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Collaboration of FlhF and FlhG to regulate polar-flagella number and localization in *Vibrio alginolyticus*

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Precise regulation of the number and placement of flagella is critical for the mono-polar-flagellated bacterium *Vibrio alginolyticus* to swim efficiently. We have shown previously that the number of polar flagella is positively regulated by FlhF and negatively regulated by FlhG. We now show that Δ*flhF* cells are non-flagellated as are most Δ*flhFG* cells; however, some of the Δ*flhFG* cells have several flagella at lateral positions. We found that FlhF–GFP was localized at the flagellated pole, and its polar localization was seen more intensely in Δ*flhFG* cells. On the other hand, most of the FlhG–GFP was diffused throughout the cytoplasm, although some was localized at the pole. To investigate the FlhF–FlhG interaction, immunoprecipitation was performed by using an anti-FlhF antibody, and FlhG co-precipitated with FlhF. From these results we propose a model in which FlhF localization at the pole determines polar location and production of a flagellum, FlhG interacts with FlhF to prevent FlhF from localizing at the pole, and thus FlhG negatively regulates flagellar number in *V. alginolyticus* cells.

INTRODUCTION

Many motile bacteria have flagella that rotate by means of a motor embedded in the cytoplasmic membrane and create a driving force by rotating a helical filament, like a screw, connected to the motor. The number and localization of flagella are different among species. *Escherichia coli*, *Salmonella typhimurium* (Macnab, 1996) and *Bacillus subtilis* (Kearns & Losick, 2003) have peritrichous flagella. *Vibrio cholerae* (Prouty *et al.*, 2001), *Caulobacter crescentus* (Brun *et al.*, 1994) and *Pseudomonas aeruginosa* (Tsuda & Iino, 1983) have a single polar flagellum. *Vibrio fischeri* (Millikan & Ruby, 2004), *Helicobacter pylori* (Niehus *et al.*, 2004) and *Pseudomonas putida* (Harwood *et al.*, 1989) have multiple flagella at the pole. *Vibrio alginolyticus* and *Vibrio parahaemolyticus* have both a single polar flagellum and peritrichous flagella (McCarter, 2001). The flagella are not essential, and cells must expend significant energy for their maintenance. Bacteria minimize the cost of producing flagella, and it seems that one strategy involves the precise regulation of flagellar number.

The flagellar genes are conserved among peritrichously flagellated and polarly flagellated bacteria, and gene regulation and morphogenesis are also similar among various species (Macnab, 1996; Wu & Newton, 1997; Prouty *et al.*, 2001; McCarter, 2001). The flagella, which are composed of a filament, hook and basal body, are formed from the proximal end towards the distal tip. During flagellar morphogenesis, first the MS ring of the basal body, which is composed of FliF, is established on the cytoplasmic membrane (Kubori *et al.*, 1992). Next, the switch complex or the C ring, which is composed of three proteins, FliG, FliM and FliN, is assembled under the MS ring. Then, the specific apparatus for protein export is assembled inside the C ring to form the entrance of the channel for flagellar proteins (Kubori *et al.*, 1997; Macnab, 2004). The rod proteins are exported by way of this apparatus and the rod structure is constructed. Then, the other proteins, such as the hook protein and flagellin are exported similarly and polymerized into tubular structures that extend from the rod. Formation of the MS ring is thought to initiate the flagellar assembly, so the number and location of MS-ring complexes are likely to determine the number and location of flagella.

The flagellar genes are hierarchically expressed under strict control. In *Vibrio* and *Pseudomonas*, they are classified into at least three classes: early genes (master regulator); intermediate genes, the expression of which depends on σ^{54} (MS ring, hook, basal body, switch, export apparatus, transcriptional regulators including FlaL, FlaM, FlhF and FlhG, *che* proteins, σ^{28} and flagellin); and late genes, the

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expression of which depends on σ^{28} (flagellins, motor proteins and FlgM) (Dasgupta *et al.*, 2003; McCarter, 2001; Prouty *et al.*, 2001). The intermediate class is further divided into two classes: the FlaK-dependent genes (homologues are FleQ in *P. aeruginosa* and FlrA in *V. cholerae*) that encode the MS ring, switch, export apparatus, FlaL, FlaM, FlhF, FlhG and σ^{28}; and the FlaLM-dependent genes (homologues are FleSR in *P. aeruginosa* and FlrBC in *V. cholerae*) that encode hook protein, rod proteins, LP-ring proteins and flagellin. These four gene classes are called class I (early genes), class II (FlaK-dependent intermediate genes), class III (FlaLM-dependent intermediate genes) and class IV (late genes).

In *V. cholerae*, *P. aeruginosa* and *P. putida*, overexpression of FlhF results in an increased number of polar flagella, and an *flhF* gene disruption gives a reduced number and aberrant placement of flagella in *V. cholerae*, *P. aeruginosa* and *P. putida* (Correa *et al.*, 2005; Murray & Kazmierczak, 2006; Pandza *et al.*, 2000). Moreover, FlhF has been reported to increase the expression of class III genes in *V. cholerae* (Correa *et al.*, 2005). Therefore, it has been suggested that FlhF increases the number of flagella by promoting the expression of flagellar genes. However, it is unclear how FlhF determines the location of the flagella. In *V. cholerae* and *P. aeruginosa*, overexpression of FlhG results in a reduced number of flagella and gives a non-flagellated phenotype, and an *flhG* gene disruption gives a hyperflagellated phenotype (Correa *et al.*, 2005; Dasgupta *et al.*, 2000). In *Pseudomonas*, it has been shown that the FleN protein (the homologue is FlhG in *Pseudomonas* sp.) binds to the flagellar-gene-specific transcriptional regulator, FleQ (Dasgupta & Ramphal, 2001), which regulates the transcription of class II genes with σ^{54} (Dasgupta *et al.*, 2002). The FleN protein represses transcription of class II genes and of the *fleN* gene by itself via FleQ. The FleQ homologue in *V. cholerae*, FlrA, also regulates transcription of class II genes (Prouty *et al.*, 2001).

We have previously reported a multi-polar flagellar mutant of *V. alginolyticus*, KK148, and we identified the mutation responsible, which is a nonsense mutation in the *flhG* gene (Kusumoto *et al.*, 2006). We cloned the upstream gene, *flhF*, and the *flhG* gene. These genes are unique to polar-flagellated bacteria, although FlhF and FlhG have similarity with the *E. coli* signal recognition particle (SRP) receptor FtsY and the *E. coli* cell division inhibitor MinD, respectively (Kusumoto *et al.*, 2006). Overexpression of FlhF results in an increased number of polar flagella; on the other hand, overexpression of FlhG results in a reduced number of polar flagella and gives a non-flagellated phenotype. These results are consistent with those of earlier studies with *V. cholerae* and *Pseudomonas* (Correa *et al.*, 2005; Dasgupta *et al.*, 2000; Murray & Kazmierczak, 2006; Pandza *et al.*, 2000). Moreover, co-expression of FlhF and FlhG reduces the number of polar flagella more significantly than expression of FlhG alone, implying that FlhG works together with FlhF to regulate the number of polar flagella (Kusumoto *et al.*, 2006).

In this study, we show that the polar localization of FlhF is reduced by FlhG, independent of other flagellar proteins, and that FlhF and FlhG interact with each other. We speculate that the FlhF–FlhG interaction inhibits FlhF from localizing at the pole and that flagellation is thus suppressed. These results contribute to our understanding of the control of flagellar number and the location of the flagellum in mono-polar flagellar systems.

METHODS

Bacterial strains and growth conditions. *V. alginolyticus* and *E. coli* strains used in this study are listed in Table 1. *V. alginolyticus* cells were cultured at 30 °C in VC medium [0.5 % (w/v) Tryptone, 0.5 % (w/v) yeast extract, 0.4 % (w/v) K_2HPO_4, 3 % (w/v) NaCl, 0.2 % (w/v) glucose] or in VPG medium [1 % (w/v) Tryptone, 0.4 % (w/v) K_2HPO_4, 3 % (w/v) NaCl, 0.5 % (w/v) glycerol]. *E. coli* cells were cultured at 37 °C in LB medium [1 % (w/v) Tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl]. When necessary, the following antibiotics were used: chloramphenicol (2.5 μg ml^{-1} for *V. alginolyticus* or 25 μg ml^{-1} for *E. coli*), kanamycin (100 μg ml^{-1}) and ampicillin (50 or 100 μg ml^{-1}).

DNA manipulations and sequencing. Routine DNA manipulations were carried out according to standard procedures. Restriction endonucleases and other enzymes for DNA manipulations were purchased from TaKaRa Shuzo, Toyobo, and New England Biolabs. Nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

Gene disruption. The *flhF* and *flhFG* deletion strains LPN1 and LPN2 were generated from VIO5 cells, which are defective for production of lateral flagella and wild-type with respect to the polar flagellum, by homologous recombination, as described previously (Terashima *et al.*, 2006). The suicide vectors containing the *sacB* gene, pKY704-Δ*flhF-sacB* and pKY704-Δ*flhFG-sacB*, were used for this construction. First, for homologous recombination of the *flhF* and *flhFG* genes, 1073 bp downstream of the *flhG* gene, containing the *fliA* gene and 313 bp from the beginning of the *cheY* gene, were cloned into a cloning vector, pGEM5Zf(+), and sequenced. Using the resultant plasmids and primers based on these sequences, we made in-frame deletions in *flhF* or *flhG* (DNA encoding 475 aa was deleted from *flhF* and DNA encoding 772 aa was deleted from *flhFG*), which were integrated into the chromosome of VIO5 cells by homologous recombination. Next, strains that had undergone the first recombination were cultured in VC medium without antibiotics overnight, and then screened for the second recombination based on their sucrose sensitivity. Finally, the *flhF* and *flhFG* deletions in the chromosome were confirmed by PCR. The *flhF* deletion and *flhFG* double deletion strains were named LPN1 and LPN2, respectively.

Transformation of *Vibrio* cells. *V. alginolyticus* cells were transformed by electroporation as described previously (Kawagishi *et al.*, 1994). The cells were subjected to osmotic shock and washed thoroughly with 20 mM $MgSO_4$. Electroporation was carried out according to the manufacturer's instructions using a Gene Pulser electroporation apparatus (Japan Bio-Rad Laboratories) at an electric field strength of 5.0–7.5 kV cm^{-1}.

High-intensity dark-field microscopy. Flagella were observed using a dark-field microscope (Olympus model BHT) equipped with a 100 W mercury lamp (Ushio USH-102). An image was recorded

Table 1. Bacterial strains and plasmids used in this study

Abbreviations: Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Rif^r, rifampicin resistant; Pof^+, possessing polar flagellum; Laf^-, lacking lateral flagella; Multi-Pof, possessing multiple polar flagella.

Strain or plasmid	Genotype or description	Reference or source
***V. alginolyticus* strains**		
VIO5	VIK4 (Rif^r Pof^+ Laf^-)	Okunishi *et al.* (1996)
KK148	VIO5 *flhG* (Rif^r Pof^+ Laf^- Multi-Pof)	Kusumoto *et al.* (2006)
YM14	YM4 *rpoN* (Pof^- Laf^-)	Kawagishi *et al.* (1997)
LPN1	VIO5 Δ*flhF* (Rif^r Pof^+ Laf^-)	This study
LPN2	VIO5 Δ*flhFG* (Rif^r Pof^+ Laf^-)	This study
***E. coli* strains**		
DH5α		Grant *et al.* (1990)
BL21(DE3)pLysS	T7 expression host	Studier (1991)
Plasmids		
pHFS401	*sacB* in pSU41	Terashima *et al.* (2006)
pKY704	Cm^r, suicide vector	Xu *et al.* (1994)
pKY704-Δ*flhF-sacB*	1425 bp-deleted *flhF* fragment in pKY704	This study
pKY704-Δ*flhFG-sacB*	2316 bp-deleted *flhFG* fragment in pKY704	This study
pET-3d	Amp^r, T7 expression vector	Novagen
pET-*flhF*	his_6-*tev-flhF* in pET-3d	This study
pGEX-6P-2	Amp^r, P_{tac}, *gst*	GE Healthcare
pGEX-*flhG*13	*gst-flhG* in pGEX-6P-2	This study
pBAD24	Amp^r, P_{BAD}	Guzman *et al.* (1995)
pBAD33	Cm^r, P_{BAD}	Guzman *et al.* (1995)
pAK322	*flhF* in pBAD33	Kusumoto *et al.* (2006)
pAK520	*flhG* in pBAD33	Kusumoto *et al.* (2006)
pAK721	*flhFG* in pBAD33	Kusumoto *et al.* (2006)
pAK325	*flhF-egfp* in pBAD33	This study
pAK541	*flhG-egfp* in pBAD33	This study

using a charge-coupled device (CCD) camera (Sony model SSC-M370) and a DVD video recorder (Panasonic model DMR-E100H).

Electron microscopy. Samples were negatively stained with 2 % (w/v) potassium phosphotungstate (pH 7.4), and then observed with a JEM-1200 EXII electron microscope (JEOL).

Fluorescence microscopy. *Vibrio* cells bearing a plasmid, pAK325 or pAK541, were cultured overnight in VC medium. The overnight culture was diluted 1 : 100 in VPG medium containing 0.02 % (w/v) arabinose and 2.5 μg chloramphenicol ml^{-1}, and incubated at 30 °C for 4 h. Fluorescence microscopy was carried out with a 'tunnel slide', which is a rudimentary flow chamber constructed from a coverslip, a microscope slide and double-sided tape. Poly-L-lysine (0.1 %, w/v) was loaded into a tunnel slide, and after 5 min the tunnel slide was washed with VPG medium. Cultures of the cells were applied by exchanging the medium, and then the tunnel slide was turned upside-down and incubated for 1 min to adhere cells to the coverslip. The tunnel slides were washed with TMK buffer (50 mM Tris/HCl, 500 mM KCl, 5 mM glucose, 5 mM $MgCl_2$, pH 7.5) and observed under a BX-50 microscope (Olympus).

To stain polar flagella, cells were treated with the following preparation before observation. VPG medium containing antiserum raised against the polar flagellum (Fukuoka *et al.*, 2005) was applied to the tunnel slide by exchanging the medium. After 3 min incubation, the tunnel slide was washed with VPG, and then medium containing a rhodamine-conjugated anti-rabbit-IgG antibody was added. After 3 min incubation, the tunnel slide was washed with VPG, then observed under a microscope. The images were recorded and processed using a digital camera (Hamamatsu Photonics, C4742-80-12AG) and imaging software (BD Bioscience, IPLab, version 3.9.5 r2 and Adobe Photoshop version 7)

Antibody raised against FlhF and FlhG. FlhF was purified from BL21 (DE3)/pLysS cells harbouring a plasmid producing hexahistidine (His_6)-tagged FlhF (His_6–FlhF), pET-*flhF*. Cells were harvested by centrifugation, washed with buffer A [10 mM Tris/HCl, 150 mM NaCl, 0.5 mM PMSF, complete EDTA-free protease inhibitor (Roche), pH 8.0], and then frozen at −80 °C. After thawing, the cells were resuspended in buffer A containing 10 μg DNase I ml^{-1} and 5 mM $MgCl_2$. The suspension was then passed twice through a French pressure cell at 500 kg cm^{-2}. After centrifugation (10 000 ***g*** for 10 min), His_6–FlhF was present in the pellet as inclusion bodies. The pellet was resuspended in buffer A containing 4 % (w/v) Triton X-100, and then sonicated. The Triton-soluble membrane fraction was removed by centrifugation (10 000 ***g*** for 10 min). To remove the membrane fraction completely, the Triton-insoluble pellet was suspended again with buffer A containing Triton X-100, sonicated, and then centrifuged (10 000 ***g*** for 10 min). After the Triton-insoluble pellet had been rinsed twice with distilled water, the pellet was suspended in buffer A containing 8 M urea, and incubated at 30 °C for 2 h with shaking. The suspension was centrifuged (7000 ***g*** for 15 min), and then His_6–FlhF was purified from the supernatant with Ni–NTA resin.

FlhG was purified from DH5α cells harbouring a plasmid producing glutathione *S*-transferase (GST)-fused FlhG, pGEX-*flhG*13. Cells were harvested by centrifugation, resuspended in buffer C (50 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 8.0) containing 0.6 mg

lysozyme ml^{-1}, and then incubated on ice for 30 min. The cell suspension was passed twice through a French press at 500 kg cm^{-2}. After removal of undisrupted cells by centrifugation (10 000 ***g*** for 10 min), the insoluble fraction was removed by ultracentrifugation (100 000 ***g*** for 1 h). GST-fused FlhG in the soluble fraction was applied to a GSTrap FF column (GE Healthcare). After removal of the GST tag by applying PreScission protease (GE Healthcare), FlhG was eluted with buffer D (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 8.0).

Purified His_6–FlhF and FlhG were separated by SDS-PAGE and stained with Coomassie blue R250. The band corresponding to each protein was excised and used to inoculate a rabbit. Rabbit anti-FlhG antibody was produced by Biogate.

Western blot analysis of flagellar proteins. *V. alginolyticus* cells were cultured overnight in VC medium. The overnight culture was diluted 1 : 100 in VPG medium, and then incubated at 30 °C for 4 h. Cells were harvested by centrifugation, and then resuspended in double-distilled water. The cell suspensions were mixed with a one-fifth volume of SDS loading buffer [0.2 M Tris/HCl (pH 6.8), 37.5 % (w/v) glycerol, 6 % (w/v) SDS, 0.004 % (w/v) bromophenol blue] and a one-twentieth volume of 2-mercaptoethanol, and then boiled for 5 min. Proteins in the samples were separated by SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Millipore) using a semi-dry blotting apparatus (Bio-craft) according to the manufacturer's instructions. Immunoblotting was performed with anti-FlhF, anti-FlhG, anti-FlgI, anti-MotX, anti-MotY, anti-PomA, anti-PomB and anti-flagellin antibodies, as described previously (Nambu & Kutsukake, 2000; Yagasaki *et al.*, 2006; Fukuoka *et al.*, 2005; Nishioka *et al.*, 1998).

Immunoprecipitation. An overnight culture of Δ*flhFG* cells harbouring a plasmid, pAK520 (*flhG*) or pAK721 (*flhFG*), was diluted 1 : 100 into VPG medium containing 0.2 % (w/v) (for pAK520) or 0.01 % (w/v) (for pAK721) arabinose and 2.5 μg chloramphenicol ml^{-1}. After 4 h incubation at 30 °C, the cells were harvested by centrifugation (10 000 ***g*** for 5 min), suspended in buffer E [50 mM Tris/HCl, 150 mM NaCl, complete protease inhibitor (Roche), pH 7.5] to an OD_{660} of 10, and sonicated to disrupt the cells. Undisrupted cells were removed by centrifugation (10 000 ***g*** for 5 min). The supernatant was ultracentrifuged (100 000 ***g*** for 1 h). The supernatant from this step was diluted 1 : 5 into buffer E containing 0.1 % (w/v) Triton X-100, and anti-FlhF antibody and protein A–Sepharose CL-4B (GE Healthcare) were added. After 3 h incubation at 4 °C, the protein A–Sepharose was washed four times with buffer E containing 0.1 % (w/v) Triton X-100, and was resuspended in buffer E containing 3 % (w/v) SDS, and boiled. The protein A–Sepharose-bound materials were separated by SDS-PAGE, and FlhF and FlhG were detected by Western blot analysis. To eliminate the signal from heavy and light chains of the anti-FlhF antibody used for immunoprecipitation, the detection of FlhF was carried out with an ExtraCruz F kit (Santa Cruz).

RESULTS

Phenotypes of the *flhF* deletion and the *flhFG* double deletion strains

To investigate the function of FlhF, *flhF* and *flhFG* deletion strains were generated by means of homologous recombination. Electron microscopic observation revealed that the parent cells (VIO5) had a single polar flagellum (Fig. 1a), but we could not find any flagella on the Δ*flhF* cells (Fig. 1c). This is consistent with the previous study, which showed that the overproduction of FlhF increases the number of polar flagella (Kusumoto *et al.*, 2006). The Δ*flhF* strain showed almost no swarming ability on 0.25 % agar VPG plates after 6 h incubation (Fig. 2a, left). After 10 h incubation, however, swarming colonies of Δ*flhF* cells showed definite expansion, although colonies of YM14 cells (an *rpoN* mutant that does not produce polar flagella) did not expand at all (Fig. 2a, right). This indicates that a very low percentage of the population of the Δ*flhF* strain have flagella.

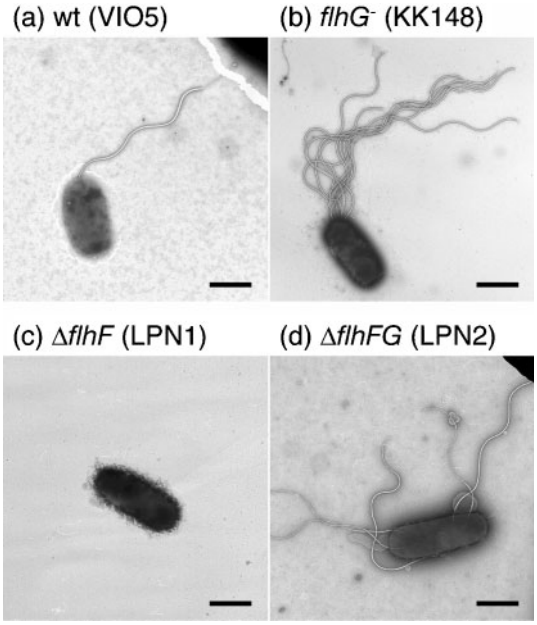

Fig. 1. Electron micrographs of (a) VIO5 (wild-type; wt), (b) KK148 (*flhG*), (c) LPN1 (Δ*flhF*), and (d) LPN2 (Δ*flhFG*) cells. Cells were negatively stained with potassium phosphotungstate. Bars, 1 μm.

High-intensity dark-field microscopic observation of Δ*flhFG* cells indicated that most (~97.5 %) were non-flagellated, although some of them (~2.5 %) had several flagella. Electron microscopic observation of the Δ*flhFG* cells also indicated that most were non-flagellated, although some had several flagella at lateral positions (Fig. 1d). Since the KK148 cells, which have a nonsense mutation in the *flhG* gene (Kusumoto *et al.*, 2006), had multiple flagella at the pole (Fig. 1b), FlhF may be involved in regulating the flagellar number as well as the placement of the polar flagellum.

Functions of the *flhF* and *flhG* genes

The motility of Δ*flhF* (LPN1) and Δ*flhFG* (LPN2) cells expressing the *flhF* and/or *flhG* genes on 0.25 % agar VPG plates was tested (Fig. 2b). The Δ*flhF* cells recovered their motility after expression of *flhF in trans* (Fig. 2b), and these cells also regained a polar flagellum, which was confirmed by high-intensity dark-field microscopy. Δ*flhFG* cells

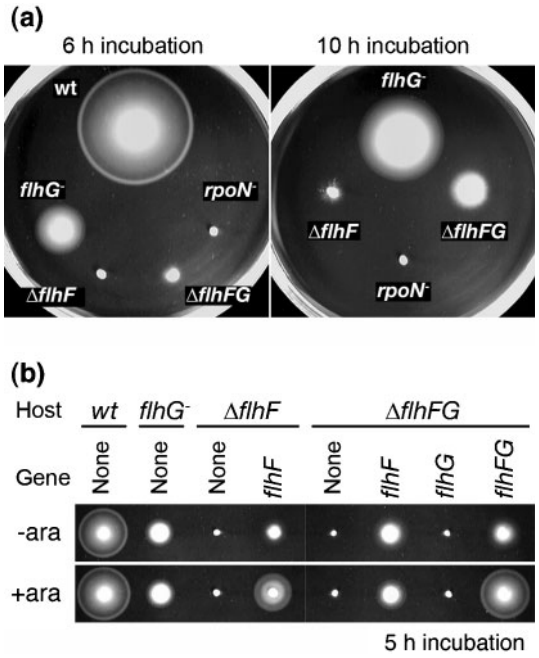

Fig. 2. Swarming ability of the Δ*flhF* and Δ*flhFG* cells. Swarming abilities are shown of the wild-type (wt; VIO5), Δ*flhF* (LPN1), Δ*flhFG* (LPN2), *flhG* mutant (KK148) and *rpoN* mutant (YM14) cells (a) without plasmids and (b) with plasmids pAK322 (*flhF*), pAK520 (*flhG*), pAK721 (*flhFG*) and pBAD33 (vector control). Aliquots (0.5 μl) of overnight cultures were spotted onto 0.25 % agar VPG plates without arabinose (a, left and right, or b, upper) or with 0.02 % arabinose (b, lower), followed by incubation at 30 °C for 6 h (a, left), 10 h (a, right) or 5 h (b).

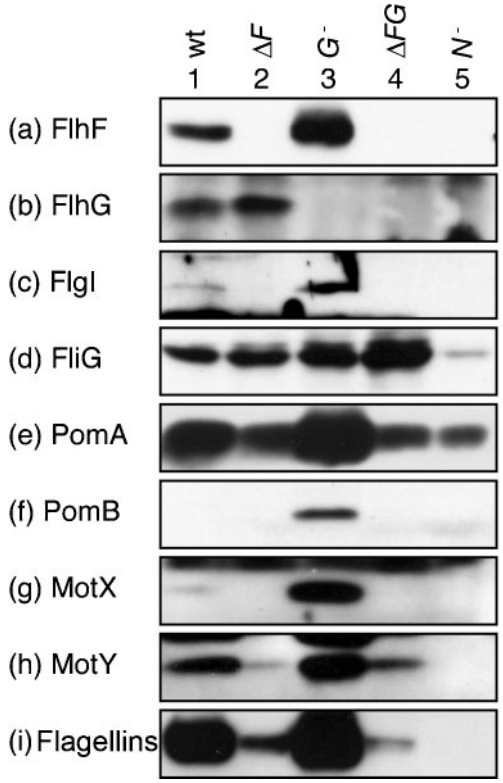

Fig. 3. Flagellar proteins in various mutant strains. Cells cultured overnight were recultured in VPG medium at 30 °C for 4 h. Cells were harvested by centrifugation and resuspended in water. Proteins were separated by SDS-PAGE and immunoblotted using anti-FlhF (a), anti-FlhG (b), anti-*Salmonella*-FlgI (c), anti-FliG (d), anti-PomA (e), anti-PomB (f), anti-MotX (g), anti-MotY (h) and anti-flagellin antibodies (i). Lanes: 1, VIO5 cells (wild-type; wt); 2, LPN1 cells (Δ*flhF*; Δ*F*); 3, KK148 cells (*flhG*; *G*⁻); 4, LPN2 cells (Δ*flhFG*; Δ*FG*); 5, YM14 cells (*rpoN*; *N*⁻).

expressing *flhF* recovered their motility even in the absence of arabinose (Fig. 2b), and the cells had multiple flagella at the cell pole, similar to the *flhG* mutant (KK148) cells. When the *flhG* gene was expressed in Δ*flhFG* cells (Fig. 2b), no flagella were produced and motility was reduced to the level observed in Δ*flhF* cells (data not shown). The expression of both *flhF* and *flhG* genes restored the motility of Δ*flhFG* cells to some extent in the absence of inducer, and to the same level as that of the wild-type in the presence of 0.2 % arabinose (Fig. 2b). Most of the cells had one or several polar flagella at the pole in the presence of 0.2 % arabinose (data not shown).

The amount of flagellar proteins in *flhF* and *flhG* mutants

We evaluated the amount of various flagellar proteins in the wild-type cells, and in the Δ*flhF*, *flhG*, Δ*flhFG* and *rpoN* mutant cells by means of Western blot analysis (Fig. 3). FlhF was not detected in the Δ*flhF*, Δ*flhFG* or *rpoN* cells (Fig. 3a), and FlhG was not detected in the *flhG*, Δ*flhFG* or *rpoN* cells (Fig. 3b). These results confirmed that the *flhF* gene, and the *flhF* and *flhG* genes in Δ*flhF* and Δ*flhFG* cells, respectively, were disrupted, and that the expression of *flhFG* genes required σ^{54}, the *rpoN* product. While a similar amount of FlhG was detected both in the wild-type and the Δ*flhF* cells (Fig. 3b), a larger amount of FlhF was detected in the *flhG* mutant (Fig. 3a). In the *flhG* mutant cells, most of the flagellar structural proteins (FlgI, PomA, PomB, MotX, MotY and flagellins), except for FliG, were detected in larger amounts than in the wild-type cells (Fig. 3c, e, f, g, h, i), which is consistent with the number of flagella on the cells. A similar amount of FliG (intermediate class) was detected in the wild-type, Δ*flhF* and *flhG* mutant cells, a larger amount was detected in the Δ*flhFG* mutant, and a smaller amount was detected in the *rpoN* mutant (Fig. 3d). These results imply that the *fliEFGHIJ* operon, which is in the intermediate class, is most likely regulated by an additional mechanism besides σ^{54} (RpoN).

Subcellular localization of FlhF and FlhG

To investigate the localization of FlhF and FlhG, GFP was fused to the C terminus of each protein. FlhF–GFP and FlhG–GFP were expressed in the Δ*flhF* and *flhG* mutant

cells, respectively, and polar flagella were visualized by means of immunofluorescence staining with an antibody raised against the polar flagellum (Fig. 4). The flagellated poles of cells showed the accumulation of FlhF–GFP as dots (Fig. 4a); on the other hand, *flhG* mutant cells producing FlhG–GFP failed to generate flagella (Fig. 4b), suggesting that FlhF–GFP and FlhG–GFP have the same ability to increase or decrease the number of flagella as the intact FlhF and FlhG. FlhF–GFP was diffused throughout the cytoplasm, with some localization at one or both poles in the Δ*flhF* cells (Fig. 4a, c). Most of the flagellated poles had FlhF–GFP foci (Fig. 4a, c). FlhG was also diffused throughout the cytoplasm in the *flhG* mutant cells (Fig. 4b). FlhG–GFP was localized at the pole in ~30 % of the cells (Fig. 4b, e, f). Most of the cells transformed with the *flhG-gfp* plasmid were not flagellated, and FlhG–GFP was not detected when the cells were flagellated (Fig. 4d).

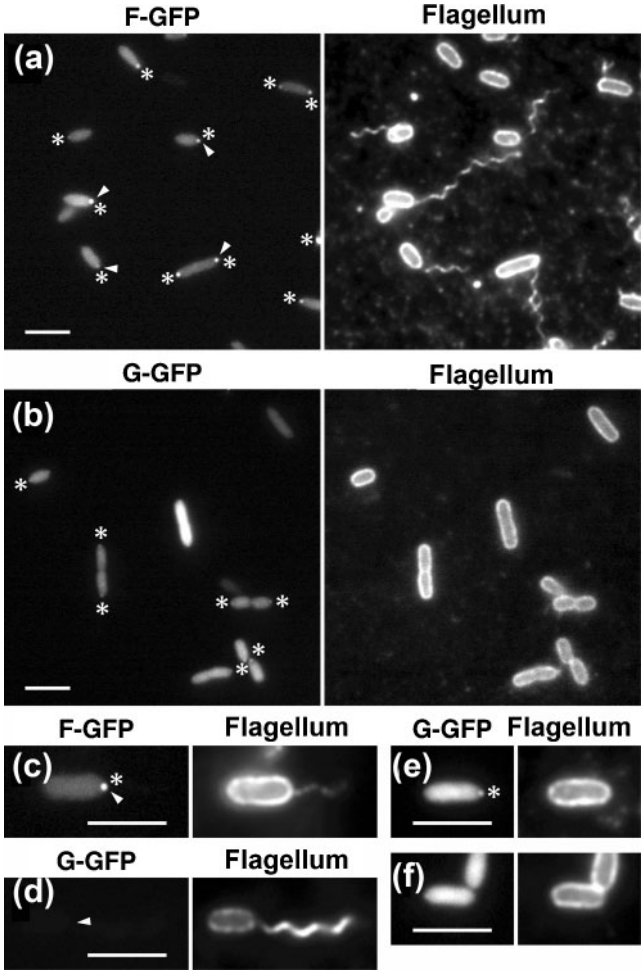

Fig. 4. Localization of GFP-fused FlhF (F-GFP) and FlhG (G-GFP). Δ*flhF* (LPN1) cells containing plasmid pAK325 (*flhF-egfp*) (a, c), and *flhG* mutant (KK148) cells containing plasmid pAK541 (*flhG-egfp*) (b, d, e, f), were observed by fluorescence microscopy. Cells cultured overnight were diluted 1 : 100 with VPG medium containing 0.02 % (w/v) arabinose. After 4 h incubation at 30 °C, the cells were attached to a poly-L-lysine-coated cover glass. To identify the flagellated pole, the cells were labelled with anti-polar-flagellum antiserum as the first antibody and rhodamine-conjugated anti-rabbit IgG antibody as the second antibody, and then observed under a fluorescence microscope. Asterisks indicate polar-localized F-GFP or G-GFP. Arrowheads indicate flagellated poles. Bars, 5 μm.

Polar localization of FlhF is affected by FlhG

The localization of FlhF–GFP and FlhG–GFP induced by arabinose was investigated in the Δ*flhFG* and *rpoN* mutant cells (Fig. 5). The amount of FlhF–GFP and FlhG–GFP in the cells did not differ among the strains, as confirmed by Western blotting (data not shown). FlhF–GFP was intensely localized at the pole in Δ*flhFG* cells (Fig. 5b), while FlhF–GFP was detected throughout the cytoplasm in addition to the poles in Δ*flhF* cells (Fig. 5a). We analysed the ratio of fluorescent signal in the cytoplasm to that at the pole in FlhF–GFP-expressing cells (Fig. 5h). The signals at the pole (denoted as 'P') and in the cytoplasm (denoted as 'C') were defined as the highest signal at the pole and the mean signal in the central cytoplasmic region of the cell, respectively (Fig. 5g). Fig. 5(h) shows the distribution of the ratio of P to C (P/C). The P/C of Δ*flhF* cells ranged from 1.8 to 15.3, and the mean ± SD P/C was 5.1 ± 3.2. The P/C of Δ*flhFG* cells ranged from 6.2 to 29.3 and the mean ± SD P/C was 14.7 ± 5.8. These results clearly show that FlhF–GFP is strongly localized at the pole in Δ*flhFG* cells compared with Δ*flhF* cells. Interestingly, the localization of FlhF in *rpoN* mutant cells, which do not express most of the flagellar proteins, including FlhF and FlhG, was similar to that in Δ*flhF* cells (Fig. 5c). The P/C of *rpoN* mutant cells ranged from 1.5 to 9.1 and the mean ± SD P/C was 3.9 ± 1.8. This may suggest that other factor(s) whose expression depends on σ^{54} are involved in the polar localization of FlhF. The FlhG–GFP signal was diffuse throughout the cytoplasm, although some FlhG–GFP formed fluorescent foci in *flhG*, Δ*flhFG* and *rpoN* mutant cells (Fig. 5d, e, f). FlhG–GFP localized at ~74 % of the *flhG* mutant cell poles, ~82 % of the Δ*flhFG* cell poles, and ~72 % of the *rpoN* mutant cell poles. The localization of FlhG–GFP in Δ*flhFG* cells was similar to that in the *flhG* mutant cells as well as that in *flhF* mutant cells. These results suggest that the polar localization of FlhF is inhibited by FlhG, while the localization of FlhG is not influenced by FlhF (see the model in Fig. 7).

To test whether the polar localization of FlhF is affected by the presence of FlhG in the absence of other *Vibrio* flagellar-related factors, GFP-fused FlhF or FlhG was produced with FlhG or FlhF in *E. coli* Δ*flhDCBA* cells, which lack the master regulators of the flagellar genes and do not express any flagellar genes, and in flagellated *E. coli* cells (RP437), which are wild-type for flagella formation

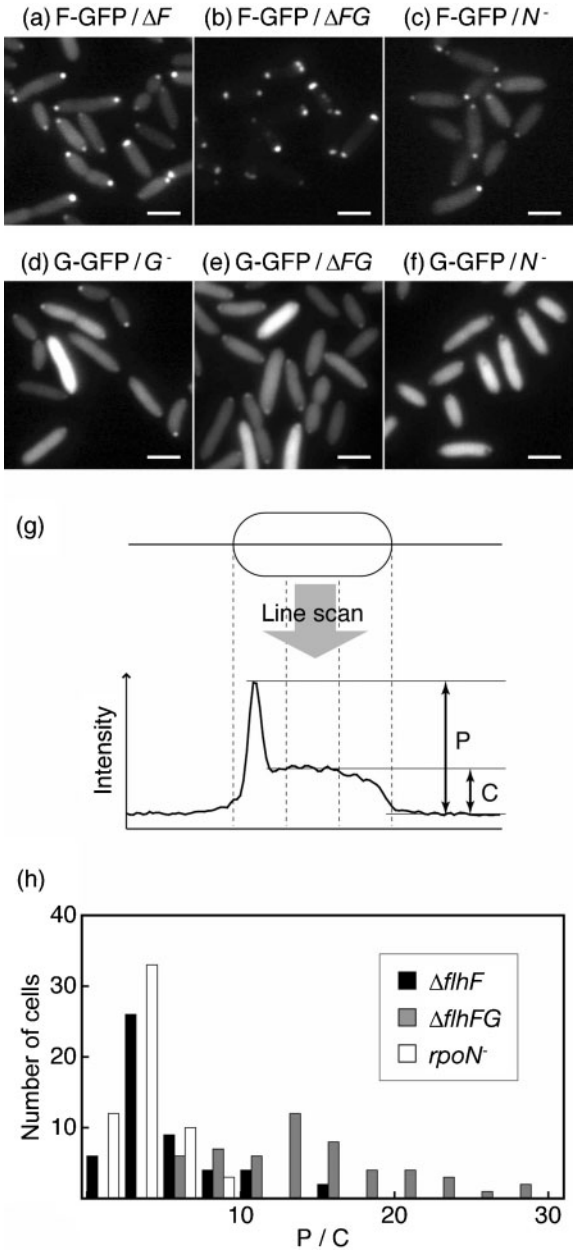

Fig. 5. The effect of FlhG on the polar localization of FlhF. The localization of FlhF–GFP (F-GFP) (a, b, c) and FlhG-GFP (G-GFP) (d, e, f) in cells was observed. The host strains were as follows: (a) Δ*flhF* (Δ*F*, LPN1); (b, e) Δ*flhFG* (Δ*FG*, LPN2); (d) *flhG* mutant (*G*⁻, KK148); (c, f) *rpoN* mutant (*N*⁻, YM14). Cells cultured overnight were diluted 1 : 100 with VPG medium containing 0.02 % arabinose. After 4 h incubation at 30 °C, the cells were bound to a poly-L-lysine-coated cover glass. Cells were observed under a fluorescence microscope. Bars, 2 μm. Quantitative analysis of the effect of FlhG on polar localization of FlhF was performed (g, h). (g) Schematic illustration of the quantification procedure. Cells were scanned for fluorescence intensity along the major axis using the line scan mode of ImageJ analysis software (ImageJ version 1.37). The fluorescence intensity at the pole ('P') for one axis was obtained by subtracting the background value from the highest intensity (in arbitrary units) within the cell. The fluorescence intensity in the cytoplasm ('C') for one axis was obtained in the same way, subtracting the background value from the average intensity of the cytoplasmic middle region (in arbitrary units) within the cell. (h) Histogram of the ratio of C to P (P/C) of 51 Δ*flhF* (LPN1) (black bars), 53 Δ*flhFG* (LPN2) (grey bars), and 58 *rpoN* mutant (YM14) (white bars) cells expressing FlhF–GFP.

(data not shown). FlhF–GFP expression was diffuse throughout the cytoplasm and a fluorescent dot was detected at the pole of ~36 % of cells. When co-expressed with FlhG, the fluorescent dot was detected in only ~2 % of cells and the fluorescence was diffuse throughout the cytoplasm, showing that FlhF–GFP failed to localize at the pole. Polar localization of FlhF and reduction of FlhF polar localization was also observed in RP437 cells. These results support the idea that FlhF can localize to the pole in the absence of other flagellar-related factors and that FlhF interacts with FlhG. FlhG–GFP was diffuse throughout the cytoplasm and was not localized at the pole in *E. coli* Δ*flhDCBA* cells or RP437 cells. Therefore, the polar localization of FlhG seems to require specific factors present in *Vibrio* cells.

The FlhF–FlhG interaction

Based on the observation that the polar localization of FlhF was inhibited by FlhG, we speculated that there is an interaction between FlhF and FlhG. Therefore, we attempted to directly demonstrate an FlhF–FlhG interaction. The Δ*flhFG* cells expressing *flhG* or *flhFG* genes were fractionated into soluble (cytoplasmic) and insoluble (membrane) fractions. FlhF was detected equally in the cytoplasmic fraction (Fig. 6a, lane 5) and in the insoluble fraction (Fig. 6a, lane 6) from Δ*flhFG* cells expressing both FlhF and FlhG. FlhG was also detected both in the cytoplasmic fraction (Fig. 6a, lanes 2 and 5) and the insoluble fraction (Fig. 6a, lanes 3 and 6), with more in the cytoplasmic fraction. There was no apparent difference in the localization profiles of FlhG when either *flhG* or *flhFG* was co-expressed in Δ*flhFG* cells.

As the cytoplasmic fraction was confirmed to contain both FlhF and FlhG, immunoprecipitation assays using an anti-FlhF antibody were performed with the cytoplasmic fraction (Fig. 6b, lane 2). As a negative control, an immunoprecipitation assay was performed with the fraction containing only FlhG (Fig. 6b, lane 1). A significant amount of FlhG was immunoprecipitated with an anti-FlhF antibody from the cytoplasmic fraction containing FlhF and FlhG (Fig. 6b, lane 2), but not from that containing FlhG alone (Fig. 6b, lane 1), indicating an interaction between FlhF and FlhG.

DISCUSSION

From previous studies in *V. cholerae* and *Pseudomonas*, it has been suggested that FlhF and FlhG regulate the number of flagella by upregulating or downregulating the expression of

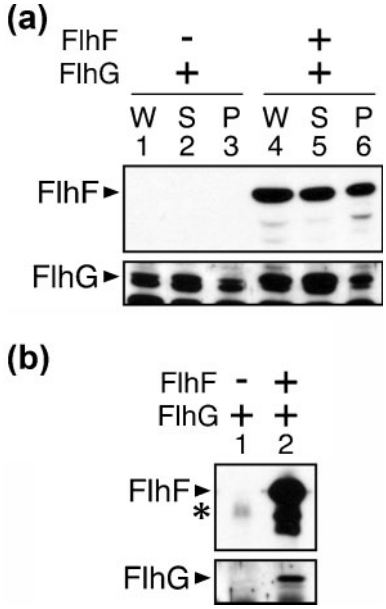

Fig. 6. Immunoprecipitation assay of the FlhF–FlhG complex using the anti-FlhF antibody. (a) Δ*flhFG* (LPN2) cells expressing FlhG alone or both FlhF and FlhG were sonicated and then ultracentrifuged. FlhF and FlhG in the whole-cell lysate (W), the cytoplasmic fraction (supernatant after ultracentrifugation; S) and the insoluble fraction (pellet after ultracentrifugation; P) were analysed by Western blotting using anti-FlhF and anti-FlhG antibodies. (b) FlhF was immunoprecipitated with anti-FlhF antibody from the cytoplasmic fraction of the Δ*flhFG* cells expressing both FlhF and FlhG (lane 2), followed by Western blotting. As a negative control, an immunoprecipitation using anti-FlhF antibody with the Δ*flhFG* cells expressing only FlhG was also performed (lane 1). The band from the heavy chain of the anti-FlhF antibody was detected in lane 1 (indicated by an asterisk).

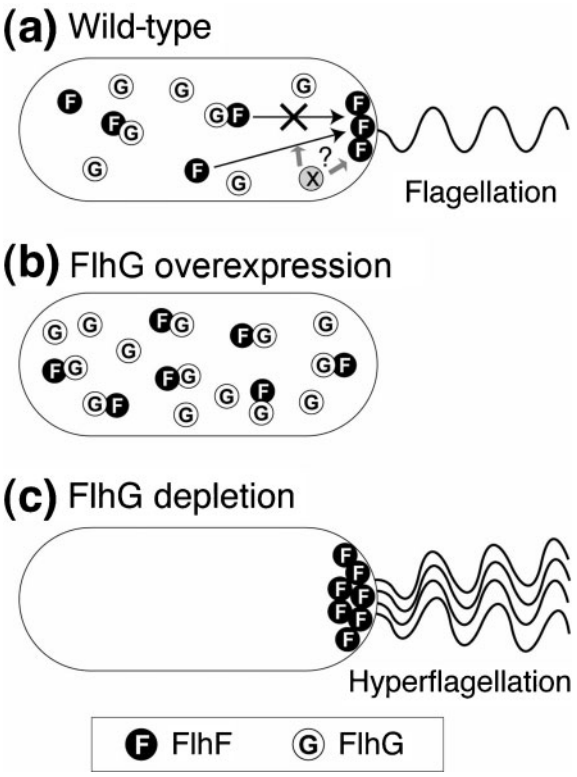

Fig. 7. Working hypothesis for the regulation of the number of polar flagella by FlhF and FlhG in *V. alginolyticus* cells. (a) In a wild-type cell, FlhF is localized at the pole and promotes the assembly of the MS ring of flagella. FlhG in the cytoplasm inhibits FlhF localization at the pole by interacting with FlhF, and thus represses excessive flagellation. Therefore, the number of polar flagella is regulated to be one by FlhF and FlhG. FlhF may need a factor 'X' to localize at the pole, as discussed in the text. The factor may be involved in the polar localization of FlhF or in the stability of FlhF at the pole. (b) When FlhG is overexpressed, almost all of the FlhF molecules are captured by FlhG. FlhG-captured FlhF does not localize at the pole, and thus FlhG-overproducing cells fail to produce a polar flagellum. (c) When FlhG is depleted, most of the FlhF is localized at the pole, and thus the cells produce multiple flagella at the pole.

flagellar genes. In this study, larger amounts of the intermediate class proteins (FlgI and MotY) and the late class proteins (PomA, PomB, MotX and flagellins) were detected in the *flhG* mutant cells (Fig. 3). The results are consistent with the earlier reports of negative feedback regulation of intermediate-class gene expression by FlhG in *V. cholerae* (Correa *et al.*, 2005) and by the FlhG homologue (FleN) in *Pseudomonas* (Dasgupta & Ramphal, 2001). The lack of FlhG causes overexpression of the intermediate-class genes, including that of σ^{28}, leading to overexpression of the late-class genes. We speculate that multiple flagellation of the *flhG* mutant cells requires the overexpression of flagellar genes.

FlhF, but not FlhG, seems to be involved in the polar placement of flagella. In electron microscopic observations, we were unable to find any flagellated Δ*flhF* cells, but did find some Δ*flhFG* cells with multiple peritrichous flagella (Fig. 1c, d). Considering that the *flhG*-defective strain has multiple flagella at the pole (Fig. 1b), FlhF appears to be one of the determinants of the polar placement of the flagellum, which is consistent with results for *P. aeruginosa* and *P. putida* (Murray & Kazmierczak, 2006; Pandza *et al.*, 2000). If FlhF collaborates with FlhG, they could be co-localized in the cell, or each protein might affect the subcellular localization of the other. To verify the collaboration between FlhF and FlhG, we constructed GFP-fusion variants of both FlhF and FlhG, and observed their localization in the cell. FlhF–GFP was localized at the flagellated pole, while most of the FlhG was diffusely expressed throughout the cytoplasm (Fig. 4). Strikingly, FlhF–GFP localization was more intense in the Δ*flhFG* cells, suggesting that FlhG may negatively affect the polar localization of FlhF (Fig. 5b). The effect of FlhG on the polar localization of FlhF was confirmed in a non-

flagellated *E. coli* strain (data not shown). This leads to the hypothesis that FlhF and FlhG interact with each other to regulate flagellar number and placement. We were able to demonstrate an interaction between FlhF and FlhG by co-immunoprecipitation (Fig. 6). From these results, we propose a working hypothesis for the role of FlhF and FlhG in controlling polar flagellar number and placement (Fig. 7). FlhF works at the pole to determine the placement of the flagellum and promotes the expression of flagellar genes (Fig. 7a). FlhG interacts with FlhF and inhibits FlhF from localizing at the pole, resulting in the suppression of flagellation (Fig. 7a). Overproduction of FlhG reduces the polar localization of FlhF, such that FlhF diffuses throughout the cytoplasm, and results in a non-flagellated phenotype (Fig. 7b). Depletion of FlhG causes strong polar localization of FlhF, leading to hyperflagellation at the pole (Fig. 7c). Consequently, the number of polar flagella is controlled to allow the production of a single flagellum by the collaboration of FlhF and FlhG. FlhG seems to act not only as an anti-coactivator of σ^{54}, as reported in *Pseudomonas*, but also an inhibitor of FlhF localization.

FlhF has a GTP-binding motif and shows similarity to *E. coli* SRP receptor FtsY, which is a membrane-associated receptor that targets the SRP/ribosome-nascent-chain complex to the translocon. The dissociation of FtsY from SRP is regulated by GTP hydrolysis of FtsY and SRP (Shan & Walter, 2005). We speculate that the FlhF–FlhG interaction is also regulated by GTP hydrolysis by analogy with FtsY. On the other hand, FlhG has an ATP-binding motif and shows similarity to *E. coli* MinD. The MinD dimer associates with the inner membrane and gathers MinC, which inhibits FtsZ polymerization, and thus inhibits the generation of the division plane (Rothfield *et al.*, 2005; Shapiro *et al.*, 2002). The MinD dimer dissociates into monomers by interacting with MinE, and monomeric MinD dissociates from the membrane, hydrolysing ATP. Therefore, it is possible that ATP hydrolysis mediates the interaction of FlhG with FlhF or with FlaK, which is the *Vibrio* homologue of *Pseudomonas* FleQ, and is similar to MinD.

Localization of FlhG–GFP was different in *Vibrio* and *E. coli* cells. FlhG–GFP diffused completely through the cytoplasm and was not localized at the pole in *E. coli* cells (data not shown), while FlhG–GFP was localized at the pole in some of the *Vibrio* cells transformed with the *flhG-gfp* plasmid (Fig. 5d, e). We also observed localization of FlhG–GFP in *Vibrio rpoN* (σ^{54}) mutant cells (Fig. 5f). These results may indicate that FlhG is localized at the pole by a certain factor which is unique to *Vibrio* and whose gene is transcribed independently of σ^{54}. Polar localization of FlhF–GFP was observed in *E. coli* cells (data not shown) and in the *rpoN* mutant cells (Fig. 5c). It seems that FlhF by itself could recognize the pole and determine where the polar flagellum is generated. FlhF–GFP was not strongly localized at the pole in *rpoN* mutant cells (Fig. 5c), although *rpoN* mutant cells did not express *flhG*, which is expressed under the control of σ^{54} (Fig. 3b). This implies that FlhF might require another factor to allow its localization at the cell pole. We know that FlhF is a key player in determining the localization of polar flagella; however, its mechanism is unclear. An investigation of the function of FlhF will lead to a better understanding of the mechanism of polar localization of flagella.

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