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Regulation of Polar Flagellar Number by the *flhF* and *flhG* Genes in *Vibrio alginolyticus*

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The number and location of bacterial flagella vary with the species. The *Vibrio alginolyticus* cell has a single polar flagellum, which is driven by sodium ions. We selected mutants on the basis of reduced swarming ability on soft agar plates. Among them, we found two mutants with multiple polar flagella, and named them KK148 and NMB155. In *Pseudomonas* species, it is known that FlhF and FleN, which are FtsY and MinD homologs, respectively, are involved in regulation of flagellar placement and number, respectively. We cloned homologous genes of *V. alginolyticus*, *flhF* and *flhG*. KK148 cells had a nonsense mutation in *flhG*; cells expressing transgenic *flhG* recovered the swarming ability and had a reduced number of polar flagella. NMB155 cells did not have a mutation in either *flhF* or *flhG*. In wild-type cells, expression of *flhF* increased the number of polar flagella; in contrast, expression of *flhG* reduced both the number of polar flagella and the swarming ability. These results suggest that FlhG negatively regulates the number of polar flagella in *V. alginolyticus*. KK148 cells expressing both *flhF* and *flhG* exhibited fewer polar flagella and better swarming ability than KK148 cells expressing *flhG* alone, suggesting that FlhG acts with FlhF.

Key words: bacterial flagella, FtsY, MinD, multiflagellate mutants.

Most motile bacteria have flagella that rotate by means of a motor embedded in the cytoplasmic membrane and generate a driving force by rotating a helical filament, like a screw, connected to the motor. There are two types of flagella: polar (mono and multi) and peritrichous. *Escherichia coli, Salmonella typhimurium* (*1*), and *Bacillus subtilis* (*2*) have peritrichous flagella. *Vibrio cholerae* (*3*), *Caulobacter crescentus* (*4*), and *Pseudomonas aeruginosa* (*5*) have a single polar flagellum. *V. fischeri* (*6*), *Helicobacter pylori* (*7*), and *P. putida* (*8*) have multiple polar flagella. *V. alginolyticus* and *V. parahaemolyticus* have both a single polar flagellum and peritrichous (or lateral) flagella (*9*). The number and localization of flagella are regulated differently in different species. Specific polar localization has been reported for several proteins and multicomponent complexes (*10*, *11*): *Shigella flexneri* IcsA and *Listeria monocytogenesis* ActA, which form actin-organizing centers to mediate bacterial movement in host cells (*12*, *13*); *E. coli* chemoreceptor complexes, which are two-component signal transduction proteins that respond to extracellular stimuli (*14*); and *E. coli* MinC and MinD, which are required for proper placement of the division septum (*15–17*). The precise mechanism of polar localization has not been clarified yet.

The flagellar genes are well conserved among peritrichously flagellated and polarly flagellated bacteria, and gene regulation and morphogenesis are also similar. Detailed studies of the regulation of flagellar assembly have been carried out for *E. coli*, *S. typhimurium* (*1*), *C. crecsentus* (*18*), *V. cholerae* (*3*) and *V. parahaemolyticus* (*9*, *19*). Flagella, which are composed of a filament, a hook and a basal body, are formed from the proximal end towards the distal tip. First, the MS ring of the basal body, which is composed of FliF, is embedded in the cytoplasmic membrane. Next, the switch complex or the C ring, which is composed of three types of protein: FliG, FliM, and FliN, is constructed on the cytoplasmic side of the MS ring. A specific apparatus for flagellar protein transport is thought to be assembled inside the C ring, which is the entrance of the channel for flagellar proteins (*20*). Rod proteins are exported by way of this apparatus and then the rod structure is constructed. Then, other proteins, such as the hook protein and flagellin, are exported similarly and polymerized into tubular structures that extend from the rods. Formation of the MS ring is thought to initiate flagellar assembly, so the number and location of the MS ring seem to determine the number and location of flagella.

In a previous study on *P. putida*, which possesses polar flagella, a *flhF* mutant showed a peritrichously flagellated phenotype, and *flhF* overexpression resulted in a multiflagellated phenotype (*21*). Thus, FlhF seems to determine flagellar placement and number. It has been shown that FleN (or MotR), the FlhG homologue in *P. aeruginosa*, negatively regulates the flagellar number (*22*, *23*). Thus, *fleN* mutants show a multiflagellated phenotype. FleN binds to a flagellar-gene-specific transcriptional regulator, FleQ (*24*), which regulates the transcription of Class II genes in a four-tiered hierarchy with σ^{54} (*25*). The Class II genes encode flagellar structural components

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and regulatory proteins, including FlhF and FleN. The FleN protein suppresses transcription of the Class II genes and the *fleN* gene by itself via FleQ. FlrA, the FleQ homologue in *V. cholerae*, also regulates transcription of the Class II genes (*3*). Both FleQ and FlrA are classified as Class II in the four-tiered hierarchy. These facts may show that the regulatory networks for flagellar biogenesis in the *Vibrio* and *Pseudomonas* species are very similar.

In *V. alginolyticus* and *V. parahaemolyticus*, two types of flagellar systems, polar flagella (Pof) and lateral or peritrichous flagella (Laf), are mainly used for movement in liquid media and on solid surfaces, respectively (*9*). Polar flagella are generated from the cell pole and the filament is sheathed with a membrane structure that is contiguous with the outer membrane (*26*). The lateral flagella are not sheathed. It has been shown that the energy sources for the polar and lateral flagellar motors are Na^+ and H^+ motive force, respectively (*27–29*). Polar flagella are produced constitutively to facilitate swimming in liquid media. When cells are transferred onto the surface of a solid medium, they produce lateral flagella, and elongate their bodies to move and spread on the surface. It has been proposed that polar flagella act as a dynamometer to sense the outer environment and thereby regulate lateral flagellar production (*30*).

In this study, we isolated swarm-deficient mutants from lateral flagella-defective (Pof^+ Laf^-) mutants that are the wild type as to polar flagellation. Among the mutants, we found multiflagellate mutants that had increased numbers of polar flagella. We attempted to determine why the number of flagella increases only at the polar region and how the number is regulated in the 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% Tryptone, 0.5% yeast extract, 0.4% K_2HPO_4, 3% NaCl, 0.2% glucose) or VPG medium (1% Tryptone, 0.4% K_2HPO_4, 3% NaCl, 0.5% glycerol). The *E. coli* strain used for DNA manipulations was cultured at 37°C in LB medium (1% Tryptone, 0.5% yeast extract, 0.5% NaCl). When necessary, the following antibiotics were added: chloramphenicol (2.5 μg/ml for *V. alginolyticus* or 25 μg/ml for *E. coli*) and ampicillin (50 μg/ml).

Isolation of Swarm-Deficient Mutants—Mutagenesis was carried out using ethyl methane sulfonate (EMS) and swarm-deficient mutants were isolated from the parental strains (Pof^+ Laf^-) on 0.25% agar VC plates as described previously (*34*). Swarm-deficient colonies were picked and streaked onto 1.25% agar VC plates for single colony isolation. Several colonies derived from each mutant candidate were streaked onto 0.25% agar VC plates and then their swarming abilities were confirmed. Flagella were observed by high intensity dark-field microscopy.

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 with a BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and an ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems).

Plasmid Construction—The plasmids used in these experiments are listed in Table 1. To express *flhF* and *flhG* in *V. alginolyticus*, the genes were inserted into arabinose-inducible vector pBAD33. Plasmids pAK322 and pAK520 carry a single copy of *flhF* and *flhG*, respectively. For coexpression, plasmid pAK721 carries a single copy of each *flhF* and *flhG*.

Transformation of Vibrio Cells—*V. alginolyticus* cells were transformed by electroporation as described (*36*).

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

Strain or plasmid	Genotype or description	Reference or source
V. alginolyticus		
138-2	Wild-type	*28*
VIO5	VIK4 (Rif^r Pof^+ Laf-)	*31*
NMB201	VIO5 (Rif^r Mpa^r Pof^+ Laf-)	*32*
KK148	VIO5 *flhG* (Rif^r Pof^+ Laf- Multi-Pof)	This study
NMB155	NMB201 (Rif^r Mpa^r Pof^+ Laf- Multi-Pof)	*33*
E. coli		
JM109	*recA1, endA1, gyrA96, thi*$^-$, *hsdR17, relA1, supE44*, λ^-, Δ(*lac-proAB*); *F*′, *traD36, proAB, lacI*^{q}, Δ*M15*	
Plasmids		
pGEM5Zf(+)	Cloning vector, Amp^r, LacZα	Promega Corp
pGEM00207	The 1.8-kb PCR product (*flhF* locus) in the *Eco*RV site of pGEM5Zf(+)	This study
pGEM03608	The 1.8-kb *flhF* fragment in opposite direction to pGEM00207	This study
pGEM00303	The 1.1-kb PCR product (*flhG* locus) in the *Eco*RV site of pGEM5Zf(+)	This study
pBAD24	Amp^r, P_{BAD}	*35*
pBAD33	Cm^r, P_{BAD}	*35*
pAK322	*flhF* of pGEM03608 in the *Sac*I/*Sph*I sites of pBAD33	This study
pAK520	*flhG* of pGEM00303 in the *Sal*I/*Sph*I sites of pBAD33	This study
pAK711	*flhF* of pGEM00207 and *flhG* of pGEM00303 in the *Nco*I/*Sph*I sites of pBAD24	This study
pAK721	*flhF* and *flhG* of pAK711 in the *Eco*RV and *Sph*I sites of pBAD33	This study

Amp^r, ampicilin-resistant; Cm^r, chloramphenicol-resistant; Rif^r, rifampicin-resistant; Mpa^r, motility resistant to phenamil.

The cells were subjected to osmotic shock and then washed thoroughly with 20 mM $MgSO_4$. Electroporation was carried out according to the manufacturer using a Gene Pulser electroporation apparatus (Japan Bio-Rad Laboratories, Tokyo) at an electric field strength of 5.0 to 7.5 kV/cm.

Electron Microscopy—Samples were negatively stained with 2% potassium phosphotungstate (pH 7.0) and then observed under a JEM-1200 EXII electron microscope (JEOL).

Counting Flagella by Dark-Field Microscopy—Overnight cultures of cells grown in VC medium were diluted 50-fold with fresh medium with or without 0.02% arabinose and then incubated for 2.5 h with shaking. Secondary cultures were then diluted 30-fold with VPG medium with or without 0.02% arabinose. After a further 2 h incubation, flagella were observed under a high intensity dark-field microscope (Olympus model BHT) equipped with a 100W mercury lamp (Ushio USH-102).

Detection of Flagellin—Secondary cultures were obtained as described in the previous section and then induced with 0.02% or 0.2% arabinose. After a further 2 h incubation, the cultures were centrifuged at 20,000 × *g* for 5 min at 4°C. The pellets were resuspended in double-distilled water. The cell suspensions were mixed with a one-fifth volume of sodium dodecyl sulfate (SDS) loading buffer (0.2 M Tris-HCl [pH 6.8], 37.5% glycerol, 6% SDS, 0.004% bromophenol blue) and a 1/20 volume of 2-mercaptoethanol, and then boiled for 5 min. Proteins in the samples were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to a polyvinylidenedifluoride (PVDF) membrane (Millipore) using a semi-dry blotting apparatus (Bio-craft, Japan) according to the manufacturer's instructions. Immunoblotting was performed with anti-flagellin antibodies as described (*37*).

RESULTS

Isolation of Multi-Pof Mutants—Among mutants whose swarm size was smaller than that of the parent strains, which were lateral flagellum-defective mutants and the wild type as to polar flagella, we found two mutants, KK148 and NMB155 (Fig. 1A), whose flagellar numbers were increased. Both mutants could swim well in VC medium although their swimming behavior and speed were different from those of the parent strains. On electron microscopic observation, while most parent cells had a single flagellum, we confirmed the multiflagellate phenotype for KK148 and NMB155 cells, which had about 15 and 7 flagella per cell, respectively. The flagella were grown not only from the cell pole but also from around it (Fig. 1B). We also observed that some KK148 and NMB155 cells had only a few flagella and these flagella had been generated from the cell pole like in the parent cells (data not shown). Therefore, these mutations seem not to be involved in the regulation of flagellar placement.

Cloning and Sequence Analysis of the flhF and flhG Genes—In *Pseudomonas* species, a multiflagellate phenotype has been observed when the *flhF* gene is overexpressed (*21*) or when the *fleN* gene is disrupted (*23*). In *Vibrio* species, such as *V. parahaemolyticus*, *V. cholerae*, and *V. vulnificus*, whose genome sequences are available (*38–40*), homologous genes, their organization, and

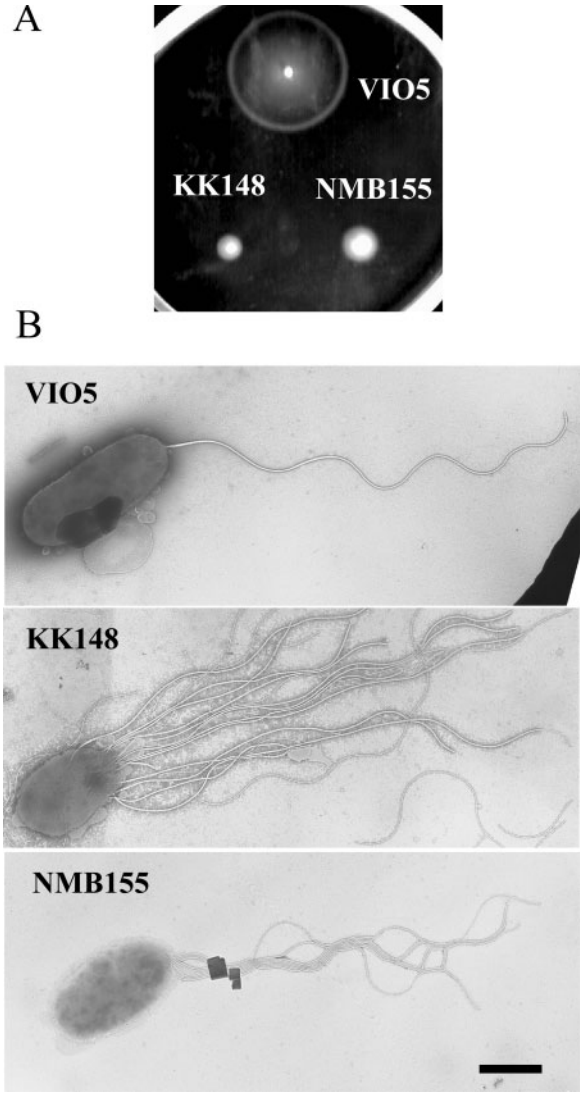


Fig. 1. **Phenotypes of multi-Pof mutants KK148 and NMB155.** (A) Swarming ability. 0.5 μl aliquots of overnight cultures were spotted onto 0.25% agar VC plates, followed by incubation at 30°C for 6h. (B) Electron micrographs of wild-type cells (VIO5) and mutant cells (KK148, and NMB155). Cells were negatively stained with potassium phosphotungstate. Bar, 1 μm.

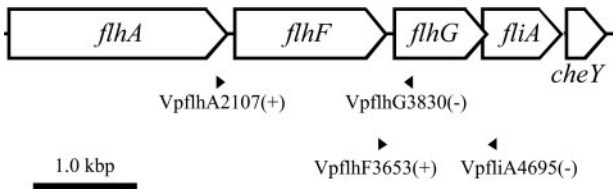
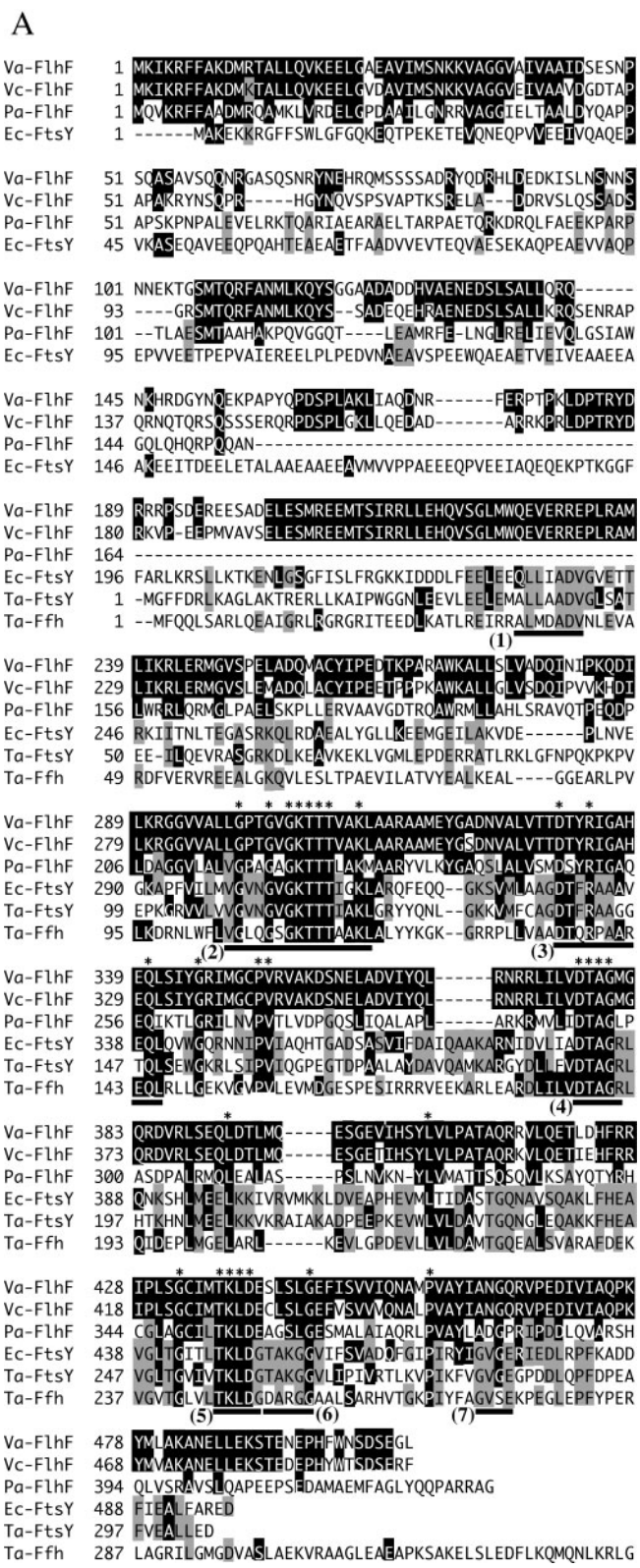


Fig. 2. **Schematic representation of the *flhF* and *flhG* locus based on the *V. parahaemolyticus* BB22 sequence (*19*).** Arrowheads indicate the positions and orientations of the primers used in this study.

neighboring genes are all similar to those in *Pseudomonas*. To clone the *flhF* and *flhG* genes of *V. alginolyticus*, PCR primers which matched regions conserved among the other three *Vibrio* species were synthesized (Fig. 2). Fragments amplified from the chromosomal DNA of strain 138–2



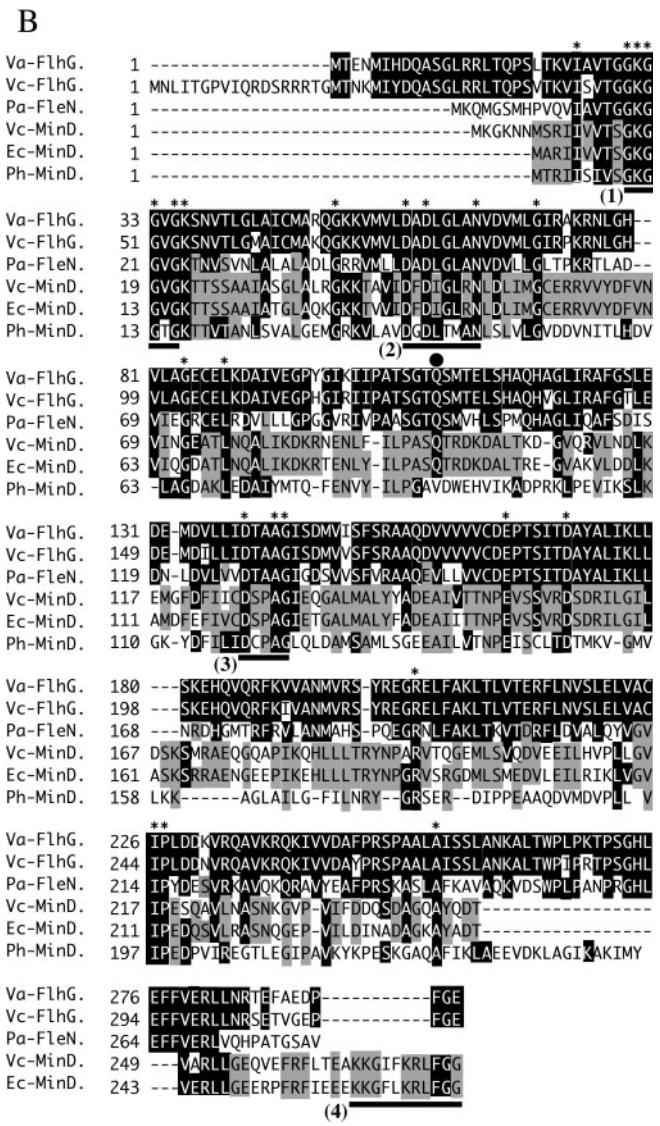


Fig. 3. **Alignment of the amino acid sequences of *V. alginolyticus* FlhF (A) and FlhG (B) with homologues from various species.** Alignment of the sequences was performed using GENETIX (version 12.1.0; GENETIX Corporation). Abbreviations: Va, *V. alginolyticus*; Vc, *V. cholerae*; Pa, *P. aeruginosa*; Ec, *E. coli*; Ph, *Pyrococcus horikoshii*; Ta, *Thermus aquaticus*. White letters in black boxes denote amino acid residues identical to those of *V. alginolyticus* FlhF or FlhG at the aligned positions. Letters in gray boxes denote amino acid residues identical to those of *E. coli* MinD or FtsY at the aligned positions. When the residues completely matched, the positions are indicated by asterisks. Thick underlines in (A) indicate (1) the ALLEADV motif, (2) motif I (P-loop), (3) motif II (insertion box domain loop), (4) motif III, and (5) motif IV, (6) the DARGG motif, and (7) the closing loop. Thick underlines in (B) indicate (1) the P loop, (2) switch I, (3) switch II, and (4) the amphipathic helix. The mutation site in KK148 is indicated by the closed circle above the sequence of Va-FlhG.

using these primers were cloned into vector pGEM5Zf(+). We determined the sequence from the end of *flhA*, which is the first gene of the operon and supposed to encode a component of the flagella-specific export apparatus essential for flagellar assembly, to the beginning of *fliA*, which encodes σ^{28}. There are non-coding regions of 28 bp between *flhA* and *flhF*, and 14 bp between *flhF* and *flhG*. The end of the *flhG* gene and the beginning of the *fliA* gene overlap by 11 bp. The nucleotide sequence has been deposited in DDBJ under accession No. AB191524.

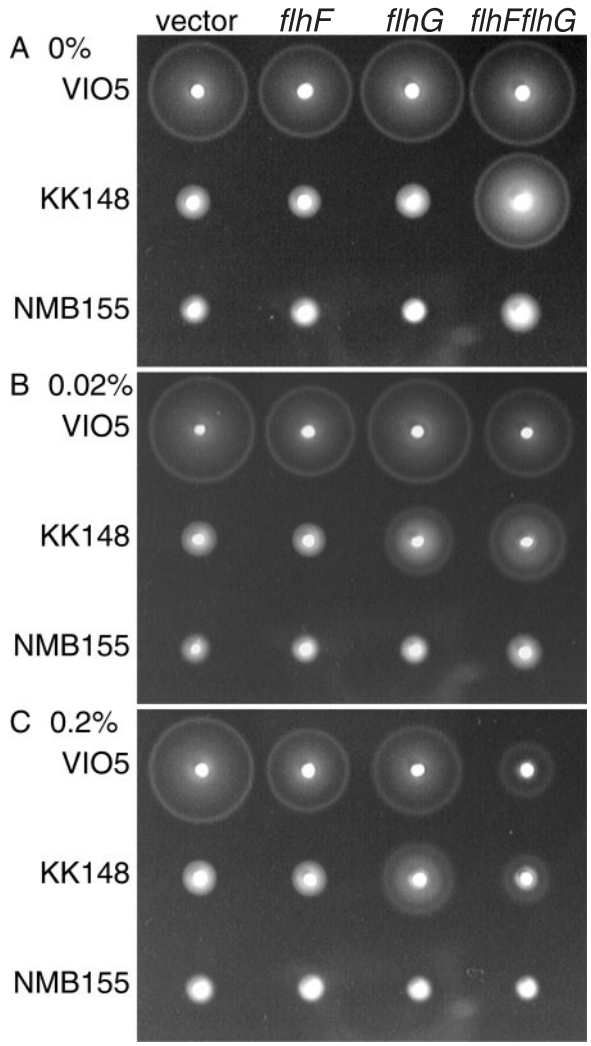


Fig. 4. **Swarming ability of the wild-type (VIO5) and multi-Pof strains (KK148 and NMB155) containing plasmid pAK322 (*flhF*), pAK520 (*flhG*), pAK721 (*flhFflhG*), or pBAD33 (vector control).** 0.5 μl aliquots of overnight cultures were spotted onto 0.25% agar VC plates with chloramphenicol, and 0% (A), 0.02% (B), or 0.2% (C) arabinose, followed by incubation at 30°C for 5 h.

The predicted sizes of FlhF and FlhG from *V. alginolyticus* are 57 kDa and 32 kDa, respectively. With the BLAST search program, the predicted amino acid sequence of FlhF was fond to be 28.3% similar to that of *E. coli* FtsY, a signal recognition particle (SRP) receptor protein (Fig. 3A). FtsY comprises three domains, two of which are called the N and G domains; the amino-terminal N domain is a four-helix bundle that is packed tightly against the G domain and the G domain adopts a clasical GTPase-fold in which there are four conserved motifs (I–IV) (*41–43*). The C-terminal G domain, which contains a GTP-binding motif, exhibits a high level of homology among proteins similar to FlhF. The N-terminal N domain, which is a four-helix bundle that is packed tightly against the G domain, is thought to play a role in regulation of the nucleotide-binding state of the protein. In SRP GTPases, it has been reported that there are conserved regions; motifs I (P-loop), II (insertion box domain loop), III, and IV, the closing loop, and the ALLEADV and DARGG motifs (*44*). Motifs I, II, III, and IV, which define the GTP binding site (*45*), are highly conserved in FlhF proteins but the N domain is not conserved. Homology among the N terminal regions of FlhF proteins is low. However, a short stretch at the very beginning of the N terminal region exhibits high similarity. The predicted amino acid sequence of FlhG is 23.9% similar to that of *E. coli* MinD, which serves as a cell division inhibitor (*46*, *47*); this similarity is distributed over the entire protein and the N-terminal region exhibits a high level of homology with the ATP-binding motif of MinD (Fig. 3B). In MinD proteins, it has been reported that there are conserved regions; the P-loop (Walker A motif), switch I, switch II, and the amphipathic helix. All these conserved regions, except the amphipathic helix one, are also highly conserved in FlhG proteins.

The *flhF* and *flhG* genes from the two multi-Pof mutants were cloned by means of the same method and then sequenced. We found no mutation in the KK148 *flhF* gene. In the KK148 *flhG* gene, we found that the CAG codon for Gln109 was changed to a TAG nonsense codon. When the mutant *flhG* of KK148 was expressed in wild-type cells, the swarming ability and flagellar number were not affected (data not shown). We found no mutation in the *flhF* and *flhG* genes from NMB155.

Effects of flhF and flhG on Swarming Ability—The effects of *flhF* and *flhG* gene expression in the wild-type and multi-Pof mutant cells on swarming ability were examined (Fig. 4). pAK322, carrying a single copy of *flhF*, reduced the swarming ability of VIO5 cells upon induction. KK148 cells expressing *flhG* recovered the swarming ability on arabinose induction, but the swarming ability of NMB155 cells was not recovered (Fig. 4). Arabinose induction of pAK520, carrying the *flhG* gene (*flhG*), reduced the swarming ability of wild-type VIO5 cells. pAK721, carrying both the *flhF* and *flhG* genes (*flhFflhG*), improved the swarming ability of KK148 cells even without arabinose induction, but did not affect the swarming of NMB155 cells. The *flhF* gene might contain some promoter activity.

Effects of flhF and flhG Expression on Flagellar Number—The polar flagella of *V. alginolyticus* can easily be observed on high intensity dark-field microscopy, so we determined the number of polar flagella per cell (Fig. 5). The number of flagella on most VIO5 cells was one (Fig. 5A). The number of flagella on VIO5 cells containing pAK322, carrying *flhF*, was increased when the *flhF* transgenes were induced (Fig. 5B). Although 77% of non-induced cells possessed a single polar flagellum, 33% of *flhF*-induced cells possessed 2 to 4 polar flagella and only 48% of them possessed a single polar flagellum. When KK148 or NMB155 was used as the host, an increase in the number of polar flagella on *flhF* expression was not observed (Fig. 5,F and J).

When *flhG* was expressed in VIO5 and KK148 cells with pAK520, the number of polar flagella was decreased (Fig. 5, C and G). Although 82% of non-induced VIO5 cells possessed a single polar flagellum, 52% of *flhG*-induced VIO5 cells did not possess any polar flagella and only 47% of them possessed a single polar flagellum. Although 63% of non-induced KK148 cells possessed more than 4 polar flagella, 25% of *flhG*-induced KK148 cells possessed a single polar flagellum, 21% of them had 2 to 4 polar

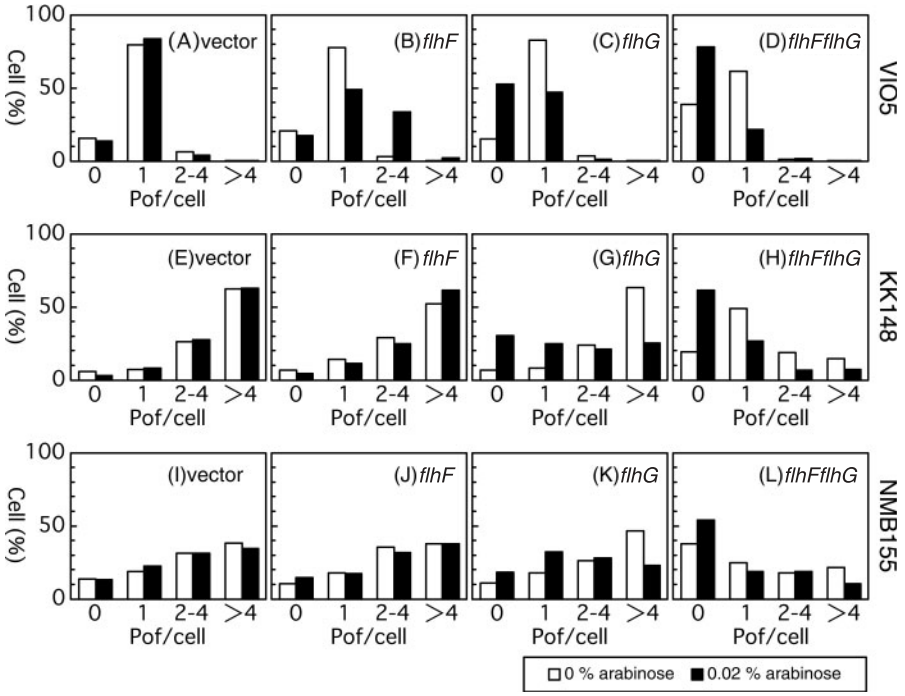


Fig. 5. **Number of polar flagella per plasmid-bearing cell.** Cells of VIO5, KK148, and NMB155 containing a plasmid, pAK322 (*flhF*), pAK520 (*flhG*), pAK721 (*flhFflhG*), or pBAD33 (vector control), from overnight cultures were re-cultured in VC medium at 30°C for 2.5 h and then diluted 1:30 with VPG medium with (filled bars) or without (open bars) 0.02% arabinose. After 2 h 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 flagella, one flagellum, two-four flagella, and more than five flagella per cell. The experiments were independently performed twice and the graphs were made constructed with the average data.

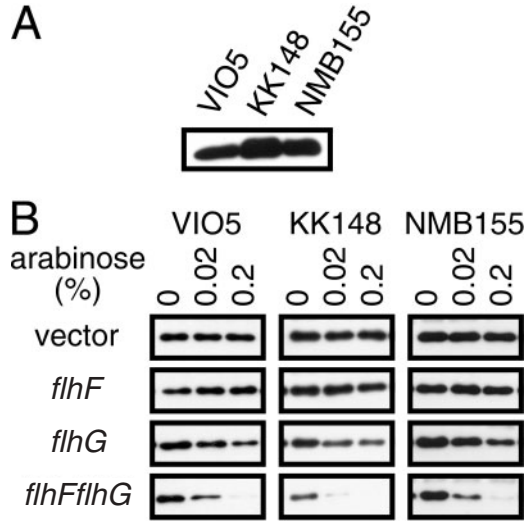


Fig. 6. **Detection of flagellin.** Western blotting analysis of the wild-type (VIO5) and multi-Pof strains (KK148 and NMB155) lacking (A) and carrying (B) a plasmid, pAK322 (*flhF*), pAK520 (*flhG*), pAK721 (*flhFflhG*), or pBAD33 (vector control), was performed using polyclonal anti-flagellin antibodies. Cells cultured overnight (*cf.* legend to Fig. 5) were re-cultured in VC medium with 0, 0.02 or 0.2% arabinose at 30°C for 2.5 h, and then diluted 1:30 with VPG medium with 0, 0.02 or 0.2% arabinose. After 2 h incubation at 30°C, the cell cultures were centrifuged, and the cell fractions were diluted to an optical density at 660 nm of 0.5 for all the strains in (A), and of 0.5 for VIO5, 0.2 for KK148, and 0.3 for NMB155 in (B). 10 μl of each sample was subjected to SDS-PAGE. Proteins were separated by SDS-PAGE and immunoblotted using anti-flagellin antibodies.

flagella, and only 25% of them had more than 4 polar flagella.

Taken together, our findings imply that FlhG is a negative regulator of the polar flagellar number and that the swarming ability of KK148 cells is reduced by the multi-flagellation caused by the nonsense mutation in the *flhG* gene. In contrast, *flhG* expression in NMB155 did not result in a significant decrease in the number of polar flagella (Fig. 5K). This implies that NMB155 exhibits its polar multiflagellate phenotype because of a mutation in some gene other than *flhG*. In all cell lines carrying pAK721, which contains both the *flhF* and *flhG* genes, the number of flagella was decreased even without arabinose induction (Fig. 5, D, H, and L). The *flhF* gene might contain some promoter activity as mentioned above.

Amount of Flagellin in Cells—We investigated the amount of flagellin in the multiflagellate mutants by means of Western analysis using polyclonal anti-flagellin antibodies. A larger amount in flagellin than that of the parent strains was detected in the multi-Pof cells, and the amount in KK148 cells was much greater than that in NMB155 ones (Fig. 6A). Cells containing the various plasmids carrying *flhF* and/or *flhG* were also examined as to the amounts of flagellin in the cells. The transgenes were induced with 0.02% or 0.2% arabinose. When pAK322, carrying the *flhF* gene, was introduced into VIO5, no significant increase in the amount of flagellin was seen (Fig. 6B). The expression of *flhG* decreased the amount of flagellin present in all strains (Fig. 6B). This is consistent with the decrease in the number of flagella induced by *flhG* gene expression (Fig. 5, C, G and K). The FlhG protein may

suppress flagellin synthesis. Coexpression of *flhF* and *flhG* dramatically decreased the amount of flagellin in all strains.

DISCUSSION

We isolated multi-Pof mutants, KK148 and NMB155, by screening for low swarming ability. The swarm sizes of the two strains were very small but their swimming abilities were not severely affected. We found a nonsense mutation, Gln109 to a stop codon, in the *flhG* gene of KK148. However, the mutant gene in the NMB155 strain remains to be identified. We focused on the *flhF* and *flhG* genes because the homologous gene products in *Pseudomonas* are known to regulate flagellar location and number, respectively (*21–23*). We cloned the *flhF* and *flhG* genes from *V. alginolyticus*. The FlhF protein exhibted 28.3% similarity to *E. coli* SRP receptor protein FtsY. In *E. coli*, membrane proteins are translated by membrane-bound ribosomes that are targeted to FtsY (*41*, *42*). The C-terminal region of FlhF exhibited especially high similarity to FtsY. The N-terminal region of FlhF is not conserved in *Vibrio* and *Pseudomonas* except for the very beginning. This might suggest that the functions of FlhF in *Vibrio* and *Pseudomonas* are slightly different. The C-terminal region of FtsY is essential for its function and contains a GTP-binding motif. These results may suggest the possibility that FlhF targets FliF, the MS-ring component, to the cell pole. In *B. subtilis*, it has been shown that FlhF is dispensable for protein secretion (*48*).

The FlhG protein showed 23.9% similarity to *E. coli* cell division inhibitor MinD. MinD functions in the cytoplasm with its C-terminal helix region attached to the cell's inner membrane, and is responsible for recruiting MinC and MinE to the membrane (*49*, *50*). MinD cooperates with MinC to form an inhibitor of septum initiation, and the MinCD complex is topologically regulated by MinE (*11*). These Min proteins show dynamic movement in the cell; MinD is a key component for oscillation with ATPase activity and dimerizes in the presence of ATP, and the polar zone of MinD starts to disassemble from the middle of the cell towards the pole (*15*). It has been reported that C-terminal amino acid residues F263, L264, L267, and F268 are essential for membrane attachment of *E. coli* MinD (*50*). These residues, however, are not conserved in FlhG. It is not known whether the FlhG protein attaches to the inner membrane or whether it forms a dimer.

Expression of *flhG* in the wild-type cells reduced their swarming ability, and decreased the amount of flagellin and the number of polar flagella. This suggests that FlhG suppresses flagellin synthesis directly or indirectly, resulting in a decrease in the number of polar flagella. FleN, the FlhG homologue in *Pseudomonas*, is thought to suppress transcription of structural components of flagella via FleQ, which is a σ^{54} (RpoN)-dependent transcriptional activator (*24*, *25*, *51*). It was speculated that FleN plays a crucial role in maintaining a single flagellum by down-regulating the FleQ activity. In *V. alginolyticus*, FlhG may also suppress transcription of flagellar components including flagellin via FleQ homologue FlaK. We have indeed demonstrated that σ^{54} is involved in the expression of polar flagellar genes in *V. alginolyticus* (*52*). In *H. pylori*, FlhF and FlhA have been characterized as functional equivalents to master regulators (*7*). Among *Vibrio*, *Helicobacter*, and *Pseudomonas* species, FlhF and FlhG homologs may not completely play the same role in flagellar gene regulation but at least they regulate the expression of the majority of flagellar genes directly or indirectly like a master regulator. When expression of all flagellar genes is elevated, does the flagellar number increase? We speculate that increases in the amounts of flagellar proteins are necessary for hyper-flagellation but are not sufficient on their own. *V. alginolyticus* should have a mechanism for maintaining a single flagellum; otherwise the flagellar number would be more variable under different culture conditions.

Expression of *flhF* in wild-type cells increased the number of polar flagella. FlhF may be involved in the regulation of the flagellar number. However, the intrinsic function of FlhF in *V. alginolyticus* remains to be elucidated. In a previous study of *P. putida*, which possesses polar flagella, it was found that FlhF determined the flagellar placement, a *flhF* mutant had a peritrichously flagellated phenotype, and *flhF* overexpression resulted in a multiflagellate phenotype (*21*). *V. alginolyticus* FlhF might also be involved in determination of the site of polar flagella. In this study, FlhF and FlhG co-expression efficiently decreased the number of polar flagella and the amount of flagellin. It is likely that FlhG acts with FlhF directly or indirectly.

FtsY is a component of the prokaryotic SRP receptor and Ffh is a prokaryotic SRP that is homologous to SRP54. Both are homologous GTPase components of the signal recognition targeting pathway for protein secretion. They interact directly with each other during cotranslational targeting of proteins to membranes. The structure of the GTP-dependent heterodimeric complex was determined and its coordinated activation was explained by the structure (*44*, *53*). The conformational changes coupled to the formation of an extensive interface may function allosterically to signal formation of the targeting site and translocon. The conserved motifs of the SRP GTPases, I to IV, which correspond to the residues directly involved in nucleotide binding and hydrolysis, are well-conserved in FlhF although several additional motifs that are restricted to the SRP GTPases are not conserved. In a structural similarity search, Ffh was found to resemble MinD (*54*). This structural similarity suggests heterodimer formation of FlhF and FlhG in a similar manner to that of Ffh and FtsY. From the structural similarity, we can speculate that FlhF and FlhG form a heterodimeric complex. From the results of this study we infer that the FlhG protein interacts with the FlhF protein either directly or indirectly. We are currently trying to obtain direct evidence of the interaction between FlhF and FlhG by means of biochemical analysis.

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