A new factor in the regulation of polar flagellum synthesis in *Vibrio parahaemolyticus*

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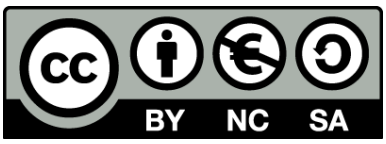

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Erklärung

Ich versichere, dass ich meine Dissertation mit dem Titel „A new factor in the regulation of polar flagellum synthesis in *Vibrio parahaemolyticus*“ selbstständig ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfsmittel bedient habe.

Diese Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Marburg, den 7.11.2022

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Table of Contents

Summary

Understanding of the inner working of living cells requires the study of the organization of their components, and how such organization arises. The bacterial flagellum is an example of such organization, since its position in the cell body is carefully regulated. Many patterns of flagellation exist in nature, indicative of the diversity of mechanisms that bacteria use to regulate it. The GTPase FlhF and the MinD-like ATPase FlhG have emerged to regulate the position and number of flagella in many species with different flagellation patterns. In γ-proteobacteria that use this system, flagella are found at the cell pole, and its production is tied to the cell cycle to ensure that daughter cells are flagellated at only one pole.

Here, we introduce *Vibrio parahaemolyticus* as a model to study the spatiotemporal regulation of flagellum synthesis. We show that FlhF and FlhG work in a similar manner to other γ-proteobacteria, although FlhF is strictly required for flagellum assembly. We also show that the main polar landmark protein HubP plays a role in regulating the localization of FlhG but not of FlhF. Furthermore, we describe a new protein, named FipA, as an interacting partner of FlhF.

We show that the phenotype of deleting FipA is very similar to that of a deletion of FlhF: they are both required to start assembly of the flagellum. Furthermore, we could demonstrate that FipA and FlhF interact directly, that this interaction is possible due to key residues in the domain of unknown function of FipA, and that this interaction is responsible for recruiting FlhF to the cell pole. In fact, we show that FipA and HubP act cooperatively to recruit FlhF to the cell pole. FipA is conserved among the γ-proteobacteria that use FlhFG, and we could show that its function is also conserved in *Pseudomonas putida.*

Finally, we used a heterologous system to show that FipA is responsible for anchoring FlhF to the membrane. We could also identify residues in FlhF that are necessary for the interaction, which suggest that FipA binds primarily to the monomeric, GDP-bound form of FlhF. This hypothesis also receives support from *in vitro* interaction studies, that suggest that GTP inhibits the interaction between FlhF and FipA.

Altogether, this work identifies a new piece in the puzzle of spatiotemporal regulation of flagella, and we propose a model incorporating our new findings to what is already known about this system in γ-proteobacteria.

Zusammenfassung

Um die inneren Funktionsweisen lebenden Zellen zu verstehen, muss man die Organisation ihrer Bestandteile und die Art und Weise, wie diese Organisation zustande kommt, untersuchen. Die bakterielle Geißel ist ein Beispiel für eine solche Organisation, da ihre Position im Zellkörper sorgfältig reguliert wird. In der Natur gibt es viele Geißelungsmuster, was auf die Vielfalt der Mechanismen hinweist, die Bakterien zu ihrer Regulierung nutzen. Die GTPase FlhF und die MinD-ähnliche ATPase FlhG haben sich herausgebildet, um die Position und Anzahl der Geißeln in vielen Arten mit unterschiedlichen Geißelungsmustern zu regulieren. Bei γ-Proteobakterien, die dieses System nutzen, befinden sich die Geißeln am Zellpol, und ihre Produktion ist an den Zellzyklus gebunden, um sicherzustellen, dass die Tochterzellen nur an einem Pol gegeißelt werden.

Hier stellen wir *Vibrio parahaemolyticus* als Modell vor, um die räumlich-zeitliche Regulierung der Flagellensynthese zu untersuchen. Wir zeigen, dass FlhF und FlhG in ähnlicher Weise funktionieren wie bei anderen γ-Proteobakterien, obwohl FlhF für die Geißelbildung unbedingt erforderlich ist. Wir zeigen auch, dass das wichtigste polare Landmarkenprotein HubP eine Rolle bei der Regulierung der Lokalisierung von FlhG, aber nicht von FlhF spielt. Darüber hinaus beschreiben wir ein neues Protein namens FipA als Interaktionspartner von FlhF.

Wir zeigen, dass der Phänotyp einer Deletion von FipA dem einer Deletion von FlhF sehr ähnlich ist: beide sind erforderlich, um den Aufbau des Flagellums zu beginnen. Darüber hinaus konnten wir zeigen, dass FipA und FlhF direkt interagieren, dass diese Interaktion aufgrund von bestimmten Aminosäuren in der Domäne mit unbekannter Funktion von FipA möglich ist und dass diese Interaktion für die Rekrutierung von FlhF am Zellpol verantwortlich ist. Tatsächlich zeigen wir, dass FipA und HubP zusammenwirken, um FlhF an den Zellpol zu rekrutieren. FipA ist unter den γ-Proteobakterien, die FlhFG verwenden, konserviert, und wir konnten zeigen, dass seine Funktion auch in *Pseudomonas putida* konserviert ist.

Schließlich haben wir ein heterologes System verwendet, um zu zeigen, dass FipA für die Verankerung von FlhF an der Membran verantwortlich ist. Wir konnten auch Rückstände in FlhF identifizieren, die für die Interaktion notwendig sind, was darauf hindeutet, dass FipA hauptsächlich an die monomere, GDP-gebundene Form von FlhF bindet. Diese Hypothese wird auch durch In-vitro-Interaktionsstudien gestützt, die nahelegen, dass GTP die Interaktion zwischen FlhF und FipA hemmt.

Insgesamt stellt diese Arbeit ein neues Element im Puzzle der raum-zeitlichen Regulierung der Geißeln dar, und wir schlagen ein Modell vor, das unsere neuen Erkenntnisse mit dem verbindet, was bereits über dieses System in γ-Proteobakterien bekannt ist.

1. Introduction

Shape is an intrinsic feature of all living organisms, which determines to a large extent the functions they can perform and the niches they can inhabit. Even in the smallest of cells that lack higher-order membranous organelles, like bacteria, the cellular components are arranged in time and space in order to permit their function and reproduction. The ability of shapes and patterns to emerge from a homogeneous mixture of cells or molecules is called morphogenesis, and it is a result of the concerted play of different molecules with particular physicochemical properties (Turing, Alan Mathison 1952). Thus, a seemingly homogenous mixture become segregated in regular patterns simply due to the diffusion and interaction of specific molecules referred to as morphogens. These diffusion-reaction mechanisms have been the basis for understanding how biological systems develop shapes, *i. e.* morphogenesis, and its research has brought together the efforts of chemical, physical and mathematical sciences.

The development of shapes and patterns has been studied thoroughly in multicellular organism, where organization between multiple components plays a more evident role. Here, morphogens are understood as molecules that act on cells in a tissue to induce certain behaviors that will produce a particular shape in the entire tissue. However, the same interaction takes place at a smaller scale, when molecules within the cell orchestrate the movement of larger molecular complexes, altering thus the shape of the cell. In fact, the interplay of morphogens that Turing initially hypothesized to describe the development of arrangements of cells can also be used to study subcellular pattern formation (Halatek, Brauns, and Frey 2018). The term morphogen now mostly refers to molecules that are active on other cells, and is therefore used in the study of multicellular organisms. The underlying principles, however, have been researched across the whole tree of life. Understanding the nature of the interactions between shape-determining molecules is therefore of great interest across all scales of living organisms.

1.1. Polar organization

The simplest organization pattern, the distinction between the poles and the middle of the cell, is common among all prokaryotes. The polar organization of a multitude of proteins is essential for DNA replication and segregation, cell division, differentiation and motility, to name a few examples. These proteins are not just tethered to the cell pole, but their localization changes as the cells grow in a coordinated fashion with the cell cycle, ensuring the inheritance of the organelles to the daughter cells.

In the model bacterium *Escherichia coli*, a distinction between the cell poles and the mid-cell is necessary for chromosome segregation and cell division. A complex of cell division proteins, called the divisome, assembles at the farthest point from both poles, and its recruitment is coordinated with the replication of DNA, ensuring that both chromosomes are separated to each daughter cell (Du and

Lutkenhaus 2017). Many other bacteria have an even more obvious polar organization. In most α- and γ-proteobacteria, the chromosome instead is tethered at the cell pole together with other organelles, like flagella or pili. Cell division therefore requires that these components are replicated and recruited to the new cell pole, in time for the new daughter cell to inherit a copy of them (Curtis and Brun 2010; Ramachandran, Jha, and Chattoraj 2014). In addition, cell growth driven by extension of the cell wall can also be restricted to the cell pole, as is the case in many *Actinobacteria* and *Rhizobiales* (Cameron, Zupan, and Zambryski 2015).

There are a few ways in which a protein can acquire a polar localization. Most polar proteins are targeted by their affinity to another protein that is already polarly localized. In this diffusion and capture mechanism, the protein in question can diffuse through the cytoplasm, but accumulates at the pole by interacting with a polar protein. Some polar proteins can recruit multiple targets, acting as landmarks in the cell. This is the case of proteins like DivIVA in *Bacillus subtilis* (Lenarcic et al. 2009; Bach, Albrecht, and Bramkamp 2014), PopZ in *Caulobacter crescentus* (Ebersbach et al. 2008; Laloux and Jacobs-Wagner 2013), and HubP in *Vibrio cholerae* (Yamaichi et al. 2012), all of which act as polar landmarks that recruit factors involved in cell division (Bramkamp et al. 2008; Eswaramoorthy et al. 2011; Tsokos, Perchuk, and Laub 2011), chromosome segregation (Ptacin et al. 2010; Lim et al. 2014), competence (dos Santos, Bisson-Filho, and Gueiros-Filho 2012), motility (Huitema et al. 2006; Green et al. 2009) and chemotaxis (Simon Ringgaard et al. 2011; Alvarado et al. 2017) in these organisms.

1.1.1. Mechanisms of polar organization

The question arises, then, of how these landmark proteins are targeted to the cell pole. In some cases, the polar localization is intrinsic to the protein. This means that the protein itself interacts preferentially with a physical feature of the cell pole. For example, DivIVA has affinity to the concave curvature of the cell membrane. Even when expressed heterologously in *Escherichia coli* (which lacks DivIVA homologues or interactors), it is found predominantly at the cell poles, or at the pre-division septa (Lenarcic et al. 2009). This is due to the presence of a membrane-binding amphipathic helix, and the formation of curved tetramers that bind preferentially to curved membranes (Oliva et al. 2010). This mechanism allows DivIVA to be localized to the cell pole, but it also allows it to change localization upon cell division, since the cell division septum has a much stronger curvature (Bach, Albrecht, and Bramkamp 2014). Thus, DivIVA acts as a spatiotemporal landmark.

It has also been proposed that the cell wall can act as a polar or lateral landmark for other proteins. Indeed, the cell wall synthesis is usually restricted to one part of the cell (Cameron, Zupan, and Zambryski 2015), suggesting that a trail of distinct features in the cell wall could emerge along the cell length as the peptidoglycan matrix matures. Differences in composition have been found between the polar and mid-cell peptidoglycan in *C. crescentus* (Billini et al. 2019), but due to the technical difficulty

of assaying the chemical structure of different parts of the cell these differences remain to be discovered in other organisms.

In addition to cellular targeting by affinity to the poles, a protein may also form structures that are excluded from the mid-cell, therefore being confined to the cell pole. For example, unfolded proteins in *E. coli* aggregate into large inclusion bodies. Due to their size and compaction, they are excluded from the tight nucleoid, and are relegated to the cell pole (Winkler et al. 2010). This improves the fitness of the cell, since in this way the inclusion bodies are inherited by one daughter cell only, instead of being caught in the middle of the cell at division and potentially inhibiting growth in both cells. Interestingly, a similar mechanism has been proposed to determine the localization of PopZ in *C. crescentus*, which also forms large aggregates due to its disordered structure (Laloux and Jacobs-Wagner 2013). PopZ is also restricted to the poles when it is expressed heterologously in *E. coli*. (Ebersbach et al. 2008). However, the localization of PopZ is likely not entirely intrinsic, since it also depends on its interaction with other membrane proteins (Bergé et al. 2016; Perez et al. 2017).

In contrast to these passive mechanisms of intracellular localization, other proteins can establish a pattern through the cell by expending energy. The best studied example is the Min system that positions the cell division ring in *E. coli*. The ATPase MinD is responsible for coupling ATP hydrolysis to the establishment of a pattern by aid of the activator MinE and MinC. In addition, many other systems have been discovered that use a similar mechanism (Lutkenhaus 2012).

1.1.2. MinDE and MinD-like proteins

The key feature of the MinD ATPase is that it changes conformation, and therefore function, when it binds ATP. The ATPase activity allows it to switch between an “active”, ATP-bound state, and an “inactive” ADP-bound state. This property occurs throughout the family of P-loop GTPases, of which MinD is a member (a specificity switch from GTP to ATP is something that occurred several times in the evolution of the family, Leipe et al. 2002). Other members of this family are the Ras-like GTPases, involved in many intracellular processes in eukaryotes, and the Signal Recognition Particle (SRP) system that targets protein synthesis to the membrane in all living organisms.

In the active state, MinD forms a homodimer and binds cooperatively to the membrane. MinE can interact with membrane-bound MinD, stimulating its ATPase activity and prompting it to bind off the membrane (Fig. 1.1A). As long as there are more MinD molecules than MinE molecules, this behavior will create a front of MinD on the membrane moving along the cell length, followed by a zone of MinE inhibiting accumulation of MinD. The movement, called an oscillating wave, is self-propagating and averages out over time to a concentration gradient with high MinD concentration at the poles and low concentration at mid-cell (Fig. 1.1B, Raskin and de Boer 1999). MinD recruits the cell-division inhibitor MinC, and this results in assembly of the divisome exclusively at mid-cell (reviewed in Lutkenhaus, 2012).

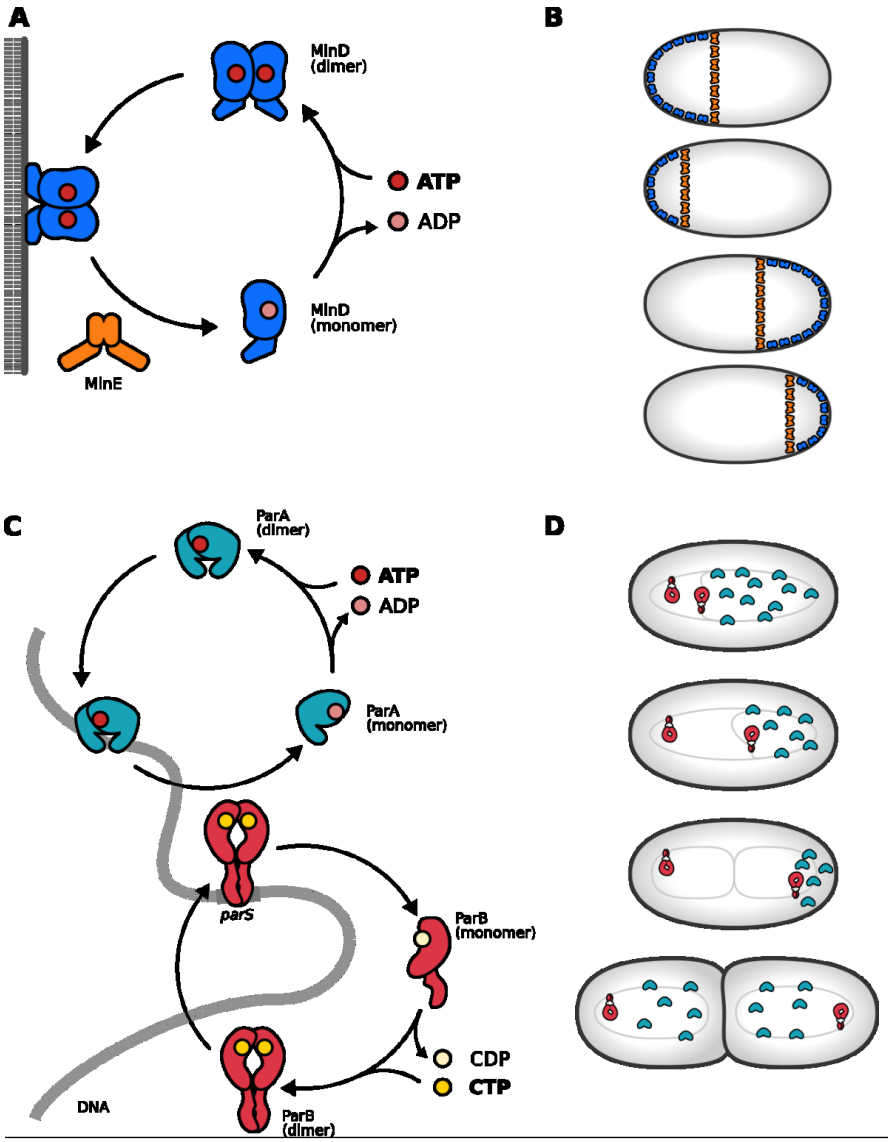

Figure 1.1. MinD and MinD-like ATPases. A) The MinD monomer dimerizes upon binding of ATP. The dimer is recruited to the membrane. The ATPase-activator MinE induces monomerization. **B)** The chase of MinD along the membrane by MinE produces oscillations along the cell, with an average minimum of MinD concentration exactly at mid-cell. **C)** The MinD-like ATPase ParA alternates between a monomer and a dimer that binds to DNA. The ATPase activator of ParA, ParB, is itself a DNA binding protein whose binding to *parS* is regulated by its CTPase activity. **D)** The chase of ParA by the ParB*S* complex results in segregation of the newly replicated chromosome across the cell. Modified from Lutkenhaus, 2012.

The MinDE gradient is an example of molecular organization that arises from very few components. It's not surprising that this system has been adapted by the evolution to coordinate many different cellular processes. The ParAB*S* system responsible for DNA segregation is also based on a P-loop ATPase ParA, which binds DNA in the active, ATP-bound state. ParB is a protein that carries DNA by binding to a specific *parS* sequence and nucleates the formation of a large ParB*S* complex, visible as distinct foci in the nucleoid. ParB is also the activator of ParA ATPase activity, inducing a gradient of ParA to form on the nucleoid (Fig. 1.1C). When the DNA cargo is replicated, the new ParB*S* complex migrates to the new cell pole, where the concentration of ParA is higher (Fogel and Waldor 2006; S. Ringgaard et al. 2009; Iniesta 2014).

The ATPase activity of ParA is necessary for segregation of the ParB*S* complex through some diffusion-ratchet mechanism that is still intensely debated. In general, ParB displaces ParA from the DNA and in doing so it pushes the ParA gradient further to the opposite side of the cell, where it will again recruit the ParB*S* complex, pulling it along (Fig. 1.1D, Lim et al. 2014).

An interesting discovery is that ParB itself has CTP-binding and hydrolysis activities (Osorio-Valeriano et al. 2019). CTP can modulate the binding of ParB to *parS* (Osorio-Valeriano et al. 2019; Soh et al. 2019), and through CTP hydrolysis, ParB can spread along the DNA and recruit more ParB molecules to *parS*, expanding the DNA partition complex (Soh et al. 2019; Jalal, Tran, and Le 2020). Both activities are necessary for DNA segregation (Jalal, Tran, and Le 2020). In this way, the ParAB*S* system works by interlocking two cycles of nucleotides hydrolysis: an ATPase cycle provides the energy to pull the ParA to the new cell pole, while a CTPase cycle provides the energy to bring the DNA to ParA (Fig. 1.1C).

1.1.3. Polar organization in C. crescentus

C. crescentus may be the most studied example of polar asymmetry in bacteria. This has been possible because cell division in *C. crescentus* produces two distinct daughter cells, a stalked cell and a flagellated cell. The two cell types are easy to separate due to their different buoyancies which has allowed the development of methods to elucidate mechanism of the differentiation process (Curtis and Brun 2010). The stalk is located in the old cell pole, and before cell division, the flagellum forms at the new cell pole, in what will become the flagellated cell. For the next cycle, the flagellated cell ejects the flagellum, assembling a stalk in its place, in what is now the old cell pole, and can now give rise to a new flagellated cell (Fig. 1.2A).

The old pole is marked by the presence of PopZ, an intrinsically disordered protein capable of forming large multimers that serve as a hub for many polar proteins (Bowman et al. 2008). PopZ tethers the chromosome to the old pole by interacting with the chromosome segregation machinery, ParAB (Bowman et al. 2008; Ptacin et al. 2014). During DNA replication, PopZ goes from unipolar localization to bipolar, forming another focus at the new cell pole. ParA is recruited by PopZ, and via interactions with ParB, forms a gradient from the new cell pole that recruits the chromosome (Ptacin et al. 2014). Cell division proceeds and in this way, each daughter cell carries a chromosome copy tethered by a PopZ-ParB complex (Bowman et al. 2008). In this way, PopZ is a polar landmark that guides localization of the ParAB proteins (Fig. 1.2A).

The new pole is also marked by the membrane protein TipN. It is called the scar protein because before cell division, TipN migrates to the division site and stays there, until each daughter cell inherits a TipN cluster at the new pole, like a cell division scar (Fig. 1.2A, Huitema et al. 2006). TipN is responsible for the localization of the flagellar marker TipF (Huitema et al. 2006). TipN can also direct ParA to the new pole, participating in chromosome segregation (Ptacin et al. 2010). In fact, PopZ and TipN can complement each other's absence, indicating a functional redundancy in the regulation of the localization of the ParAB system (Schofield, Lim, and Jacobs-Wagner 2010).

The localization of TipN at the new pole is mediated by the divisome (Huitema et al. 2006; Yeh et al. 2010), but the localization of PopZ is more elusive. PopZ can form a large matrix at the cell pole,

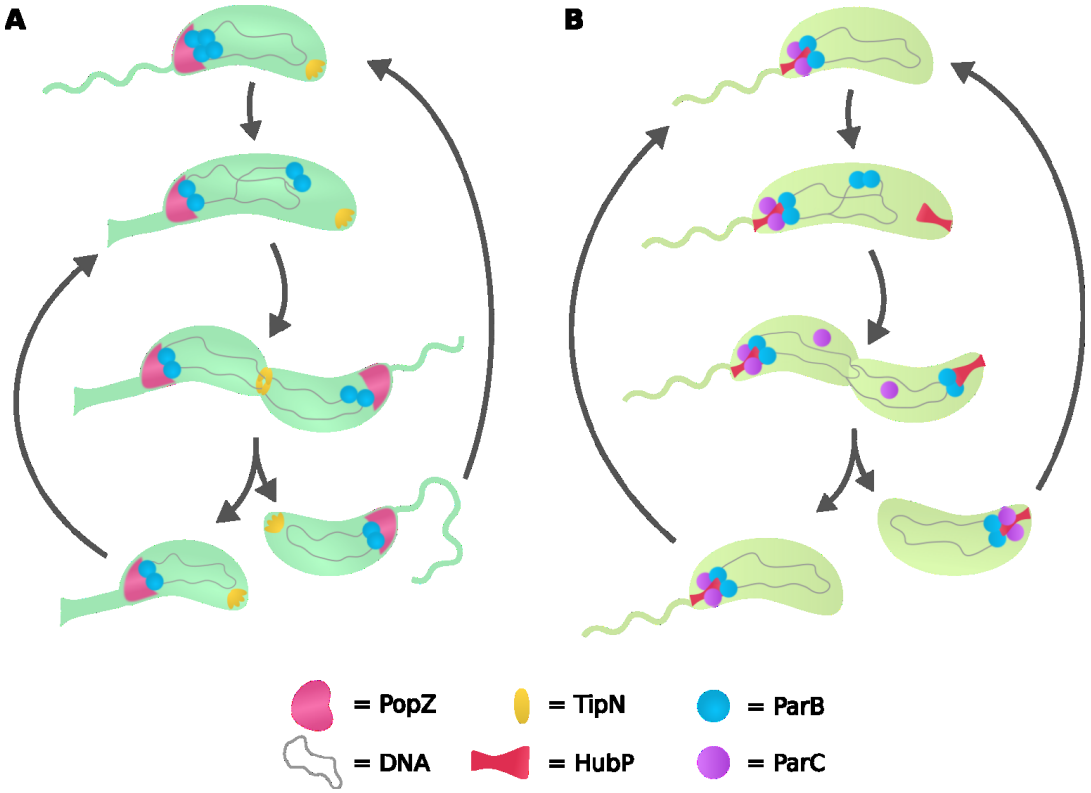

Figure 1.2. Establishment of cell polarity in *Caulobacter* and *Vibrio*. A) The cell cycle of *C. crescentus* begins with a swimming cell that loses its flagellum to become a stalked cell and start replication of the cellular components. As the chromosome replicates, ParB drags it towards the new pole, marked by TipN. Maturation of the new pole proceeds by migration of PopZ, anchoring of the new chromosome and flagellum synthesis. As cell division starts, TipN migrates to the division site. The result is a stalked cell and a swimming cell, both with TipN at their new pole and PopZ at their old pole. Each cell can restart the cycle at a different point. **B)** In *V. cholerae*, ParB and ParC are anchored at the flagellated pole by HubP. HubP is the first component to migrate to the new pole, recruiting the chromosome via ParB, and the chemotaxis clusters via ParC. Division results in a flagellated and a non-flagellated cell. It is unclear when does the daughter cell produce a new flagellum. Modified from Bergé & Viollier, 2017, and Ringgaard *et al*., 2018.

even when expressed in *E. coli*, which doesn't express any PopZ interactors (Ebersbach et al. 2008; Laloux and Jacobs-Wagner 2013). It has been suggested that the PopZ matrix is excluded from the nucleoid, much like inclusion bodies, and remains at DNA-poor regions like the cell poles of *E.* coli (Laloux and Jacobs-Wagner 2014). However, it has also been observed that upon expression of the membrane proteins SpmX or ZitP, PopZ adopts a bipolar localization (Perez et al. 2017; Bergé et al. 2016), suggesting that an anchor to the membrane is essential for PopZ migration to the new pole. Both membrane proteins can also interact and drive polar localization of PopZ *in vivo*, but they have different expression patterns throughout the cell cycle (Bergé and Viollier 2018). This is another example of redundancy in the polar organization of *C. crescentus*, and it means that cell cycle-dependent localization of PopZ can still progress if any one of these systems fail.

1.1.4. Polar organization in *Vibrio*

In many γ-proteobacteria, the old pole is marked by the flagella. In these bacteria, it is crucial to coordinate the localization and timing of flagellar components with the cell cycle, in order to ensure that newly born cells only produce a flagellum after cell division. This process is coordinated together with

the chromosome replication and the chemotaxis clusters, which migrate to the new pole before cell division to ensure that the daughter cell carries a copy of the chromosome and a set of chemotaxis proteins positioned next to what will be the site for the new flagellum (Fig. 1.2B). This contrasts with the peritrichous enterobacteria, where the chromosome is segregated at mid-cell, and the chemotaxis clusters and the flagella are distributed stochastically (Du and Lutkenhaus 2017; Thiem and Sourjik 2008).

The spatiotemporal organization of these organelles has been best studied in the human pathogen *Vibrio cholerae*. It is orchestrated by three ParA-like ATPases: ParA1 brings the chromosome I by recruiting ParB1 (Fogel and Waldor 2006), ParC the chemotaxis clusters (Simon Ringgaard et al. 2011) and FlhG regulates the flagellum (Correa, Peng, and Klose 2005). All of these proteins are recruited to the new pole, directly or indirectly by a membrane protein named HubP (Yamaichi et al. 2012). HubP consists of an N-terminal periplasmic domain similar to the peptidoglycan binding domain of LysM, a single transmembrane helix and a large number of acidic repeats in the cytosolic side. HubP is permanently localized at the old pole, together with ParA1 and ParC. As the cell cycle progresses, a new focus is formed at the new cell pole, which then attracts ParA1 and ParB1 (Galli, Paly, and Barre 2017). Only FlhG doesn't follow this pattern, neither FlhF, a GTPase that also regulates flagellar localization. Even though both proteins can be recruited by HubP in a heterologous system, flagellum assembly still occurs at the poles in a Δ*hubP* background (Yamaichi et al. 2012).

Thus, HubP resembles PopZ in its function and dynamics, although it is structurally unrelated. HubP is widespread among the polarly flagellated γ-proteobacteria, although it has poor sequence conservation. In *S. putrefaciens*, the closest homologue of HubP is also capable of guiding chromosome segregation (Rossmann et al. 2015). In *P. aeruginosa*, the HubP orthologue was named FimV because it is essential for recruitment of pilus proteins to the cell pole (Wehbi et al. 2011).

The fact that chromosome segregation still occurs to some extent in a *hubP* deletion mutant indicates that there are likely other redundant pathways, like in *C. crescentus*, awaiting to be discovered. In the same way, the polar localization of the flagellar regulator FlhF seems to be independent of HubP (Yamaichi et al. 2012; Rossmann et al. 2015; Takekawa et al. 2016), indicating that there are other still unknown factors at play.

1.2. The flagellum

Bacteria can move through many different means, but perhaps the most studied is through the flagellum. It is a long proteinaceous filament that protrudes from the cell body and is several times longer than the cell. The rotation of the filament propels the cell body through liquid or semi-solid media.

Different flagella are better suited to different environments, and therefore they are as diverse as bacteria themselves.

The position of the flagella in the cell body is also a characteristic of each species. It is determined genetically, and for a long time it had taxonomic value (Rhodes 1965). Flagella can be localized along the cell body (peritrichous), at the cell pole (polar), or right next to the cell pole (subpolar). Polar and subpolar flagella can also appear as a single filament (monotrichous) or as a tuft of multiple flagella (lophotrichous) (Fig. 1.3A, Moore 1894). The term "polar" here is used to refer mostly to the unipolar flagella, like those of *C. crescentus* or *Vibrio* already mentioned. In these species, cells only produce flagella at one pole. Thus, cell division results in a flagellated cell and a non-flagellated (or stalked, etc.) cell, that may later synthesize a flagellum. This is different from bipolar flagella, also termed amphitrichous (Fig. 1.3A), where cells have flagella on both poles, and cell division results in two unipolarly flagellated cells. This pattern is characteristic of *Campylobacter* species, and also Spirochetes in general.

Different flagellar arrangements are better suited for different environments. In general, a single flagellum can propel the cell faster through a liquid environment, whereas additional flagella can exert more force to power through more viscous environments. Indeed, the fastest swimming speeds recorded are from polar flagellates like the lophotrichous *P. aeruginosa* (70 μm/s, (Harwood, Fosnaugh, and Dispensa 1989)) or the monotrichous *V. alginolyticus* (116 μm/s, (Magariyama et al. 1995)). Even though peritrichous bacteria swim less efficiently in liquid media (*e. g.*, 36 μm/s for *E. coli*, (Lowe, Meister, and Berg 1987)), they are better equipped for semisolid environments. In fact, they often increase the number of flagella to swarm better across surfaces (reviewed in Harshey 2003). Members of the *Vibrio* genus can also swarm in surfaces by producing an entirely new set of peritrichous flagella, in addition to their fast-rotating polar flagellum (Atsumi, McCartert, and Imae 1992; L. L. McCarter 2004).

The bacterial flagellum is one of the most complex subcellular structures known. It is composed of at least 25 proteins, which have to be assembled in a spatiotemporally coordinated manner (Macnab 2003). Although there may be significant differences among species, flagella in general are composed of a motor embedded in the cytoplasmic membrane, a rod connecting it to the extracellular space, and a hook that transfers the motion to the filament (Fig. 1.3B, Macnab 2003). The basal body, which constitutes the motor, is composed of a cytoplasmic C-ring or switch complex (Francis et al. 1994), and the MS ring, which is embedded in the cytoplasmic membrane (Homma et al. 1987; Ueno, Oosawa, and Aizawa 1992). Attached to the MS ring, on the cytoplasmic side, is the export apparatus, which allows secretion of rod, hook and filament components (Minamino 2014). The C-ring interacts with stator subunits, which are protein complexes in the inner membrane that are tethered to the cell wall. These interactions allow the coupling of the electrochemical gradient of the cell to the rotation of C-ring (Nakamura and Minamino 2019). Different flagellar systems may use different ions to power the

rotation. For example, the polar flagellum of *V. parahaemolyticus* uses Na^+ ions, whereas the lateral flagella use H^+ (Atsumi, McCartert, and Imae 1992). Flagella can also be sheathed, when an extension of the outer membrane covers the filament. This is the case of the polar flagellum in most *Vibrio* species (Chen et al. 2017).

1.2.1. Transcriptional regulation of the flagellum

The construction of the flagellum follows a sequential order of synthesis, localization and assembly of the individual protein components, a process which is regulated at the transcriptional and post-transcriptional levels. The flagellar genes can be grouped in a hierarchy, where each level encodes the regulator that transcribes the genes in the next level. In this way, the rod and the hook are only synthesized when the inner rings are ready to transport them across the membranes, and the costly flagellin that constitutes the filament is only expressed when everything else is ready.

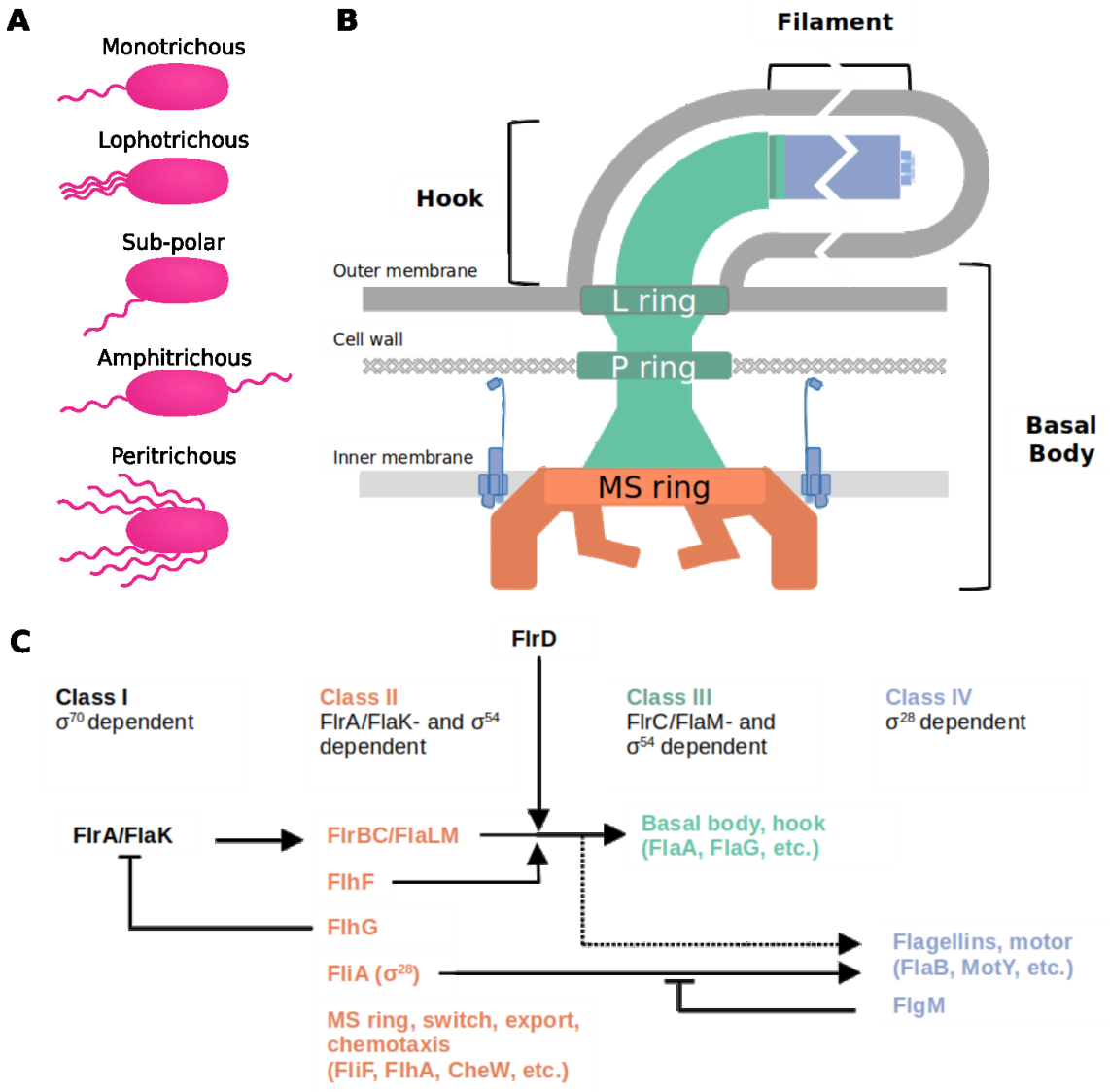

Figure 1.3. Organization and regulation of the flagellum. A) Different arrangements of flagella. **B)** General structure of the flagellum in *Vibrio*. The color of the structures follows the code of the gene classes in **C)** flagellar hierarchy in *V. cholerae*. Modified from González-Vera, 2016, and Moisi *et al.* 2009.

The best studied flagellar hierarchy is that of *Salmonella* and *E. coli*. It consists of three levels: in the class I, the transcriptional regulator $FlhD_4C_2$ is transcribed from a σ^{70} promoter, and promotes transcription of the class II genes in a σ^{70}-dependent manner. The class II proteins will form the basal body, the hook and the rod. Among these proteins is FliA (σ^{28}) together with its anti-sigma factor FlgM. When the hook is completed, a specificity change in the export apparatus makes it secrete FlgM, releasing FliA to transcribe the class III genes, which include the flagellin, the chemotaxis proteins and the stator subunits (Osterman et al. 2015).

The system differs considerably in the polarly flagellated γ-proteobacteria. In species of *Pseudomonas, Shewanella* and *Vibrio*, at least three transcriptional regulators are known, which regulate up to four hierarchical levels. The regulation by FliA-FlgM is still present at the IV level, but the expression of basal body, rod and hook proteins is σ^{54} dependent. A master regulator (named FleQ in *Pseudomonas*, FlrA in *V. cholerae* and *Shewanella* and FlaK in *V. parahaemolyticus* and *V. alginolyticus*), together with σ^{54}, transcribes the class II genes, encoding the basal body, *fliA* and also a two-component system (named FleSR, FlrBC or FlaLM). The two-component system is responsible for the transcription of class III genes, encoding the hook and rod proteins (Fig. 1.3C, Nandini Dasgupta et al. 2003; Y. K. Kim and McCarter 2000; Prouty, Correa, and Klose 2001; Kojima, Terashima, and Homma 2020; Shi et al. 2014)

Other regulators have been identified in the polar flagellar hierarchy, that are not traditional transcriptional regulators. FlhFG are two proteins with antagonic effects: FlhF is a positive activator of flagellar genes, whereas FlhG (also named FleN in *Pseudomonas*) is a negative regulator of FlrA/FleQ (Fig. 1.3C). They are both expressed in the second class of flagellar genes in the polarly flagellated γ-proteobacteria (Correa, Peng, and Klose 2005; Navarrete et al. 2019; N. Dasgupta and Ramphal 2001); the description of this regulatory system deserves its own section in this introduction. Furthermore, a small membrane protein named FlrD been described in *Vibrio* species as a positive flagellar regulator (Moisi et al. 2009; Petersen et al. 2021). Although it has the same regulatory effects as FlhF, it has its own promoter and its transcription is independent of σ^{54} and FlrA, placing it outside of the flagellar hierarchy (Fig. 1.3C).

Multiple points of regulations permit the integration of different signals to decide when and where the flagellum has to be made, more so than in the simpler hierarchy of peritrichous enterobacteria. FleQ has been shown to be sensitive to c-di-GMP (Jason W. Hickman and Harwood 2008; Matsuyama et al. 2016), which is a crucial signal in the decision to switch from a sessile to motile lifestyle in many bacteria (J. W. Hickman, Tifrea, and Harwood 2005; Teschler et al. 2015; Thormann et al. 2006). The two-component system (FleRS/FlrBC/FlaLM) has been proposed to sense the correct assembly of the export apparatus (Burnham, Kolar, and Hendrixson 2020). And the FlhFG system is essential to incorporate cues from the cell cycle to correctly position the flagellum at the poles.

1.2.2. FlhF and FlhG

The flagellation pattern is regulated in many bacteria by FlhF and FlhG. Not only in the polarly flagellated γ-proteobacteria, but also in species as diverse as the amphitrichous *Campylobacter* (Hendrixson and Dirita 2003) and spirochetes (Zhang et al. 2020; Fule et al. 2021), and even in the peritrichous *Bacillus* (Carpenter, Hanlon, and Ordal 1992; Salvetti et al. 2007). In all these species, *flhG* is always encoded downstream of *flhF*, in the same operon (Fule et al. 2021). In general, FlhF stimulates flagellar synthesis and its correct localization, and FlhG regulates the number of flagella.

As nucleotide hydrolases of the SIMIBI protein family, FlhF and G can act as molecular switches by alternating between a dimeric, nucleotide bound state, and a monomeric state after hydrolysis of the nucleotide (Fig. 1.4, Bange and Sinning 2013). It is this behavior that allows members of this family, like MinD and ParA, to establish molecular patterns and coordinate the localization of different targets across all domains of life.

FlhG is a repressor of flagellum formation. Its deletion leads to excess flagella (Schuhmacher et al. 2015; Correa, Peng, and Klose 2005; Nandini Dasgupta, Arora, and Ramphal 2000; Gulbronson et al. 2016), whereas its overexpression inhibits flagellation by repressing the expression of flagellar genes (Kusumoto et al. 2008; Correa, Peng, and Klose 2005; N. Dasgupta and Ramphal 2001). Thus, FlhG acts as a break that limits the number of flagella produced.

FlhG is a MinD-like ATPase. Upon binding ATP, it dimerizes and exposes an amphipathic helix that targets it to the membrane (Fig. 1.4, Schuhmacher et al. 2015). Dimeric FlhG can bind FleQ/FlrA and inhibit transcription of the class II flagellar genes, including itself (Chanchal, Banerjee, and Jain 2017; Chanchal et al. 2021; Blagotinsek et al. 2020). This circuit constitutes a negative feedback loop that resembles a simple genetic oscillator (Pigolotti, Krishna, and Jensen 2007). It was recently discovered that the transcriptional repression exerted by FlhG can be blocked through interaction with the C-ring component

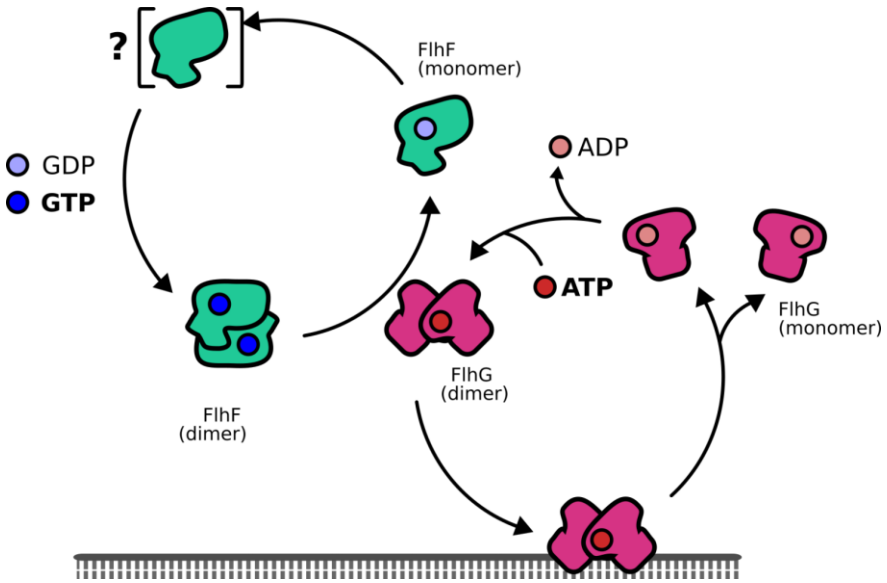

Figure 1.4. FlhF and FlhG. FlhF is a GTPase that forms a dimer upon binding of GTP. Hydrolysis produces a GDP-bound monomer. It's not clear how the GTP-bound state is regenerated. FlhG, a ParA-like ATPase, also forms a dimer upon binding of ATP, which recruits it to the membrane. The FlhG dimer can also activate GTP hydrolysis of FlhF. ATP hydrolysis induces monomerization of FlhG.

FliM, which sequesters FlhG to the cell pole during assembly of the flagellum (Blagotinsek et al. 2020). In this way, the transcriptional repression of class II genes is alleviated just as the first components of the flagellum are recruited to the pole of the daughter cell. This finding could also suggest the way in which repression of class II genes is restored after completion of the basal body.

On the other hand, FlhF is a positive regulator of flagellum formation. The deletion of *flhF* leads to reduced motility in a number of species. In *P. aeruginosa*, *S. putrefaciens* and *S. oneidensis* this is associated with mis-localization of flagella (Gao et al. 2015; Rossmann et al. 2015; Pandza et al. 2000; Murray and Kazmierczak 2006), whereas in *Vibrio* it is largely due to the absence of flagella and reduced expression of flagellar genes (Kusumoto et al. 2008; Correa, Peng, and Klose 2005). On the other hand, overexpression of FlhF can lead to bipolar flagella (Green et al. 2009).

FlhF is a GTPase. It is related to the signal-recognition particle FtsY that recruits the ribosome to the membrane during protein secretion (Bange et al. 2007). The main difference is that in addition to the GTP binding and catalytic domains (NG domains), FlhF has a longer N-terminal domain (B domain) that varies greatly between different species.

FlhF can form homodimers upon binding of GTP (Fig. 1.4). The role of the GTP binding and hydrolysis activity is not entirely clear. In many species, including *V. cholerae*, GTP hydrolysis is not essential for flagellation, but GTP binding is (Gulbronson et al. 2016; Green et al. 2009), suggesting that it's the GTP-bound FlhF dimer that promotes MS-ring formation. Furthermore, GTP hydrolysis of FlhF can be induced upon interaction with dimeric FlhG (Fig. 1.4, Bange et al. 2011; Gulbronson et al. 2016; Kondo et al. 2018), suggesting another way in which FlhG hinders flagellum formation.

The activating effect of FlhF on flagellum formation can be explained by its ability to recruit FliF to the cell pole and start assembly of the MS ring (Green et al. 2009). In this way, FlhF marks the flagellum assembly site. In *B. cereus*, the GTP-bound FlhF dimer can also help in the secretion of a virulence marker (Mazzantini et al. 2020), reminiscent of the activity of its SRP relative FtsY, and in *Aeromonas hydrophila* it permits secretion of a phage receptor (Dong et al. 2022). Whether FlhF can also aid in the secretion of the MS ring proteins remains to be seen.

In polarly flagellated bacteria, both FlhF and FlhG are found at the cell pole, and their localization is necessary for their function. In the case of FlhG, the localization is mediated directly by the general polar landmark protein HubP (Yamaichi et al. 2012; Takekawa et al. 2016; Rossmann et al. 2015). In fact, HubP is responsible for the localization of all three MinD-like ATPases in the Vibrionaceae: ParA from the chromosome segregation system, ParC from the chemotaxis clusters, and FlhG (Yamaichi et al. 2012; Rossmann et al. 2015). The delocalization of FlhG caused by the absence of HubP leads to hyper flagellation in *V. alginolyticus* (Takekawa et al. 2016), similar to an *flhG* deletion, but it leads to reduced flagellation in *S. putrefaciens* (Rossmann et al. 2015).

FlhF can also interact with HubP, but its polar localization is independent of it (Yamaichi et al. 2012; Rossmann et al. 2015). It's also independent of any flagellar protein in the FlrA regulon (Green et al. 2009). This suggests that the polar localization of FlhF could be intrinsic, or it could be mediated by an unknown factor. Mutations in FlhF predicted to disrupt GTP binding or hydrolysis do not affect polar localization nor flagellum synthesis in *V. cholerae (Green et al. 2009)*, but they do reduce polar localization in *V. alginolyticus* (Kusumoto et al. 2006). In *C. jejuni*, the GTPase activity of FlhF is required for proper localization but not for flagellum synthesis (Balaban, Joslin, and Hendrixson 2009). It's important to note that the localization of these variants of FlhF was mostly assayed by overexpression of the mutant protein, which sometimes can lead to artificial localization patterns (as was the case of HubP for example, (Galli, Paly, and Barre 2017)).

In addition, at least in *V. cholerae*, FlhF is membrane-bound (Green et al. 2009), although it lacks any recognizable membrane-targeting sequences. It is not known how is the association with the membrane mediated, whether it depends on GTP or the dimerization state, like for FlhG, or whether it is necessary for the role of FlhF stimulating flagellation. Interestingly, when FlhF from *V. alginolyticus* is expressed in *E. coli*, it is mostly found in the insoluble fraction (Kondo et al. 2018). This could be related to its membrane binding activity, or the lack of the hypothetical membrane-binding factor in the heterologous system. The addition of GTP was found to improve the solubilization (Kondo et al. 2018), suggesting that the solubility of FlhF could depend on its dimerization state.

1.3. Vibrio parahaemolyticus as a model organism

V. cholerae has been thoroughly studied for its significance to humanity as the causative agent of cholera. In fact, many members of the *Vibrionaceae* are pathogens. However, this family is also interesting because of its cell biology. This is the case of the subject of this work, *Vibrio parahaemolyticus*, which has been the subject of many clinical studies due to its causal role in food-borne gastroenteritis (Chowdhury et al. 2000), although humans are only a small part of the ecological niche of these bacteria. In fact, most strains of *V. parahaemolyticus* are not pathogenic to humans (Joseph, Colwell, and Kaper 1982).

V. parahaemolyticus is a curved rod-shaped bacterium that lives in estuarine environments (Joseph, Colwell, and Kaper 1982). It is a member of the *Harveyi* clade (Jiang et al. 2022), together with *V. alginolyticus* and *V. campbellii*, and not so closely related to *V. cholerae*. Like other members of this clade (and unlike *V. cholerae*), *V. parahaemolyticus* possesses two sets of flagellar genes, which are structurally unrelated and expressed under different conditions (L. L. McCarter 2004). The polar flagellum, which is the focus of this work, is constitutively expressed and is especially suited for swimming in liquid environments (Y. K. Kim and McCarter 2000; L. McCarter 1999). The lateral flagella are expressed as part of a larger differentiation program that sets into motion when the cells

encounter solid surfaces (Gode-Potratz et al. 2011). Cell division stops and the cells elongate up to 30 μm (Muraleedharan et al. 2018). In solid or semisolid environments, the elongated cells can use the lateral flagella to swarm and colonize surfaces more efficiently than swimming cells (Atsumi, McCartert, and Imae 1992; Freitas, Glatter, and Ringgaard 2020). Each flagellar system can operate independently and is fully functional in the absence of the other. However, some cross-talk does happen between the two regulatory networks (Y.-K. Kim and McCarter 2004).

All members of the *Vibrio* genus have two chromosomes. The larger one, or chromosome I, contains most essential genes, and its partition machinery is more closely related to that of single-chromosome γ-proteobacteria. Chromosome II resembles a plasmid in its mode of replication and segregation, and in that it encodes mostly accessory genes (Espinosa, Barre, and Galli 2017). Interestingly, the genes for the polar flagellum are encoded in chromosome I, and the genes for the lateral flagella in chromosome II (Makino et al. 2003). The chromosome II also encodes most pathogenicity genes of *V. parahaemolyticus*, which include two type III secretion systems, which are not found in *V. cholerae* ((Makino et al. 2003). This further highlights the difference between these two human pathogens.

The differences between *V. parahaemolyticus* and other model γ-proteobacteria merit its study to understand the molecular basis of the broad diversity of phenotypes observed in these bacteria.

1.4. Aims of this study

The spatiotemporal organization of intracellular components underlies the functions that the cells can perform. There is a molecular basis to the intracellular organization, and its study allows us to understand how cells develop, reproduce and perform their functions. Furthermore, by comparing different organisms, we can gain a better understanding of their function, and of the origin of their differences. The flagellum, for example, is one of such cellular functions present in a wide variety of organisms, with an equally varied number of mechanisms. Different molecular agents account for some functional differences, but sometimes one set of molecules can produce vastly different forms in different species.

The FlhFG system is responsible for the spatiotemporal regulation of the flagellum in bacteria with very different flagellation patterns. In polarly flagellated bacteria that use this system, like the *Vibrionaceae*, FlhFG restrict the flagella to the cell pole. HubP is the landmark protein that recruits FlhF and FlhG to the cell pole and promote their segregation to the daughter cell after division, together with the other ParA-like proteins that position the chromosome and the chemotaxis proteins. Nevertheless, the polar localization of FlhF is independent of HubP in *V. cholerae* and *V. alginolyticus*. With this work, we sought to investigate whether this is also true for *V. parahaemolyticus*, and to use it as a model to explore factors that determine the localization of FlhF in addition to HubP.

In the first chapter of this thesis, we describe the role of the FlhFG system in the regulation of the number and placement of the flagella in our *V. parahaemolyticus*, their dependence on HubP and on each other. We discuss the similarities and differences to other model organisms with polar flagella, like *V. cholerae.*

In the second chapter, we describe new functions of a protein that regulates flagellum formation, and we show that this protein allows us to explain some aspects of the behavior of FlhF. We show that this protein is an interaction partner of FlhF, and that this interaction is responsible for the localization of FlhF. In addition, we show that this interaction is conserved in the γ-proteobacteria *Pseudomonas putida.*

In the third chapter, the interaction between FlhF and this new protein is scrutinized at the molecular level, by comparing the behavior of heterologously expressed protein variants *in vivo* and *in vitro*. We present some evidence indicating that the interaction of FlhF with this new polar regulator depends on its state on the GTPase cycle, and speculate on the implications that this could have for the function of the system.

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2. Interdependent polar localization of FlhF and FlhG and their importance for flagellum formation of *Vibrio parahaemolyticus*

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2.1. Introduction

It is essential to understand the mechanisms required for dissemination of bacteria in the environment and for many bacteria, the primary means of motion is flagella-mediated swimming motility. Correct swimming behaviour heavily depends on the production of the correct number and proper placement of the flagella within the cell (Schuhmacher et al., 2015b; Blagotinsek et al., 2020; Kojima et al., 2020).

The localization of flagella in several species has been demonstrated to be mediated by landmark proteins. In particular, two proteins have been implicated in regulating the number (the ATPase FlhG) and positioning (the GTPase FlhF) of flagella in several bacterial species (Schuhmacher et al., 2015b). Interestingly, the FlhF/G system is responsible for the positioning of flagella in peritrichously, lophotrichously and monotrichously flagellated bacteria. In some γ-proteobacteria, such as *Pseudomonas sp*., *Shewanella sp*. and *Vibrio sp*., the flagella are positioned and formed only at the old cell pole. At cell division, one daughter cell inherits these flagella at its old cell pole, whereas the second daughter is non-flagellated, but begins to produce a flagellum at its old cell pole shortly after division is finalized.

In *Vibrio alginolyticus*, *S. putrefaciens* and *P. aeruginosa*, the absence of *flhG* results in hyper-flagellated cells (Campos-García et al., 2000; Hulko et al., 2006; Kusumoto et al., 2006, 2008; Schuhmacher et al., 2015a). Hyper-flagellation may be a result of increased flagellar protein production, as many flagellar genes have been shown to be upregulated in the absence of FlhG in these organisms

(Dasgupta et al., 2000; Dasgupta and Ramphal, 2001; Correa et al., 2005). Deletion of *flhF* has been shown to result in swimming defects due to the absence and/or mis-localization of flagella (Pandza et al., 2000; Correa et al., 2005; Kusumoto et al., 2006; Green et al., 2009). In *C. jejuni*, *V. alginolyticus* and *V. cholerae*, the absence of *flhF* results mostly in non-flagellated non-motile cells, however, in the rare cases in which a flagellum is formed nevertheless, it is no longer positioned at the cell pole (Correa et al., 2005; Kusumoto et al., 2008; Balaban et al., 2009). A different phenotype is observed in in *Pseudomonas sp.* (Pandza et al., 2000; Murray and Kazmierczak, 2006) and *S. putrefaciens* (Rossmann et al., 2015), where a single mis localized non-polar flagellum is produced in the absence of FlhF. Importantly, FlhF is thought to establish the site of flagellum assembly by recruiting the earliest flagellar structural component FliF, which constitutes the MS-ring (Green et al., 2009; Kojima et al., 2020; Terashima et al., 2020). Fluorescence microscopy studies have shown that FlhF is localized to the bacterial cell poles in several monotrichous bacterial species, including *P. aeruginosa* (Murray and Kazmierczak, 2006), *S. putrefaciens* (Rossmann et al., 2015), *V. alginolyticus* (Kusumoto et al., 2008), and *V. cholerae* (Green et al., 2009; Yamaichi et al., 2012; Takekawa et al., 2016). In all cases, FlhF shows a specific spatiotemporal localization pattern that is cell cycle-dependent. Particularly, FlhF localizes uni-polarly to the old flagellated cell pole in young short cells and displays a bi-polar localization in older longer cells and as a consequence each daughter cell inherits FlhF localized to its old cell pole when cell division is completed (Murray and Kazmierczak, 2006; Kusumoto et al., 2008; Rossmann et al., 2015; Takekawa et al., 2016).

FlhG has been shown to negatively regulate the intracellular localization of FlhF and positively influences flagellar production by regulating FlhF GTP hydrolysis (Bange et al., 2011). In *V. alginolyticus* and *V. cholerae* species, FlhG too localizes to the cell poles (Kusumoto et al., 2008; Yamaichi et al., 2012), which fits with its function in regulating FlhF localization at this site. However, data suggests that the recruitment of FlhG to the cell pole is independent of FlhF and instead depends on interactions with the polar landmark protein HubP (Yamaichi et al., 2012).

In summary, many different bacteria use FlhF and FlhG to regulate the localization and positioning of their flagella. The system's general function as positive and negative regulators of the flagellum synthesis, respectively, is largely conserved even among different bacterial phyla (Schuhmacher et al., 2015b; Kojima et al., 2020). Nevertheless, differences in the details of the system account for individual differences in the flagellar assembly even between members of the same genus (Schuhmacher et al., 2015b; Kojima et al., 2020). Because of these differences on the effect of the FlhF-FlhG system, also between closely related organisms, it is important to study this flagellum positioning system in different bacterial species in order to understand its importance for flagellum formation in the specific bacterium of interest.

Here, we have analyzed the importance of the FlhF-FlhG system for flagellum formation and swimming motility in the bacterium *Vibrio parahaemolyticus*. *V. parahaemolyticus* is an important human pathogen and the principal course of acute seafood-borne gastroenteritis in the world

(Letchumanan et al., 2014). Furthermore, it causes substantial problems in the aquaculture industry with early mortality syndrome (EMS) of shrimps, which is an important shrimp disease particularly in Southeast Asia (Tran et al., 2013). *V. parahaemolyticus* exhibits a dimorphic life-style depending on its environmental conditions – particularly as a swimmer or a swarmer cell. Swimmer cells are monotrichously flagellated and optimized for life in liquid environments. On solid surfaces it lives as a swarmer cell, which is a cell type specialized for colonization of solid environments. Swarmer cells are highly elongated and express a distinct flagellum system in addition to the polar flagellum of swimmer cells, which results in a multiple peritrichous flagella positioned along the length of the swarmer cell (Baumann and Baumann, 1977; McCarter, 2004; Böttcher et al., 2016). In liquid environments *V. parahaemolyticus* exists as a short motile swimmer cell that is propelled by a single polar flagellum, which is positioned at the old cell pole. Swimming motility is essential for the dissemination of *V. parahaemolyticus* in the environment and for its resistance to phage attacks (Zhang et al., 2016; Freitas et al., 2020). Consequently, it is essential to study the mechanisms regulating polar flagellum formation in this specific species in order to fully understand the forces driving its spreading and survival in the environment. Here, we show that FlhF and FlhG are required for proper formation of the polar flagellum and swimming motility in *V. parahaemolyticus*. We further analyze the intracellular localization of FlhF and FlhG during the cell cycle. Both proteins localize to the bacterial cell pole in a dynamic and cell cycle-dependent manner, however, importantly their patterns of localization are distinct from each other and FlhG undergoes a different localization pattern as that of FlhF. Interestingly, their localization patterns depend on each other and in the case of FlhG also on the cell pole determinant HubP.

2.2. Materials and Methods

2.2.1. Strains and growth media

All strains were grown in LB medium at 37 °C. When needed, indicated antibiotics were added. Genetic modifications in *V. parahaemolyticus* RIMD 2210633 were performed using standard allele exchange methods with plasmids derived from pDM4 (Milton et al., 1996). All *V. parahaemolyticus* strains were generated in a Δ*lafA* background to eliminate any cellular movement through the lateral flagella system of *V. parahaemolyticus*. *E. coli* DH5αλpir was used for cloning and SM10λpir for introducing plasmids into *V. parahaemolyticus* by conjugation. All strains and plasmids used are listed in Supplementary Table S1. Primers used are listed in Supplementary Table S2. A description of each plasmid is also included as Supplementary Information.

2.2.2. Swimming assay

Swimming assays were performed as described in (Ringgaard et al., 2013; Alvarado et al., 2017).

2.2.3. Fluorescence Microscopy

Fluorescence microscopy was carried out essentially as described by (Ringgaard et al., 2013; Heering and Ringgaard, 2016; Alvarado et al., 2017; Heering et al., 2017). Bacterial strains for fluorescence microscopy analysis were inoculated in LB medium and cultivated at 37 °C and shaking to an OD_{600} = 0.5-0.6. Cells were then spotted on a pad of 1 % agarose in 50 % PBS + 1 0% LB on a microscope slide, covered with a coverslip and imaged immediately. All microscopy was performed on a Nikon Eclipse Ti inverted Andor spinning-disc confocal microscope equipped with a 100x lens and an Andor Zyla sCMOS cooled camera and an Andor FRAPPA system. Microscopy images were analyzed using ImageJ imaging software (http://rsbweb.nih.gov/ij) and Metamorph Offline (version 7.10.2.240, Molecular Devices). FlhF-sfGFP fusion was imaged at 400 ms exposure, and sfGFP-FlhG at 1000 ms for all backgrounds. Demographs were constructed by measuring the fluorescence intensity profiles in Fiji and processing the data in R (3.0.1, R Foundation for Statistical Computing), using a script described by (Cameron et al., 2014; Alvarado et al., 2017; Heering et al., 2017; Muraleedharan et al., 2018)

2.2.4. Transmission Electron Microscopy

Cellular cultures were propagated using identical growth conditions as those used for fluorescence microscopy analysis. Cells were grown to an OD600 = 0.5-0.6. Samples were subsequently treated as described by (Ringgaard et al., 2007) and spotted on a plasma-discharged carbon-coated copper grid (Plano, Cat#S162-3) and rinsed with 0.002% uranyl acetate, blotted dry with Whatman filter paper, and further dried. TEM images were obtained with a JEOL JEM-1400 Plus 120 KV transmission electron microscope at 80 kV.

2.2.5. Western blot

Whole-cell extracts from the same cultures as used for microscopy were normalized by cell density, and equal amounts were loaded on an SDS-PAGE, blotted and probed with JL-8 anti-GFP monoclonal antibody (Takara Bio Cat# 632380, RRID:AB_10013427), and detected with horse-radish-peroxidase-conjugated anti-mouse IgG antibodies (Thermo Fisher Scientific Cat# 45-000-680, RRID:AB_2721110).

2.2.6. Sample size and statistical analysis

For microscopy experiments, a minimum of three biological replicates were performed, with >200 cells measured per replicate. Western blots were performed with samples from the same replicates as used for the microscopy analysis. The mean values of the replicates were plotted ± standard deviation. Statistical significance was evaluated with an ANOVA test with post hoc Tukey's test. Demographs were plotted using the cellProfiles R package (Cameron et al., 2014). Ten replicates of the swimming

assays were performed. The statistical significance was calculated with an ANOVA with different petri dishes as blocks. All calculations were done in R (R Development Core Team, 2008).

2.3. Results

In order to understand the importance of the FlhF-FlhG-system and HubP in motility of *V. parahaemolyticus*, we generated strains bearing in-frame deletions of either *flhF, flhG* and *hubP* (*ΔflhF*, *ΔflhG* and *ΔhubP*), and their effect on motility was analyzed by measuring swimming motility in soft-agar plates. As a control for no motility, we included a strain lacking the chemotaxis protein CheW (*ΔcheW*). Wild-type *V. parahaemolyticus* spread through the soft-agar, resulting in large swimming colonies, whilst no displacement was observed for the *ΔcheW* strain (Fig. 2.1A, B). The absence of FlhF resulted in a complete lack of displacement, similar to the *ΔcheW* strain (Fig. 2.1A, B). The absence of FlhG also significantly reduced swimming displacement by ~50% when compared to wild-type, however, cells were still more proficient swimmers than cells lacking FlhF (Fig. 2.1A, B). A strain lacking HubP was also significantly reduced in swimming displacement by ~65% when compared to wild-type, however, it was significantly more swimming proficient that a strain lacking FlhF (Fig. 2.1A, B). Interestingly, the absence of HubP resulted in a significantly stronger reduction in swimming ability than for cells lacking FlhG (Fig. 2.1A, B).

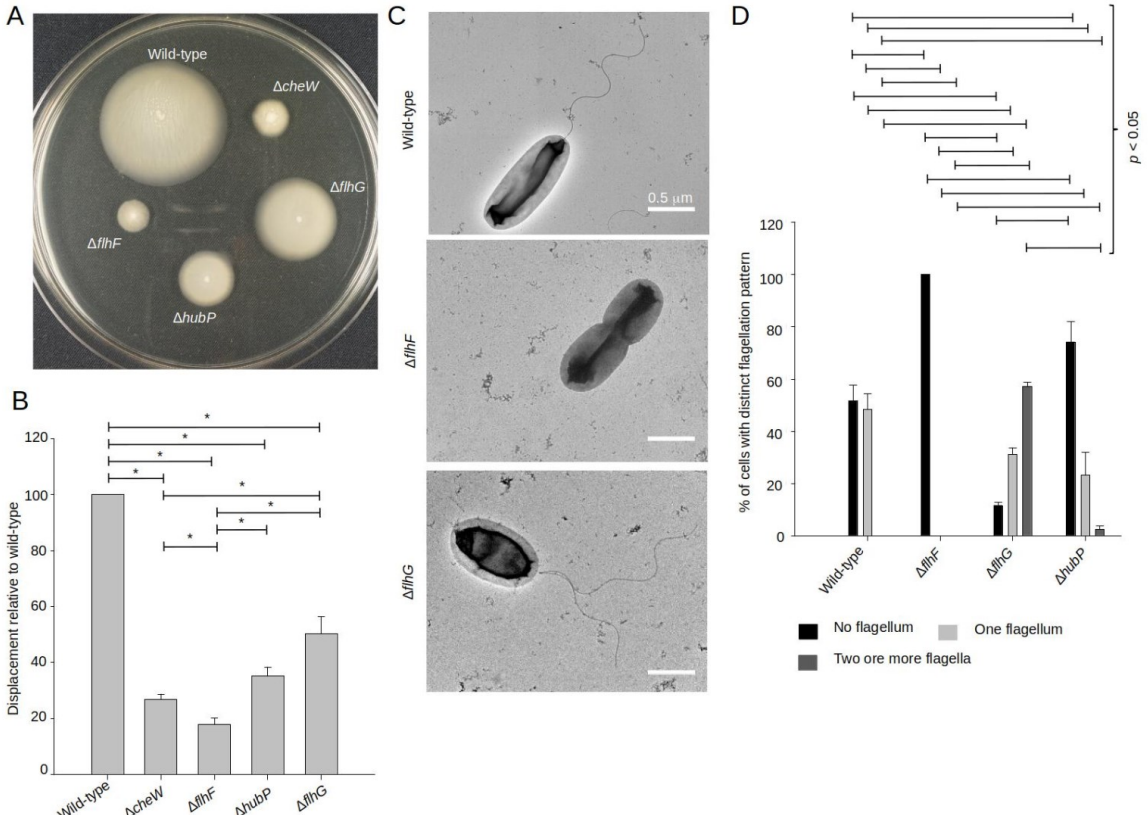

Figure 2.1. FlhF and FlhG regulate swimming and flagellum production in *V. parahaemolyticus*. (A) Representative image of a swimming assay in soft agar of indicated *V. parahaemolyticus* strains. **(B)** Bar graph showing the average diameter of swimming colonies of the indicated *V. parahaemolyticus* strains relative to wild-type cells. **(C)** Representative transmission electron micrographs of the indicated *V. parahaemolyticus* strains stained with uranyl acetate. **(D)** Bar graph depicting the average percentage of cells with distinct flagellation patterns, n=200 cells. **(B, D)** Asterisk, *, indicates p<0.05, Tested with ANOVA in blocks + Tukey HSD. Error bars indicate standard deviation.

Upon analysis of the above strains using transmission electron microscopy (TEM), it became clear that the observed swimming defects in the absence of FlhF, FlhG or HubP was due to abnormalities in synthesis of the polar flagellum (Fig. 2.1C, D). A polar flagellum was observed for ~50% of wild-type cells whilst the other 50% were non-flagellated. This was in contrast to cells lacking FlhF where 100% were non-flagellated, thus showing that FlhF is required for swimming motility and flagellum production in *V. parahaemolyticus* (Fig. 2.1C, D). A different result was obtained for cells lacking FlhG, which on the contrary is a negative regulator of flagellum synthesis, as in its absence there was a significant increase in flagellated cells, with only ~15% of *ΔflhG* cells being non-flagellated (Fig. 2.1C, D). Furthermore, ~60% of *ΔflhG* cells displayed multiple flagella positioned at the same cell pole, which is virtually never seen for the wild-type. Importantly, no mis localized non-polar flagella were observed in any case. Interestingly, the absence of *hubP* increased both the numbers of non-flagellated cells (~70%) and multi flagellated cells (Fig. 2.1C, D).

2.3.1. FlhF and FlhG display distinct cell-cycle dependent polar localization patterns

To further elucidate the role that FlhF and FlhG have in determining the number and position of the polar flagellum, the proteins were tagged with super folder GFP (FlhF-sfGFP and sfGFP-FlhG) and their intracellular localization visualized by fluorescence microscopy. Importantly, both fusion proteins could either completely (FlhF-sfGFP) or partially (sfGFP-FlhG) complement their respective deletion strains when the native locus was replaced by the gene encoding the fusion protein (Fig. S2.1A, B). This indicates that FlhF-sfGFP is fully functional while sfGFP-FlhG is at least partial functional, and thus are likely to reflect the true localization of the proteins in vivo. Both proteins localized in three distinct patterns: diffuse, unipolar, bipolar. Both proteins localized as discreet foci at one of the cell poles (Fig. 2.2A, white arrows). In approximately 45% of cells FlhF was diffusely localized, whilst a significantly larger proportion (80%) of cells showed diffuse localization of FlhG (Fig. 2.2A, B). About 60% of the cells had at least one focus of FlhF at one of the poles (Fig. 2.2A, B), however, interestingly FlhF experienced two types of polar localization – uni-polar (~40%, Fig. 2.2A orange arrow, B) and bi-polar (~15%, Fig. 2.2A green arrow, B). Time-lapse microscopy showed that the two types of polar localization, was a result of a cell-cycle dependent transition in the polar localization pattern of FlhF. Particularly, time-lapse microscopy showed that in young new-borne cells FlhF localized uni-polarly at the old flagellated cell pole. Then, later in the cell cycle FlhF was recruited to the new non-flagellated cell pole, resulting in a bi-polar localization pattern. In consequence, each daughter cell inherited an FlhF cluster localized to its old pole upon completion of cell division (Fig. 2.3A). Occasionally, we observed that FlhF became diffuse after cell division, resulting in cells with no visible foci.

Similarly, FlhG was localized at the cell poles. However, the proportion of cells with polar FlhG foci was significantly lower than that observed for FlhF. Particularly, over 80% of the population had no visible FlhG foci, which instead was localized diffusely in the cytoplasm (Fig. 2.2A red arrow, B). When localized to the cells pole, FlhG was primarily localized in a uni-polar manner (~18%), while only

in a very small percentage (~2%) of cells was FlhG localized in a bi-polar manner (Fig. 2.2A, B) and observed primarily in cells very close to completing cell division. We next analysed the temporal localization pattern of FlhG during the cell-cycle using time-lapse microscopy. During the majority of the cell cycle, FlhG did not form polar foci but was instead localized diffusely in the cytoplasm (Fig. 2.3B). Interestingly, very close to completion of cell division FlhG was recruited to both cell poles

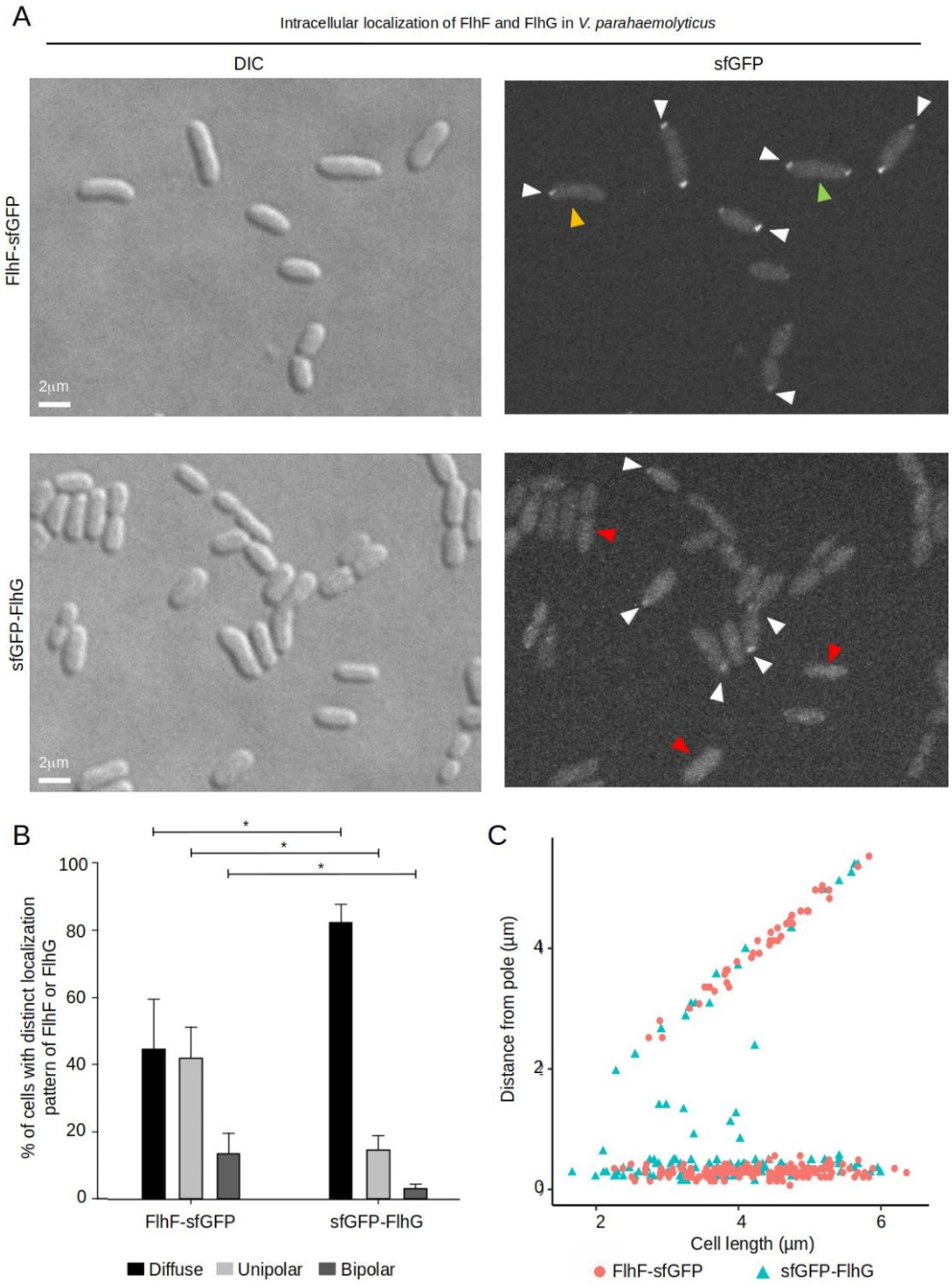

Figure 2.2. The intracellular localization of FlhF and FlhG in *V. parahaemolyticus*. (A) DIC and fluorescence microscopy of *V. paraharmolyticus* strains expressing FlhF-sfGFP or sfGFP-FlhG fusion proteins. White arrows indicate polar foci, orange arrows = unipolar foci, green arrows = bipolar foci, red arrow = difuse. **(B)** Bar graph showing the percentages of cells with fluorescent foci at one, two or no poles. **(C)** Graph depicting the distance of FlhF-sfGFP clusters from the cell poles as a function of cell length.

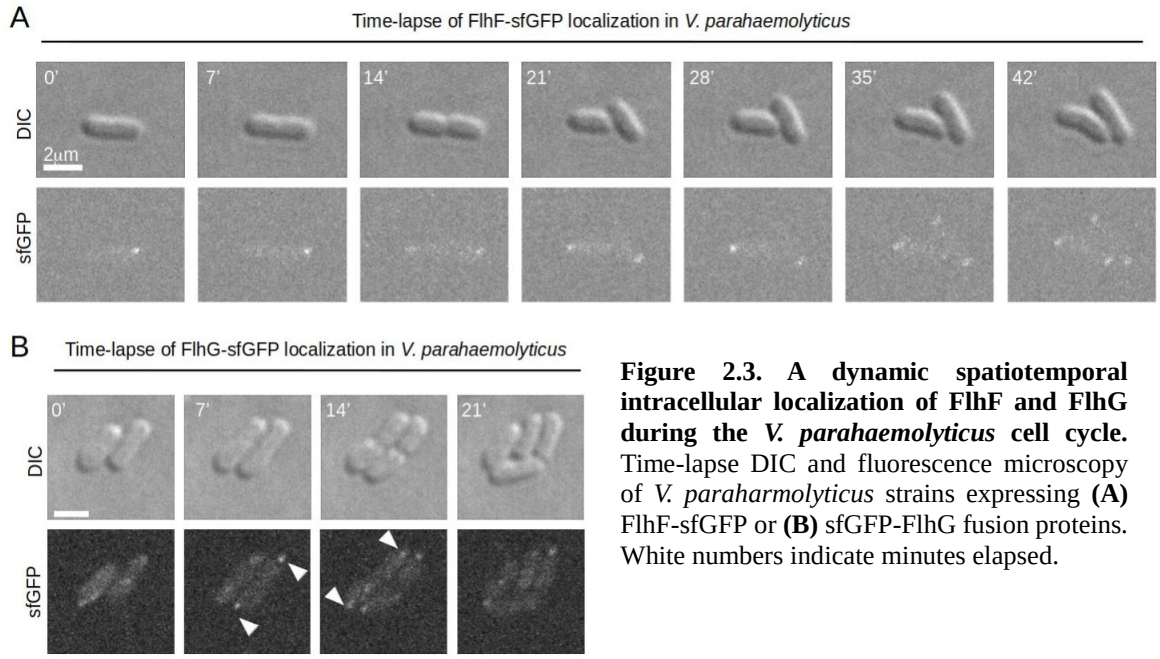

Figure 2.3. A dynamic spatiotemporal intracellular localization of FlhF and FlhG during the *V. parahaemolyticus* cell cycle. Time-lapse DIC and fluorescence microscopy of *V. paraharmolyticus* strains expressing **(A)** FlhF-sfGFP or **(B)** sfGFP-FlhG fusion proteins. White numbers indicate minutes elapsed.

resulting in a bi-polar localization pattern and as a consequence each daughter cell inherited FlhG localized to their respective old cell poles. Soon after completion of cell division, FlhG disappeared from the cell pole and was only localized diffusely in the cytoplasm.

2.3.2. FlhG is required for proper polar localization of FlhF

We next aimed to analyse how FlhF and FlhG might influence each other's intracellular localization and the importance of HubP on their recruitment to the cell pole. To this effect, the localization of FlhF was analysed in a Δ*hubP* and a Δ*flhG* background, respectively. FlhF was still capable of forming foci and localizing to the cell pole in the absence of HubP and no significant difference was observed in FlhF localization between wild-type and Δ*hubP* cells (Fig. 2.4A-C). Absence of FlhG, on the other hand, had a very clear effect on the intracellular localization of FlhF localization. Particularly, there was a significant increase in the percentage of cells with polarly localized FlhF and a concomitant decrease in cells with diffusely localized FlhF, with ~90% of cells with polarly localized FlhF in the absence of FlhG compared to ~55% of wild-type cells (Fig. 2.4A-C). Particularly, there was a striking increase in the number of cells with a bi-polar localization of FlhF in the absence of FlhG (~60%) compared to wild-type (~12%, Fig. 2.4A-C). Interestingly, demographic analysis showed that FlhF was recruited to the new pole earlier in the cell cycle in the absence of FlhG when compared to wild-type (Fig. 2.4B). Furthermore, analysis of the fluorescence intensity polar FlhF clusters, showed that polar FlhF clusters were significantly brighter in a Δ*flhG* background, when compared to wild-type and Δ*hubP*, suggesting an increased level of FlhF localized to the cell pole in the absence of FlhG (Fig. 2.4D). Consistently, Western-blot analysis determined that the level of FlhF-sfGFP was ~8.8 fold higher in the absence of FlhG, compared to wild-type and Δ*hubP* (Fig. 2.4E). These results, show that FlhG is required for the proper polar localization of FlhF in *V. parahaemolyticus*. They further indicate that FlhG negatively regulates the intracellular protein level of FlhF and its spatiotemporal localization and

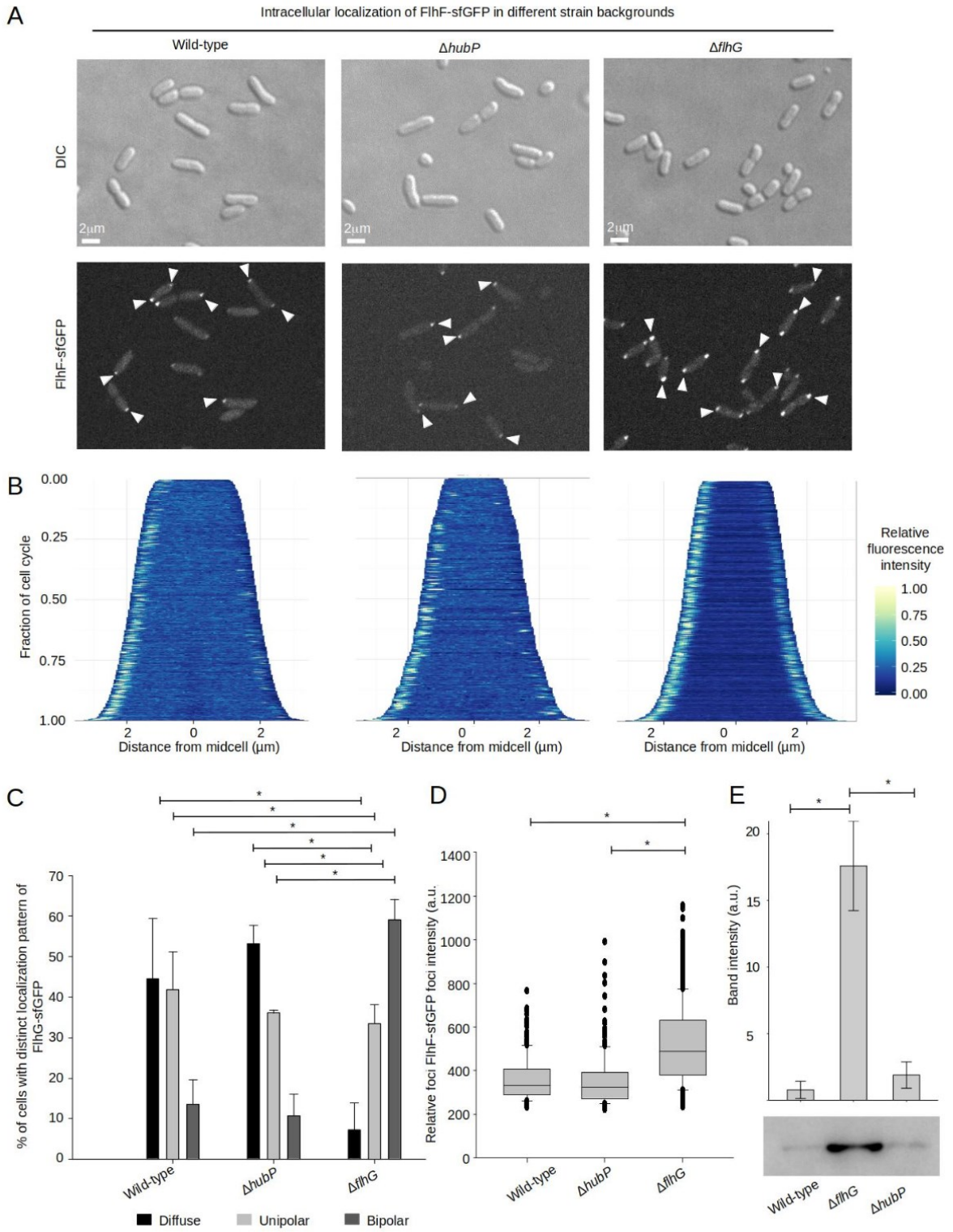

Figure 2.4. FlhG is required for proper intracellular localization of FlhF. (A) DIC and fluorescence microscopy of indicated *V. parahaemolyticus* strains expressing FlhF-sfGFP. White arrows indicate polar FlhF-sfGFP foci. **(B)** Demographs showing the fluorescence intensity of sfGFP along the cell length in a population of V. parahaemolyticus cells relative to cell length. Demographs include date from >600 cells pooled from three distinct experiments. **(C)** Bar graph showing the percentage of cells with distinct FlhF-sfGFP localization patterns in the indicated *V. parahaemolyticus* strain backgrounds. Asterisk, *, indicates $p<0.05$, tested with ANOVA in blocks + Tukey HSD. Error bars indicate standard deviation. **(D)** Box plot showing the fluorescence intensity of polar FlhF-sfGFP foci of the indicated *V. parahaemolyticus* strains. Asterisk, *, indicates $p<0.05$, tested with ANOVA + Tukey HSD. (E) Western blot with anti-GFP monoclonal antibody against whole cell extract of strains expressing FlhF-sfGFP. The bar-graph depicts the quantification of the signal detected from three biological replicates. Error bars indicate standard deviation and asterisk, *, indicates $p<0.05$, tested with student's t-test.

suggest that HubP has very little or no influence on the intracellular localization of FlhF.

2.3.3. HubP and FlhF are required for proper recruitment of FlhG to the cell pole

Next, we analysed the importance of FlhF and HubP on the intracellular localization of FlhG. Both FlhF and HubP were individually required for proper intracellular localization of FlhG and in the absence of either protein there was a significant reduction in the percentage of cells with polarly localized FlhG (Fig. 2.5A, B). Where FlhG was localized as clusters in ~15% of wild-type cells, only ~2-3% of cells showed polarly localized FlhG in the absence of HubP or FlhF (Fig. 2.5A, B). Interestingly, even though FlhG no longer localized as clusters at the cell pole in the absence of HubP or FlhF, it was observed to localize as distinct foci along the length of the cell in ~10% of cells (Fig. 2.5A blue arrows, B-C), while such foci only were observed in ~2% of wild-type cells. However, our results also suggested that sfGFP-FlhG was unstable in the absence of FlhF. Particularly, after culturing of the strain, the fluorescent signal of the sfGFP-FlhG in a Δ*flhF* background, always faded in the population until it was no longer possible to detect. However, a PCR assay in all cases confirmed the gene encoding *sfGFP-flhG* in its correct locus. Western-blot analysis showed that in the Δ*flhF* background the level of sfGFP-FlhG was significantly lower than in wild-type and Δ*hubP* cells (Fig. 2.5D). These results together suggest that both HubP and FlhF are required for the proper polar localization of FlhG and that in their absence FlhG is capable of forming non-polar clusters along the length of the cell.

2.4. Discussion

In this study, we have investigated the spatiotemporal localization of the polar flagellum determinants FlhF and FlhG in *V. parahaemolyticus*. We showed that both FlhF and FlhG are required for proper swimming of *V. parahaemolyticus* and that absence of either protein results in a significant defect in swimming ability. Particularly, TEM analysis showed that deleting FlhF resulted in a complete absence of flagella, similar to what has been observed in *V. cholerae* and *V. alginolyticus* (Correa et al., 2005; Kusumoto et al., 2008). This shows that FlhF is essential for flagellum formation in *V. parahaemolyticus* and suggests that the function of FlhF is similar in the three Vibrio species (Correa et al., 2005; Kusumoto et al., 2008). Absence of FlhG in *V. parahaemolyticus* resulted in a hyper flagellation phenotype, again similar to what has been observed for other γ-proteobacteria (Correa et al., 2005; Kusumoto et al., 2008; Gao et al., 2015), suggesting that FlhG is a negative regulator of flagellum synthesis and acts to ensure that only one flagellum is formed at the cell pole in *V. parahaemolyticus*. This further supports that the FlhF-FlhG system works in very similar ways, particularly in Vibrio species.

We further showed that both FlhF and FlhG undergo a dynamic intracellular localization, where both proteins localized to the cell pole in a cell cycle-dependent manner. FlhF and FlhG displayed very distinct patterns of localization throughout the cell cycle. Particularly, FlhF showed a localization pattern

that has been reported for FlhF in other polarly flagellated bacteria and Vibrio species as well (Correa et al., 2005; Murray and Kazmierczak, 2006; Rossmann et al., 2015). In young cells, FlhF was uni-polarly localized at the old flagellated cell pole. Then, later in the cell cycle FlhF was recruited to the new non-flagellated cell pole, resulting in a bi-polar localization pattern and as a result, each daughter cell inherited an FlhF cluster localized to its old cell pole. This is further supporting that the function of FlhF is identical within Vibrio species and similar to that reported in other polar flagellated bacteria.

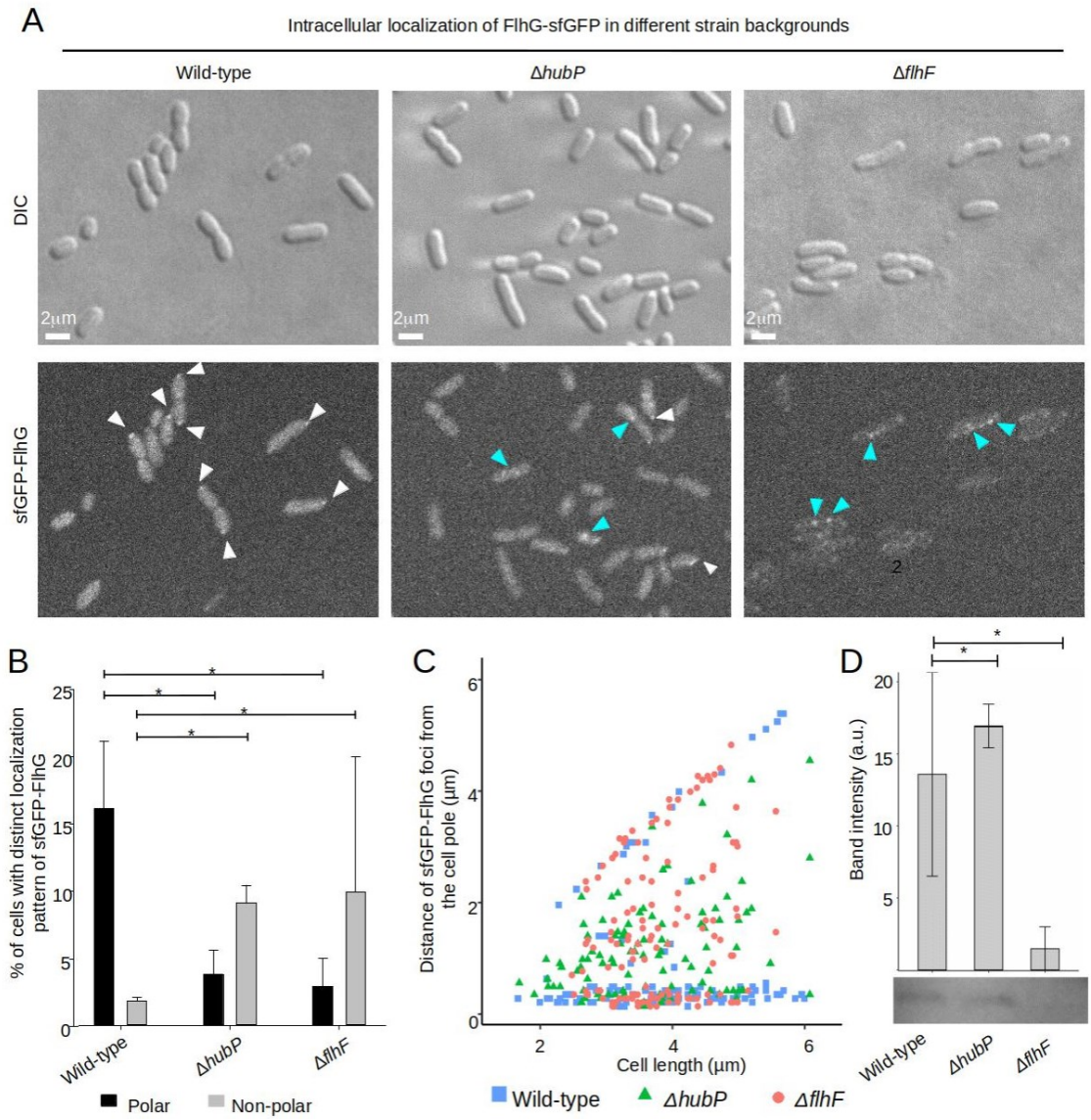

Figure 2.5. Proper intracellular localization of FlhG is regulated by HubP and FlhF. (A) DIC and fluorescence microscopy of sfGFP-FlhG in the indicated *V. parahaemolyticus* strains. White arrows indicate polar foci of sfGFP-FlhG and blue arrows indicate cytoplasmic clusters. **(B)** Bar graphs showing the percentage of cells with distinct localization pattern of sfGFP-FlhG in the indicated strains of *V. parahaemolyticus*. Error bars indicate standard deviation and asterisk, *, indicates $p<0.05$, tested with ANOVA + Tukey HSD. **(C)** Graph depicting the distance of sfGFP-FlhG clusters from the cell poles as a function of cell length in the indicated *V. parahaemolyticus* strain backgrounds. (D) Western blot with anti-GFP monoclonal antibody against whole cell extract of strains expressing sfGFP-FlhG. The bar-graph depicts the quantification of the signal detected from three biological replicates. Error bars indicate standard deviation and asterisk, *, indicates $p<0.05$, tested with student's t-test.

Despite the wealth of knowledge in regard to the intracellular localization of FlhF, it remains an open question how it is recruited to the cell pole. In other organisms, it has been shown that FlhG relies on the cell pole determinant protein HubP for its recruitment to the cell pole. Here, we show that FlhG in *V. parahaemolyticus* also depends on the protein HubP for its recruitment to the cell pole. But, in contrast to FlhG, the recruitment of FlhF to the pole seemed to be independent of HubP – again consistent with what has been observed for FlhF and FlhG in other Vibrio species (Yamaichi et al., 2012; Rossmann et al., 2015; Takekawa et al., 2016). Our data does suggest that, similar to what is observed in *V. alginolyticus* (Kusumoto et al., 2006, 2008), recruitment of FlhF to the cell pole is negatively regulated by FlhG. Particularly, in the absence of FlhG, a much larger proportion of cells showed polarly localized FlhF and there was particularly an increase in the percentage of cells with a bi-polar localization pattern of FlhF in the absence of FlhG. Additionally, FlhF foci at the cell poles were brighter in the absence of FlhG, suggesting an increased recruitment of FlhF to the cell poles in this background. In other *Vibrio* species, deleting *flhG* increases the transcription of flagellar genes, including *flhF* (Correa et al., 2005). It is likely that the increased size and number of FlhF foci observed in our *V. parahaemolyticus* strain was due to an increase in the amount of FlhF molecules present in the cell. Indeed, a Western-blot confirmed that the protein levels of FlhF-sfGFP were much higher in the Δ*flhG* strain compared to the wild-type *V. parahaemolyticus*. Furthermore, we were not able to tell if FlhG directly influences localization of FlhF at the cell poles, however, given data of the system from other organisms, which have shown that FlhG directly interacts with and regulates FlhF's GTP hydrolysis and nucleotide bound state (Balaban et al., 2009; Bange et al., 2011; Kazmierczak and Hendrixson, 2013; Schniederberend et al., 2013), it is likely that this is also the situation in *V. parahaemolyticus*. Thus, the effect of FlhG on FlhF localization is likely a combination of its regulatory function on FlhF's protein level within the cell and FlhG-dependent regulation of FlhF's nucleotide cycle.

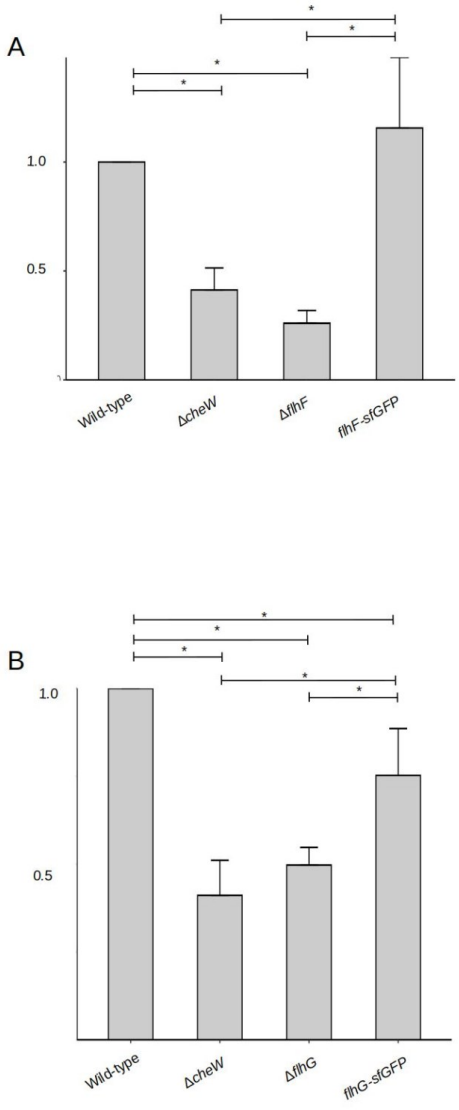

Figure S2.1. FlhF-sfGFP and sfGFP-FlhG fusion proteins are functional for swimming behavior of *V. parahaemolyticus*. (A) Complementation of a *ΔflhF* strain with *flhF-sfGFP*. Bar graph shows the average diameter of swimming colonies of the indicated *V. parahaemolyticus* strains relative to wild-type cells. **(B)** Complementation of a *ΔflhG* strain with *sfGFP-flhG*. Bar graph shows the average diameter of swimming colonies of the indicated *V. parahaemolyticus* strains relative to wild-type cells. (A, B) Error bars indicate standard deviation. Asterisk, *, indicates $p<0.05$, tested with ANOVA in blocks + Tukey HSD

FlhG, too, has been reported to localize to the bacterial cell pole in other *Vibrio* species (Kusumoto et al., 2008; Ringgaard et al., 2011; Yamaichi et al., 2012; Rossmann et al., 2015). However, here we show that FlhG, in contrast to FlhF, remained diffusely localized in the cytoplasm for the majority of the cell cycle in *V. parahaemolyticus*, and only when the cell was close to completion of cell division was FlhG recruited to both cell poles, resulting in a bi-polar localization pattern immediately before cell division was finalized. As a result, FlhG localized to the old-cell pole of each daughter cell immediately after cell division, whereafter it was delocalized from the pole and again diffusely localized in the cytoplasm. Thus, not only did FlhF and FlhG show distinct localization patterns, but also relied on different mechanisms for their recruitment to the cell pole. Polar localization of FlhG was strictly dependent on the cell pole-determinant HubP, while polar localization of FlhF was HubP independent. This distinct dependency on HubP for their recruitment to the cell pole has been shown in other related bacterial organisms and Vibrio species as well (Kojima et al., 2020). This again supports the notion that FlhF and FlhG work in *V. parahaemolyticus*, in a manner similar to that reported in other Vibrio species. The mis-localization of FlhG from the cell pole, could be responsible for the increase in flagellation observed in the *ΔflhG* and *ΔhubP* strains. It remains to be investigated whether this effect is caused by the diminished presence of FlhG at the pole, where in wild-type it interacts with components of the flagellum assembly, or the increased presence of FlhG in the cytoplasm, where it could regulate expression of flagellar genes. A combination of both mechanisms is also possible.

Furthermore, as FlhF still localizes properly to the cell pole in the absence of HubP, where FlhG is mis-localized and found only in the cytoplasm, our data suggest that FlhG does not need to be localized to the cell pole in order to carry out its effect on the localization of FlhF. We further show that the protein levels of FlhF is similar to wild-type levels in the absence of HubP, while there is a significant increase in FlhF levels in the absence of FlhG. Altogether, these results suggest that polar localization of FlhG is not directly to regulate FlhF localization dynamics and protein levels, and thus might serve an additional purpose related to FlhG's function in regulating proper flagellation pattern. Interestingly, we show that in the absence of either HubP or FlhF, FlhG forms non-polar foci in the cytoplasm of the cell, suggesting a secondary localization site for FlhG within the cell, besides its recruitment to the cell poles. In the absence of FlhF, there was an unstable expression of sfGFP-FlhG, which we were unable to explain. Consequently, we are not able to tell for sure if the effect on the localization of sfGFP-FlhG in the absence of FlhF, was a result of this unstable expression of the fusion protein itself or due to the lack of a direct regulatory role of FlhF on FlhG activity via FlhF-FlhG protein-protein interactions. However, as there was no change in sfGFP-FlhG expression level in the absence of HubP and FlhG formed non-polar foci in this background too, we think that these non-polar foci reflect a true secondary localization site of FlhG, which is more prevalent upon its delocalization from the cell pole. Other ParA-like ATPases are known for binding DNA. This ability is essential for their role as spatiotemporal regulators of cell components (Hester and Lutkenhaus, 2007; Ringgaard et al., 2009; Roberts et al., 2012). The FlhG homolog in *P. aeruginosa*, FleN, interacts with the master transcriptional regulator of flagella

FleQ. Together they bind specific sites on the chromosome, regulating the transition between biofilm and motile lifestyles (Navarrete et al., 2019). In *V. cholerae*, both FlhF and FlhG are known transcriptional regulators of flagellar genes (Correa et al., 2005). Indeed, very recently it was shown that FlhG plays a very direct role in regulating the expression of flagellum genes in *S. putrefaciens* by connecting the initial phases of flagellum formation with the activity of the transcriptional regulator FlrA (Blagotinsek et al., 2020), which in *V. parahaemolyticus* is referred to as FlaK. It would be interesting to study whether a similar regulatory mechanism exists in *V. parahaemolyticus*. We find it noteworthy that FlhG so directly interacts with a transcriptional regulator (Blagotinsek et al., 2020) and we speculate that perhaps its localization in non-polar foci, which we observe in the absence of HubP, could be related to its function in transcriptional regulation and possibly reflect an interaction with transcriptional regulators on the chromosome – hereby giving rise to the distinct focus localization sites that are particularly enhanced in the absence of HubP in *V. parahaemolyticus*. In this way, we would like to hypothesize that the localization of FlhG to the cell pole might not only reflect a function in regulating FlhF activity, but possibly to sequester it spatially to prevent its action on transcriptional regulation in the cytoplasm as a specific cell cycle check point.

Lastly, we would again like to address the polar localization of FlhF. Interestingly, our data show that in the absence of FlhG, FlhF is recruited earlier in the cell cycle to the new cell pole, resulting in an earlier establishment of its bi-polar localization. However, despite FlhF always being bi-polarly localized before cell division, and this occurring even earlier in the cell cycle in the absence of FlhG, *V. parahaemolyticus* is never flagellated at both cell poles at any point during the cell cycle – only ever at its old cell pole. This, indicates that localization of FlhF at the new cell pole is not sufficient to initiate a complete and finalized flagellum formation at this site before cell division has been completed. This further suggests that a so-far unknown factor is required for stimulation of flagellum production at the old cell pole only. Or the presence of an unknown factor prevents or inhibits FlhF function, when FlhF is positioned at the new cell pole. Thus, further studies are required in order to understand how FlhF is recruited to the cell pole and how monotrichously flagellated bacteria inhibit a flagellum to form at their new cell pole during the progression of the cell cycle, despite the flagellum determinant FlhF being bi-polarly localized for a significant part of the cell-cycle. Ultimately, knowledge of these distinct differences between species will help to shed light on the molecular details that allow bacteria to count and position their motility system in many sorts of different arrangements.

2.5. Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

2.6. Author Contributions

EEA-P carried out the experimental work. SR conceived the study. EEA-P and SR designed the research and experiments, analyzed the data and wrote the manuscript.

2.7. Funding

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2.8. Acknowledgments

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3. A conserved cell-pole determinant organizes proper polar flagellum formation

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This chapter is written in manuscript style, and is a work in preparation for submission. My contribution to this work was designing and performing the experiments in Vibrio parahaemolyticus, *and the FlhF Co-IP experiment, as well as analyzing the general data and writing the manuscript.*

3.1. Introduction

Many cellular processes depend on a specific spatiotemporal organization of its components. The DNA replication machinery, cell division proteins and motility structures are some examples that need to be positioned at a particular site in the cell in a coordinated manner to ensure adequate cell division or proper function of the structures.

To carry out this task, many cellular components of bacteria have a polar organization, *i. e.* they are asymmetrically positioned inside the cell, which is particularly apparent in, *e. g.*, monopolarly flagellated bacteria. In the marine bacteria *Vibrio*, which constitutively express a single polar flagellum (McCarter 1995; Kim and McCarter 2000), it is crucial to coordinate the localization and timing of flagellar components, in order to guarantee that newly born cells only produce a flagellum around cell division. This process is coordinated with the chromosome replication and the chemotaxis clusters through a supramolecular hub that is tethered at the old cell pole (Takekawa et al. 2016; Yamaichi et al.

2012). This organization depends on ATPases of the ParA/MinD family, which regulate the migration of the hub components to the new cell pole as the cell cycle progresses. In this way, the new cell has a copy of the chromosome and a set of chemotaxis clusters positioned next to what will be the site for the new flagellum. Central to this process is the protein HubP, which recruits to the pole the three ATPases responsible for the localization of these organelles: ParA1 for the chromosome (Fogel and Waldor 2006), ParC for the chemotaxis clusters (Simon Ringgaard et al. 2011) and FlhG for the flagellum (Correa, Peng, and Klose 2005; Arroyo-Pérez and Ringgaard 2021). Orthologs of HubP occur in several species, e.g., *Pseudomonas* (here called FimV), *Shewanella* or *Legionella*, where they similarly act as organizers (hub) of the cell pole (Wehbi et al. 2011; Rossmann et al. 2015; Coil and Anné 2010).

The bacterial flagellum is one of the most complex subcellular structures known. It is composed of around 25 proteins, which have to be assembled in a spatiotemporally coordinated manner (Macnab 2003). Although there may be significant differences among species, flagella in general are composed of a motor attached to the cytoplasmic membrane, a rod connecting it to the extracellular space, a hook and a filament. The basal body is composed of a cytoplasmic C-ring or switch complex (Francis et al. 1994), which receives the signal from the chemotaxis proteins and transfers it to the MS ring, which is embedded in the cytoplasmic membrane (Homma et al. 1987; Ueno, Oosawa, and Aizawa 1992). Attached to it, on the cytoplasmic side, is the export apparatus, which allows secretion of rod, hook and filament components (Minamino 2014). Flagella can also be sheathed, if an extension of the outer membrane covers the filament, as is the case in many *Vibrio* species (Chen et al. 2017).

The positioning of the flagellum is regulated in many bacteria by the interplay between FlhF, an SRP-type GTPase, and FlhG. Studies in a wide variety of proteobacteria indicate that FlhF is a positive regulator of flagellum synthesis and necessary for proper localization, whereas FlhG is a negative regulator that restricts the number of flagella synthesized. In organisms such as *Campylobacter jejuni*, *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Pseudomonas putida*, *Shewanella putrefaciens* and *Shewanella oneidensis*, the absence of *flhF* results reduced number of flagella, and mis-localization (Hendrixson and Dirita 2003; Correa, Peng, and Klose 2005; Arroyo-Pérez and Ringgaard 2021; Pandza et al. 2000; Rossmann et al. 2015; Gao et al. 2015). In contrast, the absence of *flhG* results in hyper-flagellated cells in strains of *Vibrio*, *Shewanella putrefaciens* and *Pseudomonas aeruginosa* (Kusumoto et al. 2006; Arroyo-Pérez and Ringgaard 2021; Schuhmacher et al. 2015; Campos-García et al. 2000; Murray and Kazmierczak 2006).

FlhF is responsible for marking the site for the new flagella and recruiting the components of the MS ring (Green et al. 2009; Zhang et al. 2020). However, it remains a mistery how the localization of FlhF is established in the first place. In all species tested, it is independent of any known factor, including HubP, or any other flagellar component (Green et al. 2009; Yamaichi et al. 2012; Takekawa et al. 2016; Rossmann et al. 2015; Arroyo-Pérez and Ringgaard 2021).

In this study, we characterize a small membrane protein, FipA, that interacts with FlhF to promote flagellar synthesis in *Vibrio parahaemolyticus*. By also studying the lophotrichous model *P. putida*, we show that the activating effect of FipA in FlhF is broadly conserved. The spatiotemporal localization behavior of FipA, as well as its relationship to FlhF support its role as a licensing protein that enables flagellum synthesis.

3.2. Results

3.2.1. Identification of an FlhF protein interaction partner, FipA

In order to identify potential factors required for FlhF function and its recruitment to the cell pole, we performed affinity purification of a super folder green fluorescent protein (sfGFP)-tagged FlhF (FlhF-sfGFP) ectopically expressed in wild-type *V. parahaemolyticus* cells followed by tandem mass spectrometry (MS/MS) analysis. Several proteins were significantly co-purified with FlhF-sfGFP, of which eight were structural components of the flagellum (Fig. 3.1A, supplementary table S1). Interestingly, the protein VP2224 was also significantly co-purified with FlhF (Fig. 3.1A, supplementary table S1). The homologue of VP2224 in *V. cholerae* was named FlrD because it acts as a positive regulator of transcription of class III genes, just like FlhF (Moisi et al. 2009). We were surprised to find it in the interaction network of FlhF, indicating that it has a more direct role in flagellar regulation.

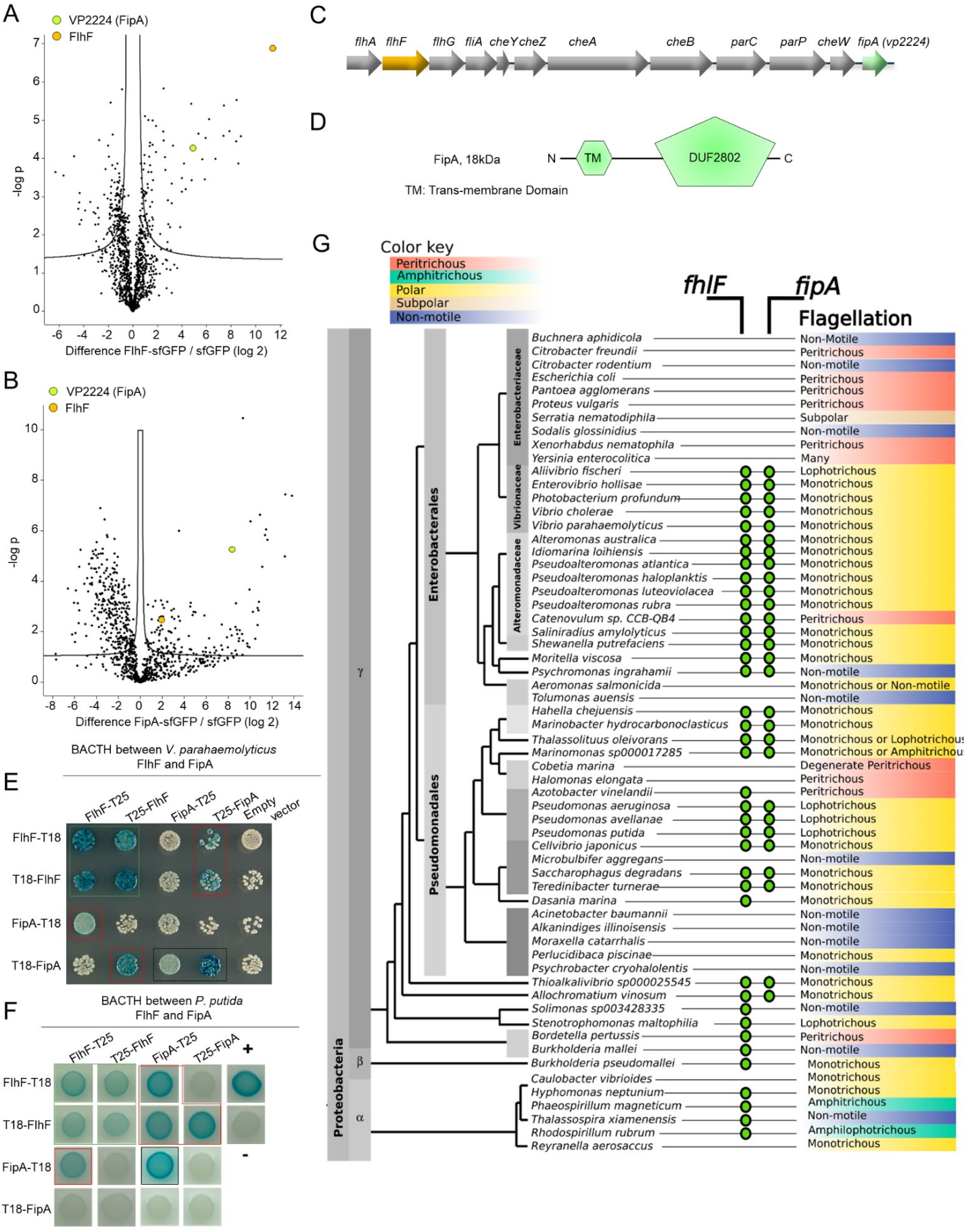
FlhF was also significantly co-purified in the reciprocal co-IP-MS/MS experiment using VP2224-sfGFP as bait (Fig. 3.1B). Furthermore, bacterial two-hybrid (BACTH) assays in the heterologous host *E. coli* also showed an interaction between FlhF and the cytoplasmic part of VP2224. We also observed self-interaction of both proteins (Fig. 3.1E). Altogether, these data indicate that FlhF and VP2224 form an interaction complex in both the native and a heterologous host organism, and that they both self-interact. After these findings, we refer to VP2224 as FipA, standing for Flh**F I**nteraction **P**artner **A**.

3.2.2. FipA constitutes a new family of FlhF interaction partners

The gene encoding FipA is located immediately downstream of the flagellar operon that encodes FlhAFG, FliA and the chemotaxis proteins (Fig. 3.1C). In *V. parahaemolyticus*, FipA consists of 163 amino acids with a molecular mass of 18.4 kDa. *In silico* analysis and membrane topology mapping predicted that FipA consists of a very short periplasmic N-terminal part consisting of aa 1-5 followed by transmembrane region (6-28), and a cytoplasmic part consisting of a coiled region (aa 31-58) and a

Figure 3.1. FipA constitutes a new family of FlhF interaction partners. A) Log-ratios of mass-spectrometry-identified pulled down targets using FlhF-sfGFP vs sfGFP used as control. Full list of targets in Table S1. **B)** Reciprocal pull-down using FipA-sfGFP as bait. **C)** Genomic context of *fipA* in *V. parahaemolyticus*. **D)** Domain organization of FipA. **E)** BACTH between the cytoplasmic fragment of FipA and FlhF from *V. parahaemolyticus.* **F)** BACTH between FipA and FlhF of *P. putida*. **+** indicates positive interaction control (T18-zip vs T25-zip), **-** indicates negative control (T18 vs T25). **G)** Dendrogram of proteobacteria, indicating the presence of FlhF or FipA homologues, and the flagellation pattern. Extended version and sources available in Table S2. →

domain of unknown function, DUF2802, positioned in the C-terminal half of the protein (aa 68-135) (Fig. 3.1D, Supplementary Fig. S3.1).



A search on the InterPro database found that the domain architecture of FipA (*i. e.* a transmembrane domain followed by a single cytoplasmic DUF2802 repeat) are widespread among γ-proteobacteria. The *Enterobacteriaceae* are the exception, encoding no copies of either FipA nor of FlhFG. In fact, FipA is only present in genomes that also encode FlhFG (Fig. 3.1G), which prompts the question of whether FipA is also involved in regulating the flagellation pattern via the FlhFG system. By adding the flagellation pattern reported in the literature to the comparison between these bacteria, we could see that the species that encode FipA are all polar flagellates, either monotrichous or lophotrichous (Fig. 3.1G). FipA homologues are absent from bacteria that use the FlhFG system to produce different flagellation patterns, like the peritrichous *Bacillus* or the amphitrichous ε-Proteobacteria or Spirochetes (supplementary table S2). Interestingly, it is also absent from the α-Proteobacteria, where the presence of FlhF is the exception rather than the rule (Fig. 3.1G).

Based on these analyses, we hypothesized that FipA represents a new family of FlhF interaction partners important for the γ-proteobacteria. To test this hypothesis, we decided to analyze FipA function in our monotrichously flagellated model organism *V. parahaemolyticus* and in the distantly related and lophotrichously flagellated *Pseudomonas putida*. BACTH analysis showed that the FipA orthologue from *P. putida* (PpFipA, PP_4331) interacted with FlhF of their respective species and that they self-interacted (Fig. 3.1F) – a result similar to that of FipA from *V. parahaemolyticus* (VpFipA). Altogether, this supports our hypothesis and indicates that FipA represents a new class of FlhF-interaction partners.

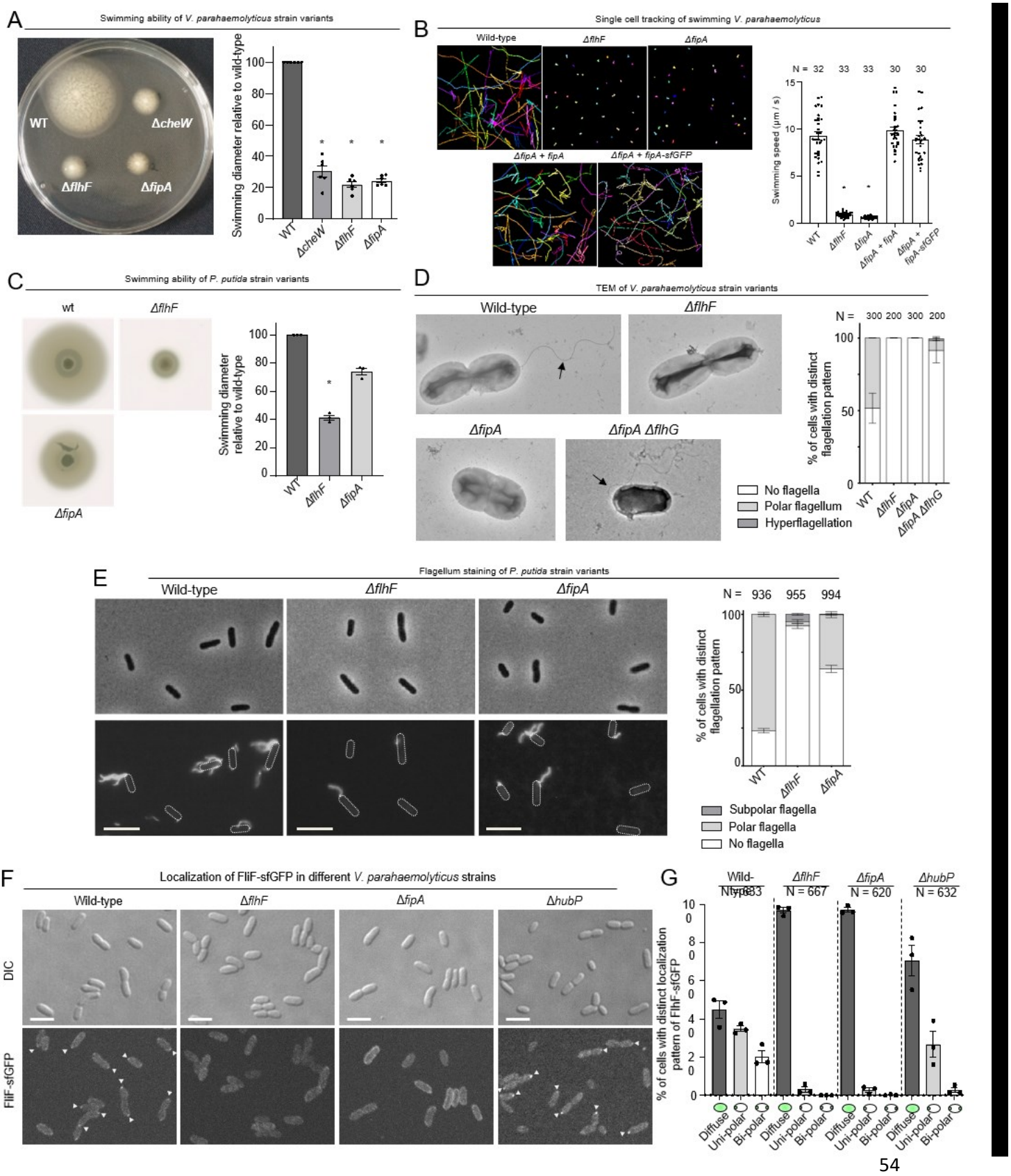
3.2.3. FipA is required for proper swimming motility and flagellum formation

To obtain a more comprehensive understanding of FipA function in relation to FlhF in different organisms, we continued our studies of FipA in both organisms, *V. parahaemolyticus* and *P. putida* in parallel. For the swimming assays in soft agar, a strain of *V. parahaemolyticus* deleted in the lateral flagellin gene (*ΔlafA*) was used as wild-type strain.

We first generated strains with individual deletions of the *fipA* and *flhF* genes. Strikingly, absence of FipA in *V. parahaemolyticus* completely abolished swimming motility in soft-agar medium to the same degree as cells lacking FlhF (Fig. 3.2A). Furthermore, single cell tracking of planktonic *V. parahaemolyticus* cells confirmed that cells lacking FipA were completely non-motile and behaved identical to cells lacking FlhF, as opposed to the fast and highly motile wild-type cells (Fig. 3.2B).

Figure 3.2. FipA is required for correct flagellum formation. Representative plate of swimming assay in soft agar of *V. parahaemolyticus* **(A)** or *P. putida* **(C)** strains, and halo diameter measurements normalized to the halo of the wild type on each plate. Six (A) or three (C) independent replicates, p-value <0.05, ANOVA + Tukey test. **B)** Single cell tracking of *V. parahaemolyticus*. Representative swimming trajectories and quantification of swimming speed. N indicates number of cells tracked among 3 biological replicates. ANOVA + Tukey test. **D)** Representative electron micrographs of *V. parahaemolyticus* strains stained with uranyl acetate and quantification of flagellation pattern in the population. Black arrows point at the flagellum filament. **E)** Flagellum stain of *P. putida* strains with Alexa 488 C5-maleimide and quantification in the population. N indicates number of cells counted among 3 biological replicates. **F)** Localization of FliF-sfGFP in different backgrounds of *V. parahaemolyticus* and **(G)** quantification of localization patterns in the population, from 3 biological replicates. White arrows indicate polar foci. All plots represent the mean ± SD + squares indicating the mean value from each replicate, except for (B), where the squares represent individual cells. →

Importantly, ectopic expression of FipA in the *ΔfipA* strain, restored the strain's swimming ability to wild-type levels (Fig. 3.2B), further supporting that it is the deletion of *fipA* that results in the phenotype and not polar effects resulting from the *fipA* deletion. The C-terminal FipA-sfGFP fusion used throughout this paper also restored the phenotype, indicating that the fusion protein is fully functional.



A similar phenotype was observed for *P. putida* cells lacking FipA, however, the phenotype was not as pronounced as that observed in *V. parahaemolyticus*. While cells lacking FlhF were severely affected in their ability to spread through soft agar (around 40 % of wild-type spreading, Fig. 3.2C), *fipA* deletion strains of *P. putida* were still able to spread, with a ~25% reduction (Fig. 3.2C). Altogether, these results indicate that FipA is necessary for flagella-mediated swimming motility in both model species, albeit to a different extent.

3.2.4. FipA is required for proper assembly of the polar flagellum

The swimming phenotypes in the absence of FipA could result from defects in either flagellar assembly or in the motor that propels flagellar movement. To differentiate between these possibilities, we examined *V. parahaemolyticus* by transmission electron microscopy (TEM, Fig. 3.2D) and *P. putida* by fluorescent labeling of the flagellar filament (Fig. 3.2E). Planktonic *V. parahaemolyticus* cells lacking FlhF or FipA, respectively, showed a complete absence of flagella on the bacterial surface, whilst a single polar flagellum was observed in ~50 % of wild-type cells (Fig. 3.2D). We also explored the effect of additionally deleting *flhG* in the Δ*fipA* background. Since the absence of FlhG produces hyper-flagellation, we were interested to see which phenotype would be dominant in a double mutant. Surprisingly, the deletion of FlhG suppressed the need of FipA for flagellum assembly in ~10% of the cells in a Δ*fipA* Δ*flhG* strain (Fig. 3.2D). A small proportion even produced multiple flagella on one cell pole, like in a single Δ*flhG* background.0

Flagellum quantification in *P. putida* showed that the deletion of PpFlhF almost completely abolished flagellar formation, but in contrast to *V. parahaemolyticus*, not completely. Mis-localization of flagella was also observed in this strain (Fig. 3.2E). The deletion of PpFipA also had a more modest effect than in *V. parahaemolyticus*, reducing the number of flagellated cells to about half of the wild-type (down to ~36% from ~77 %, Fig. 3.2G).

Thus, FipA and FlhF independently are essential for normal formation of the polar flagellum in both model species.

3.2.5. FipA is required for the early stages of flagellum basal body formation in *V. parahaemolyticus*

The assembly of the flagellum follows a very specific hierarchy, with the initial formation of the basal body to the generation of the filament and the formation of a mature flagellum complex (Kim and McCarter 2000; Dasgupta et al. 2003). However, it is not clear precisely at which stage of flagellum formation FipA acts. In order to analyze this, we fluorescently tagged the flagellum protein FliF (FliF-sfGFP) that constitutes the MS-ring, and that is recruited by FlhF in the formation of the basal body (Green et al. 2009). FliF-sfGFP localized as distinct foci in ~55 % of wild-type cells (Fig. 3.2F-G. In contrast, FliF-sfGFP was delocalized from the cell pole and showed a diffuse localization in ~97 % of cells lacking FlhF (Fig. 3.2F-G), indicating that FlhF is required for proper polar localization of FliF.

Strikingly, FliF-sfGFP was also completely delocalized from the cell pole in the absence of FipA and instead showed a non-specific diffuse localization, similar to that in cells lacking FlhF (Fig. 3.2F-G), thus, suggesting that both FipA and FlhF are required for the first stage of flagellum basal body formation in *V. parahaemolyticus*.

3.2.6. FipA and HubP ensure correct localization of FlhF to the cell pole

We analyzed if the intracellular localization of FlhF was influenced by FipA. In this regard, we used fully functional translational fusions of FlhF to fluorescent proteins expressed from its native site on the chromosome (Arroyo-Pérez and Ringgaard 2021). In *V. parahaemolyticus*, there was a significant delocalization of FlhF from the cell pole in the absence of FipA (Fig. 3.3A-C). As previously reported

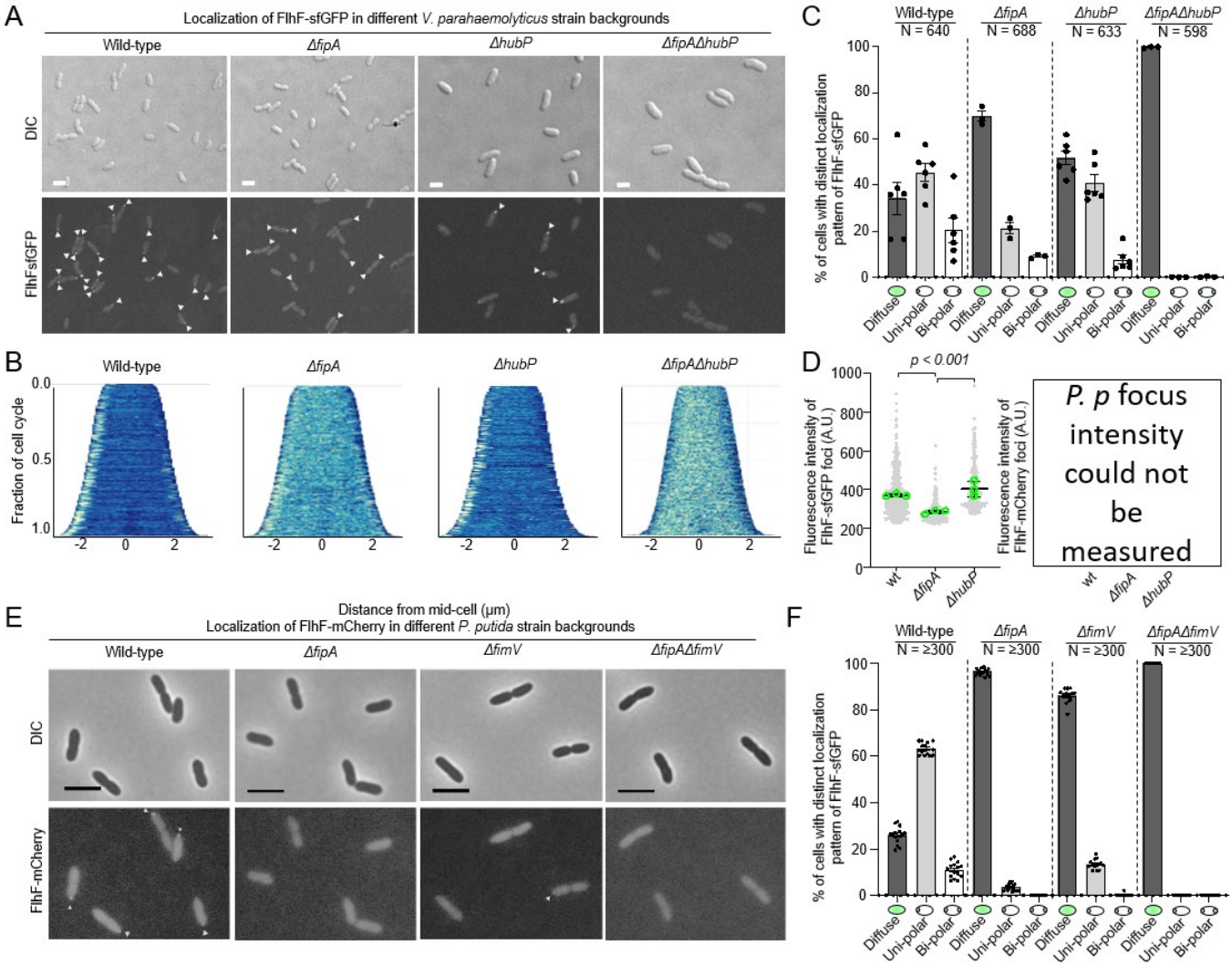


Figure 3.3. Localization of FlhF depends on FipA and HubP. A) Representative micrographs of *V. parahaemolyticus* expressing FlhF-sfGFP from its native promoter. Scale bar = 2 μm. White arrows indicate polar foci. **B)** Demographs displaying FlhF-sfGFP fluorescence intensity along the cell length. **C)** quantification of localization patterns and **(D)** foci fluorescence intensity. The gray dots represent each individual focus measured, the green circles represent the mean of each biological replicate. Data combined from three biological replicates. **E)** Representative micrographs of *P. putida* expressing FlhF-sfGFP from its native promoter. White arrows indicate foci. Scale bar = 5 μm. **F)** Quantification of localization patterns. Plots represent the mean ± SD + squares indicating the mean value from each replicate.

FlhF localized either diffusely in the cytoplasm or to the cell pole (Arroyo-Pérez and Ringgaard 2021). Particularly, FlhF was diffusely localized in ~37 % of cells or localized to the cell pole in a uni- and bi-polar manner in ~45 % and ~19 %, respectively, of wild-type cells. Absence of FipA significantly reduced localization of FlhF to the cell pole with a concomitant increase in diffusely localized FlhF (70%, Fig. 3.3B-C). Furthermore, the foci of FlhF at the cell pole in the absence of FipA were significantly dimmer compared to wild-type FlhF foci (Fig. 3.3D). This indicates that the amount of FlhF localized to the cell pole is much lower in the absence of FipA than in wild-type cells (Fig. 3.3D). Importantly, even though FlhF was still able to localize to the cell pole in a certain number of cells in the absence of FipA, no flagellum was formed in this background (Fig. 3.2D), thus indicating that FipA not only is required for proper polar recruitment of FlhF but also for the stimulation of flagellum formation.

It was previously shown that the land-mark protein HubP interacts with FlhF (Yamaichi et al. 2012). Thus, given the role of HubP in cell pole organization and its interaction with FlhF, we also analyzed the localization of FlhF in the absence of HubP. The recruitment of FlhF to the cell pole was reduced in the absence of HubP (Fig. 3.3A-C). Strikingly, in the double deletion strain *ΔfipA ΔhubP*, FlhF did not localize as foci at the cell pole but was instead localized diffusely in the cytoplasm in 100% of cells (Fig. 3.3A-C). We corroborated the expression levels of FlhF-sfGFP in the different backgrounds by Western Blot (Fig. S3.2). Although there were some differences in the expression levels, FlhF was clearly expressed and stable in all three mutants, indicating that the absence of foci was not due to the absence of protein.

The requirement for FipA and FimV (HubP) individually on FlhF-mCherry localization in *P. putida* was even stronger, with ~95 and ~90% cells with diffuse FlhF-mCherry signal in their absence, respectively (Fig. 3.3E-F). FlhF was also completely delocalized in the double mutant *ΔfipA ΔfimV*. Unfortunately, the signal from the mCherry fusion was too weak to measure the foci intensities in these strains (Fig. 3.3D).

These data show that both FipA and HubP are necessary for the polar localization of FlhF.

3.2.7. A conserved domain in FipA mediates interaction with FlhF

In order to identify the regions of FipA mediating its role in flagellum regulation, we analyzed in detail the DUF2802 domain. We identified a motif of conserved amino acids, and at least three of them, corresponding to residues G110, E126 and L129 of VpFipA, were 100% conserved among FipA homologues (Fig. 3.4A); these amino acids were chosen for mutagenesis.

Alanine substitutions of residues G110 and L129 abolished the interaction between VpFipA and VpFlhF in BACTH, while the E126A substitution did not affect the interaction (Fig. 3.4B). These variants were still able to self-interact with wild-type VpFipA (Fig. 3.4B).

Similarly, the substitutions G104A and L123A in PpFipA (corresponding to G110A and L129A in VpFipA) also abolished the interaction between PpFipA and PpFlhF, as did the substitution L116A (not tested in *V. parahaemolyticus*, but corresponding to L122). The mutation in the glycine had a less pronounced effect as in *V. parahaemolyticus*. None of these mutations affected the self-interaction of FipA (Fig. 3.4C). Altogether, these results suggest that the conserved residues in the DUF2802 domain support the interaction between FipA and FlhF, especially the residue L129A in VpFipA/L123 in PpFipA. In addition, the results suggest that the regions mediating the interaction with FlhF and the self-interaction with FipA are different.

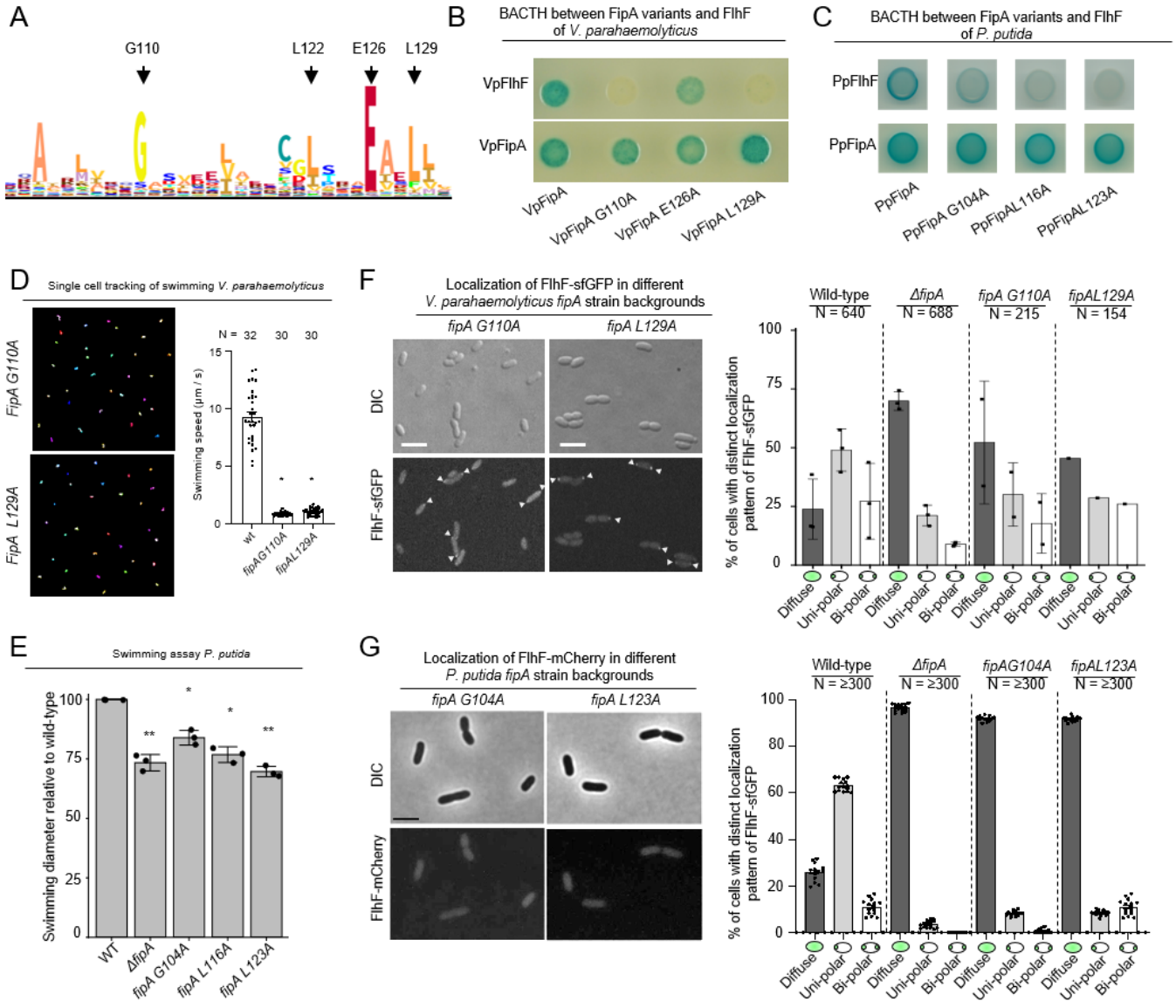


Figure 3.4. Conserved residues in the domain of unknown function of FipA are essential for interaction with FlhF. A) Consensus sequence of the conserved region of DUF2802 from 481 species. The numbers indicated correspond to the residues in VpFipA. Bacterial two-hybrid assay of FipA variants of *V. parahaemolyticus* **(B)** or *P. putida* **(C)** with an alanine substitution in the conserved residues indicated in (A). **D)** Swimming speed from single cell tracks of *V. parahaemolyticus* bearing those alanine substitutions in the native *fipA* locus. N is the number of cells tracked from three biological replicates. ANOVA + Tukey test, $p<0.05$. **E)** Swimming halo in soft agar of *P. putida* strains bearing the respective mutations in *fipA*. ANOVA + Tukey test, * = $p<0.05$, ** = $p<0.01$. Representative micrographs showing the localization of FlhF in **(F)** *V. parahaemolyticus* **(G)** or *P. putida*, and quantification of the localization pattern in the population. White arrows indicate polar foci. The data for wild-type and Δ*fipA* strains is the same as in Fig. 3.3C-F. For (E), (F) and (G), the plots represent the mean ± SD + squares indicating the mean value from each replicate.

3.2.8. Interaction between FipA and FlhF is required for FipA function on regulating flagellum formation and FlhF localization

We proceeded to evaluate the role of these residues *in vivo*. After introducing the mutations in the *fipA* gene in its native loci, the effects on motility were almost indistinguishable from a Δ*fipA* mutation. In *V. parahaemolyticus*, this resulted in completely immotile cells in planktonic cultures (Fig. 3.4D). In *P. putida*, the effect was less pronounced: there was a reduction in the swimming halo of the same magnitude as in the Δ*fipA* background. Only the G104A mutation had a less pronounced phenotype, but still significantly different from the wild-type (Fig. 3.4E).

The localization of FlhF was also affected by the point mutations in *fipA*. Cells of *V. parahaemolyticus* expressing VpFlhF-sfGFP natively had reduced polar localization when VpFipA was substituted with either G110A or L129A (Fig. 3.4F), with almost 50% of cells presenting only diffuse VpFlhF-sfGFP signal. The effect was more pronounced in *P. putida*, with almost 90% percent of cells presenting diffuse PpFlhF-mCherry signal in the presence of $PpFipA_{G104A}$ or $PpFipA_{L123A}$, very close to the ~95% of cells in the Δ*fipA* background (Fig. 3.4G). Indeed, it seems that the DUF2802 domain of FipA is responsible for the effect of FipA on motility, and that it its effects occur primarily through the interaction with FlhF. The interruption of this interaction seems to impede the recruitment of FlhF to the cell pole.

3.2.9. Cell cycle-dependent polar localization of FipA

Given FipA's function on regulating correct recruitment of FlhF to the cell pole, we next analyzed the intracellular localization of FipA in our model organisms *V. parahaemolyticus* and *P. putida* using stable and fully functional translation fusions of the respective FipA proteins to a fluorescent protein (Fig. 3.2B and Supplementary Fig. S3.3). In both organisms FipA formed distinct foci at the cell pole, either uni- or bi-polarly (Fig. 3.5A-B). No delocalized foci were ever observed. The polar localization of FipA at the cell pole is consistent with its function in regulating flagellum formation and recruitment of FlhF to this site. A remarkable difference between the organisms is that in *P. putida*, FipA was seen as foci in virtually all cells, whereas in *V. parahaemolyticus*, it remained diffuse in half of the population (Fig. 3.5A-B). The ratio of bi-polar to uni-polar foci was also greater in *P. putida* than in *V. parahaemolyticus*. This behavior was explored further by following the proteins through the cell cycle. In *V. parahaemolyticus*, FipA foci disappear frequently (Fig. 3.5E, 14'). A new focus appears at the opposite pole, sometimes before cell division (Fig. 3.5E, 56'), sometimes after (Fig. 3.5E, 42'). On rare cases, the first focus persists until after the second focus appears, resulting in bipolar foci (Fig. 3.5E, 63'). On the other hand, in *P. putida*, the FipA foci were more stable, and foci at both poles often persisted until cell division. In fact, appearance of the second focus at the new pole frequently occurred right after cell division (Fig. 3.5F, 50'), explaining the greater bi-polar to uni-polar ratio of FipA foci in *P. putida* vs. *V. parahaemolyticus*. These results are consistent with a protein that is recruited to the cell

pole right before the start of the flagellum assembly after cell division, with the lophotrichous *P. putida* apparently recruiting more FipA units earlier in the cell cycle than the monotrichous *V. parahaemolyticus.*

3.2.10. The interaction between FipA and FlhF is required for proper polar localization of FipA

FlhF was deleted in the strains expressing FipA-sfGFP from its native promoter, to determine the dependency of FipA on FlhF. Almost no FipA foci were detected in this background at all, for both *V. parahaemolyticus* (Fig. 3.6A-C) and *P. putida* (Fig. 3.6D-F), even though the protein levels of FipA were comparable to WT (Fig. S3.3).

Furthermore, the FipA variants that do not interact with FlhF (Fig. 3.4B-C), were also labeled in the native *fipA* locus with sfGFP. Both VpFipA$_{G110A}$ and VpFipA$_{L129A}$ were incapable of forming polar foci (Fig. 3.6B-C). The corresponding variant in *P. putida*, PpFipA$_{L123A}$ was also mostly distributed in

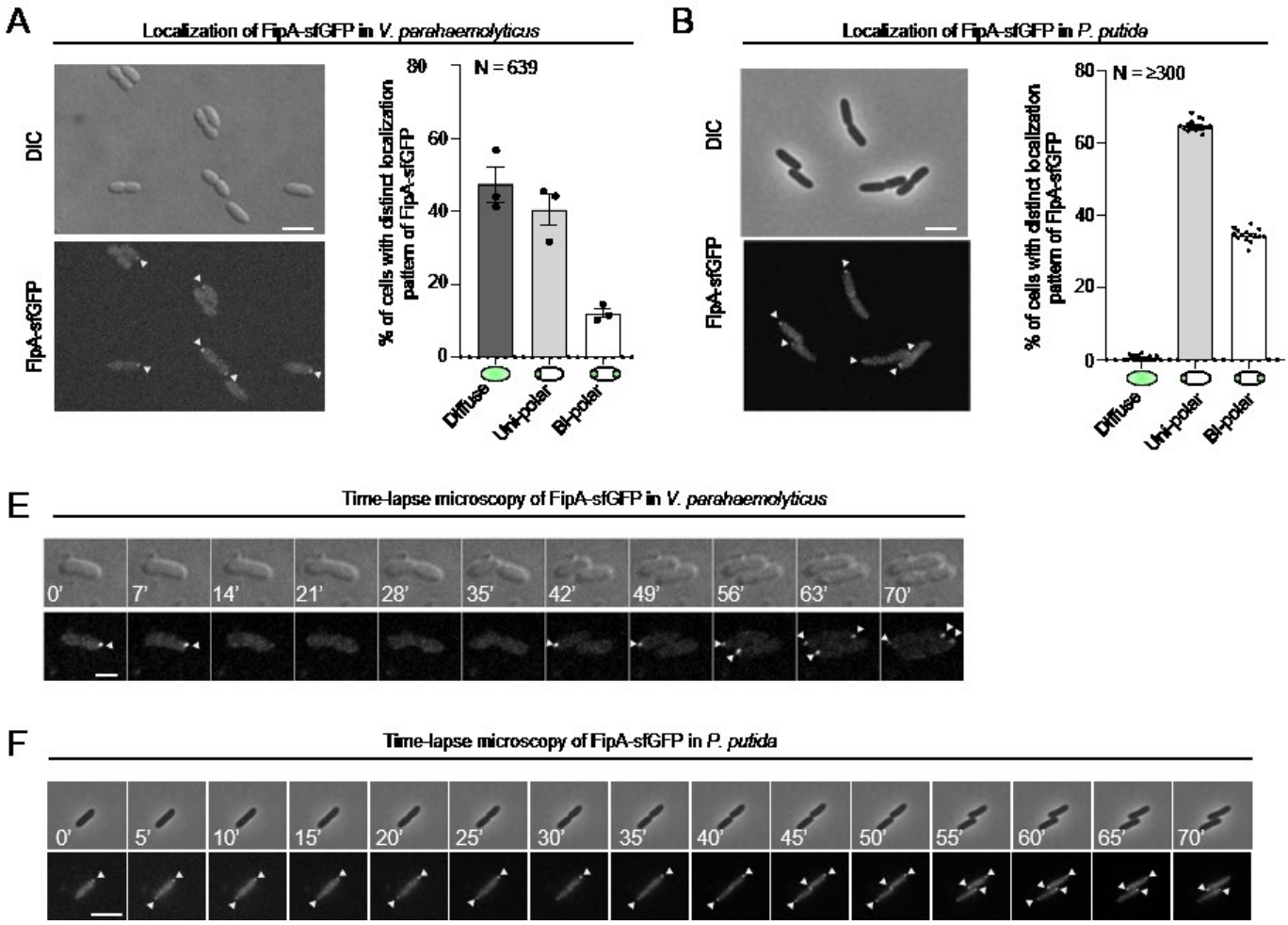


Figure 3.5. Localization of FipA. Representative micrograph and quantification of the cell localization pattern of **A)** *V. parahaemolyticus* or **B)** *P. putida* expressing FipA-sfGFP from its native promoter. White arrows indicate the foci. Scale bar = 5 µm. The plots represent the mean ± SD + squares indicating the mean of each replicate. N is the total number of cells measured. Time lapse of a dividing **C)** *V. parahaemolyticus* or **D)** *P. putida* cell expressing FipA-sfGFP from its native loci. Scale bar = 2 µm. White arrows indicate polar foci.

the cytoplasm, whereas the variant PpFipA$_{G104A}$ did exhibit some polar localization, although reduced (Fig. 3.6E-F). This is the variant that still exhibits some interaction with PpFlhF (Fig. 3.4C-G)

Altogether, these results suggest that a direct interaction with FlhF, mediated by the residues in the DUF2802, is responsible for recruiting FipA to the pole.

3.2.11. Membrane anchoring of FipA is required for its function in regulating flagellum formation and proper FlhF localization

We next analyzed the importance of FipA membrane anchoring for its function in mediating proper flagellum production. When expressed in *E. coli*, VpFipA-sfGFP showed a clear membrane localization, while a FipA variant deleted for the predicted transmembrane domain (FipA$_{\Delta TM}$) was diffusely localized in the cytoplasm (Fig. 3.7A), showing that FipA is indeed a membrane protein anchored by the predicted transmembrane domain.

The native *fipA* locus was replaced by a gene encoding a FipA variant lacking the N-terminal TM domain, and the resulting strains (*fipA ΔTM*) were analyzed for swimming ability and flagellum production. The *V. parahaemolyticus* strain carrying this mutation did not produce any flagella at all (Fig. 3.7C), as did the Δ*fipA* strain (Fig. 3.2D). Similarly, the *fipA ΔTM* mutation in *P. putida* had the same swimming defect as the deletion of *fipA* (Fig. 3.7B), indicating that the attachment to the membrane is essential for FipA function. In fact, this truncated version of PpFipA-sfGFP was completely unable to form polar foci (Fig. 3.7D).

Finally, the localization of FlhF was equally affected in the deletion of the transmembrane domain of PpFipA as in the complete deletion of FipA (Fig. 3.7E), showing that the recruitment of FlhF is also completely dependent on membrane-anchored FipA.

3.3. Discussion

Many bacteria use the FlhFG system to coordinate the localization and number of flagella. FlhG is a MinD-like ATPase, and just like MinD, it can shift from a monomeric, soluble conformation to a membrane-associated dimer when it binds ATP (Schuhmacher et al. 2015). FlhF is also membrane associated (Green et al. 2009; Kondo, Homma, and Kojima 2017), and it was speculated to have a partner that binds it to the membrane. Furthermore, it was not known how FlhF adopts its polar localization. In this work, we have identified FipA, a small integral membrane protein with a domain of unknown function that is responsible for recruiting FlhF to the membrane at the cell pole.

This protein is conserved among polar flagellates that use FlhFG to position their flagella, mostly γ-proteobacteria (Fig. 3.1G), which already suggests that FipA most likely evolved together with FlhFG to regulate the flagellation pattern. In this study, we investigated two distantly related γ-proteobacteria, a monotrichous and a lophotrichous species, and found that FipA is conserved as a regulatory factor of FlhF in both of them.

The interaction between FipA and FlhF was confirmed by reciprocal co-immuno precipitations with each protein as bait, as well as with a bacterial two-hybrid assay (Fig. 3.1). The latter also showed self-interaction of both proteins. This was expected of FlhF, which is known to form dimers in the presence of GTP (Bange et al. 2007; Kondo et al. 2018), but it is interesting that FipA is also capable of forming oligomers.

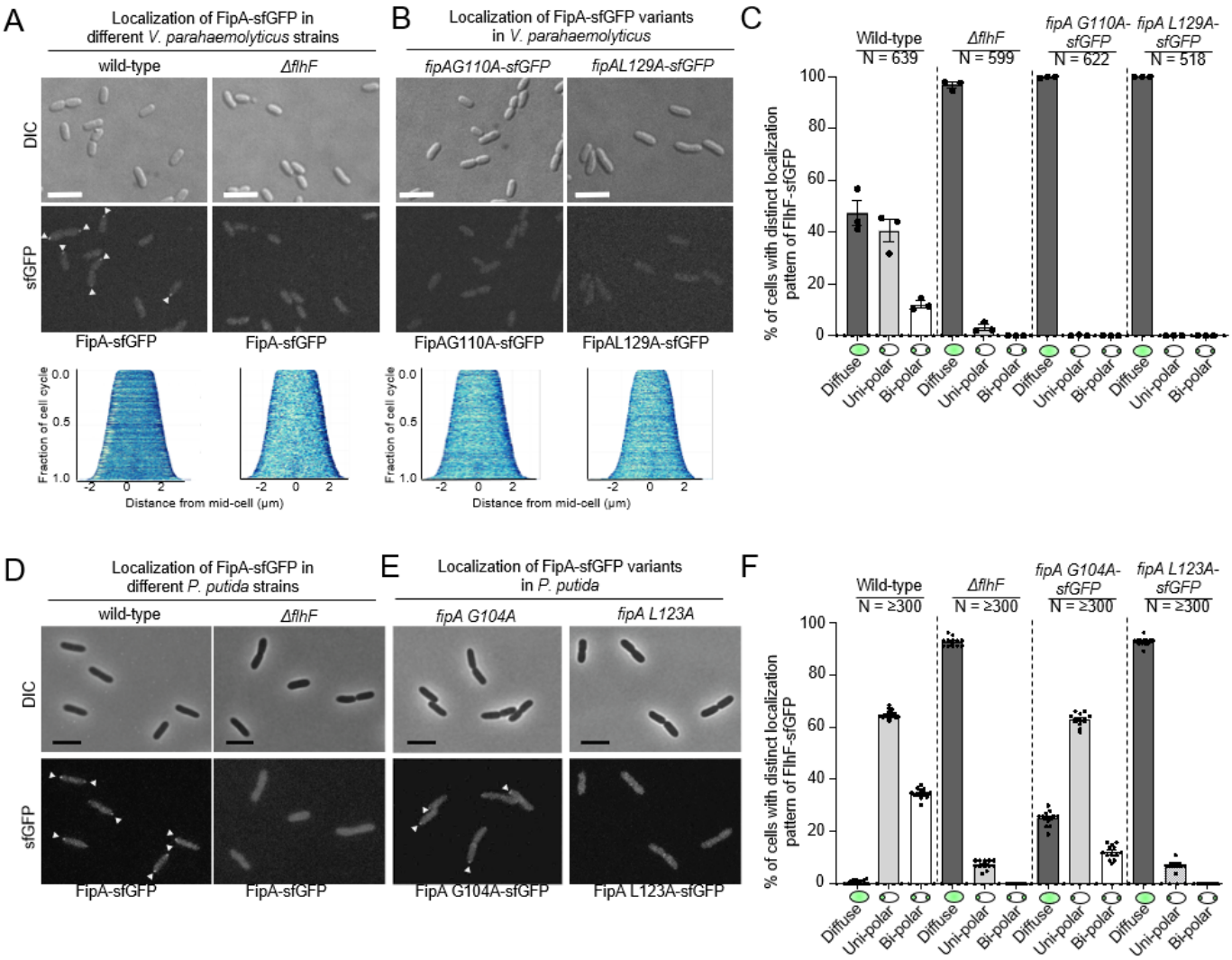


Figure 3.6. The localization of FipA depends on the interacting with FlhF. Localization of FipA-sfGFP expressed from the native promoter in wild type and Δ*flhF* backgrounds of *V. parahaemolyticus* **(A)** and *P. putida* **(D)**, and localization of FipA-sfGFP variants expressed from the native promoter in *V. parahaemolyticus* **(B)** and *P. putida* **(E)**. White arrows indicate polar foci. Quantification of localization patterns **(C, F)**. Combined data from three biological replicates. Data for wild type is the same as in Fig. 3.5A-B. Demographs represent the average population of each strain of *V. parahaemolyticus*. The plots represent the mean ± SD + squares indicating the mean of each replicate. N is the total number of cells measured.

The bacterial two-hybrid assay also showed that the FlhF-FipA interaction is dependent on conserved residues in the DUF2802 domain (Fig. 3.2). It remains to be discovered which region of FlhF is involved in the interaction. Furthermore, the substitutions in FipA did not affect its ability to interact with itself, suggesting that the binding to FlhF and the multimerization of FipA occur on different interfaces.

FipA is essential for the flagellum synthesis. A Δ*fipA* mutation causes the same phenotype as Δ*flhF* in *V. parahaemolyticus*: cells no longer make flagella (Fig. 3.2D), and they are not capable of starting assembly of FliF into the MS ring (Fig. 3.2F), the first step in flagellar synthesis. This may be partially due to an effect on the localization of FlhF. Indeed, in the absence of FipA in *V. parahaemolyticus*, the number of cells with diffuse FlhF localization doubled to more than two thirds (Fig. 3.3C). However, even the remaining 30% of cells where FlhF is still localized at the pole were not able to form flagella

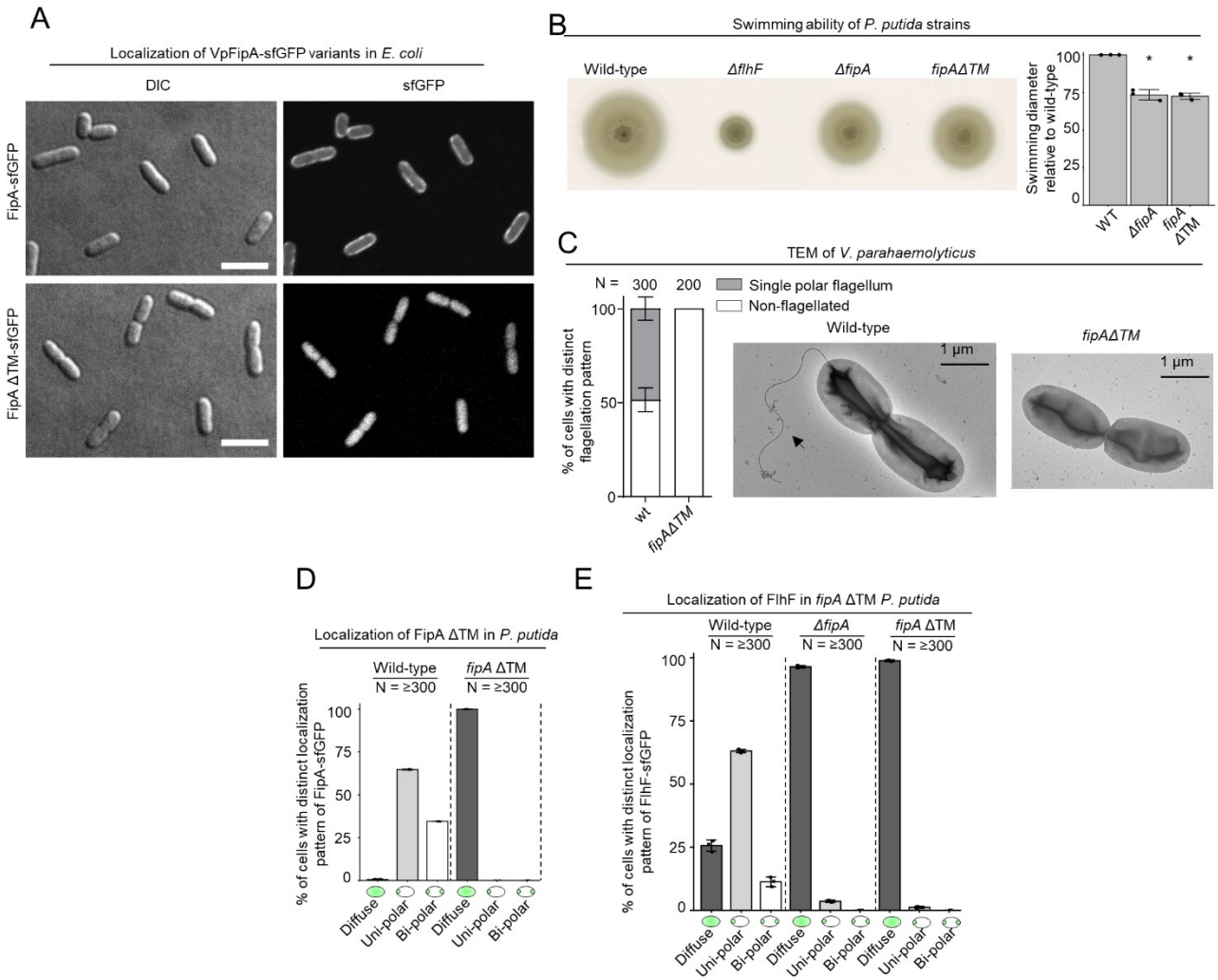


Figure 3.7. The activity of FipA depends on its transmembrane domain. A) Micrographs of *E. coli* cells expressing VpFipA-sfGFP from an inducible plasmid, and a truncated version lacking the transmembrane domain (ΔTM). Scale bar = 5 µm. **B)** Swimming assay of *P. putida* strains carrying the deletion of the transmembrane domain in the *fipA* locus. **C)**Electron micrographs of *V. parahaemolyticus* cells lacking the transmembrane domain in *fipA*, and quantification of flagellation pattern. Data for wild type is the same as in Fig. 3.2D. **D)** Localization of $FipA_{\Delta TM}$-sfGFP expressed natively in *P. putida*. **E)** localization of FlhF-sfGFP in a strain carrying the *fipA ΔTM* mutation. The plots represent the mean ± SD + squares indicating the mean of each replicate

(Fig. 3.2D), nor to recruit FliF to the cell pole (Fig. 3.2F-G). This means that localization of FlhF to the pole is not enough to trigger flagellum synthesis, but FipA is also needed.

In *P. putida*, the requirement for FlhF and FipA on flagellation was less strict. In fact, *V. parahaemolyticus* is the only organism known so far where FlhF is strictly required for flagellum synthesis. In contrast, Δ*flhF* cells of *P. putida* can still produce flagella and swim (Fig. 3.2C-E). *P. putida* cells lacking FipA can also produce flagella (Fig. 3.2E), despite FlhF being mostly delocalized in this background (Fig. 3.3E-F), further supporting that FlhF is not strictly required for flagellum assembly in this organism, but it stimulates it, together with FipA.

Removing HubP/FimV had a similar effect as removing FipA on the localization of FlhF, although to a lesser extent: more cells still had polar FlhF in the Δ*hubP/fimV* mutant than in the Δ*fipA* mutant, and when they did, the foci were brighter (Fig. 3.3D). This is probably due to the effect of the delocalization of FlhG, which leads to increased expression of flagellar genes (Arroyo-Pérez and Ringgaard 2021; Correa, Peng, and Klose 2005). Furthermore, the unipolar to bipolar ratio of FlhF foci increased in the Δ*hubP* mutant compared to the wild-type or the Δ*fipA* background. In the demographs, it appears that deleting *fipA* does not affect the time in the cell cycle at which FlhF shifts from unipolar to bipolar; there are simply more cells with diffuse localization. On the other hand, deleting *hubP* delays the time at which FlhF goes bipolar (Fig. 3.3B). This indicates that HubP and FipA act through different pathways to bring FlhF to the cell pole. In addition, both pathways are essential for FlhF localization, since the double mutant Δ*hubP* Δ*fipA* is the only case known so far where FlhF becomes completely delocalized. Thus, we can think of two pathways that bring FlhF to the cell pole: the general HubP-dependent cell pole maturation pathway, and a FipA-dependent pathway. Even though each one is sufficient to recruit FlhF, both are needed to bring sufficient (active) FlhF molecules to trigger MS ring formation and start flagellum assembly.

Interestingly, FipA is not intrinsically polarly localized, but instead it depends entirely on FlhF for its localization. In *V. parahaemolyticus*, FipA is localized at the cell pole in fewer cells than FlhF (Fig. 3.5 & 3), and it appears to arrive at the new cell pole only around cell division (Fig. 3.5C). Thus, FipA cannot be considered a landmark protein guiding FlhF, but rather a necessary component of a FipA-FlhF complex that together can be recruited to the pole in a HubP-independent manner. The discovery of FipA provides a new point where spatiotemporal organization is coordinated. It remains to be explored if the complex is anchored through yet another protein or through an intrinsic feature of the cell envelope or cytoplasm during cell division.

Even if FipA plays a role in FlhF localization, it could be another regulatory point for the activation of FlhF. The deletion of *fipA* resulted in the same phenotype as mutations that impede the interaction with FlhF (Fig. 3.4), as well as deleting only the transmembrane domain (Fig. 3.7). Therefore, the effects of FipA on flagellum formation must act primarily through the interaction with FlhF, and at the interface

with the membrane. It is likely that FipA not only recruits FlhF to the cell pole, but specifically to the membrane, and this additional step is needed to "activate" FlhF in order to recruit FliF. This could be by promoting the formation of the GTP-bound FlhF dimer, which has been proposed to be the molecular species responsible for flagellar recruitment that gets disassembled by FlhG (Kojima, Terashima, and Homma 2020), or simply by bringing FlhF to the membrane where FliF needs to be inserted. Support for this hypothesis comes from the fact that a Δ*flhG* mutation suppresses the absence of flagella in a Δ*fipA* background (Fig. 3.2D), indicating that FipA and FlhG have antagonic effects. Thus, the role of FipA could be to shift the equilibrium to the active state of FlhF in order to start assembly of the MS ring.

Since the transcription of FipA is independent of the rest of the flagellar genes in other species of *Vibrio* (Moisi et al. 2009; Petersen et al. 2021), its transcription could provide another regulatory point, where both branches have to converge to lead to the synthesis of a flagellum (*e. g.* a "mature cell pole" signal provided by HubP, and a "divided cell" signal provided by FipA). The discovery of this new protein provides more points to study the mechanism of FlhFG and their interaction with other cellular signals.

3.4. Materials and methods

3.4.1. Growth conditions and media

In all experiments *V. parahaemolyticus*, and *E. coli* were grown in LB medium or on LB agar plates at 37°C containing antibiotics in the following concentrations: 50 μg/mL kanamycin, 100 μg/mL ampicillin and 20 μg/mL chloramphenicol for *E. coli*; and 5 μg/mL for *V. parahaemolyticus*. *P. putida* was grown in Tryptone broth (Tryptone 10g/L, NaCl 5g/L) at 30° C.

3.4.2. Strains and plasmids

The strains and plasmids used in this study are listed in Tables S3 and S4, respectively. Primers used are listed in Table S5. *E. coli* strain SM10λ*pir* was used to transfer DNA into *V. parahaemolyticus* by conjugation (Miller and Mekalanos 1988). *E. coli* strains DH5αλ*pir* and SM10λ*pir* were used for cloning. Construction of *V. parahaemolyticus* deletion mutants was performed with standard allele exchange techniques using derivatives of plasmid pDM4 (Donnenberg and Kaper 1991), with the parental strain and plasmid combinations indicated in Table S4.

3.4.3. Bacterial-two-hybrid experiments, BACTH

BTH101 cells were made competent with calcium chloride. 15 μL aliquots were spotted in a 96 well plate, to which 2.5 μL of the corresponding pUT18(C) and pK(N)T25 derivative plasmids were added. After 30 minutes on ice, a heat shock at 42° C was applied for 30 s. The transformed cells were

allowed to recover for 1 hour, after which the were grown in selective LB broth for another 3 hours. The resulting cultures were spotted on plates containing kanamycin, ampicillin, IPTG (0.25 mM) and X-Gal (80 µg/mL). The plates were photographed after 48-72 hours at 30° C.

3.4.4. Soft-agar swimming assays

Swimming assays were essentially performed as described in (S. Ringgaard et al. 2014) with the following modifications. Late exponential cultures of the required strains were used to prick LB plates with 0.3% agar, and incubated for 30 hours at 30° C.

3.4.5. Video tracking of swimming cells

Video tracking of swimming cells was performed essentially as described previously (S. Ringgaard et al. 2014). Swimming cells were recorded using the streaming acquisition function in the Metamorph software and the swimming paths of individual cells were tracked using the MTrackJ plug-in for ImageJ. The swimming speed, displacement, and number of reversals of individual cells were then measured and the average plotted with error bars indicating the standard deviation. Video tracking was performed using a Zeiss Axio Imager M1 fluorescence microscope. Images were collected with a Cascade:1K CCD camera (Photometrics), using a Zeiss αPlan-Fluar 40x/1.45 Oil phase contrast objective.

3.4.6. Transmission-electron-microscopy (TEM) analysis

Cell cultures grown to an OD600 = 0.5–0.6 were spotted on a plasma-discharged carbon-coated copper grid (Plano, Cat#S162-3) and rinsed with 0.002% uranyl acetate. Afterwards they were rinsed with water and blotted dry with Whatman filter paper. TEM images were obtained with a JEOL JEM-1400 Plus 120 KV transmission electron microscope at 80 kV.

3.4.7 Flagellum labeling

Staining of the flagellum was carried out as described in (Hintsche et al. 2017). Cultures of *P. putida* strains bearing the mutation *fliC* S267C were washed twice and resuspended in 1/20 of the volume in motility buffer with glucose (11.2 g/1 K_2HPO_4, 4.8 g/1 KH_2PO_4, 3.93 g/l NaCl, 0.029 g/1 EDTA and 0.5 g/1 glucose; pH 7.0). A DMSO solution of Alexa 488 C5-maleimide was added to a final concentration of 0.1 mg/mL and the cells were shaken at 40 rpm for 15 minutes in the dark. Afterwards, the stained cells were washed twice with motility buffer and used for imaging.

3.4.8. Fluorescence microscopy

Florescence microscopy of *V. parahaemolyticus* was carried out essentially as previously described (Muraleedharan et al. 2018), using a Nikon eclipse Ti inverted Andor spinning-disc confocal microscope equipped with a 100x lens, an Andor Zyla sCMOS cooled camera, and an Andor FRAPPA system. Cultures were grown at 37° C after diluting an overnight culture 200-fold, until OD_{600}=0.60. 4 µL of the culture were spotted on a 0.5X PBS, 10% LB, 1% agarose pad and observed immediately. Microscopy

images were analyzed using ImageJ imaging software (Schneider, Rasband, and Eliceiri 2012; Schindelin et al. 2012) and Metamorph Offline (version 7.7.5.0, Molecular Devices). Demographs were generated as described by (Cameron et al. 2014); foci were counted manually. Foci intensity was determined using the MicrobeJ plugin (Ducret, Quardokus, and Brun 2016).

Images of *P. putida* were taken in an inverted wide-field Olympus IX71 microscope and analyzed with BacStalk (Hartmann et al. 2020).

3.4.9. Mapping interaction partners using immunoaffinity purification and mass spectrometry (IP-MS)

For sample preparation of the IP-MS experiments we used a modified version of the protocol presented by (Turriziani et al. 2014). Cultures of *V. parahaemolyticus* overexpressing the bait proteins from a pBAD-derivative plasmid were centrifuged and cell pellets were washed with cold PBS, then resuspended in lysis buffer (50 mM HEPES (pH 7.5), 150 mM NaCl, 0.5 % NP50, 5 mM EDTA, compete mini protease inhibitors (Complete Mini (Roche)). Cell lysis was performed by repetitive sonication. After removing cell debris by centrifugation, 10ul GFP-trap Sepharose (Chromotek) slurry was added to the lysate and incubation was carried out for 1.5 hours on a rotating shaker at 4 °C. The beads were pelleted, the supernatant removed and the beads washed 4x with 100 mM NH_4CO_3. 200 µl elution buffer (1 M urea, 100 mM NH_4CO_3, 0.2 µg/mL Trypsin (Promega)) was added to the beads and incubated at 1000 rpm on a thermomixer at 27°C for 45min. Beads were centrifuged and supernatant was collected. In order to increase peptide recovery, 2 washing steps with 100µl elution buffer 2 (1 M urea, 100 mM NH_4CO_3, 5 mM Tris(2-caboxyethyl)phosphine)) were performed and the individual bead supernatants were collected into one tube. Tryptic digest was carried out overnight at 30 °C. After digestion, alkylation was performed with 10 mM Iodoacetamide at 25 °C in the dark. Samples were then acidified (1 % Trifluoroacetic acid (TFA)) and the peptides purified on C18 Microspincolumns (Harvard Apparatus) according to the manufacturer's instruction. The samples were dried and recovered in 0.1 % TFA and applied to liquid chromatography-mass spectrometry (LC-MS) analysis.

LC-MS analysis of digested lysates was performed on a Thermo QExactive Plus mass spectrometer (Thermo Scientific), which was connected to an electrospray ion source (Thermo Scientific). Peptide separation was carried out using an Ultimate 3000 RSLCnano with Proflow upgrade (Thermo Scientific) equipped with a RP-HPLC column (75 µm x 42 cm) packed in-house with C18 resin (2.4 µm; Dr. Maisch) on an in-house designed column heater. The following separating gradient was used: 98 % solvent A (0.15 % formic acid) and 2 % solvent B (99.85 % acetonitrile, 0.15 % formic acid) to 35 % solvent B over 90 at a flow rate of 300 nL/min. The data acquisition mode was set to obtain one high resolution MS scan at a resolution of 70,000 full width at half maximum (at m/z 200) followed by MS/MS scans of the 10 most intense ions. To increase the efficiency of MS/MS attempts, the charged state screening modus was enabled to exclude unassigned and singly charged ions. The dynamic

exclusion duration was set to 30 sec. The ion accumulation time was set to 50 ms (MS) and 50 ms at 17,500 resolution (MS/MS). The automatic gain control (AGC) was set to 3x106 for MS survey scan and 1x105 for MS/MS scans.

Raw data was searched against the UniProt *V. parahaemolyticus* RIMD 2210633 protein database (www.uniprot.org) with Mascot 2.5 (Matrix Science) via the Proteome Discoverer 1.4 (Thermo Fisher Scientific) environment. False discovery rate and statistical testing (Student´s t-Test with multiple testing correction) was done in Scaffold 4 (Proteome Software). The search criteria were set as follows: full tryptic specificity was required (cleavage after lysine or arginine residues); two missed cleavages were allowed; carbamidomethylation (C) was set as fixed modification; oxidation (M) as variable modification. The mass tolerance was set to 10 ppm for precursor ions and 0.02 Da for fragment ions.

3.4.10. Genome comparisons

Homologues of FipA were searched using BLAST against the KEGG database, with the sequence of the *V. parahaemolyticus* protein. All homologues containing a DUF2802 were included. The search was later expanded to species known to encode a FlhF homologue (defined as the highest scoring result from a BLAST search using FlhF of *V. parahaemolyticus* as a query, that was also encoded upstream of an FlhG homologue).

The flagellation phenotype was later corroborated in the description registered at the List of Prokaryotic names with Standing in Nomenclature (Parte et al. 2020) or other sources (Table S2). The species were sorted according to the taxonomy in the Genome Taxonomy Database (https://gtdb.ecogenomic.org/).

3.4.11. Construction of plasmids

Plasmid pSW022: The regions flanking *vp2224* (*fipA*) were cloned with primers VP2224-del-a/ b and VP2224-del-c/ d, using *V. parahaemolyticus* RIMD 2210633 chromosomal DNA as template. The resulting products were fused in a third PCR using primers VP2224-del-a/ VP2224-del-d. The end product was digested with XbaI and ligated in the equivalent site of vector pDM4, resulting in plasmid pSW022. The mutation in *V. parahaemolyticus* was confirmed with a PCR using primers VP2224-del-a/VP2224-check.

Plasmid pPM123: The regions flanking aa 7-27 of *vp2224* (*fipA*) were cloned with primers VP2224-del-a/ del AA7-27 vp2224-b and del AA7-27 vp2224-c/ d, using *V. parahaemolyticus* RIMD 2210633 chromosomal DNA as template. The resulting products were fused in a third PCR using primers VP2224-del-a/ VP2224-del-d. The end product was digested with XbaI and ligated in the equivalent site of vector pDM4, resulting in plasmid pPM123. The mutation in *V. parahaemolyticus* was confirmed with a PCR using primers VP2224-del-a/VP2224-check.

Plasmid pPM178: The gene *vp2224* (*fipA*) was amplified from *V. parahaemolyticus* RIMD 2210633 chromosomal DNA with primers C-term sfGFP-vp2224-a/-b; the downstream region with primers C-term sfGFP-vp2224-e/f; the gene encoding sfGFP with C-term sfGFP-vp2224-c/d from plasmid pJH036. The three products were fused together in another PCR using primers C-term sfGFP-vp2224-a/f. The obtained product, encoding FipA fused in frame to sfGFP via a 5-residue linker, was digested with SpeI and SphI and ligated in vector pDM4 digested with XbaI and SphI, resulting in plasmid pPM178. The mutation in *V. parahaemolyticus* was confirmed with a PCR using primers C-term sfGFP-vp2224-f/VP2224-check.

Plasmid pPM179 & pPM180: The region upstream of *vp2224* (*fipA*) were amplified as for pSW022. The downstream region was amplified with downstream vp2224-cw/VP2224-del-d from *V. parahaemolyticus* RIMD 2210633. The gene *vp2224* itself was amplified with primers vp2224 cw restore deletion/vp2224 ccw restore deletion, from plasmids pEP005 & pEP006, carrying the mutations G110A and L129A. The products were fused in a third PCR using primers VP2224-del-a/ VP2224-del-d. The end product was digested with XbaI and ligated in the equivalent site of vector pDM4, resulting in plasmids pPM179 & pPM180. The the re-insertion in *V. parahaemolyticus* SW01 was confirmed with a PCR using primers VP2224-del-a/VP2224-check.

Plasmid pPM187 & pPM191: The insertion of sfGFP at the C-terminus of FipA was cloned with the same strategy for pPM178, but using pPM179 & pPM180 as templates for the *vp2224* sequence, resulting in plasmids pPM191 & pPM187, respectively.

Plasmid pPM146: The gene *vp2224* (*fipA*) was amplified from *V. parahaemolyticus* RIMD 2210633 chromosomal DNA with primers vp2224-cw-pBAD/vp2224-ccw-pBAD. The product was digested with enzymes XbaI and SphI, and inserted in the corresponding site in plasmid pBAD33.

Plasmid pPM159: The gene *vp2224* (*fipA*) was amplified from *V. parahaemolyticus* RIMD 2210633 chromosomal DNA with primers C-term sfGFP-vp2224-a/-b, and the gene encoding sfGFP with C-term sfGFP-vp2224-c/sfGFP-1-ccw from plasmid pJH036. The resulting products were fused in another reaction with primers C-term sfGFP-vp2224-a/sfGFP-1-ccw, and this final product was digested with XbaI and cloned in pBAD33.

Plasmid pPM194: Same as pPM159, but *vp2224 ΔTM* was amplified from pPM123 using primers vp2224 AA1-6/28-end w/o Stop/sfGFP-1-ccw.

Plasmid pPM181: The gene *vp2249* (*fliF*) was amplified with primers vp2249(fliF)-cw/vp2249 end w/o stop-linker ccw, from *V. parahaemolyticus* RIMD 2210633 chromosomal

DNA. The gene encoding sfGFP was amplified from pJH036 with primers C-term sfGFP-vp2224-c/sfGFP-1-ccw. The resulting products were fused in another reaction with primers vp2249(fliF)-cw/sfGFP-1-ccw, and this final product was digested with XbaI and cloned in pBAD33.

Plasmids pSW74 & pSW119: The region of the gene *vp2224* (*fipA*) encoding the cytoplasmic part (residues 28-163) was amplified with primers tr2224 put18C cw/ pUT18C/pKT25-vp2224-ccw from *V. parahaemolyticus* chromosomal DNA. The product was digested with KpnI and XbaI and ligated in the corresponding site in pKT25, to generate pSW74, and in pUT18C, to generate pSW119.

Plasmid pPM118 & pPM119: The cytoplasmic part of the gene *vp2224* (*fipA*) was amplified with primers pUT18/pKNT25-tr-vp2224-cw & pUT18/pKNT25-vp2224ccw from *V. parahaemolyticus* chromosomal DNA. The product was digested with KpnI and XbaI and ligated in the corresponding site in pUT18, to generate pPM118, and in pKNT25, to generate pPM119.

Plasmid pPM124 & pPM128: The gene *vp2234* (*flhF*) was amplified with primers pUT18/pKNT25- vp2234-cw & pUT18/pKNT25-vp2234 -ccw from *V. parahaemolyticus* chromosomal DNA. The product was digested with KpnI and XbaI and ligated in the corresponding site in pUT18, to generate pPM124, and in pKNT25, to generate pPM128.

Plasmid pPM132 & pPM136: The gene *vp2234* (*flhF*) was amplified with primers pUT18C/pKT25- vp2234-cw & pUT18C/pKT25-vp2234 -ccw from *V. parahaemolyticus* chromosomal DNA. The product was digested with KpnI and XbaI and ligated in the corresponding site in pUT18C, to generate pPM132, and in pKT25, to generate pPM136.

Plasmids pPM160, pPM161 & pPM162: Site directed mutagenesis was performed on plasmid pSW74 by the QuickChange method (Zheng, Baumann, and Reymond 2004), using primers vp2224-Gly-110Ala-cw/ccw, vp2224-Glu 126Ala-cw/ccw or vp2224-Leu 129Ala-cw/ccw. After digesting the template with DpnI, the products were transformed into *E. coli* and the mutations were confirmed by sequencing.

Plasmid pPM106: The gene vp2224 (fipA) was amplified with primers pUT18C/pKT25-vp2224-cw & pUT18C/pKT25-vp2224ccw from V. parahaemolyticus chromosomal DNA. The product was digested with KpnI and XbaI and ligated in the corresponding site in pKTop, to generate pPM106.

Plasmid pPM109 & pPM112: The *phoA-lacZα* fragment of pKTop was amplified with primers vp2224 C-term PhoA-LacZ cw & end -LacZ w/o STOP ccw. The full length *vp2224(fipA)* gene was amplified from plasmid pPM146 using primers LacZ to vp2224 w/o

ATG & end vp2224 ccw. The *vp2224* ΔTM allele was amplified from plasmid pPM123 using primers vp2224 AA1-6/28-end w/o Stop & end vp2224 ccw. The PCR products were fused in a second PCR using primers vp2224 C-term PhoA-LacZ cw & end vp2224 ccw. The fusion product was digested with XbaI and HindIII and cloned in the corresponding site of plasmid pBAD33.

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3.7. Supplementary material

3.7.1 Supplementary figures

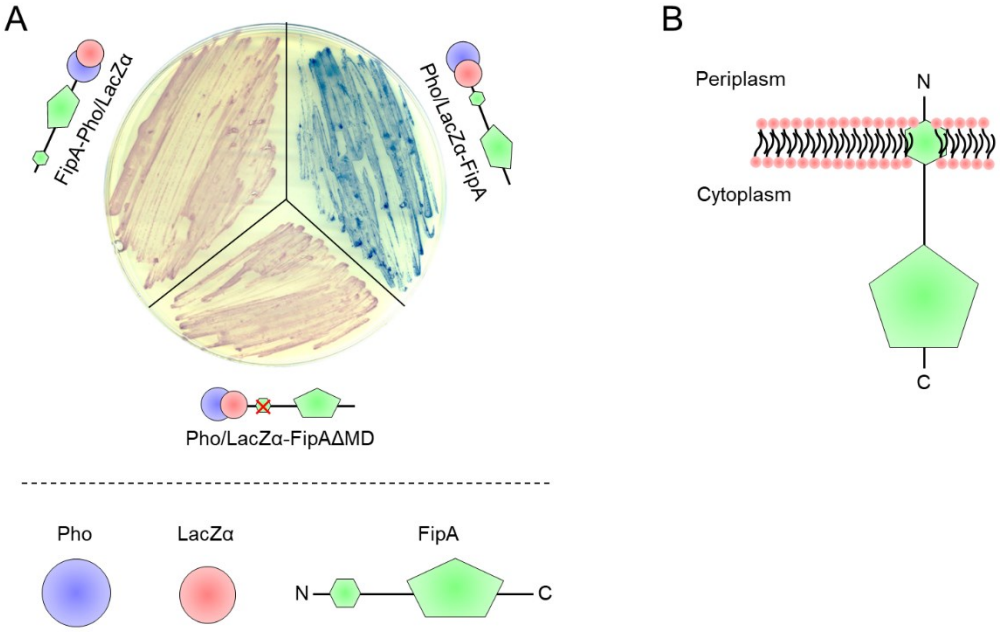

Figure S3.1. Membrane topology mapping of FipA

We experimentally determined the membrane orientation of VpFipA in the membrane using the dual pho-lac reporter system (Karimova, Robichon, and Ladant 2009), which consists of a translational fusion of the *E. coli* alkaline phosphatase fragment PhoA22-472 and the α-peptide of *E. coli* β-galactosidase, LacZ4-60. A periplasmic localization of the reporter leads to high alkaline phosphatase activity and low β-galactosidase activity, whereas a cytosolic location of the reporter results in high β-galactosidase activity and low alkaline phosphatase activity. Pho-Lac-FipA and FipA-Pho-Lac fusion proteins were ectopically expressed in *E. coli* DH5α grown on a dual-indicator LB medium containing a blue indicator for phosphatase activity (X-Phos) and red indicator for β-galactosidase activity (Red-Gal).

Expression of Pho-Lac-FipA resulted in blue colonies whereas expression of FipA-Pho-Lac resulted in red colonies (A), indicating that the FipA N-terminus is positioned in the periplasm and the C-terminus in the cytoplasm. Deletion of the predicted membrane spanning domain in Pho-Lac-FipA (aa 6-28, Pho-Lac-FipA$_{\Delta TM}$) resulted in red *E. coli* colonies (A), indicating that the domain between residues 6-28 is targeting FipA to the membrane. These results indicate that the N-terminus of FipA is located in the periplasm and the C-terminus in the cytoplasm (B).

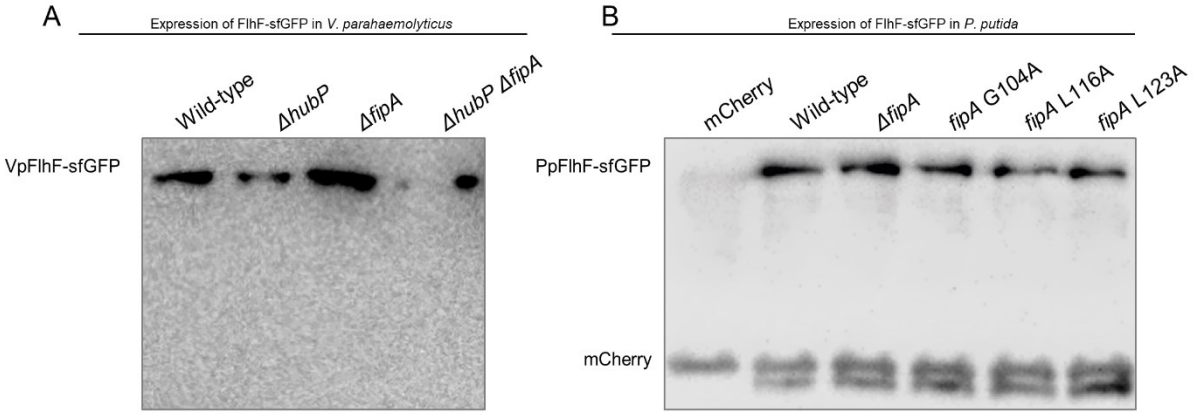

Figure S3.2. Expression levels of FlhF. Western blots of whole-cell extracts of the indicated strains expressing FlhF-sfGFP or -mCherry from the native promoter, of **(A)** *V. parahaemolyticus* and **(B)** *P. putida*, grown in the same conditions used for microscopy. After blotting, the samples were probed with an anti-GFP antibody (A) or an anti-mCherry antibody and HRP-conjugated secondary antibody

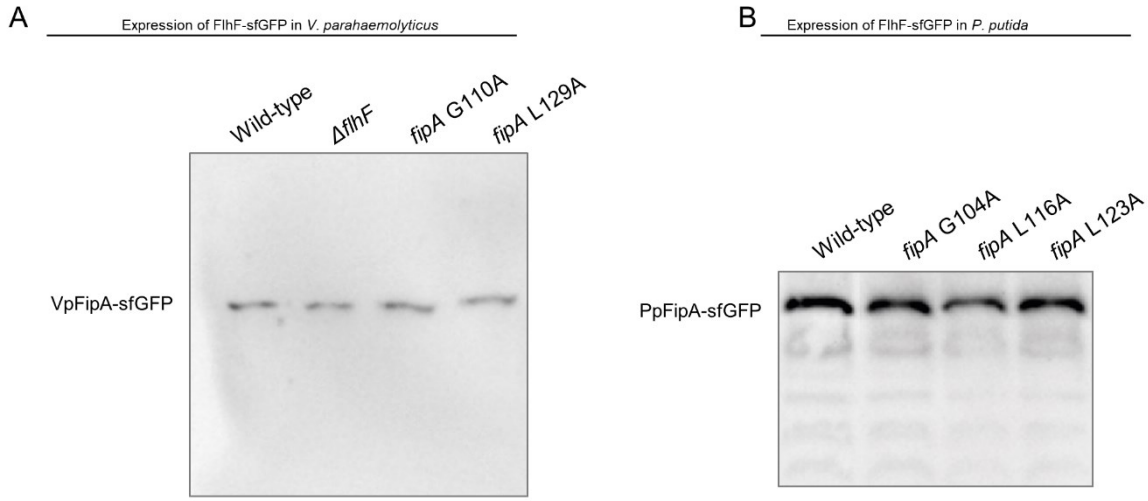

Figure S3.3. Expression levels of FipA. Western blots of whole-cell extracts of the indicated strains expressing FipA-sfGFP from its native promoter, of **(A)** *V. parahaemolyticus* and **(B)** *P. putida*, grown in the same conditions used for microscopy. After blotting, the samples were probed with an anti-GFP antibody and HRP-conjugated secondary antibody

3.7.2. Supplementary Tables

Table S1. Enriched proteins in Co-IP FlhF-sfGFP vs. sfGFP

Protein name	Gene name	Difference FlhF-GFP
FlhF	VP2234	11.33
	VP0127	8.78
	VPA0809	8.59
FliF	VP2249	8.43
	VPA0808	8.40
	VPA0807	7.85
	VP0700	7.42
FliG	VP2248	7.39
FliE	fliE	6.72
FlgB	VP0775	6.18
	VP1353	5.91
FlhA	VP2235	5.87
	VP0944	5.65
	VPA0337	5.32
FipA	VP2224	4.85
FtsI	VP0454	4.77
FlgC	VP0776	4.74
	VP0974	4.31
DedD	VP2187	4.17
	VPA1077	4.00
FliL	VP2243	3.97
	VP1100	3.24
	VP0646	3.17
IbpA	VP0018	2.65
SecD	secD	2.61
TolC	VP0425	2.51
FlgF	VP0780	2.50
DnaJ	dnaJ	2.47

Table S2. Conservation of FlhF and FipA among bacteria with different flagellation pattern

Name	*flhF*	*fipA*	Flagellation pattern	Reference
Buchnera aphidicola JF99	No	No	non motile	https://doi.org/10.1099/00207713-41-4-566
Citrobacter freundii	No	No	peritrichous	https://doi.org/10.1128%2Fjb.23.2.167-182.1932
Citrobacter rodentium	No	No	non motile	https://doi.org/10.1128/jcm.33.8.2064-2068.1995
Escherichia coli K12 MG1655	No	No	peritrichous	
Pantoea agglomerans C410P1	No	No	peritrichous	https://doi.org/10.1099/00207713-39-3-337
Proteus vulgaris	No	No	peritrichous	https://doi.org/10.1128/jb.90.5.1337-1354.1965
Serratia marcescens WW4	No	No	subpolar	https://doi.org/10.1016/j.resmic.2008.07.003
Sodalis glossinidius	No	No	non motile	https://doi.org/10.1099/00207713-49-1-267
Xenorhabdus nematophila	No	No	peritrichous	https://doi.org/10.1099/00207713-29-4-352
Yersinia enterocolitica WA	No	No	peritrichous	https://doi.org/10.1111/j.1699-0463.1969.tb04259.x
Aliivibrio fischeri ES114	Yes	Yes	lophotrichous	https://doi.org/10.1128/jb.187.6.2058-2065.2005
Grimontia hollisae	Yes	Yes	monotrichous	https://doi.org/10.1099/ijs.0.02660-0
Photobacterium profundum	Yes	Yes	monotrichous	https://doi.org/10.1007/s007920050036
Vibrio cholerae O1 El Tor N16961	Yes	Yes	monotrichous	
Vibrio parahaemolyticus	Yes	Yes	monotrichous	
Alteromonas australica H 17	Yes	Yes	monotrichous	doi.org/10.1099/00207713-45-4-755
Idiomarina loihensis L2TR	Yes	Yes	monotrichous	https://doi.org/10.1099/ijs.0.02701-0
Pseudoalteromonas atlantica	Yes	Yes	monotrichous	doi.org/10.1099/00207713-45-4-755
Pseudoalteromonas haloplanktis	Yes	Yes	monotrichous	doi.org/10.1099/00207713-45-4-755
Pseudoalteromonas luteoviolacea	Yes	Yes	monotrichous	doi.org/10.1099/00207713-45-4-755
Pseudoalteromonas rubra	Yes	Yes	monotrichous	doi.org/10.1099/00207713-45-4-755
Catenovulum sp. CCB-QB4	Yes	Yes	peritrichous	https://doi.org/10.1099/ijs.0.027565-0
Salinimonas sp. HMF8227	Yes	Yes	monotrichous	https://doi.org/10.1099/ijs.0.63279-0
Shewanella putrefaciens 200	Yes	Yes	monotrichous	https://doi.org/10.1016/S0723-2020(85)80051-5
Moritella viscosa	Yes	Yes	monotrichous	https://doi.org/10.1099/00207713-50-2-479
Psychromonas ingrahamii	No	No	non motile	https://doi.org/10.1099/ijs.0.64068-0
Aeromonas salmonicida	Yes	Yes	monotrichous	https://doi.org/10.1099/00207713-17-3-273
Tolumonas auensis	No	No	non motile	https://doi.org/10.1099/00207713-46-1-183
Hahella chejuensis	Yes	Yes	monotrichous	https://doi.org/10.1099/00207713-51-2-661
Marinomonas sp. MWYL1	Yes	Yes	monotrichous or amphitrichous	https://doi.org/10.1128/jb.110.1.402-429.1972
Cobetia marina	No	No	Subpolar	https://doi.org/10.1099/00221287-62-2-159
Halomonas elongata	No	No	lophotrichous or peritrichous	https://doi.org/10.1099/00207713-30-2-485
Azotobacter vinelandii DJ	Yes	No	Peritrichous	https://doi.org/10.1099/mic.0.2008/017665-0
Pseudomonas aeruginosa PAO1	Yes	Yes	Lophotrichous	
Pseudomonas syringae	Yes	Yes	Lophotrichous	
Pseudomonas putida F1	Yes	Yes	Lophotrichous	
Cellvibrio japonicus	Yes	Yes	Monotrichous	https://doi.org/10.1099/ijs.0.02271-0
Microbulbifer aggregans	No	No	non-motile	https://doi.org/10.1099/ijsem.0.002258
Saccharophagus degradans	Yes	Yes	Monotrichous	https://doi.org/10.1099/ijs.0.63627-0
Teredinibacter turnerae	Yes	Yes	Monotrichous	https://doi.org/10.1099/00207713-52-6-2261
Dasania marina	Yes	No	Monotrichous	https://pubmed.ncbi.nlm.nih.gov/18176532/
Acinetobacter baumanii	No	No	non-motile	https://doi.org/10.1007/978-1-4939-9118-1_17
Alkanindiges illinoisensis	No	No	non-motile	https://doi.org/10.1099/ijs.0.02568-0

Moraxella catarrhalis	No	No	non-motile	https://doi.org/10.1099/00221287-51-3-387
Perlucidibaca piscinae	No	No	Monotrichous	https://doi.org/10.1099/ijs.0.65039-0
Psychrobacter cryohalolentis	No	No	non-motile	https://doi.org/10.1099/ijs.0.64043-0
Thioalkalivibrio sp. K90 mix	Yes	Yes	Monotrichous	https://doi.org/10.1099/00207713-51-2-565
Allochromatium vinosum	Yes	Yes	Monotrichous	https://doi.org/10.1099/00207713-48-4-1129
Solimonas sp. K1W22B-7	Yes	No	non-motile	https://doi.org/10.1099/ijs.0.64938-0
Stenotrophomonas maltophilia R5513	Yes	No	Lophotrichous	https://doi.org/10.1099/00221287-26-1-123
Bordetella bronchiseptica RB50	Yes	No	Peritrichous	https://doi.org/10.1128%2Fjb.94.4.1216-1224.1967
Burkholderia mallei ATCC 23344	Yes	No	Degenerate flagellum	https://doi.org/10.1111/j.1348-0421.1992.tb02129.x
Burkholderia pseudomallei K96243	Yes	No	Monotrichous	https://doi.org/10.1128/IAI.71.4.1622-1629.2003
Caulobacter crescentus	No	No	monotrichous	
Hyphomonas neptunium	Yes	No	monotrichous	https://doi.org/10.1099/00207713-34-1-71
Magnetospirillum magneticum	Yes	No	amphitrichous	https://doi.org/10.1099/00207713-31-4-452
Thalassospira xiamenensis	Yes	No	monotrichous	https://doi.org/10.1099/ijs.0.64544-0
Rhodospirillum rubrum ATCC 11170	Yes	No	Amphilophotrichous	
Enhydrobacter aerosaccus	No	No	non-flagellated	https://doi.org/10.1099/00207713-37-3-289
Phycisphaera mikurensis	Yes	No	Monotrichous	https://doi.org/10.2323/jgam.55.267
Gimesia maris	Yes	No	polar to subpolar	https://doi.org/10.1186/1944-3277-9-10
Planctopirus ephydatiae	Yes	No	monotrichous	https://doi.org/10.1016/j.syapm.2019.126022
Rubinisphaera brasiliensis	Yes	No	monotrichous	https://doi.org/10.1016/S0723-2020(89)80008-6
schlesneria paludicola	Yes	No	subpolar	https://doi.org/10.1186/1944-3277-9-10
Thermogutta terrifontis	Yes	No	monotrichous	https://doi.org/10.1099/ijs.0.000009
Spirochaeta africana	Yes	No	spirochaete	https://doi.org/10.1099/00207713-46-1-305
Salinispira pacifica	Yes	No	spirochaete	https://doi.org/10.1186/1944-3277-10-7
Oceanispirochaeta sp. K2	Yes	No	spirochaete	https://doi.org/10.1099/ijsem.0.002130
Lentibacillus amyloliquefaciens	No	No	non-motile	https://doi.org/10.1007/s10482-015-0618-9
Virgibacillus halodenitrificans	Yes	No	polar and lateral	https://doi.org/10.1099/00207713-39-2-145
Bacillus subtilis	Yes	No	peritrichous	
Halobacillus halophilus	Yes	No	peritrichous	https://doi.org/10.1016/s0723-2020(83)80007-1
Psychrobacillus sp. AK 1817	Yes	No	peritrichous	https://doi.org/10.1128/jb.94.4.889-895.1967
Clostridioides difficile 630	Yes	No	peritrichous	https://doi.org/10.1128/iai.69.12.7937-7940.2001
Selenomonas sputigena	No	No	Mid-cell	https://doi.org/10.1128/br.18.3.165-169.1954
Selenomonas ruminantium	Yes	No	Mid-cell	https://doi.org/10.1128/aem.00286-11

Table S3. Strains

Strains	**Genotype**	**Reference**	Construction
E. coli SM10λpir	KmR, thi-1, thr, leu, tonA, lacY, supE, recA::RP4-2-Tc::Mu, pir		
E. coli DH5pir	*sup E44, ΔlacU169 (ΦlacZΔM15), recA1, endA1, hsdR17, thi-1, gyrA96, relA1, λpir phage lysogen*		
E. coli BTH101	*F-, cya-99, araD139, galE15, galK16, rpsL1 (StrR), hsdR2, mcrA1, mcrB1, relA1*	(Karimova et al. 1998)	
Vibrio parahaemolyticus RIMD 2210633	Clinical isolate, wild type	(Makino et al. 2003)	
Vibrio parahaemolyticus EP12	*Δvp2234 (ΔflhF), Δvpa1548 (ΔlafA)*	(Arroyo-Pérez and Ringgaard 2021)	
Vibrio parahaemolyticus SR58	*Δvp2225 (ΔcheW)*	(S. Ringgaard et al. 2014)	
Vibrio parahaemolyticus JH2	*Δvpa1548 (ΔlafA)*	(Arroyo-Pérez and Ringgaard 2021)	
Vibrio parahaemolyticus EP15	*Δvp2224 (ΔfipA), Δvpa1548 (ΔlafA)*	This work	SW01 + pJH003
Vibrio parahaemolyticus PM60	*Δvp2234 (ΔflhF)*	(Arroyo-Pérez and Ringgaard 2021)	
Vibrio parahaemolyticus SW01	*Δvp2224 (ΔfipA)*	This work	WT + pSW022
Vibrio parahaemolyticus PM49	*vp2224(fipA) ΔTM*	This work	SW01 + pPM123
Vibrio parahaemolyticus JH4	*Δvp2191 (ΔhubP)*	(Arroyo-Pérez and Ringgaard 2021)	
Vibrio parahaemolyticus PM69	*Δvp2234::vp2234-sfgfp (ΔflhF::flhF-sfgfp)*	(Arroyo-Pérez and Ringgaard 2021)	
Vibrio parahaemolyticus PM77	*Δvp2234::vp2234-sfgfp (ΔflhF::flhF-sfgfp), Δvp2224 (fipA)*	This work	SW01 + pPM188fip
Vibrio parahaemolyticus EP11	*Δvp2234::vp2234-sfgfp (ΔflhF::flhF-sfgfp), Δvp2191 (hubP)*	(Arroyo-Pérez and Ringgaard 2021)	PM69 + pPM039
Vibrio parahaemolyticus EP49	*Δvp2233 (ΔflhG), Δvp2224 (ΔfipA), Δvp2234::vp2234-sfgfp (ΔflhF::flhF sfgfp)*	This work	
Vibrio parahaemolyticus EP09	*Δvp2234::vp2234-sfgfp (ΔflhF::flhF-sfgfp), Δvp2224 (fipA), Δvp2191 (hubP)*	This work	PM77 + pPM039
Vibrio parahaemolyticus PM65	*vp2224 (fipA) L129A*	This work	SW01 + pPM180
Vibrio parahaemolyticus PM66	*vp2224 (fipA) G110A*	This work	SW01 + pPM179
Vibrio parahaemolyticus EP16	*Δvp2234::vp2234-sfgfp (ΔflhF::flhF-sfgfp), vp2224 (fipA) L129A*	This work	PM65 + pPM188fip
Vibrio parahaemolyticus EP17	*Δvp2234::vp2234-sfgfp (ΔflhF::flhF-sfgfp), vp2224 (fipA) G110A*	This work	PM66 + pPM188fip
Vibrio parahaemolyticus EP13	*vp2224 L129A, Δvpa1548(lafA)*	This work	PM65 + pJH003
Vibrio parahaemolyticus EP14	*vp2224 G110A, Δvpa1548(lafA)*	This work	PM66 + pJH003
Vibrio parahaemolyticus PM64	*Δvp2224::vp2224-sfgfp (ΔfipA::fipA-sfgfp)*	This work	WT + pPM178
Vibrio parahaemolyticus PM68	*Δvp2224::vp2224-sfgfp (ΔfipA::fipA-sfgfp), Δvp2234 (flhF)*	This work	PM60 + pPM178
Vibrio parahaemolyticus PM71	*Δvp2224::vp2224 L129A-sfgfp (ΔfipA L129A ::fipA-sfgfp)*	This work	WT + pPM187
Vibrio parahaemolyticus PM72	*Δvp2224::vp2224 G110A-sfgfp (ΔfipA G110A ::fipA-sfgfp)*	This work	WT + pPM191

Table S4. Plasmids

Plasmids	Description	Reference
pDM4	Suicide vector for gene deletions	Milton et al., 1996
pJH081	pDM4-derived suicide vector for gene deletions	
pJH036	pBAD33 derivative for sfGFP C-terminal fusion	
pJH003	For deletion of vpa1548(lafA)	Heering & Ringgaard, 2016
pSW022	For deletion of vp2224(fipA)	This work
pPM123	For deletion of aa 7-27 (TM) of vp2224(fipA)	This work
pPM188fip	For insertion of vp2234-sfgfp (flhF-sfgfp), replacing native flhF	Arroyo-Pérez and Ringgaard 2021
pPM178	For insertion of vp2224-sfgfp (fipA-sfgfp), replacing native fipA	This work
pPM179	For insertion of vp2224 (fipA) G110A point mutation in the chromosome in the native locus	This work
pPM180	For insertion of vp2224 (fipA) L129A point mutation in the chromosome in the native locus	This work
pPM191	For insertion of vp2224 (fipA) G110A fused to sfGFP in the chromosome in the native locus	This work
pPM187	For insertion of vp2224 (fipA) L129A fused to sfGFP in the chromosome in the native locus	This work
pPM039	For deletion of vp2191 (hubP)	Arroyo-Pérez and Ringgaard 2021
pPM194	For overexpression of VP2224(FipA)Δ7-27 -sfGFP	This work
pPM146	For overexpression of VP2224(FipA)	This work
pPM159	For overexpression of VP2224(FipA)-sfGFP	This work
pPM181	For overexpression of VP2249(FliF)-sfGFP	This work
Plasmids for Bacterial Two Hybrid and Membrane Topology assay		
pKT25	For C-terminal fusion of genes to T25 adenylate cyclase fragment	Karimova et al. 1998
pUT18C	For C-terminal fusion of genes to T18 adenylate cyclase fragment	Karimova et al. 1998
pUT18	For N-terminal fusion of genes to T18 adenylate cyclase fragment	Karimova et al. 1998
pKNT25	For N-terminal fusion of genes to T25 adenylate cyclase fragment	Karimova et al. 1998
pSW74	T25-vp2224(fipA)Δ1-27	This work
pSW119	T18-vp2224(fipA)Δ1-27	This work
pPM118	vp2224(fipA)Δ1-27-T18	This work
pPM119	vp2224(fipA)Δ1-27-T25	This work
pPM124	vp2234(flhF)-T18	This work
pPM128	vp2234(flhF)-T25	This work
pPM132	T18-vp2234(flhF)	This work
pPM136	T25-vp2234(flhF)	This work
pPM160	T18-vp2224(fipA)Δ1-27 G110A	This work
pPM161	T18-vp2224(fipA)Δ1-27 E126A	This work
pPM162	T18-vp2224(fipA)Δ1-27 L129A	This work
pKTop	For *phoA-lacZα* fusions	Karimova, Robichon, and Ladant 2009

pPM106	vp2224(fipA)-phoA-lacZα	This work
pPM109	phoA-lacZα-vp2224(fipA)	This work
pPM112	phoA-lacZα-(vp2224(fipA) ΔTM)	This work

Table S5. Oligos

Name	Sequence
sfGFP-1-ccw	ccccc tctaga tttgtagagctcatccatgccatg
VP2224-del-a	CCCCC tctaga ACGTTGTCATGCTTGGTGAAAGCA
VP2224-del-b	AGTCTCTTCAGCCATCGTCATTC
VP2224-del-c	gaatgacgatggctgaagagact cgacgataaagagaataaaaagaagc
VP2224-del-d	CCCCC tctaga ACGCGACGCTGCTGACCCGCAGAA
VP2224-check	acaaactccgtggggatgaatac
del AA7-27 vp2224-b	AAAAGTCTCTTCAGCCATCGTCATTC
del AA7-27 vp2224-c	GAATGACGATGGCTGAAGAGACTTTT CTGCGCATTCGTGCTAGTTTGC
tr2224 put18C cw	ccccc tctaga G ATG cgcattcgtgctagtttgcaaaa
VP2224 pKNT25 ccw 25.8.	Ccccc ggtacc cg TCGTCGACGCCCACGTGG
C-term sfGFP-vp2224-a	CCCCC actagt ATGGCTGAAGAGACTTTTTTATCTGTAC
C-term sfGFP-vp2224-b	gagctcgaggatgtc TCGTCGACGCCCACGTGG
C-term sfGFP-vp2224-c	gacatcctcgagctc atgagcaaaggagaagaacttttcac
C-term sfGFP-vp2224-d	tta tttgtagagctcatccatgcc
C-term sfGFP-vp2224-e	ggcatggatgagctctacaaa taa AGAGAATAAAAAGAAGCTTCGG
C-term sfGFP-vp2224-f	ccccc gcatgc TTTGTTTGTCGATTGCTGTTAGTGG
pUT18C/pKT25-vp2224-cw	ccccc tctaga G gctgaagagacttttttatctgtac
pUT18C/pKT25-vp2224ccw	ccccc ggtacc ttatcgtcgacgcccacgtg
pUT18/pKNT25-tr-vp2224-cw	ccccc TCTAGA ATG cgcattcgtgctagtttgc
pUT18/pKNT25-vp2224ccw	ccccc GGTACC CG tcgtcgacgcccacgtg
pUT18/pKNT25-vp2234 -cw	ccccc TCTAGA ATGaaaataaagcgatttttgccaaagac
pUT18/pKNT25-vp2234 -ccw	ccccc GGTACC CG GAGTCCTTCGTTGTCACTGTTC
pUT18C/pKT25-vp2234 -cw	ccccc tctaga G aaaataaagcgatttttgccaaagac
pUT18C/pKT25-vp2234 -ccw	ccccc ggtacc ctagagtccttcgttgtcactg
vp2224-cw-pBAD	CCCCC tctaga atggctgaagagacttttttatctg
vp2224-ccw-pBAD	CCCCC gcatgc ttatcgtcgacgcccacg
vp2224-Gly110Ala-cw	gagcaaccaaaatggtgcagttaGCGgctgatatcaacgagctaatcg
vp2224-Gly110Ala-ccw	CGATTAGCTCGTTGATATCAGCcgcTAACTGCACCATTTTGGTTGCTC
vp2224-Glu126Ala-cw	agagtgtgaactgccaaaagcaGCAgcagagttgatgctctctttgc
vp2224-Glu126Ala-ccw	GCAAAGAGAGCATCAACTCTGctgCTGCTTTTGGCAGTTCACACTCT
vp2224-Leu129Ala-cw	tgaactgccaaaagcagaagcagag GC gatgctctctttgcagaaaaaactg
vp2224-Leu129Ala-ccw	CAG TTT TTT CTG CAA AGA GAG CAT CGC CTC TGC TTC TGC TTT TGG CAG TTC A
vp2224 cw restore deletion	ACCTATAATTGGCTGAATGACG ATGGCTGAAGAGACTTTTTTATCTGTAC
vp2224 ccw restore deletion	GCCGAAGCTTCTTTTTATTCTCT TTATCGTCGACGCCCACGTG
downstream vp2224 cw	AGAGAATAAAAAGAAGCTTCGGC
vp2249(fliF)-cw	ccccc tctaga ATGgcagacaagtctacagatttaac
vp2249 end w/o stop linker ccw	gagctcgaggatgtc gccattttcgttcatccagtttttc
vpa1548-del-d	Ccccc ctcgag TTATGTGTTCCGCCTTCCTCTC
vpa1548-del-chk	aagtagccacatcccaaacgc
VP2191-del-d	ccccc tctaga GACAATGCGCTGCACGGAAT
VP2191-del-chk	gatggaaaacggctacacca
del vp2234(FlhF)-d	CCCCC tctaga GAATACATGCTACGAGCTCAAGG
del vp2234(FlhF)-chk	GTTTACGGCATGATTGATGGCG
del AA7-27 vp2224-b	AAAAGTCTCTTCAGCCATCGTCATTC
del AA7-27 vp2224-c	GAATGACGATGGCTGAAGAGACTTTT CTGCGCATTCGTGCTAGTTTGC
vp2224 C-term PhoA-LacZ cw	CCCCC tctaga g atggcccggacaccagaaatg
end -LacZ w/o STOP ccw	gcgccattcgccattcaggctgc
LacZ to vp2224 w/o ATG	CCTGAATGGCGAATGGCGC GCTGAAGAGACTTTTTTATCTGTACC
end vp2224 ccw	CCCCC aagctt ttatcgtcgacgcccacgtgg
vp2224 AA1-6/28-end w/o Stop	ccccc ctcaga atg gctgaagagacttttctgcgc

4. Molecular aspects of the interaction between FlhF and FipA

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This chapter is written in manuscript style, and is a work in preparation for submission. My contribution to this work was designing the experiments, training A. Tomasevic to perform the microscopy assays, purifying the proteins, performing the biochemical characterizations and analyzing the data, as well as writing the manuscript. The microscopy results were published as part of the Bachelor Thesis of Anella Tomasevic (Tomasevic 2021).

4.1. Introduction

Many bacteria regulate the number and position of flagella by means of the proteins FlhFG (Schuhmacher, Thormann, and Bange 2015). Related ATPases, like MinD or ParA, are known to regulate the localization of cellular components through their ATPase activity and their interaction with different elements when they are bound to ATP or ADP (Lutkenhaus 2012). The FlhFG system is especially interesting because not only FlhG is a ParA-like ATPase (Schuhmacher et al. 2015), but FlhF is also a GTPase with a similar behavior (Bange et al. 2007). FlhF regulates the position of the flagella in the cell, whereas FlhG limits the number of flagella produced (Schuhmacher, Thormann, and Bange 2015).

The ATPase activity of FlhG allows it to switch between a monomeric, soluble form, and a dimeric, membrane-bound form (Schuhmacher et al. 2015). The monomeric form interacts preferentially with the flagellar components, whereas the dimer can bind to the flagellar regulator FlrA and repress its activity (Blagotinsek et al. 2020). In this way, FlhG represses flagellar gene expression, limiting the number of flagella synthesized. As a GTPase, FlhF can also alternate between a GTP-bound dimer and a GDP-bound monomer (Bange et al. 2011). The role the GTPase activity of FlhF has been harder to elucidate, partly because its poor solubility makes it difficult to analyze biochemically. Even though it

has no predicted membrane-targeting sequences, FlhF is membrane bound in *V. cholerae* (Green et al. 2009). Furthermore, when it is expressed in *E. coli*, it is found in the insoluble fraction (Kondo et al. 2018). The addition of GTP during the lysis improved the solubility of FlhF, suggesting that the nucleotide may play a role altering its hydropathy and membrane affinity (Kondo et al. 2018).

The recent discovery of FipA, a membrane protein that interacts directly with FlhF to induce flagellum formation, offered a new opportunity to study FlhF *in vitro*. By co-expressing both proteins in a heterologous host, we could restore the membrane localization of FlhF and purify it as a membrane protein, and demonstrating that FipA acts as the membrane anchor of FlhF. By studying variants of FlhF predicted to be deficient in GTP binding or hydrolysis, we found evidence that suggests that it is the monomeric, GDP bound state of FlhF that interacts with the membrane

4.2. Materials and methods

4.2.1. Microscopy

E. coli MG1655 cells carrying the indicated plasmids were grown overnight in LB containing the corresponding antibiotics. This culture was diluted 1:100 in fresh medium containing 0.1% L-arabinose and incubated at 37° C until OD_{600}=0.6. Cells were spotted on an agarose pad and photographed immediately in a Delta Vision Elite Deconvolution Microscope using filters for CFP (exposure 0.5 s, 100% intensity) and YFP (exposure 0.6 s, 100% intensity). Each observation was carried out at least three different times from cultures from independent transformants.

4.2.2. Protein expression and preparation of cell membrane

BL21(DE3) RIL cells carrying the expression plasmid pEP077 (FipA), pEP078 (FlhF + FipA), pEP079 (FipA L129A) were grown overnight in LB + glucose 0.1%. This was diluted 1:100 in Terrific broth containing kanamycin 30 µg/mL. At OD_{600}=0.6 cultures were induced for 3 hours with 50 µM IPTG. The cells were harvested and kept at -80° C until later use.

The cell pellet was resuspended in lysis buffer (20 mM Tris-HCl pH=8.0 (for FlhF) or 8.8 (for FipA), 200 mM NaCl, 1 mM DTT, 10 mM $MgCl_2$, 10% glycerol v/v) to 200 mg of cells per mL. Lysozyme (1 mg/mL), DNase (50 µg/mL) and 1 mM PMSF were added and stirred 15 minutes at 4° C. The suspension was then passed through a Continuous Flow Cell Disrupter (Constant Systems) at 1.35 kPa. The lysate was centrifuged 30 minutes at 5 000 g. The pellet was collected as Cell debris, the supernatant was centrifuged at 20 000 g for 20 minutes. The pellet was collected as Inclusion bodies, the supernatant was centrifuged at 154 000 g for 45 minutes. The supernatant was collected as the Cytoplasmic fraction, the pellet containing the cell membranes was resuspended in lysis buffer and aliquots containing 10-15 mg of protein (measured by Bradford) were frozen in liquid nitrogen and stored at -80° C.

4.2.3. FipA purification

The membrane suspension was diluted to a protein concentration of 1 mg/mL in the presence of 20 mM Tris-HCl pH=8.8, 250 mM NaCl, 0.5 mM DTT, 5% glycerol v/v, 1 mM PMSF and 6.4 mM F-12, for 30 minutes at 4° C. After sedimentation of insoluble material at 261 000 g for 30 minutes, the sample was diluted 5-fold with FipA purification buffer (20mM Tris-HCl pH=8.8, 150 mM NaCl, 1 mM DTT, 10% glycerol v/v, 0.4 mM DDM (Neo Biotech, NB-19-0056-25G)) and incubated for one hour with equilibrated StreptActin® Sepharose® (IBA, 2-1201-010). It was washed with 10 volumes of FipA purification buffer, and eluted by addition of 5 mM D-desthiobiotin (IBA, 2-1000-002).

4.2.4. FlhF purification

The same solubilization steps were carried out as for FipA, but at pH=8.0. The clarified sample was incubated undiluted with HisPur™ Ni-NTA resin (Thermo Scientific, #88222) equilibrated with FlhF buffer (20mM Tris-HCl pH=8.0, 350 mM NaCl, 0.5 mM DTT, 10% glycerol v/v, 0.4 mM DDM) for one hour. The resin was washed with FlhF buffer containing 20 mM imidazole, and eluted with 100 mM imidazole.

The eluted fractions were concentrated in an Amicon® Ultracel® 10K Centrifugal Filter (Merck Millipore, UFC501024), and then rinsed with imidazole-free FlhF buffer.

4.2.5. Pull-down assay

Protein concentration was determined with the Bradford method (Bradford 1976) using Bioquant® reagent (Merck, 1.10306.0500). FlhF was diluted to 2 µM with FlhF buffer, and FipA and FipA L129A to 6 µM with FipA buffer, then mixed in equal volumes. To 45 µL of the mixture 5 µL were added of corresponding nucleotide solution (10 mM Tris pH=8.0, 200 mM $MgCl_2$, with or without 20 mM GTP or GDP), and incubated for 1 hour with His Mag Sepharose® Ni beads (Cytiva, 28-9799-17) previously equilibrated with FlhF buffer, at 4° C under constant shaking. After binding, the supernatant was collected as “flow-through” and the beads were washed with 45 µL FlhF buffer plus 5 µL of the corresponding nucleotide solution for 10 minutes at 4° C. The wash was collected and the rest of the protein was eluted in 50 µL of FlhF elution buffer.

Equal volumes of the samples were run on a 10-12% discontinuous gradient SDS-PAGE, blotted and probed first with monoclonal anti-StrepTag antibody (Sigma, SAB2702216) or monoclonal anti-histidine antibody (Invitrogen, WE323793), and anti-mouse IgG-HRP (Sigma, LNXA931V/AF). The blot was visualized with the chemiluminescent reagent Immobilon® Forte (Merck Millipore, WBLUF0100) in a Fusion FX Imager (Vilber), and quantified in ImageJ (Schneider, Rasband, and Eliceiri 2012). Each replicate was performed with independently purified protein.

4.2.6. Plasmids

Plasmids	Description	Reference
pBAD33	pBR322-derivative for arabinose-inducible expression, CmR	Guzman et al. 1995
pBAD18kan	pACYC184-derivative for arabinose-inducible expression, KanR	Guzman et al. 1995
pET28b+	For heterologous expression in BL21 cells	Novagen
pCOLA-Duet-1	For dual expression under Plac	Novagen
pEP054	pBAD33 - *vp2234(flhF)-eCFP*	This work
pEP058	pBAD18kan - *vp2224(fipA)-eYFP*	This work
pEP059	pBAD18kan - *vp2224(fipA)-eYFP* G110A	This work
pEP060	pBAD18kan - *vp2224(fipA)-eYFP* L129A	This work
pAT005	pBAD33 - *vp2234(flhF)-eCFP* K303A	This work
pAT006	pBAD33 - *vp2234(flhF)-eCFP* D375A	This work
pAT007	pBAD33 - *vp2234(flhF)-eCFP* D437A	This work
pPM173	pET28b+ - vp2234(flhF)-8His	This work
pPM159	pBAD33 - *vp2224(fipA)-sfGFP*	Chapter 3
pEP077	pCOLADuet-*vp2224(fipA)-STII*	This work
pEP078	pCOLADuet-*vp2224(fipA)-STII*, *vp2234(flhF)-6His*	This work
pEP079	pCOLADuet-*vp2224(fipA)-STII* L129A	This work

Plasmid pEP054: The gene *vp2234(flhF)* was amplified with primers pET28b+_vp2234(flhF)-8HIS cw & C-term sfGFP-vp2234-b (flhF) from *Vibrio parahaemolyticus* chromosomal DNA. The gene encoding eCFP was amplified using primers CFP-3 & sfGFP-ccw using pAA75 (Alvarado et al., 2017) as a template, and a permissive PCR program. The primers overlap by a sequence encoding a flexible 5-residue DILEL linker. Both products were fused in a third PCR using primers pET28b+_vp2234(flhF)-8HIS cw & sfGFP-ccw. The resulting fusion product was digested with enzymes XbaI and SphI and cloned in the corresponding sites in pBAD33, resulting in plasmid pEP054.

Plasmids pEP058, pEP059 & pEP060: The gene *vp2224(fipA)*, and the variants G110A and L129A, were amplified with primers vp2224 cw w/o linker & C-term sfGFP-vp2224-b, using plasmids pPM178, pPM191 or pPM187 (Chapter 3) as templates, respectively. The gene encoding eYFP was amplified using primers CFP-3 & sfGFP-ccw using pAA79 (Alvarado et al., 2017) as template, and a permisive PCR program, which resulted in fusion of the genes via the same DILEL linker used in pEP054. The resulting products were fused in a third PCR using primers vp2224 cw w/o linker & sfGFP-ccw. The resuling fusion product was digested with enzymes XbaI and SphI and cloned in the corresponding sites in pBAD18kan, resulting in plasmids pEP058, pEP059, pEP060.

Plasmids pAT005, pAT006 and pAT007: The mutations K303A, D375A and D437A were introduced in plasmid pEP054 by the QuickChange™ method (Zheng, Baumann, and Reymond, 2004)

and the primer pairs vp2234-Lys303Ala-cw/ccw, vp2234-Asp375Ala-cw/ccw and vp2234-D437A-for/rev, respectively.

Plasmid pPM173: The gene *vp2234(flhF)* was amplified from *Vibrio parahaemolyticus* chromosomal DNA with primers pET28b+_vp2234(flhF)-8HIS cw & ccw, which add an 8-histidine tag at the C-terminus. The PCR product was digested with ENZYMES and cloned in the corresponding sites of vector pET28b+, resulting in vector pPM173.

Plasmids pEP077 & pEP79: The gene *vp2224 (fipA)* and the mutant allele L129A were cloned from plasmid pEP058 and pEP060, respectively, using primers vp2224(FipA)-NcoI-cw and linker-StrepTag-stop-BamHI-ccw, that add the StrepTagII to the C-terminus of the gene, after the DILEL linker. The fragment was digested with NcoI and BamHI, and cloned in the second site in vector pCOLADuet-1.

Plasmids pEP078: The gene *vp2234 (flhF)* was cloned from plasmid pEP054, respectively, using primers vp2234(FlhF)-NdeI-cw and linker-6His-stop-KpnI-ccw, that add a 6-histidine tag to the C-terminus of the gene, after the DILEL linker. The fragment was digested with NdeI and KpnI, and cloned in the first site of pCOLADuet-1-derivatie pEP077.

4.2.7. Oligos

Name	Sequence
pET28b+_vp2234(flhF)-8HIS cw	ccccc TCTAGA ATGAAAATAAAGCGATTTTTTGCCAAAGAC
C-term sfGFP-vp2234-b (flhF)	gagctcgaggatgtc GAGTCCTTCGTTGTCACTGTTCC
CFP-3	GACATCCTCGAGCTC atggtgagcaagggcgagga
sfGFP-ccw	ccccc gcatgc TCATTTGTAGAGCTCATCCATGCC
vp2224 cw w/o linker	ccccc tctaga atggctgaagagacttttttatctg
C-term sfGFP-vp2224-b	gagctcgaggatgtc TCGTCGACGCCCACGTGG
pET28b+_vp2234(flhF)-8HIS ccw	ccccc CTCGAG TTA gtgatggtgatgatgatgatgatg GAGTCCTTCGTTGTCACTGTTC
linker-6His-stop-KpnI-ccw	ccc ggtacc ttagtggtgatgatggtgatg gagctcgaggatgtc
linker-StrepTag-stop-BamHI-ccw	ccc ggatcc ttatttttcgaactgcgggtggctcca gagctcgaggatgtc
vp2234(FlhF)-NdeI-cw	ccc catATG AAAATAAAGCGATTTTTTGCCAAAGAC
vp2224(FipA)-NcoI-cw	ccc ccatgg ctgaagagacttttttatctg

4.3. Results

4.3.1. FipA and FlhF interact in the membrane when expressed heterologously

E. coli lacks any of the polar determinants that are known to be important in *V. parahaemolyticus*. We figured that expressing FlhF and FipA heterologously in this species would allow us to explore the nature of the interaction between FlhF and FipA and their localization, without the confounding factors of the *Vibrio* cell pole. We labeled FlhF with eCFP and FipA with eYFP, at the C-terminus, to be able

to track each one independently *in vivo*, and cloned them in two compatible plasmids, under the inducible promoter P_{BAD}.

In *E. coli*, FipA was correctly expressed and localized uniformly along the membrane (Fig4.1A). FlhF, on the other hand, concentrated entirely as static foci at one cell pole (Fig. 4.1B), reminiscent of inclusion bodies. Interestingly, the localization of both FipA and FlhF changed upon co-expression. In this case, both proteins appear together as several foci along the cell membrane (Fig. 4.1C). This is not the case when the $FipA_{G110A}$ or $FipA_{L129A}$ are expressed (Fig. 4.1D, E), which were shown to be unable to interact with FlhF (Fig. 3.4), indicating that the colocalized foci along the membrane are a result of a specific FipA-FlhF interaction.

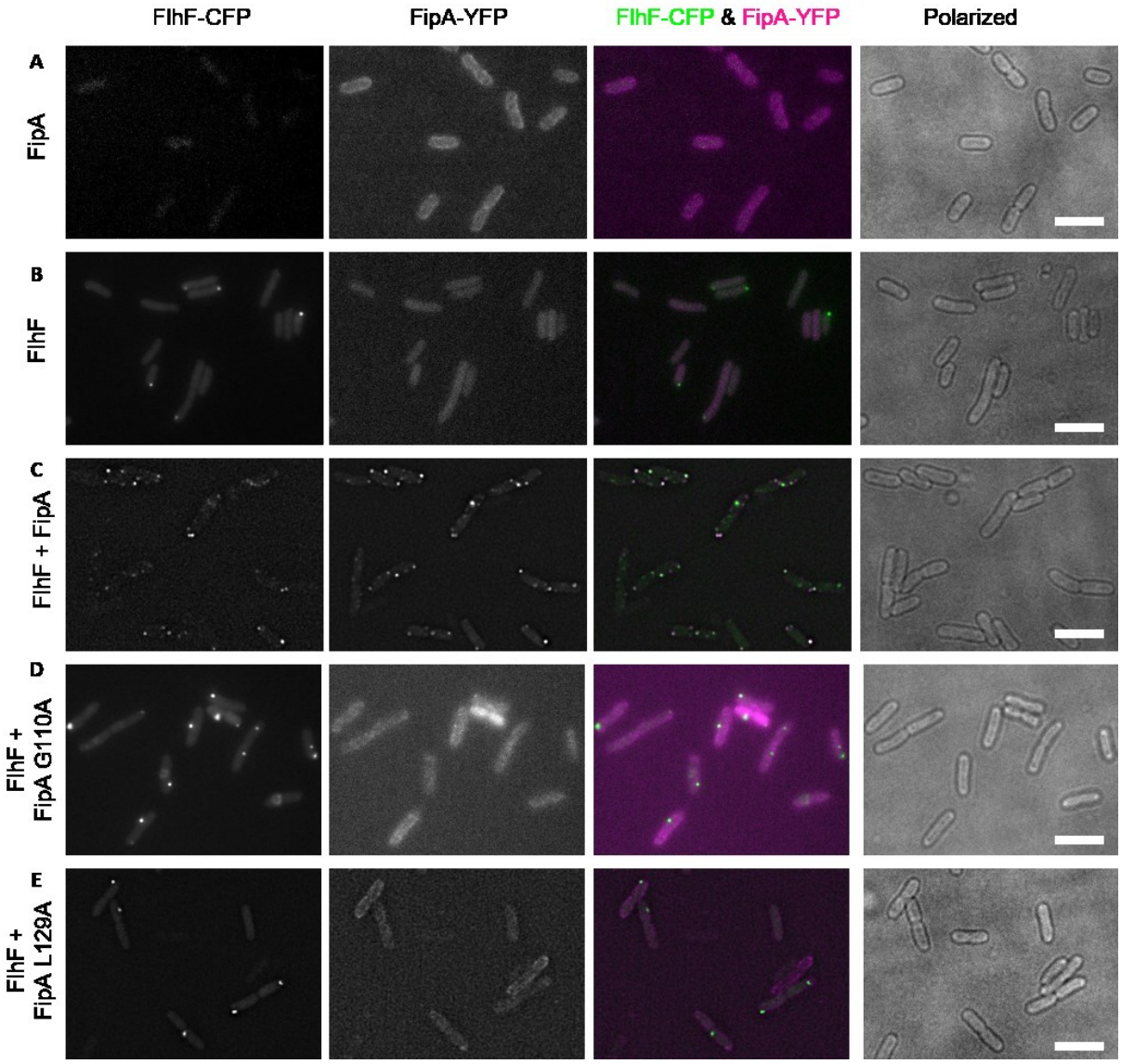

Figure 4.1. Heterologous co-expression of FlhF with FipA. Epifluorescence microscopy of *E. coli* expressing FipA-eYFP, FlhF-eCFP, or both, or the indicated FipA variants, from a plasmid under the PBAD promoter. Scale bar = 5 µm.

4.3.2. Catalytic mutations in FlhF prevent FipA interaction

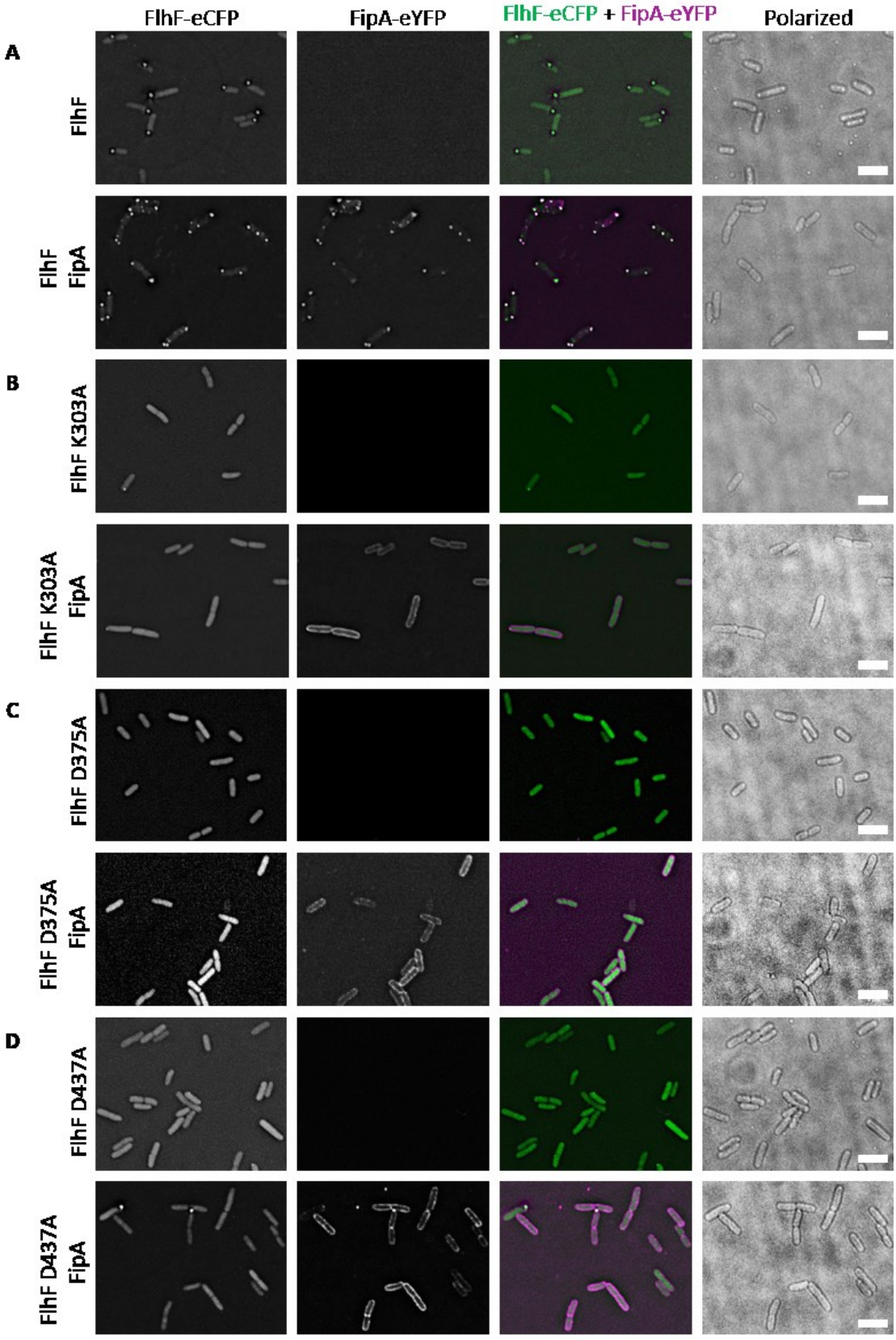

Figure 4.2. FlhF variants and their interaction with FipA. Epifluorescence microscopy of *E. coli* expressing FlhF-eCFP or the indicated variants, with or without FipA-eYFP. The plasmids used were derivatives of the ones used in Fig. 4.1. Scale bar = 5 µm. Figure expanded from Tomasevic, 2021.

A set of amino acid substitutions in FlhF were screened for their ability to interact with FipA. The sites tested were residues in the G1 and G3 loops, K303 and D375, that are essential for activity of FlhF in *V. cholerae* (Green et al. 2009), and D437, in the G4 loop, which is necessary for nucleotide specificity and GTP binding (Bange et al. 2007, Fig. 4.3A).

The variants were labeled with eCFP to conduct the same double-labeling experiment in *E. coli*. Upon co-expression with FipA, no colocalization was observed: FipA remained uniformly distributed along the membrane, while FlhF remained in the cytoplasm (Fig. 4.2A-D). Interestingly, all the variants were mostly found in the cytoplasm and not as insoluble foci, even in the absence of FipA (Fig. 4.2B-C). Only $FlhF_{D437A}$, the variant deficient in GTP binding, was occasionally seen as foci in some cells. These results suggest that the FipA-binding activity of FlhF is dependent on the nucleotide that is bound to it, and also related to the insoluble form of FlhF in *E. coli*.

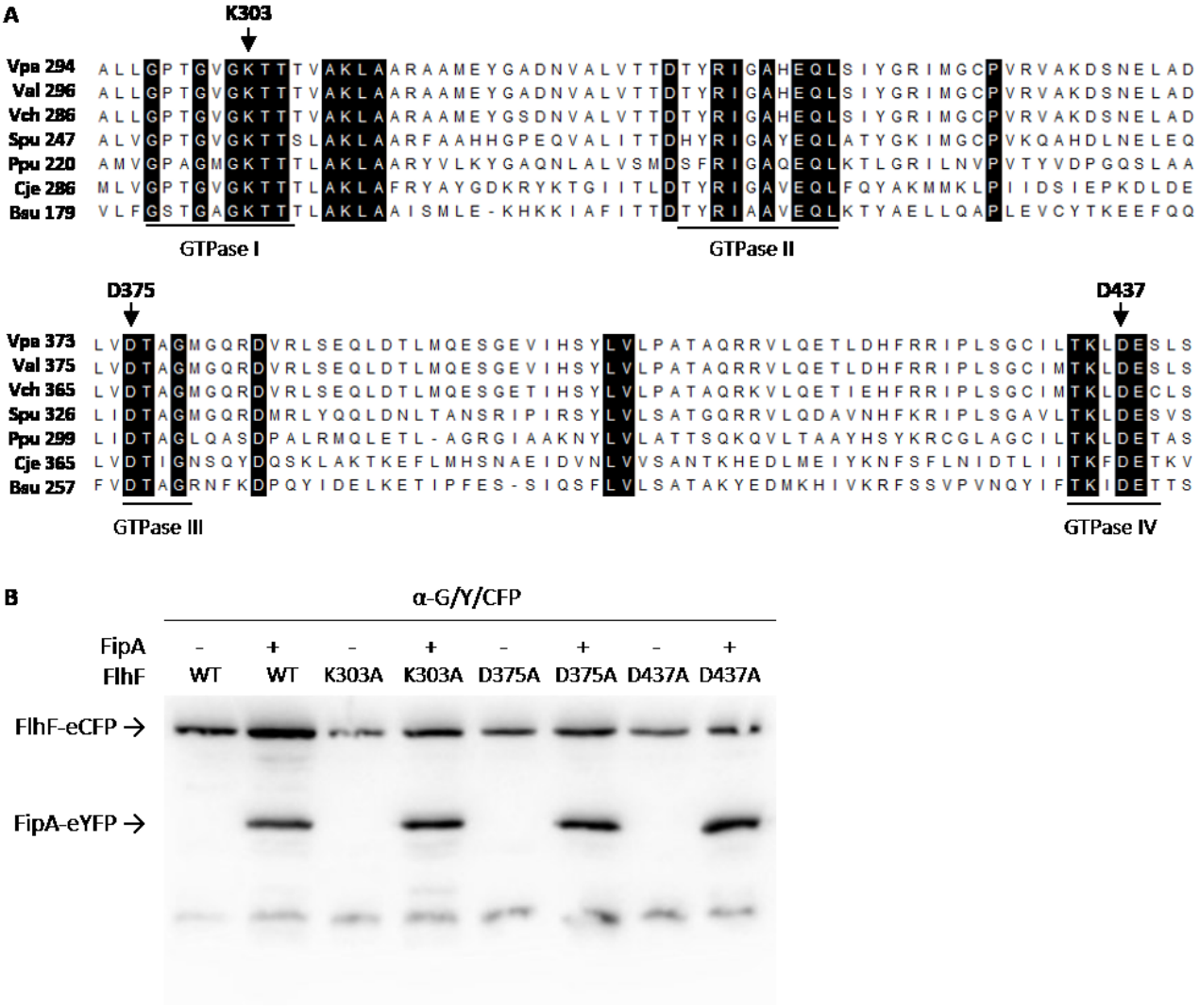

Figure 4.3. FlhF variants. A) Alignment of FlhF variants from *V. parahaemolyticus, V. alginolyticus, V. cholerae, S. putrefaciens, P. putida, C. jejuni* and *B. subtilis.* The mutations used in this study are indicated. **B)** Samples from the cultures for microscopy were lysed and separated on an SDS-PAGE, blotted and probed with JL8 anti-GFP antibody (Takara Bio).

A western blot was performed on lysates of cultures used in this assay, with an anti-GFP monoclonal antibody that can detect both eCFP and eYFP labels, to discard the possibility that the mutations rendered FlhF unstable. No significant degradation was observed in the FlhF variants compared to the WT, in the presence or absence of FipA (Fig. 4.3B).

4.3.3. FlhF can be solubilized with the membrane in the presence of FipA

The fact that FlhF can be recruited to the membrane by the expression of FipA in *E. coli* led us to believe that it could be purified from the cellular membrane by co-expressing the two proteins together. To test this hypothesis, we analyzed the cellular fractions of *E. coli* expressing histidine-tagged FlhF from a P_{lac} promoter. We compared these with cells carrying an arabinose-inducible plasmid encoding FipA-eYFP. Expression cultures were lysed and fractionated by ultracentrifugation. Cell debris was separated at 5000 rcf, inclusion bodies at 20 000 rcf, and cell membranes at 150 000 rcf. FlhF was found primarily in the inclusion bodies when expressed alone, but when co-expressed with FipA, it was detected primarily in the membrane fraction (Fig. 4.4A).

We constructed a pCOLA-Duet1 plasmid that encodes both FipA and FlhF genes, each one under an inducible P_{lac} promoter. FlhF was tagged with 6 histidines, and FipA with the StrepTag II, both at the C-terminus, to facilitate independent purification and detection.

A variety of detergents were tested to solubilize FipA and FlhF. FipA (19 kDa) was effectively solubilized by incubating the membrane fraction with 5 mM dodecyl maltoside (DDM), a non-ionic detergent, at pH=8.9. FlhF solubilization could be further improved by treating first with the zwitterionic detergent foscholine-12 (F12) at pH=8.0, and switching back to DDM for the rest of the purification. Purification of FipA was performed by means of a StreptActin resin, washing with FipA purification buffer (25 mM Tris pH=8.9, 350 mM NaCl, 1 mM DTT, 10% glycerol w/v, 0.4 mM DDM) and eluting in the presence of 5 mM desthiobiotin (Fig. 4.4B).

Purification of FlhF (56 kDa) was performed with a Ni-NTA resin and FlhF solubilization buffer (25 mM Tris pH=8.0, 350 mM NaCl, 0.5 mM DTT, 10% glycerol w/v, 0.4 mM DDM). 20 mM imidazole was added to the buffer for washing, and 100 mM for elution of FlhF (Fig. 4.4C). Both methods of solubilization (DDM alone or F12 and then DDM) resulted in dissociation of the FlhF-FipA protein complex during the purification: FlhF could not be detected in the purification of FipA, nor vice versa (Fig. 4.4D, E). This indicates that the interaction between FlhF and FipA is rather weak, and it can be easily disrupted by detergent treatment.

4.3.4. Restoration of the FlhF-FipA complex *in vitro*

In order to investigate whether the FlhF-FipA interaction could be restored, we mixed both purified proteins with a three-fold molar excess of FipA, and performed a pull-down assay using magnetic Ni-NTA beads that bind the histidine tag in FlhF. We were able to detect FipA in the eluate (Fig. 4.5),

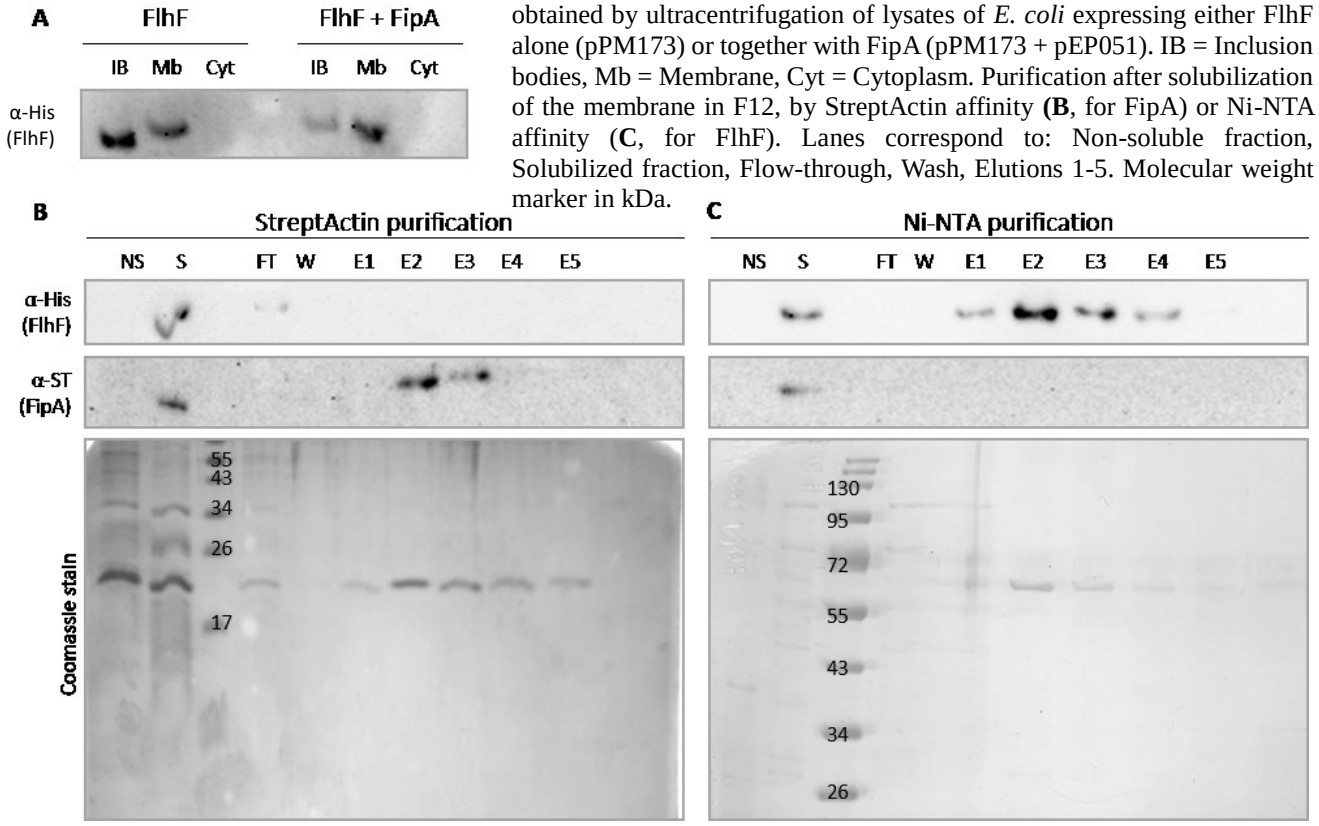

Figure 4.4. Purification of membrane-bound FlhF. A) Cell fractions obtained by ultracentrifugation of lysates of *E. coli* expressing either FlhF alone (pPM173) or together with FipA (pPM173 + pEP051). IB = Inclusion bodies, Mb = Membrane, Cyt = Cytoplasm. Purification after solubilization of the membrane in F12, by StreptActin affinity **(B**, for FipA) or Ni-NTA affinity (**C**, for FlhF). Lanes correspond to: Non-soluble fraction, Solubilized fraction, Flow-through, Wash, Elutions 1-5. Molecular weight marker in kDa.

indicating that FipA could bind to the beads in the presence of FlhF. Furthermore, there was considerably less binding of $FipA_{L129A}$ (Fig. 4.5) which cannot interact with FlhF (Fig. 3.4, 4.1E), demonstrating that what we observed was a specific interaction between wild-type FipA and FlhF.

We tried to explore with this method different conditions that can affect the interaction. We tested different nucleotides by adding them to the mixture with the beads throughout the pull-down incubations. Interestingly, in the presence of 2 mM GTP, FipA was no longer pulled down with FlhF (Fig. 4.5). In the presence of 2 mM GDP, FipA was still enriched compare to the non-interacting L129A variant (Fig. 4.5). However, the differences were not found to be statistically significant (Two-way ANOVA), most likely due to the small sample size, high variance and particularly high background in one of the replicates. Altogether these results show that the FipA-FlhF interaction can be restored *in vitro* after disruption with detergent, and they suggest that FipA cannot interact with GTP-bound FlhF.

4.4. Discussion

FlhF is a membrane-associated protein. When it was first isolated in *V. cholerae*, it was localized in the membrane fraction (Green et al. 2009), although it lacks any recognizable transmembrane or membrane-targeting domains. Although it was speculated that another protein could anchor FlhF to the membrane, its localization persisted when the transcription of all flagellar genes was suppressed. In this study, we have shown that FipA serves as the membrane anchor of FlhF.

The interaction between FlhF and FipA, that was thoroughly explored in chapter 2 of this thesis, was found to occur in *E. coli* as well, where there is no HubP or any polar flagellar protein.

Expression of FlhF in *E. coli* produces large aggregates near the poles. The same happened when *V. cholerae* FlhF was heterologously expressed, and it was ascribed to FlhF's supposed intrinsic polar localization (Yamaichi et al. 2012). However, the aggregates resemble inclusion bodies, which are found at the poles due to exclusion from the nucleoid (Winkler et al. 2010). Something similar occurred with the polar marker in *C. crescentus* PopZ, which also forms apparent polar aggregates in *E. coli*, but adopts its native localization pattern upon co-expression of its membrane anchors (Bergé and Viollier 2018; Perez et al. 2017; Bergé et al. 2016).

Expression of FipA was sufficient to recruit FlhF to the membrane, whereas the FipA variants that cannot interact with FlhF could not. Interestingly, the interaction between FipA and FlhF was enough to prevent the latter from forming inclusion bodies (Fig. 4.1), as FlhF from *V. alginolyticus* has been observed to do when expressed in *E. coli* (Kondo et al. 2018).

As a member of the SIMIBI family of nucleotide hydrolases (Bange and Sinning 2013), FlhF can alternate between a dimeric, GTP bound state, and a monomeric GDP bound-state (Bange et al. 2007; Kondo et al. 2018). It is not clear if an apo-monomeric state, without nucleotide, is also possible. Mutations in the catalytic site would therefore lock the protein in the dimeric, GTP bound state, whereas mutations in the GTP binding site could lock the protein in the monomeric state, either GDP-bound or without nucleotide. In this work we included mutations in the loops responsible for interacting with GTP during catalysis: the mutations K303A and D375A potentially abolish GTP hydrolysis by preventing interactions with the phosphates, and the mutation D437A interferes with binding of the guanosine moiety (Bange et al. 2007, Fig. 4.3A). Both types of mutations abolished the interaction with FipA (Fig. 4.2.). Interestingly, all the mutations that inhibited this interaction also prevented formation of inclusion

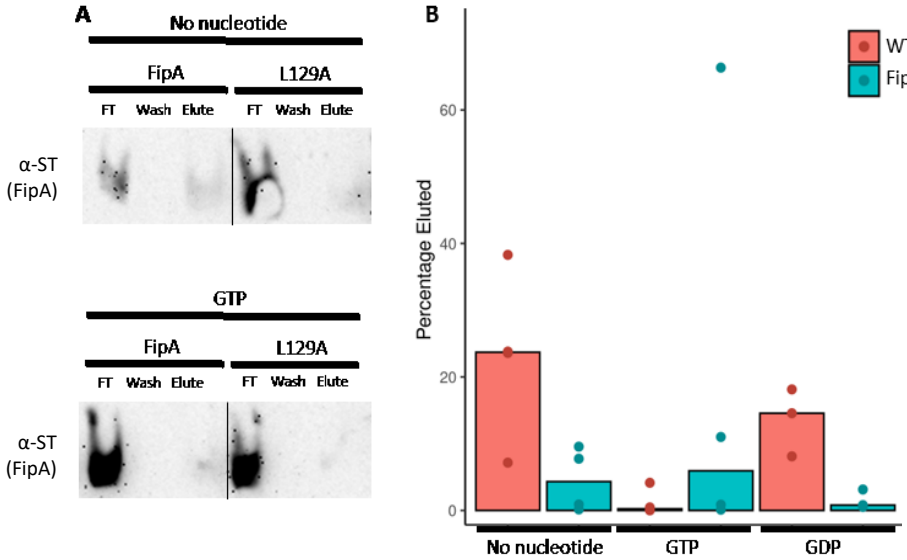

Figure 4.5. Pull down of FipA by FlhF. Ni-NTA beads were incubated with a mixture of purified FlhF and FipA, in the presence or absence of GTP or GDP. The not-bound fraction (FT), the wash and the elution were collected and analyzed by western blot. **A)** Representative blot. **B)** quantification of the amount of FipA protein eluted from the beads (median and individual values from each replicate). $FipA_{L129A}$ was used as non-interacting control.

bodies in *E. coli*, even without co-expression of FipA (Fig. 4.2.), and didn't cause instability of the protein (Fig. 4.3B).

The variants in the catalytic site $FlhF_{K303A}$ and $FlhF_{D375A}$ are expected to remain locked as a GTP dimer, whereas $FlhF_{D437A}$, that cannot bind GTP, FlhF is expected to remain in the apo-monomeric form. Therefore, it would seem that FlhF is insoluble in only one of the states of its GTP hydrolysis cycle: the GDP-bound state (Fig. 4.6.). Since the expression of FipA can prevent the aggregation of FlhF altogether (Fig. 4.1, 4.4), FipA seems to be responsible for stabilizing the insoluble GDP-bound state (Fig. 4.6.).

Further evidence for this model comes from the *in vitro* pull-down experiments, where GTP was found to inhibit the FlhF-FipA interaction. This indicates that FipA binds to the monomeric form of FlhF, and not to the GTP-bound dimer (Fig. 4.6.). This also explains why the solubility of FlhF from *V. alginolyticus* could be improved by adding GTP during lysis of the cells (Kondo et al. 2018), since in this model, the GTP-bound dimer is the soluble form of FlhF. These results should be taken with caution, however, since our assay lacked the power to detect a significant difference with the addition of the nucleotides. This is probably due to the low sample size and variable background. A confirmatory study needs to be carried out to test the hypothesis that GTP inhibits FlhF-FipA interaction, either by repetition of the pull-down or by an alternative method. Also, the catalytic and GTP-binding mutations are only presumed to be such, but we have yet to measure GTPase and GTP binding activity in any of them. Even reports of FlhF homologues in other species have difficulty determining the effect of these mutations due to the difficulties in purifying FlhF with high specific activity (Kusumoto et al. 2009; Balaban, Joslin, and Hendrixson 2009; Kondo, Homma, and Kojima 2017).

It's important to mention that although our evidence seems to suggest that the FlhF dimer doesn't bind to FipA, we don't know how the monomer interacts with FipA, through which residues, or if it does it in the GDP-bound or the *apo*-monomeric form. In fact, it's not clear if the *apo*-monomeric form is found in the cells, since it hasn't been crystalized without any nucleotide (Bange et al. 2007). It's also possible that the interaction with FipA proceeds through the G-loops. This would explain why the variant $FlhF_{D437A}$, expected to

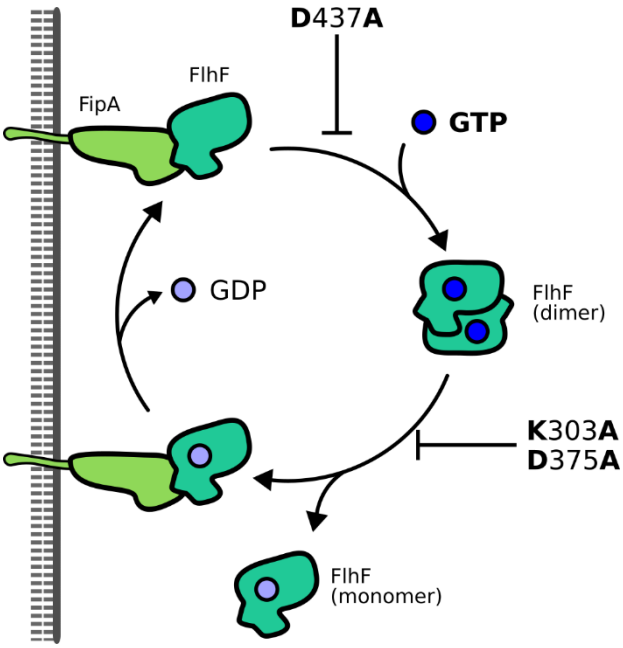

Figure 4.6. Model of FlhF interacting with the cell membrane. FlhF is soluble as a GTP-bound dimer. Hydrolysis produces a GDP-bound monomer. FipA can interact either with this monomer or with the *apo* form, or maybe both. Mutations in the catalytic site lock FlhF as a soluble dimer that does not interact with FipA. Mutations that prevent GTP binding lock the protein as a monomer and may also form inclusion bodies in *E. coli*.

prevent nucleotide binding, did not colocalize with FipA (Fig. 4.2).

Under our conditions, the interaction of FlhF with FipA is not very stable. The FlhF-FipA complex separated with either DDM or F-12 treatment (Fig. 4.4). It is possible that the complex only exists briefly during the transition of FlhF from GDP- to GTP-bound. Interestingly, FlhF was reported to remain membrane-bound after treatment with 0.5 % Triton X-100 when expressed in *V. cholerae* (Green et al. 2009). It is possible that in the native conditions there are other membrane components that interact with FlhF in a detergent-resistant manner (*e. g.* HubP).

The discovery of FipA as the membrane anchor of FlhF permits us to propose this model in which FlhF interacts with the membrane in its monomeric state. We're sure that this model can provide many hypotheses to be tested by other biochemical and structural methods. We also expect that the discovery of the membrane localization of FlhF in a heterologous host by co-expression of FipA can open up new roads for the biochemical and structural characterization of these proteins.

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5. General discussion

5.1. FlhF and FlhG have different localization patterns

We started this study by replicating key experiments about the role of FlhFG in *V. parahaemolyticus*. We looked at the effect of each protein on flagellum synthesis, and found that they play a similar role as in other species, especially *V. cholerae.* FlhG acts as a negative regulator of flagellar number, since its deletion produced multi-flagellated cells (Fig. 2.1). FlhF acted as a positive regulator, since in its absence cells were completely unable to produce flagella (Fig. 2.1). This means that the system is largely conserved between different *Vibrio* species, and likely beyond that. A notable difference from other *Vibrio* species is that FlhF appears to be strictly necessary for flagellum formation, since no flagellated cells were detected at all. In *V. cholerae* and *V. alginolyticus*, some cells can still produce flagella in the absence of FlhF (Correa, Peng, and Klose 2005; Kusumoto et al. 2006), and in other γ-proteobacteria, FlhF is only necessary to determine the position of the flagellum (Pandza et al. 2000; Gao et al. 2015; Rossmann et al. 2015). For this reason, *V. parahaemolyticus* could be a better candidate to study the function of FlhF in γ-proteobacteria, since its deletion has a stronger phenotype that makes it easier to study.

To follow the localization of FlhF and FlhG throughout the cell cycle, we labeled the proteins in their native locus in the chromosome, because heterologous expression can result in non-native expression levels. The regulation of these cellular processes can be sensitive to the number of molecules present in the cell (Galli, Paly, and Barre 2017), and therefore heterologous expression can result in artificial phenotypes. As expected, FlhF and FlhG were localized at the cell pole, but they followed different patterns. FlhF is present as a polar focus in more than half of the cells in the population, and it is more likely to become bipolar as the cells progress through the cell cycle (Fig. 2.4). This is consistent with its role as a marker and recruiter of the early flagellar proteins, although its presence alone is not equivalent to presence of a flagellum filament. Indeed, the existence of cells with bipolar FlhF foci shows that FlhF can be present at the cell pole without necessarily starting the synthesis of the flagellum, since flagella are never observed at both poles in *V. parahaemolyticus* (Fig. 2.1). Similarly, in the absence of FipA, where FlhF is still recruited to the cell pole, there's no flagellar synthesis and no recruitment of FliF (Fig. 3.2). Even upon deletion of FlhG, which results in excess FlhF recruitment at both poles (Fig. 2.4), no flagella are synthesized at the new pole, indicating that the presence and number of FlhF is not sufficient to trigger the recruitment of the MS-ring components; something else is required to "activate" FlhF.

While FlhG was found as polar foci as well, in the majority of cells such foci could not be detected (Fig. 2.5). Furthermore, in a notable proportion of cells, FlhG foci were seen in the cytoplasm (Fig. 2.5), and even more so in the absence of FlhF or HubP. The cytoplasmic foci were mostly seen in younger cells, and as the cell cycle progresses, FlhG is found only at the poles (Fig. 2.2). This is the first detailed

description of the localization of FlhG, and it is beautifully coherent with the model emerging from biochemical evidence of the interactions between FlhG, FlrA and FliM (Blagotinsek et al. 2020; Chanchal et al. 2021). In this model, FlhG represses transcription of the flagellar genes by interacting with FlrA at their promoter as an ATP-bound dimer (Fig. 5.1A). ATP hydrolysis can potentially disrupt this interaction by inducing monomerization of FlhG. When the basal body is being assembled at the new cell pole, monomeric FlhG is brought there by FliM (Fig. 5.E), allowing transcription of the genes necessary for the basal body and the hook. Thus, FlhG spends most of the cell cycle on the DNA with FlrA, and only when the transcription of class II genes is required it is sequestered at the cell pole (Fig. 5.1C). When assembly of the basal body is not possible due to mis-localization or complete absence of FlhF, there are more cytoplasmic foci of FlhG, since FliM is not recruited at the cell pole and cannot sequester FlhG (Fig. 2.5).

Interestingly, FliM is not sufficient to recruit FlhG to the cell pole. The deletion of HubP is sufficient to abolish polar recruitment of FlhG (Fig. 2.5), as is the case in other *Vibrio* species (Yamaichi et al. 2012; Rossmann et al. 2015; Takekawa et al. 2016). In this genetic background, we can deduce that FliM is still present at the cell pole by the presence of functional flagella (Fig. 2.1), and polar FlhF and FliF (Fig. 2.4 and 3.2). Therefore, even if FliM can keep FlhG sequestered at the cell pole, it most likely reaches the pole by the action of HubP. In this way, HubP provides a signal linking maturation of the new pole with the start of flagellum synthesis by recruiting FlhG to the pole and allowing transcription of FlrA-dependent promoters (Fig. 5.1C).

Furthermore, we could confirm that the polar localization of FlhF is independent of HubP also in *V. parahaemolyticus*. FlhF has thus the potential to connect an independent signal from the pole maturation with the assembly of the flagellum.

An interesting question that arises from the observation of cytoplasmic FlhG clusters, is what is the stoichiometry of the FlhG-FlrA complex. By the intensity of the foci, they are likely composed of several molecules (Fig. 2.5). It would be interesting to investigate as well the localization of FlrA, which according to this model should co-localize with the cytoplasmic FlhG, and to see if the formation of these clusters depends on the DNA-binding activity of FlrA.

5.2. FipA, a new interacting node at the cell pole

In this work, we identified FipA as new factor driving localization of FlhF. FipA had only been described as a regulatory protein named FlrD, in *V. cholerae* (Moisi et al. 2009). It's role at the cell pole had not been described until now. In fact, our evidence indicates that FipA is not a transcriptional regulator. The transcriptional effects of FipA observed by Moisi *et al.* are entirely explained by the activating role of FipA on FlhF. The deletion of FipA has the same phenotype as the deletion of FlhF. Since the deletion of either of them impedes basal body assembly (Fig. 3.2F, G), activation of FlaLM never occurs, and therefore there's no expression of class III genes (Fig. 1.3C).

Our evidence also shows that FipA is the missing factor that anchors FlhF to the membrane (Green et al. 2009). Even though it is encoded downstream of a flagellar operon, *fipA* can be transcribed from a different promoter that does not depend on FlrA (Moisi et al. 2009; Petersen et al. 2021). For this reason, Green *et al.* found FlhF at the cell pole even in a Δ*flrA* background.

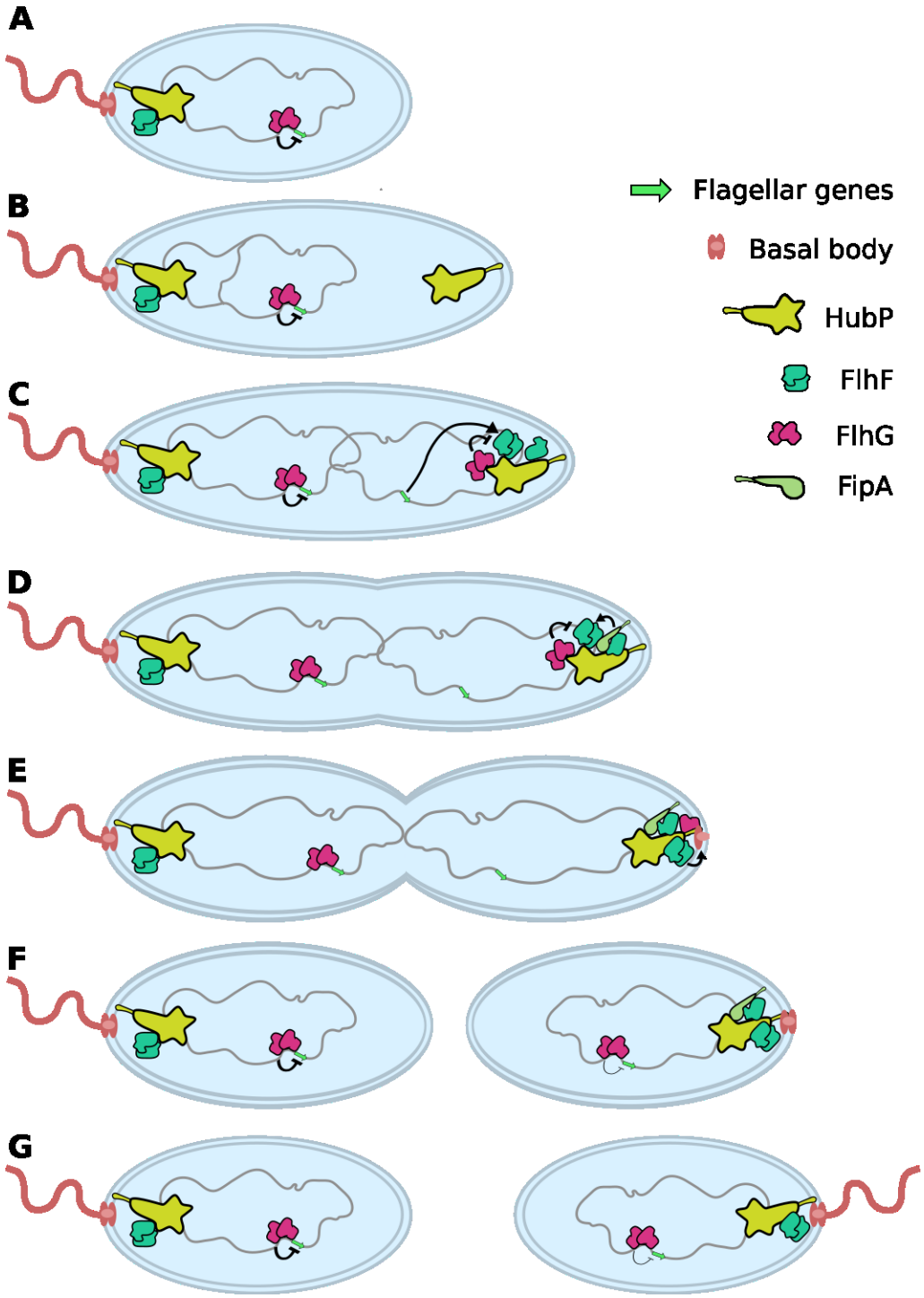

Figure 5.1. Model of FipA, FlhF and FlhG in the cell cycle. A) A freshly divided cell has FlhF at the old pole and transcription of flagellar genes is repressed by cytoplasmic FlhG. **B)** HubP is the first protein to migrate to the new pole. **C)** Once the flagellar genes are replicated, it is class II flagellar proteins are produced. FlhF and FlhG are recruited by HubP to the new pole. FlhF is repressed from starting basal body assembly by FlhG. **D)** Late in the cell cycle, FipA is recruited to the cell pole and counter acts the repressor effect of FlhG on FlhF. **E)** FlhF recruits FliF and starts basal body assembly. FlhG is sequestered by the basal body proteins. **F)** Once the basal body is completed, FlhG becomes cytoplasmic again and represses class II flagellar genes. **G)** After cell division, FipA disappears from the pole, and flagellum elongation proceeds.

The interaction between FipA was confirmed by a reciprocal pull-down (Fig. 3.1B), by bacterial two-hybrid assays with proteins from *V. parahaemolyticus* and *P. putida* (Fig. 3.1E, F) and by co-localization in *E. coli* (Fig. 4.1). We could even discover that the interaction hinges on conserved residues inside the DUF2802, since mutations in these residues abolished the interaction (figs. 3.4, 4.1 and 4.5). It's tempting to say that these residues mediate the interaction with FlhF. However, it's also possible that they are essential to the structure of the domain, in a way that doesn't abolish self-interaction of FipA (Fig. 3.4B, C). This could very well be the case for the G110 in VpFipA, corresponding to G104 in PpFipA, since its change to an alanine could have significant impacts on the structure. In addition, the FlhF-FipA interaction could be restored *in vitro* in the presence of low (0.4 mM) concentrations of the non-ionic detergent DDM (Fig. 4.5), but high concentrations (5 mM) of DDM or the zwitterionic F12 separated the complex (Fig. 4.4). This indicates that the interaction between the proteins is rather weak, as would be expected from an interaction between hydrophobic residues like the glycine and leucine that we tested in this study. Further characterization of the interaction between FlhF and FipA and its variants is needed to discern these possibilities.

5.3. Different factors drive polar localization of FlhF

FlhF remained as the only polar protein in *Vibrio* whose localization could not be traced back to the landmark protein HubP. Even though HubP and FlhF were shown to interact directly in *V. cholerae* (Yamaichi et al. 2012), FlhF forms polar foci in the absence of HubP in a variety of organisms, namely *V. cholerae* (Yamaichi et al. 2012), *V. alginolyticus* (Takekawa et al. 2016), *S. putrefaciens* (Rossmann et al. 2015) and now *V. parahaemolyticus* (Fig. 2.4). This meant that at least one additional factor is recruiting FlhF to the cell pole.

The localization of FlhF was shown to depend on FipA and HubP. In fact, FlhF appeared completely delocalized when both FipA and HubP were deleted in *V. parahaemolyticus* and *P. putida* (Fig. 3.3). However, FipA is unlikely to be a polar landmark on its own, since its own localization is entirely dependent on FlhF (Fig. 3.6). It seems rather that the polar localization of FlhF is an emergent property of its interaction with FipA. There are most likely other factors present in the cells of polar flagellates that are necessary for the localization of FipA FlhF, since their expression in a heterologous host result in non-polar colocalization (Fig. 4.1). Other landmark proteins with a truly intrinsic polar localization are found thus also in *E. coli*, namely HubP (Yamaichi et al. 2012), PopZ (Bergé et al. 2016; Perez et al. 2017) and DivIVA (Bach, Albrecht, and Bramkamp 2014). It is also to be expected that many independent factors regulate the intracellular organization, which would make the system more robust. We would expect that as the exploration of the intracellular organization of ɣ-proteobacteria continues, more redundancy in the regulatory network will be discovered, as has happened in the field of *C. crescentus (Bergé* and Viollier 2018).

5.4. FipA as an activator of FlhF

The function of FipA goes beyond recruiting FlhF to the cell pole. Indeed, FlhF can still induce flagellum synthesis in the absence of HubP as long as FipA is present (Fig. 2.1), but FipA is strictly required for flagellum synthesis even when FlhF is present at the pole via HubP (Fig. 3.2, 3.3).

One possibility is that even though FlhF can be recruited to the cell pole by HubP, FipA recruits even more FlhF units, and a threshold of FlhF concentration at the pole needs to be crossed to trigger MS FliF recruitment and MS ring assembly. This could also explain why the lophotrichous *P. putida* still produces flagella in the absence of FipA, if this threshold is lower for *P. putida* than for *V. parahaemolyticus*. In light of this hypothesis, it would be interesting to find out if the self-interacting potential of FipA that we observed in the bacterial two-hybrid (Fig. 3.1E, F) translates to formation of larger multimers in combination with FlhF, which are more stable and last longer when recruited to the pole. The formation of such complexes is also supported by the appearance of the colocalization of both proteins in *E. coli* as defined points (Fig. 4.1), as opposed to a smooth line along the membrane.

But in addition to bringing FlhF to the cell pole, we have shown in Chapter 4 that FipA is the bridge to the membrane specifically (Fig. 5.2). This is probably the main role of FipA, since a deletion of the transmembrane domain in *fipA* results in the same phenotype as whole-gene deletion (Fig. 3.7). The role of FlhF in the membrane seems natural, since it aids in the recruitment of FliF, a membrane protein. In fact, it has been suggested that FlhF participates in the secretion of other proteins, in *B. cereus* (Mazzantini et al. 2020) and in the γ-proteobacterium *A. hydrophila* (Dong et al. 2022). However, protein secretion mediated by SRP requires its GTPase activity (and actually also the general FlhF-dependent protein secretion seen in *A. hydrophila*), whereas FlhF seems to require GTP binding but not GTPase activity (Green et al. 2009; Gulbronson et al. 2016; Mazzantini et al. 2020). Thus, GTPase

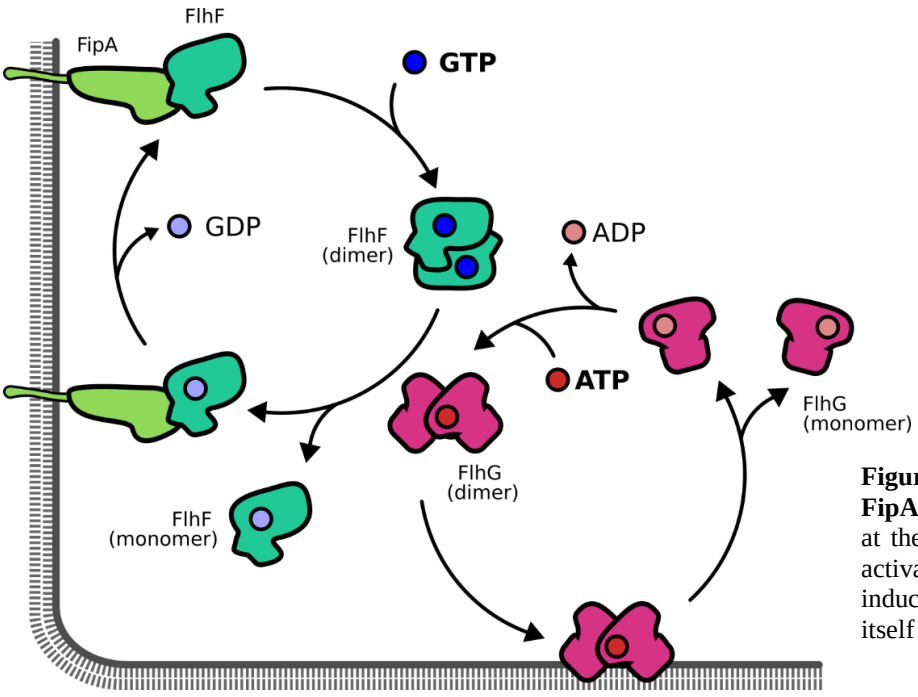

Figure 5.2. Integrated model of FipA and FlhF. FipA anchors FlhF at the membrane and promotes its activation. FlhG inactivates FlhF by inducing GTP hydrolysis, and is itself regulated by its ATPase cycle.

activity rather acts as an "off" switch for FlhF in flagellum assembly, and this is accelerated by FlhG (Fig. 5.2). There is an interesting comparison to draw here to the other double-NTPase cycle of ParAB (Fig. 1.1C). In that case, ATPase activity of ParA drives movement across the cell, and CTPase activity of ParB provides energy to push ParB along the DNA. However, the orthologue of ParB in *M. xanthus*, PadC, has lost the need for the CTPase activity (Osorio-Valeriano et al. 2019). It seems that these regulatory loops could have evolved alternative mechanisms in addition to the pure NTPase switch in some cases.

Following this, in the model proposed in Chapter 4, the membrane-binding role of FipA towards FlhF promotes the active, dimeric state (Fig. 5.2). It remains to be seen if this is simply by increasing the permanence of FlhF at the cell pole long enough for it to dimerize again, or by actively acting as a nucleotide exchange factor.

In this model, FlhG is an antagonist of FipA. Thus, FlhG is not only a regulator at the transcriptional level, but also locally at the cell pole. This would explain why the deletion of *flhG* can suppress the deletion of *fipA* in some cells (Fig. 3.2D): in a Δ*fipA* strain, GDP-bound FlhF accumulates by the catalytic action of FlhG. The deletion of *flhG* allows accumulation of basal FlhF-GTP dimers, which can produce flagella in at least a small percentage of the cells.

As a consequence of its role in this model, FipA could represent a new point in the network that licenses flagellum formation upon its arrival. Thus, new signals can be integrated in the flagellar hierarchy by regulating the expression of FipA, which is transcribed independently of the flagellar genes, or by regulating its localization through as-yet unidentified factors. The localization of FipA was also aligned with this hypothesis, since it was found to migrate to the new pole shortly before or after cell division (Fig. 3.5). At this time only is when flagellum synthesis is required in the unipolar flagellates. Therefore, if the signals recruiting FipA are related to the maturation of the new pole and separation of the daughter cells, it would explain why this regulatory level is necessary exclusively in the unipolarly flagellated species (Fig. 3.1), where it is necessary to start flagellum assembly only after cell division is completed.

5.5. References

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6. Conclusions and future perspectives

In this work, we have found that the function of FlhF and FlhG is conserved in *V. parahaemolyticus* as in other members of the genus. We described in detail the localization of these proteins in living cells throughout the cell cycle, and expressed in native conditions.

We discovered that the seemingly intrinsic polar localization of FlhF is in fact a result of its interaction with FipA, a new polar determinant of flagellum formation. FipA is conserved among γ-proteobacteria that use FlhFG to regulate the position of their flagella. We identified residues in the conserved cytoplasmic domain of unknown function of FipA that are essential to mediate activation of FlhF and recruit the components of the flagellar basal body, and show that these interactions are also conserved and functional in *P. putida*.

We also showed that this novel protein is responsible for the membrane localization of FlhF, and that its co-expression can help the production and purification of FlhF in a heterologous host. Furthermore, we observed that mutations affecting the GTPase activity of FlhF affect its interaction with FipA, and that GTP itself prevents the interaction in an *in vitro* setting. With this data, we generated a model in which FipA promotes flagellum synthesis by recruiting FlhF to the cell pole and increasing the amount of active, GTP-bound FlhF. FipA therefore arrives at a late time point in the cell cycle to counter act the inhibitory effect of FlhG.

Many hypotheses derive from this model that still remain to be tested. The “active” and “inactive” states of FlhF, assigned to the dimeric and monomeric forms, respectively, need to be confirmed by expressing the proteins from native promoters and evaluating their effects in flagellum formation, as well as their localization *in vivo*. This work could receive help from a structural characterization of the interaction interface between FlhF and FipA. Ideally, by finding the residues on FlhF that are essential for the interaction with FipA, we could find point mutations that are catalytically active but not interactive. According to this model, such variants should not be able to produce flagella, due to inactivation of FlhF by FlhG that cannot be restored by FipA.

Analysis of the structure, stoichiometry and kinetics of the FipA-FlhF complex could provide further clues to the mechanism of its formation through the cell cycle, and whether it depends on *de novo* synthesis of FipA and FlhF (as would be our prediction). Structural data from FipA alone would also help in this regard, as well as the identification of mutations that abolish FipA self-interaction but not FipA-FlhF interaction.

A question that arises from the comparison of *V. parahaemolyticus* to the other organisms, is why is FlhF strictly necessary in this organism. Insights into the differences between FlhF and FipA from this bacterium and other species could reveal information on the molecular basis of the diversity of flagella.

Finally, the potential of FipA as a new regulatory point in the flagellar cascade is a very interesting research topic, which should lead to the investigation of the transcriptional regulation of its gene, and hopefully to the identification of its promoter, its transcription factors and the signals which they respond to. This will surely open up new pathways in the research of intracellular organization of these bacteria.

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