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Mutational analysis of the GTP-binding motif of FlhF which regulates the number and placement of the polar flagellum in *Vibrio alginolyticus*

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Summary

Precise regulation of the number and placement of flagella is critical for the mono-flagellated bacterium *Vibrio alginolyticus* to swim efficiently. We previously proposed a model in which the putative GTPase FlhF determines the polar location and generation of the flagellum, the putative ATPase FlhG interacts with FlhF to prevent FlhF from localizing to the pole, and thus FlhG negatively regulates the flagellar number in *V. alginolyticus* cells. To investigate the role of the GTP-binding motif of FlhF, we generated a series of alanine-replacement mutations at the positions that are highly conserved among homologous proteins. The results indicate that there is a correlation between the polar localization and the ability to produce flagella in the mutants. We investigated whether the mutations in the GTP-binding motif affected the ability to interact with FlhG. In contrast to our prediction, no significant difference was detected in the interaction with FlhG between the wild-type and mutant FlhFs. We showed that the GTP-binding motif of FlhF is important for polar localization of the flagellum but not for the interaction with FlhG.

Keyword: bacterial flagella / polar flagella / GTP binding motif

Introduction

Many motile bacteria have a locomotive organelle, called the flagellum which has a motor embedded in the cytoplasmic membrane to allow rotation. The helical filament of the flagellum as a screw, which is connected to the motor, rotates and makes it possible to swim. The number and localization of flagella differ among species; for example, various bacteria have peritrichous (or lateral) flagella, a single polar flagellum, or multiple flagella at the pole. *V. alginolyticus* and *V. parahaemolyticus* each have both a single polar flagellum and peritrichous flagella (1). The flagella are not essential, but cells must expend significant energy for their maintenance. Bacteria should minimize the cost of producing flagella, and one strategy appears to involve the precise regulation of flagellar number.

It has been reported that a pair of the *flhF* and the *flhG* genes are unique to polarly-flagellated bacteria and FlhF and FlhG regulate the number of flagella in *Vibrio* and *Pseudomonas*. In *V. cholerae*, *V. alginolyticus, P. aeruginosa*, and *P. putida*, overexpression of FlhF results in an increased number of polar flagella, and a *flhF* gene disruption gives reduced the number of flagella and aberrant placement of flagella in *V. cholerae*, *P. aeruginosa* and *P. putida* (2-6). Interestingly, disruption of the *flhF* gene led to reduction in the number of flagella and abnormal placement (peri-polar position) of flagella in the peritrichously-flagellated bacterium, *Bacillus cereus* (7). The regulation of the number and placement of flagella by FlhF may be conserved among polarly-flagellated bacteria and peritrichously-flagellated bacteria. Moreover, FlhF is reported to increase the expression of class III genes in *V. cholerae* (2). Therefore, it was speculated 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 a *flhG* gene disruption causes a hyperflagellated phenotype (2, 8). In *Pseudomonas*, it has been shown that the FleN protein (homologue of FlhG in *Pseudomonas* sp.) binds to the flagellar-gene specific transcriptional regulator, FleQ (9), which regulates the transcription of class II genes together with σ^{54} (10). The FleN protein represses transcription of class II genes and the *fleN* gene by itself via FleQ. The FleQ homologue of *V. cholerae*, FlrA also regulates transcription of class II genes (11).

We reported that the polar localization of FlhF is reduced by FlhG, which is independent of other flagellar proteins, and that FlhF and FlhG interact with each other in *V. alginolyticus* (4). We speculate that the FlhF-FlhG interaction inhibits FlhF from localizing at the pole and thus flagellation is suppressed. FlhF has a GTP-binding motif and shows similarity to *E. coli* signal recognition particle (SRP), Ffh, and its receptor, FtsY. FtsY is a membrane-associated receptor that targets the Ffh/ribosome-nascent-chain complex to the translocon. The both Ffh and FtsY are related at the sequence as well as the structural level, and they contain three domains, named the N (amino-terminal), G (ras-type GTPase), IBD (insertion box) domains. The G domain adopts a classical GTPase fold, in which four conserved sequence motifes (I-IV) are arranged around the nucleotide-binding site (12). It has been shown that the dissociation of FtsY from Ffh is regulated by GTP hydrolysis of FtsY and Ffh (13). In this study, we investigated the role of the GTP-binding motif in FlhF using a series of alanine-replaced FlhF mutants.

Materials and Methods

***Bacterial strains and growth conditions*-**The strains of *V. alginolyticus* and *E. coli* 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*).

***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 (Japan), TOYOBO (Japan), and New England Biolabs (USA). 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).

Transformation of Vibrio cells*-V. alginolyticus* cells were transformed by electroporation as described previously (14). 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, Tokyo) at an electric field strength of 5.0 to 7.5 kV/cm.

***High-intensity dark-field microscopy*-**Flagella were observed using a dark-field microscope (Olympus model BHT) equipped with a 100W mercury

lamp (Ushio USH-102). An image was recorded using a CCD camera (Sony model SSC-M370) and a DVD video recorder (Panasonic model DMR-E100H).

Fluorescence microscopy-*Vibrio* cells bearing plasmids were cultured in VC medium overnight. The overnight culture was diluted 1:100 in VPG medium containing 0.02% (w/v) arabinose and 2.5 μg ml^{-1} chloramphenicol, and then incubated at 30°C for 4 h. Fluorescence microscopy to observe GFP was performed as described previously (4).

Immunoprecipitation-An overnight culture of *ΔflhFG* cells harboring plasmids, was diluted 1:100 into VPG medium containing 0.2% (w/v) arabinose and 2.5 μg ml^{-1} chloramphenicol. After 4 h incubation at 30°C, the cells were harvested by centrifugation (10,000 x g for 5 min). Immunoprecipitation assays were performed as described previously (4).

Results

Constructs of alanine-replaced FlhFs-GTP binding proteins have wide-ranging roles in cell proliferation, signal transduction, protein synthesis, protein targeting, cell differentiation, cytoskeleton formation and membrane trafficking. GTP binding proteins contain consensus motifs, termed motif I, II, III and IV, which are directly related to GTP hydrolysis (15). FlhF and its homologous proteins, SRP protein, Ffh, and its receptor protein, FtsY, each has a GTP-binding motif at the C-terminal region (3). Complex formation between FtsY and Ffh regulates protein targeting to the Sec export apparatus and is regulated by GTP hydrolysis (13). To investigate the role of the GTP-binding motif of FlhF in flagellation and flagellar placement, we generated a series of eight mutant FlhFs, in which alanine-replacements were introduced at highly

conserved amino acid residues among FlhFs and their homologous proteins, FtsY, Ffh, $P21^{Ras}$ and EF-Tu, from various species (Fig. 1). $P21^{Ras}$ has an important role in the signal transduction pathway of cell proliferation and differentiation (16). EF-Tu is a prokaryotic elongation factor which mediates the entry of the aminoacyl tRNA into a free site of the ribosome (17). GTP hydrolysis by these proteins is important for their function as well as FtsY and Ffh.

Swarming ability of mutant FlhFs-We investigated whether the mutant FlhFs restored the swarming ability of the *ΔflhF* cells, which were almost non-motile due to the lack of a flagellum (Fig. 2). Wild-type FlhF from the plasmid with 0.02% arabinose induction restored the swarming ability of *ΔflhF* cells slightly less than that of the wild-type cells; stronger induction seemed to reduce the swarming ability. FlhF-G299A, FlhF-K305A, FlhF-G380A and FlhF-K437A restored the swarming ability of *ΔflhF* cells less than the wild-type FlhF. FlhF-G304A and FlhF-D377A slightly restored the swarming ability of *ΔflhF* cells with 0.02% arabinose induction and significantly with 0.2% arabinose induction. FlhF-T306A and FlhF-D439A did not confer swarming ability to the *ΔflhF* cells even with 0.2% arabinose induction. We have reported that the overproduction of the wild-type FlhF causes excess of flagellation and, as a result, reduces swarming ability in the wild-type cells (3). The overproduction of FlhF-G380A notably reduced the swarming ability in the wild-type cells more than overproduction of the wild-type FlhF. The other mutant proteins conferred a phenotype similar to the wild-type FlhF or seemed not to reduce swarming at all.

Most of the *ΔflhFG* cells are not flagellated, but some possess multiple flagella around the cell, and they are not generated at the pole as shown

previously (3). Therefore, the *ΔflhFG* cells show a slight swarming ability. The swarming ability of the *ΔflhFG* cells expressing the mutant FlhFs was examined. FlhF-G299A, FlhF-G304A, FlhF-K305A, FlhF-D377A and FlhF-G380A restored the swarming ability of the *ΔflhFG* cells to the same level as the wild-type FlhF even without induction. It is notable that FlhF-T306A and FlhF-D439A, which failed to restore the swarming ability to the *ΔflhF* cells, slightly restored the swarming ability of the *ΔflhFG* cells. This may suggest that FlhG inhibits the function of the mutant FlhFs.

Flagellation by mutant FlhFs-To investigate the flagellation in the presence of the mutant FlhFs, we determined the number of flagella per cell by high intensity dark-field microscopy (Fig. 3). With 0.02% arabinose induction, 17% of the *ΔflhF* cells expressing the wild-type FlhF possessed a single flagellum at the pole, and 35% of them possessed two or more flagella at the pole. FlhF-G299A, FlhF-K305A, FlhF-G380A and FlhF-K437A conferred flagella at the pole in the *ΔflhF* cells with 0.02% arabinose induction (Fig. 3C, 3E, 3G, 3H and 3I); however, the flagellar number was much less than that in cells containing the wild-type FlhF. FlhF-G304A did not confer the ability to produce a flagellum with 0.02% arabinose induction but 3% of the cells did produce a flagellum at the pole in the presence of 0.2% arabinose (Fig. 3D). FlhF-T306A and FlhF-D439A did not allow any flagellar production even with 0.2% arabinose induction (Fig. 3F and 3J). These results are consistent with the swarming ability of the *ΔflhF* cells expressing the mutant FlhFs (Fig. 2). It seems that the function of FlhF is completely eliminated by the T306A and D439A mutations.

We have shown that overproduction of wild-type FlhF causes excess flagellation in the wild-type cells (3). About 80% of wild-type cells with the

vector control possessed a single flagellum at the pole, and a few cells possessed two or more flagella (Fig. 3K). In the wild-type cells expressing the wild-type FlhF from a plasmid with 0.2% arabinose induction, ca. 30% and ca. 40% of the cells had a single flagellum and two or more flagella, respectively (Fig. 3L). With 0.02% arabinose induction, about 55% of the wild-type cells expressing FlhF-G380A possessed a single polar flagellum, and about 15 % possessed 2-4 flagella. Approximately 10% of the cells had five flagella or more, whereas none of the wild-type cells expressing the wild-type FlhF possessed more than five flagella (Fig. 3L and M). Therefore, excess flagellation was somewhat enhanced by heterologous expression of mutant (G380A) and wild-type FlhF.

Effect of alanine replacement of GTP binding motif on the polar localization of FlhF-To investigate the localization of mutant FlhFs , each was fused to EGFP and produced in the *ΔflhF* and *ΔflhFG* cells (Fig. 4). The fraction of mutant FlhFs localized at the pole was determined by enumerating fluorescent dots (summarized in Fig. 4S). In the *ΔflhF* cells, the wild-type FlhF diffused throughout the cytoplasm and some localized at the pole (Fig. 4A). Most of the FlhF-G299A diffused throughout the cytoplasm; some localized at the pole in the *ΔflhF* cells (Fig. 4C). FlhF-G304A, FlhF-K305A, FlhF-T306A, FlhF-D377A, FlhF-K437A and FlhF-D439A were diffuse throughout the cytoplasm, and the fraction localized at the pole was less than the wild-type FlhF (Fig. 4E, 4G, 4I, 4K, 4O and 4Q). FlhF-G380A was intensely localized at the pole and the diffuse fluorescent signal in a cell was much lower than the others (Fig. 4M). This intense localization was more similar to that of the wild-type FlhF in the *ΔflhFG* cells (Fig. 4B). The localization of FlhF-G299A, FlhF-G380A and FlhF-K437A was not obviously different between the *ΔflhF* and the *ΔflhFG* cells (Fig. 4C, 4D, 4M, 4N, 4O and 4P). The polar localization ratio of the fluorescent dots by

FlhF-G304A, FlhF-T306A, FlhF-D377A and FlhF-D439A in the *ΔflhFG* cells was less than that in the *ΔflhF* cells (Fig. 4E, 4F, 4I, 4J, 4K, 4L, 4Q and 4R). On the other hand, the fraction of FlhF-K305A that localized to the pole in *ΔflhFG* cells was more than that in the *ΔflhF* cells (Fig. 4G and 4H).

The interaction with FlhG-FlhF-G299A and FlhF-G380A, which allowed formation of the flagella, were localized at the pole in most of the *ΔflhF* cells. On the other hand, FlhF-T306A and FlhF-D439A, which did not allow flagella production, failed to localize at the pole in most of the cells. These results may indicate that there is a correlation between the ability to produce flagella and to localize at the pole. Thus we predicted that mutations in FlhF affect the interaction with FlhG because we have shown that FlhG directly interacts with FlhF to prevent FlhF localization at the pole (4). To test this possibility, immunoprecipitation assays were performed using an anti-FlhF antibody with the cytoplasm fraction of the *ΔflhFG* cells expressing the wild-type or the mutant FlhF and FlhG (Fig. 5). Almost the same amounts of FlhG were immunoprecipitated using anti-FlhF antibody with the mutant FlhFs and the wild-type FlhF. This suggests that the mutations do not affect the interaction with FlhG.

Discussion

We have presented a model in which the FlhF localization at the pole determines polar location and production of the flagellum, FlhG interacts with FlhF to prevent FlhF from localizing at the pole, and thus FlhG negatively regulates flagellar number in *V. alginolyticus* cells (4). However, how FlhF recognizes the

pole and how FlhF promotes flagellation are still unclear. To address this question, we focused on the GTP binding motif of FlhF because this motif is often critical in regulating the function of the proteins (15).

We speculated that the GTP-binding motif of FlhF is involved in the polar localization of flagella. However, none of the mutations seemed to affect the localization of flagella. It has been reported that the FtsY/Ffh heterodimer contains GTP in the GTP-binding motifs, motif I, II, III and IV (13). Recently, the crystal structure of the *Bacillus subtilis* FlhF with bound GTP was reported (18). The structure of the FlhF homodimer with bound GTP is similar to that of the FtsY/Ffh heterodimer with GTP bound. *V. alginolyticus* FlhF may also form a homodimer complex with bound GTP.

Motif I is also called the P loop and binds to β, γ-phosphate of GTP or GDP. The residue in *B. subtilis* FlhF and FtsY/Ffh corresponding to T306 of the *V. alginolyticus* FlhF is oriented to Mg^{2+}. FtsY with a mutation at this position fails to hydrolyze GTP and to form a complex with Ffh. FlhF-T306A might lose the GTPase activity and the ability to produce flagella. Some of the FlhF mutants with substitutions in motif I (G299A, G304A, and K305A, but not T306A) still had the ability to produce flagella. The residues of *B. subtilis* FlhF equivalent to G304 and K305 seems to interact directly with the phosphate group of GTP from the structural data (18). The substitutions in Ffh at the residues equivalent to G299 led to loss of complex formation with Ffh as well as the ability to hydrolyze GTP.

Motif III plays an important role in the conformational change from the GTP-bound form to the GDP-bound form (15). The orientation of the C-terminal α2 helix, which lies adjacent to motif III, significantly differs between the GTP-bound form and the GDP-bound form. A mutation at residue D377 in motif

III of FlhF impaired the ability of cells to produce flagella. The corresponding residues in *B. subtilis* FlhF and FtsY/Ffh have been shown to bind to Mg^{2+} via a H_2O molecule. The mutation of this residue in FtsY led to the failure to make a complex with Ffh and hydrolyze GTP. On the other hand, a mutation at G380 seemed not to impair the ability to produce flagella. The corresponding residue of $p21^{ras}$ has been shown to form a hydrogen bond with the γ-phosphate of GTP (15). The G380 residue in Ffh binds to a glutamate residue in the closing loop (GVGE) of FtsY (19). When expressed in the *ΔflhF* cells, FlhF-G380A produced less flagella than the wild-type FlhF. When expressed in the wild-type cells, however, FlhF-G380A produced more flagella than the wild-type FlhF. FlhF carrying the G380 mutation might enhance the function of wild-type FlhF.

Motif IV is believed to determine nucleotide specificity (15). Generally, the nucleotide specificity of GTPase is achieved by two hydrogen bonds between the aspartate residue in motif IV (D439 in *V. alginolyticus* FlhF) and the guanine base of the nucleotide. The ability to produce flagella was severely defective in FlhF and was completely lost in FlhF-D439A. Mutations corresponding to these residues in FtsY led to a decreased binding to GTP, impaired ability to hydrolyze GTP, and thus FtsY fails to make a complex with Ffh. If FlhF is functionally similar to FtsY, FlhF-K437A and FlhF-D439A may fail to bind to GTP and thus may lead to a decrease in its function.

The localization profiles of FlhF-G304A, FlhF-K305A, FlhF-T306A, FlhF-D377A and FlhF-D439A are different in the *ΔflhF* cells and the *ΔflhFG* cells. FlhF-T306A and FlhF-D439A slightly restored the swarming ability of the *ΔflhFG* cells, while they failed to restore the swarming ability of the *ΔflhF* cells. In a previous study we reported that FlhF requires an additional factor to strongly localize at the pole and this factor may play a role in the interaction and

explain these results. In this study we found that there is a correlation between the ability to produce flagella and to localize at the pole by the *flhF* mutations used in this study. Table 2 summarizes the restoration of the swarming ability of the *ΔflhF* cells by the mutant FlhFs, the ability to produce flagella, and the fraction of their polar localization. FlhF-G299A and FlhF-G380A confer the ability to produce flagella in the *ΔflhF* cells, and they are localized at the pole in most or all of the cells, while FlhF-T306A and FlhF-D439A failed to produce flagella in the *ΔflhF* cells and failed to localize at the pole in most of the cells. This may indicate that there is a correlation between the ability to produce flagella and to localize at the pole. We predicted that the mutations in the GTP-binding motif may affect the interaction with FlhG, and thus, performed immunoprecipitation assays. However, we found that there was no significant difference in the interaction between FlhG and either the the wild-type FlhF or the mutants. It is inferred that the putative interaction between FlhF and FlhG that regulates the polar localization of FlhF is independent of the function of the GTP-binding motif. Our results showed the mutations in GTP-binding motif of FlhF affected the number and polar localization of flagellum, however, it is not ruled out the possibility that these mutations just cause the structural distortion of protein, and lead to the deactivation. To clarify the effects of mutations, we have to directly measure the GTP-binding or the hydrolysis activity of FlhF and to solve the structure of *Vibrio* FlhF.

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CONFLICT OF INTEREST

None declared.

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Table 1. **Bacterial strains and plasmids used in this study.**

Strain or plasmid	Genotype or description	Reference or source
V. alginolyticus		
VIO5	VIK4 (Rif^r Pof^+ Laf^-)	(20)
LPN1	VIO5 *ΔflhF* (Rifr Pof^+ Laf^-)	(4)
LPN2	VIO5 *ΔflhFG* (Rifr Pof^+ Laf^-)	(4)
E. coli		
JM109		
Plasmids		
pBAD33	Cm^r, P_{BAD}	(21)
pAK323	*flhF (wt)* in pBAD33	(3)
pAK331	*flhF (G299A)* in pBAD33	This study
pAK332	*flhF (G304A)* in pBAD33	This study
pAK333	*flhF (K305A)* in pBAD33	This study
pAK334	*flhF (T306A)* in pBAD33	This study
pAK335	*flhF (D377A)* in pBAD33	This study
pAK336	*flhF (G380A)* in pBAD33	This study
pAK337	*flhF (K437A)* in pBAD33	This study
pAK338	*flhF (D439A)* in pBAD33	This study
pAK325	*flhF-egfp (wt)* in pBAD33	(4)
pAK341	*flhF-egfp (G299A)* in pBAD33	This study
pAK342	*flhF-egfp (G304A)* in pBAD33	This study

pAK343	*flhF-egfp (K305A)* in pBAD33	This study
pAK344	*flhF-egfp (T306A)* in pBAD33	This study
pAK345	*flhF-egfp (D377A)* in pBAD33	This study
pAK346	*flhF-egfp (G380A)* in pBAD33	This study
pAK347	*flhF-egfp (K437A)* in pBAD33	This study
pAK348	*flhF-egfp (D439A)* in pBAD33	This study
pAK721	*flhF (wt), flhG* in pBAD33	(3)
pAK731	*flhF (G299A), flhG* in pBAD33	This study
pAK732	*flhF (G304A), flhG* in pBAD33	This study
pAK733	*flhF (K305A), flhG* in pBAD33	This study
pAK734	*flhF (T306A), flhG* in pBAD33	This study
pAK735	*flhF (D377A) , flhG* in pBAD33	This study
pAK736	*flhF (G380A), flhG* in pBAD33	This study
pAK737	*flhF (K437A), flhG* in pBAD33	This study
pAK738	*flhF (D439A), flhG* in pBAD33	This study
Cm^r, chloramphenicol-resistant; Rif^r, Rifampicin-resistant; Pof^+, possessing polar flagellum; Laf^-, lack of lateral flagella		

Table 2. **Summary of the mutant FlhF properties.**

Mutation		Swarming[1)]	Flagellation[2)]	Polar localization[3)]
wt		+++	+++	100 %
Motif I	G299A	++	++	93 %
	G304A	+	+	23 %
	K305A	+	++	15 %
	T306A	-	-	17 %
Motif III	D377A	+	++	45 %
	G380A	++	+++	100 %
Motif IV	K437A	++	+	54 %
	D439A	-	-	16 %

[1)]From the data of Fig. 2 in which the swarm ring size of *ΔflhF* cells producing the mutant FlhF was measured at 0.02% arabinose induction. +++; the swarming ability of the *ΔflhF* cells by the wt FlhF, ++; the swarming ability less than by the wt FlhF, +; the slight swarming ability, -; no swarming ability.

[2)]From the data of Fig. 3. +++; more than 30% of cells were flagellated at 0.02% arabinose induction, ++; less than 30% of cells were flagellated at 0.02% arabinose induction, +; no cell was flagellated at 0.02% arabinose induction but flagellated cells were observed at 0.2% arabinose induction, -; no cell was flagellated at any condition.

[3)]From the data of Fig. 4S.

Figure legends

Fig. 1. **GTP-binding motifs of FlhF.** (A) Alignment of the amino acid sequences of the GTP-binding motifs, motif I, III and IV, conserved among various GTPase, FlhF, FtsY, $p21^{ras}$, and EF-Tu proteins. Abbreviations: Va, *Vibrio alginolyticus*; Vc, *V. cholerae*; Pa, *Pseudomonas aeruginosa*; Bs, *Bacillus subtilis*; Bc, *B. cereus*; Ec, *Escherichia coli*; Ta, *Thermus aquaticus*; Hs, *Homo sapiens*. White letters in black boxes are amino acids that are identical among FlhFs and their homologues. Black letters in gray boxes are amino acids that are identical among FlhFs. The amino acid residues that were replaced by alanine are indicated above their sequences. (B) Structure of *B. subtilis* FlhF with GTP (2PX3) are shown by ribbon representation (green) using PyMOL, and the motif I, III, IV and GTP are shown by purple, red, orange and cyan, respectively. The amino acid residues replaced by alanine are indicated by dots.

Fig. 2. **The swarming ability of the *flhF* mutants.** (A) The wild-type (VIO5), (B) *ΔflhF* (LPN1) and (C) *ΔflhFG* (LPN2) cells bearing a plasmid, pAK322 (*flhF wt*), pAK331 (*flhF G299A*), pAK332 (*flhF G304A*), pAK333 (*flhF K305A*), pAK334 (*flhF T306A*), pAK335 (*flhF D377A*), pAK336 (*flhF G380A*), pAK337 (*flhF K437A*), pAK338 (*flhF D439A*), or pBAD33 (empty vector control) were grown in VC medium containing chloramphenicol. 0.5μl aliquots of the overnight cultures were spotted onto 0.25% agar VPG plates with chloramphenicol, and 0, 0.02 or 0.2% arabinose, followed by incubation at 30°C for 5h.

Fig. 3. **Number of flagella per plasmid-bearing cell.** The wild-type (VIO5) and *ΔflhF* (LPN1) cells bearing a plasmid, pAK322 (*flhF wt*), pAK331 (*flhF G299A*), pAK332 (*flhF G304A*), pAK333 (*flhF K305A*), pAK334 (*flhF T306A*), pAK335 (*flhF D377A*), pAK336 (*flhF G380A*), pAK337 (*flhF K437A*), pAK338 (*flhF D439A*), or pBAD33 (empty vector control), from overnight cultures were inoculated in VPG medium with 0 % (open bars), 0.02% (gray bars) or 0.2% (filled bars) arabinose. After 4h incubation at 30°C, the cells were observed under a high intensity dark-field microscope. The flagellar number per cell was determined and classified into one of four categories, no flagellum, one flagellum, two-four flagella, and more than five flagella per cell. The experiments were independently performed twice and the graphs were made with the average data.

Fig. 4. **Localization of the GFP-fused FlhF (*flhF-egfp*).** The *ΔflhF* (LPN1) (A, C, E, G, I, K, M, O and Q) and *ΔflhFG* (LPN2) (B, D, F, H, J, L, N, P and R) cells containing a plasmid, pAK325 (*flhF-egfp wt*) (A and B), pAK341 (*flhF-egfp G299A*) (C and D), pAK342 (*flhF-egfp G304A*) (E and F), pAK343 (*flhF-egfp K305A*) (G and H), pAK344 (*flhF-egfp T306A*) (I and J), pAK345 (*flhF-egfp D377A*) (K and L), pAK346 (*flhF-egfp G380A*) (M and N), pAK347 (*flhF-egfp K437A*) (O and P), pAK348 (*flhF-egfp D439A*) (Q and R), were observed by means of fluorescence microscopy. Cells cultured overnight were diluted 1:100 with VPG medium containing 0.02% (w/v) arabinose. After 4 h incubation at 30ºC, the cells were attached to a poly-L-lysine-coated cover glass, and then observed under a fluorescence microscope. The populations of the *ΔflhF* (open bars) and *ΔflhFG* (filled bars) cells with polar-localized FlhF were counted (S).

Fig. 5. **Immunoprecipitation assay of the FlhF-FlhG complex using the anti-FlhF antibody.** The *ΔflhFG* (LPN2) cells expressing the wild-type or the mutant FlhF with FlhG were sonicated and then ultracentrifuged. FlhF was immunoprecipitated using anti-FlhF antibody from the cytoplasmic fraction (supernatant after ultracentrifugation) and Western blot analysis was carried out using anti-FlhF antibody and anti-FlhG antibody. As a negative control, an immunoprecipitaion without anti-FlhF antibody was also performed.

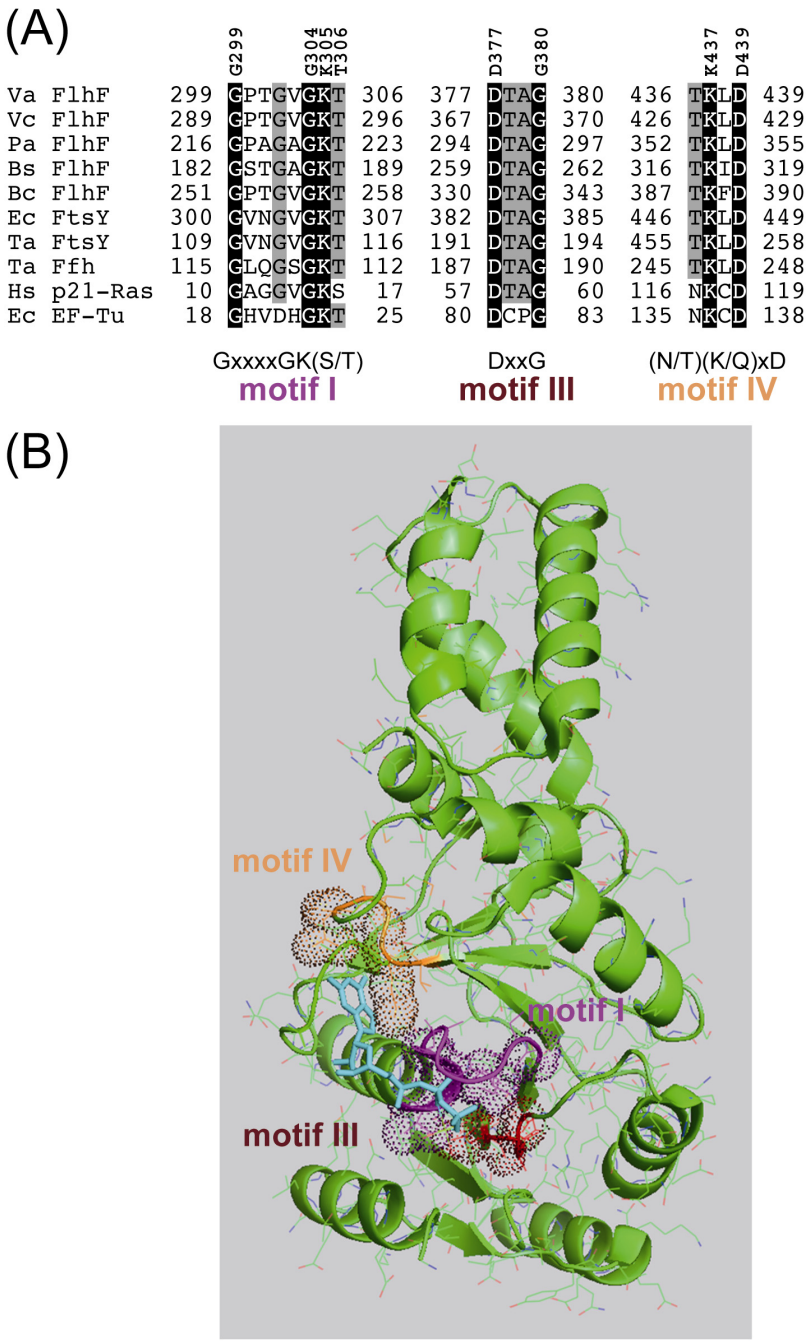

Figure 1. Kusumoto et al.

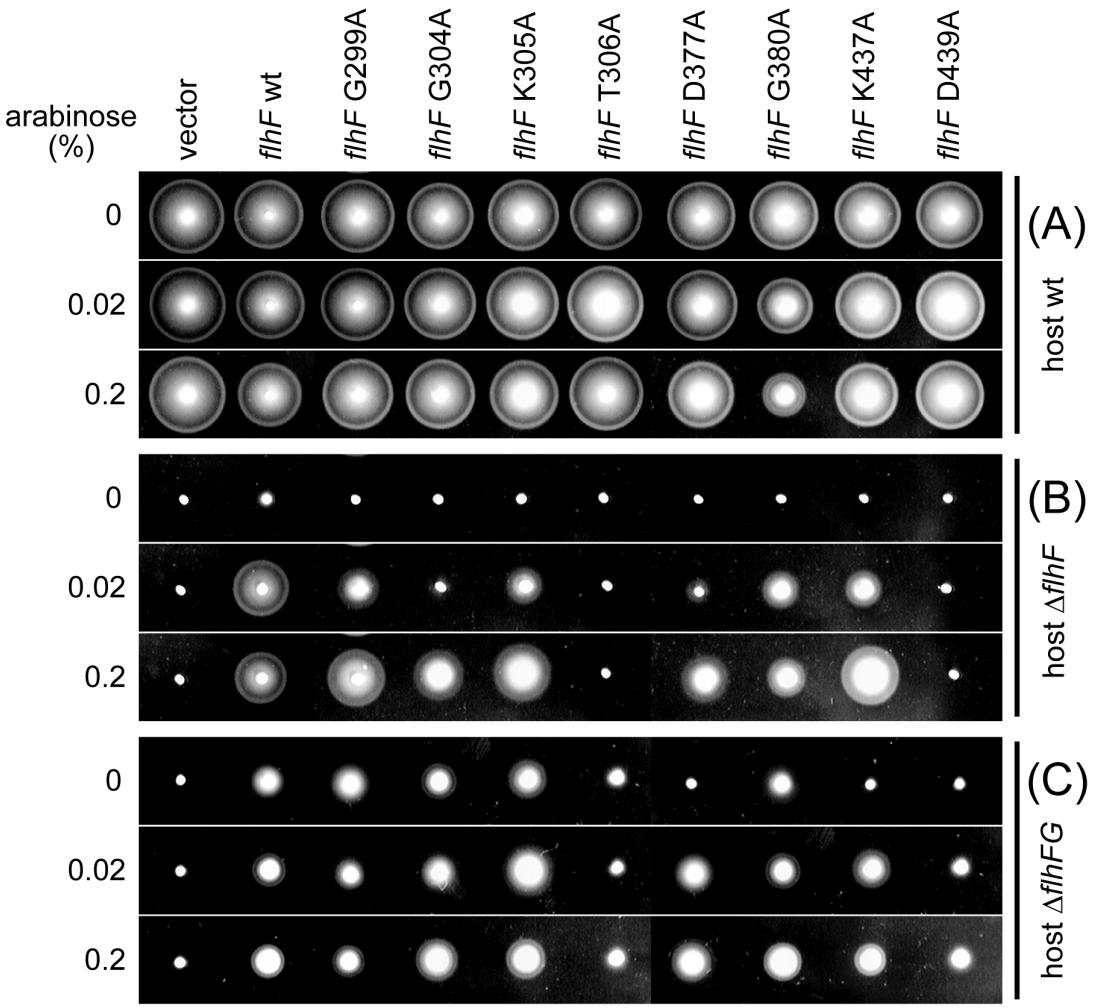

Figure 2. Kusumoto et al.

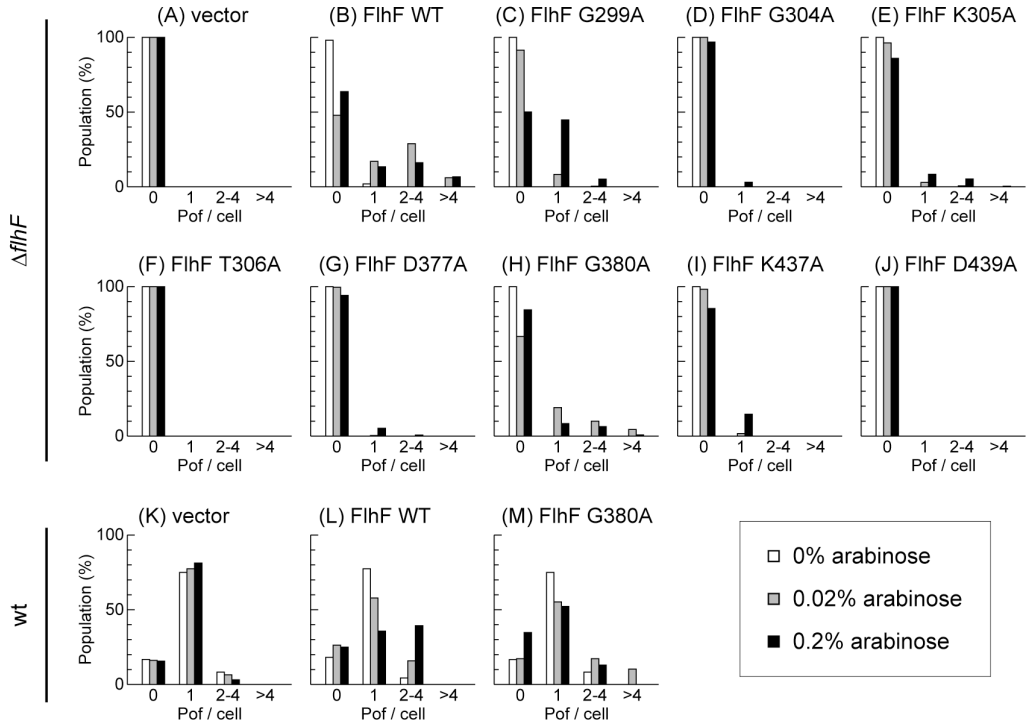

Figure 3. Kusumoto et al.

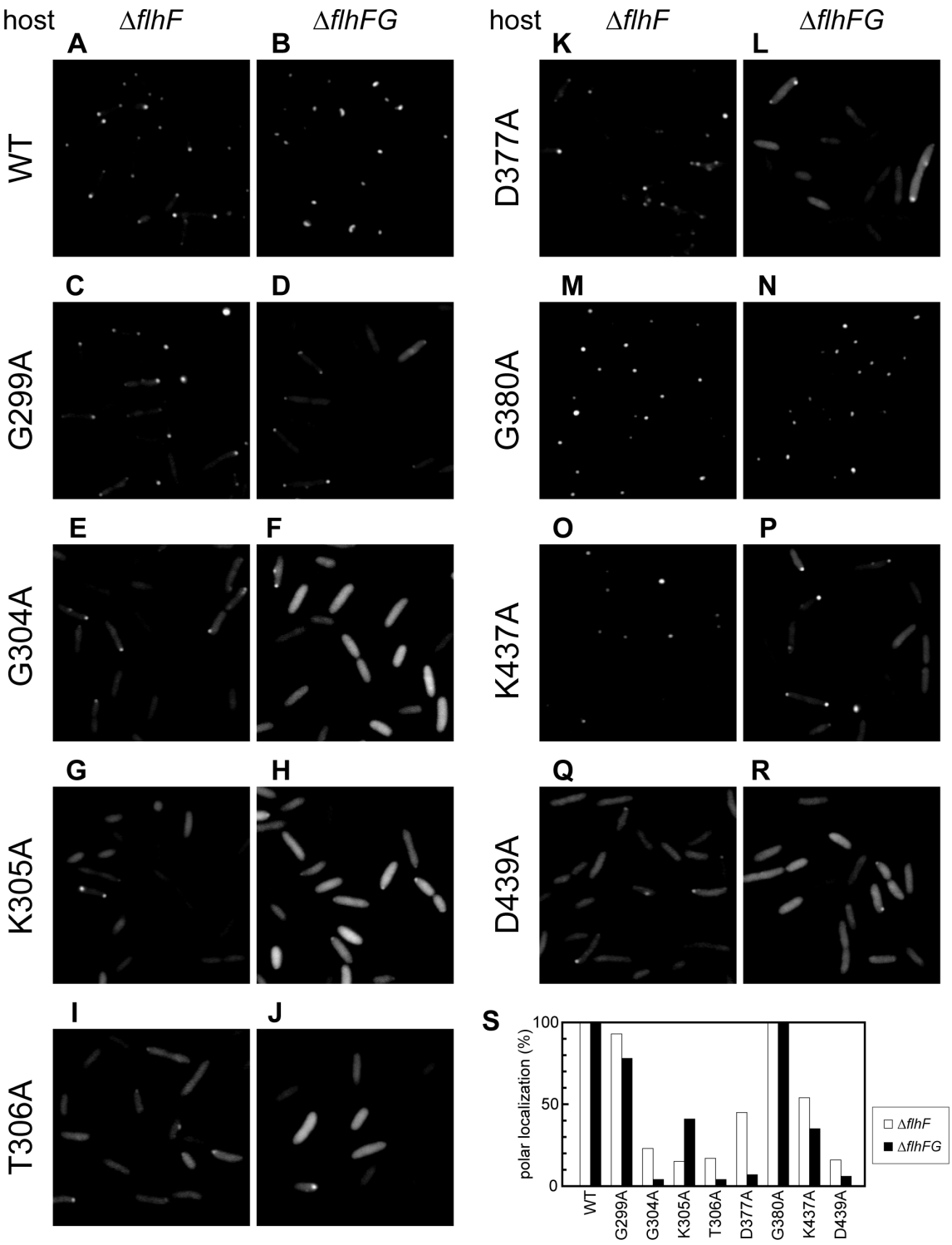

Figure 4. Kusumoto et al.

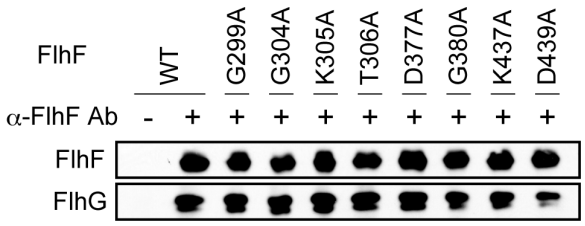

Figure 5. Kusumoto et al.