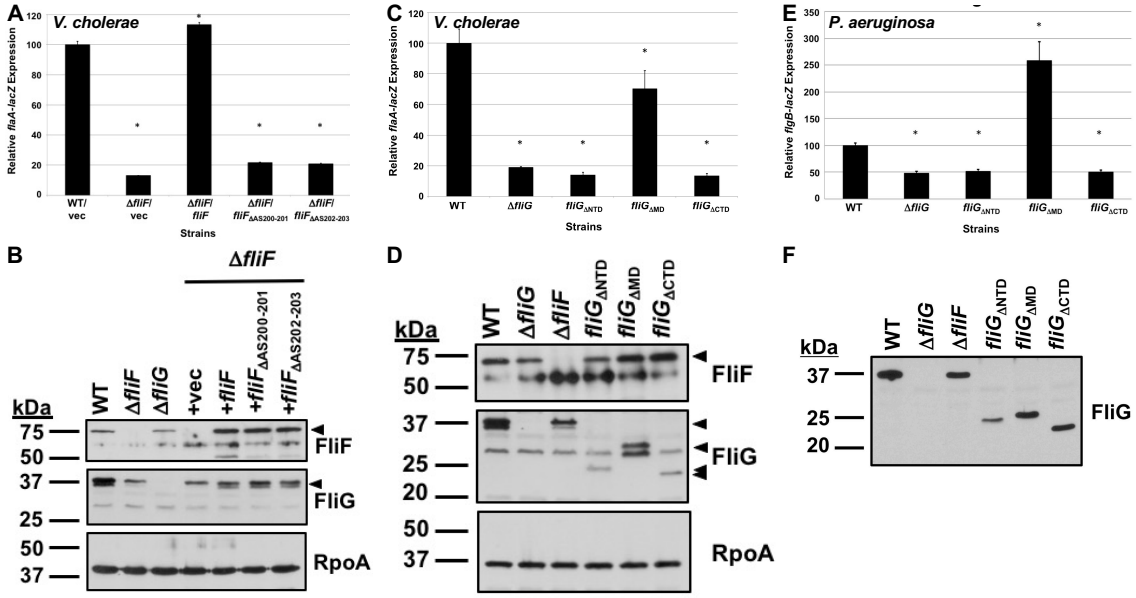

Figure 12: **Requirements of *V. cholerae* and *P. aeruginosa* for TCS activation and flagellar gene expression.** (A) Expression of *flaAp-lacZ* transcriptional fusion in WT *V. cholerae* and isogenic Δ*fliF* mutant. Both the WT and *fliF* mutant strain contained vector alone (VEC). The Δ*fliF* mutant also contained vectors to express WT *fliF* (WT) or *fliF* containing in-frame deletions within the ASASVXL motif (depicted as deletion of the AS residues from positions 200 and 201 or positions 202 and 203). (B) Immunoblot analysis of FliF and FliG in whole-cell lysates of WT *V. cholerae* and isogenic mutants. Specific antiserum to FliF or FliG was used to detect each protein. Detection of RpoA served as a control to ensure equal loading of proteins across strains. (C) Expression of *flaAp-lacZ* transcriptional fusion in WT *V. cholerae* and isogenic fliG mutants lacking the N-terminal domain (NTD), middle domain (MD), or C-terminal domain (CTD). (D) Immunoblot analysis of FliF and FliG in whole-cell lysates of WT *V. cholerae* and isogenic mutants. E) Expression of *flgBp-lacZ* transcriptional fusion in WT *P. aeruginosa* and isogenic fliG mutants lacking the N-terminal domain (NTD), middle domain (MD), or C-terminal domain (CTD). (F) Immunoblot analysis of FliG in whole-cell lysates of WT *P. aeruginosa* and isogenic mutants. For panels A, C, and E, the level of expression of the transcriptional reporter in each strain is relative to the level of expression in WT *V. cholerae* or *P. aeruginosa*, which was set to 100 U. Results from a representative assay, with each sample analyzed in triplicate, are shown. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates increased or decreased reporter activity of a mutant relative to that of the WT strain ($p<0.05$).

The *V. cholerae* FliF MS ring protein contains a motif in its periplasmic region (ASASVXL from residues 200 to 206) similar to that of *Salmonella* and *C. jejuni* FliF. We expressed WT FliF, FliF$\Delta_{AS200-201}$, and FliF$\Delta_{AS202-203}$ from plasmids in *V. cholerae* Δ*fliF* and monitored expression of *flaAp-lacZ* to assess FlrBC TCS activity. WT FliF restored *flaAp-lacZ* expression to the Δ*fliF* mutant, but FliF$\Delta_{AS200-201}$ and FliF$\Delta_{AS202-203}$ did not (Figure 12A). Consistent with these observations, WT FliF restored flagellation and motility, but the mutant FliF proteins did not (data not shown). Immunoblot analysis verified that the WT FliF and FliF mutant proteins were produced at similar levels, as were the FliG rotor proteins in these strains (Figure 12B). Of note, we did observe that FliG is dependent upon FliF for stability, as FliG levels were reduced in the *V. cholerae* Δ*fliF* mutant with or without empty vector (Figure 12B). If the ASVXV motif in *V. cholerae* FliF is required for recruitment to the fT3SS core and/or FliF multimerization into the MS ring around the fT3SS core, as in other flagellates, these data, along with our analysis of mutants lacking FliF, FliG, and fT3SS core proteins, support a model that the formation of the *V. cholerae* MS ring-rotor-fT3SS complex, rather than the production of unassembled fT3SS, MS ring, and rotor proteins, is a cue and regulatory checkpoint influencing FlrBC activity. We were unable to perform a similar analysis in *P. aeruginosa*, as we could not construct mutations in the FliF ASVXV motif.

We next explored whether differences in the requirements of FliG rotor domains for activity of the *V. cholerae*, *P. aeruginosa*, and *C. jejuni* flagellum-associated TCSs existed. Typical FliG proteins possess three major domains: an amino-terminal domain (NTD) that interacts with the cytoplasmic caboxy-terminal domain (CTD) of FliF and is required for multimerization of the MS ring and rotor; a middle domain (MD) to interact with FliM for assembly of the switch complex and the lower portion of the C ring; and a CTD to interact with stator proteins that generate torque for flagellar rotation [18, 202, 203]. In *C. jejuni*, only the FliG NTD was required with FliF to form a signal detected by the FlgSR TCS for flagellar rod and hook gene expression [44]. We generated *V. cholerae* and *P. aeruginosa fliG* mutants with deletions of the NTD, MD, or CTD and then assessed TCS- and σ^{54}-dependent flagellar gene expression.

FliG mutants lacking the NTD or CTD were unable to restore FlrBC- and σ^{54}- dependent flagellar gene expression in *V. cholerae* (Figure 12C). However, these proteins were produced at reduced levels compared to those of WT FliG (Figure 12D), tempering interpretations that these domains are essential for FlrBC TCS activity. In contrast, the expression of FliGΔ_{MD} restored gene expression to 70% of the WT level (Figure 12C), suggesting that at least the middle domain of FliG is not required for FlrBC TCS-dependent flagellar gene expression.

For analysis of *P. aeruginosa* FliG, WT *fliG* was replaced on the chromosome with *fliG* mutants encoding in-frame deletions of the NTD, MD, or CTD. FliG levels were reduced only modestly with removal of the NTD, but levels of FliGΔ_{MD} and FliGΔ_{CTD} were comparable to those of WT FliG (Figure 12F). Expression of the FleSR- and σ^{54}-dependent *flgB-lacZ* reporter was reduced to the same level in the *fliG*Δ_{NTD} and *fliG*Δ_{CTD} mutants as in the Δ*fliG* mutant (Figure 12E). However, a 2.5-fold increase in *P. aeruginosa* FleSR activity and flagellar gene expression was observed with *fliG*Δ_{MD}. These observations suggest that at least the *P. aeruginosa* FliGΔ_{CTD} is required for FleSR TCS activity, whereas the requirement for the NTD is less clear. Considering that only the *C. jejuni* FliG$_{NTD}$ was required to activate flagellar gene expression [44], our findings indicated different FliG domains, along with FliF, are required in *P. aeruginosa* (and possibly *V. cholerae*) for flagellum-associated TCS activity. While we did not analyze MS ring-rotor-fT3SS complex formation directly in *V. cholerae* and *P. aeruginosa*, these differences in FliF and FliG domains required for activity of the flagellar TCSs suggest requirements for the assembly of functional fT3SSs vary in these organisms. Additionally, the different sensor domains within FleS and FlrB relative to *C. jejuni* FlgS may be needed to detect the distinctive signal composed by the different FliF and FliG domains from the respective bacteria.

3.6 *The polar flagellar transcriptional program and the FlhF/FlhG system are linked.*

Our analysis presented above suggested a connection with many polar flagellates possessing (i) a FlhF/FlhG regulatory system, for spatial and numerical control of polar flagellar

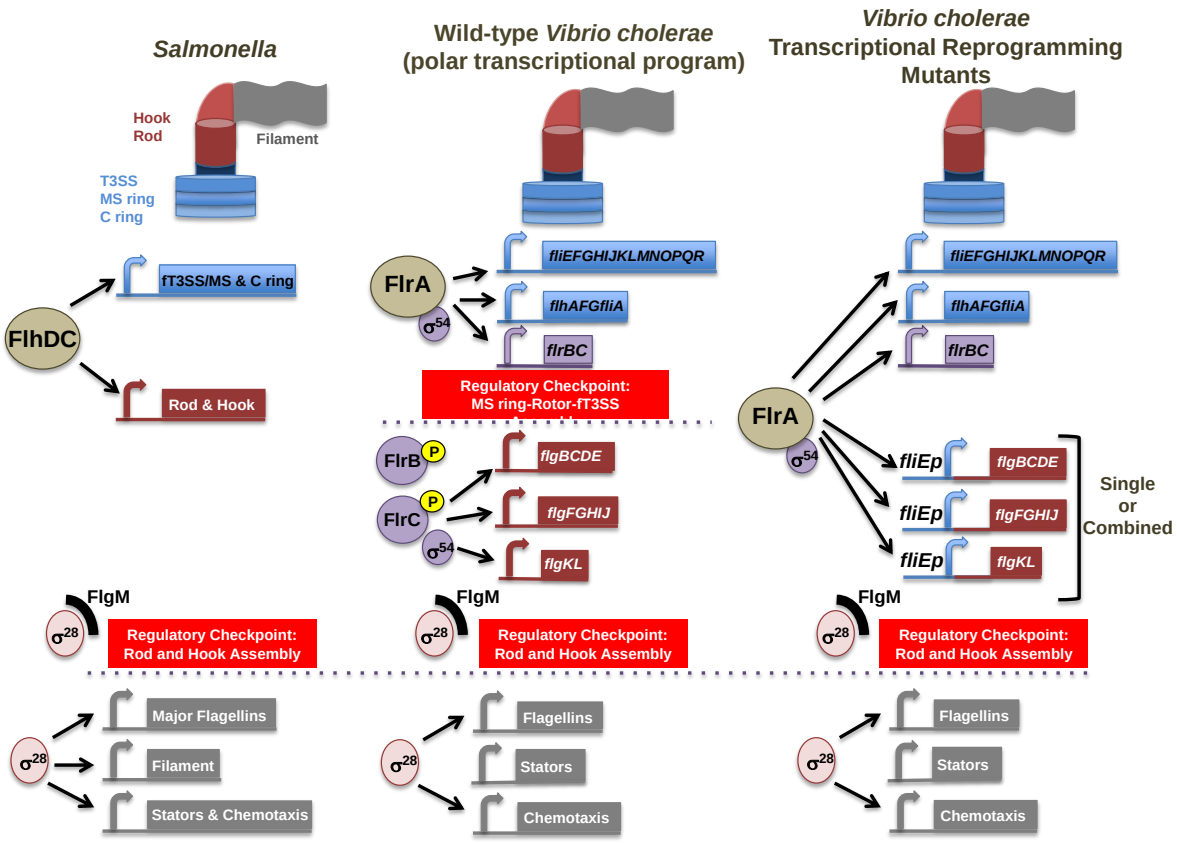

Figure 13: **Construction of *V. cholerae* mutants for altering a flagellar transcriptional program.** The normal peritrichous flagellar transcriptional program for *Salmonella* (left) and the normal polar flagellar transcriptional program for WT *V. cholerae* (middle) are shown. Note the WT *V. cholerae* polar flagellar transcriptional program (middle) includes both FlrA-dependent transcription of initial flagellar genes, the regulatory checkpoint associated with MS ring, rotor, and fT3SS core proteins discovered in this work, and FlrBC- and σ^{54}-dependent transcription of flagellar rod and hook genes. Full *V. cholerae* operons for FlrA- or FlrBC-/σ^{54}-dependent operons are shown. For creation of transcriptional reprogramming mutants (right), the native σ^{54}- and FlrBC TCS-dependent promoters for the *flgB*, *flgF*, and *flgK* operons encoding flagellar rod and hook genes were replaced with the FlrA-dependent *fliE* promoter (*fliEp*). Promoter mutations were made individually or in different combinations to create a full array of *V. cholerae* mutants with up to all three flagellar rod and hook operons dependent on FlrA for transcription (right), rather than on FlrBC TCS and σ^{54} (middle), to resemble a peritrichous flagellar transcriptional program (left).

Strain	% Of population[a]		% Hyperflagellated	Strain	% Of population[a]		% Hyperflagellated[b]
	Flagellated	Aflagellated			Flagellated	Aflagellated	
WT	54.2 ± 2.5	45.9 ± 2.5	1.4 ± 2.5	Δ*flhG*	67.5 ± 11.1	32.5 ± 11.1	61.4 ± 11.6
fliEp-flgB operon	56.6 ± 4.3	43.4 ± 4.3	0 ± 0	Δ*flhG fliEp-flgB* operon	8.3 ± 4.1[c]	91.7 ± 4.1[c]	0 ± 0[c]
fliEp-flgF operon	48.2 ± 4.0	51.8 ± 4.0	0.8 ± 1.3	Δ*flhG fliEp-flgF* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]
fliEp-flgK operon	53.4 ± 5.6	46.6 ± 5.6	0.9 ± 0.9	Δ*flhG fliEp-flgK* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]
fliEp-flgB operon *fliEp-flgF* operon	48.1 ± 6.3	51.9 ± 6.3	0.6 ± 0.5	Δ*flhG fliEp-flgB* operon *fliEp-flgF* operon	13.9 ± 2.1[c]	86.1 ± 2.1[c]	9.5 ± 4.8[c]
fliEp-flgB operon *fliEp-flgK* operon	55.6 ± 2.0	44.4 ± 2.0	2.0 ± 0.6	Δ*flhG fliEp-flgB* operon *fliEp-flgK* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]
fliEp-flgF operon *fliEp-flgK* operon	50.9 ± 6.4	49.1 ± 6.4	4.4 ± 6.3	Δ*flhG fliEp-flgF* operon *fliEp-flgK* operon	0.4 ± 0.6[c]	99.6 ± 0.6[c]	0 ± 0[c]
fliEp-flgB operon *fliEp-flgF* operon *fliEp-flgK* operon	53.0 ± 3.5	47.0 ± 3.5	2.1 ± 1.9	Δ*flhG fliEp-flgB* operon *fliEp-flgF* operon *fliEp-flgK* operon	0 ± 0[c]	100 ± 0[c]	0 ± 0[c]

[a]For each strain, three independent samples with at least 100 bacterial cells per sample were analyzed. Values are presented as averages ± standard deviations.
[b]The values for the hyperflagellated population represent the percentages of the flagellated population that had at least two or more flagella at one pole of the cell.
[c]The level of flagellation, aflagellation, or hyperflagellation of individual *V. cholerae* Δ*flhG* transcriptional reprogramming mutants was statistically significantly different from the *V. cholerae* Δ*flhG* mutant with normal flagellar gene transcription ($P < 0.05$). No significant differences were noted among WT *V. cholerae* and the respective transcriptional reprogramming mutants.

Table 1: **Measurement of flagellation in WT *V. cholerae*, *V. cholerae* Δ*flhG*, and transcriptional reprogramming mutants**.

biogenesis; (ii) a flagellum-associated TCS whose activity is dependent on MS ring, rotor, and fT3SS proteins; and (iii) a polar flagellar transcriptional program that requires MS ring-rotor-fT3SS protein production and possibly assembly for subsequent flagellar rod and hook gene expression. The FlhF/FlhG flagellar biogenesis regulatory systems and flagellum-associated TCSs are absent from peritrichous bacteria (with *Bacillus subtilis* as an exception for FlhF and FlhG). In these peritrichous bacteria, a master transcriptional regulator promotes the peritrichous flagellar transcriptional program by expressing MS ring, C ring, rod, and hook genes simultaneously to result in efficient creation of multiple flagella across the surface (Figure 13) [3, 10, 169, 204]. The cooccurrence of the FlhF/FlhG system, flagellum-associated TCSs, and a transcriptional program that separates MS ring-rotor-fT3SS complex gene expression from that of rod and hook genes raises interesting questions. (i) Do polar flagellates require the specific polar flagellar transcriptional program to build flagella in general or to specifically construct polar flagella? (ii) Is ordering rod and hook gene transcription after MS ring-rotor-fT3SS assembly required for an FlhF/FlhG-dependent activity for flagellation? (iii) Can polar flagellates produce flagella (polar or otherwise) if reprogrammed to transcribe flagellar genes similarly to a peritrichous organism in the presence or absence of the FlhF/FlhG flagellar biogenesis regulatory system?

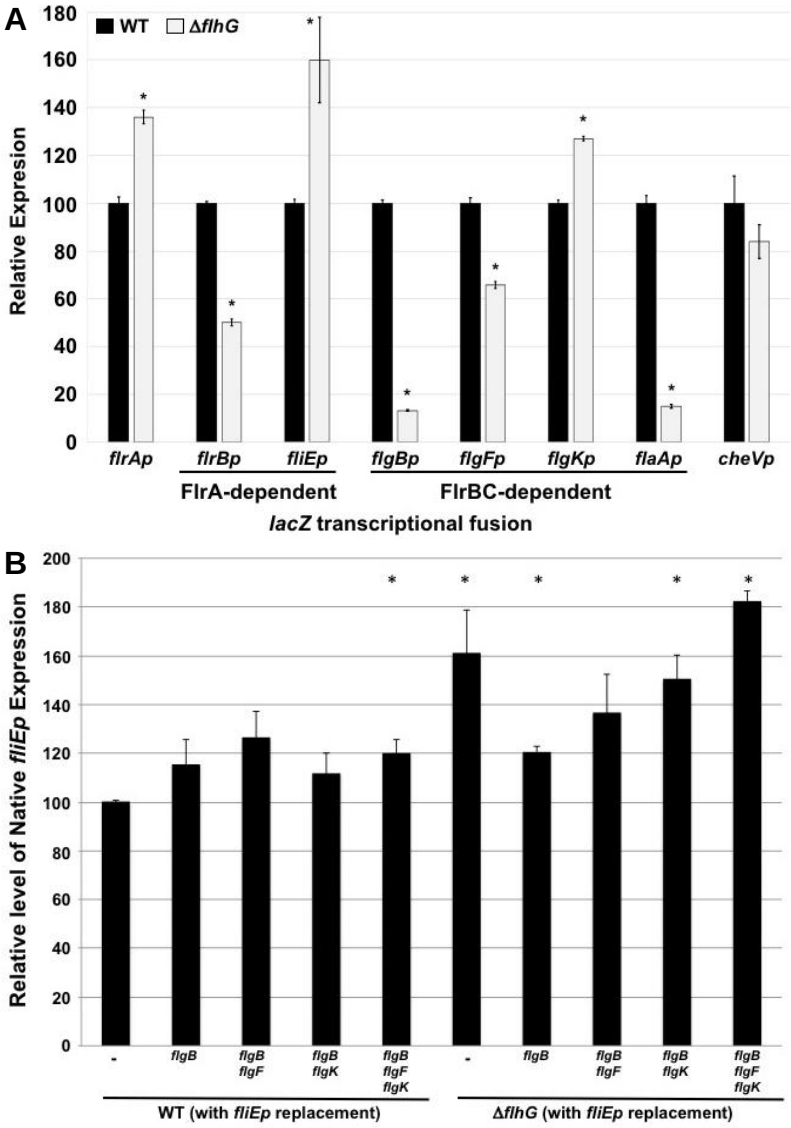

Figure 14: **Activity of flagellar promoters in WT *V. cholerae* and Δ*flhG* mutant strains.** (A) Expression of *lacZ* transcriptional fusions from different flagellar promoters in WT *V. cholerae* C6706 and an isogenic Δ*flhG* mutant. *flrAp-*, *flrBp-*, *fliEp-*, *flgBp-*, *flgF-*, *flgKp-*, *flaAp-*, and *cheVp-lacZ* transcriptional reporters were maintained on plasmids in *V. cholerae* strains. The level of expression of each transcriptional reporter in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Results from a representative assay with each sample analyzed in triplicate are shown. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates significant difference in expression from the WT containing vector alone ($p<0.05$). (B) *fliE* promoter activity in WT *V. cholerae* and the Δ*flhG* mutant with either a normal polar flagellar transcriptional program or transcriptionally reprogrammed toward a peritrichous pattern. An *fliEp-lacZ* transcriptional reporter was introduced on a plasmid in WT *V. cholerae* or the *V. cholerae* Δ*flhG* mutant with a normal polar flagellar transcriptional program (indicated by a dash) and select transcriptional reprogramming mutants. In the transcriptional reprogramming mutants analyzed, the promoter for one or more rod and hook operons that was replaced with the *fliE* promoter is indicated. The level of expression of the *fliEp-lacZ* transcriptional reporter in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Results from a representative assay, with each sample analyzed in triplicate, are shown. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates significant difference in expression relative to that of WT *V. cholerae* ($p<0.05$).

For these analyses, we developed a transcriptional reprogramming strategy in *V. cholerae* so that expression of one, two, or all three FlrBC- and σ^{54}-dependent flagellar rod and hook operons (*flgBCDE*, *flgFGHIJ*, and *flgKL*) were under the control of the FlrA master transcriptional regulator that normally only controls expression of MS ring, C ring, fT3SS, and FlrBC TCS genes (Figure 13). By replacing the FlrBC- and σ^{54}-dependent *flgB*, *flgF*, and *flgK* promoters with the FlrA-dependent *fliE* promoter at the native locations on the *V. cholerae* chromosome (Figure 13), the requirement to detect the regulatory checkpoint centered around MS ring-rotor-fT3SS protein production for rod and hook gene expression would be bypassed [114]. Thus, *V. cholerae* would produce some or all rod and hook proteins earlier than normal and at the same time as the MS ring, C ring, and fT3SS proteins, which shifts the normal *V. cholerae* polar flagellar transcriptional program to one more closely following the peritrichous transcriptional program that normally exists in *E. coli* and *Salmonella* species (Figure 13) [8, 10].

In these experiments, we retained *flaA* expression under the control of its natural FlrBC- and σ^{54}-dependent promoter. By doing so, the FlaA major flagellin in these *V. cholerae* transcriptional reprogramming mutants was produced either simultaneously with some rod and hook proteins as normal (if the mutant contained only one or two promoter alterations) or after all rod and hook proteins (if the mutant contained all three promoter alterations) (Figure 13). Transcription of *flaA* after rod and hook protein production in these *V. cholerae mutants* would be temporally similar to how most polar and peritrichous flagellates naturally express major flagellins from a σ^{28}-dependent promoter after formation of the flagellar rod and hook (Figure 6 and Figure 13). As shown below, maintaining *flaA* expression under its natural FlrBC- and σ^{54}-dependent promoter allowed for sufficient flagellin production for filament assembly and motility in many transcriptionally reprogrammed mutants.

We also deleted *flhF* and *flhG* from these *V. cholerae* transcriptional reprogramming mutants to examine any potential link between the activity of the FlhF/FlhG regulatory system for monotrichous flagellation in *V. cholerae* and alteration in the timing of rod and hook gene transcription relative to MS ring, C ring, and fT3SS expression. As reported previously, FlhF

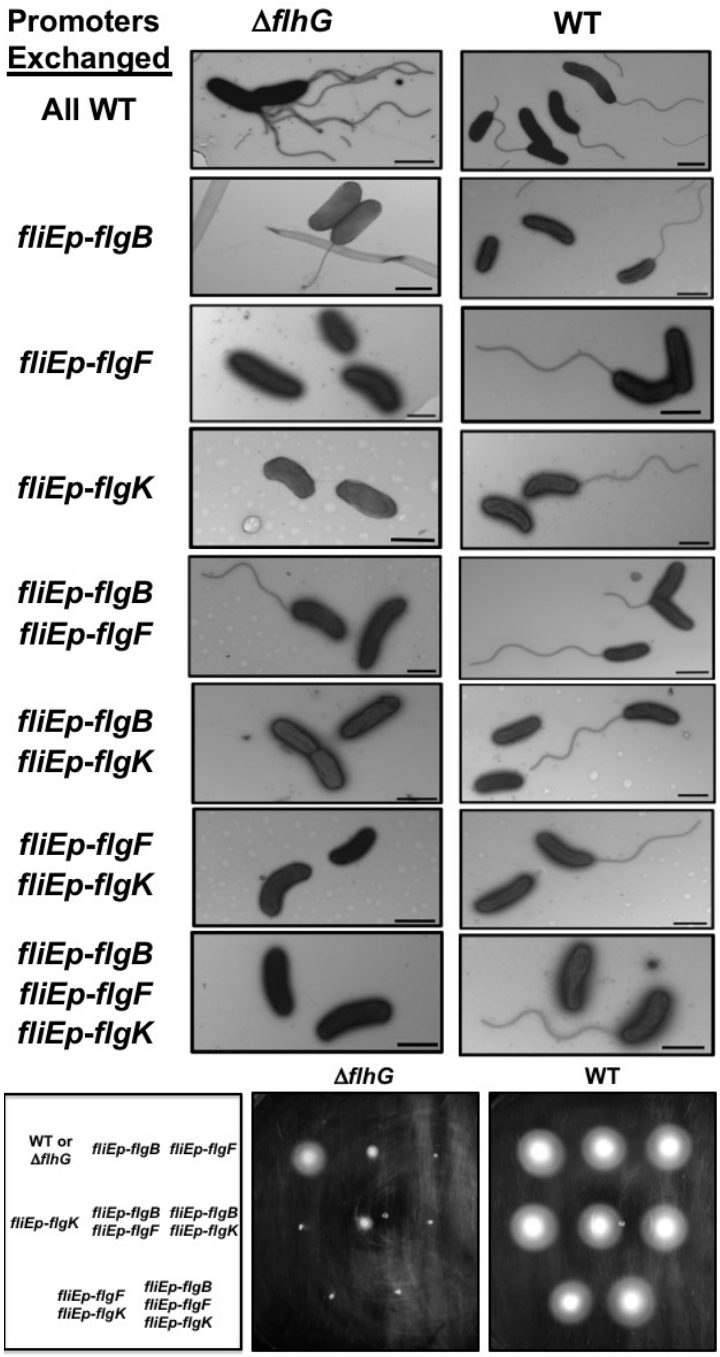

Figure 15: **Effect of transcriptional reprogramming of flagellar genes on *V. cholerae* flagellation.** Shown are electron micrographs (A) and motility phenotypes (B) of WT *V. cholerae*, the *V. cholerae* Δ*flhG* mutant, or isogenic transcriptional reprogramming mutants in which one or more operons with an FlrBC TCS and σ^{54} dependent promoter was replaced with the *fliE* promoter as indicated to transition flagellar gene transcription toward the peritrichous flagellar program. All combinations of transcriptional reprogramming mutants were made in *V. cholerae* Δ*flhG* and WT *V. cholerae* strains (left and right columns, respectively). In panel A, the bar represents 1 μm. In panel B, motility was assessed after inoculating overnight cultures in LB with 0.3% agar and incubation at 37°C for 8 hours. The box on the left panel B is a map depicting how the Δ*flhG* or WT strain and their corresponding transcriptionally reprogrammed mutants were inoculated into motility agar in the center and right panels.

is required for flagellar biogenesis in *V. cholerae*, and we confirmed that V. cholerae Δ*flhF* lacked flagella (Figure 14 and data not shown); thus, we could not analyze flagellation in our transcriptional reprogramming mutants in the Δ*flhF* background. However, deletion of *flhG* from the classical *V. cholerae* O395 strain allowed for the production of polar flagella but with hyperflagellation due to the lack of proper numerical control of flagellar biogenesis (Figure 14). Hyperflagellation was proposed to be due to increased *flrA* expression to cause overexpression of all flagellar genes, resulting in multiple flagella. However, this hyperflagellated phenotype was unstable in *V. cholerae* O395 Δ*flhG* and after subsequent *in vitro* passaging, flagellar gene expression was reduced and the monotrichous phenotype returned (Figure 14).

We verified the hyperflagellation phenotype of a Δ*flhG* mutant in *V. cholerae* C6706 (the strain used throughout this study). In our analysis, 54% of the WT population produced exclusively monotrichous flagella, with 46% lacking a flagellum (Table 1 and Figure 15A). Only a small minority of the WT flagellated population was hyperflagellated (1.4%). *V. cholerae* Δ*flhG* cells produced flagella in a higher percentage of the population (67.5% versus 54.2% for the WT). Furthermore, 61% of flagellated Δ*flhG* cells were hyperflagellated by producing 2 to 7 polar flagella at a single pole (Table 1 and Figure 15A). The flagella of *V. cholerae* Δ*flhG* cells occasionally appeared thinner in structure with possible defects in flagellar sheath formation compared to the monotrichous flagellum of WT *V. cholerae*. Despite hyperflagellation, the Δ*flhG* mutant was motile, although modestly less so than the WT (Figure 15B). Contrary to a previous report, hyperflagellation was stable in the *V. cholerae* C6706 Δ*flhG* strain [151]. Expression of *lacZ* transcriptional reporters linked to promoters from different classes of flagellar genes were generally altered in the Δ*flhG* strain but with either increased or decreased expression depending on the promoter examined (Figure 14A). Thus, we could not link hyperflagellation to gross overexpression of flagellar genes in the Δ*flhG* strain, as previously hypothesized, suggesting that FlhG regulates flagellar number by another means, such as controlling *in vivo* FlhF activity by modulating its GTP-binding state, as postulated for *Vibrio alginolyticus* and *C. jejuni* [36, 151, 164, 165]. In summary, the *V. cholerae* Δ*flhG* mutant with a WT polar flagellar transcriptional program efficiently produced polar flagella (possibly due to dysregulated FlhF

activity), as observed by an increase in polarly flagellated cells and in the number of polar flagella per cell (hyperflagellation).

Upon alteration of the polar flagellar transcriptional program in the *V. cholerae* Δ*flhG* mutant to more closely resemble a peritrichous transcriptional program by replacing the FlrBC- and σ^{54}-dependent promoter for a single rod and hook operon with the FlrA-dependent *fliE* promoter (Figure 13), we observed almost complete elimination of polar flagellar biogenesis and the prominent Δ*flhG* hyperflagellation phenotype (Table 1 and Figure 15A). Further shifts toward the peritrichous transcriptional program in which two or all three rod and hook promoters were replaced with *fliEp* were also mostly or completely aflagellated. The only Δ*flhG* transcriptional reprogramming mutants that were flagellated were those with *fliEp* expressing the *flgB* operon alone or both the *flgB* and *flgF* operons, but a significantly smaller population of cells were flagellated than those of the Δ*flhG* mutant with the normal polar flagellar transcriptional program (8.3% to 13.9%) (Table 1 and Figure 15A). These mutants also were severely reduced for motility relative to *V. cholerae* WT and Δ*flhG* strains (Figure 15B). The flagella of the Δ*flhG* *fliEp-flgBCDE* mutant that were produced tended to be shorter than those of WT *V. cholerae* and the Δ*flhG* mutant (Figure 15A).

In select Δ*flhG* transcriptional reprogramming mutants, we did not observe gross decreases in FlrA activity, which was driving rod and hook gene expression in these mutants, as monitored by *fliEp-lacZ* expression relative to that of WT *V. cholerae* (Figure 14B). Instead, FlrA activity was comparable to or modestly greater in Δ*flhG* than in WT strains. Thus, reduced or absent flagellation in the Δ*flhG* transcriptional reprogramming mutants was not due to impaired FlrA activity and expression of rod and hook genes from the *fliE* promoter. Our data indicate that polar flagellar biogenesis efficiently occurs in *V. cholerae* with altered activity of the FlhF/FlhG flagellar biogenesis system (as in a Δ*flhG* mutant), albeit with hyperflagellation, as long as the WT polar flagellar transcriptional program is maintained by the FlrBC TCS to order rod and hook gene transcription after MS ring-rotor-fT3SS production. Any alteration toward a peritrichous flagellar transcriptional program without an intact FlhF/FlhG polar flagellar

regulatory system leads to severe reduction or loss of flagellation.

We next addressed whether flagellar biogenesis was affected in a *V. cholerae* hybrid that contained an intact FlhF/FlhG flagellar biogenesis regulatory system but with alterations to follow more closely a peritrichous flagellar transcriptional program. Contrary to the *V. cholerae* Δ*flhG* transcriptional reprogramming mutants, the level of monotrichous flagellation in any population of transcriptional reprogramming mutants with a WT FlhF/FlhG regulatory system did not change relative to that of WT *V. cholerae* (Table 1 and Figure 15A), even in the *V. cholerae* mutant with all three *fliE* promoter substitutions (*fliEp-flgB*, *fliEp-flgF*, and *fliEp-flgK*), to most closely resemble a peritrichous flagellar transcriptional program. We also did not detect differences in flagellar filament length, appearance, or function in motility *in vitro* (Figure 15A and Figure 15B). Thus, *V. cholerae* with a peritrichous flagellar transcriptional program produces polar flagella normally during *in vitro* growth, as long as the FlhF/FlhG flagellar biogenesis regulatory system is fully operational and intact. Disruption of the FlhF/FlhG system in such a transcriptionally reprogrammed *V. cholerae* cell abolishes or greatly reduces flagellation and motility. Thus, we propose that flagellum-associated TCSs of polar flagellates mediate the polar flagellar transcriptional program, characterized by the ordered transcription of flagellar rod and hook genes after the MS ring-rotor-fT3SS regulatory checkpoint during flagellar assembly, to allow polar flagellates flexibility in producing flagella and retaining motility (even with hyperflagellation) when the FlhF/FlhG system may not function properly.

3.7 *FlhF, FlhG, polar flagellar checkpoint, and the evolution of polar flagella.*

Polarly flagellated bacteria are present in a wide range of proteobacterial classes. To achieve species-specific flagellation patterns for optimal motility, each polar flagellate must have transcriptional mechanisms to correctly control flagellar gene expression and biogenesis regulators to create specific flagellation patterns composed of the correct number of flagella at one or both poles. However, little is known regarding how broadly conserved these transcriptional and biosynthetic regulatory mechanisms are and how they may be intertwined for correct

biogenesis of polar flagella and polar flagellation patterns.

To explore what may contribute to the conserved polar flagellar transcriptional program for creating a specific ordering of transcription of different classes of flagellar genes, we showed by *in silico* analysis that Gram-negative polar flagellates can be divided into two distinct groups. One group produces an FlhF/FlhG flagellar biogenesis regulatory system with a flagellum-associated TCS, and another is composed of α-proteobacteria that lack an FlhF/FlhG system. We found that FlhF/FlhG- and flagellum-associated TCS-positive polar flagellates may be subdivided into two or more groups based on the sensory domains of the TCS kinases. The different kinases are best represented by *C. jejuni* FlgS, which contains a predicted coiled-coiled (CC) domain in the sensory region, and *V. cholerae* FlrB/*P. aeruginosa* FleS, which contain PAS domains.

Despite their identification years ago, the actual signals detected by the *V. cholerae* FlrB and *P. aeruginosa* FleS sensor kinases had not been analyzed. We previously discovered that the amphitrichous polar flagellate *C. jejuni* detects a regulatory checkpoint formed by the MS ring and rotor assembling around the fT3SS core by FlgS of its flagellum-associated FlgSR TCS [44]. In our current work, we found that MS ring, rotor, and fT3SS proteins are broadly required for the activity of the *V. cholerae* FlrB and *P. aeruginosa* FleS sensor kinases to result in rod and hook gene transcription. Additionally, we found that *V. cholerae* FliF mutants that likely fail to form an MS ring were defective in FlrBC TCS activity. These findings are similar to our previous studies in *C. jejuni* and what others have reported in *H. pylori* [44, 205–207]. Furthermore, we accumulated evidence that for at least *P. aeruginosa* (and possibly *V. cholerae*), more domains of the FliG rotor were required for flagellum-associated TCS activity than in *C. jejuni*. These combined data build support for a general conserved mechanism in which flagellum-associated TCSs of polar flagellates broadly detect a regulatory checkpoint centered around MS ring-rotor-fT3SS assembly as a signal to facilitate the polar flagellar transcriptional program that orders rod and hook gene expression as a subsequent step for the progression of flagellar biogenesis. We suspect that the FliF MS ring, FliG rotor, and fT3SS core proteins (FlhA, FlhB, FliP, FliQ,

and FliR) would be required for the activity of flagellum-associated TCSs for rod and hook gene expression in many other polar flagellates that have yet to be explored.

A caveat to our work presented here is that we have not yet been able to detect a direct interaction between the FlrB or FleS kinase with the FliF MS ring or FliG rotor proteins, like we observed with *C. jejuni* FlgS, as a mechanism to monitor formation of the MS ring and rotor around the fT3SS core [44]. This indicates that the interactions between the kinases and flagellar proteins are weaker or more transient than in *C. jejuni* or that FlrB and FleS monitor fT3SS assembly indirectly through an unidentified factor. Differences in FliG domains potentially required for signal formation and detection, the abilities of the kinases to interact with MS ring and rotor components, and potential sensory domains within the kinases may reflect varied mechanisms for how these flagellum-associated TCSs monitor the formation of the regulatory checkpoint. Regardless, our findings continue to support that bacteria have mechanisms to monitor the formation of intracellular macromolecular structures and to relay information that influences behavior. Verification and deeper investigation of how each flagellum-associated TCS might detect MS ring-rotor-fT3SS assembly will be insightful for how these regulatory systems function.

When also considering the α-proteobacterial polar flagellates that lack the FlhF/FlhG flagellar biogenesis regulatory system, monitoring formation of a competent fT3SS by different systems to influence subsequent rod and hook gene expression emerges as a common strategy across Gram-negative polar flagellates for the development of the conserved polar flagellar transcriptional program. Of the α-proteobacterial polar flagellates in the reference collection, the flagellar system of *C. crescentus* is the best characterized [208]. *C. crescentus* executes the polar flagellar transcriptional program so that transcription of MS ring, C ring, and fT3SS genes occurs prior to σ^{54}-dependent transcription of rod and hook genes [209]. *C. crescentus* σ^{54} requires FlbD, an enhancer-binding protein similar to the *C. jejuni* FlgR, *V. cholerae* FlrC, and *P. aeruginosa* FleR response regulators of the flagellum-associated TCSs, to activate flagellar rod and hook gene expression [210–214]. MS ring, C ring, and fT3SS proteins are also

required for FlbD activity and FlbD- and σ^{54}-dependent rod and hook gene expression [212, 214]. Thus, FlbD activity is linked to MS ring-rotor-fT3SS complex formation, yet FlbD lacks a cognate sensor kinase like FlgS, FlrB, or FleS to monitor flagellar assembly and control its activity. The FliX transactivating factor has been identified as the link that relays the status of fT3SS assembly to positively or negatively control the activity of FlbD to bind to target flagellar rod and hook promoters [215–218]. Although its regulatory mechanism is not understood, FliX does not function as a kinase to transduce signals regarding fT3SS assembly.

As diverse polar flagellates have evolved different mechanisms to create and maintain the polar flagellar transcriptional program so that rod and hook gene expression occurs after formation of the regulatory checkpoint at fT3SS assembly, we hypothesized that this program is beneficial for biogenesis of polar flagella. *E. coli* and *Salmonella*, as models with the peritrichous flagellar transcriptional program, do not recognize this checkpoint and transcribe most basal, rod, and hook genes simultaneously to efficiently build peritrichous flagella [10, 169, 204]. Since, in this study, we show a *V. cholerae* cell containing the WT FlhF/FlhG regulatory system engineered with a peritrichous flagellar transcriptional program produced a monotrichous flagellum efficiently, the type of flagellar transcriptional program itself does not seem to determine the peritrichous or polar flagellation pattern of the species.

Instead, we discovered that possessing a polar transcriptional program and the FlhF/FlhG flagellar biogenesis regulatory system allows polar flagellation while retaining motility to a modest extent when perturbations to FlhF and FlhG activity occur. For example, the *V. cholerae* Δ*flhG* mutant with a polar flagellar transcriptional program was hyperflagellated, indicating high proficiency in producing flagella. However, the Δ*flhG* mutant was less motile than the WT monotrichous strain, likely due to the inability of the Δ*flhG* mutant to coordinate multiple rotating polar flagella for optimal swimming motility. In contrast, flagellation was severely diminished or even abolished in *V. cholerae* mutants that more closely resembled most peritrichous bacteria by lacking a properly functioning FlhF/FlhG flagellar biogenesis system (through deletion of *flhG*) and engineered with a peritrichous flagellar transcriptional program. Thus, the

peritrichous transcriptional program is much more affected by alterations in FlhF/FlhG activity in a polar flagellate, resulting in greatly decreased flagellation, motility, and, likely, fitness in nature. Currently, it is unknown whether FlhF/FlhG activity is naturally regulated or altered by extrinsic factors or metabolic capacity. We have observed hyperflagellation in WT polarly flagellated systems in a small minority of cells (in *V. cholerae* in this work and previously in *C. jejuni* [36, 41]. Thus, the FlhF/FlhG flagellar biogenesis regulatory system is likely affected by stochastic influences on a cell-to-cell basis that may at least alter FlhG activity and likely its ability to regulate FlhF. It remains to be determined whether the polar flagellar transcriptional program also provides an advantage to *C. crescentus* that has a different collection of determinants to produce polar flagella during developmental stages and asymmetrical division [219–222].

Our analysis of *V. cholerae* flagellar transcriptional reprogramming mutants provided some intriguing observations, but many questions remain. One such question is what advantage exactly does the polar flagellar transcriptional program provide for the FlhF/FlhG regulatory system to enable efficient flagellation when FlhF or FlhG activity is altered that the peritrichous flagellar transcriptional program does not provide. It is currently unclear how FlhG controls flagellum numbers in *V. cholerae*. FlhG orthologs regulate flagellum number by at least two different processes, including influencing the activity of FlhF or a master regulator of flagellar gene transcription, such as FlrA [6, 36, 151, 153, 154, 163–165, 223]. In contrast to a previous report, we did not observe a broad increase in transcription across classes of flagellar genes in the *V. cholerae* Δ*flhG* mutant that would explain the consistent hyperflagellation phenotype we observed [151]. Thus, hyperflagellation in the *V. cholerae* Δ*flhG* mutant may be due to a dysregulated, hyperactive FlhF, which has been proposed in *C. jejuni* and other *Vibrio* species [36, 164, 165].

The molecular mechanism by which the FlhF GTPase influences polar flagellation has not been determined in many bacteria. One hypothesis includes that FlhF localizes MS ring, C ring, and fT3SS core proteins at a pole or facilitates interactions between these proteins to create

a new flagellum [6, 163]. FlhG presumably functions in some polar flagellates to transition FlhF from a GTP-bound "on" state competent for a function to initiate flagellation to a GDP-bound "off" state. Tight control of FlhF by FlhG may be required so that FlhF can organize flagellar proteins properly for MS ring-rotor-fT3SS assembly either with the stepwise production of MS ring, C ring, fT3SS, rod, and hook proteins provided by the polar flagellar transcriptional program or with their simultaneous production facilitated by the peritrichous flagellar transcriptional program. However, a dysregulated FlhF in the Δ*flhG* mutant may be unable to perform its natural function in flagellation when MS ring, C ring, fT3SS, rod, and hook proteins are produced at the same time. Consistent with this, we observed a great reduction or abolishment of flagellation when some or all rod and hook proteins were simultaneously produced with MS ring, C ring, and fT3SS proteins in the Δ*flhG* mutant engineered to follow more closely a peritrichous transcriptional flagellar program.

Our findings may point toward a more expansive role for FlhF: in addition to its hypothesized role in assisting polar assembly of the MS ring-rotor-fT3SS complex, FlhF may also organize flagellar proteins, such as the rod and hook proteins, for secretion via the fT3SS. If so, production of multiple FlhF-interacting proteins (fT3SS complex proteins and their secretion substrates) simultaneously may overwhelm a dysregulated FlhF so that flagellar biogenesis does not occur. Other possibilities exist, including that the peritrichous flagellar transcriptional program in a Δ*flhG* mutant disrupts flagellar protein stoichiometry. In this case, there may not be enough rod and hook proteins produced for the multiple fT3SSs that may form in the Δ*flhG* mutant. Undoubtedly, there are functions for FlhF and FlhG that are not yet adequately understood to reveal how the polar flagellar transcriptional program contributes to the FlhF/FlhG flagellar biogenesis system for efficient polar biogenesis. Continued exploration will likely further reveal how transcriptional and biosynthetic processes are integrated in polar flagellates to construct the ideal number and positioning of these macromolecular machines for motility in bacterial cells. Our findings raise some questions regarding how different flagellar transcriptional programs formed across flagellated species. One prominent question is whether polar and peritrichous flagellar transcriptional programs developed independently of each other or if

one evolved from the progenitor of another. It is clear that flagellar structural components are largely conserved across bacterial species. Even the mechanism to detect rod and hook formation as a late regulatory checkpoint required for activation of σ^{28} and expression of terminal flagellar genes is widely conserved [27, 167, 168]. However, regulatory factors and mechanisms required for expression of flagellar components required for formation of the fT3SS, rod, and hook differ in peritrichous and polar flagellates. Most peritrichous bacteria (albeit with *B. subtilis* as a Gram-positive exception) have a seemingly less complex flagellar transcriptional program so that MS ring, C ring, fT3SS core, rod, and hook proteins are produced simultaneously by the activity of a flagellar master regulator, and these bacteria do not require FlhF or FlhG to efficiently construct multiple flagella across their surfaces.

Polar flagellates may have originated from a peritrichous progenitor but also could have developed independently. Comparisons between *C. crescentus* and many other Gram-negative polar flagellates as discussed above clearly show that different polar flagellar biogenesis systems exist in the presence of somewhat conserved regulatory mechanisms to facilitate the polar flagellar transcriptional program, indicating convergent evolution of polar flagellates. Regardless, our findings suggest that a species needs to acquire a polar flagellar biogenesis system (such as the FlhF/FlhG system) and a mechanism to order flagellar genes for the polar flagellar transcriptional program (such as a flagellum-associated TCS) to become an efficient polar flagellate. Possessing only the FlhF/FlhG system with the peritrichous program does not guarantee optimal flagellation and motility if FlhF/FlhG activity is affected by extrinsic, stochastic factors.

It is unknown which came first in a polar flagellate, the FlhF/FlhG polar flagellar biogenesis system or the flagellum-associated TCSs, to drive the polar flagellar transcriptional program. Both the FlhF GTPase and FlhG ATPase are members of the SIMIBI class of nucleotide-binding proteins that commonly function in cellular organization and protein targeting [224, 225]. FlhF is related to the Ffh GTPase of the signal recognition particle system, whereas FlhG is closely associated with the MinD and ParA ATPases that generally perform partitioning functions related to division and DNA segregation [166, 224–226]. Development of the

FlhF/FlhG flagellar biogenesis regulatory system, perhaps from Ffh and MinD/ParA superfamilies, could have caused the emergence of a polar flagellate in a Gram-negative organism. The motile, monotrichous *V. cholerae* strain we engineered with an intact FlhF/FlhG system and a peritrichous flagellar transcriptional program might resemble this ancestor. As revealed in this work, this bacterium is heavily reliant on a precisely functioning FlhF/FlhG system to form any flagella and retain some level of motility; perturbations to FlhF or FlhG activity severely reduce or completely abolish flagellation. By possessing a mechanism mediated by the flagellum-associated TCSs to order rod and hook gene transcription after production of MS ring, rotor, and fT3SS proteins (and possibly assembly of a functional fT3SS), a bacterium can produce polar flagella with some alterations to FlhF/FlhG activity. In this bacterium, an optimally functioning FlhF/FlhG system allows for the correct number and placement of polar flagella and WT motility; an impaired FlhF/FlhG system (at least by altering FlhG) results in polar flagellation with extra flagella produced and at least modest motility. This hyperflagellated bacterium has an advantage over one with the FlhF/FlhG system and a peritrichous program that cannot maintain flagellation and motility with perturbations to the FlhF/FlhG system.

Modulations in FlhF and FlhG activity in different species with flagellum-associated TCSs to maintain the polar flagellar transcriptional program and flagellar biogenesis may have facilitated the emergence of different polar flagellation patterns, amphitrichous, lophotrichous, and monotrichous. An example of this is *C. jejuni* and *H. pylori*, which, while closely related, produce amphitrichous and lophotrichous flagella, respectively, yet have the FlhF/FlhG flagellar biogenesis regulatory system and similar flagellum-associated FlgSR TCSs. A study comparing FlhF and FlhG biochemical activity and biological function between these two bacterial species has not been conducted. Although many details remain to be discovered for how FlhF and FlhG function in polar flagellates, our results indicate regulatory links between the FlhF/FlhG flagellar biogenesis regulatory systems and the order of flagellar protein production controlled by the flagellum-associated TCSs for polar flagellar biogenesis.

4

FEEDBACK-INHIBITION IN EARLY POLAR FLAGELLAR FORMATION

The flagellum is a costly structure to express and involves dozens of proteins to produce (See Section 1.1). Additionally, many bacteria have life cycles with a stage where flagella are not produced, such as when some motile bacteria attach to surfaces and produce biofilms [119, 146, 227–233]. For these reasons and others, many flagellated bacteria tightly control initiation of flagellar gene expression using a master flagellar regulator such as *E. coli* FlhDC (See Section 1.1), *V. cholerae* FlrA, or *P. aeruginosa* FleQ (See Section 1.3) [37, 114, 148]. But how is flagellar gene expression turned off? Some bacteria such as *C. jejuni* seem to express flagellar genes constitutively, but *C. jejuni* notably lacks a master flagellar regulator. Completion of the flagellum is a logical signal to repress flagellar gene expression and *E. coli*, *V. cholerae*, and *P. aeurginosa* likely have evolved different signaling mechanisms to achieve this by linking completion of the flagellum to master transcriptional regulators of flagellar genes.

4.1 *c-di-GMP represses flagellar gene transcription in V. cholerae and P. aeruginosa*

Completion of the flagellum in *V. cholerae* and *P. aeruginosa* lays the foundation for a transition from a motile to sessile lifestyle [234, 235]. This transition is complex and multifaceted, involving many competing and complementary regulators, but in general c-di-GMP and the enzymes that produce and degrade c-di-GMP appear to be central [227–229, 236–242]. c-di-GMP represses FlrA- and FleQ-dependent expression of flagellar genes in *V. cholerae* and *P. aeruginosa*, respectively, by repressing FlrA and FleQ activity directly or via FlhG (See Section 1.3.3) [149–152]. As a result, increasing c-di-GMP levels via DGC activity can repress flagellar gene expression and promote biofilm-related gene expression while decreasing c-di-GMP

levels via PDE activity can increase flagellar gene expression and repress biofilm-related gene expression [240, 242]. This simplistic paradigm belies the apparent specificity of how DGCs and PDEs repress flagellar gene expression [228, 239, 243].

4.1.1 *DGCs and PDEs that influence flagellar motility in V. cholerae*

c-di-GMP signaling in *V. cholerae* appears specific in that biofilm formation does not correlate well to global c-di-GMP levels, but rather to the degree of induced expression of genes encoding some enzymatically active DGCs and not others [243]. How this specificity is achieved remains a large unanswered question in c-di-GMP research within bacteriology, but our understanding of DGC and PDE specificity is likely sufficient to warrant treating each *V. cholerae* DGC or PDE as distinct and likely not interchangeable. The *V. cholerae* C6706 genome has 53 genes that encode proteins that synthesize or hydrolyze c-di-GMP. These include 28 genes with a GGDEF domain (required for DGC activity), 16 genes with an EAL domain (often required for PDE activity), 5 genes with a HD-GYP motif domain (sometimes required for PDE activity) and 4 genes with both GGDEF and EAL domains (See Section 1.3.2 for a review) [245]. Of these 53 genes, only a subset influence *V. cholerae* motility (Figure 24) [246]. Most of the work describing putative *V. cholerae* DGCs or PDEs that alter motility has been via genetic knockouts and only a few have had their putative DGC or PDE activity verified via an *in vitro* biochemical assay [241, 243–246]. This is an important note for caution, as GGDEF and EAL domains can be retained by enzymatically inactive proteins (no discernible DGC or PDE activity) to serve a c-di-GMP binding platform [119].

DGC or PDE mutants that alter *V. cholerae* motility can be divided into two groups, those that increase or decrease *V. cholerae* motility in soft agar. *V. cholerae* Δ*cdgD*, Δ*cdgH*, Δ*cdgL* and Δ*cdgK* mutants showed increased motility in soft agar and decreased biofilm formation (Figure 24) [241, 244–246]. Only CdgH DGC activity has been demonstrated *in vitro* [244]. *cdgD* expression is disrupted in flagellar gene deletion mutants, which suggests that flagellar biosynthesis may regulate *cdgD* through an unknown mechanism [39]. These putative DGCs

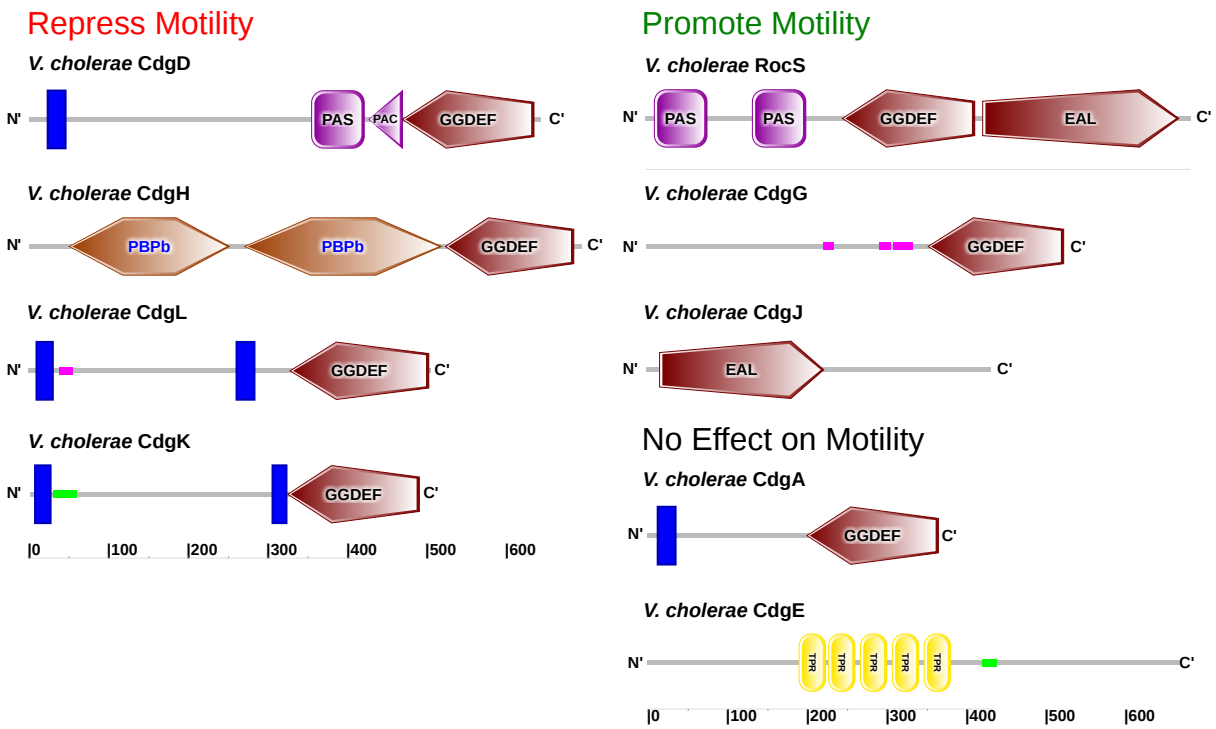

Figure 16: **Domain analysis of proteins with GGDEF and EAL domains that alter *V. cholerae* motility.** Protein domains shown are predicted by SMART. *V. cholerae* CdgD, CdgH, CdgL, CdgK, RocS, and CdgG have GGDEF domains, which are represented as elongated brown pentagrams (pointing right to left), that are required for c-di-GMP synthesis via DGC activity. Some GGDEF domains, such as the GGDEF motif in CdgG, are not catalytically active and may be retained to bind c-di-GMP or GMP monomers [244]. SMART was unable to identify the GGDEF motif described in Syed *et. al.*, but the UNIPROT database annotates a GGDEF domain in the C-terminal residues of CdgE similar to CdgD [39]. *V. cholerae* RocS and CdgJ both have EAL domains, which are represented as brown pentagrams (pointing left to right) and are required for c-di-GMP hydrolysis. Transmembrane regions in *V. cholerae* CdgD, CdgL, and CdgK are represented as blue rectangles. *V. cholerae* CdgD and RocS have PAS domains which are represented as purple squares. The PAS domain-associated PAC domain is represented as a purple triangle in *V. cholerae* CdgD. Prokaryotic periplasmic binding protein domains (PBPb) in CdgH are represented as elongated brown hexagons. Coiled-coil regions of *V. cholerae* CdgK and CdgE are represented with green rectangles. The tetratricopeptide (TPR) repeats of CdgE are represented as yellow ovals. Areas of low structural complexity or that have an "unknown region" according to SMART are represented with pink rectangles in *V. cholerae* CdgL and CdgG.

likely repress motility one of three possible ways. They either alter *Vibrio* polysaccharide production (which interferes with flagellar function), change *V. cholerae* behavior near surfaces (which is the case for CdgD and CdgH), or alter FlrA activity [245].

V. cholerae Δ*rocS*, Δ*cdgG*, and Δ*cdgJ* mutants showed decreased motility in soft agar and increased biofilm formation (Figure 24) [241, 244, 246]. RocS likely functions primarily as a PDE and overexpressing RocS via a multicopy number plasmid decreased colony rugosity in *V. cholerae* [241]. Interestingly, Δ*rocS* has an additive effect on the flagellum-dependent biofilm regulatory response in a Δ*flaA* background, which suggests that RocS could either be linked to flagellar assembly before the flagellar filament forms or could be participating in another unknown signaling pathway independent of flagellar filament formation [239]. CdgJ contains an EAL domain and an *in vitro* analysis suggests that it has PDE activity [246]. CdgG lacks DGC activity and may act as a c-di-GMP-binding effector through a degenerate GGDEF site [244].

4.1.2 *Flagellar assembly regulates biofilm formation in V. cholerae and P. aeruginosa*

In *V. cholerae*, we have known for almost two decades that disrupting flagellar assembly increases biofilm formation [235]. Specifically, any *V. cholerae* flagellar mutant that prevents flagellar filament formation (i.e fT3SS, MS and C ring, rod and hook mutants, but not stator mutants) adopt a rugose colony morphology indicative of increased biofilm formation [235]. In *P. aeruginosa*, the relationship between flagellar assembly and biofilm-related gene expression has mainly focused on the flagellum as a mechanosensor via MotC/SadC to promote an increase in c-di-GMP levels or FleQ-dependent expression of Pel exopolysaccharides when c-di-GMP levels are high, but a relationship between early flagellar formation and biofilm-related gene expression (likely through DGCs and PDEs activity) in *P. aeruginosa* has not been demonstrated [147, 247]

Recently, a study by the Yildiz group found that disrupting the flagellar filament, flagellar rod and hook, and flagellar MS ring-rotor-fT3SS all increased c-di-GMP levels and biofilm

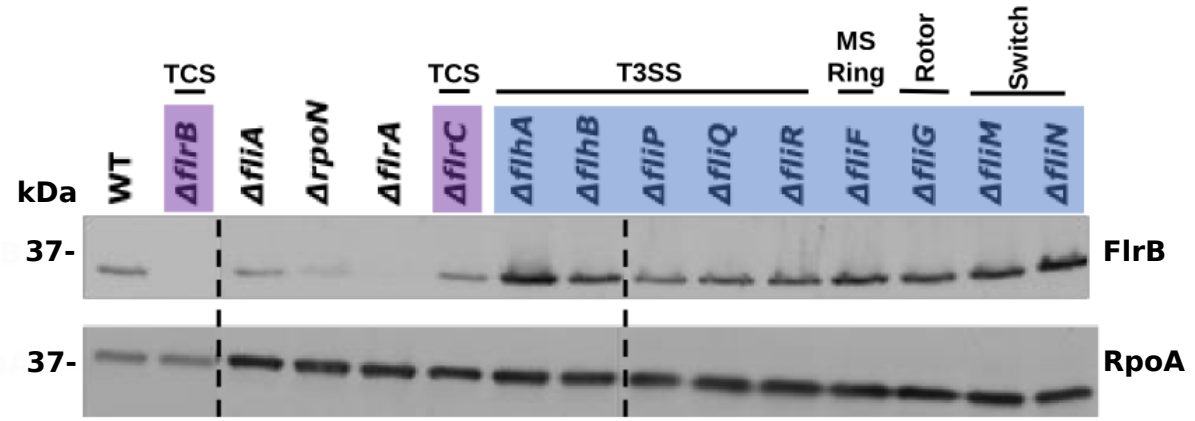

Figure 17: **fT3SS, MS ring, and C ring mutants increase FlrB expression.** Immunoblot analysis of FlrB in whole-cell lysates of *V. cholerae* isogenic mutants. TCS genes are indicated with purple boxes and MS ring-rotor-fT3SS genes are indicated with blue boxes. Specific antiserum to FlrB was used to detect FlrB protein. Detection of RpoA served as a control to ensure equal loading of proteins across each strain

formation [239]. Additionally, CdgA, CdgL, and CdgO were responsible for the increase in c-di-GMP in this flagellum-dependent biofilm regulatory response [239]. This establishes the link between flagellar formation and biofilm-related gene expression through the activity of specific DGCs. What impact this relationship between stages of flagellar assembly and DGC activity has on flagellar gene expression remains to date uncharacterized.

4.2 *Early flagellar formation represses FlrA activity*

Over the course of our work characterizing the MS ring-rotor-fT3SS regulatory checkpoint in *V. cholerae*, we found a potentially novel signaling pathway where MS ring-rotor-fT3SS formation may shut down flagellar gene expression by repressing the FlrA master regulator. Our first piece of evidence for this pathway was via an immunoblot we performed to examine the effect of flagellar mutations on FlrB expression (Figure 17). FlrB production was clearly visible in WT *V. cholerae*, but FlrB was absent in Δ*flrB*, Δ*flrA* and Δ*rpoN* ($\Delta\sigma^{54}$) strains, which was expected as *flrB* expression requires FlrA and σ^{54}. Removing σ^{28} (Δ*fliA*) and FlrC (Δ*flrC*) did not alter FlrB expression as no mechanism for σ^{28} and FlrBC regulation of *flrB* gene expression has been described to date. Interestingly, fT3SS mutants (Δ*flhA*, Δ*flhB*, Δ*fliP*, Δ*fliQ*, Δ*fliR*), MS ring (Δ*fliF*), rotor (Δ*fliG*), and switch mutants (Δ*fliM* and Δ*fliN*) all showed evidence of

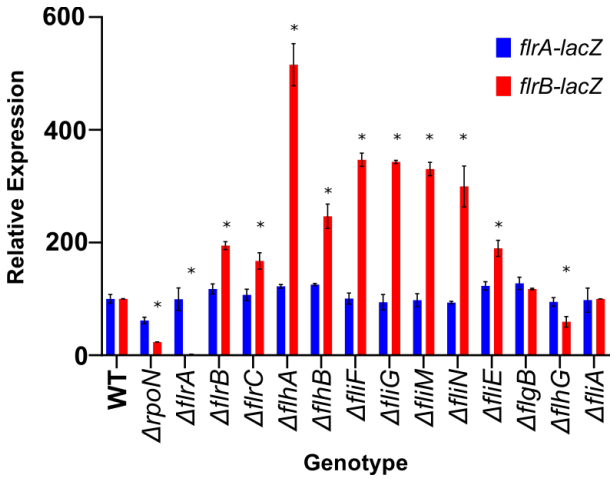

Figure 18: **Analysis of *flrA* expression and FlrA activity in fT3SS, MS ring, and C ring mutants increase FlrA activity** Analysis of *flrA* gene expression and FlrA- and σ^{54}-dependent flagellar gene expression in flagellar mutants of *V. cholerae*. *flrA-lacZ* and *flrB-lacZ* transcriptional reporters were electroporated into *V. cholerae* mutants.The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates the mutant had a significantly increased or decreased reporter expression relative to that of the WT strain ($p<0.05$).

increased FlrB production. This increase in FlrB production was particularly pronounced in Δ*flhA*. Since *flrB* expression is FlrA-dependent, we suspected that fT3SS, MS ring, and C ring deletion mutants may increase FlrA levels or FlrA activity, both of which could further link a stage of flagellar assembly (MS ring-rotor-fT3SS formation) to changes in flagellar gene expression.

To determine whether disrupting MS ring-rotor-fT3SS formation formation alters FlrA levels or FlrA activity, we generated a *flrA-lacZ* transcriptional reporter to measure *flrA* expression and a *flrB-lacZ* transcriptional reporter to measure FlrA activity. We electroporated these reporters into MS ring, C ring, fT3SS mutants separately and measured *flrA-lacZ* and *flrB-lacZ* expression using β-galactosidase assays (Figure 18). *flrA-lacZ* expression decreased in a Δ*rpoA* mutant by roughly half of WT. It is unclear why this is the case as a role for σ^{54} (*rpoA*) in *flrA-lacZ* expression has not previously been described. *flrA-lacZ* expression did not change in Δ*flrA*, Δ*flrB*, Δ*flrC* or MS ring-rotor-fT3SS mutants (Figure 18). This suggests that MS ring-rotor-fT3SS formation likely does not increase FlrB expression by increasing FlrA levels. We observed that *flrB-lacZ* expression was disrupted in Δ*rpoN* (σ^{54}) and Δ*flrA* mutants as expected since *flrB-lacZ* expression is FlrA- and σ^{54}-dependent (Figure 18). *flrB-lacZ* expression roughly doubled in Δ*flrB* and Δ*flrC*, which is unexpected since we did not see an appreciable difference in FlrB production in Δ*flrC* mutant (Figure 17). Interestingly, fT3SS mutants

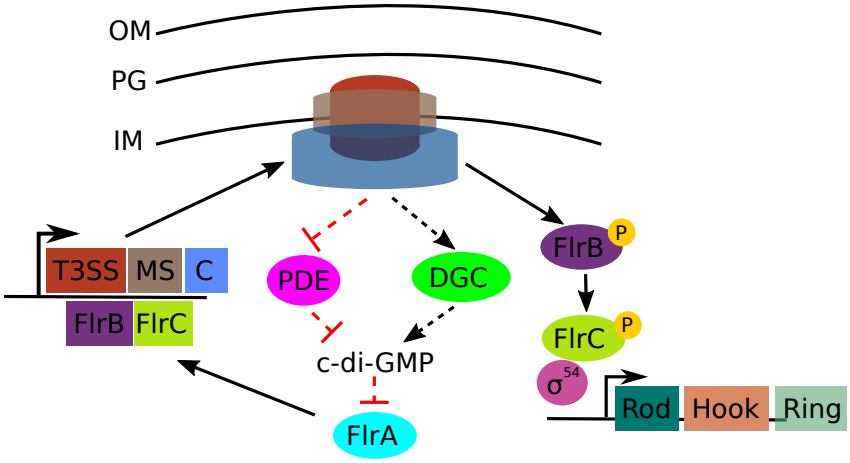

Figure 19: **Model of how *V. cholerae* MS ring-rotor-fT3SS formation stimulates rod and hook gene expression and may repress FlrA activity through c-di-GMP.** Flagellar gene transcription in *V. cholerae* starts with FlrA, which stimulates σ^{54}-dependent expression of *flrBC*, fT3SS, MS ring, and C ring genes. The fT3SS forms first, which enables multimerization of the MS ring and C ring. We demonstrated in Section 3.3 that *V. cholerae* MS ring-rotor-fT3SS formation stimulates FlrBC- and σ^{54}-dependent gene expression. Disrupting MS ring-rotor-fT3SS formation also may repress FlrA activity possibly through c-di-GMP signaling. This stage of flagellar assembly may stimulate one or multiple DGCs or repress one or multiple PDEs to increase c-di-GMP levels. This increase in c-di-GMP may repress FlrA activity to shut off the expression of *flrBC*, fT3SS, MS ring, and C ring genes.

(Δ*flhA*, Δ*flhB*, Δ*fliP*, Δ*fliQ*, Δ*fliR*), MS ring (Δ*fliF*), rotor (Δ*fliG*), and switch mutants (Δ*fliM* and Δ*fliN*) all increased *flrB-lacZ* expression (Figure 18). This suggests that the increased FlrB levels in MS ring-rotor-fT3SS mutants we observed (in Figure 17) was likely due to an increase in *flrB* transcription. Since FlrA is required for *flrB* transcription, this suggests that disrupting MS ring-rotor-fT3SS formation increases FlrA activity.

Previously, *V. cholerae* Δ*flhG* was shown to increase both *flrA* and early flagellar gene expression [151]. We also examined whether FlhG had an effect on FlrA expression (*flrA-lacZ*) or FlrA activity (*flrB-lacZ*) in Figure 18. In our assay, Δ*flhG* did not alter *flrA-lacZ* expression relative to WT, but did decrease *flrB-lacZ* expression by roughly half relative to WT (Figure 18). This was surprising given that it has been previously reported that FlhG inhibits *flrA* expression [151]. It is possible that FlhG may increase or decrease FlrA activity under different conditions although a mechanism for how FlhG may be required for WT levels of FlrA activity has not been described.

How could *V. cholerae* MS ring-rotor-fT3SS formation influence FlrA activity? We suspected it may be through c-di-GMP signaling. This hypothesis was based off two indirect lines of evidence. The first was that we had previously observed that in motility assays where *V. cholerae* were stabbed into 0.3% LB motility agar and grown overnight at 30°C (instead of 8 hours at 37°C), Δ*fliF* and Δ*fliG* mutants were motile (but less than WT). Since Δ*fliF* and Δ*fliG* mutants prevent flagellar formation, the motility we observed was likely a non-flagellar form of motility. Most non-flagellar forms of motility are positively regulated by c-di-GMP [119]. Thus, we suspected that Δ*fliF* and Δ*fliG* mutants may increase c-di-GMP levels. The second line of evidence is that c-di-GMP is a known negative regulator of FlrA activity [152]. Taken together, this made us suspect that MS ring-rotor-fT3SS formation may alter c-di-GMP levels to regulate FlrA activity as a negative feedback-inhibition loop.

Our model for how MS ring-rotor-fT3SS formation influences FlrA activity is illustrated in Figure 19. First, FlrA drives the expression of MS ring, rotor, fT3SS and the FlrBC TCS genes. The MS ring-rotor-fT3SS complex forms which stimulates FlrBC-dependent expression of flagellar rod and hook genes expression as described in Chapter 3. We suspect that MS ring-rotor-fT3SS formation also increases c-di-GMP levels either by increasing DGC levels or activity or by repressing PDE levels or activity. Either scenario would increase c-di-GMP levels, possibly localized to the poles, which decreases FlrA activity, thereby shutting off early flagellar gene expression.

4.3 Δ*flhA increases FlrA activity independently of FlhA-associated DGCs and PDEs*

We developed a model where MS ring-rotor-fT3SS formation influences FlrA activity through c-di-GMP via DGCs and/or PDEs (Figure 19). To determine the validity of our model, we first sought to identify DGCs and PDEs mutants that alter *flrB-lacZ* expression, a measure of FlrA activity. Our Δ*flhA* mutant increased *flrB-lacZ* expression roughly 4.5-fold relative to WT. Based on our model, we suspected that c-di-GMP levels were significantly lowered in Δ*flhA* relative to WT. A recent publication by the Yildiz group found that c-di-GMP levels actually in-

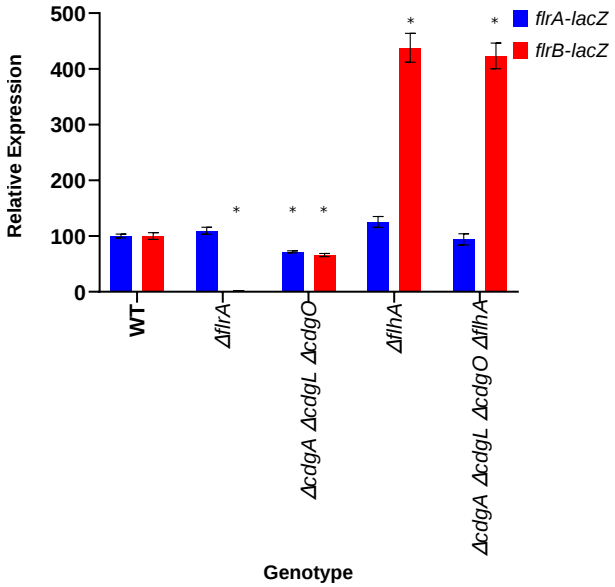

Figure 20: **Analysis of FlrA activity in FlhA-associated DGCs and PDEs mutants.** *flrA-lacZ* and *flrB-lacZ* transcriptional reporters were electroporated into *V. cholerae* mutants. The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates the mutant had a significantly increased or decreased reporter expression relative to that of the WT strain ($p<0.05$).

creased in Δ*flhA* mutant and that three DGCs CdgA, CdgL, and CdgO were responsible [239]. This is surprising given that none of these DGCs apart from CdgL were found to impact motility by themselves [246]. Single Δ*cdgA*, Δ*cdgL*, or Δ*cdgO* mutants did not reduce c-di-GMP levels in Δ*flhA* similar to WT, but a Δ*cdgA* Δ*cdgL* Δ*cdgO* triple mutant did reduce c-di-GMP level in a Δ*flhA* background [239]. How these three DGCs function together is unknown. While the observation that these enzymes likely increase c-di-GMP level in Δ*flhA* works against our model, we thought that these three DGCs may affect the increase in FlrA activity we observed in Δ*flhA*. We acquired the Δ*cdgA* Δ*cdgL* Δ*cdgO* triple mutant generated in both WT C6706 *V. cholerae* and Δ*flhA* from the Yildiz group and electroporated either a *flrA-lacZ* reporter (to measure *flrA* expression) or *flrB-lacZ* reporter (to measure FlrA activity) into each strain. We then performed β-galactosidase assays to measure *flrA-lacZ* or *flrB-lacZ* reporter expression (Figure 20).

As we observed in Figure 18, Δ*flrA* had a similar level of *flrA-lacZ* expression as WT (Figure 20). The Δ*cdgA* Δ*cdgL* Δ*cdgO* triple mutant decreased *flrA-lacZ* expression relative to WT although it is unclear if this decrease is biologically relevant. The Δ*flhA* mutant and the Δ*flhA* Δ*cdgA* Δ*cdgL* Δ*cdgO* quadruple mutant each had a similar levels of *flrA-lacZ* expression as WT (Figure 20). In Δ*flrA*, *flrB-lacZ* expression was reduced as expected. The Δ*cdgA* Δ*cdgL* Δ*cdgO* triple mutant decreased *flrB-lacZ* expression relative to WT although it is unclear if this decrease is biologically relevant (Figure 20). Although Δ*flhA* showed a roughly 4.5-fold

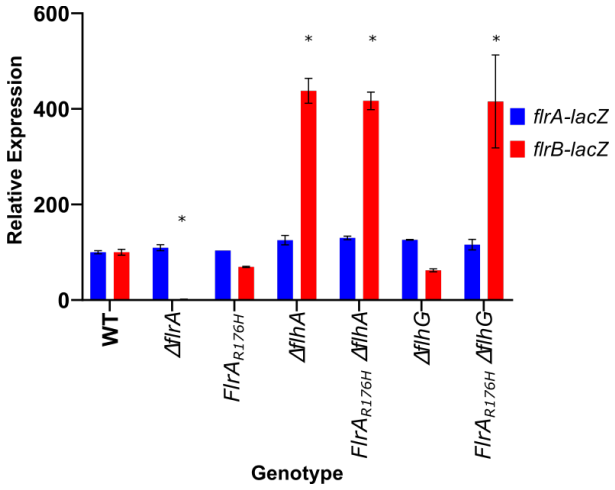

Figure 21: **Analysis of FlrA activity in c-di-GMP blind FlrA mutants.** *flrA-lacZ* and *flrB-lacZ* transcriptional reporters were electroporated into *V. cholerae* mutants. The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates the mutant had a significantly increased or decreased reporter expression relative to that of the WT strain ($p<0.05$).

increase in *flrB-lacZ* expression, the Δ*flhA* Δ*cdgA* Δ*cdgL* Δ*cdgO* quadruple mutant showed a similar level of *flrB-lacZ* expression as Δ*flhA*. Taken together, these observations suggest that deletion of *cdgA*, *cdgL*, and *cdgO* does not impact FlrA activity and these DGCs do not contribute to how Δ*flhA* increased FlrA activity. While Δ*flhA* may increase c-di-GMP levels through CdgA, CdgL and CdgO, this may not be sufficient to explain how the Δ*flhA* mutant increases FlrA activity [239]. The activity of other DGCs or PDEs may be responsible for the increase in FlrA activity in the Δ*flhA* mutant or this increase in FlrA activity may be independent of c-di-GMP signaling.

4.4 *c-di-GMP-blind FlrA mutants do not increase FlrA activity*

Although CdgA, CdgL, and CdgO were reported to increase c-di-GMP levels in Δ*flhA*, we did not observe an effect on FlrA activity with a Δ*cdgA* Δ*cdgL* Δ*cdgO* triple mutant (as measured by *flrB-lacZ* reporter expression) [239]. We decided to take another approach to determine if MS ring-rotor-fT3SS formation influences FlrA activity through c-di-GMP by generating c-di-GMP-blind FlrA mutants. The *flrA*$_{R176H}$ mutant had previously been shown to render FlrA blind to c-di-GMP [152]. According to our model, we expected the *flrA*$_{R176H}$ Δ*flhA* double mutant to decrease *flrB-lacZ* expression relative to Δ*flhA* if a change in c-di-GMP concentration (and a subsequent change in FlrA activity) is responsible for the roughly 4.5-fold increase in *flrB-lacZ* expression we observed in Δ*flhA* relative to WT (Figure 19).

We generated the *flrA*$_{R176H}$ mutation in WT, Δ*flhA*, and Δ*flhG* backgrounds and then electroporated either a *flrA-lacZ* reporter (to measure FlrA levels) or a *flrB-lacZ* reporter (to measure FlrA activity). We performed a β-galactosidase assay to measure the effect of FlrA$_{R176H}$ on the expression of *flrA-lacZ* or *flrB-lacZ* reporters in WT, Δ*flhA*, and Δ*flhG* backgrounds (Figure 21). FlrA$_{R176H}$ did not to significantly alter *flrA-lacZ* expression in WT, Δ*flhA*, and Δ*flhG* backgrounds (Figure 21).

As we observed in Figure 18, FlrA is required for *flrB-lacZ* expression. In Figure 21, FlrA$_{R176H}$ decreased *flrB-lacZ* expression by roughly half (Figure 18), rather than increasing *flrB-lacZ* expression, which would be expected in the WT background if c-di-GMP repressed FlrA activity in the WT strain. However the *flrA*$_{R176H}$ mutation did not have much of an effect on *flrB-lacZ* expression in the Δ*flhA* background as the *flrA*$_{R176H}$ Δ*flhA* double mutant only had slightly lower *flrB-lacZ* expression than the Δ*flhA* single mutant (Figure 21). Taken together, these observations lend further support that a c-di-GMP-independent mechanism may contribute to increased FlrA activity and FlrB levels in Δ*flhA*.

We had previously observed that Δ*flhG* decreased *flrB-lacZ* expression in Figure 18, which we also see in Figure 21. Surprisingly, we observed that FlrA$_{R176H}$ substantially increased *flrB-lacZ* expression in the Δ*flhG* background relative to Δ*flhG* alone. It is unclear to us why Δ*flhG* decreases FlrA activity, while a presumably c-di-GMP-blind FlrA$_{R176H}$ produced much higher FlrA activity when FlhG is absent. Regardless, these observations suggest a surprising possilbity that c-di-GMP through FlhG, but independent of fT3SS formation, influences FlrA activity.

4.5 *FlhG, CdgE, and RocS may regulate FlrA activity*

We previously determined that CdgA, CdgL, and CdgO are not responsible for the increase in *flrB-lacZ* expression in the Δ*flhA* mutant (Figure 20) and are thus unlikely to participate in the MS ring-rotor-fT3SS formation negative feedback-inhibition pathway (Figure 19).

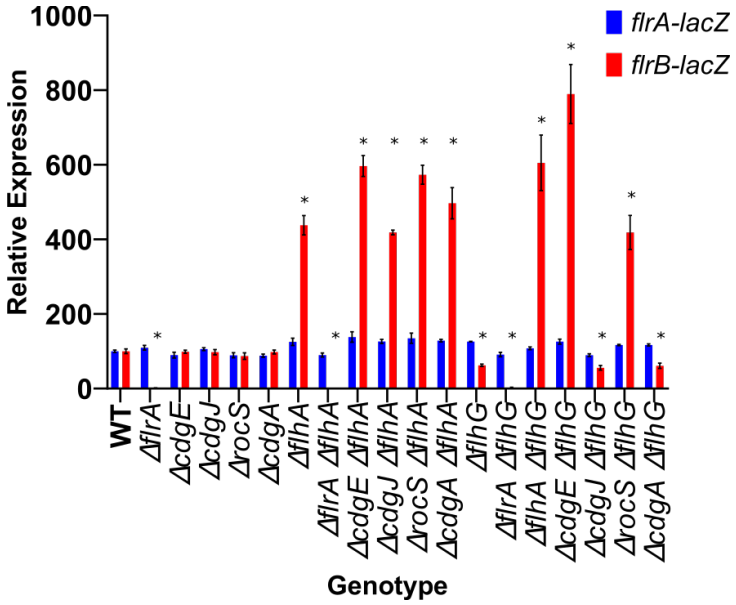

Figure 22: **Analysis of FlrA activity in FlhG, DGC, and PDE mutants.** *flrA-lacZ* and *flrB-lacZ* transcriptional reporters were electroporated into *V. cholerae* mutants. The level of expression of transcriptional reporters in each mutant is relative to the level of expression in WT *V. cholerae*, which was set to 100 U. Error bars indicate standard deviations of the average level of expression from three samples. An asterisk indicates the mutant had a significantly increased or decreased reporter expression relative to that of the WT strain ($p<0.05$).

Additionally, there may be a role for FlhG regulation of FlrA activity in *V. cholerae* as was previously observed in *P. aeruginosa* (Figure 21) [152]. With these two parallel lines of inquiry in mind, we decided to examine other DGCs and PDEs (CdgJ and RocS) that were previously found to regulate motility. We were also interested in determining if there was specificity in DGCs or PDEs that regulate FlrA activity as we expected CdgE and CdgA (which do not regulate *V. cholerae* motility) to not regulate FlrA activity [241]. Therefore, we constructed Δ*cdgE*, Δ*cdgJ*, Δ*rocS*, and Δ*cdgA* mutants in WT, Δ*flhA*, and Δ*flhG* backgrounds before electroporating in either a *flrA-lacZ* reporter (to measure FlrA levels) and a *flrB-lacZ* reporter (to measure FlrA activity). We then performed β-galactosidase assays to measure *flrA-lacZ* expression or *flrB-lacZ* expression in these mutants.

We observed that Δ*cdgE*, Δ*cdgJ*, Δ*rocS*, and Δ*cdgA* mutants did not alter *flrA-lacZ* expression relative to WT (Figure 22). Similarly, we found that Δ*cdgE*, Δ*cdgJ*, Δ*rocS*, and Δ*cdgA* mutants in Δ*flhA* or Δ*flhG* backgrounds did not alter *flrA-lacZ* expression relative to Δ*flhA* or Δ*flhG* mutants alone (Figure 22). Δ*cdgE*, Δ*cdgJ*, Δ*rocS*, and Δ*cdgA* mutants alone did not alter *flrB-lacZ* expression relative to WT (Figure 22). It is possible we would only see an effect with these DGCs or PDEs in double or triple mutants (similar to how CdgA, CdgL, and CdgO together altered c-di-GMP levels in a Δ*flhA* mutant). In a Δ*flhA* background, the Δ*cdgE* and Δ*rocS* mutants modestly increased *flrB-lacZ* expression relative to Δ*flhA* alone, which

may suggest an unexpected synergistic effect between Δ*cdgE* and Δ*rocS* mutants and Δ*flhA*. Since Δ*cdgE* Δ*flhA* and Δ*rocS* Δ*flhA* were unable to decrease *flrB-lacZ* expression to WT levels, which we would expect if they mediated c-di-GMP-dependent regulation of FlrA activity in Δ*flhA*, a c-di-GMP-independent mechanism likely explains the increase in FlrA activity in Δ*flhA*.

In Figure 18, Δ*flhG* showed a decrease in *flrB-lacZ* expression relative to WT. A Δ*flhA* Δ*flhG* double mutant increased *flrB-lacZ* expression above Δ*flhA* alone, which suggests that Δ*flhA* and Δ*flhG* may also have a synergistic effect (Figure 22) that is not explained by our model (Figure 19). Unexpectedly, while deletion of *flhG* alone did not increase *flrB-lacZ* expression relative to WT, deleting *cdgE* or *rocS* in the Δ*flhG* background increased *flrB-lacZ* expression more than 8-fold and 4-fold over WT, respectively (Figure 22).

These observations were surprising to us. We did not expect Δ*cdgE* Δ*flhG* to have an effect on *flrB-lacZ* expression as Δ*cdgE* did not alter *V. cholerae* motility [241]. However, this outcome suggests an interesting avenue to explore as *cdgE* expression is σ^{28}-dependent and thus requires flagellar rod and hook formation [39]. It would be interesting to determine if *rocS* expression is disrupted in Δ*flrB* and Δ*flhA* mutants. It is possible that mutating *cdgE* and *rocS* does not have an effect in Δ*flhA* if *cdgE* and *rocS* expression is already repressed indirectly in Δ*flhA* as σ^{28} activity is dependent on FlrBC, σ^{54}, and FlhA (See Section 1.1.1.6 and Figure 9). CdgE is a suspected DGC although its activity has not been demonstrated *in vitro* [241]. It is important to determine if the CdgE GGDEF domain is catalytically active or if is retained as a c-di-GMP-binding domain because Δ*cdgE* Δ*flhG* and Δ*rocS* Δ*flhG* both increase *flrB-lacZ* expression relative to Δ*flhG* alone [241]. RocS has been described as a PDE and so we did not expect the *cdgE* and *rocS* mutants to behave similarly in a Δ*flhG* background [241].

4.6 *Closing thoughts*

It is unclear to us whether the increase in *flrB-lacZ* expression we observe in Δ*flhA*, Δ*flhG* Δ*rocS*, and Δ*flhG* Δ*cdgE* (Figure 22) is due to disruption of a single, very complex

c-di-GMP-dependent flagellar feedback-inhibition pathway or rather involves two flagellar feedback-inhibition pathways: a FlhA-centered c-di-GMP-independent pathway and a c-di-GMP-dependent pathway involving FlhG, CdgE, and RocS. Mutating *flhA* does not appear to increase FlrA activity directly via c-di-GMP-signaling as the c-di-GMP-blind $FlrA_{R176H}$ mutant and DGC or PDE mutants in a Δ*flhA* background were unable to reduce FlrA activity to WT levels (Figure 21 and Figure 22). These data suggest that a c-di-GMP-independent mechanism is likely responsible for the increase in FlrA activity we observed in Δ*flhA* and supports the two flagellar feedback-inhibition pathways hypothesis. However, we know that *cdgE* expression is σ^{28}-dependent [39]. This opens the possibility that by disrupting early flagellar formation in Δ*flhA*, the transcription of genes encoding DGCs and PDEs linked to the flagellar transcriptional cascade such as the σ^{28}-dependent expression of *cdgE* (and possibly *rocS*) were repressed, which would support the one flagellar feedback-inhibition pathway hypothesis. Only until we examine if Δ*flhA* is having an effect on *cdgE* and *rocS* transcription, can we determine if our data is indicative of one or two negative flagellar feedback-inhibition pathways. Additionally, we could engineer *V. cholerae* strains with constitutive expression of *cdgE* and *rocS* in Δ*flhA* mutant to determine if c-di-GMP plays a role in influencing FlrA activity in a Δ*flhA* mutant.

Whether c-di-GMP regulates FlrA activity in the Δ*flhG* mutant as part of a flagellar feedback-inhibition pathway remains an open question. The increase in *flrB-lacZ* expression in a $flrA_{R176H}$ Δ*flhG* double mutant suggests that Δ*flhG* may be repressing FlrA activity through c-di-GMP (Figure 21). Additionally, our data suggests that CdgE and RocS are important for regulating FlrA activity in Δ*flhG* (Figure 22), but DGC activity has not been demonstrated for CdgE *in vitro* and RocS likely functions primarily as a PDE [241].

Reconciling CdgE and RocS with the role *V. cholerae* FlhG likely has in repressing FlrA activity based on the FleN/FleQ regulatory paradigm in *P. aeruginosa* has been challenging [37, 114, 148]. *P. aeruginosa* FleN binds to FleQ and enhances c-di-GMP inhibition of FleQ activity via the AAA+ ATPase domain without altering FleQ DNA binding activity [149, 150, 154]. A similar role for *V. cholerae* FlhG on FlrA activity has not been demonstrated although

c-di-GMP does appear to inhibit FlrA activity by binding to the AAA+ ATPAse domain [152]. Whether or not FlhG enhances or represses c-di-GMP binding to *V. cholerae* FlrA has not yet been examined. We may need to test our model for a flagellar feedback-inhibition pathway in *P. aeruginosa*, both because more is known about how FleN regulates FleQ activity and *P. aeruginosa* possesses a different c-di-GMP signaling network that might make it easier to link early flagellar formation to specific DGCs or PDEs (See Section 5.3) [149–152]. Comparing flagellar feedback-inhibition in *V. cholerae* and *P. aeruginosa* may help us identify conserved or species-specific DGCs or PDEs in each species to resolve and refine our model (Figure 19) and explain our early observations in *V. cholerae* regarding altered FlrA activity in Δ*flhA* and Δ*flhG* mutants.

5

CONCLUSIONS AND FUTURE DIRECTIONS

My thesis work has contributed to our understanding of how polarly-flagellated bacteria use MS ring-rotor-fT3SS assembly to both stimulate the expression of downstream flagellar genes and repress early flagellar gene expression via a feedback-inhibition pathway. These findings suggest that MS ring-rotor-fT3SS formation is a major signaling event across many polar flagellates, a role that was not characterized previously. My thesis work has also shown that there are more species-specific differences in how polarly-flagellated bacteria regulate flagellar gene expression than we had appreciated previously, both in how they stimulate flagellar gene expression through distinct TCSs and in how some polar flagellates repress flagellar gene expression through novel feedback-inhibition pathways. Understanding the intricate and different transcriptional steps bacteria use to build flagella not only informs our understanding of how some bacterial pathogens build an essential machine to cause human disease, but also informs our broader understanding of how bacteria evolved transcriptional cascades around flagellar synthesis to transition between motile and sessile lifestyles.

5.1 *Finding the signal sensed by polar flagellar TCSs*

When work on this thesis began, we knew that several polar flagellates required TCSs for σ^{54}-dependent rod and hook gene expression including *C. jejuni*, *V. cholerae*, *P. aeruginosa*, and *H. pylori* [37, 38, 114, 248]. After determining that the *C. jejuni* FlgSR TCS detects formation of the MS ring-rotor-fT3SS complex, we were interested in determining if other polar flagellar TCSs also appear to detect this stage of flagellar formation [44]. Once we found that *V. cholerae* FlrB and *P. aeruginosa* FleS both likely detect MS ring-rotor-fT3SS formation (Section 3.3 and Section 3.4), we then sought to understand how *C. jejuni* FlgS and *V. cholerae* FlrB

detect MS ring-rotor-fT3SS formation and which regions of *C. jejuni* FliF and FliG contribute to a signal detected by FlgS. These data did not clearly establish a model for how FliF and FliG stimulate FlgSR- and FlrBC-dependent gene expression, but did provide us some valuable insights.

5.1.1 *Examining the sensor regions of C. jejuni FlgS and V. cholerae FlrB*

The FlgS sensor region comprises the first 131 N-terminal amino acids and contains a predicted coiled-coil domain (Figure 6). The FlgS sensor region does not closely resemble either the *V. cholerae* FlrB or *P. aeruginosa* FleS sensor regions (Figure 6). We initially generated FlgS mutants that deleted 10 amino acids sequentially starting with $flgS_{\Delta 2-10}$ to $flgS_{\Delta 120-131}$. We found that $flgS_{\Delta 41-50}$ abolished FlgSR-dependent gene expression without negatively impacting FlgS stability via immunoblot (data not shown). $FlgS_{41-50}$ falls within the predicted coiled-coil domain, suggesting that the coiled-coil domain was required for FlgS sensing activity. We made a series of double alanine substitutions starting with $flgS_{N41A-Y42A}$ to $flgS_{V49A-D50A}$ and found that every mutant abrogated FlgSR-dependent gene expression without altering stability, which suggests that these mutations are likely disrupting the coiled-coil domain rather than key amino acids essential for sensing and that the coiled-coil domain is likely the key sensing region of FlgS (data not shown).

The FlrB sensor region comprises the first 133 N-terminal amino acids and contains a predicted PAS domain, which is also present in the *P. aeruginosa* FleS sensor region (Figure 6). We generated FlrB mutants that deleted 10 amino acids sequentially starting with $flrB_{\Delta 2-10}$ to $flrB_{\Delta 120-133}$. However, all mutants decreased FlrB stability as measured by immunoblot except $flrB_{\Delta 120-133}$, which could have impaired the HisKA domain (data not shown). Thus, it is difficult to make conclusions about which parts of the *V. cholerae* FlrB sensor domain are important for detecting a signal.

5.1.2 *FliF-FliG interface, stability, and multimerization influence C. jejuni FlgS activity*

We made a series of alanine-scanning mutants that covered the cytoplasmic C-terminus of *C. jejuni* FliF and the N-terminus of *C. jejuni* FliG and examined the effect of these mutations on FlgSR-dependent gene expression in order to find areas of these proteins that contribute to a signal detected by FlgS. Most FliF or FliG mutants that decreased FlgSR-dependent gene expression reduced FliF or FliG stability. The FliF and FliG mutants that did decrease FlgSR-dependent gene expression, but did not alter FliF or FliG stability fell within three α-helical domains in FliF and eight α-helical domains in FliG. These FliF and FliG α-helices both form interactions between between FliF and FliG and the interfaces between FliF monomers in the MS ring or FliG monomers in the rotor based on a crystal structure of FliF and FliG in *H. pylori* [249]. This confirmed our early observation that FliF and FliG multimerization was ultimately important for FlgS activity and not FlgS interacting with FliF or FliG monomers [44]. In order to separate residues important for MS ring and rotor formation from those important for MS ring and rotor interaction with FlgS, we will likely need a crystal structure of FlgS bound to FliF and FliG.

5.1.3 *Identifying a signal for the V. cholerae FlrBC TCS*

Although we have evidence that *V. cholerae* likely detects FliF and or FliG multimerization as a cue to initiate signal transduction and σ^{54}-dependent rod and hook gene expression, we have been unable to demonstrate that *V. cholerae* FlrB interacts with FliF or FliG (data not shown). Given how distinct the sensor domain of *V. cholerae* FlrB is from *C. jejuni* FlgS, it is entirely possible that FlrB detects a different signal linked to MS ring-rotor-fT3SS assembly. An important possiblity to consider is that *V. cholerae* FlrB might detect FliF and FliG multimerization indirectly through another factor.

An experiment we have not performed, but could be useful, is a transposon mutagenesis screen in *V. cholerae* to identify mutants that increase FlrBC-dependent gene expression, espe-

cially in a rod or hook deletion mutants to prevent hook assembly from initiating the next stage of flagellar gene transcription. A transposon mutagensis screen could identify a protein acting as a direct cue for FlrB or a protein that generates a small molecule to act as a signal for FlrB that is dependent on FliF and FliG multimerization. We could also perform a mass-spectrometry analysis of the cytoplasmic fraction of WT *V. cholerae*, Δ*fliF*, and Δ*flgB* to identify any small molecules or proteins that decreases in concentration in Δ*fliF* relative to WT that also increases in concentration in Δ*flgB*. We assume that FlrB signal may accumulate if we prevent flagellar assembly from proceeding to rod and hook assembly. If we identified a likely factor, we could then perform an *in vitro* phosphorylation assay with purified FlrB and FlrC to demonstrate an increase in FlrB autophosphorylation and subsequent phosphotransfer to FlrC in the presence of such a factor.

5.2 *FlhG and the unique polar flagellar regulatory checkpoint*

We demonstrated that *V. cholerae* transcriptional reprogramming mutants could successfully build flagella while bypassing the unique polar flagellar regulatory checkpoint involving FlrB detection of fT3SS formation, but *V. cholerae* FlhG was required for this bypass (See Section 3.6). Disrupting FlhG (and presumably altering FlhF activity as well) abolished flagellation when the polar flagellar regulatory checkpoint was bypassed. There are several reasons why flagellar assembly may collapse in a *V. cholerae* transcriptional reprogramming mutant when *flhG* is removed. The simplest explanation is a stoichiometric defect. By placing rod and hook genes under the control of the *fliE* promoter, their expression may have decreased relative to their native expression. This speculation is based off of the relative strengths of the *fliEp-lacZ* and *flgB-lacZ* reporters used in our β-galactosidase assays where *fliEp-lacZ* expression was lower in WT *V. cholerae* than *flgB-lacZ*. Rod and hook gene expression in WT *V. cholerae* may be above the minimum required to build a single polar flagellum and reducing rod and hook gene expression from the *fliE* promoter might not produce a visible phenotype. This decrease in rod and hook expression may be consequential when FlhG is disrupted. A *V. cholerae* Δ*flhG* mutant produces a pronounced hyperflagellation phenotype without showing an increase in

flagellar gene expression, which may mean that most flagellar components expressed at a level necessary to build multiple polar flagella. However, by decreasing rod and hook gene expression under the *fliE* promoter and by increasing flagellar number in a Δ*flhG* mutant, flagellar assembly may collapse as there may be too few rod and hook components split across an excess of nascent flagellar fT3SS complexes.

We do not understand how bypassing the MS ring-rotor-fT3SS regulatory checkpoint affects the speed at which flagella are built or any transcriptional steps tied to specific stages of flagellar assembly. We found that MS ring-rotor-fT3SS assembly seemed to acts a feedback-inhibition pathway that represses FlrA- and σ^{54}-dependent early flagellar gene expression through several unknown mechanisms, one of which may involve FlhG (Chapter 4). It is possible that allowing rod and hook expression to occur simultaneously with MS ring, C ring, and fT3SS genes, reduces the amount of time MS ring-rotor-fT3SS complex assembly can participate in a negative feedback mechanism before rod and hook assembly completes. Since a *V. cholerae* transcriptional reprogramming mutant in a WT background is able to produce flagella as well as a WT strain, we assume that the MS ring-rotor-fT3SS feedback-inhibition pathway is either functioning normally or some other FlrA regulatory mechanism is compensating. Reprogramming mutants in a *V. cholerae* Δ*flhG* background may have a disrupted feedback-inhibition pathway that leads to a scenario where FlrA activity remains high and unable to be repressed so that flagellar assembly collapses either because of unwanted protein aggregations or too few flagellins are expressed for the number of nascent flagellar structures being built.

5.2.1 *The evolution of polar flagella, FlhF and FlhG, and the unique polar flagellar checkpoint*

The purpose of the a polar regulatory checkpoint that links MS ring-rotor-fT3SS assembly to flagellar rod and hook gene expression could lie in the evolution of different flagellation patterns among polar flagellates. Polar flagellates may have developed from a peritrichous progenitor, but could also have developed independently. Comparisons between *C. crescentus* with

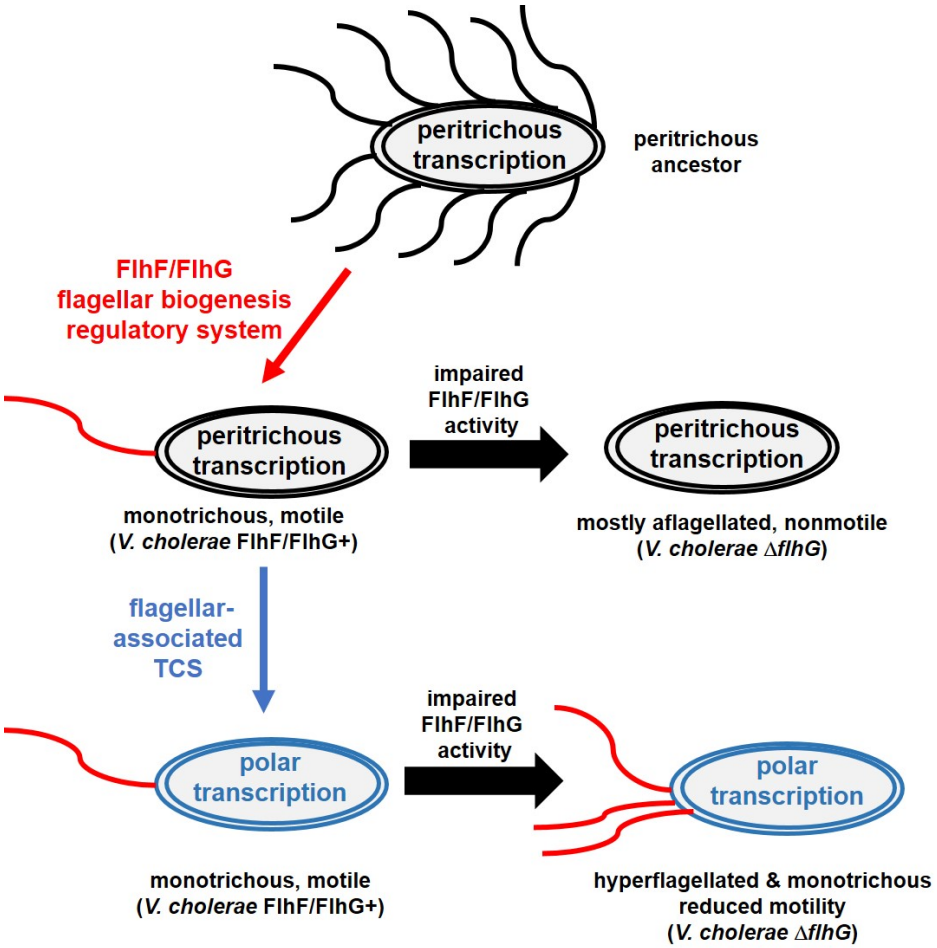

Figure 23: **Model for the influence of flagellar transcriptional and biogenesis regulatory systems on polar flagellation and potential evolution of polar flagellates.** To transition an ancestor with peritrichous flagellation and a peritrichous flagellar transcriptional program to a polar flagellate, the ancestor likely evolved or acquired a FlhF/FlhG flagellar biogenesis regulatory system. This species is best represented by a monotrichous *V. cholerae* mutant containing a FlhF/FlhG system and a peritrichous transcriptional program in this work. This bacterium is dependent on a FlhF/FlhG system to produce flagella as a mutation of *flhG* caused aflagellation. Development of a flagellar-associated TCS to function with MS ring, C ring, and fT3SS proteins to reorder transcription of flagellar rod and hook genes after formation of a competent fT3SS would continue to allow for monotrichous flagellation (resembling modern WT *V. cholerae* in this study), but also maintain production of flagella, albeit with a hyperflagellation phenotype, and modest motility when the FlhF/FlhG system is partially impaired (as indicated by *V. cholerae* Δ*flhG*). This combination of a functional FlhF/FlhG system with a polar flagellar transcriptional programw ould provide a fitness advantage over a species with a FlhF/FlhG system and a peritrichous flagellar transcriptional program that is aflagellate and non-motile with any alterations in FlhF and/or FlhG activity.

many other Gram-negative polar flagellates (as discussed in Section 3.7) clearly show that different polar flagellation systems exist with more similar regulatory mechanisms to facilitate the polar flagellar transcriptional program, suggesting possible convergent evolution of polar flagellates. Regardless, our findings suggest that a species needs to acquire a polar flagellar biogenesis system (such as the FlhF/FlhG system) and a mechanism to order flagellar genes for the polar flagellar transcriptional program (such as a flagellar-associated TCS) to become an efficient polar flagellate.

It is unknown which came first in a polar flagellate, the FlhF/FlhG polar flagellar biogenesis system or the flagellar-associated TCSs to drive the polar flagellar transcriptional program. Both the FlhF GTPase and FlhG ATPase are members of the SIMIBI-class of nucleotide-binding proteins that commonly function in cellular organization and protein targeting [30, 166]. FlhF is related to the Ffh GTPase of the signal-recognition particle system, whereas FlhG is closely associated with the MinD and ParA ATPases that generally perform partitioning functions related to division and DNA segregation [30, 106, 166]. Development of the FlhF/FlhG flagellar biogenesis regulatory system, perhaps from Ffh and MinD/ParA superfamilies, could have caused the emergence of a polar flagellate in a Gram-negative organism. The motile, monotrichous *V. cholerae* strain we engineered with an intact FlhF/FlhG system and a peritrichous flagellar transcriptional program might resemble this ancestor (Figure 23). As revealed in this work, this bacterium is heavily reliant on a precisely functioning FlhF/FlhG system to form any flagella and retain some level of motility; perturbations to FlhF or FlhG activity severely reduces or completely abolishes flagellation.

By possessing a mechanism mediated by the TCS to order rod and hook gene transcription after production of MS ring, rotor, and fT3SS proteins (and possibly assembly of a functional fT3SS), a bacterium can produce polar flagella with some alterations to FlhF/FlhG activity. In this bacterium, an optimally functioning FlhF/FlhG system allows for correct number and placement of polar flagella and WT motility; an impaired FlhF/FlhG system (at least by altering FlhG) results in polar flagellation with extra flagella produced and at least modest

motility. This hyperflagellated bacterium has an advantage over one with the a peritrichous program that cannot maintain flagellation and motility with perturbations to the FlhF/FlhG system. Modulations in FlhF and FlhG activity in different species with flagellar-associated TCSs to maintain the polar flagellar transcriptional program and flagellar biogenesis may have facilitated the emergence of different polar flagellation patterns. An example of this is *C. jejuni* and *H. pylori*, which while closely related, produce amphitrichous and lophotrichous flagella, respectively, yet have the FlhF/FlhG flagellar biogenesis regulatory system and similar FlgSR TCS. A study comparing FlhF and FlhG biochemical activity and biological function between these two bacterial species has not been conducted such as a study examining differences in FlhF and FlhG interactions and activity and their impact on amphitrichous or lophotrichous flagellation. Although many details remain to be discovered for how FlhF and FlhG function in polar flagellates, our results indicate regulatory links between the FlhF/FlhG flagellar biogenesis regulatory systems and the order of flagellar protein production controlled by the TCS for polar flagellar biogenesis.

5.3 *Flagellar feedback-inhibition in P. aeruginosa*

One of the advantages for bacteria placing early flagellar gene expression under the control of a master regulator such as $FlhD_4FlhC_2$ in *E. coli* and *Salmonella*, FlrA in *V. cholerae*, and FleQ in *P. aeruginosa* is that it allows for conditional transcription of flagella. Whereas *C. jejuni* appears to be locked into perpetual expression of flagellar components, other flagellated bacteria can limit flagellar gene expression based on environmental conditions or to promote a sessile lifestyle within a biofilm. I provided evidence in Chapter 4 that flagellar formation may inhibit *V. cholerae* gene expression by repressing FlrA activity via one or two feedback-inhibition pathways (a FlhA-centered pathway and a FlhG, RocS, and CdgE-centered pathway). While it currently unclear how either pathway functions in *V. cholerae*, we suspect analogous flagellar feedback-inhibition pathways exist in *P. aeruginosa*. The *P. aeruginosa* FlhG homologue, FleN, promotes c-di-GMP-mediated repression of FleQ activity by increasing FleQ sensi-

tivity to c-di-GMP binding [149, 150].

An unexpected finding in our investigation into whether or not MS ring-rotor-fT3SS formation was required for *P. aeruginosa* FleSR- and σ^{54}-dependent gene expression was that Δ*fliM* showed a 3-fold increase in *flgB-lacZ* expression relative to WT (Figure 11). We did not observe a similar effect on FlgSR-dependent gene expression in *C. jejuni* Δ*fliM* (Figure 10) or FlrBC-dependent gene expression in *V. cholerae* Δ*fliM* (Figure 9). This striking increase in FleSR-dependent gene expression in *P. aeruginosa* Δ*fliM* suggests two possible mechanisms. FliM binds to FliG in the C ring and FliM binding could alter the conformation of FliG in the C ring to shut off the signal formed by MS ring-rotor-fT3SS formation likely detected by FleS. Another possibility is that FliM could play a role in repressing FleQ activity by altering c-di-GMP levels as part of a flagellar feedback-inhibition pathway and Δ*fliM* increases FleQ-dependent expression of *fleS* and *fleR*, which leads to an increase in FleSR-dependent gene expression. For these reasons, FliM may be an important piece in unraveling how early flagellar biogenesis in *P. aeruginosa* regulates flagellar gene expression, perhapbs by FliM incorporation into the C ring by stimulating DGC or PDE activity.

5.3.1 *DGCs that influence flagellar motility in P. aeruginosa*

We began our analysis of DGCs and PDEs that could play a role in our flagellar inhibition pathway by first examining DGCs and PDEs mutants that altered *V. cholerae* motility. Similar analyses in *P. aeruginosa* have identified DGCs and PDEs mutants that altered *P. aeruginosa* motility, only one of which (a RocS homologue ProE), resembles any of the *V. cholerae* DGCs and PDEs that we examined [250]. We were able to identify a *P. aeruginosa* RocS homologue ProE, but were unable to identify a *P. aeruginosa* DGC that resembles CdgE [251, 252]. This suggests that if *P. aeruginosa* flagellar formation inhibits early flagellar gene expression by repressing FleQ activity through FleN, *P. aeruginosa* may use both a PDEs conserved with *V. cholerae* (ProE) and possibly some species-specific DGCs or c-di-GMP binding proteins.

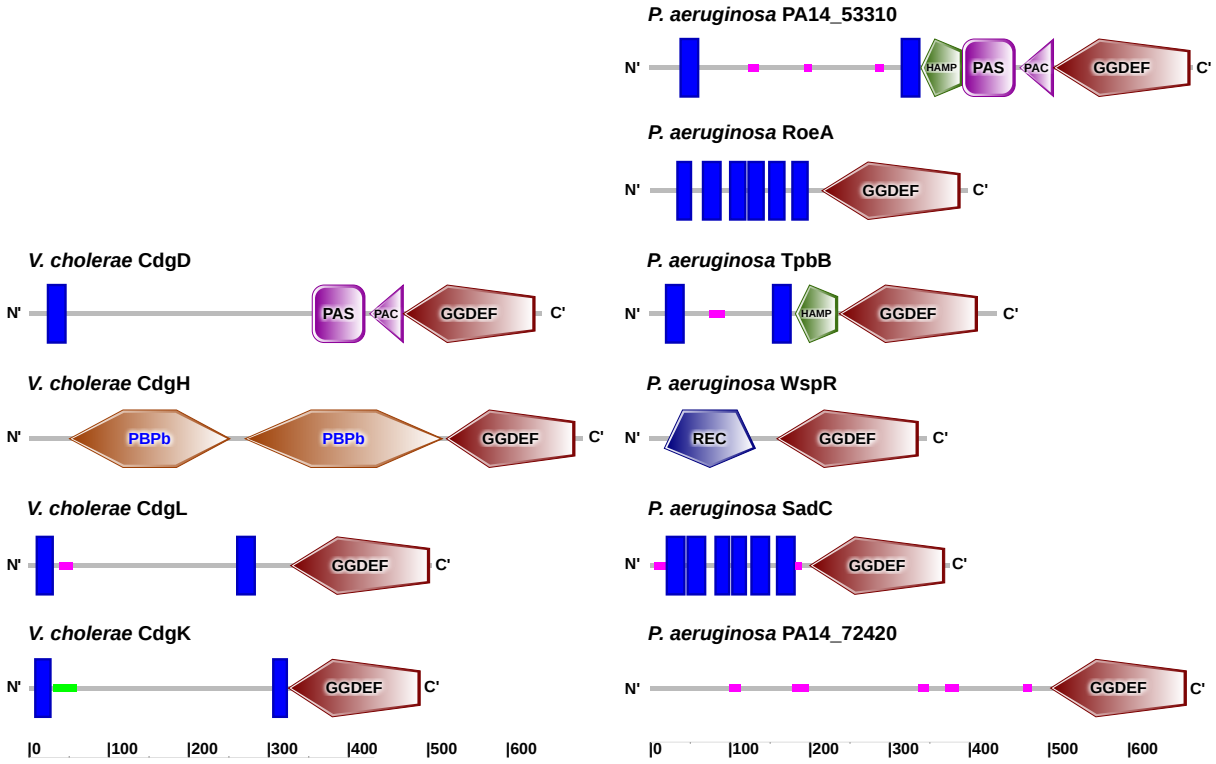

Figure 24: **Domain analysis of proteins with GGDEF and EAL domains that repress *V. cholerae* or *P. aeruginosa* motility.** Protein domains shown are predicted by SMART. *V. cholerae* CdgD, CdgH, CdgL and CdgK and *P. aeruginosa* PA14 53310, RoeA, TpbB, WspR, SadC, PA14 72420 have GGDEF domains, which are represented as elongated brown pentagrams (pointing right to left), are required for c-di-GMP synthesis via DGC activity. Transmembrane regions in *V. cholerae* CdgD, CdgL, CdgK and *P. aeruginosa* PA14 53310, RoeA, TpbB, and SadC are represented as blue rectangles. *V. cholerae* CdgD and *P. aeruginosa* PA14 53310 have PAS domains which are represented as purple squares. The PAS domain-associated PAC domain is represented as a purple triangle in *V. cholerae* CdgD and *P. aeruginosa* PA14 53310. Prokaryotic periplasmic binding protein domains (PBPb) in CdgH are represented as elongated brown hexagons. HAMP-linkers (described in Section 1.2.1) found in *P. aeruginosa* PA14 53310 and TpbB are represented as green pentagrams. *P. aeruginosa* WspR has a REC domain (described in Section 1.2.1), which is represented by a blue pentagram. Coiled-coil regions of *V. cholerae* CdgK are represented with a green rectangle (described in Section 3.2). Areas of low structural complexity or that have an "unknown region" according to SMART are represented with pink rectangles in *V. cholerae* CdgL and *P. aeruginosa* PA14 53310, TpbB, SadC, and PA14 72420.

The *P. aeruginosa* PA14 genome contain 17 genes with a GGDEF domain, 5 genes with an EAL domain, and 16 genes with both GGDEF and EAL domains [250]. Overexpression of PA3702 (*wspR*), PA5487 (*PA14 72420*), PA0847 (*PA14 53310*), PA1120 (*tpbB*), PA4332 (*sadC*), PA1107 (*roeA*), and PA1727 reduced swimming motility in *P. aeruginosa* (Figure 24) [250]. These can further be divided into probable cytoplasmic- and membrane-localized groups based on the presence of transmembrane domains. WspR and PA14 72420 lack any predicted transmembrane domains and are likely cytoplasmic while PA14 53310, RoeA, TpbB, and SadC are likely attached to the inner membrane. How cytoplasmic DGCs can generate a specific c-di-GMP-mediated response apart from simply increasing global c-di-GMP levels is an open question in the c-di-GMP field. One possibility is that cytoplasmic DGCs interact with a protein whose activity is regulated by c-di-GMP directly (bypassing the need to alter c-di-GMP levels locally or globally) or that cytoplasmic DGCs may interact with a membrane bound protein to "hitch" a ride to a specific area of the bacterial cell to establish a local c-di-GMP microgradient. WspR is a cytoplasmic GGDEF-containing protein that resembles the CheY RR [253]. WspR phosphorylation at its REC domain increases its DGC activity, similar to the role REC domains play in DNA-binding RRs (See Section 1.2.2). A Δ*wspR* mutant does not alter *fleQ* expression or flagellin expression, but increases *P. aeruginosa* motility through unknown means [236]. The other cytoplasmic GGDEF-containing protein, PA14 72420, has not been well characterized to date apart from appearing in screens of GGDEF and EAL containing proteins that alter motility and biofilm formation [250, 254].

Of the membrane-bound proteins with GGDEF and EAL domains that alter motility in *P. aeruginosa*, the least is known about PA14 53310. PA14 53310 is an enzymatically active DGC and a Δ*PA14 53310* mutant shows increased swimming motility, but there is no apparent change in biofilm formation [255]. TpbB has a periplasmic region that likely links periplasmic stimuli to cytoplasmic c-di-GMP levels and is uniquely regulated by serine/tyrosine phosphatase TpbA, which controls TpbB DGC activity through Ser/Thr phosphorylation [256–258]. SadC plays an important role in the ability of *P. aeruginosa* to detect surfaces via pili as contact with surfaces stimulates an increase in c-di-GMP through SadC activity which promotes biofilm formation

and represses motility [228, 254, 259]. SadC membrane localization seems important for SadC DGC activity as SadC mutants that lack its transmembrane regions display reduced DGC activity [260]. SadC interacts with the alternate stator MotC (MotCD stators replaces MotAB stators when *P. aeruginosa* comes in contact with a surface) and further increases c-di-GMP levels when *P. aeruginosa* interacts with a surface [147]. Not as much is known about RoeA, but interestingly, both Δ*sadC* or Δ*roeA* deletion mutants lead to a nearly 50% decrease in c-di-GMP levels relative to WT *P. aeruginosa*. However, Δ*sadC* leads to a pronounced hypermotile phenotype, while Δ*roeA* mutant has a more modest hypermotile phenotype and near total loss of *pel* transcription, which may be a good example of the specificity DGCs have in regulating particular phenotypes in *P. aeruginosa* [261]. Future work should examine the effect of these DGCs on FleQ activity or if these DGCs play a role in the increase in FleSR-dependent gene expression we observe in *P. aeruginosa* Δ*fliM*

5.4 *The polar flagellum as a signaling platform*

This thesis project began as a project where I wanted to understand how some polar flagellates use TCSs to enable σ^{54}-dependent rod and hook gene expression. We were interested in this question because flagella are critical for virulence in many human pathogens and figuring out how polar flagellar TCSs function would unravel a major step in flagellar biogenesis and transcription. Over time my interests gradually shifted. It became clear that the MS ring-rotor-fT3SS likely directly or indirectly stimulated *V. cholerae* FlrBC- and *P. aeruginosa* FleSR-dependent flagellar gene expression, which meant that this early step in flagellar assembly was broadly acting as an important cue to stimulate further flagellar gene expression as we had previously observed in *C. jejuni* [44]. When I demonstrated that the unique polar regulatory checkpoint in *V. cholerae* could be bypassed when the FlhFG system remained intact, I began to shift my focus from how bacterial signaling mechanisms lead to the completion of flagellar assembly to how the polar flagellum acts acting as an important signaling platform itself. Perhaps some of the value of polar flagellates having an additional step in flagellar gene transcription relative to peritrichous bacteria has less to due with these additional steps being necessary for flagellar

assembly because of some structural reason, but that it may enable MS ring-rotor-fT3SS formation to be linked to transcriptional events, such as rod and hook gene expression. I found evidence for a novel way that MS ring-rotor-fT3SS may influence flagellar gene expression when I observed that *V. choleare* FlrB expression increased in MS ring-rotor-fT3SS mutants. This suggested that MS ring-rotor-fT3SS formation was stimulating an undescribed signaling pathway not necessary for flagellar biogenesis, but rather by repressing early flagellar gene expression as a feedback-inhibition pathway. Through further experimentation, we developed an initial hypothesis where MS ring-rotor-fT3SS represses FlrA activity via c-di-GMP. *V. cholerae* FlhG, RocS, and CdgE may function together to regulate FlrA activity through an unknown c-di-GMP-dependent mechanism. While this flagellar feedback-inhibition pathway needs more characterization, I propose that this is a good example of how polar flagella can act as a signaling platform that stimulates bacterial signaling pathways not necessarily tied to flagellar biogenesis, but to motile and sessile lifestyles, which remain a less explored area of flagellar biology.

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