Mutations in The Stator Protein PomA Affect Switching of Rotational Direction in Bacterial Flagellar Motor

Hiroyuki Terashima

Nagasaki University

Kiyoshiro Hori

Nagoya University

Kunio Ihara

Nagoya University

Michio Homma (✉ g44416a@cc.nagoya-u.ac.jp)

Nagoya University

Seiji Kojima

Nagoya University

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Abstract

The flagellar motor rotates bi-directionally in counter-clockwise (CCW) and clockwise (CW) directions. The motor consists of a stator and a rotor. Recent structural studies have revealed that the stator is composed of a pentameric ring of A subunits and a dimer axis of B subunits. The stator interacts with the rotor through conserved charged and neighboring residues, and the rotational power is generated by their interactions through a gear-like mechanism. The rotational direction is controlled by chemotaxis signaling transmitted to the rotor, with no evidence for the stator being involved. In this study, we found novel mutations that affect the switching of the rotational direction at the putative interaction site of the stator to generate rotational force. Our results highlight a novel aspect of flagellar motor function that appropriate switching of the interaction states between the stator and rotor is critical for controlling the rotational direction.

Introduction

Bacteria can swim in an aqueous environment or swarm on a surface through screw-like rotation of a flagellum. The flagellar motor consists of stator and rotor elements. The stator, which functions as an ion channel, conducts ion influx, such as the H^+ influx in *Escherichia coli* or the Na^+ influx in *Vibrio alginolyticus*, depending on the transmembrane electrochemical potential. The ion influx induces conformational changes in the stator, followed by changing the interaction between the stator and rotor to generate rotation power. Being bidirectional, the flagellar motor can rotate in either the counter-clockwise (CCW) or clockwise (CW) direction. The rotational direction is associated with chemotaxis, the ability of a bacterium to sense the environment around itself and migrate towards a more suitable location, away from unfavorable stimuli ^{1,2}. The cells swim straight (forward) using the flagellum with CCW rotation. Once the cells sense an unfavorable chemical or temperature, they change the direction of flagellar rotation from CCW to CW, resulting in changes in the direction of the cell body.

The stator is composed of two kinds of membrane proteins, MotA and MotB for *E. coli* or PomA and PomB for *V. alginolyticus* ^{3,4}. MotA and PomA are membrane proteins with four transmembrane segments (TM) and a large cytoplasmic region (Loop₂₋₃) between TM2 and TM3. MotB and PomB are membrane proteins with a single TM and a periplasmic peptidoglycan-binding (PGB) domain in the C-terminal region. The atomic structures of the stator were determined by two different groups ^{5,6}. The MotA/MotB and PomA/PomB complexes, previously proposed as 4:2 hetero-hexamers, were shown to be 5:2 hetero-heptamers. From the structure in which two molecules of MotB are inserted in the center of the MotA five-molecule ring, a model was proposed in which the MotA ring rotates with respect to the axis of MotB due to the influx of ions.

The rotor is a huge ring complex composed of a transmembrane MS-ring in which FliF assembles and a cytoplasmic C-ring in which FliG, FliM, and FliN assemble. The stator-rotor interaction arises between the C-terminal region of FliG and the cytoplasmic region of the stator A subunit and should generate torque ^{7,8}. Previous genetic studies have shown that conserved charged residues in the A subunit (MotA R90 and

E98 in *E. coli* and PomA R88 and E96 in *V. alginolyticus*) are associated with those in FliG (FliG R281 and D288 in *E. coli* and FliG R301 and D308 in *V. alginolyticus*) through electrostatic interaction^{9–13}. Recently, we showed the physical interaction between PomA and FliG biochemically by using site-directed *in vivo* photo-crosslinking and cysteine disulfide crosslinking. Moreover, we identified that additional residues, PomA-D85, K89, G90, F92, and L93, were close to FliG¹⁴. The region interacting with the rotor was located at the external and membrane-distal portion of the A subunit. Current knowledge suggests a gear-like rotation model between the stator and rotor^{5,6,14,15}.

The direction of rotation is controlled by the C-ring (also called the switch complex), which is the cytoplasmic surface of the flagellar rotor and is attached to the MS-ring formed by 34 molecules of the two-transmembrane FliF protein^{16–19}. In the C-ring, many of the three constituent proteins, FliG, FliM, and FliN, assemble to form an inverted cup-like structure in the cytoplasm^{20–22}. The chemotaxis protein (CheY) is converted into a phosphorylated state via intracellular signaling pathways from the receptors^{23,24}. The phosphorylated CheY (CheY-P) binds to a well-conserved motif in the N-terminal region of FliM (FliM_N)^{25–29}. CheY-P binding induces structural changes in the middle domain of FliM³⁰. As a result, a structural change occurs at the FliG-stator interaction site switching the direction of rotation of the flagellar motor from CCW to CW³¹. After the dissociation of phosphorylated CheY, the rotational direction returned to CCW. In *V. alginolyticus*, a single transmembrane protein called ZomB is necessary for the CW direction, depending on CheY³².

Cryo-electron tomography of intact flagellar motors in *Borrelia burgdorferi* and *V. alginolyticus* showed that the diameter of the C-ring expands during CW rotation^{15,33}. The C-terminal region of FliG appears to interact with the rotor-proximal portion of the stator with CCW rotation, whereas it appears to interact with the rotor-distal portion of the stator in CW rotation. Therefore, the rotation direction of the flagellar motor is determined by changing the portion of the stator interacting with the rotor. Previous genetic studies have reported that mutations in the stator confer only the Mot[−] phenotype (non-motile) but not the Che[−] phenotype (no chemotaxis or inhibited switching of rotational direction). Therefore, the rotational direction is thought to be determined only by the conformational change of the C-ring.

In this study, we focused on the PomA-F92 and L93 residues that a previous report identified as interacting with FliG¹⁴. We also focused on the PomA-L95 residue, which is highly conserved and is located next to the conserved glutamate residue, an important charged residue that generates torque. The substitution of these residues to a charged residue decreases the switching frequency of the motor and is biased toward CCW rotation. Interestingly, combination with a CW-biased mutation in FliG was biased more toward CW rotation than in wild-type FliG. This study showed that the mutation in the stator affects the rotational direction, providing novel insights into the gear rotation model of the flagellar motor.

Materials And Methods

Bacterial strains and plasmids. The bacterial strains and plasmids are listed in Table S1. *E. coli* was cultured in LB broth (1% [w/v] bactotryptone, 0.5% [w/v] yeast extract, 0.5% [w/v] NaCl) at 37°C. *V. alginolyticus* was cultured in VC broth (0.5% [w/v] polypeptone, 0.5% [w/v] yeast extract, 3% [w/v] NaCl, 0.4% [w/v] K₂HPO₄, 0.2% [w/v] glucose), or VPG broth [1% (w/v) polypeptone, 3% (w/v) NaCl, 0.4% (w/v) K₂HPO₄, 0.5% (w/v) glycerol] at 30°C. Chloramphenicol (Cm) was added to a final concentration of 25 µg/mL for *E. coli* and 2.5 µg/mL for *V. alginolyticus*. Ampicillin (Amp) was added at a final concentration of 100 µg/mL for *E. coli*. Kanamycin was added at a final concentration of 50 µg/mL for *E. coli* and 250 µg/mL for *V. alginolyticus*. Arabinose for protein expression from pHFAB was added at a final concentration of 0.02% (w/v) for *V. alginolyticus*.

Swimming assay in soft agar plates. *V. alginolyticus* NMB191 cells harboring pHFAB were inoculated on VPG 0.25% (w/v) agar plates with 0.02% (w/v) arabinose and 2.5 µg/mL Cm incubated at 30°C for the desired time.

Mutagenesis. Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis method, as described by Agilent Technologies (Santa Clara, USA). Transformation of *V. alginolyticus* by plasmids pHFAB or pYA303 was carried out by electroporation³⁷. Transformation of *V. alginolyticus* by plasmid pNT1 was carried out by conjugational transfer from *E. coli* S17-1³⁸.

Measurement of swimming speed and motile fraction. *V. alginolyticus* cells, cultured overnight, were inoculated into fresh VPG broth with antibiotics and incubated by shaking at 30°C for 4 h. The cells were suspended at a 100-fold dilution in V buffer (50 mM Tris-HCl pH 7.5, 300 mM NaCl, 5 mM MgCl₂), and then spotted onto glass slides for microscopic observation. The motile fraction was calculated by dividing the number of motile cells by the total number of cells. All experiments were performed three times, and the averages of the motile fraction and standard deviation (SD) were calculated. To measure the swimming speed of an individual cell, the cell was suspended at a 100-fold dilution in V buffer, and serine was added at a final concentration of 5 mM to allow the cell to swim straight. The motion of the cells was captured at 30 frames/s for 5 s under a microscope. The swimming speed of individual 10 cells was measured using ImageJ software (Rasband W.S., Bethesda, USA), and the average swimming speed and SD were calculated.

Measurement of switching frequency and ratio of CCW/CW rotation. *V. alginolyticus* cells, cultured overnight, were inoculated into fresh VPG broth with antibiotics and incubated by shaking at 30°C for 4 h. The cells in the 200 µL culture were collected by centrifugation at 2,000 × *g* for 5 min. The precipitated cells were resuspended in 200 µL of V buffer. The cells were then spotted onto glass slides for high-intensity dark-field microscopic observation. The motion of the cells was captured at 30 frames/s for 10 s under a microscope. The number of frames of either CCW or CW rotation was recorded, and the ratio of CCW/CW rotation and switching events was calculated for 1 s using ImageJ software.

Whole genome analysis of swimming ring-restored mutants in soft agar plates. The genomic DNA of swimming ring-restored mutants was prepared using a Monarch Genomic DNA Purification Kit (New

England Biolabs, Ipswich, USA). The concentration of the purified genomic DNA was adjusted to approximately 50 ng/μL. Genomic DNA libraries were constructed by the tagmentation method on magnetic beads using the Nextera DNA Flex Library Prep kit (Illumina, San Diego, USA) with some modifications. One μL of genomic DNA (~ 10 ng) was mixed with 6.5 μL of tagmentation Reaction mix (beads linked transposome 0.5 μL, X5 TAPS buffer 1.5 μL, and miliQ water 4.5 μL; X5 TAPS buffer containing 50 mM TAPS-NaOH, pH = 7.5, 50 mM MgCl₂, 50% (w/v) DMF), incubate at 55°C for 25 min and transposases were inactivated by incubation at 80°C for 3 min. Magnetic beads with tagmented DNA were washed with 100 μL of tagmentation wash buffer (10 mM Tris-HCl pH 8.0, 30 mM NaCl, 0.1% [w/v] Triton X-100) and recovered by setting on a magnetic plate. After discarding the tagmentation wash buffer, 20 μL of PCR reaction mix (KAPA HiFi HS Ready mix; Nippon Genetics) and a set of indexed primers (2.5 μL each) were mixed with the magnetic beads and processed under the following conditions: 72°C for 5 min, 98°C for 30 s, (98°C for 20 s, 62°C for 15 s, 72°C for 1 min) x 15 cycles, and at 72°C for 1 min. Amplified genomic DNA libraries were size-selected around 300 ~ 1000bp using ProNex beads (Promega, Madison, USA), and their sizes were confirmed by the microtip electrophoresis analyzer MultiNA (Shimadzu, Kyoto, Japan). DNA concentration was determined using SYBR Green I (Takara Bio, Kusatsu, Japan). Twenty DNA library samples were pooled to even out the amount of DNA in each library. A mixed library sample was sequenced using HiSeq-X (150 bp PE; Illumina, San Diego, USA), and 20 samples were separated by their index sequences.

Identification of putative mutation sites using bresEq. Genome sequences of the mutants were analyzed using breseq v0.35³⁹ with default parameters. For the reference sequence, we used *V. alginolyticus* VIO5 DNA, chromosome 1, complete sequence (accession number: AP022861), and *V. alginolyticus* VIO5 DNA, chromosome 2, complete sequence (accession number: AP022862).

Results

Residues of PomA mutations are involved in stator-rotor interaction involved in motility. A previous report showed that PomA-F92 and L93 of the stator are located very close to the C-terminal region of FlhG in the rotor and suggested that these residues were involved in the interaction between the stator and rotor in addition to the conserved charged residues¹⁴. These residues were highly conserved to hydrophobic residues among many species in multiple sequence alignments of PomA and MotA, implying functional significance of hydrophobicity at this position (Fig. S1). Furthermore, PomA-L95 is also highly conserved in leucine (or the similar residue of isoleucine) among many species and is located next to PomA-E96, which is a conserved and important charged residue for motor function. Thus, we substituted these residues with alanine, glutamate, or arginine, and characterized the motility of cells expressing the mutant PomA. We first examined the expansion of cells in a soft agar plate (Fig. 1A). The substitutions of PomA-F92 did not affect motility, suggesting that hydrophobicity at this position is not involved in motility. On the other hand, the glutamate-substituted mutant at the position of PomA-L93 severely decreased the swimming ring in the soft agar plate, and the arginine-substituted mutant moderately decreased the swimming ring, suggesting that the charged side chain at this position affects motility. In the substituted

mutants at the position of PomA-L95, the alanine and phenylalanine mutants retained motility, whereas the threonine mutant moderately inhibited motility, and the arginine and glutamate mutants severely decreased the swimming ring in the soft agar plate. These results suggest that motility is affected by the charged or hydrophilic side chains at this position.

We next examined the protein expression levels of the mutant PomA using immunoblotting for anti-PomA and anti-PomB antibodies (Fig. 1B). All the mutated PomA proteins were expressed at a similar level as the wild-type PomA produced from plasmid pHFAB, although the band intensities of L95E, L95R, and L95T were reduced, suggesting that the motility defects of the mutants in the soft agar plate were not due to the decrease in the expression level of the mutant proteins.

We examined the effects of temperature on motility. Cells treated with wild-type PomA, L93E, or L95R were incubated at 20°C, 30°C and 40°C in a soft agar plate (Fig. S2). Wild-type PomA conferred a larger swimming ring at 40°C than at 30°C. On the other hand, the two mutants conferred a smaller swimming ring at 40°C than at 30°C. At 20°C, the swimming abilities were much worse than at 40°C and 30°C. The relative swimming abilities of wild-type PomA and mutant PomA were similar under each temperature condition. These results implied that the mutant PomA protein is more sensitive to high temperatures than the wild-type protein.

PomA-L93 and L95 mutants conferred a rotationally direction-biased phenotype. We next characterized the motility of the cells expressing PomA-L93E, L93R, L95E, or L95R, as these mutants severely affected motility in the soft agar plate. We captured cell motion under the microscope, and then analyzed motility properties: motile fraction, swimming speed, switching frequency, and ratio of rotational direction (CCW/CW). Although the mutants showed a decreased motile fraction compared to wild-type PomA, the mutants sufficiently retained their swimming ability in the liquid (Fig. 2A). Swimming speed of the mutants was not significantly different from that of wild-type PomA (Fig. 2B). On the other hand, the ratio of the rotational direction of PomA-L93E, L93R, or L95R, but not L95E, was significantly biased in the counter-clockwise direction compared to that of wild-type PomA (Fig. 2C). Moreover, the switching frequency of all mutants was significantly decreased compared to that of wild-type PomA (Fig. 2D). Therefore, these results indicated that these mutations to the position of the stator A subunit confer the *Che⁻* phenotype, showing defects in the rotational direction and switching frequency. However, the *che* function was not completely lost by the mutations because the response of phenol to the CW rotation of flagella remained active (Fig. 3). Since all mutations in the stator proteins described previously have been *Mot⁻* phenotype but not *Che⁻* phenotype, we found novel mutations in the stator protein conferring the *Che⁻* phenotype. This result implies that the structural changes in the stator affect the switching of the rotational direction.

PomA CCW mutants combined with a CW-biased FliG mutant. PomA L93 and L95 mutants, as described above, exhibited motility properties biased toward the CCW direction. Next, we examined whether PomA mutations are dominant for switching the rotational direction in combination with CW-locked FliG-G215A mutation or CW-biased FliG-Q147H mutation. PomA L93E or L95R combined with FliG-G215A showed a

CW-locked phenotype similar to wild-type PomA with FliG-G215A (Fig. 4), suggesting that the switching of the rotational direction is predominantly determined by the rotor. Wild-type PomA combined with FliG-Q147H showed the CW biased rotation with CCW: CW = 1:9, but a few switching events occurred. In contrast, PomA-L93E or L95R combined with FliG-Q147H showed more CW-biased rotation and a much lower switching frequency than wild-type PomA with FliG-Q147H (Fig. 4). This result suggests that the mutation at the position of PomA L93 or L95 biases the rotational direction determined by the rotor and inhibits the switching of the rotational direction.

Swimming ring-restored mutants on soft agar plate. Next, we isolated mutants that restored the swimming ring in a soft agar plate from the *V. alginolyticus pomAB* null mutant cells expressing PomA-L93E, L95E or L95R, and wild-type PomB. We obtained a total of 23 mutants: 10 mutants from PomA L93E (named L93Es1-L93Es10), nine mutants PomA-L95E (named L93Es1-L95Es9), and four mutants PomA L95R (named L95Rs1-L95Rs4) (Fig. S3). When the plasmid extracted from the swimming ring-restored mutants was re-transformed into the parent strain, the swimming ring of the re-transformants, except for L95Rre3, returned to the same level as that of the original cell (Fig. S3). This result indicated that a mutation exists in the genome, but not in the plasmid. Next, we examined the motility of swimming ring-restored mutants (Fig. S4-S6). Most of the mutants did not exhibit a significant difference from the parent strain on the motile fraction, swimming speed, switching frequency, and ratio of rotational direction. The swimming speed of L93Es3 and L95Es6-8 was significantly faster than that of the parent strain (Fig. S4). L95Rs3 and L95Rs4 restored the ratio of the rotational direction, which was similar to that of the cells expressing wild-type PomA (Fig. S5). The mutants, L93Es6, L95Es3, L95Es9, L95Rs3, and L95Rs4, showed significantly restored switching frequency, whereas some mutants, L93Es2, L93Es4, and L95Es8, experienced a decreased frequency (Fig. S6). L95Rs3 possessed an L95S mutation in *pomA* encoded in the plasmid pHFAB.

Identification of mutation positions by whole genome sequencing. To identify the mutations of the swimming ring-restored mutants, we determined the whole genome sequences of the mutants by short-read sequencing. Using Breseq, we identified suppressor mutations in the 19 mutants (Table S2). In L93Es1, L93Es3, L93Es5-10, and L95Rs3-4, candidate mutations exist in genes encoding proteins involved in ATP synthase production. In L95Es1-3 and L95Rs1-2, candidate mutations exist in genes encoding ATPase proteins of the type II secretion system. In L95Es6, candidate mutations exist in genes encoding transketolase 2. In L95Es7, candidate mutations exist in genes encoding ATP-binding proteins in the hypothetical ABC transporter. These genes may be involved in the switching events of the stator and rotor interactions.

Discussion

It is thought that the rotational direction of the flagellar motor is mainly controlled by the phosphorylated CheY (CheY-P) and the C-ring proteins, FliG, FliM, and FliN. In this study, we found that mutations in the stator protein PomA affect regulation in the rotational direction. These mutations were the residues involved in the stator-rotor interaction (PomA-L93) and the highly conserved hydrophobic residue (PomA-

L95) adjacent to the important charged residue (PomA-E96). Since the positions of PomA-L93 and L95 are conserved in hydrophobic residues among various species, hydrophobicity may be important for motor function. In fact, PomA L93E, L93R, L95E, and L95R showed a significant decrease in swimming expansion in the soft-agar plate, indicating that these mutations affect motor function. Further microscopic observation of these mutations identified a Che^- phenotype, characterized by a decrease in the switching frequency and CCW-biased rotation rather than a decrease in swimming speed.

It is thought that the rotational direction of the flagellar motor is determined by the structural change of the rotor. Cryo-electron tomography has revealed that the diameter of the C-ring expands when the rotational direction is changed from CCW rotation to CW rotation^{15,33}. Furthermore, the rotor interacts with the proximal side of the stator toward the rotor during CCW rotation, whereas it interacts with the distal side of the stator during CW rotation. Atomic-resolution structures of the stator were represented as a barrel-like shape of the pentamer of the A subunits surrounding the dimer of the B subunits^{5,6}. Our site-directed crosslinking experiments indicated that the stator and rotor interaction resembles a simple mechanism, similar to a spur gear¹⁴. Recent studies have proposed a novel model of flagellar rotation: the pentamer of the A subunit rotates in the CW direction around the dimer of the B subunits and the rotation is transmitted to the rotor C-ring. In other words, the A subunits and the C-ring function like a small gear and a large gear, respectively. The outer and inner teeth (A subunits) of the small gear engage during CCW rotation and CW rotation, respectively (Fig. 5). The rotational direction is determined by which side of the stator gear, outer or inner, engages according to the change in rotor size of the large gear.

In this study, we propose that the stator plays an important role in rotational switching of the flagellar motor. The mutations at the position of PomA-L93 or L95 decreased the switching frequency and biased to CCW direction, indicating inhibition of the stator in switching from ring gear to internal gear of the rotor. This hypothesis is supported by the motility property exhibited by a combination of switching frequency-decreased PomA mutation and CW-biased FliG-Q147H mutation. The resultant mutants with FliG-Q147H showed more CW-biased rotation, but still showed fewer switching events than the sole FliG Q147H mutant. This suggests that the mutation at the position of PomA-L93 or L95 inhibits the shift of the stator from switching the inner teeth to the outer teeth of the small gear, not allowing interaction with the large gear of the rotor when the rotor ring adopts the CW-type conformation. How are PomA-L93 and L95 involved in switching rotor gears? When PomA-L93 was substituted with the charged residues, the switching was affected, implying that the charged residues at the position of PomA-L93 participate in the electrostatic interaction of the rotor gear teeth, in addition to PomA-R88, K89, and E96 which are thought to be residues of a part of gear teeth. Because the position of PomA-L95 is located inside the stator structure like the tooth flank, the charged residues at this position may change the local conformation of the interaction surface with the rotor. These results suggest that to achieve the electrostatic interaction for stator assembly and torque generation, the conserved hydrophobic residues may act as effective insulators.

We obtained swimming ring-restored mutants in the soft agar plate from the cells expressing PomA-L93E, L95E, or L95R, to identify the factors involved in switching frequency and ratio of CCW/CW rotation.

Analysis considered two perspectives: video analysis to characterize motility properties and genomic analysis to identify the position of the mutation. L95Rs3 and L95Rs4 cells showed significant recovery of switching frequency and ratio of CCW/CW rotation. The putative suppressor mutation in L95Rs3 caused a frame shift by a 1 bp deletion in the β -subunit of ATP synthase, probably inducing loss-of-function of the ATP synthase. However, other mutations in components of ATP synthase induced no significant changes in the switching frequency and ratio of CCW/CW rotation. Previous studies have shown that mutations in *uncB*, which encodes the α -subunit of ATP synthase, induced increased motility in soft agar plates^{34,35}. It has been suggested that defects in ATP synthase increase the proton gradient generated by the respiratory chain and eventually increase the sodium-motive force. Therefore, it is more likely that the swimming ring-restored mutants with the mutation in the component of ATP synthase allow motility to be restored by increasing the motive force and continuous swimming time, rather than by directly reversing the defect on switching frequency caused by the mutation. Whole genome sequence analysis showed that the putative suppressor mutations occurred in genes encoding ATP synthase, an ATPase of the Type II secretion system, such as transketolase 2. It is not known whether these mutations are directly involved in motility. Previous studies have reported that the β -subunit of ATP synthase interacts with FlhG³⁶. Therefore, it may be possible to identify a novel motor-interacting protein by examining ATP synthase. We propose that these proteins affect the condition of the membrane or intracellular metabolic states and eventually influence the interaction between PomA and FlhG.

In this study, we demonstrated for the first time that mutations in the stator result in defective motor switching. This finding suggests that proper switching of the interaction between the stator and rotor is critical for controlling the rotational direction. If the electrostatic interaction becomes too strong due to the additional charge of the mutation, changing the interaction site between the stator and rotor becomes difficult. On the other hand, if the interaction is too weak, the gear of the stator and rotor is easy to release, resulting in a rotation defect of the motor.

Declarations

Author contributions:

H.T. and M.H. designed research; H.T., K.H., K.I. M.H. and S.K. performed research; H.T., K.H., K.I. and M.H. analyzed data; and H.T., M.H. and S.K. wrote the paper.

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References

1. Porter, S. L., Wadhams, G. H. & Armitage, J. P. Signal processing in complex chemotaxis pathways. *Nat. Rev. Microbiol*, **9**, 153–165 (2011).
2. Zhu, S., Kojima, S. & Homma, M. Structure, gene regulation and environmental response of flagella in *Vibrio*. *Front. Microbiol*, **4**, 410 (2013).
3. Silverman, M., Matsumura, P. & Simon, M. The identification of the mot gene product with *Escherichia coli*-lambda hybrids. *Proc. Natl. Acad. Sci. USA* **73**, 3126–3130(1976).
4. Asai, Y. *et al.* Putative channel components for the fast-rotating sodium-driven flagellar motor of a marine bacterium. *J. Bacteriol*, **179**, 5104–5110 (1997).
5. Santiveri, M. *et al.* Structure and Function of Stator Units of the Bacterial Flagellar Motor., **183**, 244–257 (2020).
6. Deme, J. C. *et al.* Structures of the stator complex that drives rotation of the bacterial flagellum. *Nat. Microbiol*, **5**, 1553–1564 (2020).
7. Blair, D. F. Flagellar movement driven by proton translocation. *FEBS Lett*, **545**, 86–95 (2003).
8. Takekawa, N., Imada, K. & Homma, M. Structure and energy-conversion mechanism of bacterial Na⁺-driven flagellar motor. *Trends Microbiol.*(2020).
9. Yakushi, T., Yang, J., Fukuoka, H., Homma, M. & Blair, D. F. Roles of charged residues of rotor and stator in flagellar rotation: comparative study using H⁺-driven and Na⁺-driven motors in *Escherichia coli*. *J. Bacteriol*, **188**, 1466–1472 (2006).
10. Takekawa, N., Kojima, S. & Homma, M. Contribution of many charged residues at the stator-rotor interface of the Na⁺-driven flagellar motor to torque generation in *Vibrio alginolyticus*. *J. Bacteriol*, **196**, 1377–1385 (2014).
11. Zhou, J. D., Lloyd, S. A. & Blair, D. F. Electrostatic interactions between rotor and stator in the bacterial flagellar motor. *Proc. Natl. Acad. Sci. USA* **95**, 6436–6441(1998).
12. Lloyd, S. A. & Blair, D. F. Charged residues of the rotor protein FliG essential for torque generation in the flagellar motor of *Escherichia coli*. *J. Mol. Biol*, **266**, 733–744 (1997).
13. Zhou, J. D. & Blair, D. F. Residues of the cytoplasmic domain of MotA essential for torque generation in the bacterial flagellar motor. *J. Mol. Biol*, **273**, 428–439 (1997).
14. Terashima, H., Kojima, S. & Homma, M. Site-directed crosslinking identifies the stator-rotor interaction surfaces in a hybrid bacterial flagellar motor. *J. Bacteriol*, **9**, e00016–00021 (2021).
15. Carroll, B. L. *et al.* The flagellar motor of *Vibrio alginolyticus* undergoes major structural remodeling during rotational switching. *eLife*(2020).
16. Yamaguchi, S. *et al.* Genetic evidence for a switching and energy-transducing complex in the flagellar motor of *Salmonella typhimurium*. *J. Bacteriol*, **168**, 1172–1179 (1986).
17. Francis, N. R., Sosinsky, G. E., Thomas, D. & Derosier, D. J. Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. *J. Mol. Biol*, **235**, 1261–1270 (1994).
18. Kawamoto, A. *et al.* Native flagellar MS ring is formed by 34 subunits with 23-fold and 11-fold subsymmetries. *Nat. Commun*, **12**, 4223 (2021).

19. Takekawa, N. *et al.* Two distinct conformations in 34 FliF subunits generate three different symmetries within the flagellar MS-ring. *mBio*, **12**, e03199–03120 (2021).
20. Zhao, R. B., Amsler, C. D., Matsumura, P. & Khan, S. FliG and FliM distribution in the Salmonella typhimurium cell and flagellar basal bodies. *J. Bacteriol*, **178**, 258–265 (1996).
21. Zhao, R. H., Pathak, N., Jaffe, H., Reese, T. S. & Khan, S. FliN is a major structural protein of the C-ring in the Salmonella typhimurium flagellar basal body. *J. Mol. Biol*, **261**, 195–208 (1996).
22. Francis, N. R., Irikura, V. M., Yamaguchi, S., DeRosier, D. J. & Macnab, R. M. Localization of the Salmonella typhimurium flagellar switch protein FliG to the cytoplasmic M-ring face of the basal body. *Proc. Natl. Acad. Sci. USA* **89**, 6304–6308(1992).
23. Hess, J. F. & Bourret, R. B. & Simon, M. I. Histidine phosphorylation and phosphoryl group transfer in bacterial chemotaxis. *Nature*, **336**, 139–143 (1988).
24. Hess, J. F., Oosawa, K., Kaplan, N. & Simon M. I. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis., **53**, 79–87 (1988).
25. Welch, M., Oosawa, K., Aizawa, S. I. & Eisenbach, M. Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. *Proc. Natl. Acad. Sci. USA* **90**, 8787–8791(1993).
26. Toker, A. S. & Macnab, R. M. Distinct regions of bacterial flagellar switch protein FliM interact with FliG, FliN and CheY. *J. Mol. Biol*, **273**, 623–634 (1997).
27. Bren, A. & Eisenbach, M. The N terminus of the flagellar switch protein, FliM, is the binding domain for the chemotactic response regulator, CheY. *J. Mol. Biol*, **278**, 507–514 (1998).
28. Sarkar, M. K., Paul, K. & Blair, D. Chemotaxis signaling protein CheY binds to the rotor protein FliN to control the direction of flagellar rotation in Escherichia coli. *Proc. Natl. Acad. Sci. USA* **107**, 9370–9375(2010).
29. Lee, S. Y. *et al.* Crystal structure of an activated response regulator bound to its target. *Nat. Struct. Biol*, **8**, 52–56 (2001).
30. Wheatley, P. *et al.* Allosteric priming of E. coli CheY by the flagellar motor protein FliM. *Biophys. J*, **119**, 1108–1122 (2020).
31. Paul, K., Brunstetter, D., Titen, S. & Blair, D. F. A molecular mechanism of direction switching in the flagellar motor of Escherichia coli. *Proc. Natl. Acad. Sci. USA* **108**, 17171–17176(2011).
32. Takekawa, N. *et al.* ZomB is essential for chemotaxis of *Vibrio alginolyticus* by the rotational direction control of the polar flagellar motor. *bioRxiv*, doi: <https://doi.org/10.1101/2021.07.06.451403>
33. Chang, Y. *et al.* Molecular Mechanism for Rotational Switching of the Bacterial Flagellar Motor. *Nat. Struct. Mol. Biol*, **27**, 1041–1047 (2020).
34. Terashima, H., Kojima, S. & Homma, M. Functional transfer of an essential aspartate for the ion-binding site in the stator proteins of the bacterial flagellar motor. *J. Mol. Biol*, **397**, 689–696 (2010).
35. Terashima, H. *et al.* Mutation in the a-subunit of F1FO-ATPase causes an increased motility phenotype through the sodium-driven flagella of Vibrio. *J. Biochem*, **154**, 177–184 (2013).

36. Zarbiv, G. *et al.* Energy complexes are apparently associated with the switch-motor complex of bacterial flagella. *J. Mol. Biol*, **416**, 192–207 (2012).

37. Kawagishi, I., Okunishi, I., Homma, M. & Imae, Y. Removal of the periplasmic DNase before electroporation enhances efficiency of transformation in a marine bacterium *Vibrio alginolyticus*., **140**, 2355–2361 (1994).

38. Okunishi, I., Kawagishi, I. & Homma, M. Cloning and characterization of *motY*, a gene coding for a component of the sodium-driven flagellar motor in *Vibrio alginolyticus*. *J. Bacteriol*, **178**, 2409–2415 (1996).

39. Deatherage, D. E. & Barrick, J. E. Identification of mutations in laboratory-evolved microbes from next-generation sequencing data using bresEq. *Methods Mol Biol*, **1151**, 165–188 (2014).

Figures

Figure 1

Motility and protein stability of PomA F92, L93 and L95 mutants. (A) Motility of *V. alginolyticus* ΔpomAB strain NMB191 cells expressing PomA and PomB from the plasmid pHFAB in a soft-agar plate. The cells were inoculated in VPG 0.25% (w/v) agar plate with 0.02% (w/v) arabinose and chloramphenicol at 30 °C for 4 h. (B) Detection of the mutant *V. alginolyticus* PomA and PomB, expressed from the plasmid pHFAB in the NMB191 cells. The sample for SDS-PAGE was prepared from whole cell lysates. Upper and lower panels showed immunoblot images by using anti-PomA and anti-PomB antibodies, respectively.

Figure 2

The profile of the switching in rotation direction of PomA mutants. *V. alginolyticus* ΔpomAB strain NMB191 cells expressing PomA and PomB, from the plasmid pHFAB, and the mutant plasmids were observed by high-intensity dark-field microscopy or by standard dark-field microscopy. (A) The cells were video-recorded four times, then swimming fraction of the cells was calculated by dividing the number of the motile cells into the number of the total cells. All experiments were performed three times, and the average and SD of swimming fraction were calculated. (B) The cells were video-recorded at 30 frames/s for 5 s, the swimming speed of the individual 10 cells measured, then the average and SD of the

swimming speed were calculated. The ratio of CCW/CW rotation (C) and the switching frequency (D) were calculated from video motion of the cells captured for 10 s. At least 10 cells were tracked in all the experiments, then average of the ratio of CCW/CW rotation with SD, and average of switching events with SD, was calculated. Asterisks indicated $P < 0.05$ (*) and $P < 0.01$ (**) for wild-type PomA versus the mutants of PomA by the Welch's t-test.

Figure 3

The effect of phenol for the switching of rotation direction. The switching frequency was calculated similarly to Fig. 2D, from video motion of the cells captured for 10 s, with and without phenol.

Figure 4

The profile of switching and rotation direction bias of PomA/FliG double mutants. *V. alginolyticus* Δ pomAB and fliG strain NMB301 cells expressing PomA and PomB from the plasmid pYA303, and FliG from the plasmid pNT1, were observed by high-intensity dark-field microscopy. The ratio of CCW/CW rotation (A) and the switching frequency (B) were calculated from video motion of the cells captured for 10 s. At least five cells were tracked in all the experiments, then average of the ratio of CCW/CW rotation with SD was calculated. Asterisks indicated $P < 0.05$ (*) and $P < 0.01$ (**) for wild-type PomA versus the mutants of PomA by the Welch's t-test.

Figure 5

Switching model of flagellar motor rotation. The stator rotational force is transmitted to the rotor through a gear motion. In the default, the rotor rotates with counter-clockwise direction engaged by the stator rotation with clockwise direction. The rotor interacts with the inner tooth of stator gear for CCW rotation and changes with the outer tooth of stator gear to CW rotation. When the rotor receives the switching signal by CheY-P, the rotor size expands for the CW structure.

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