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Spatial and Numerical Regulation of Flagellar Biosynthesis in Polarly-Flagellated Bacteria

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Summary

Control of surface organelle number and placement is a crucial aspect of the cell biology of many Gram-positive and Gram-negative bacteria, yet mechanistic insights into how bacteria spatially and numerically organize organelles are lacking. Many surface structures and internal complexes are spatially restricted in the bacterial cell (e.g., Type IV pili, holdfasts, chemoreceptors), but perhaps none show so many distinct patterns in terms of number and localization as the flagellum. In this review, we discuss two proteins, FlhF and FlhG (also annotated FleN/YlxH), which control aspects of flagellar assembly, placement, and number in polar flagellates, and may influence flagellation in some bacteria that produce peritrichous flagella. Experimental data obtained in a number of bacterial species suggest that these proteins may have acquired distinct attributes influencing flagellar assembly that reflect the diversity of flagellation patterns seen in different polar flagellates. Recent findings also suggest FlhF and FlhG are involved in other processes, such as influencing the rotation of flagella and proper cell division. Continued examination of these proteins in polar flagellates is expected to reveal how different bacteria have adapted FlhF or FlhG with specific activities to tailor flagellar biosynthesis and motility to fit the needs of each species.

Keywords

FlhF; FlhG; FleN; polar flagella

Introduction

Flagella are complex molecular machines that facilitate swimming motility in many bacteria. Bacteria evolved elegant transcriptional control mechanisms to ensure temporal production of subsets of flagellar proteins conducive for proper flagellar biosynthesis (for extensive reviews, see (Chilcott and Hughes, 2000; Chevance and Hughes, 2008)). Although core flagellar components and basic principles for regulating flagellar gene expression are fairly conserved across motile bacteria, bacteria produce different flagellation patterns characteristic for each species. These patterns range from peritrichous flagellation (production of many flagella along the cell body) to polar flagellation (production of flagella exclusively at bacterial poles). Among polar flagellates, flagellation patterns include monotrichous (a single flagellum at one pole), amphitrichous (a flagellum at each pole), or lophotrichous flagellation (a few flagella at a single pole). Determining how bacteria -

particularly polar flagellates - exquisitely control spatial and numerical parameters of flagellar biosynthesis is one of the more recent challenges in the field of bacterial motility. Two proteins implicated in spatial and numerical control of flagellar biosynthesis in polar flagellates are the FlhF GTPase and FlhG (a putative ATPase also annotated as FleN or YlxH). Both FlhF and FlhG are members of the SIMIBI class of nucleotide-binding proteins, which often are involved in partitioning or localizing other factors within prokaryotic or eukaryotic cells (Leipe *et al.*, 2002). Recent investigation has revealed that FlhF and FlhG are produced by *B. subtilis* and influence spatial aspects of peritrichous flagellation in this bacterium (Guttenplan *et al.*, 2013). However, these proteins are not produced by other peritrichous organisms, such as *E. coli* or *Salmonella* species, which raises questions regarding how broadly these proteins influence peritrichous flagellation.

Initial studies in *Vibrio cholerae* and *Pseudomonas aeruginosa* suggested that FlhF is involved in spatial regulation of flagellar biosynthesis whereas FlhG numerically controls flagellation (Dasgupta *et al.*, 2000; Dasgupta and Ramphal, 2001; Correa *et al.*, 2005; Murray and Kazmierczak, 2006; Green *et al.*, 2009). However, more recent studies have shown that FlhF and FlhG interact in some species, namely *Campylobacter jejuni*, *Vibrio alginolyticus*, and *B. subtilis* (Parrish *et al.*, 2007; Kusumoto *et al.*, 2008; Bange *et al.*, 2011). Thus, these interactions may influence FlhF and FlhG activities *in vivo* so that each protein can function properly to regulate spatial and numerical parameters of flagellar biosynthesis. Furthermore, data is accumulating that FlhF-FlhG cognate pairs likely possess differing biochemical properties, which may contribute to the different flagellation phenotypes across bacterial species. In this review, we attempt to summarize previous findings regarding the activities of FlhF and FlhG proteins and present new questions for future investigations of how FlhF, FlhG, or pairs of these proteins may mediate spatial and numerical parameters of flagellar biosynthesis and other cellular activities in different polar flagellates.

Biochemical Properties of FlhF and FlhG

FlhF, the signal sequence-binding protein Ffh (SRP54 in Archaea and Eukarya) and the signal-recognition particle (SRP) receptor FtsY (SR α in Eukarya) comprise the SRP-GTPase subfamily of SIMIBI-class of nucleotide-binding proteins (Leipe *et al.*, 2002). In biology, GTPases often function as molecular switches, in which transitions between inactive GDP-bound and active GTP-bound conformations are integral to the regulation of many fundamental cell processes. Ffh (in complex with SRP RNA) and FtsY together constitute the universally conserved machinery that targets ribosomes in complex with nascent proteins to membrane channels for translocation (reviewed in (Doudna and Batey, 2004)). When bound to GTP, the Ffh and FtsY proteins form a heterodimer with a composite active site (Egea *et al.*, 2004). GTP hydrolysis is reciprocally activated within this heterodimer, and SRP RNA and membrane lipids play crucial roles in this activation process (Zhang *et al.*, 2009). Recent elegant work has revealed how dimerization and conformational changes of Ffh and FtsY are integrated with binding of “cargo” (complexes of ribosome and nascent chain protein) and its delivery to the membrane translocation machinery (Saraogi *et al.*, 2011). Much less is known, however, about the regulation and function of the FlhF GTPase.

FlhF proteins share significant homology with Ffh and FtsY within their N and G domains, but have amino-terminal extensions (or B domains) that are unique (Figure 1A). The first and only crystal structure to date of a FlhF family member was solved in 2007 (Bange *et al.*, 2007). In this study, the crystal structure of *B. subtilis* FlhF lacking the B domain (NG-FlhF) formed a composite active site via a dimer interface composed entirely by interactions between G domains. In contrast, the FtsY-Ffh heterodimer interface is mediated by N

domain interactions from each protein and regulated by three motifs that are absent from FlhF.

FtsY and Ffh both possess conserved arginines in the G2 subdomain of the GTPase domain that form an 'arginine finger' to stimulate GTP hydrolysis (Egea *et al.*, 2004). Asymmetry of the side chain conformation of these two arginines within the heterodimer may allow for sequential hydrolysis of nucleotide. No such asymmetry exists within the NG-FlhF homodimer, and the arginine finger is disordered within the GTP bound structure ((Bange *et al.*, 2007); Figure 1A). Subsequently, Bange *et al.* demonstrated that FlhG (originally annotated as YlxH) associates with the GTP-bound NG-FlhF dimer and stimulates its GTPase activity approximately five-fold (Bange *et al.*, 2011). Such regulation of GTPase activity by external factors, though not observed for Ffh-FtsY, is commonly described for eukaryotic signaling GTPases (Bourne *et al.*, 1991). A co-crystal structure consisting of NG-FlhF and the N-terminal 23 residues of FlhG, which are necessary and sufficient for stimulation of GTPase activity, revealed that the Gln8 side chain of FlhG inserts into the composite active site of the NG-FlhF homodimer and repositions the G2 arginine finger residue to stabilize the transition state geometry of the nucleotide substrate ((Bange *et al.*, 2011); Figure 1B). A similar mechanism of activation by regulator of G protein signaling (RGS) proteins occurs for eukaryotic monomeric G α proteins (Ross and Wilkie, 2000). RGS binding locks the flexible switch regions containing catalytic residues of G α into their active conformation with a resulting GTP-hydrolysis rate enhancement of 100- to 1000-fold (Ross and Wilkie, 2000).

Even though FlhG stimulates the GTPase activity of *B. subtilis* FlhF *in vitro*, the consequences of this activation for peritrichous flagellation are not known. In polar flagellates, the ability of FlhG to influence the GTPase activity of FlhF has yet to be explored. Furthermore, the ATPase activity for any FlhG protein of a polar flagellate has yet to be verified. For many polar flagellates, it is not known whether FlhF-FlhG cognate pairs interact *in vivo* and if these interactions influence respective GTPase or ATPase activities to regulate the function of each protein in flagellar assembly or organization. It is possible that differences in specific domains of FlhF and/or FlhG in polar flagellates give rise to different *in vivo* activities such as nucleotide binding or hydrolysis that ultimately result in different flagellation patterns. Overall, the FlhF proteins that have been analyzed in polar flagellates are most similar within their GTPase domains (27–39% identity; 49–62% similarity) and are more diverse in other regions of the proteins. Additionally, FlhG orthologs in polar flagellates display homology (30–59% identity; 55–79% similarity) across approximately 80% of the length of the proteins, with the putative ATPase domains being the most highly conserved (Figure 1B). The FlhG N-terminal extension with the DQAxLR motif encompassing the Gln8 residue is conserved among many FlhG orthologs ((Bange *et al.*, 2011); Figure 1B). However, this N-terminal domain is lacking from the FlhG proteins of the Pseudomonads, Xanthomonads, and Stenotrophomonads. Thus, it is unclear whether *P. aeruginosa* FlhF and FleN (the *P. aeruginosa* FlhG ortholog) do interact and whether FleN or some other protein stimulates the GTPase activity and biological function of FlhF. It is conceivable that FlhF-FlhG cognate pairs may interact in some polar flagellates but not in others, which consequently results in the different flagellation patterns produced by these bacteria.

Contributions of FlhF to Polar Flagellar Biogenesis

FlhF appears to be required for proper spatial arrangement of flagella at the poles in many polar flagellates, but mutation of *flhF* results in additional effects on flagellation in some bacteria (Pandza *et al.*, 2000; Murray and Kazmierczak, 2006; Balaban *et al.*, 2009; Green *et al.*, 2009). *P. aeruginosa* and *Pseudomonas putida* $\Delta flhF$ mutants produce a flagellum that is

no longer restricted to the pole (Pandza *et al.*, 2000; Murray and Kazmierczak, 2006). This misplaced flagellum rotates and powers swimming motility, but swimming paths are no longer straight and motility is impaired as viscosity increases (Murray and Kazmierczak, 2006). Whether this is simply due to the increased drag associated with movement off the longitudinal axis of the cell, or to alterations between the assembled flagellum and proteins that dynamically associate with it (e.g., flagellar motor components) is not known. In other organisms such as *Campylobacter jejuni*, *Vibrio alginolyticus*, and *V. cholerae*, deletion of *flhF* causes aflagellation in most cells, which is likely attributed to the requirement for FlhF to stimulate wild-type levels of flagellar gene expression (Hendrixson and DiRita, 2003; Correa *et al.*, 2005; Kusumoto *et al.*, 2008; Balaban *et al.*, 2009; Green *et al.*, 2009). However, it is not known why FlhF is required for transcription of certain flagellar genes. Of note, in a small number of *V. cholerae flhF* mutant cells that produced a flagellum, the flagellum was not localized to the pole, which was similar to the misplaced flagellum phenotype of a *P. aeruginosa* $\Delta flhF$ mutant (Murray and Kazmierczak, 2006; Green *et al.*, 2009).

For monotrichous flagellation in *V. cholerae*, it is thought that a new flagellum is synthesized at the old pole of a daughter cell after division. Like in other polar flagellates, FlhF is targeted to the bacterial pole in *V. cholerae* (Murray and Kazmierczak, 2006; Kusumoto *et al.*, 2008; Ewing *et al.*, 2009; Green *et al.*, 2009; Kusumoto *et al.*, 2009). Polar localization of FlhF in *V. cholerae* is likely a prerequisite for FlhF mediating the polar localization of FliF, which composes the inner membrane MS ring of the flagellum (Green *et al.*, 2009). The N domain of *V. cholerae* FlhF was required, but not sufficient to localize FlhF to the pole. FlhF truncations lacking the N-terminal B domain or C-terminal G domain were polarly localized in *V. cholerae*, but these proteins did not support motility or recruit FliF to the pole, indicating that all domains are required for FlhF-dependent activities in flagellation (Green *et al.*, 2009). Furthermore, production of a FlhF mutant hindered for GTP binding accumulated a significant level of FliF at a pole, suggesting that the GTPase activity of FlhF is not essential for the polar targeting of FliF. However, it is unclear what FlhF may be recognizing at the pole for its localization or how FlhF mediates the polar localization of the MS ring.

It is not clear why deletion of *flhF* has such varied consequences for flagellar assembly in different polarly-flagellated organisms. Many of these observations are consistent with a model in which FlhF positively influences flagellar assembly, presumably at the site where FlhF itself resides. The work in *V. cholerae* would argue that FlhF might exert its role in many polar flagellates by recruiting components such as FliF to form essential flagellar substructures necessary for initial stages in flagellar biosynthesis (Green *et al.*, 2009). However, a single flagellum - albeit mislocalized - is consistently produced in *flhF* mutants in *Pseudomonas* species, which could reflect a lower barrier to flagellar assembly initiation in these organisms relative to other polar flagellates (Pandza *et al.*, 2000; Murray and Kazmierczak, 2006).

Correlating FlhF GTPase Activity and Function

B. subtilis, *C. jejuni*, and *P. aeruginosa* FlhF proteins bind and hydrolyze GTP *in vitro*, indicating at least a low level of FlhF GTPase activity without any exogenous activating factors (Balaban *et al.*, 2009; Bange *et al.*, 2011; Schniederberend *et al.*, 2013). Additionally, *V. cholerae* FlhF binds GTP, but GTPase activity has yet to be verified (Green *et al.*, 2009). Many works have examined how amino acid mutations in FlhF predicted or documented to alter GTPase activity and/or GTP binding affect flagellation phenotypes or motility. In general, the observations are consistent with the idea that FlhF function, like that of eukaryotic GTPases, is determined by its nucleotide-bound state. However, mutations that

alter GTP binding or hydrolysis by FlhF have vastly different effects on flagellar motility, assembly, number, or placement in different polar flagellates, suggesting that different FlhF proteins may promote create specific flagellation patterns or alter flagellar function that are suitable to the needs of each species.

In *C. jejuni*, mutation of D321 and R324 (the G2 arginine finger residue) in FlhF altered flagellar assembly and reduced GTPase activity, although it is unclear if these FlhF mutants were specifically defective in GTP binding or hydrolysis ((Balaban *et al.*, 2009); Figure 1A). Unlike wild-type *C. jejuni*, which mainly produces bacteria with a single flagellum at one or both poles, these mutants demonstrated diverse flagellation phenotypes ranging from a significant number of wild-type flagellated bacteria, aflagellated bacteria, and bacteria with non-polar flagella (Balaban *et al.*, 2009). Additionally, a small number of bacteria produced extra polar flagella. One interpretation of these data is that the normal GTPase activity of FlhF is required for *C. jejuni* to exclusively construct polar flagella. In contrast, *V. cholerae* FlhF with D321A or R324A mutations, which are also predicted to reduce its GTPase activity, supported motility, while mutations in other residues (K295, D367 and D429) that reduced binding to GTP agarose abolished flagellar assembly and motility (Green *et al.*, 2009). Of note, GFP fusions to FlhF_{K295A} and FlhF_{D367A} localized to the pole, suggesting that polar localization of FlhF was independent of GTP binding or hydrolysis, but GTP binding appears to be required to assemble polar flagella.

In *P. aeruginosa*, FlhF, but not its GTPase activity, is required for polar flagellar assembly. Instead, FlhF GTPase activity affects flagellar function for the swimming motility of *P. aeruginosa*. Point mutations that rendered purified *P. aeruginosa* FlhF enzymatically inactive *in vitro* (K222A and D294A in the G2 and G3 subdomains, respectively), or altered its hydrolytic activity (R251G, the arginine finger residue) all supported polar flagellar assembly in the $\Delta flhF$ mutant background ((Schniederberend *et al.*, 2013); Figure 1A). FlhF_{D294A} did not bind GTP or dimerize, while FlhF_{K222A} bound GTP, but dimerized less well than wild-type FlhF. Single-cell analysis of swimming velocity revealed that FlhF_{D294A} supported faster swimming in a greater proportion of bacteria than FlhF_{K222A}, while FlhF_{R251G}-expressing bacteria were predominantly non-motile. FlhF_{R251G} bound GTP and dimerized, but this mutant protein demonstrated a defect in GTP/GDP exchange, suggesting that the mutation causes a delay in homodimer dissociation and product release (Schniederberend *et al.*, 2013). These findings are consistent with a model in which FlhF in a GTPase-independent manner functions as a “place setter” for flagellar assembly, but the GTPase activity is required for flagellar function as a motility organelle, perhaps via regulating interactions with components of the flagellar motor. Any possible *P. aeruginosa* FlhF interaction partners associated with the flagellar motor, however, remain unknown. Taken together, these varied phenotypes of *flhF* mutants in polar flagellates highlight the diverse means by which FlhF in GTPase-dependent and -independent activities shapes flagellation patterns and/or flagellar function in individual polarly-flagellated species.

Numerical Regulation of Flagellar Biosynthesis

Initial studies of FlhG homologs in various polar flagellates suggested that the protein controls numerical parameters of flagellar biosynthesis. A *P. aeruginosa* mutant lacking *flaN* (an *flhG* ortholog) produced three to six flagella at a pole instead of only one (Dasgupta *et al.*, 2000; Dasgupta and Ramphal, 2001). These flagella conferred movement, but not correct directional swimming. In *V. cholerae*, mutation of *flhG* also caused a switch from monotrichous to lophotrichous flagellation with eight to ten flagella at a single pole and, in some cells, amphitrichous flagellation with flagella at both poles (Correa *et al.*, 2005).

These *P. aeruginosa* and *V. cholerae* *flhN/flhG* mutants overexpressed many classes of flagellar genes, suggesting that hyperflagellation may be a result of increased flagellar protein production (Dasgupta *et al.*, 2000; Dasgupta and Ramphal, 2001; Correa *et al.*, 2005). In *P. aeruginosa*, FleN interacts with the FleQ master transcriptional regulator that governs flagellar gene expression (Dasgupta and Ramphal, 2001), which likely allows FleN to finely control FleQ as a transcriptional activator. An elevated expression of the master transcriptional regulator FlrA (a FleQ ortholog) was observed in *V. cholerae* $\Delta flhG$, which is believed to contribute to overexpression of flagellar genes and hyperflagellation (Correa *et al.*, 2005). Although increased flagellar gene expression via a hyperactive or overexpressed master transcriptional regulator may dysregulate numerical control of flagellation in some *flhG* mutants, this explanation is not valid for all polar flagellates. For example, *C. jejuni* lacks such a master transcriptional regulator yet deletion of *flhG* caused *C. jejuni* cells to shift from producing a single flagellum at one or both poles to assembling multiple polar flagella with only a mild increase in the expression of a subset of flagellar genes (Balaban and Hendrixson, 2011).

Interactions between FlhF and FlhG may occur *in vivo* in some polar flagellates to suggest an alternate hypothesis for numerical control of flagellar biosynthesis. Regulation of flagellar number may be achieved by FlhG modulating some activity of FlhF important for flagellation, such as its GTPase activity, polar localization, or interaction with flagellar components. In most polar flagellates, FlhF positively influences flagellar biosynthesis. FlhF is required for flagellar gene expression in *V. cholerae*, *C. jejuni*, and *H. pylori* (Hendrixson and DiRita, 2003; Niehus *et al.*, 2004; Correa *et al.*, 2005; Balaban *et al.*, 2009), while overexpression of FlhF in *Pseudomonas* and *Vibrio* species causes hyperflagellation that may or may not be restricted to the poles, depending on the species (Pandza *et al.*, 2000; Kusumoto *et al.*, 2006; Green *et al.*, 2009; Schniederberend *et al.*, 2013). In contrast, overproduction of FlhG in *P. aeruginosa* or *V. alginolyticus* reduces flagellation (Dasgupta and Ramphal, 2001; Kusumoto *et al.*, 2006). If FlhG influences FlhF GTPase activity *in vivo*, FlhG modulation of cycles of GTP binding and hydrolysis by FlhF might influence whether FlhF is competent for some step in flagellar assembly, such as polar localization, interaction with flagellar subunits, or nucleating flagellar assembly. Indeed, *V. alginolyticus* FlhG affects the amount of FlhF that can accumulate at a pole (Kusumoto *et al.*, 2008). The finding that FlhF GTPase mutants alter flagellation differently among polar flagellates provides some support to the idea that differences in FlhF-FlhG interactions and possible resultant effects on their biochemical properties may underlie the variety of flagellation patterns and numbers observed in polar flagellates.

What Does FlhF Do?

Currently, specific mechanistic roles of FlhF in flagellar biosynthesis or motility are unknown. Because flagella either mislocalize or are not produced in *flhF* mutants, FlhF may affect the assembly, activity, and/or polar localization of flagellar substructures such as the MS ring or the flagellar type III secretion system (T3SS). Like the MS ring, the flagellar T3SS is one of the earliest formed flagellar substructures and is responsible for secretion of almost all remaining flagellar proteins. Thus, its placement is crucial in deciding where a flagellum will form. However, most peritrichous bacteria do not have FlhF or FlhG homologs and assemble flagella nonetheless. Thus, some unique aspect of the assembly or activity of the flagellar T3SSs in polar flagellates may exist and require FlhF for these T3SSs to be formed or secretion-competent at poles. If so, FlhF may directly interact with T3SS components or assist in nucleation of these components into a mature system with other flagellar structures such as the MS ring. Furthermore, in the case of monotrichous flagellation, the bacterial poles may possess discernable features after division that either allow for or prevent flagellation so that the production of a flagellum occurs only at one

pole. An intriguing possibility is that flagellar biosynthesis may be linked to cell division cues that generate structurally different old and new poles, with FlhF perhaps differentiating between the poles to begin flagellar biosynthesis specifically at one pole.

In addition to its flagellar polar-positioning function, FlhF may have other regulatory roles that have diverged in some species. In *P. aeruginosa*, certain FlhF mutant proteins facilitate polar flagellation, but alter flagellar function (Schniederberend *et al.*, 2013). An effect on flagellar rotation might be mediated by the ability of FlhF to associate with the flagellar motor and other components that “tune” rotation, such as stator units, or molecular brakes and clutches (Delalez and Armitage, 2009).

Even though most peritrichous organisms, including *E. coli*, *Salmonella* species, and many others, do not produce FlhF and FlhG, these proteins are found in *B. subtilis*. By analyzing the distribution of basal bodies, peritrichous flagellation along the cell body of *B. subtilis* was found to be fairly ordered, rather than random (Guttenplan *et al.*, 2013). Basal bodies in wild-type cells were dispersed in a grid-like pattern, symmetrically distributed on either side of the midcell, and discouraged at poles. Furthermore, flagellar basal bodies were separated from each other by a minimum distance greater than that predicted by chance. Thus, peritrichous flagellation in at least *B. subtilis* is specifically programmed by the cell. It is currently unknown if peritrichous flagellation in other bacterial species is similarly ordered, rather than a random distribution of the organelles. In an $\Delta flhF$ mutant, basal bodies accumulated towards a pole, suggesting that FlhF discourages polar flagellation in *B. subtilis*, which is opposite to its function in many polar flagellates (Guttenplan *et al.*, 2013). Additionally, mutation of *flhG* resulted in aggregation of flagellar basal bodies and a loss of the normal spacing distance between flagella (Guttenplan *et al.*, 2013). From these data, Guttenplan *et al.* proposed that FlhG may determine the distance between individual FlhF proteins in *B. subtilis*, which allows formation of flagellar basal bodies at some distance from each other. Although FlhF and FlhG spatially influenced flagellation in *B. subtilis*, they did not influence numerical parameters of flagellation. These results further suggest that FlhF-FlhG pairs may have co-evolved in individual motile bacterial species to facilitate flagellation patterns and numbers specific for each species.

A Role for FlhG in Influencing Cell Division in *C. jejuni*

In addition to numerically regulating flagellar biosynthesis, *C. jejuni* FlhG influences spatial parameters of division (Balaban and Hendrixson, 2011). A striking phenotype of the *C. jejuni flhG* mutant was the abundant presence of minicells during normal growth. Minicells are products of cell division when the FtsZ polymerizes into the Z-ring at a pole to divide the cell, rather than at the cellular midpoint to promote symmetrical division (Adler *et al.*, 1967; Davie *et al.*, 1984). In many bacteria, spatial regulation of Z-ring formation is controlled by the Min system (for detailed reviews, see (Barak and Wilkinson, 2007; Lutkenhaus, 2007)). The MinD ATPase is a SIMIBI family member with homology to FlhG proteins (Figure 1B). However, MinD itself does not inhibit Z-ring formation. Instead, MinD complexes with MinC, the actual inhibitor of FtsZ polymerization. MinCD complexes form at a pole to inhibit Z-ring formation and dissociate due to stimulation of MinD-mediated ATP hydrolysis by MinE. After dissociating from a pole, MinCD complexes form at the other pole and are eventually dissociated at this pole. The net effect of this process is that MinCD complexes oscillate between poles and inhibit Z-ring formation at polar regions. Because MinCD concentrations are minimal at the cellular midpoint, FtsZ forms the Z-ring at the midpoint to promote symmetrical division.

Whereas many bacteria encode a complete Min system, *Campylobacter* species lack these genes. As such, it is possible that *Campylobacter* species have adapted FlhG with some

properties common to MinD that allows it to influence inhibition of division at poles. Expression of *Helicobacter pylori* or *V. cholerae* FlhG proteins, but not the respective MinD proteins moderately restored normal division to *C. jejuni* $\Delta flhG$ (Balaban and Hendrixson, 2011). These findings raise the question of whether FlhG, the Min system, or both contribute to spatial regulation of division in other polar flagellates. It is currently unknown if FlhG or another as yet unidentified protein directly inhibits Z-ring formation.

Further examination of *C. jejuni* revealed that flagellar components function with FlhG to inhibit division at poles. In mutants lacking FlhF, FliF (the inner membrane MS ring component), FliM, or FliN (the latter two proteins are cytoplasmic C ring components that form part of the flagellar switch complex), minicells formed at similar levels as an *flhG* mutant (Balaban and Hendrixson, 2011). Furthermore, minicell formation in these mutants was suppressed by overexpression of *flhG*, which suggested that FlhG works in a mechanism with and possibly downstream of these flagellar proteins to prevent division at poles. Currently, it is not known how flagellar proteins may influence an FlhG-dependent mechanism to inhibit division at poles. FliF, FliM, and FliN compose the base of the flagellum with domains accessible to the cytoplasm, which is where FlhG is predicted to reside. It is possible that FlhG may require MS and C ring structures to localize to poles or be spatially arranged to function in a mechanism to inhibit division.

Proposed Models for How FlhF and FlhG Function in Different Polar Flagellates

The most pronounced effect of *flhF* or *flhG* mutation in polar flagellates is a gross alteration in flagellar placement or number, but the existence of a single model for how these proteins function to mediate specific flagellation patterns across polarly-flagellated species is unlikely. FlhF and FlhG are present in many polar flagellates, yet as a group, these bacteria produce monotrichous, lophotrichous, and amphitrichous flagellation. It is possible that intrinsic biochemical properties of each FlhF-FlhG cognate pair may create the specific flagellation pattern for each polar flagellate. For instance, whether FlhG regulates the GTPase activity of its FlhF partner may dictate how many flagella form or whether flagella form at a specific pole. In the case of the lophotrichous flagellation pattern of *H. pylori*, FlhG may only weakly regulate the GTPase activity of its cognate FlhF, thereby resulting in a relatively hyperactive FlhF with a prolonged GTP-bound 'ON' state relative to monotrichous organisms, which may facilitate construction of multiple flagella at one pole. Presented below are two proposed models for how FlhF and FlhG may function in *C. jejuni* and *P. aeruginosa* to mediate flagellation patterns or contribute to flagellar function. Future research will be necessary to test and further refine such models to accurately detail how these organisms achieve specific flagellation patterns and flagellar activity required by each species.

C. jejuni

Data indicate that FlhF, FlhG, the MS ring and C ring components of *C. jejuni* are linked together in mechanisms that impact not only spatial and numerical parameters of flagellation, but also spatial regulation of cell division (Balaban *et al.*, 2009; Balaban and Hendrixson, 2011). One possible model for how these components may work together to mediate these biological processes is presented in Figure 2.

Immediately after division, a *C. jejuni* daughter cell possesses a flagellum only at the old pole. Thus, flagellar biosynthesis must occur at the new pole to complete amphitrichous flagellation. FlhF, but not its GTPase activity, is required for expression of σ^{54} -dependent flagellar genes (Hendrixson and DiRita, 2003). However, *C. jejuni* FlhF is polarly localized

and alteration of its GTPase activity affected flagellar placement (Balaban *et al.*, 2009; Ewing *et al.*, 2009; Balaban and Hendrixson, 2011), indicating that the GTPase activity is important for normal amphitrichous flagellation. One possible scenario in wild-type bacteria is that FlhF, in a GTPase-dependent manner, polarly localizes and promotes one round of flagellar biosynthesis thereby completing amphitrichous flagellation. FlhF may help decide where flagellation occurs by influencing an early step in flagellar biosynthesis, such as promoting the flagellar T3SS, the MS ring and/or the C ring to form at the new pole. FlhG also polarly localizes, but it is unknown if its localization is dependent on FlhF or flagellar structures (Balaban and Hendrixson, 2011). Once at the pole, FlhG may subsequently alter GTP binding or hydrolysis by FlhF so that FlhF is incompetent for another round of flagellar assembly, ensuring that only one flagellum is produced at the new pole. Positioning of FlhG at the new pole is likely a prerequisite for the protein to function with the MS and C rings to prevent division at this pole and encourage Z-ring formation at the cellular midpoint for symmetrical division.

This unusual mechanism to spatially control division may have contributed a biological advantage for *C. jejuni* to produce amphitrichous flagellation, which is a fairly rare flagellation pattern in motile bacteria. By having a flagellum-influenced cell division system, *C. jejuni* must produce a flagellum at each pole to inhibit division at both poles via FlhG. If *C. jejuni* possessed monotrichous flagellation, division could occur at an aflagellated pole to result in minicells, which decreases the number of viable daughter cells generated per a round of division. If the bacterium produced peritrichous flagella, division may be inhibited throughout the cell. Thus, amphitrichous flagellation allows *C. jejuni* to not only promote motility, but also prevent polar division and encourage symmetrical division to guarantee production of viable daughter cells.

P. aeruginosa

The biology of *P. aeruginosa* FlhF and FleN differs from that of *C. jejuni* in several significant regards. FlhF appears to play a minor role, if any, in influencing flagellar gene expression. The primary phenotype of *P. aeruginosa* $\Delta flhF$ bacteria is the assembly of a single, mislocalized flagellum that can power swimming motility (Murray and Kazmierczak, 2006). FleN, unlike *C. jejuni* FlhG, likely controls flagellar number by modulating expression of flagellar genes via a master transcriptional regulator (Dasgupta *et al.*, 2000; Dasgupta and Ramphal, 2001). Cell division defects are also not reported for $\Delta flhF$ or $\Delta fleN$ mutants, suggesting that a MinD-like function is not a feature of FleN. Lastly, FlhF mutant proteins with altered nucleotide binding and hydrolysis functions do not alter the monotrichous flagellation pattern, although they do influence the motility of the assembled organelle. Since overexpression of wild-type or mutant FlhF alleles is associated with increased numbers of bacteria expressing more than one polar flagellum, the protein may interact with, guide or localize an early component of the flagellar T3SS or MS ring to the pole. In this regard, it is interesting that *flhF* is located immediately downstream from and co-transcribed with the flagellar T3SS component *flhA* (Wurtzel *et al.*, 2012). However, it is unknown if these two proteins interact.

Ultimately, data regarding FlhF in the biology of *P. aeruginosa* largely suggest a polar-positioning function independent of GTPase activity, but a GTPase-dependent effect on flagellar motility. The notion that FlhF behavior is altered by its nucleotide-bound state is consistent with the observations that FlhF dimerization depends on nucleotide binding, similar to what is reported for the FtsY-Ffh heterodimer. Whether FleN can influence the GTP hydrolysis rate of *P. aeruginosa* FlhF is not clear because the activating helix of FlhG is not present in FleN (Figure 1B). The observation that FlhF mutants with slowed GDP/GTP exchange do not support flagellar rotation does suggest, however, that FlhF maintains

ongoing interactions with the assembled flagellum (and perhaps more specifically, motor and stator components) that are sensitive to the nucleotide-bound state of FlhF.

Conclusions

Understanding spatial and numerical regulation of flagella and other organelles are new areas for exploring how bacteria genetically program the assembly and distribution of surface-associated structures. Undoubtedly, FlhF and FlhG influence spatial and numerical parameters of flagellar biosynthesis, yet these proteins appear to function in a species-specific manner among the polar flagellates to result in different flagellation patterns. Thus, future analysis will likely uncover different mechanisms by which these proteins function in the various polar flagellates. It is expected that answering questions regarding how flagella are organized in motile bacteria will add to our growing knowledge of how bacteria organize and assign regions of the cell to enable the formation of structures with specific activities critical for bacterial fitness, such as locomotion, protein secretion, adherence, environmental sensing, and proper cell division.

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References

- Adler HI, Fisher WD, Cohen A, Hardigree AA. Miniature *Escherichia coli* cells deficient in DNA. Proc Natl Acad Sci U S A. 1967; 57:321–326. [PubMed: 16591472]
- Balaban M, Hendrixson DR. Polar flagellar biosynthesis and a regulator of flagellar number influence spatial parameters of cell division in *Campylobacter jejuni*. PLoS Pathog. 2011; 7:e1002420. [PubMed: 22144902]
- Balaban M, Joslin SN, Hendrixson DR. FlhF and its GTPase activity are required for distinct processes in flagellar gene regulation and biosynthesis in *Campylobacter jejuni*. J Bacteriol. 2009; 191:6602–6611. [PubMed: 19717591]
- Bange G, Kummerer N, Grudnik P, Lindner R, Petzold G, Kressler D, Hurt E, Wild K, Sinning I. Structural basis for the molecular evolution of SRP-GTPase activation by protein. Nat Struct Mol Biol. 2011; 18:1376–1380. [PubMed: 22056770]
- Bange G, Petzold G, Wild K, Parlitz RO, Sinning I. The crystal structure of the third signal-recognition particle GTPase FlhF reveals a homodimer with bound GTP. Proc Natl Acad Sci U S A. 2007; 104:13621–13625. [PubMed: 17699634]
- Barak I, Wilkinson AJ. Division site recognition in *Escherichia coli* and *Bacillus subtilis*. FEMS Microbiol Rev. 2007; 31:311–326. [PubMed: 17326815]
- Bourne HR, Sanders DA, McCormick F. The GTPase superfamily: conserved structure and molecular mechanism. Nature. 1991; 349:117–127. [PubMed: 1898771]
- Chevance FF, Hughes KT. Coordinating assembly of a bacterial macromolecular machine. Nat Rev Microbiol. 2008; 6:455–465. [PubMed: 18483484]
- Chilcott GS, Hughes KT. Coupling of flagellar gene expression to flagellar assembly in *Salmonella enterica* serovar *typhimurium* and *Escherichia coli*. Microbiol Mol Biol Rev. 2000; 64:694–708. [PubMed: 11104815]
- Correa NE, Peng F, Klose KE. Roles of the regulatory proteins FlhF and FlhG in the *Vibrio cholerae* flagellar transcription hierarchy. J Bacteriol. 2005; 187:6324–6332. [PubMed: 16159765]
- Dasgupta N, Arora SK, Ramphal R. *flaN*, a gene that regulates flagellar number in *Pseudomonas aeruginosa*. J Bacteriol. 2000; 182:357–364. [PubMed: 10629180]
- Dasgupta N, Ramphal R. Interaction of the antiactivator FleN with the transcriptional activator FleQ regulates flagellar number in *Pseudomonas aeruginosa*. J Bacteriol. 2001; 183:6636–6644. [PubMed: 11673434]

- Davie E, Sydnor K, Rothfield LI. Genetic basis of minicell formation in *Escherichia coli* K-12. *J Bacteriol.* 1984; 158:1202–1203. [PubMed: 6327636]
- Delalez N, Armitage JP. Parts exchange: tuning the flagellar motor to fit the conditions. *Mol Microbiol.* 2009; 71:807–810. [PubMed: 19170878]
- Doudna JA, Batey RT. Structural insights into the signal recognition particle. *Annu Rev Biochem.* 2004; 73:539–557. [PubMed: 15189152]
- Egea PF, Shan SO, Napetschnig J, Savage DF, Walter P, Stroud RM. Substrate twinning activates the signal recognition particle and its receptor. *Nature.* 2004; 427:215–221. [PubMed: 14724630]
- Ewing CP, Andreishcheva E, Guerry P. Functional characterization of flagellin glycosylation in *Campylobacter jejuni* 81–176. *J Bacteriol.* 2009; 191:7086–7093. [PubMed: 19749047]
- Green JC, Kahramanoglou C, Rahman A, Pender AM, Charbonnel N, Fraser GM. Recruitment of the earliest component of the bacterial flagellum to the old cell division pole by a membrane-associated signal recognition particle family GTP-binding protein. *J Mol Biol.* 2009; 391:679–690. [PubMed: 19497327]
- Guttenplan SB, Shaw S, Kearns DB. The cell biology of peritrichous flagella in *Bacillus subtilis*. *Mol Microbiol.* 2013; 87:211–229. [PubMed: 23190039]
- Hendrixson DR V, DiRita J. Transcription of σ^{54} -dependent but not σ^{28} -dependent flagellar genes in *Campylobacter jejuni* is associated with formation of the flagellar secretory apparatus. *Mol Microbiol.* 2003; 50:687–702. [PubMed: 14617189]
- Kusumoto A, Kamisaka K, Yakushi T, Terashima H, Shinohara A, Homma M. Regulation of polar flagellar number by the *flhF* and *flhG* genes in *Vibrio alginolyticus*. *J Biochem.* 2006; 139:113–121. [PubMed: 16428326]
- Kusumoto A, Nishioka N, Kojima S, Homma M. Mutational analysis of the GTP-binding motif of FlhF which regulates the number and placement of the polar flagellum in *Vibrio alginolyticus*. *J Biochem.* 2009; 146:643–650. [PubMed: 19605463]
- Kusumoto A, Shinohara A, Terashima H, Kojima S, Yakushi T, Homma M. Collaboration of FlhF and FlhG to regulate polar-flagella number and localization in *Vibrio alginolyticus*. *Microbiology.* 2008; 154:1390–1399. [PubMed: 18451048]
- Leipe DD, Wolf YI, Koonin EV, Aravind L. Classification and evolution of P-loop GTPases and related ATPases. *J Mol Biol.* 2002; 317:41–72. [PubMed: 11916378]
- Lutkenhaus J. Assembly dynamics of the bacterial MinCDE system and spatial regulation of the Z ring. *Annu Rev Biochem.* 2007; 76:539–562. [PubMed: 17328675]
- Murray TS, Kazmierczak BI. FlhF is required for swimming and swarming in *Pseudomonas aeruginosa*. *J Bacteriol.* 2006; 188:6995–7004. [PubMed: 16980502]
- Niehus E, Gressmann H, Ye F, Schlapbach R, Dehio M, Dehio C, Stack A, Meyer TF, Suerbaum S, Josenhans C. Genome-wide analysis of transcriptional hierarchy and feedback regulation in the flagellar system of *Helicobacter pylori*. *Mol Microbiol.* 2004; 52:947–961. [PubMed: 15130117]
- Pandza S, Baetens M, Park CH, Au T, Keyhan M, Matin A. The G-protein FlhF has a role in polar flagellar placement and general stress response induction in *Pseudomonas putida*. *Mol Microbiol.* 2000; 36:414–423. [PubMed: 10792727]
- Parrish JR, Yu J, Liu G, Hines JA, Chan JE, Mangiola BA, Zhang H, Pacifico S, Fotouhi F, DiRita VJ, Ideker T, Andrews P, Finley RL Jr. A proteome-wide protein interaction map for *Campylobacter jejuni*. *Genome Biol.* 2007; 8:R130. [PubMed: 17615063]
- Ross EM, Wilkie TM. GTPase-activating proteins for heterotrimeric G proteins: regulators of G protein signaling (RGS) and RGS-like proteins. *Annu Rev Biochem.* 2000; 69:795–827. [PubMed: 10966476]
- Saraogi I, Akopian D, Shan SO. A tale of two GTPases in cotranslational protein targeting. *Protein Sci.* 2011; 20:1790–1795. [PubMed: 21898651]
- Schniederberend M, Abdurachim K, Murray TS, Kazmierczak BI. The GTPase activity of FlhF is dispensible for flagellar localization, but not motility, in *Pseudomonas aeruginosa*. *J Bacteriol.* 2013; 195:1051–1060. [PubMed: 23264582]
- Wurtzel O, Yoder-Himes DR, Han K, Dandekar AA, Edelheit S, Greenberg EP, Sorek R, Lory S. The single-nucleotide resolution transcriptome of *Pseudomonas aeruginosa* grown in body temperature. *PLoS Pathog.* 2012; 8:e1002945. [PubMed: 23028334]

Zhang X, Schaffitzel C, Ban N, Shan SO. Multiple conformational switches in a GTPase complex control co-translational protein targeting. *Proc Natl Acad Sci U S A*. 2009; 106:1754–1759. [PubMed: 19174514]

Figure 1. Domain organization of the FlhF, FlhG, and MinD proteins

(A) The FlhF GTPases are divided into three domains: an N-terminal B domain (grey), a central N domain (black), and a C-terminal G domain with GTPase activity (blue). Five conserved G domain motifs (G1–G5) are shown in red. The consensus sequence that defines each G domain motif are shown below, except for the G5 motif, which is much less conserved among G domains. The G2 motif contains the arginine finger of the FlhF proteins. (B) The ATPase domain of MinD is shown in brown and the predicted ATPase domains of the FlhG/FleN proteins are indicated as tan boxes. The green boxes at the C-termini of some FlhG proteins are predicted amphipathic helices that are similar to the membrane-targeting sequence of MinD, which interacts with the cytoplasmic face of the inner membrane at poles. In other FlhG proteins, the amphipathic helix is not strongly predicted and these C-terminal regions are shown in white. The N-terminal extensions of FlhG proteins conserving the DQAXXLR motif that may stimulate the GTPase activity of cognate FlhF proteins are shown in yellow. Bacterial species are indicated as: *B. subtilis* (*Bs*), *C. jejuni* (*Cj*), *H. pylori* (*Hp*), *V. cholerae* (*Vc*), *P. aeruginosa* (*Pa*), and *E. coli* (*Ec*).

Figure 2. Proposed model for FlhG-mediated regulation of amphitrichous flagellar biosynthesis and division site formation in *C. jejuni*

After division, a *C. jejuni* daughter cell lacks a flagellum at the new pole. FlhF is proposed to localize to the new pole in a GTPase-dependent manner to determine the site of flagellar biosynthesis (Step 1). FlhF may subsequently polarly organize the MS ring, C ring (including the rotor and switch complex), and/or the flagellar T3SS to initiate flagellar biosynthesis (Step 2). Flagellar biosynthesis is completed by construction of the rod, hook and filament. FlhG polarly localizes to the pole, perhaps through interactions with FlhF or flagellar components. At the pole, FlhG may prevent FlhF from initiating a second round of flagellar biosynthesis at the new pole (Step 3). Once at the new pole, FlhG functions with the MS ring and switch complex of the C ring to inhibit division only at the new pole. As a result, the Z-ring forms at the cellular midpoint due to low FlhG concentrations or activity (Step 4). Symmetrical division results in two daughter cells of equal sizes that lack a flagellum at the new poles (Step 5).