A positive charge region of \textit{Salmonella} FliI is required for ATPase formation and efficient flagellar protein export

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The FliH$_2$FliI complex is thought to pilot flagellar subunit proteins from the cytoplasm to the transmembrane export gate complex for flagellar assembly in \textit{Salmonella} enterica. FliI also forms a homo-hexamer to hydrolyze ATP, thereby activating the export gate complex to become an active protein transporter. However, it remains unknown how this activation occurs. Here we report the role of a positively charged cluster formed by Arg-26, Arg-27, Arg-33, Arg-76 and Arg-93 of FliI in flagellar protein export. We show that Arg-33 and Arg-76 are involved in FliI ring formation and that the \textit{fliI(R26A/R27A/R33A/R76A/R93A)} mutant requires the presence of FliH to fully exert its export function. We observed that gain-of-function mutations in FlhB increased the probability of substrate entry into the export gate complex, thereby restoring the export function of the \textit{ΔfliH fliI(R26A/R27A/R33A/R76A/R93A)} mutant. We suggest that the positive charge cluster of FliI is responsible not only for well-regulated hexamer assembly but also for substrate entry into the gate complex.

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The flagellum of *Salmonella enterica* (hereafter referred to as *Salmonella*) is a supramolecular motility machine consisting of the basal body, the hook, and the filament. For construction of the flagellum on the cell surface, a specialized protein export apparatus uses ATP and a proton motive force (PMF) across the cytoplasmic membrane to transport flagellar building blocks from the cytoplasm to the distal end of the growing flagellar structure. The flagellar export apparatus is composed of a PMF-driven transmembrane export gate complex made of FliA, FliB, FliP, FliQ, and FliR and a cytoplasmic ATPase ring complex consisting of FliH, FliI, and FliJ (Fig. 1). The PMF-driven export gate complex is located inside the basal body MS ring formed by a transmembrane protein, FliF (Fig. 1). FliP, FliQ, and FliR form a tubular structure with a stoichiometry of five FliP, four FliQ, and one FliR with a helical symmetry similar to those of the rod, hook, and filament, and the central pore of this FliP₅FliQ₄FliR₁ complex is thought to be a polypeptide channel for the translocation of flagellar building blocks across the cytoplasmic membrane. The N-terminal transmembrane domain of FliB (FliB₉₉) associates with the FliP₅FliQ₄FliR₁ complex to form the FliP₅FliQ₄FliR₁FliB₁ complex and is proposed to coordinate opening of the export gate for substrate entry into the polypeptide channel. FliA assembles into a homo-nonamer through its C-terminal cytoplasmic domain (FliAC) and forms a pathway for the transit of protons across the cytoplasmic membrane and the C-terminal cytoplasmic domain of FliB (FliB₉₉) project into the cytoplasmic cavity of the basal body C ring and form a docking platform for the cytoplasmic ATPase complex, flagellar export chaperones, and flagellar building blocks.

The cytoplasmic ATPase ring complex is composed of 12 copies of FliH, 6 copies of FliI, and a single copy of FliJ (Fig. 1). FliI forms a homo-hexamer to hydrolyze ATP at an interface between Fli subunits and the FliI ring structure is localized to the flagellar base through interactions of the extreme N-terminal region of FliH (FliH₉₉) with FliA and a C ring protein, FliN. FliJ binds to the center of the FliI ring and plays an important role in an ATP-dependent gate activation mechanism.

FliI also exists free in the cytoplasm as a heterotrimeric complex formed with a FliH dimer. This FliH–FliI complex binds to FliJ, export substrates and flagellar chaperone/substrate complexes, and facilitates their docking to the FliAC–FliBC docking platform for efficient and robust flagellar protein export.

The C-terminal domain of FliI (FliIN) is responsible not only for the interaction with FliH but also for FliI₉₉ ring formation. The C-terminal domain of FliH (FliHC) binds to the extreme N-terminal α-helix consisting of residues 2–21 of FliI (FliIN) and a positively charged cluster formed by Arg-26, Arg-27, Arg-30, Arg-33, Arg-76, and Arg-93. The binding of FliH to FliIN not only inhibits FliI₉₉ ring formation but also reduces the FliI ATPase activity considerably. FliI ATPase seems to play a regulatory role in the energy coupling mechanism of the flagellar protein export apparatus. However, it remains unknown how FliH regulates ATP hydrolysis by the FliI ATPase for flagellar protein export. To clarify this question, we performed mutational analysis of the positively charged cluster of FliIN. We show that Arg-33 and Arg-76 of FliIN are involved in well-regulated FliI ring formation. We also show that this positive charge cluster is required for efficient entry of flagellar building blocks into the transmembrane export gate complex.

**Results**

**Effect of alanine substitution in the positive charge cluster on motility.** Arg-26, Arg-27, Arg-30, Arg-33, Arg-76, and Arg-93 of *Salmonella* FliI ATPase form a positive charge cluster on the molecular surface of the Fli hexamer and Arg-26, Arg-30, Arg-33, Arg-76, and Arg-93 of FliIN are conservationally well conserved among FliI homologs. To clarify the role of these Arg residues in flagellar protein export, we constructed the following nine mutants: FliI(R26A), FliI(R27A), FliI(R30A), FliI(R33A), FliI(R76A), and FliI(R93A) (hereafter referred to as FliI-5A), and FliI(R26A/R27A/R33A/R76A), and FliI(R26A/R27A/R33A/R76A/R93A) (hereafter referred to as FliI-5A, FliI-3A, FliI-4A, and FliI-5A, respectively). All of these mutant variants except FliI-5A fully restored the motility of a *Salmonella* ΔfliI mutant when they were expressed even at a relatively low copy level from pET19b-based plasmids (Supplementary Fig. 1a). In contrast, the motility of the fliI-5A mutant was lower than that of...
wild-type cells (Fig. 3a). Consistently, the flII-5A mutation reduced the secretion level of FlgD (Fig. 3b). Because the flII-5A mutation did not affect the steady cellular level of FliI (Supplementary Fig. 1b), we conclude that this mutation affects the protein export function of FliI.

To investigate whether the overexpression of FliI-5A improves the motility, we cloned this flII allele into the pTrc99A vector. When FliI-5A was overexpressed from a pTrc99A-based plasmid, FliI-5A restored the motility of the ΔfliI mutant to the wild-type level (Fig. 3a). Consistently, the secretion level of FlgD was also at the wild-type level (Fig. 3b), indicating that the reduced export activity of FliI-5A is restored to the wild-type level by an increase in its expression level.

Effect of alanine substitution in the positive charge cluster on FliH–FliI interaction. The positive charge cluster of FliIN is involved in the interaction with FliHC (Fig. 2a)16. To test whether the flII-5A mutation reduces the binding affinity of Fli for FliH, we carried out pull-down assays by Ni affinity chromatography (Supplementary Fig. 2). Untagged FliH co-purified with His-FliI, in agreement with a previous report25. In contrast, the flII-5A mutation reduced the binding affinity of Fli for FliH (Supplementary Fig. 2). Although the flII-2A, flII-3A and flII-4A mutations did not affect motility at all (Supplementary Fig. 1a), these three mutations also reduced the binding affinity of Fli for FliH (Supplementary Fig. 2). Therefore, we conclude that electrostatic interactions between the positive charge cluster of FliC and FliH are dispensable for flagellar protein export although these interactions stabilize an interaction between FliIEN and FliHC. Because a complete loss of the positive charges by flII-5A mutation reduced the protein transport activity (Fig. 3), we hypothesize that this positive charge cluster may be involved in the interaction with other export apparatus components for efficient flagellar protein export.

Multicopy effect of FliI(R33A) and FliI-3A on Salmonella cell growth. When FliI(R33A) and FliI-3A (R26A/R27A/R33A triple mutation) were expressed at a relatively high copy level from pTrc99A-based plasmids, the motility ring of these two FliI mutants were smaller than the wild-type ring (Supplementary Fig. 1c). However, they fully restored the motility of the ΔfliI mutant when they were expressed at a relatively low copy level from pET19b-based plasmids (Supplementary Fig. 1a). As the motility ring size also depends on the growth rate of Salmonella motile cells, we investigated whether the higher expression level of FliI with either R33A or FliI-3A substitution reduces the cell growth at all (Supplementary Fig. 3a). In contrast, when FliI(R33A) and FliI-3A were expressed...
from pTrc99A-based plasmids, they reduced the cell growth (Supplementary Fig. 3a) even though their expression levels were lower than the wild-type level (Supplementary Fig. 3b). To confirm this, we measured the growth rate of *Salmonella* cells overexpressing wild-type FliI, FliI(R33A), or FliI-3A (Fig. 4a). Overexpression of wild-type FliI slightly reduced the cell growth rate compared to the vector control. In contrast, cell growth was totally inhibited by induction of FliI(R33A) and FliI-3A (Fig. 4a), even though their expression level was lower than the wild-type level (Fig. 4b).

FliH suppresses FliI ring formation in solution, thereby suppressing the ATPase activity of FliI. Therefore, we next tested whether FliH relieves the growth inhibition caused by the fliI(R33A) and fliI-3A mutations. When FliI(R33A) or FliI-3A was co-expressed with FliH from the pTrc99A-based plasmids, no growth inhibition occurred (Supplementary Fig. 3a) although their expression levels were increased (Supplementary Fig. 3b, c). As described above, the fliI-3A mutation reduced the binding affinity of FliI for FliH, whereas the R33A mutation did not (Supplementary Fig. 2). Because FliH strongly binds to FliI<sub>EN</sub> to suppress FliI ring formation<sup>16,25,32</sup>, we suggest that the binding of FliH to FliI<sub>EN</sub> inhibits premature FliI ring formation in the cytoplasm, thereby not only suppressing the growth inhibition caused by these two fliI mutations but also increasing the steady cellular level of FliI (R33A) and FliI-3A (Supplementary Fig. 3b, c).

**Multicopy effect of the fliI-3A and fliI-4A mutations on the ATPase activity of FliI.** The ATPase activity of FliI displays a protein concentration-dependent positive cooperativity, indicating that FliI ring formation is required for efficient ATP hydrolysis by the FliI ATPase<sup>17,33</sup>. This raises the possibility that the fliI (R33A) and fliI-3A mutations increase the probability of premature FliI ring formation in the cytoplasm, thereby reducing the cytoplasmic ATP level enough to inhibit the cell growth. To test...
this, we measured the ATPase activity of FliI(R33A) and FliI-3A proteins at several distinct protein concentrations (Fig. 4c and Table 1). The ATPase activity of FliI increased from 0.001 ± 0.0001 to 0.888 ± 0.222 [mean ± standard deviation (SD), n = 3] nmol of phosphate min⁻¹ μg⁻¹ with an increase in the protein concentration from 17 to 680 nM. The ATPase activity of FliI (R33A) and FliI-3A also showed a protein concentration-dependent positive cooperativity (Fig. 4c and Table 1) but was greatly higher than that of the wild type even at a very low protein concentration. At FliI concentration of 85 nM, the ATPase activity of FliI increased from 0.001 ± 0.0001, 1.211 ± 0.09, and 1.898 ± 0.036 nmol of phosphate min⁻¹ μg⁻¹, respectively.

To test whether FliI-3A forms homo-hexamer, we analyzed the ability of FliI ring formation in the presence of Mg2⁺-ADP-AlF₄, which is a non-hydrolyzable ATP analog (Fig. 5). At a FliI concentration of 1 μM, many ring-like particles were observed in the FliI-3A sample but not in the wild-type FliI one. Only end-on images were selected, aligned in a reference-free mode and averaged, which yielded a ring structure with an approximate sixfold symmetry (Fig. 5, inset). Because the fliI(R26A), fliI(R27A), and fliI-2A (R26A/R27A double mutation) mutations did not affect the cell growth, we suggest that the fliI(R33A) mutation increases the probability of autonomous FliI ring formation even at a low protein concentration.

FliI-4A also contains the R26A/R27A/R33A triple mutation in addition to the R76A substitution, but overexpression of FliI-4A did not inhibit the cell growth in a way similar to wild-type FliI (Fig. 4a), raising the possibility that the fliI(R76A) substitution may reduce the ring formation efficiency of FliI-3A. To clarify this possibility, we purified FliI-4A and measured its ATPase activity at different protein concentrations (Fig. 4c and Table 1). The ATPase activity of FliI-4A was 0.021 ± 0.004 nmol of phosphate min⁻¹ μg⁻¹ at a protein concentration of 85 nM. This ATPase activity of FliI-4A was higher than the wild-type level at the same protein concentration but was much lower than that of FliI-3A. Consistently, FliI-4A formed a hexamer but the ring formation efficiency was lower than FliI-3A albeit higher than the wild type (Fig. 5). These suggest that the fliI(R76A) substitution partially suppresses efficient FliI ring formation caused by the fliI(R33A) mutation, thereby suppressing the growth inhibition caused by overexpression of FliI-3A. Because Arg-33 and Arg-76 are located at an interface between Flg subunits, and Arg-76 forms a hydrogen bond with Asn-73 of its neighboring subunit (Fig. 2a), we suggest that Arg-33, Asn-73, and Arg-76 are critical for well-regulated FliI ring formation.

**fliH deletion effect on motility of fli mutants.** To clarify the role of the positive charge cluster of FliI in flagellar protein export, we investigated motility of fli mutants in the absence of FliH. Because overexpression of FliI bypasses the FliH defect to a significant degree, we transformed a Salmonella fliH-fliI double null mutant (fliH-fliI) with pTrc99A-based plasmids encoding the FliI mutant variants we studied above and analyzed the motility of the resulting transformants. Except for FliI(R30A) and FliI(R33A), single alanine substitutions in the positive charge cluster of FligH30 reduced motility in soft agar (Supplementary Fig. 4a, left panel). Consistently, the secretion level of FlgD was lower than the wild-type level (Supplementary Fig. 4b). Interestingly, the fliI-2A mutation reduced flagella-driven motility compared to the R26A and R27A single mutations, and the R33A mutation improved the motility defect of the fliI-2A mutant to a considerable degree (Supplementary Fig. 4a, right panel). A complete loss of the positive charges of FliI by fliI-5A mutation inhibited motility of and the secretion of FlgD by the ΔfliH-fliI mutant (Fig. 3c, d), suggesting that FliI-5A exerts an inhibitory effect on flagellar protein export in the absence of FliH. These observations suggest that the positive charge cluster of FligH30 is involved in well-regulated flagellar protein export.

The FliI ATPase plays an important role in substrate recognition. Addition of purified FlhA30FlhI30 complex at a final concentration of 1.5 μM to the in vitro assay solution increases the level of FlgD transported to the inside of inverted membrane vesicles by 20-fold, indicating that the FlhA30FlhI30 complex facilitates the export of FlgD. To clarify why the fliI-5A mutation abolishes the secretion of FlgD in the absence of FliH, we analyzed the FliI–FlgD interaction by glutathione S-transferase (GST) affinity chromatography. A very small amount of FlgD was detected in the elution fractions derived from GST alone presumably due to its non-specific binding to the column (Fig. 6a). In contrast to GST alone, much higher amounts of FlgD co-purified with GST–FliI (Fig. 6b), indicating a specific interaction between FliI and FlgD. The fliI-5A mutation did not inhibit the interaction of FlI with FlgD (Fig. 6c).

FliI also interacts with FlhA30, FlhB30, and FliJ during flagellar protein export. To investigate whether the fliI-5A mutation...
affects the interactions of FliI with these three proteins, we performed pull-down assays by GST affinity chromatography. Small amounts of FliI co-purified with GST-FlhAC, GST-FlhBC, and GST-FliJ but not with GST alone (Fig. 7a). Furthermore, compared to the GST control, elution of FliI was clearly observed as a delayed wash out, reflecting weak and highly dynamic interactions of FliI with GST-FlhAC, GST-FlhBC, and GST-FliJ (Fig. 7a). Two-tailed Student’s t tests revealed that the amounts of FliI-5A co-purified with GST-FlhBC and GST-FliJ were significantly higher than those of wild-type FliI (p < 0.05; Fig. 7b), indicating that the fliI-5A mutation increases the binding affinities of FliI for FlhBC and FliJ. In contrast, this fliI-5A mutation reduced the binding affinity of FliI for FlhAC (p < 0.05; Fig. 7b). Because the level of FlgD secreted by the ΔfliH fliI-5A mutant was lower than that by the ΔfliH-fliI mutant (Fig. 3d), we suggest that FliI-5A may bind to FlhBC and FliJ to block the flagellar protein export process in the absence of FlhH. Since the overexpression of FliI-5A restored motility to the wild-type level in the presence of FlhH (Fig. 3a), we propose that an interaction between the positive charge cluster of FliI and FlhAC may be involved in the flagellar protein export process.

Fig. 4 Multicopy effect of FliI mutant proteins on cell growth. a Growth curve of MKM30 (ΔfliI) cells transformed with pTrc99A (V), pMM1702 (WT), pMM1702(R33A) [pTrc99A/His-FliI(R33A), indicated as R33A], pMM1702-3A [pTrc99A/His-FliI(R26A/R27A/R33A), indicated as FliI-3A], pMM1702-4A [pTrc99A/His-FliI(R26A/R27A/R33A/R76A), indicated as FliI-4A], or pMM1702-5A [pTrc99A/His-FliI(R26A/R27A/R33A/R76A/R93A), indicated as FliI-5A]. Cells were grown in L-broth containing ampicillin at 30 °C for 3 h and then IPTG was added at a final concentration of 0.1 mM. The OD600 value of each culture was monitored every hour. These data are the average of three independent biological replicates. The experimental errors are within 10%. b Effect of the fliI(R3A), fliI-3A, fliI-4A, and fliI-5A mutations on the expression level of FliI. Immunoblotting, using polyclonal FliI antibody, of whole cellular (W), soluble (S), and insoluble (P) fractions prepared from the above transformants. The regions of interest were cropped from original immunoblots shown in Supplementary Fig. 10. c Effect of FliI mutations on dependence of the FliI ATPase activity on protein concentration. The ATPase activity of purified His-FliI (WT), His-FliI(R33A) (R33A), His-FliI(R26A/R27A/R33A) (FliI-3A), His-FliI(R26A/R27A/R33A/R76A) (FliI-4A), or His-FliI(R26A/R27A/R33A/R76A/R93A) (FliI-5A) was measured at different protein concentrations in the presence of 4 mM ATP by using the Malachite Green assay. The activity is expressed as nmol of Pi released per min per μg of FliI.
Effect of the fliI-5A mutation on the ATPase activity of FliI.

FliI₆ ring formation is required for efficient flagellar protein export by the PMF-driven export gate complex even in the absence of FliH₁₉, raising the possibility that the fliI-5A mutation might suppress FliI oligomerization at the FlhAC-FlhBC docking platform. To clarify this possibility, we measured the ATPase activity of FliI-5A at various protein concentrations (Fig. 4c and Table 1). The ATPase activity of FliI-5A was higher than the

| Protein concentration (nM) | FliI ATPase activity (nmol of phosphate min⁻¹ μg⁻¹) (mean ± SD) |
|---------------------------|---------------------------------------------------------------|
|                           | Wild type | R33A       | FliI-3A | FliI-4A | FliI-5A |
| 17                        | 0.001 ± 0.0001 | 0.008 ± 0.002 | 0.011 ± 0.004 | 0.002 ± 0.001 | 0.001 ± 0.002 |
| 34                        | 0.0014 ± 0.0001 | 0.041 ± 0.001 | 0.075 ± 0.019 | 0.0033 ± 0.002 | 0.0065 ± 0.003 |
| 51                        | N.D. | 0.079 ± 0.001 | 0.231 ± 0.007 | N.D. | N.D. |
| 68                        | 0.0019 ± 0.001 | 0.155 ± 0.049 | 0.643 ± 0.017 | 0.0086 ± 0.001 | 0.0083 ± 0.002 |
| 85                        | 0.008 ± 0.001 | 1.211 ± 0.090 | 1.398 ± 0.036 | 0.021 ± 0.004 | 0.024 ± 0.012 |
| 170                       | 0.043 ± 0.015 | N.D. | N.D. | 0.115 ± 0.050 | 0.093 ± 0.033 |
| 255                       | N.D. | N.D. | N.D. | 0.254 ± 0.104 | 0.196 ± 0.052 |
| 340                       | 0.258 ± 0.049 | N.D. | N.D. | 0.467 ± 0.114 | 0.333 ± 0.049 |
| 510                       | 0.527 ± 0.163 | N.D. | N.D. | N.D. | N.D. |
| 680                       | 0.888 ± 0.222 | N.D. | N.D. | N.D. | N.D. |

The ATPase activity of each FliI protein was measured using the Malachite Green assay. These data are the average of three independent measurements.
N.D. not determined.

Fig. 5 Electron micrographs showing the oligomerization ability of FliI, FliI-3A, FliI-4A, and FliI-5A. Each purified FliI sample (1 μM) was preincubated with 5 mM MgCl₂, 5 mM ADP, 5 mM AlCl₃, and 15 mM NaF for 20 min at room temperature, and then the mixture was stained with 1% uranyl acetate and visualized by electron microscopy. Electron micrographs were recorded at a magnification of ×50,000. Insets indicate reference-free 2D class average images calculated by RELION3.0.7.
Isolation of pseudorevertants from the ΔfliH fliI-5A mutant.
To clarify our hypothesis described above, we isolated three pseudorevertants from the ΔfliH fliI-5A mutant. Motility of these pseudorevertants was better than that of their parental mutant (Fig. 8a). DNA sequencing revealed two insertion mutations, fliBSP2 and fliBSP3, at the end of the C-terminal α-helix of FlhBC and a P361A missense mutation, named fliBSP3, in the flexible C-terminal tail of FlhB (FlhB\text{CC}T) (Fig. 8b). The inserted LKRWQ and WQLKR sequences of the ΔfliH fliI-5A fliBSP2 and ΔfliH fliI-5A fliBSP3 suppressor mutants, which are presumably caused by gene duplication, are located between Glu-349 and Leu-350 and between Arg-352 and Thr-353, respectively.

To test whether the fliBSP2, fliBSP3 and fliBSP4 mutations display allele specificity, we transduced these fliB alleles into the ΔfliH fliI mutant using P22 phage to produce the ΔfliH fliI flhBSP4 mutant (Supplementary Fig. 5a). Consistently, much larger amounts of FlgD were detected in the culture supernatants of the ΔfliH fliI flhBSP2, ΔfliH fliI flhBSP3, and ΔfliH fliI flhBSP4 mutants than in that of the ΔfliH fliI fliI flhBSP4 mutant (Supplementary Fig. 5b). These results indicate that these fliB mutations are able to bypass both FliH and FliI defects. Overexpression of wild-type FliI enhanced the motility of the ΔfliH fliI flhBSP4 mutant (Fig. 8c). Consistently, FliI overexpression increased the secretion level of FlgD (Fig. 8d). In contrast, overexpression of FliI-5A reduced motility of and the secretion level of FlgD by the ΔfliH fliI flhBSP4 mutant (Fig. 8d), indicating that FliI-5A still exerts an inhibitory effect on flagellar protein export even in the presence of fliBSP4 mutation. Similar results were obtained with the ΔfliH fliI flhBSP2 and the ΔfliH fliI flhBSP3 mutants (Supplementary Fig. 6). The second-site fliB mutations by themselves displayed no phenotype (Supplementary Fig. 5c, d). Because FliI-5A was fully functional at a relatively high copy level in the presence of FliH but not in its absence (Fig. 3), we suggest that the docking of FlI-5A to FlhBC inhibits substrate entry into the polypeptide channel in the absence of FliH.

Effect of fliB mutations on the interaction between FlhB\text{C} and FlgD. The N-terminal export signal of FlgD binds to a conserved hydrophobic patch formed by Ala-286, Pro-287, Ala-341, and Leu-344 residues of FlhB. To test whether the fliB bypass mutations affect the interaction of FlhB\text{C} with FlgD, we carried out pull-down assays by GST affinity chromatography. FlgD copurified with GST-FlhBC (Fig. 8e, first row). The fliBSP2, fliBSP3, and fliBSP4 mutations did not reduce the binding affinity of FlhB\text{C} for FlgD (Fig. 8e), suggesting that these fliB mutations increase the probability of substrate entry into the FliP, FliQ, FlrI, polypeptide channel complex and that FlhB\text{C} may play a regulatory role in the substrate entry mechanism.

Membrane topology of FlhB and FlhA. The fliB (P13T), fliB (A21T/V), flhB (I27N), and flhB (P28T) mutations, which are postulated to be located in the N-terminal cytoplasmic tail of FlhB (FlhB\text{NCT}) (Fig. 8b), also bypass both FliH and FliI defects. Recent genetic analysis has suggested that an interaction between FlhB\text{NCT} and FlhB\text{EN} may facilitate the entry of export substrates into the FlipP, FliQ, FlrI, polypeptide channel complex. However, it remains unclear whether FlhB\text{NCT} and FlhB\text{EN} are close to the cytoplasmic entrance of the polypeptide channel because the densities corresponding to these two parts of FlhB in the cryo-electron microscopic (cryoEM) structure of the core of the type III export apparatus were not high enough to build their atomic models. Therefore, we analyzed the topology of FlhB\text{TM} by PhoA fusion assays using L-broth agar plate containing a chromogenic substrate of alkaline phosphatase, 5-bromo-4-chloro-3-indolyl phosphate (BCIP). We constructed five FlhB–PhoA fusions, PhoA–FlhB, FlhB\text{TM}–PhoA, FlhB\text{I–132}–PhoA, FlhB\text{I–184}–PhoA, and FlhB\text{I–212}–PhoA (Fig. 9a). We used a precursor form of PhoA (prePhoA) and its mature form (mPhoA) as the positive and negative controls, respectively, because prePhoA contains a signal peptide, allowing PhoA to be secreted via the Sec translocon into the periplasm to become the active form, whereas mPhoA does not. We also used a PhoA–FlhQ fusion as a positive control because the cryoEM structure of FliQ has shown that the N-terminus of FliQ is located in the periplasm. We used a Salmonella ΔphoN strain as a host. When prePhoA and the PhoA–FlhQ fusion were expressed in the ΔphoN cells, the colonies became blue on the BCIP indicator plates (Supplementary Fig. 7). In contrast, the ΔphoN colonies expressing mPhoA remained white (Supplementary Fig. 7). These results indicate that the fusion of FliQ to the C-terminus of PhoA does not affect the PhoA activity. The ΔphoN cells expressing FlhB\text{TM}–PhoA or FlhB\text{I–184}–PhoA formed blue colonies on...
the BCIP indicator plates, whereas the cells expressing FlhB(1–132)–PhoA formed white colonies (Fig. 9a), in agreement with the cryoEM structure of FlhB. The PhoA–FlhB and FlhB(1–212)–PhoA fusions showed no PhoA phosphatase activity (Fig. 9a), indicating that both FlhBNCT and FlhBC are located in the cytoplasm. Therefore, we suggest that they are in close proximity to each other in the export gate complex.

The FlhA(I21T), FlhA(L22F), and FlhA(V404M) mutations have been identified as gain-of-function mutations that are able to overcome both FliH and FliI defects. The FlhA(I21T) and FlhA(L22F) mutations lie in the N-terminal cytoplasmic tail of FlhA(FlhANCT), whereas the FlhA(V404M) mutation is located in FlhAC (Fig. 9b). The FlhAC ring structure has been visualized to project into the cytoplasmic cavity of the C ring (Fig. 1), but structural information of FlhATM containing putative eight TM helices is lacking. Therefore, we constructed nine PhoA fusions, PhoA–FlhA, FlhA(1–44)–PhoA, FlhA(1–65)–PhoA, FlhA(1–93)–PhoA, FlhA(1–196)–PhoA, FlhA(1–236)–PhoA, FlhA(1–278)–PhoA, FlhA(1–306)–PhoA, and FlhA(1–339)–PhoA (Fig. 9b). The FlhA(1–44)–PhoA, FlhA(1–93)–PhoA, FlhA(1–236)–PhoA, and FlhA(1–306)–PhoA fusions exhibited the phosphatase activity. However, the PhoA–FlhA, FlhA(1–65)–PhoA, FlhA(1–196)–PhoA, FlhA(1–278)–PhoA, and FlhA(1–339)–PhoA showed no phosphatase activity. These results are consistent with the membrane topology of FlhA predicted based on the primary sequence of FlhA. Because FlhANCT was in the cytoplasm (Fig. 9b), we suggest that FlhANCT is presumably close to FlhAC, FlhBNCT, and FlhBC.

Discussion

FliI forms a homo-hexamer at the flagellar base and hydrolyzes ATP to activate the PMF-driven export gate complex to drive flagellar protein export in a PMF-dependent manner (Fig. 1). FliI shows an extensive structural similarity with the α and β subunits of F1-ATPase and hydrolyzes ATP with the mechanism similar to that of F1-ATPase. The αβ3 hetero-hexamer of F1-ATPase is stabilized by interactions between the N-terminal
Fig. 8 Isolation of pseudorevertants from the Δflh-5A mutant. a Motility of MMHI001 (Δflh-5A) cells transformed with pTrc99A (indicated as Δflh-5A), pMM1702 (indicated as Δflh), or pMM1702-5A (indicated as Δflh flh-5A) and MMHI001-5A-SP2 transformed with pMM1702-5A (indicated as Δflh flh-5A flhBSP2), MMHI001-5A-SP3 transformed with pMM1702-5A (indicated as Δflh flh-5A flhBSP3), and MMHI001-5A-SP4 transformed with pMM1702-5A (indicated as Δflh flh-5A flhBSP4) in soft agar. The plate was incubated at 30 °C for 6 h. Scale bar, 0.5 cm. b Location of gain-of-function mutations in FlhBC. FlhBC undergoes autocatalytic cleavage between Asn-269 and Pro-270 residues to generate two distinct FlhBCN (CN) and FlhBCC (CC) polypeptides. FlhBC has a highly flexible C-terminal cytoplasmic tail (FlhBCCT). All gain-of-function mutations identified in this study are located in FlhBCCT. The flhB(P13T), flhB(A21V), flhB(A21T), flhB(I27N), and flhB(P28T) mutations in the N-terminal cytoplasmic tail of FlhB (FlhBNCT) have been identified as gain-of-function mutations that overcome the FliH and FliI defects to a considerable degree. c Motility of the Δflh-5A mutant carrying with pTrc99A (V), pMM1702 (WT), or pMM1702-5A (FliI-5A) in soft agar. The plate was incubated at 30 °C for 8 h. The diameter of the motility ring of five colonies of each strain was measured. The average diameter of the motility ring of each transformant was calculated. Dots indicate individual data points. Vertical bars indicate standard deviations. Scale bar, 0.5 cm. d Immunoblotting, using polyclonal anti-FlgD antibody, of whole-cell proteins and culture supernatant fractions prepared from the same transformants. The regions of interest were cropped from original immunoblots shown in Supplementary Fig. 13. Relative secretion levels of FlgD were measured. These data are average of three independent experiments. Dots indicate individual data points. Vertical bars indicate standard deviations. Comparisons between datasets were performed using a two-tailed Student’s t test. A P value of <0.05 was considered to be statistically significant difference. **P < 0.01; ***P < 0.001; ND, no statistical difference. e Effect of flhB mutations on the interaction of FlhBC with FlgD. Whole-cell lysates (L) prepared from Salmonella SJW1368 (ΔcheW-flhD) cells expressing GST-FlhBC, GST-FlhBC-SP2, GST-FlhBC-SP3, or GST-FlhBC-SP4 were mixed with those from E. coli BL21 (DE3) cells producing FlgD, followed by GST affinity chromatography. Elution fractions were analyzed by CBB staining (upper panel) and immunoblotting with anti-FlgD antibody (lower panel). The regions of interest were cropped from original CBB-stained gels and immunoblots shown in Supplementary Fig. 14. Three independent assays were performed.
domains of the α and β subunits. The core structure of FliI can be superimposed relatively well onto the N-terminal domains of the α and β subunits. However, the FliI ring model, which is generated by fitting the crystal structure of FliI onto the α and β subunits of the F1-ATPase, shows steric hindrances of FliI N at the subunit interface, suggesting that a conformational change of FliI N is required for FliI ring formation. Here, we showed that the flI(R33A) and flI-3A (R26A/R27A/R33A triple mutation) mutations increased the probability of FliI ring formation at a relatively low protein concentration compared to wild-type FliI and that the flI(R76A) mutation partially reduced the FliI ring formation efficiency of FliI-3A (Figs. 4 and 5 and Table 1). These suggest that Arg-33 and Arg-76 are involved in well-regulated FliI ring formation. The flI-3A mutation reduced the binding affinity of FliH for FliI (Supplementary Fig. 2). However, when FliH was co-expressed with FliI-3A, it suppressed the growth defect caused by FliI-3A (Supplementary Fig. 3). Because FliHC also binds to FliI N, we suggest that the interaction between FliHC and FliI N suppresses premature FliI ring formation in the cytoplasm. Therefore, we propose that FliI requires FliH for well-regulated conformational rearrangements of FliI N to form a homo-hexamer at the flagellar base and that Arg-33 and Arg-76 of FliI play important roles in the hexamer assembly.

Fig. 9 PhoA fusion assays. a Membrane topology of FliB. Salmonella TH12991 (ΔphoN) cells were transformed with pMKM10001 (pTrc99A/mPhoA), pMKM10002 (pTrc99A/prePhoA), pMKM10004 (pTrc99A/PhoA-FliB, indicated as B1), pMKM10005 (pTrc99A/ FliB1-59-PhoA, indicated as B2), pMKM10006 (pTrc99A/FliB1-132-PhoA, indicated as B3), pMKM10007 (pTrc99A/FliB1-184-PhoA, indicated as B4), or pMKM10008 pTrc99A/ FliB1-232-PhoA, indicated as B5), and then fresh transformants were inoculated onto BCIP indicator plates containing 100 μg ml⁻¹ ampicillin, followed by incubation at 30 °C for 18 h. In the periplasm, PhoA adopts an active conformation to hydrolyze BCIP, thereby generating blue color colonies on BCIP indicator plates. As a result, the colonies remain white. Scale bar, 1.0 cm. b Membrane topology of FliA. FliA consists of the N-terminal transmembrane domain with eight putative transmembrane helices (TM1-TM8) (FliA NCT) and the C-terminal cytoplasmic domain (FliA C). The flhA(I2I1T) and flhA(L22F) mutations in the N-terminal cytoplasmic tail of FliA (FliA NCT) and the flhA(V404M) mutation in FliA C have been identified as gain-of-function mutations to partially rescue motility of the ΔfliH and Δflih-flii mutant cells. Fresh TH12991 cells carrying pMKM10001, pMKM10002, pMKM10009 (pTrc99A/PhoA-FliA, indicated as A1), pMKM10010 (pTrc99A/ FliA1-44-PhoA, indicated as A2), pMKM10011 (pTrc99A/FliA1-65-PhoA, indicated as A3), pMKM10012 (pTrc99A/FliA1-93-PhoA, indicated as A4), pMKM10013 (pTrc99A/FliA1-132-PhoA, indicated as A5), pMKM10014 (FliA1-236-PhoA, indicated as A6), pMKM10015 (pTrc99A/FliA1-278-PhoA, indicated as A7), pMKM10016 (pTrc99A/FliA1-306-PhoA, indicated as A8), or pMKM10017 pTrc99A/FliA1-339-PhoA, indicated as A9) were inoculated onto BCIP indicator plates, and the plates were incubated at 30 °C for 18 h. Scale bar, 1.0 cm.
FlhI is not essential for flagellar protein export37,40,41, and the FlhI defect can be overcome to a considerable degree by many factors, such as bypass mutations in FlhA and FlhB, an increase in the expression level of export substrates, and an increase in total PMF37,41. Recently, in vitro reconstitution experiments using inverted membrane vesicles have demonstrated that the FlhI-Flh complex facilitates the docking of export substrates and flagellar chaperone/substrate complexes to the transmembrane export gate complex so that the gate complex can efficiently unfold and transport export substrates in a PMF-dependent manner30,42. Based on these observations, FlhI is thought to be involved in at least three distinct steps. First, FlhI recognizes export substrates in the cytoplasm and delivers them to the FlhAC-FlhB docking platform along with FlhIEC37,39,43. Second, ATP hydrolysis by FlhI activates the export gate complex to become an active PMF-driven protein transporter23,24. Finally, FlhI promotes export substrate entry into the FliP5FliQ4FliR1 polypeptide channel complex37. Here, we showed that the flii-5A (R26A/R27A/R33A/R76A/R93A) mutation caused a loss-of-function phenotype in the absence of FlhH but not in its presence (Fig. 3). Interestingly, the level of FlgD secreted by the ΔfliHI-flii-5A mutant was lower than that by the ΔfliHI-flii double null mutant (Fig. 3d). Furthermore, overexpression of FliI-5A reduced the secretion level of FlgD by the ΔfliHI-flii-5A mutant compared to the vector control (Fig. 3d). These observations indicate that FlhI-5A exerts an inhibitory effect on flagellar protein export in the absence of FlhH. The binding affinities of FlhI-5A for FlhB and FlhI were higher than that of wild-type FlhI (Fig. 7). In contrast, the binding affinity of FlhI-5A for FlhAC was lower than that of wild-type FlhI (Fig. 7). Because FlhI-5A retained the ability to bind to FlgD (Fig. 6c), we suggest that the binding of FliI-5A to FlhBC and FlhI may inhibit the flagellar protein export process in the absence of FlhH. Therefore, we propose that a positive charge cluster of FlhI may regulate the binding affinities of FlhI for FlhAC, FlhB, and FlhI to facilitate the subsequent entry of flagellar building blocks into the FliP5FliQ4FliR1 polypeptide channel complex and that the dissociation of FlhI from FlhB and FlhI may be required for efficient substrate entry into the polypeptide channel. Because FlhI-5A was functional in the presence of FlhH but not in its absence (Fig. 3), we also propose that an interaction between FlhH2 and FlhI, in addition to FlhI-5A, may allow the positive charge cluster of FlhI to undergo its proper conformational changes coupled with the substrate entry process.

Gain-of-function mutations in FlhB_CCT enhanced motility of the ΔfliHI-flii-5A mutant cells (Fig. 8a). These flihBCCT mutations did not affect the binding affinity of FlhB for FlgD (Fig. 8e). Furthermore, they did not display allele specificity (Supplementary Fig. Sc, d). These suggest that these flihBCCT mutations considerably increase the probability of export substrate entry into the FliP5FliQ4FliR1 polypeptide channel complex in the absence of FlhH and FlhI. Therefore, we propose that FlhB_CCT may suppress spontaneous gate opening of the polypeptide channel to avoid undesirable leakage of small salts through the channel. How does FlhB_CCT control gate opening? FlhBTM associates with the FliP5FliQ4FliR1 complex and the cytoplasmic loop connecting helices 2 and 3 (FlhB-loop) wraps around the cytoplasmic face of the FliP5FliQ4FliR1 complex through interactions of FlhB-loop with each FliQ subunit, having proposed that FlhB-loop may be involved in the gating mechanism of the polypeptide channel9. The flihB(E230A) mutation in FlhB_CCT reduces the protein transport activity of the flihB(E230A) mutant to the wild-type level38, suggesting that an interaction between FlhB_CCT and FlhB_CCT is required for flagellar protein export. We found that the flihB(E230A) mutation did not reduce the binding affinities of FlhB for either FlhI or FlgD (Supplementary Fig. 8). PhoA fusion assays showed that both FlhB_CCT and FlhB_CCT are in the cytoplasm (Fig. 9a), suggesting that they are close to FlhB-loop. Recent photo-crosslinking experiments have shown that Pro-28 of FlhB_CCT is in very close proximity to FlhIΔ. These suggest that conformational rearrangements of FlhB_CCT, FlhB-loop, and FlhB_CCT occur in a FlhI-dependent manner, allowing the polypeptide channel to be opened and that FlhB_CCT may regulate the FlhB_CCT-FlhB_CCT interaction to interfere with premature gate opening.

The flhB(P13T), flhB(A21V), flhB(A21T), flhB(I27N), and flhB (P28T) mutations in FlhB_CCT, the flha(A121T), and flha(A122F) mutations in FlhA_CCT, and the flaA(V404M) mutation in FlhA_CCT overcome both FlhI and FlhI defects33,37. PhoA fusion assays revealed that FlhA_CCT is located in the cytoplasm (Fig. 9b), suggesting that FlhA_CCT is in close proximity to FlhA_CCT, FlhB_CCT, and FlhB. Therefore, we propose that gain-of-function mutations in FlhA and FlhB may mimic FliI-bound state of the cytoplasmic entrance of the polypeptide channel so that an interaction between flagellar building block and FlhB may trigger the gate opening in the absence of FlhI and FlhI.

Methods

Bacterial strains, plasmids, transductional crosses, and DNA manipulations

Bacterial strains and plasmids used in this study are listed in Supplementary Table 1. P22-mediated transductional crosses were performed with P22HTini. Site-directed mutagenesis was carried out using the QuikChange Site-Directed Mutagenesis method as described in the manufacturer’s instructions (Stratagene). DNA sequencing reactions were carried out using BigDye v3.1 (Applied Biosystems), and then the reaction mixtures were analyzed by a 3130 Genetic Analyzer (Applied Biosystems).

Motility assays in soft agar

Fresh colonies were inoculated into soft agar plates [1% (w/v) triptone, 0.5% (w/v) NaCl, 0.35% Bacto agar] with or without 100 µg ml⁻¹ ampicillin and incubated at 30°C. A diameter of the motility ring was measured using the ImageJ software version 1.52 (National Institutes of Health). At least five different colonies were measured.

Secretion assays

S. enterica wild-type and mutant cells were grown at 30°C with shaking until the cell density had reached an optical density of 600 nm (OD600) of ca. 1.4–1.6. Cultures were centrifuged to obtain cell pellets and culture supernatants. The cell pellets were resuspended in a sample buffer solution [62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.001% bromophenol blue] containing 1 µl of 2-mercaptoethanol. Proteins in the culture supernatants were precipitated by 10% trichloroacetic acid and suspended in a Tris/SDS loading buffer (one volume of 1 M Tris, nine volumes of 1x sample buffer solution) containing 1 µl of 2-mercaptoethanol. Both whole cellular proteins and culture supernatants were normalized to a cell density of each culture to give a constant cell number. After boiling proteins in both whole cellular and culture supernatant fractions at 95°C for 3 min, these protein samples were separated by SDS–polyacrylamide gel (normal 12.5% acrylamide) electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Bio-Rad) using a transblotting apparatus (Hofer). Then immunoblotting with polyclonal anti-FlgD antibody was carried using iBand Flex Western Device (Thermo Fisher Scientific) as described in the manufacturer’s instructions. Detection was performed with Amersham ECL. Primary western blotting detection reagent (Cytiva). Chemiluminescence signals were captured by a LuminoImage analyzer LAS-3000 (GE Healthcare). The band intensity of each blot was analyzed using an image analysis software, CS Analyzer 4 (ATTO, Tokyo, Japan). All image data were processed with the Photoshop software (Adobe). At least three independent measurements were performed.

Pull-down assays by Ni affinity chromatography

The S. enterica SJW1368 strain carrying pTec9A-based plasmids co-expressing of untagged FlhI with His-FlhI or its mutant variants were grown overnight at 30°C in 100 ml L-broth containing ampicillin. Cell lysates were loaded onto a nickel-nitroacetic acid (Ni-NTA) agarose column (QIAGEN). After washing the column with 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 50 mM imidazole, proteins were eluted with 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl containing imidazole by a stepwise increase in the imidazole concentration of 100, 250, and 300 mM. Fractions were analyzed by SDS-PAGE with Coomassie Brilliant blue (CBB).

Cell growth measurements

Overnight cultures of S. enterica cells were diluted 100-fold into fresh L-broth containing 100 µg ml⁻¹ ampicillin, and the cells were grown at 30°C for 3 h with shaking. After adding IPTG at a final concentration of 0.1 mM, the incubation was continued for another 4 h. The cell growth was...
monitored at an OD_{600} every hour. Three different cells were measured and averaged.

**Purification of wild-type and point mutant variants of FliI.** *Escherichia coli* BL21 (DE3) Star cells carrying an appropriate PET19b-based plasmid encoding His-FliI, His-FliI(R33A), or His-FliI-3A, were grown overnight at 30°C in 250 ml of L-broth containing 100 μg ml⁻¹ ampicillin. His-FliI and its variant mutants were purified from cell lysates by Ni affinity chromatography with a Ni-NTA agarose column (QiAGEN), followed by size exclusion chromatography with a Hi-Load Superdex 200 (26/60) column (GE Healthcare)⁴³. For purification of His-FliI-4A and His-FliI-5A, the *S. enterica* SJW1368 cells transformed with pMMK1702-4AIIH or pMMK1702-5AIH, which encodes His-FliI-4A + FlhI or His-FliI-5A + FlhI on the pTc99A vector, were grown overnight at 30°C in 250 ml of L-broth containing ampicillin. His-FliI-4A/FlhI and His-FliI-5A/FlhI complexes were purified from the soluble fractions by Ni affinity chromatography, followed by size exclusion chromatography to remove FlhI. Fractions containing His-FliI or its variant mutants were dialyzed overnight against 50 mM Tris-HCL, pH 8.0, 150 mM NaCl, and 1 mM EDTA at 4°C.

**Measurements of the FliI ATPase activity.** Wild-type FliI, FlhI(R33A), FlhI-3A, FlhI-4A, and FlhI-5A were concentrated to 40, 5, 5, 20, and 20 μM, respectively, and then each purified sample was added to a buffer containing 30 mM HEPES-NaOH, pH 8.0, 30 mM KCl, 30 mM NH₄Cl, 5 mM MgCl₂, 5 mM ADP, 5 mM AlCl₃, and 15 mM NaF assay⁴⁴. At least three measurements were carried out at each FliI protein concentration.

**In vitro reconstruction of the Fli ring structure.** Purified proteins (final concentration 1 μM) was incubated in 50 mM Tris-HCL, pH 8.0, 113 mM NaCl, 0.8 mM EDTA, 1 mM DTT, 5 mM MgCl₂, 5 mM ADP, 5 mM AlCl₃, and 15 mM NaF at room temperature for 20 min. Samples were applied to carbon-coated copper grids and negatively stained with 2% (w/v) uranyl acetate. Electron micrographs were recorded at a magnification of ×50,000 with a JEM-1011 transmission electron microscope (JEOL, Tokyo, Japan) operated at 100 kV. To carry out two-dimensional class averaging of the Flh-I, Flh-4A, and Flh-5A ring structures, 154, 100, and 103 particle images were picked manually, aligned, classified, and averaged using the RELION3.0.7 program⁴⁵. At least three measurements were carried out at each FlhI protein concentration.

**Pull-down assays by GST affinity chromatography.** To investigate the effect of the flh-5A mutation on interactions of Flh with FlhAC, FlhBC, and Flh pull-down assays by GST affinity chromatography were carried out as described previously⁴⁶. Purified His-FliI or His-FliI-5A was mixed with purified GST, GST-FlhAC, GST-FlhBC, or GST-FlhI, and then each mixture was dialyzed overnight against PBS at 4°C with three changes of PBS. A 5 μl of each mixture was loaded onto a glutathione Sepharose 4B column (GE Healthcare). After washing with phosphate-buffered saline (PBS, 8 g of NaCl, 0.2 g of KCl, 3.63 g of Na₂HPO₄·12H₂O, 0.24 g of KH₂PO₄, pH 7.4 per liter), proteins were eluted with 50 mM Tris-HCL, pH 8.0, and 10 mM reduced glutathione. Fractions containing GST-tagged proteins were pooled and dialyzed overnight against PBS at 4°C with three changes of PBS.

**PhoA fusion assays.** Fresh transformants were inoculated onto BCIP indicator plates (1% (w/v) tripton, 0.5% (w/v) NaCl, 0.35% (w/v) Bacto agar, 50 μg ml⁻¹ BCIP) containing 100 μg ml⁻¹ ampicillin and incubated at 30°C for 18 h. At least seven independent measurements were performed.

**Multiple sequence alignment.** Multiple sequence alignment was carried out using CLUSTAL-Ω (http://www.ebi.ac.uk/Tools/msa/clustalo/).
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Author contributions

M.K., K.N., and T.M. designed research; M.K. and T.M. performed research; M.K. and T.M. analyzed data; M.K., K.N., and T.M. wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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