The FlhA linker mediates flagellar protein export switching during flagellar assembly

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Abstract

The flagellar protein export apparatus switches export specificity from hook-type to filament-type upon completion of hook assembly, thereby initiating filament assembly at the hook tip. The C-terminal cytoplasmic domain of FlhA (FlhA_C) forms a homo-nonameric ring structure that serves as a docking platform for flagellar export chaperones in complex with their cognate filament-type substrates. Interactions of the flexible linker of FlhA (FlhA_L) with its nearest FlhA_C subunit in the ring allow the chaperones to bind to FlhA_C to facilitate filament-type protein export, but it remains unclear how it occurs. Here, we report that FlhA_L acts as a switch that brings the order to flagellar assembly. The crystal structure of FlhA_C(E351A/D356A) showed that Trp-354 in FlhA_L bound to the chaperone-binding site of its neighboring subunit. We propose that FlhA_L binds to the chaperone-binding site of FlhA_C to suppress the interaction between FlhA_C and the chaperones until hook assembly is completed.

Introduction

The flagellum of *Salmonella enterica* (hereafter referred to *Salmonella*) is a supramolecular motility machine consisting of the basal body, the hook and the filament. For construction of the flagella on the cell surface, a type III protein export apparatus (FT3SS) transports flagellar building blocks from the cytoplasm to the distal end of the growing flagellar structure. The FT3SS is divided into three structural parts: a transmembrane export gate complex made of FlhA, FlhB, FliP, FliQ and FliR, a docking platform composed of the cytoplasmic domains of FlhA and FlhB (FlhA_C and FlhB_C), and a cytoplasmic ATPase ring complex consisting of FliH, FliI and FliJ¹. The FlhA_C-FlhB_C docking platform switches its substrate specificity from hook-type export substrates (FlgD, FlgE and FliK) to filament-type ones (FlgK, FlgL, FlgM, FliC and FliD) when the hook reaches its mature length of about 55 nm in *Salmonella*, thereby terminating hook assembly and initiating filament formation².

FliK is secreted via FT3SS during hook-basal body (HBB) assembly not only to measure the hook length but also to switch export specificity of the FlhA_C-FlhB_C docking platform². This has been recently verified by *in vitro* reconstitution experiments using inverted membrane vesicles^{3,4}. The N-terminal domain of FliK (FliK_N) serves as a secreted molecular ruler to measure the hook length⁵⁻⁸. When the hook length reaches about 55 nm, a flexible linker region of FliK connecting FliK_N and the C-terminal domain (FliK_C) promotes a conformational rearrangement of FliK_C to interact with FlhB_C, thereby

terminating hook-type protein export^{9,10}.

FlhA_C consists of four domains, D1, D2, D3 and D4, and a flexible linker (FlhA_L) connecting FlhA_C with the N-terminal transmembrane domain of FlhA (Fig. 1)¹¹. FlhA_C forms a homo-nonamer ring^{12,13} and provides binding-sites for flagellar export chaperons (FlgN, FliS and FliT) in complex with their cognate filament-type substrates^{14–17}. Interactions of FlhA_C with the flagellar chaperones facilitate the filament-type substrates to enter into the export gate complex for efficient protein export and assembly^{15,16}. The FliK_C-FlhB_C interaction is thought to induce structural remodeling of the FlhA_C ring through interactions of FlhA_L with its nearest FlhA_C subunit, thereby initiating the export of filament-type proteins^{13,18,19}. However, it remains unknown how.

In the present study, to clarify the role of FlhA_L in the export switching mechanism of FT3SS, we analyzed the interaction between FlhA_L and FlhA_C and provide evidence suggesting that the interaction of FlhA_L with the chaperone-binding site of FlhA_C inhibits the binding of the flagellar export chaperones to FlhA_C to keep the export specificity in the hook type during hook assembly.

Results

Isolation of pseudorevertants from the *flhA(E351A/W354A/D356A)* mutant. Glu-351, Trp-354 and Asp-356 of FlhA_L bind to the D1 and D3 domains of its neighboring FlhA_C

79 subunit to stabilize FlhA_C ring structure in solution^{11,13}. The *flhA(E351A/D356A)* and
80 *flhA(W354A)* mutants produces the HBBs without the filament attached although their
81 hook length is not controlled properly¹³. The W354A and E351A/D356A mutations inhibit
82 the interaction of FlhA_C with flagellar chaperones in complex with their cognate
83 filament-type substrates, suggesting that the interaction between FlhA_L and the D1 and
84 D3 domains of its neighboring FlhA_C subunit keeps the chaperone binding site of FlhA_C
85 open to allow the chaperones to bind to FlhA_C to facilitate the export of the filament-type
86 substrates¹³. However, the *flhA(E351A/W354A/D356A)* mutant do not produce the HBBs
87 at all, raising a question of why the E351A/W354A/D356A triple mutation inhibits HBB
88 assembly¹³. To clarify this question, we isolated 14 pseudorevertants from the
89 *flhA(E351A/W354A/D356A)* mutant. Motility was somewhat restored by these
90 pseudorevertant mutations but it was much poorer than that of wild-type cells (Fig. 2a).
91 Export substrates such as FlgD, FlgE, FlgK and FliD were detected in the culture
92 supernatants of these pseudorevertants (Fig. 2b). In agreement with this, these
93 pseudorevertants produced a couple of flagella on the cell surface (Fig. 2c). DNA
94 sequencing revealed that all suppressor mutations are located in the *flgMN* operon. One
95 was the M1I mutation at the start codon of the *flgM* gene (isolated twice), presumably
96 inhibiting FlgM translation. Two suppressor mutations produced a stop codon at position
97 of Gln-52 or Ser-85 of FlgM, resulting in truncation of the C-terminal region of FlgM. Nine
98 suppressor mutations were large deletions in *flgM*. We also found that there was a large

deletion in the *flgM* and *flgN* genes, thereby disrupting both FlgM and FlgN. A loss-of-function of FlgM results in a considerable increment in the transcription levels of flagellar genes^{20,21}. Consistently, the cellular levels of FlgK and FliD seen in the pseudorevertants were higher than those in its parental strain (Fig. 2b, 3rd and 4th rows). It has been shown that an interaction between FliJ and FlhA_L brought about by FliH and FliI fully activates the transmembrane export gate complex of σ^{54} SS to utilize proton motive force (PMF) across the cell membrane to drive flagellar protein export²². Because the E351A/W354A/D356A triple mutation reduces the binding affinity of FlhA_C for FliJ¹³, this suggests that these *flgM* mutations considerably increase the cytoplasmic levels of FliH, FliI, FliJ and export substrates to allow the *flhA(E351A/W354A/D356A)* mutant to export flagellar building blocks for producing a small number of flagella on the cell surface. Therefore, we propose that Glu-351, Trp-354 and Asp-356 of FlhA_L also play an important role in the activation mechanism of the PMF-driven export gate complex.

We found that the secretion levels of FlgD and FlgE by the pseudorevertants were about 1.5-fold and 4-fold higher than those by the wild-type whereas the secretion levels of FlgK and FliD were much lower (Fig. 2b), raising the possibility that *flgM* suppressor mutations do not efficiently promote export switching of σ^{54} SS from hook-type substrates to filament-type ones in the *flhA(E351A/W354A/D356A)* mutant. To clarify this, we introduced the $\Delta flgM::km$ allele to the *Salmonella* NH001 ($\Delta flhA$) strain to produce the $\Delta flgM::km$ and *flhA(E351A/W354A/D356A)* $\Delta flgM::km$ cells (Supplementary

Fig. 1). The $\Delta flgM::km$ allele restored motility of the *flhA(E351A/W354A/D356A)* mutant in a way similar to other *flgM* suppressor mutations. Then, we isolated flagella from the $\Delta flgM::km$ and *flhA(E351A/W354A/D356A) \Delta flgM::km* cells and measured their hook length. The hook length of the $\Delta flgM::km$ strain was 52.0 ± 5.1 nm (mean $\pm$ SD, $n = 157$) (Fig. 2d), which is nearly the same as that of the wild-type strain (51.0 ± 6.9 nm)¹³. This indicates that the loss-of-function mutation of FlgM does not affect the hook length control. In contrast, the average hook length of the *flhA(E351A/W354A/D356A) \Delta flgM::km* strain was 68.8 ± 30.9 nm (mean $\pm$ SD, $n = 157$) (Fig. 2d), indicating that the hook length control becomes worse in the presence of the E351A/W354A/D356A triple mutation. These suggest that this triple mutation affects not only the initiation of filament-type protein export but also the termination of hook-type protein export. Therefore, we propose that conformational rearrangements of FlhA_L are required for well-regulated export switching of fT3SS.

Effect of FlhA linker mutations on the hydrodynamic properties of FlhA_C in solution. A well conserved hydrophobic dimple of FlhA_C containing Asp-456, Phe-459 and Thr-490 residues is located at the interface between domains D1 and D2 and is involved in the interactions with the FlgN, FliS and FliT chaperones in complex with their cognate filament-type substrates (Fig. 1)^{15–17}. The W354A, E351A/D356A and E351A/W354A/D356A mutations significantly reduce the binding affinity of FlhA_C for

these chaperone/substrate complexes¹³, raising the possibility that FlhA_L carrying these *flhA* mutations binds to the hydrophobic dimple of FlhA_C and blocks the FlhA_C-chaperone interaction. If true, FlhA_C with these mutations would show distinct hydrodynamic properties compared with wild-type FlhA_C. To clarify this possibility, we performed size exclusion chromatography with a Superdex 75 column HR 10/30 column. Wild-type His-FlhA_C appeared as a single peak at an elution volume of 10.3 mL, which corresponds to the deduced molecular mass of His-FlhA_C (about 43 kDa) (Fig. 3a). His-FlhA_C(W354A), His-FlhA_C(E351A/D356A) and His-FlhA_C(E351A/W354A/D356A) appeared as a single peak at an elution volume of 10.3 mL, 10.5 mL and 10.4 mL, respectively (Fig. 3a), indicating that these mutant variants exist as a monomer in solution. His-FlhA_C(E351A/D356A) exhibited a slightly delayed elution behavior compared with the wild-type. Furthermore, His-FlhA_C(E351A/D356A) showed a slightly faster mobility on SDS-PAGE gels. Far-UV CD measurements revealed that the E351A/D356A double mutation did not affect the secondary structures of FlhA_C (Supplementary Fig. 2). These suggest that FlhA_C(E351A/D356A) adopts a more compact conformation than wild-type FlhA_C. The elution peak position of His-FlhA_C(E351A/W354A/D356A) was between those of the wild-type and FlhA_C(E351A/D356A) (Fig. 3a). Because His-FlhA_C(E351A/W354A/D356A) showed two different bands on SDS-PAGE gels, with a slower mobility band corresponding to wild-type FlhA_C and a faster one corresponding to FlhA_C(E351A/D356A) (Fig. 3a, inset), we suggest that FlhA_C(E351A/W354A/D356A)

exists in an equilibrium between the wild-type conformation and the compact conformation. Since FlhA_C(W354A) adopted the wild-type conformation (Fig. 3a), we suggest that the E351A/D356A double mutation is required to make FlhA_C more compact and that Trp-354 of FlhA_L is needed to stabilize the compact conformation.

Effect of FlhA linker mutations on methoxypolyethylene glycol 5000 maleimide (mPEG-maleimide) modifications of Cys-459 and Cys-548. FlhA_C structures have shown that it adopts three distinct, open, semi-closed and closed conformations^{11,14,17,18,23}. Phe-459 and Lys-548 are fully exposed to solvent on the molecular surface of the open conformation of FlhA_C but are in close proximity to each other in the closed conformation^{11,18,23}. To test whether mutations in FlhA_L bias FlhA_C towards the closed structure, we performed Cys modification experiments with mPEG-maleimide, which adds a molecular mass of ~5 kDa to a target protein. FlhA_C(F459C/K548C) modified by mPEG-maleimide showed much slower mobility shift, indicating that both Cys459 and Cys548 are exposed to the solvent. The W354A, E351A/D356A and E351A/W354A/D356A mutations did not inhibit Cys modifications with mPEG-maleimide at all, indicating that FlhA_C with these mutations does not adopt the closed conformation.

Crystal structure of FlhA_C(E351A/D356A). To investigate whether Trp-354 of FlhA_L

binds to the hydrophobic dimple of FlhA_C to makes FlhA_C(E351A/D356A) more compact, we explored crystallization conditions of FlhA_C(E351A/D356A) for a molecular packing distinct from the open (PDB code: 3A5I)¹¹ and semi-closed (PDB code: 6AI0)¹⁸ forms of wild-type FlhA_C. We found a new orthorhombic crystal that diffracted up to 2.8 Å resolution, with unit cell dimensions $a = 71.7$ Å, $b = 96.2$ Å, $c = 114.1$ Å (Table 1) and the asymmetric unit containing two FlhA_C molecules (A and B). Mol-A adopts an open conformation similar to the 3A5I structure whereas Mol-B shows a semi-closed conformation similar to the 6AI0 structure (Supplementary Fig. 3). The residues from Val-349 to Val-357 in FlhA_L of Mol-A form an α -helix, which interacts with the hydrophobic dimple of a neighboring Mol-A molecule related by a crystallographic symmetry (Fig. 4a). Trp-354 fits into the hydrophobic dimple, and Ala-351 hydrophobically contacts with Pro-442 on the periphery of the dimple (Fig. 4b). These interactions resemble the interaction between the N-terminal α -helix of FliS and the hydrophobic dimple of FlhA_C (PDB ID: 6CH3)¹⁷ (Fig. 4c). Ile-7 and Tyr-10 of the N-terminal α -helix of FliS is in the corresponding position of Ala-351 and Trp-354 of FlhA_L, respectively. Tyr-10 fits into the hydrophobic dimple of FlhA_C, and Ile-7 interacts with Pro-442 of FlhA_C (Fig. 4d). These observations suggest that FlhA_L and flagellar chaperones bind competitively to a common binding site on FlhA_C and that the dissociation of FlhA_L from this binding site is required for the binding of the flagellar chaperones to FlhA_C.

Discussion

The FlhA_C ring serves as the docking platform for flagellar export chaperones in complex with their cognate substrates and facilitates the export of filament-type proteins to form the filament at the hook tip after completion of hook assembly^{14–17}. The FlhA_C ring also ensures the strict order of flagellar protein export, thereby allowing the huge and complex flagellar structure to be built efficiently on the cell surface^{13,14,16,18,19}. Interactions of FlhA_L with its neighboring FlhA_C subunit in the nonamer ring is required for the initiation of filament-type protein export upon completion of hook assembly. However, it remained unclear how the FlhA_C ring mediates such hierarchical protein export during flagellar assembly.

In this study, we first performed genetic analyses of the *flhA(E351A/W354A/D356A)* mutant and found that the E351A/W354A/D356A triple mutation caused a loose hook-length control (Fig. 2d), indicating that the E351A/W354A/D356A mutation significantly affects the termination of hook-type protein export. Furthermore, this triple mutation also reduced the secretion levels of the filament-type proteins considerably (Fig. 2b), thereby reducing the number of flagellar filaments per cell (Fig. 2c). These results suggest that FlhA_L serves as a structural switch for substrate specificity switching of fT3SS from hook-type to filament-type and that Glu-351, Trp-354 and Asp-356 of FlhA_L are directly involved in this export switching mechanism.

It has been reported that the W354A, E351A/D356A and E351A/W354A/D356A mutations inhibit interactions between FlhA_C and flagellar chaperones in complex with their cognate filament-type substrates¹³, suggesting that FlhA_L regulates the binding of flagellar chaperones to FlhA_C. The crystal structure of FlhA_C(E351A/D356A) showed that Trp-354 of one Mol-A molecule bound to the hydrophobic dimple of the flagellar chaperone binding site of its nearest Mol-A in the crystal (Fig. 4). Although the relative orientations of these Mol-A molecules in the crystal differs from those in the FlhA_C nonameric ring, FlhA_L can bind to the hydrophobic dimple of FlhA_C in the nonamer ring structure because of a highly flexible nature of FlhA_L (Fig. 5). The C-terminal region of FlhA_L is flexible enough to allow such subunit orientations without changing the essential interaction between FlhA_L and the chaperone binding site of FlhA_C, as it has been shown to have various conformations in the known FlhA_C structures¹⁸. Therefore, we propose that an interaction between FlhA_L and the hydrophobic dimple of its neighboring FlhA_C subunit suppresses the docking of flagellar chaperones to the FlhA_C ring platform during HBB assembly and that the hook completion induces the detachment of FlhA_L from the dimple through an interaction between FliK_C and FlhB_C and its attachment to the D1 and D3 domains to induce structural remodeling of the FlhA_C ring, thereby terminating hook assembly and initiating filament formation (Fig. 5). Because Trp-354 of FlhA_L stabilized a more compact conformation of the FlhA_C(E351A/D356A) monomer compared to the wild-type FlhA_C and FlhA_C(W354A) monomers (Fig. 3), it is also possible that FlhA_L may

block the docking of the flagellar chaperones to FlhA_C by covering the binding site of the same FlhA_C molecule.

Methods

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are listed in [Supplementary Table 1](#).

DNA manipulations. DNA manipulations and site-directed mutagenesis were carried out as described previously²⁴. 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. Fresh colonies were inoculated into soft agar plates [1% (w/v) triptone, 0.5% (w/v) NaCl, 0.35% Bacto agar] and incubated at 30°C.

Secretion assays. Details of sample preparations have been described previously²⁵. After SDS-polyacrylamide gel electrophoresis (PAGE), immunoblotting with polyclonal anti-FlgD, anti-FlgE, anti-FlgK, or anti-FliD antibody was carried out as described previously²⁶.

258

259 **Hook length measurements.** The HBBs were purified from NH004gM carrying pMM130
 260 or pYI003 as described previously²⁷. The HBBs were negatively stained with 2%(w/v)
 261 uranyl acetate. Electron micrographs were recorded with a JEM-1011 transmission
 262 electron microscope (JEOL, Tokyo, Japan) operated at 100 kV and equipped with a F415
 263 CCD camera (TVIPS, Gauting, Germany). Hook length was measured by ImageJ version
 264 1.48 (National Institutes of Health).

265

266 **Protein purification.** *E. coli* BL21 (DE) Star cells carrying a pET15b-based plasmid
 267 encoding His-FlhA_C or its mutant variants were grown overnight at 30°C in 250 mL of
 268 L-broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl] containing
 269 ampicillin. His-FlhA_C and its mutant variants were purified by affinity chromatography,
 270 followed by size exclusion chromatography as described previously²⁴.

271

272 **Far-UV CD spectroscopy.** Far-UV CD spectroscopy of His-FlhA_C or its mutant variants
 273 was carried out at room temperature using a Jasco-720 spectropolarimeter (JASCO
 274 International Co., Tokyo, Japan) as described previously²⁸.

275

276 **Cystein modification by mPEG-maleimide.** His-FlhA_C(F459C), His-FlhA_C(K548C),
 277 His-FlhA_C(F459C/K548C), His-FlhA_C(W354A/F459C/K548C),

His-FlhA_C(E351A/D356A/F459C/K548C) and
His-FlhA_C(E351A/W354A/D356A/F459C/K548C) were dialyzed overnight against 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) at
4°C, followed by cysteine modification by mPEG-maleimide (Fluka) as described
previously¹⁸. Each protein solution was run on SDS-PAGE and then analyzed by
Coomassie Brilliant blue (CBB) staining.

X-ray crystallographic study of FlhA_C(E351A/D356A). Initial crystallization screening
was performed at 20°C by the sitting-drop vapor-diffusion method using Wizard Classic I
and II, Wizard Cryo I and II (Rigaku Reagents, Inc.), Crystal Screen and Crystal Screen 2
(Hampton Research). Crystals suitable for X-ray analysis were obtained from drops
prepared by mixing 0.5 µL protein solution with 0.5 µL reservoir solution containing 0.1 M
Tris-HCl (pH 8.5), 20% (v/v) PEG 8000, and 200 mM MgCl₂. 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.
2016B2544 and 2018A2568). The FlhA_C(E351A/D356A) crystal was soaked in a solution
containing 90% (v/v) of the reservoir solution and 10% (v/v) glycerol for a few seconds
and was directly transferred into liquid nitrogen for freezing. The X-ray diffraction data
were collected under nitrogen gas flow at 100 K. The diffraction data were processed

with MOSFLM²⁹ and were scaled with Aimless³⁰. The initial phase was determined by molecular replacement using the software package Phenix³¹ with the wild-type FlhAc structure in the orthorhombic crystal form (PDB code: 6Al0) as a search model. The atomic model was constructed with COOT³² and refined with Phenix³¹. During the refinement process, iterative manual modification was performed. The diffraction data statistics and refinement statistics are summarized in [Table 1](#).

Accession code

The atomic coordinates have been deposited in Protein Data Bank under the accession code 7CTN.

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Acknowledgements

We thank beamline staffs at SPring-8 for technical help in use of beamlines BL41XU. This work was supported in part by JSPS KAKENHI Grant Numbers JP18K14638 and

JP20K15749 (to M.K.), JP16J01859 (to N.T.), 25000013 (to K.N.), 15H02386 (to K.I.) and JP26293097 and JP19H03182 (to T.M.). This work has also been partially supported by JEOL YOKOGUSHI Research Alliance Laboratories of Osaka University to K.N.

Author Contributions

K.N. K.I. and T.M. conceived and designed research; Y.I., M.Kida, M.Kinoshita, N.T., K.I. and T.M. performed research; Y.I., M.Kida, M.Kinoshita, N.T., K.I. and T.M. analysed the data; and K.N., K.I. and T.M. wrote the paper based on discussion with other authors.

Competing interests

The authors declare no competing interests.

Data availability

All data generated during this study are included in this published article and its Supplementary Information files.

Table 1. X-ray data collection and refinement statistics

X-ray data collection		
Space group	$P2_12_12_1$	
Cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	71.7, 96.2, 114.1	
Wavelength (Å)	1.0000	
Resolution (Å)	73.5-2.80	(2.95-2.80)
R_{merge}	0.074	(0.317)
R_{pim}	0.065	(0.283)
CC(1/2)	0.995	0.915
$I/\sigma I$	8.1	(2.8)
Completeness (%)	97.1	(94.6)
Redundancy	3.4	(3.1)
Refinement statistics		
Resolution range (Å)	73.5-2.80	(2.87-2.80)
No. of reflections working	17,398	(1,182)
No. of reflections test	1,903	(112)
R_w (%)	23.2	(33.5)
R_{free} (%)	29.0	(42.1)
Rms deviation bond length (Å)	0.003	
Rms deviation Bond angle (°)	0.680	
B-factors		
Protein atoms	70.0	
Solvent atoms	-	
Ramachandran plot (%)		
Most favored	96.0	
Allowed	3.9	
Disallowed	0.1	
No. of protein atoms	5, 252	
No. of solvent atoms	0	

Values in parentheses are for the highest resolution shell.

$R_w = \sum ||F_o| - |F_c|| / \sum |F_o|$, $R_{free} = \sum ||F_o| - |F_c|| / \sum |F_o|$

Fig. 1. FlhA_C structure in a crystal (PDB ID: 3A5I). FlhA_C consists of four domains, D1, D2, D3 and D4 and a flexible linker (FlhA_L). Glu-351, Trp-354 and Asp-356 of FlhA_L binds to the D1 and D3 domains of its neighboring subunit. A well-conserved hydrophobic dimple including Phe-459 is responsible for the interaction of FlhA_C with flagellar export chaperones in complex with filament-type substrates. Phe-459 and Lys-548 are exposed to solvent on the molecular surface when FlhA_C adopts the open conformation. The interactions between the two FlhA_C molecules in this crystal also represent those in the FlhA_C nonameric ring of the export apparatus.

Fig. 2. Isolation pseudorevertants from the *flhA*(E351A/W354A/D356A) mutant. (a) Motility of the *Salmonella* NH001 strain transformed with pTrc99A ($\Delta flhA$), pMM130 (WT), or pYI003 [FlhA(E351A/W354A/D356A) indicated as EWD], YI1003-4 (EWD $\Delta flgM$) or YI1003-13 (EWD $\Delta flgMN$) in soft agar. (b) Immunoblotting using polyclonal anti-FlgD (1st row), anti-FlgE (2nd row), anti-FlgK (3rd row) or anti-FliD (4th row) antibody, of whole cell proteins (Cell) and culture supernatants (Sup) prepared from the above strains. (c) Electron micrographs of the above cells. (d) Histogram of hook length distribution of NH001gM ($\Delta flhA$ $\Delta flgM::km$) carrying pMM130 ($\Delta flgM$) or pYI003 (EWD $\Delta flgM$).

Fig. 3. Effect of FlhA linker mutations on the FlhAc conformation. (a) Size exclusion chromatography using a Superdex 75HR 10/30 column. Elution positions of His-FlhAc (WT, black), His-FlhAc(W354A) (red), His-FlhAc(E351A/D356A) (blue) and His-FlhAc(E351A/W354A/D356A) (orange) are 10.3 ml, 10.3 ml, 10.5 ml and 10.4 ml, respectively. Arrow indicates the elution peak of ovalbumin (43 kDa). Inset, CBB-stained gels of purified FlhAc proteins. (b) Effect of FlhA mutations on mPEG-maleimide modification of Cys459 and Cys548. His-FlhAc(F459C), His-FlhAc(K548C), His-FlhAc(F459C/K548C), His-FlhAc(W354A/F459C/K548C), His-FlhAc(E351A/D356A/F459C/K548C) and His-FlhAc(E351A/W354A/D356A/F459C/K548C) were incubated with (+) or without (-) mPEG-maleimide. After centrifugation at 20,000 g for 20 min to remove any aggregates, supernatants were analyzed by SDS-PAGE with CBB staining. Cyan and magenta dots indicate positions of FlhAc-(mPEG) and FlhAc-(mPEG)₂, respectively.

Fig. 4. Interaction between FlhA_L and a well conserved hydrophobic dimple of its neighboring FlhA_C in the crystal of FlhA_C(E351A/D356A). (a) Mol-A of FlhA_C(E351A/D356A) (magenta) interacts with neighboring Mol-A (cyan) related by a crystallographic symmetry. (b) Close-up view of the interaction between FlhA_L and the hydrophobic dimple. Residues that form the hydrophobic dimple are indicated by balls. Ala-351 and Trp-354 in FlhA_L are shown in stick models. (c) Interaction between FlhA_C (green) and FliS (orange) fused with the C-terminal region of FliC (yellow) (PDB code: 6CH3). (d) Close-up view of the interaction between FliS and the hydrophobic dimple. The residues that form the hydrophobic dimple are indicated by ball. Ile-7 and Tyr-10 of FliS are shown in stick models.

Fig. 5. Structural rearrangements of FlhA_L responsible for export switching of ft3SS. Trp-359 of FlhA_L binds to a well-conserved hydrophobic dimple containing Asp-456, Phe-459 and Thr-490 of its neighboring FlhA_C subunit in the FlhA_C ring to inhibit the interaction of FlhA_C with flagellar chaperones in complex with their cognate filament-type substrates during hook assembly. When the hook reaches its mature length of about 55 nm, an interaction between FliK_C and FlhB_C triggers a conformational rearrangement of the FlhA_C ring so that FlhA_L dissociates from the hydrophobic dimple and binds to the D1 and D3 domains of the neighboring FlhA_C subunit, allowing the chaperones to bind to FlhA_C to facilitate the export of their cognate substrates for filament assembly.