Structure of Vibrio FliL, a New Stomatin-like Protein That Assists the Bacterial Flagellar Motor Function

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ABSTRACT Many motile bacteria swim or swarm using a filamentous rotating organelle, the flagellum. FliL, a component protein of the flagellar motor, is known to enhance the motor performance under high-load conditions in some bacteria. Here, we determined the structure of the periplasmic region of FliL (FliLPeri) of the polar flagellum of Vibrio alginolyticus. FliLPeri shows a remarkable structural similarity to the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain of stomatin family proteins, some of which are involved in modulation of ion channel activities in various organisms. FliLPeri forms a ring assembly in the crystal with an inner diameter of around 8 nm, which is comparable to the size of the stator unit. Mutational analyses suggest that the FliL ring forms a complex with the stator unit and that the length of the periplasmic linkers of FliL and the stator B-subunit is essential for the complex formation. We propose a model of the FliL-stator complex to discuss how Vibrio FliL modulates stator function in the bacterial flagellar motor under conditions of high viscosity.

IMPORTANCE Some flagellated bacteria regulate motor torque in response to the external load change. This behavior is critical for survival, but the mechanism has remained unknown. Here, we focused on a key protein, FliL of Vibrio alginolyticus, and solved the crystal structure of its periplasmic region (FliLPeri). FliLPeri reveals striking structural similarity to a conserved domain of stomatin, which is involved in ion channel regulation in some organisms, including mammals. FliLPeri forms a ring with an inner diameter that is comparable in size to the stator unit. The mutational analyses suggested that the presence of the ring-like assembly of FliL around the stator unit enhances the surface swarming of Vibrio cells. Our study data also imply that the structural element for the ion channel regulation is conserved from bacteria to mammals.

KEYWORDS bacterial flagellar motor, crystal structure, mechanosensor, stator, stomatin

Many bacteria move in physically distinct environments, such as soil, mucus, host cell surface, and planktonic environments, for survival in nature using the flagellum. The flagellum is one of the most common organelles for bacterial motility and consists of a filamentous propeller structure protruding from the cell surface and a rotational motor embedded in the cell envelope. To adjust their motility to the various physical environments, some bacteria, such as Escherichia coli and Bacillus subtilis, modify the flagellar motor power according to the external load (1–3). However, how they sense the external condition and optimize the motor output to the environment is not well understood.
The flagellar motor is made up of about 20 different types of proteins and is composed of two functionally distinct parts, the rotor and the stator. The motor is driven by electrochemical potential across the cell membrane. Coupling ion flow (e.g., H\(^+\), Na\(^+\), or K\(^+\) flow) through a channel in the stator induces the rotor-stator interaction to generate torque (4–6). The rotor consists of the rod, the MS-ring and the C-ring. The MS-ring is embedded in the cell membrane, and the C-ring is located beneath the MS-ring. The MS-ring is a homomultimer of a two-transmembrane protein, FliF. The C-ring is composed of three cytoplasmic soluble proteins, FliG, FliM, and FliN. The stator is a heterohexameric complex composed of four A-subunits and two B-subunits, PomA\(_4\)PomB\(_2\) in the Na\(^+\)-driven polar flagellar motor of *Vibrio alginolyticus* and MotA\(_4\)MotB\(_2\) in the H\(^+\)-driven flagellar motor of *E. coli* and *Salmonella* and the lateral flagellar motor of *V. alginolyticus* (7, 8). Up to about a dozen of stator units surround the rotor (9, 10).

The motor torque is generated by electrostatic interaction between FliG and the stator A-subunit (11). The stator B-subunit interacts with the peptidoglycan layer via the periplasmic region to anchor the stator unit (12, 13). The periplasmic region of the B-subunit contains a plug region that regulates the ion influx through the stator (14). The stator units are not static but dynamically assemble into and disassemble from the motor (11, 15). Although each single stator unit generates enough torque to rotate the filament under low-load conditions, a number of stators assemble around the rotor under high-load conditions to generate sufficient torque, suggesting that the flagellar motor somehow senses the load and adjusts its torque to the load by changing the number of stators around the rotor (1–3, 16, 17). A recent mutational study showed that the long cytoplasmic loop region of the A-subunit is responsible for the load-dependent assembly of the stator unit in *Salmonella* (18).

A flagellar protein, FliL, which is a single-transmembrane protein with a large periplasmic region and associates with the flagellar basal body, has been found to be important for the function of the motor under high-load conditions, such as the highly viscous environments in some species. FliL is essential for the swimming of *Caulobacter crescentus* and *Rhodobacter sphaeroides* and for the surface swarming of *E. coli* and *Salmonella*, whereas deletion of FliL does not strongly affect the swimming motility of *E. coli*, *Salmonella*, *B. subtilis*, *Borrelia burgdorferi*, *Proteus mirabilis*, and *V. alginolyticus* (19–26). A cryo-electron tomography analysis of *B. burgdorferi* showed that FliL is located close to the rotor and the stator (22). FliL interacts with various motor components, such as the stator proteins, the MS-ring protein FliF, and the periplasmic ring component FlgT (20, 21, 24, 27). Among them, the interaction with the stator seems to be most important for the function of FliL because some of the swimming defects caused by *fliL* mutations were suppressed by mutations in the plug region of the stator B-subunit (21, 24). Moreover, FliL did not assemble into the motor without the stator (25). Other mutational studies revealed various roles of FliL in addition to the motor function, including stabilization of the rod structure in the motor, correct positioning of the flagellum, and surface sensing (20, 22, 23, 28). However, most of the phenotypes of these mutants were diverse and species specific. Recently, a contradictory result has been reported that indicated that the torque of the *E. coli* motor is not changed by the deletion of FliL (29). Thus, the specific function of FliL is rather unclear.

In this study, we performed structural and mutational analyses of FliL to elucidate its molecular mechanism in *V. alginolyticus*. This bacterium has two different sets of flagella, i.e., a polar flagellum and multiple lateral flagella, and each set of the flagella has its own *fliL* gene. The polar flagellum is used for swimming in low-viscosity liquid (e.g., seawater), whereas the lateral flagella are used for surface swarming under conditions of very high loads (e.g., fish mucus) (30). Deletion of polar *fliL* did not strongly affect the swimming motility provided by polar flagellum under low-load conditions but reduced the swimming speed provided by polar flagellum in high-load conditions (25). On the other hand, the function of lateral FliL is unknown. Here we determined the structure of the periplasmic region of polar FliL (FliL\(_{\text{peri}}\)). FliL\(_{\text{peri}}\) forms a ring or a helical assembly in crystal, and the subunit structure resembles the SPFH
domain of the stomatin superfamily, which includes proteins involved in mechanosensory transduction and ion channel regulation, implying that FliL is involved in the regulation of the ion-channel activity of the stator in response to some kinds of mechanical signals. We also found that FliL is essential for the swarming provided by lateral flagellar motors. On the basis of the structure and the following mutational analyses, we propose a model for how FliL associates with the stator and regulates the flagellar motor function.

RESULTS

The length of the flexible linker of PomB is important for the FliL localization around the motor. FliL interacts with the stator proteins and localizes at the motor. Deletion of the stator genes, i.e., pomA and pomB, completely disrupted the localization of polar FliL at the polar flagellar motor in *V. alginolyticus* (25). Here we introduced internal deletion mutations in the periplasmic flexible linker (residues 60 to 121) of PomB (Fig. 1A and B). It has been shown that the flexible linker region is not essential for the motor function but involved in motor efficiency (31). We prepared a series of
in-frame deletion mutants which lacked 20 amino acid residues in the linker region and observed cell motility as well as the intracellular localization of polar FilL fused with green fluorescent protein (GFP) at its cytoplasmic N terminus (GFP-FilL) by fluorescence microscopy (Fig. 1C). As described previously, GFP-FilL was detected as a dot on the flagellated cell pole in the strain expressing wild-type PomB (Fig. 1C) (25). In contrast, GFP-FilL was not localized at the cell pole in all of the in-frame deletion mutants of PomB, even though these mutants showed swimming rings that were similar in size to those seen with the wild type in a soft-agar plate (Fig. 1D). These results indicate that the linker region of PomB is essential for FilL to localize at the motor and suggest that it is not a specific region but the length of the linker that is important for the FilL localization.

The periplasmic region of FilL consists of the N-terminal linker and the C-terminal core region. Polar FilL is composed of an N-terminal short cytoplasmic region (residues 1 to 17), a single transmembrane region (TM) (residues 18 to 40), and a periplasmic region (FilLPeri) (residues 41 to 167) (25). To determine the core region of FilLPeri, we performed limited proteolysis of purified FilLPeri with trypsin. FilLPeri was immediately digested into a fragment composed of residue 58 to 167 that is named FilLC, and FilLC is highly resistant to trypsin digestion (see Fig. S1A to C in the supplemental material). Thus, we concluded that FilLC is a core region of FilLPeri and that the N-terminal 17 residues (residues 41 to 58; FilLL) act as a linker connecting the TM region with FilLC (Fig. 1E). To evaluate the importance of FilLL to the FilL function, we prepared mutants with various deletions in FilLL and observed the FilL localization at the cell pole (Fig. 1F and G). FilLΔ40-59 did not localize at the cell pole, whereas FilLΔ40-49, FilLΔ46-55, and FilLΔ50-59 did. These results indicate that FilLL is essential for the FilL localization but suggest that any 7 of the 17 residues are sufficient.

Structure of the periplasmic region of FilL. We crystallized and determined the structures of FilLC and FilLPeri at 2.1-Å and 3.4-Å resolution, respectively (Fig. 2A to D; see also Fig. S2A and B and Table S1 in the supplemental material). The initial phase was obtained by molecular replacement using a FilLC structure predicted by the Robetta server (32), because no Se-Met-labeled crystals or heavy-atom-derivative crystals were obtained. The FilLC crystal belongs to the space group of P61 and contains two molecules in an asymmetric unit. The final refinement R factor and the free R factor values were 20.6% and 25.8%, respectively. FilLC consists of a single domain with a bean shape containing four α-helices (α1 to α4) and one β-sheet composed of four β-strands (β1 to β4). The β-sheet is highly twisted at the middle and therefore can be divided into two subsheets, subsheet 1 (S1) and S2 (Fig. 2B). S1 consists of β1, β3, and β4, and S2 is composed of β2, β3, and β4. The FilLPeri crystal belongs to the space group of P42212 and contains 20 molecules in an asymmetric unit. FilLPeri is 17 residues longer at its N terminus than FilLC, but these residues are invisible in the electron density map of FilLPeri. Therefore, we determined the structure of FilLPeri with the same region as that of FilLC. All molecules in the asymmetric units of both FilLC and FilLPeri crystals showed no significant structural difference except for slight differences in the N-terminal region (residues 58 to 63) and in the loop between β2 and β3 (residues 77 to 84) (Fig. S2C).

FilLPeri forms a ring complex. FilLPeri forms a head-to-head stack of two decameric rings in the crystal asymmetric unit (Fig. 2C). The bean-shaped subunits are vertically arranged in a parallel manner in the ring. The two rings weakly interact with each other through α1. The N terminus locates near the ring-to-ring interface and points toward the outer side of the ring. The C terminus of each subunit is arranged on the inner surface of the ring on the opposite side of the ring-to-ring interface. The loop between β2 and β3 surrounding the hole of the ring contributes to the crystal contacts with the neighboring ring related by crystallographic symmetry. Since the N terminus of FilLPeri is connected to the transmembrane helix, the decameric ring may be formed on the inner membrane (Fig. S2D).

The side-by-side subunit interaction in the ring is primarily hydrophobic. The subunit interface is formed by characteristic hydrophobic belts on both sides of the
subunit along the longitudinal direction of the molecule (Fig. 2E and F; see also Fig. S2E). P108, L109, and L115 on N9251 form a hydrophobic belt on one side of the subunit surface, and Y66 and M93 on subsheet S1 and L160, F161, and F164 on N9252 form another on the opposite surface (Fig. 2F; see also Fig. S2E). L109, L115, and L160 contribute to the intersubunit mainly by hydrophobic interaction. L115 interacts with F161 and F164. L109 tightly interacts with L160 and slightly with M93 and Y66. The side-by-side interaction is further stabilized by hydrogen bonds around the hydrophobic belts (Fig. S2E). N76 forms a hydrogen bond with the main-chain NH group of I166. Q71, Y105, and D163 form hydrogen bonds with the main-chain carbonyl group consisting of T162, I64, and V74, respectively. The side chain of H106 forms hydrogen bonds with the OH group of Y66. Similar subunit interactions can be observed in the FliL crystal. The two molecules in the asymmetric unit of the FliL crystal are related by 30° rotation around the crystallographic 61 screw axis with a translation of 1/12 of the repeat along the c axis; thus, the molecules form a helical tube with 121 screw symmetry along the c axis in the FliL crystal (Fig. 2D). The interaction between the subunits related by the 121 screw symmetry is almost the same as that in the FliLperi ring except for the lack of hydrogen bonds between T162 and Q71 and between D163 and V74. The neighboring subunit in the FliLperi crystal is slightly tilted compared with that in the FliLperi ring, resulting in the formation of the helical tube rather than the ring (Fig. S2F).

The common side-by-side subunit interaction implies that assembly formation through the hydrophobic belt is important for the FliL function. To analyze the

FIG 2 Structure of the periplasmic region of FliL. (A) Schematic representation of the molecular architecture of FliL, FliLperi, and FliLC. (B) Ribbon drawing of FliLperi (FliLperi) colored as a rainbow from the N terminus (blue) to the C terminus (red). (C and D) Higher-order structure of FliLC in crystal. A ribbon representation of the two decameric rings in the P42212 crystal (C) and the 121 helical assembly in the P61 crystal (D) are shown. A single subunit is colored red. (E) Mapping of the mutation sites that disrupt the FliL assembly. L109, L115, and L160 are indicated by red balls. (F) Cut-open view of the subunit interface indicated by the blue dashed line in panel E. L109, L115, and L160 are colored in orange, and other hydrophobic residues are colored in yellow. (G) Fluorescence images of cells expressing GFP-FliL or GFP-FilL with the L109D, L115K, or L160D mutation.
significance of the hydrophobic interaction, we replaced the residues that mainly contribute to the hydrophobic interaction with hydrophilic residues (L109D, L115K, or L160D) and observed the localization of GFP-FliL (Fig. 2E to G). The polar localization of GFP-FliL was completely disrupted by these mutations. Other mutations of the residues on the outer, top, and bottom surfaces of the FliL ring did not affect the polar localization of FliL (Fig. S3A and B). We further confirmed that FliL interacts with itself without any help from other flagellar proteins in the cell by two-hybrid assay (Fig. S3C). These results indicate that the FliL ring formation by the hydrophobic interaction is essential for the FliL assembly into the motor.

The FliLC structure resembles the SPFH domain of stomatin. The Dali database search revealed that FliLC shows remarkable structural similarity (Z-score of 9.5) to the stomatin/prohibitin/flotillin/HflK/C (SPFH) domain of stomatin from Mus musculus (Mm-stomatin) (Fig. 3A and B) (PDB identifier [ID]: 4FVJ) (33), although the amino acid sequence identity is only 17% (Fig. 3C). The SPFH domain is conserved in various membrane-associated proteins of eukaryotes, archaea, and bacteria, such as stomatin, prohibitin, flotillin, HflK/C, and podocin (34). The SPFH domain is composed of four alpha helices and a highly twisted beta-sheet, and its folding topology is exactly the same as that of FliLC (Fig. 3B and C).
The SPFH domain is often involved in multimer formation of the SPFH superfamily proteins. In spite of the structural similarity, the subunit arrangement and the subunit interface in the FliLC multimer differ from those in the SPFH family protein multimer. The Mm-stomatin assembles into a tail-to-tail dimer by forming an intersubunit β-sheet via the C-terminal β-strands (Fig. S4A) (33). However, FliLC does not form the tail-to-tail dimer. The Mm-stomatin dimers form a helical higher-order assembly in the crystal through the interaction between the N-terminal loop and the hydrophobic surface of the neighboring subunit. Although FliLC also forms a helical assembly in the P6₁ crystal, the FliLC assembly is formed by the lateral subunit interaction and the corresponding hydrophobic surface of FliLC is not accessible because it is covered by the N-terminal β-strand (Fig. 3B). The stomatin from Pyrococcus horikoshii (Ph-stomatin) assembles into a trimer formed by head-to-tail interaction of the unique N-terminal β-strand, β₀, with the C-terminal β-strand of the neighboring subunit (Fig. S4B) (35). However, FliLC does not form a trimer and does not have the counterpart of β₀. The SPFH domains of the major vault protein are laterally arranged in the vault particle in a manner similar to that seen with the FliLC subunits in the ring structure, but the interaction surface to the neighboring subunit is different (Fig. S4C) (36). Therefore, the positions of the neighboring molecules differ by about 60 degrees.

**DISCUSSION**

The flexible linker of the stator B-subunit following the plug region is not essential for the motor rotation (31) but is necessary to control the number of active stator units around the rotor in response to the external load (3, 16). Since the linker in PomB is required for the FliL localization at the motor, FliL is expected to directly interact with the PomB linker region to regulate the motor output. On the other hand, all of the mutants with 20-residue deletions in various regions of the PomB linker lost the FliL localization ability at the motor. This suggests that FliL does not interact with a specific region of the PomB linker and that the length of the linker is important for the interaction with FliL. FliL also has a flexible linker (FliL₁) that is essential for the FliL localization. FliL₁ connects the transmembrane helix with FliLₐ. The analysis of various deletion mutants in FliL₁ suggests that it is not a specific region in FliL₁ but the length of the linker that is critical for the FliL localization, as in the case of the PomB linker. It is probable that the positional flexibility of the periplasmic domains of PomB and FliL attributed to the linker regions with appropriate length is needed for the PomB-FliL interaction.

We have determined the structure of the periplasmic core region of polar FliL (FliLₐ). FliLₐ shows remarkable structural similarity to the SPFH domain despite the low similarity of amino acid sequence (Fig. 3B and C). Although the physiological function of the SPFH domain has not yet been well characterized, some of the SPFH family proteins are known to interact with various ion channels and transporters to modulate their activities (33, 37, 38). These proteins are involved in mechanosensory transduction in mammalian sensory neurons and in mechanosensor formation in Caenorhabditis elegans (39, 40), but the mechanosensing mechanism is still unknown (41). The structural similarity between FliLₐ and the SPFH domain implies that FliL is involved in mechanosensing by the flagellum and in regulation of the channel activity of the stator. In fact, FliL of P. mirabilis is involved in the mechanosensing pathway (23, 28). It is possible that FliL and these SPFH family proteins share a mechanism for mechanosensing and mechanoresponse.

The periplasmic domain of FliL forms a decameric ring or a helical tube complex with 12 screw symmetry in the crystals, and the two complexes share similar lateral subunit interactions. The mutations of the hydrophobic residues on the subunit interface to hydrophilic ones disrupted the FliL localization at the motor. This suggests that FliL forms a ring-like oligomer on the cell membrane (see Fig. S2D in the supplemental material) and that the oligomerization is essential for the function of FliL. The idea of the assembly formation of FliL is also supported by the results of two-hybrid assays (Fig. S3C). Moreover, it is consistent with our previous reports of findings.
indicating that the periplasmic region of FliL oligomerizes at high concentrations (42). Similar ring structures have been reported in the SPFH family proteins, such as human stomatin and mitochondrial prohibitin of *Saccharomyces cerevisiae* (43, 44). They form ring complexes composed of 9 to 12 subunits on the cell membrane. On the other hand, some other SPFH family proteins whose structures are known have shown diverse assembly states and subunit arrangements in multimer (Fig. S4). Mm-stomatin assembles into a tubular complex composed of antiparallel dimers (Fig. S4A) (33), and Ph-stomatin forms a triangular complex of three molecules (Fig. S4B) (35). The SPFH domain of the rat liver vault forms a 39-fold ring through the lateral interaction in the vault (Fig. S4C) (36). The subunit interactions of FliLC completely differ in those SPFH domains (Fig. 2C and D). The SPFH domains do not have the hydrophobic belts on the interface, which is important for the ring formation of FliL (Fig. S4D). The subunit arrangement and the interaction manner of the SPFH domains may be optimized for their individual function and/or their regulation partner proteins.

FliL modulates the motor torque by direct interaction with stator proteins in *E. coli* and *Salmonella* (24) and requires the stator to localize at the motor in *V. alginolyticus* (25). A motility defect in *R. sphaeroides* caused by the *fliL* mutation was suppressed by mutation in the plug region of MotB (21). These lines of evidence suggest that FliL forms a complex with the stator. Our crystal structure revealed that FliLC forms a ring with an inner diameter of around 8 nm (Fig. 2C), which is a size comparable to that of the stator unit. We then built a FliL-stator complex model by fitting the FliL ring structure to the stator model constructed by the electron microscopy (EM) density of MotA at 25-Å resolution (EMDB ID: 3417) (45) and the crystal structure of the periplasmic domain of PomB (PDB ID: 3WPW) (46). The stator was nicely accommodated within the FliL ring (Fig. 4A), and the plug region of the stator B-subunit locateds in close proximity to the inner surface of the FliL ring (Fig. 4B).

This model is supported by results of a mutation analysis of the residues on the inner surface of the ring (Fig. 4C to F). We replaced conserved valine residues on the inner surface of the FliL ring (V74 and V165) with hydrophilic ones and analyzed the FliL localization. These mutations did not affect the GFP-FliL localization (Fig. 4D). We next replaced these valine residues with p-benzoyl-L-phenylalanine (pBpa), which is a photoreactive unnatural amino acid and forms a covalent bond with nearby protein molecules upon UV irradiation. The mutated FliL protein was coexpressed with *V. alginolyticus* PomA/PomB proteins in *E. coli* BL21(DE3) cells, and UV light was used for irradiation of the cells for photo-cross-linking. The mutated FliL was cross-linked to PomB by the use of UV light (Fig. 4E), indicating that V74 and V165 of FliL are close to PomB in *vivo*. Since *E. coli* BL21(DE3) cells have no flagellum, this result suggests that FliL can assemble around the stator without the flagellar basal body. We further examined the effect of a corresponding mutation of lateral FliL on the surface motility under high-load conditions. Deletion of lateral *fliL* in the YM19 strain, which forms only lateral flagella, resulted in loss of swarming ability on a 1.25% agar plate (Fig. 4F; see also Fig. S5A) but did not strongly affect swimming in a 0.25% agar plate (Fig. S5A). We confirmed that lateral flagellar formation was not affected by the deletion of *fliL* in the wild-type strain (strain 138-2) by electron microscopy and Western blotting (Fig. S5B and C). The replacement of the corresponding valine residues of lateral FliL (V73 and V164) with the hydrophilic ones disrupted the swimming motility by lateral flagella (Fig. 4F). These results suggest that the conserved valine residues, located at the inner side of the FliL ring, are not essential for the complex formation with the stator but are close to PomB and important for activation of the stator in the swarming cells.

A recent fluorescence microscopic analysis using FliL fused with GFP at its N terminus (GFP-FliL) suggested that the stoichiometry of a stator unit and FliL protein in a functional motor would be 1:1 (47), which is inconsistent with the model presented in this study. We found, however, that GFP-FliL impairs the motor function (Fig. S5D). The mutant cells expressing GFP-FliL reduced the motility less than the Δ*fliL* mutant cells. This motility reduction was suppressed by the L109D mutation or the L160D mutation, each of which disrupts the localization of FliL (Fig. S5D). It is possible that the...

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number of stators around the motor is reduced by the presence of GFP. Therefore, it would be difficult to estimate the number of FliL proteins in a functional motor using GFP-FliL.

A previous cryo-electron tomographic study of an in situ flagellar basal body of *B. burgdorferi* resulted in a report that FliL is located between the stator and the rotor (22). However, the stator unit of *B. burgdorferi* is flanked by a collar, which is a basal-body component specific for spirochetes. Therefore, we cannot directly compare the *Borrelia* basal body structure with that of *Vibrio*, but another extra density is present on the outer side of the stator density in their tomogram (22) which is consistent with our ring model.

On the basis of our results together with the previous reports, we propose a model for the regulation mechanism of motor output by FliL (Fig. 4B). The FliL molecules may surround the stator to form the FliL-stator complex at the motor. The conformational flexibility attributed to the linker regions of FliL and PomB may be important to adjust each subunit in the appropriate position to form the FliL-stator complex. The inner-surface residues of the FliL ring may then interact with the periplasmic region of PomB, presumably representing the plug region that is present to keep the ion influx, to increase the motor torque, especially under swarming conditions. The structural simi-
larity to the SPFH domains of the stomatin family proteins implies that FliL may regulate the ion channel activity of the stator in response to some mechanical signals. However, mechanosensing mechanism is still unknown for all stomatin family proteins. Another possible mechanism of regulation of motor output is that the FliL ring acts as the catch-bond proposed by Nord et al. (17). FliL may assist the binding of the B-subunit to the peptidoglycan layer under high-load conditions and may thus prevent dissociation of the stator unit from the motor. Further studies are required to clarify the regulation mechanism of motor output in response to external load as well as the load-sensing mechanism.

MATERIALS AND METHODS

Bacterial strains, media, and plasmids. The bacterial strains and plasmids used in this study are listed in Table S2 in the supplemental material. *V. alginolyticus* was cultured at 30°C in VC medium (0.5% [wt/vol] peptone, 0.5% [wt/vol] yeast extract, 0.4% [wt/vol] K₂HPO₄, 3% [wt/vol] NaCl, 0.2% [wt/vol] glucose) or VPG medium (1% [wt/vol] polypeptone, 0.4% [wt/vol] K₂HPO₄, 3% [wt/vol] NaCl, 0.5% [wt/vol] glycerol). If needed, chloramphenicol was added at a final concentration of 2.5 μg ml⁻¹ for *Vibrio* culture. *E. coli* was cultured in LB medium (Lennox; Nacalai Tesque, Kyoto, Japan). If needed, chloramphenicol or ampicillin was added at a final concentration of 25 μg ml⁻¹ or 50 μg ml⁻¹ for *E. coli* culture.

To construct the plasmids, PCR-amplified DNA fragments were cloned into each plasmid vector by using T4 DNA ligase (New England Biolabs) or in vivo cloning (48). To delete the *fliL* genes in *V. alginolyticus*, the upstream sequence (ca. 500 bp) and downstream sequence (ca. 500 bp) of the *fliL* gene were cloned into pSW7848 and homologous recombination was performed as previously described (49). Point mutations and internal deletion mutations in the plasmids were introduced by QuickChange site-directed mutagenesis (Agilent Technologies).

Transformation of *E. coli* was performed using a standard heat shock method. Transformation of *V. alginolyticus* was performed by the use of the electroporation method described below. Overnight cultures grown in VC medium were inoculated at a 100-fold dilution into 5 ml of VC medium and cultivated for 90 min at 30°C. The cells were harvested and suspended in 1 ml of ice-cold SNT buffer (30 mM Tris-HCl [pH 8.0], 20% [wt/vol] sucrose, 0.4 M NaCl, 1 mM EDTA), followed by gentle shaking for 10 min at 4°C. The cells were precipitated and washed by 1 ml of ice-cold SP buffer (14% [wt/vol] sucrose, 3.5 mM K₂HPO₄, 3.5 mM NaH₂PO₄). The cells were precipitated again, suspended in 40 μl of ice-cold SP buffer containing plasmid, and transferred into a 0.1-cm-gap electroporation cuvette. Electroporation was carried out with a gene pulser (MicroPulse Electroporator; Bio-Rad Laboratories, Hercules, CA, USA) at 7 kV cm⁻¹ of electric field strength. After electroporation, 1 ml of VC medium was added immediately and incubated at 30°C for 45 min. The cells were precipitated, spread on a selective plate containing antibiotics, and incubated overnight at 30°C.

Soft-agar and hard-agar plate assays for motility. A 2-μl volume of overnight culture was spotted onto VPG soft-agar plates (VPG containing 0.25% [wt/vol] agar) or VC hard-agar plates (VC containing 1.25% [wt/vol] agar) followed by incubation at 30°C for various time periods as described in the text. Motility ring diameters were measured relative to those of the wild-type cells. The averaged values and standard deviations of data from more than three independent experiments were plotted in the graphs.

Observation of subcellular localization of GFP-FliL with fluorescence microscopy. Overnight cultures grown in VC medium were inoculated at a 100-fold dilution into VPG medium containing 0.006% (wt/vol) arabinose and cultivated at 30°C. The cells were harvested and centrifuged and resuspended in TMNS500 buffer (50 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 5 mM glucose, 500 mM NaCl). The cells were then loaded into the space between a coverslip and a microscope slide, both of which are covered with polyl-lysine, and incubated for 10 min for attachment to the coverslip surface. After washing away unbound cells by the use of TMNS500 buffer, the cells were observed by the use of a fluorescence microscope (BX53; Olympus, Japan) equipped with a 100-W high-pressure mercury lamp (U-RFL-T; Olympus). Images were recorded using a charge-coupled-device (CCD) camera and an acquisition program (INFINITY2-1RM and INFINITY CAPTURE; Argo Corporation, Japan). Strain ZSW1 harboring pZSW6 was used for the observation of the FliL mutant cells, and strain ZSW2 harboring pZSW81 was used for the observation of the PomB mutant cells.

Purification of periplasmic fragments of FliL. Expression and purification of FliL_exn were performed as previously described (42). FliL_exn was prepared from FliL_exn by limited proteolysis (50). His₂FliL_exn, purified by nickel-nitrotriacetic acid (Ni-NTA) affinity chromatography, was digested using trypsin at the protease/protein ratio of 1/100 (wt/wt) for 30 min at 27°C, and the products were separated by size exclusion chromatography (Superdex 75 10/300 GL; GE Healthcare, Chicago, IL, USA).

Crystallization and structure determination. Crystallization was carried out using the sitting-drop vapor-diffusion method. Details of the crystallization screening were previously described (42). FliL_exn crystals used for X-ray data collection were grown at 20°C from drops prepared by mixing 0.5 to 1 μl protein solution (70 mg ml⁻¹) containing 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl with the equivalent volume of reservoir solution containing 0.1 M sodium acetate (pH 6.0), 25% (vol/vol) polyethylene glycol (PEG) 200, 0.15 M CaCl₂ and 1.4% (vol/vol) ethyl alcohol (EtOH). The crystals belong to the space group of P₂₁2₁2 with unit cell dimensions of a = b = 162.2 and c = 302.2 Å. The FliL_exn crystals used for X-ray data collection were obtained at 20°C from drops prepared by mixing 0.5 to 1 μl protein solution (10 mg ml⁻¹) containing 20 mM Tris-HCl (pH 8.0) and 150 mM NaCl with the equivalent volume of reservoir solution

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containing 0.1 M sodium acetate (pH 4.4), 22.5% (wt/vol) PEG 4000, and 0.2 M ammonium sulfate. The crystals belong to the space group of P6, with unit cell dimensions of a = b = 104.2 Å and c = 40.1 Å.

X-ray diffraction data were collected at synchrotron beamline BL41XU in SPring-8 (Harima, Japan) with the approval of the Japan Synchrotron Radiation Research Institute (JASRI) (Proposal no. 2015A1084, 2015B1084, 2016A2541, 2016B2541, 2017A2588, and 2017B2588). Crystals were frozen in liquid nitrogen and mounted in nitrogen gas flow at 100 K for X-ray data collection. The diffraction data were processed with MOSFLM (51) and were scaled with AIMLESS (52). The statistics of the diffraction data are summarized in Table S1.

The initial phase of the FliL crystal was calculated using the molecular replacement method with Phenix software (33) and a predicted FliL structure model produced by the Robetta server (http://robeta.bakerlab.org/) (32) as a search model. The atomic model of FliL was built with Coot (54) and refined to 2.1 Å resolution with Phenix (53). The structure of FliL was determined by molecular replacement with Phenix using the atomic model of FliL as a search model. The model was modified with Coot (54) and refined to 3.4 Å resolution with Phenix. The refinement statistics are summarized in Table S1.

In vivo photo-cross-linking. BL21(DE3) cells carrying two different plasmids, pEVO-Lp8pF and pNT70 with or without the V74amber or V165amber mutation, were cultured in LB medium containing 1 mM p-benzoyl-L-phenylalanine (pBpa) (Bachem AG, Switzerland) for 2.5 h at 30°C from an initial optical density of 600 nm of 0.1. Then, arabinose and isopropyl-β-D-thiogalactopyranoside (IPTG) were added to reach final concentrations of 0.02% (wt/vol) and 0.1 mM, respectively, and the cultivation was continued for 4 h. Cells were collected by centrifugation (3,000 g) suspended in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.76 mM KH2PO4), collected again by centrifugation, and resuspended in PBS buffer. The UV irradiation was performed with B-100AP UV lamp (Analytik Jena US, Upland, CA, USA) for 5 min. The cells were then disrupted by sonication and centrifuged at 15,000 × g for 5 min. The supernatant was ultracentrifuged at 100,000 × g for 30 min. The precipitant was suspended in TN buffer containing 0.1% (wt/vol) DDM and 10 mM imidazole, and the proteins were subsequently eluted with TN buffer containing 0.1% (wt/vol) DDM and 300 mM imidazole. The proteins were precipitated by trichloroacetic acid precipitation and suspended in sodium dodecyl sulfate (SDS) loading buffer (62.5 mM Tris-HCl [pH 6.8], 2% [wt/vol] SDS, 10% [wt/vol] glycerol, 0.01% [wt/vol] bromophenol blue) containing 5% (vol/vol) β-mercaptoethanol and were boiled at 95°C for 3 min. Samples were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. The proteins were detected using mouse anti-His antibody and rabbit anti-PomBC antibody (55).

Bacterial two-hybrid assay. We used the bacterial two-hybrid system developed by Karimova et al. (56). DHM1 cells, carrying the plasmids derived from pKT25 and pUT18C, were grown in LB medium containing 50 μg ml⁻¹ ampicillin and 50 μg ml⁻¹ kanamycin at 37°C for about 24 h, and cell cultures were spotted on a BacTH plate (LB containing 1.25% [wt/vol] agar, 0.5 mM IPTG [isopropyl-β-D-thiogalactopyranoside], 40 μg ml⁻¹ 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside [X-Gal], 50 μg ml⁻¹ ampicillin, and 50 μg ml⁻¹ kanamycin) and incubated at 30°C for about 24 h.

Detection of lateral flagella by immunoblotting. Overnight cultures grown in VC medium were inoculated into VPG medium at a 100-fold dilution and were cultivated at 30°C for 4 h. Cells were harvested by centrifugation, suspended to an optical density at 660 nm of 10 in SDS loading buffer containing 5% (vol/vol) β-mercaptoethanol, and boiled at 95°C for 5 min. Samples were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The proteins were detected using rabbit anti-lateral flagellum polyclonal antibody raised against the purified lateral flagelin.

Data availability. The atomic coordinates have been deposited in the Protein Data Bank (www.pdb.org) (PDB ID: 6AHP and 6AHQ).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00292-19.

FIG S1, PDF file, 0.2 MB.
FIG S2, PDF file, 0.2 MB.
FIG S3, PDF file, 0.2 MB.
FIG S4, PDF file, 0.2 MB.
FIG S5, PDF file, 0.2 MB.
TABLE S1, PDF file, 0.1 MB.
TABLE S2, PDF file, 0.1 MB.

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