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The Flagellar Basal Body-Associated Protein FlgT Is Essential for a Novel Ring Structure in the Sodium-Driven *Vibrio* Motor[▽]

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In *Vibrio alginolyticus*, the flagellar motor can rotate at a remarkably high speed, ca. three to four times faster than the *Escherichia coli* or *Salmonella* motor. Here, we found a *Vibrio*-specific protein, FlgT, in the purified flagellar basal body fraction. Defects of FlgT resulted in partial Fla^- and Mot^- phenotypes, suggesting that FlgT is involved in formation of the flagellar structure and generating flagellar rotation. Electron microscopic observation of the basal body of Δ*flgT* cells revealed a smaller LP ring structure compared to the wild type, and most of the T ring was lost. His_6-tagged FlgT could be coisolated with MotY, the T-ring component, suggesting that FlgT may interact with the T ring composed of MotX and MotY. From these lines of evidence, we conclude that FlgT associates with the basal body and is responsible to form an outer ring of the LP ring, named the H ring, which can be distinguished from the LP ring formed by FlgH and FlgI. *Vibrio*-specific structures, e.g., the T ring and H ring might contribute the more robust motor structure compared to that of *E. coli* and *Salmonella*.

The bacterial flagellar motor is a rotary nanomotor, which converts the electrochemical potential difference of the coupling ion (H^+ or Na^+) into rotational energy. *Escherichia coli* and *Salmonella* spp. have H^+-driven motors, and *Vibrio alginolyticus* has Na^+-driven motors. The rotation speed of the *Vibrio* motor is remarkably fast, 1,100 Hz on average and up to 1,700 Hz maximum, which is more than four times faster than that of the *E. coli* motor (24, 27).

The flagellum is coordinately and hierarchically constructed from more than 30 related proteins and is composed of rotor, stator, universal joint (hook), and helical filament (22, 43). The rotor part (also called the basal body) contains several rings and a drive shaft, which are named the L, P, MS, and C rings and the rod (1, 14). The L, P, MS, and C rings are thought to be located in positions corresponding to the outer membrane, peptidoglycan layer, cytoplasmic membrane, and cytoplasm, respectively (Fig. 1). Because the LP ring is thought to be a bushing for rotation of the rod, the LP ring seems not to rotate. Analyses of the basal body components of *Salmonella* were carried out in detail, thereby identifying all of the gene products that are responsible for the substructures. The L, P and MS rings are composed of FlgH, FlgI, and FliF, respectively, while the C ring is composed of three different proteins, FliG, FliM, and FliN, and the rod is composed of FlgB, FlgC, FlgF, and FlgG (14, 17, 18, 39, 44).

The stator part is responsible for torque generation. The torque generation unit of the stator is composed of MotA and MotB in *E. coli* or PomA and PomB in *Vibrio* spp. and is a hexamer of four A subunits and two B subunits. They assemble around the rotor and transfer the coupling ions (H^+ in *E. coli* and Na^+ in *Vibrio*) across the membrane due to the electrochemical potential (2, 4, 11, 15, 37, 38, 40, 41). MotX and MotY are species-specific (e.g., *Vibrio* and *Shewanella* spp.) stator proteins, and defects in these proteins result in a *mot* phenotype in which flagellar morphogenesis is normal but the flagella cannot rotate (21, 30, 31, 33, 36). *Pseudomonas* spp. have only MotY but not MotX; MotY is required for flagellar rotation (12). In *Vibrio alginolyticus* it has been shown that MotX and MotY are produced as precursor proteins with signal sequences and are translocated to the periplasmic space by a general secretion pathway (35). MotX and MotY form a ring structure called the T ring in addition to the LP ring (Fig. 1). The N-terminal domain of MotY has been suggested to directly associate with the basal body, probably the P ring and MotX (23, 42), and MotX has been suggested to interact with PomB (34). Based on these lines of evidence, the T ring was proposed to be involved in the incorporation and/or stabilization of the PomA/B complex into the motor and provide a connection between the rotor and PomA/B in *Vibrio* (42).

When flagellar basal bodies were purified from various species, the basic structures were similar but the details were different. When we compared the structures from *Vibrio* cells and *E. coli* cells, the *Vibrio* LP rings were bigger than those of *E. coli* (42). We speculated that additional proteins were present in the *Vibrio* LP rings. In the present study, we recognized a novel ring structure on the basal body of *V. alginolyticus*, and it was composed of the product of a recently identified motility gene, *flgT*. It was reported in that in *Vibrio cholerae* FlgT is somehow involved in motility and flagellar formation (9, 29). Furthermore, *V. cholerae* strains with defects in FlgT develop outer membrane blebbing and release the flagellum into the medium, suggesting that FlgT is involved in anchoring the flagellar base on the cell surface (29). We found that FlgT is necessary to form an outer ring of the LP ring, named the H ring (for holding ring of the flagellar base on the cell surface). The H ring is thought to be involved in assembly of MotX and MotY to the basal body.

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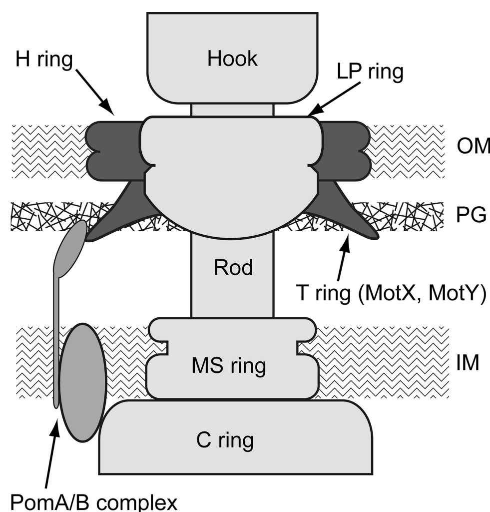

FIG. 1. Model of the flagellar basal body in *Vibrio*. The H ring and the T ring are shown in dark gray. The LP ring and the other basal body parts are shown in light gray. The PomA/B complex is shown in the medium gray. OM, outer membrane; PG, peptidoglycan layer; IM, inner membrane.

MATERIALS AND METHODS

Bacterial strains, plasmids and growth condition. Bacterial strains and plasmids used in the present study are listed in Table 1. *V. alginolyticus* was cultured in VC medium (0.5% [wt/vol] Bacto tryptone, 0.5% [wt/vol] yeast extract, 0.4% [wt/vol] K_2HPO_4, 3% [wt/vol] NaCl, 0.2% [wt/vol] glucose) or in VPG500 medium (1% [wt/vol] Bacto tryptone, 0.4% [wt/vol] K_2HPO_4, 500 mM NaCl, 0.5% [wt/vol] glycerol) at 30°C. *E. coli* was cultured in LB broth (1% [wt/vol] Bacto tryptone, 0.5% [wt/vol] yeast extract, 0.5% [wt/vol] NaCl). In the second selection to isolate mutants, *V. alginolyticus* was cultured at 30°C in plates containing sucrose (1% [wt/vol] polypeptone, 30 mM NaCl, 55 mM KCl, 10% [wt/vol] sucrose, 1.25% [wt/vol] agar). Chloramphenicol was added to final concentrations of 2.5 μg/ml for *V. alginolyticus* and 25 μg/ml for *E. coli*. Kanamycin was added to final concentrations of 100 μg/ml for *V. alginolyticus* and 50 μg/ml for *E. coli.* Ampicillin was added to a final concentration of 50 μg/ml for *E. coli*.

Swarming assay. VPG500 semisolid agar (1% [wt/vol] Bacto tryptone, 0.4% [wt/vol] K_2HPO_4, 500 mM NaCl, 0.5% [wt/vol] glycerol, 0.25% [wt/vol] Bacto agar) was used for motility assays of *V. alginolyticus*. A 1-μl aliquot of an overnight culture was spotted onto VPG500 semisolid agar, followed by incubation at 30°C for the desired time.

Immunoblotting. The samples were suspended with sodium dodecyl sulfate (SDS) loading buffer and boiled at 95°C for 5 min, and SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed as described previously (49). Antisera against PomA (PomA1312), PomB (PomB93), MotX (MotXB0080), MotY (MotYB0079), and FliF (FliFB0424) were prepared previously (47, 49; R. Ogawa et al., unpublished data). Antisera against FlgT (VA15390B0472) were prepared (see below), and a His probe (Santa Cruz) was purchased. Horseradish peroxidase-linked goat anti-rabbit IgG (Santa Cruz) was used as the secondary antibody.

Introduction of plasmids into *V. alginolyticus*. Transformations were carried out by electroporation as described previously (19).

Isolation of flagellar basal bodies. The isolation of the flagellar basal bodies was carried out as described previously with several modifications (42). EDTA for forming spheroplasts was modified to a final concentration of 5 mM and, in the following step, $MgSO_4$ was added to a final concentration of 10 mM.

Electron microscopy. The isolated flagellar structures were negatively stained with 2% uranyl acetate and observed with a JEM-2010 electron microscope (JEOL, Japan).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description[a]	Source or reference
Strains		
V. alginolyticus		
VIO5	VIK4 *laf* (Rif^r Pof^+ Laf^-)	36
NMB191	VIO5 *pomAB* (Mot^-)	49
KK148	VIO5 *flhG* (multi-Pof^+)	25
TH3	KK148 Δ*motX* Δ*motY*	42
TH6	VIO5 Δ*flgT*	This study
TH7	KK148 Δ*flgT*	This study
YM14	YM4 *rpoN* (Pof^- Laf^-)	19a
E. coli		
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4−2-Tc::Mu Km λpirRK6	32
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *lacZ*ΔM15)	48
Plasmids		
pSU41	P_{lac} *lacZa*; Km^r	3
pGEM-T	Cloning vector to TA cloning method; Amp^r	Promega
pET3a	Expression vector; Amp^r	Novagen
pKY704	Suicide vector; Cm^r	46
pHFS401	*sacB* in pSU41	42
pKJ502	*motY* (SalI-XbaI) in pSU41	35
pTH103	*flgT* in pGEM-T	This study
pTH104	*flgT* (BamHI-SacI) in pSU41	This study
pTH105	*flgT*-His_6 (BamHI-SacI) in pSU41	This study
pTH106	*flgT* (BamHI-SacI) in pKJ502	This study
pTH107	*flgT*-His_6 (BamHI-SacI) in pKJ502	This study
pTH108	*flgT*-His_6 (NdeI-BamHI) in pET3a	This study
pTH109	Deletion fragment of *flgT* in pGEM-T	This study
pTH110	Deletion fragment of *flgT* (SacI-SacI) and *sacB* (XbaI-XbaI) in pKY704	This study

[a] Rif^r, rifampin resistant; Pof^+, normal polar flagellar formation; Laf^-, defective in lateral flagellar formation; Mot^-, nonmotile; multi-Pof^+, multiple polar flagellar formation; Amp^r, ampicillin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; P_{lac}, *lac* promoter.

High-intensity dark-field microscopy. Flagella were observed by using a dark-field microscope (Olympus model BHT) equipped with a 100-W mercury lamp (Ushio USH-102). Images was recorded by using a charge-coupled device camera (Sony model SSC-M370) and a DVD video recorder (Panasonic model DMR-E100H).

***flgT* gene cloning, plasmid construction, and disruption.** The *flgT* gene and flanking regions (−541 to +1660) were amplified from purified chromosomal DNA from strain VIO5 by PCR and then subcloned in pGEM-T (Promega), and the resultant plasmid was named pTH103. The *flgT* gene and upstream sequence (−30 to −1) with a BamHI site at the 5′ end and SacI site at the 3′ end was cloned in pSU41 or pKJ502, and the resultant plasmids were named pTH104 and pTH106, respectively. The DNA sequence of a hexahistidine tag was attached by site-directed mutagenesis (Stratagene), and the resultant plasmids were named pTH105 and pTH107, respectively. For purification of FlgT proteins, the *flgT*-His_6 gene with an NdeI site at the 5′ end and a BamHI site at the 3′ end was cloned in pET3a (Novagen), and the resultant plasmid was named pTH108. The *flgT* deletion mutant was generated by homologous recombination using a suicide vector as described previously (42). The sequence upstream of the *flgT* coding region (−541 to −1) with a SacI site at the 5′ end and the downstream sequence (+1135 to +1144) at the 3′ end, and the downstream sequence of the *flgT* coding region (+1135 to +1660) with upstream sequence (−10 to −1) at the 5′ end and a SacI site at the 3′ end were amplified by PCR. Their PCR products were used as primer and template in the following PCR. A deletion fragment was amplified by PCR and subcloned into pGEM-T, and the resultant plasmid was named pTH109. The *flgT* deletion fragment was inserted into the SacI site of pKY704, followed by insertion of the *sacB* gene, which was obtained from pHFS401, in the XbaI site, and the resultant plasmid was named pTH110. pTH110 was used to transform *E. coli* SM10λpir. The deletion allele was introduced into VIO5 or KK148 by a conjugation-based method. The resultant strains were named TH6 and TH7, respectively.

Determination of N-terminal amino acid sequence. The proteins were separated by SDS-PAGE and transferred electrophoretically to a polyvinylidene difluoride membrane (Millipore) and stained with Coomassie blue R250. The MotX, MotY, and FlgI bands were excised. The N-terminal amino acid sequences were determined by Aproscience (Tokushima, Japan), using the Edman degradation method.

Antibody raised against FlgT. BL21(DE3)/pLysS cells harboring pTH108 were cultured in LB broth at 30°C, and FlgT proteins were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at early exponential phase. Cells were collected by centrifugation, resuspended in buffer A (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 25% [wt/vol] sucrose), incubated on ice for 10 min, diluted with 2 volumes of buffer B (100 mM NaCl, 1.5 mM sodium-EDTA [pH 8]), and incubated on ice for 20 min. The supernatant was recovered by centrifugation (10,000 × *g* for 20 min), $MgCl_2$ and imidazole were added to final concentrations of 2 and 20 mM, respectively, and then the periplasmic fraction was recovered by ultracentrifugation (100,000 × *g* for 60 min). The periplasmic fraction was applied to HisTrap FF (GE Healthcare), and the bound proteins were eluted with a 20 to 500 mM linear gradient of imidazole (using the buffers 20 mM Tris-HCl [pH 7.5] and 100 mM NaCl containing 20 or 500 mM imidazole). FlgT-His_6 was purified with a HiTrap Q column (GE Healthcare) and Superdex 200HR 10/30 (GE Healthcare) as necessary. Purified FlgT was separated by SDS-PAGE, stained with Coomassie blue R250, and excised. Rabbit anti-FlgT antibody was produced by Biogate (Tokushima, Japan).

Coelution assay to detect the interaction between FlgT and MotY. The coelution assay was carried out by the purification protocol described above with slight modifications. YM14 cells, which are $sigma54^-$ mutant (*rpoN* mutant) cells that do not express the flagellar genes, harboring pTH106 or pTH107 were cultured in VPG500 broth at 30°C. The pH of Tris buffer was changed from 7.5 to 8.0.

RESULTS

Identification of a novel component in the *Vibrio* basal body. We purified the wild-type hook-basal bodies from the *flhG* mutant (KK148), which produces multiple polar flagella (25). The fractions of purified hook-basal bodies were analyzed by SDS-PAGE and stained (Fig. 2). We obtained a similar band profile, and the most intense band of 50 kDa was the hook protein, FlgE, as previously reported (42). To confirm the basal body components, we determined the N-terminal amino acid sequences of some of the protein bands, which appeared to be MotX, MotY and FlgI (Fig. 2). The sequences of the 25- and 30-kDa bands were NVADV and VMGKR, which correspond to the sequences of MotX and MotY without the putative N-terminal signal sequences, respectively. The 38-kDa band contained two sequences, ARIKD and SWYEV, which correspond to the sequences of FlgI without the signal sequence and a hypothetical gene product (VA15390 of *V. alginolyticus* strain 12G01). This result suggests that the hypothetical gene product, VA15390, is a component of the *Vibrio* basal body. A homolog of VA15390 has been identified as a gene required for motility in *V. cholerae* and was named FlgT (9). FlgT homologs from various species were aligned, and the secondary structure of FlgT from *V. alginolyticus* strain VIO5 was predicted by using PSIPRED (8) (Fig. 3). FlgT homologs are likely to exist in only *Vibrio*, *Shewanella* and related species, which are known to possess MotX and MotY. The deduced *flgT* products have a signal sequence for secretion and the amino acid sequence of FlgT detected from the basal body fraction had lost the signal sequence. This suggests that FlgT is cleaved between Ala^{23} and Ser^{24} and is translocated into the periplasmic space. FlgT was predicted to have an α/β mixed structure in the N-terminal half and be rich in β-strand structures in the C-terminal half. There are two conserved cysteine residues that might form a disulfide bond for protein stabilization.

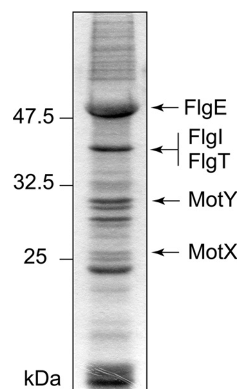

FIG. 2. Identification of the proteins containing the purified hook-basal body fraction. The proteins of the hook-basal body from KK148 were separated by SDS-PAGE. The N-terminal amino acid sequences of the putative MotX, MotY or FlgI bands were determined by Edman degradation.

Motility of Δ*flgT* cells. To examine the role of FlgT in the *Vibrio* flagellar system, we deleted the *flgT* gene and examined the motility of the Δ*flgT* cells based on swimming ability in semisolid agar and in liquid medium (Fig. 4 and data not shown). The Δ*flgT* cells scarcely showed any ability to swim in 4 h (Fig. 4A) and slightly expanded in semisolid agar by 9 h (Fig. 4B) compared to strain NMB191 (Mot^-, Δ*pomAB*). Although most of the Δ*flgT* cells did not have a flagellum (Fla^-), only a small fraction had a flagellum (Fla^+) (Fig. 4C). We did not observe released flagella with attached basal bodies as reported previously for *V. cholerae* (29). This might suggest that the strength of the membrane between the two species is not the same. When we observed flagella using high-intensity dark-field microscopy, cells of wild type (VIO5) were more

FIG. 3. Alignment of the amino acid sequences of *V. alginolyticus* strain VIO5 FlgT and homologous proteins from various species. The sequence alignment was generated with Genetyx software (Genetyx Corp.). Abbreviations: VaFlgT, FlgT of *Vibrio alginolyticus* strain VIO5; VP0767, hypothetical protein of *Vibrio parahaemolyticus* RIMD 2210633; VC2208, FlgT of *Vibrio cholerae* El Tor N16961; Il1154, hypothetical protein of *Idiomarina loihiensis* L2TR; SO_3258, hypothetical protein of *Shewanella oneidensis* MR-1. White letters in black boxes indicate residues that are identical in all sequences. Letters in gray boxes show residues that matched in at least three of the five sequences. The arrowhead shows the site of signal sequence cleavage. Asterisks show the conserved cysteine residues. Green boxes indicate β-strands predicted by PSIPRED, and orange wavy lines indicate predicted α-helixes.

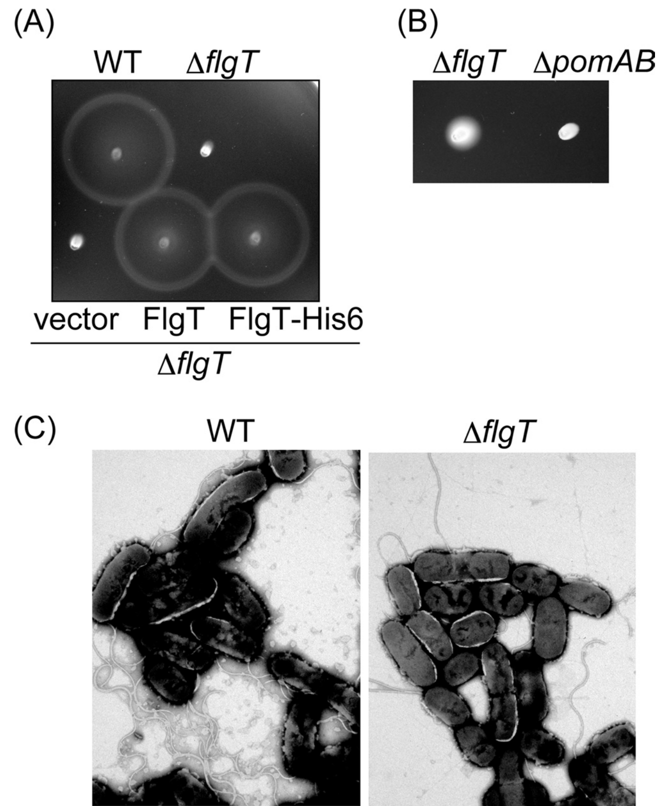

FIG. 4. Motility of Δ*flgT* cells in semisolid agar. (A) Cells were inoculated into VPG500 semisolid agar and incubated at 30°C for 4 h. WT, VIO5; Δ*flgT*, TH6; vector, pSU41; FlgT, pTH104; FlgT-His6, pTH105. (B) Cells were inoculated into VPG500 semisolid agar and incubated at 30°C for 9 h. Δ*flgT*, TH6; Δ*pomAB*, NMB191. (C) Cells of VIO5 (WT) and TH6 (Δ*flgT*) grown in panel A were negatively stained with 2% potassium phosphotungstate and observed with a JEM-2010 electron microscope (JEOL, Japan). Bar, 2 μm.

than 90% flagellate, and all of the flagellate cells were motile; on the other hand, the *flgT* mutant cells were ca. 30% flagellate, and only ca. 10% of the flagellate cells were motile but very slow. Therefore, FlgT seems to partially (not critically) contribute to both flagellar formation and rotation.

Basal body structure purified from the Δ*flgT* cells. Because FlgT seemed to be a component of the basal body, we speculated that FlgT forms a substructure in the basal body. To investigate this possibility, we purified the basal bodies from the Δ*flgT* cells of a multiple-flagellated strain (KK148) and observed them by electron microscopy. The basal bodies of the Δ*flgT* cells had smaller LP rings than those of wild type, and the T ring beneath the LP ring was lost, suggesting that FlgT is a component protein of the ring structure outside of the LP ring (Fig. 5). Therefore, the ring that has been referred to as the *Vibrio* LP ring could be separated into a ring that is lost in the *flgT* mutant and the conventional LP ring formed by FlgH and FlgI. The new ring was named the H ring (Fig. 1). Next, to examine whether depletion of FlgT affects the other components of the basal body (MotX, MotY, and FliF), we analyzed whole-cell lysates or basal body fractions by immunoblotting using anti-MotX, MotY, FliF, or FlgT antibodies. In whole-cell lysates, MotY and FliF were expressed at similar levels regardless of the presence or absence of FlgT, but MotX was decreased in the absence of FlgT (Fig. 6). In the basal body fraction, FliF in the Δ*flgT* cells was detected at lower levels than in the wild-type cells. We measured the band densities of FliF from the wild-type and the Δ*flgT* cells. The intensity of the band from the Δ*flgT* cells was reduced to 18 or 51% compared to the intensity of wild type in two independent experiments.

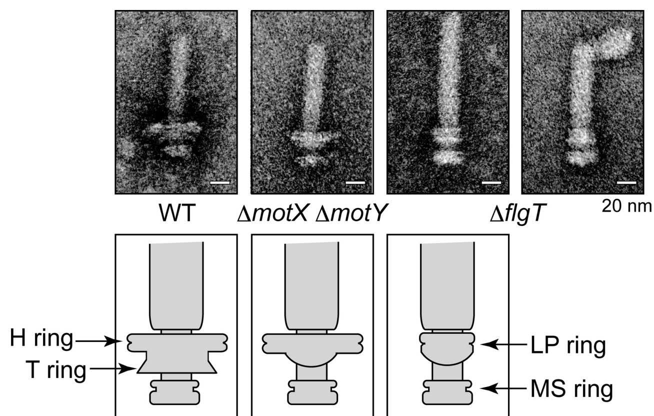

FIG. 5. Electron microscopy images of the hook-basal bodies purified from *V. alginolyticus*. The hook-basal bodies were negatively stained with 2% (wt/vol) uranyl acetate and observed with a JEM-2010 electron microscope (JEOL, Japan). Bar, 20 nm. WT, KK148; Δ*motX* Δ*motY*, TH3; Δ*flgT*, TH7. The diagrams of the flagellar basal bodies isolated from the various strains are shown below the pictures.

FliF in the basal body fraction was detected as smeared bands, suggesting that the N- or C-terminal region of FliF was degraded during the purification step. On the other hand, MotX and MotY were barely detectable in Δ*flgT* cells. These results suggest that the H ring is involved in basal body formation and is necessary to assemble the T ring composed of MotX and MotY (Fig. 6).

Interaction between FlgT and MotY. We speculated that FlgT interacts directly with MotX or MotY, which has been suggested to directly interact with the basal body (23). To determine whether this interaction occurs, a hexahistidine tag-fused FlgT and MotY were produced in sigma54^{-} mutant (mutant *rpoN*) cells that do not produce the other flagellar components (Fig. 7). As for MotX, we cannot purify it as a soluble protein (34, 35), and thus we could not test for interaction between MotX and FlgT. After the periplasmic fraction was applied to a HisTrap column and washed, the bound proteins were eluted with imidazole. Although most of the MotY protein was detected in the flowthrough fraction, MotY was also coeluted with FlgT-His$_6$ but not with plain FlgT. The elution profile of MotY corresponded to that of FlgT-His$_6$ eluted by the liner gradient of imidazole. The present results support a direct interaction of MotY with FlgT. Under these experimental conditions, only FlgT and MotY were produced, so the interaction between them may be weak, and other flagellar proteins might be necessary for more stable interaction to form the ring structures.

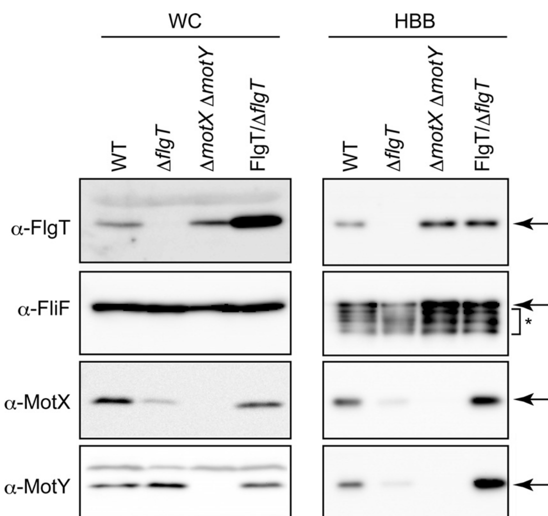

FIG. 6. Detection of FlgT, FliF, MotX, and MotY in the purified hook-basal body fraction. Whole-cell lysate (WC) and the purified hook-basal bodies (HBB) of *V. alginolyticus* were subjected to SDS-PAGE, followed by immunoblotting with anti-FlgT, FliF, MotX, and MotY antibodies. Asterisks show degraded products of FliF proteins in the basal body. WT, KK148; Δ*flgT*, TH7; Δ*motX* Δ*motY*, TH3; FlgT/Δ*flgT*, pTH104/TH7.

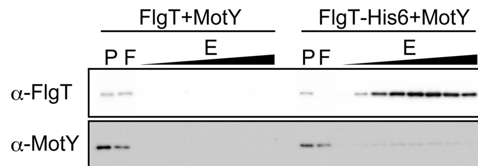

FIG. 7. Coelution assay of MotY with FlgT-His$_6$. FlgT or FlgT-His$_6$ proteins with MotY proteins were expressed from pTH106 or pTH107 in strain YM14 (*rpoN* mutant). The samples were subjected to SDS-PAGE, followed by immunoblotting with anti-FlgT and MotY antibodies. P, periplasmic fraction; F, flowthrough fraction; E, eluted fraction.

DISCUSSION

A hypothetical gene, *flgT*, was identified in *V. cholerae*, and *flgT* mutant cells were very rarely flagellated (9). FlgT was predicted to have structural homology to TolB, a protein involved in maintaining outer membrane integrity. Since the sheath of the *V. cholerae* flagellum appears to be derived from the cell's outer membrane, it has been speculated that FlgT may play a role in flagellar sheath formation (9). Taylor and coworkers (29) reported that the flagellar base of *V. cholerae* is released into the culture medium or is associated with membrane blebs in a *flgT* deletion mutant, and these researchers speculated that FlgT interacts with different components of the basal body. Transcriptional analysis of the *flgT* mutant has previously been carried out in *V. cholerae* (9), and it was determined that the mutant was specifically stalled at the class III/IV assembly checkpoint, which is controlled by FlgM, the anti-sigma factor. In the present study we observed that the number of complete basal bodies decreased significantly in the *flgT* mutant and that the amount of MotX, whose gene belongs to the class IV transcriptional hierarchy (20), was decreased in whole-cell lysates. Our results are consistent with the previous speculation that FlgT affected not only anchoring of the flagellum base on the membrane but also the construction of the rod/hook structure (29).

In the present study, we showed that FlgT is isolated with the basal body fraction of *V. alginolyticus* and that FlgT of *V. alginolyticus* is involved in flagella formation and rotation similar to *V. cholerae*. We found that FlgT is necessary to form a new ring structure, named the H ring (for holding ring of the flagellar base on the cell surface), which has been recognized as the outermost part of the LP rings (Fig. 1). Therefore, the

ring of *Vibrio* looks obviously bigger than that of *E. coli*. These observations might imply that the H ring reinforces the association of the flagellar base with the outer membrane or peptidoglycan layer in *Vibrio* spp. It is possible that the H ring is composed of other flagellar proteins in addition to FlgT. Because FlgT is a periplasmic protein, a region corresponding to the outer membrane in the H ring may be formed by outer membrane proteins, such as FlgO and FlgP, which are somehow involved in the motility of *V. cholerae* (28). The *flgO* and *flgP* genes are located in the region next to the *flgT* gene on genome, and it has been reported that flagella in a *flgO* or *flgP* mutant are shorter in length than wild-type flagella (28). Further work needs to be done to identify all of the components contained in the purified basal body fraction.

MotX and MotY were poorly associated with the basal body of the Δ*flgT* mutant. We demonstrated that FlgT interacts directly with MotY by coelution assays. These results suggest that FlgT is a primary target for assembly of MotY into the basal body and the H ring is a scaffold for forming the T ring structure. On the other hand, a component other than FlgT, e.g., the P ring component FlgI, might be a second target for assembly of MotX and MotY to the basal body because very small amounts of MotX and MotY are associated with the basal body in the Δ*flgT* mutant. If the T ring, which is composed of MotX and MotY, is formed in the basal body of Δ*flgT*, the stators can be assembled around the rotor. Consequently, a small fraction of the Δ*flgT* cells seems to be able to swim.

Using the program, Phyre, it was predicted that FlgT has structural homology with the N-terminal domain of TolB in the Tol-Pal system, which is a supramolecular complex required for outer membrane integrity and resistance to antibiotics (9). TolB has an N-terminal mixed α/β domain and a C-terminal six-bladed β-propeller domain (5, 26). The α/β domain of TolB interacts with the periplasmic domain of TolA, which is a monotropic cytoplasmic membrane protein (6, 13, 45). The β-propeller domain of TolB interacts with the peptidoglycan-binding (PGB) domain of Pal, which is an outer membrane lipoprotein (6, 7). Furthermore, TolB is capable of interacting with OmpA and Lpp, which are outer membrane proteins (10). Therefore, TolB is thought to act as the "network hub" for the Tol-Pal complex (5). We have shown that the PGB region of Pal is interchangeable with the PGB region of MotB in *E. coli* (16). We suggested that FlgT interacts directly with MotY and the LP ring of the basal body in the present study and we have shown that MotX interacts with MotY and PomB (34). Based on this information, we speculate that FlgT, as well as MotY or MotX, also serves as a "network hub" to connect between the outer membrane LP ring of the basal body and the PomA/B complex.

FlgT, MotX, and MotY are specific flagellar components in *Vibrio*, *Shewanella*, and related species. Why are they present in only these species? The flagellar motor of *V. alginolyticus* can achieve remarkably fast rotation. To allow such rapid rotation, FlgT might be required to hold the flagellar base on the cell surface and as a scaffold to form the T ring. The H ring might reinforce the bushing robustness, thereby, might protect against physical breaking of the basal body. MotX interacts with PomB and thereby is involved in incorporation and stabilization of the PomAB complex. These specific components might contribute a robust motor structure compared to that of *E. coli* and *Salmonella*.

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