Interaction of the Extreme N-Terminal Region of FliH with FlhA Is Required for Efficient Bacterial Flagellar Protein Export

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The flagellar type III protein export apparatus plays an essential role in the formation of the bacterial flagellum. FliH forms a complex along with Flh ATPase and is postulated to provide a link between Flh ring formation and flagellar protein export. Two tryptophan residues of FliH, Trp7 and Trp10, are required for the effective docking of the FliH-FliI complex to the export gate made of six membrane proteins. However, it remains unknown which export gate component interacts with these two tryptophan residues. Here, we performed targeted photo-cross-linking of the extreme N-terminal region of FliH (FliHEN) with its binding partners. We replaced Trp7 and Trp10 of FliH with p-benzoyl-phenylalanine (pBPA), a photo-cross-linkable unnatural amino acid, to produce FliHEN(W7pBPA) and FliHEN(W10pBPA). They were both functional and were photo-cross-linked with one of the export gate proteins, FlhA, but not with the other gate proteins, indicating that these two tryptophan residues are in close proximity to FlhA. Mutant FlhA proteins that are functional in the presence of FliH and FlhI but not in their absence showed a significantly reduced function also by N-terminal FliH mutations even in the presence of FlhI. We suggest that the interaction of FliHEN with FlhA is required for anchoring the Fli hexamer ring to the export gate for efficient flagellar protein export.

Bacteria such as Salmonella enterica serovar Typhimurium swim in liquids and swarm over surfaces by using flagella. The Salmonella cells employ a variety of proteins and gene regulation circuits to efficiently produce flagella in a temporally and spatially regulated manner. Upon the assembly of the flagellar basal body MS-C ring complex, which acts as a rotor of the flagellar motor, the flagellar type III protein export apparatus transports the flagellar axial proteins from the cytoplasm to the distal end of the growing flagellar structure, where their self-assembly occurs with the help of a capping structure. The export apparatus consists of a membrane-embedded export gate made of six membrane proteins, FlhA, FlhB, FlhC, FlhD, FlhM, and FlhR, and a water-soluble ATPase complex consisting of FliH, FliI, and FliJ (22, 28). The C-terminal cytoplasmic domains of FlhA (FlhAC) and FlhB (FlhBC) provide binding sites for the ATPase complex, flagellar substrate-specific chaperone, and export substrates (1, 25, 29, 32, 34, 50). FlhB acts as the export specificity switch to mediate the ordered export of the axial flagellar components during flagellar assembly (19). The specific roles of FlhO, FlhP, FlhQ, and FlhR remain unknown, although it has been shown that FlhO, FlhP, and FlhQ interact with FlhA (24) and that FlhR interacts with FlhA and FlhB (13, 45). Recently, it has been shown that the fliO null mutation, which leads to a drastic reduction in motility, is bypassed by the extragenic fliP suppressor mutation, proposing that FlhO has an important functional role in regulating FliP stability (2). FlhI is the ATPase (9) and forms a homohexameric ring to fully exert its enzyme activity (4). FliH acts as the regulator of FlhI ATPase to provide a link between Flh ring formation and protein export (25, 33). FliI is involved in the energy transduction mechanism along with FlhA (34). These proteins are evolutionarily related to those of the type III secretion system of pathogenic bacteria, which directly injects virulence factors into their host cells, although the FlhO homologue is apparently missing in virulence type III secretion systems (5).

Export substrates and chaperone-substrate complexes bind to the FliH-FliI-FliJ complex through their interactions with FliH and FliJ (16, 30, 44). The FliH-FliI complex binds to the C ring through a specific interaction of FliH with a C ring protein, FliN (12, 23, 37, 41). The overexpression of Flh alone or the FliH-FliI complex significantly enhances the reduced secretion activity of the fliN null mutant, suggesting that the FliH-FliN interaction increases the local concentration of the FliH-FliI-FliJ-chaperone-substrate complex around the export gate, thereby allowing the complex to efficiently associate with the export gate (8, 18, 23, 37). Then, a specific association between the FliH-FliI-FliJ complex and the FlhAC-FlhBC docking platform on the export gate induces the formation of the FliHX-FliI6-FliJ ring complex onto the platform, thereby facilitating the initial entry of the substrates into the export gate (35). The export gate utilizes proton motive force (PMF) across the cytoplasmic membrane to drive protein translocation into the central channel of the growing flagellar structure (35, 40). The export gate by itself is a proton-protein antiporter that uses the two components of PMF, namely, the electric potential difference (ΔΦ) and the proton concentration difference (ΔpH), for different steps of the protein export process (34). An interaction of FlhI with a linker region of FlhA between its N-terminal transmembrane region (FlhATM) and FlhAC is brought about by the FlhX-FliI6 ring complex, and this FlhI binding turns the export gate into a highly efficient ΔΨ-driven export apparatus (34).

FliH is a 235-amino-acid-residue protein, which can be divided into three regions: an N-terminal region (residues 1 to 100, FliHEN), a central region (residues 101 to 140, FlhAC), and a C-terminal region (residues 141 to 235, FlhHC) (11). Only the first 10
residues in FliH (FliH<sub>EN</sub>) are critical for the export function of FliH (11). Two tryptophan residues of FliH<sub>EN</sub>, Trp7 and Trp10, are absolutely required not only for the interaction with FliN but also for the effective docking of the FliH-Flii complex to the export gate (37). FliH<sub>N</sub> and FliH<sub>C</sub> are responsible for self-imerization and the interaction with Flii, respectively (11, 26). The amino acid sequences of FliH<sub>N</sub> and FliH<sub>C</sub> are homologous to those of the b and /β subunits of F<sub>1</sub>-F<sub>r</sub>-ATP synthase, respectively (39). The b and /β subunits form the peripheral stalk that links the water-soluble F<sub>1</sub>-ATPase unit to the transmembrane proton-translocating F<sub>r</sub> unit (46, 47). The crystal structures of FliI and FliJ show remarkable structural similarities to the /αβ/β subunits (17) and the γ subunit (15) of the F<sub>r</sub> unit, respectively. FliI penetrates the central hole of the FliI hexameric ring in a way similar to an antiparallel /α-helical coiled coil of the amino and carboxyl termini of the γ subunit penetrating into the central cavity of the /αβ<sub>3</sub> ring (15). These observations raise the possibility that FliH may act as a peripheral stalk that connects the FliH-Flii ring complex with the export gate. However, it remains unknown which component of the export gate is directly involved in the interaction with FliH<sub>EN</sub>.

In this paper, we performed site-directed photo-cross-linking of FliH<sub>EN</sub> with its binding partners. We show that FliH<sub>EN</sub> is in relatively close proximity to FlhA. We also show that the interaction between FliH and FlhA allows Flii to efficiently exert its export function.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and media.** Bacterial strains and plasmids used in this study are listed in Table 1. *Salmonella* ΔfliH ΔflaN and ΔflH ΔflfA double null strains were constructed using the λ Red homologous recombination system (6) as described previously (13). L-broth (LB), T-broth (TB), and motility agar plates were prepared as described previously (31, 32). Ampicillin, kanamycin, and chloramphenicol were added to LB at a final concentration of 50 μg/ml, 50 μg/ml, and 30 μg/ml, respectively.

**Construction of plasmids.** Single cysteine and /amber mutations of FliH were generated through PCR-based site-directed mutagenesis as described previously (13). All FliH substitutions were confirmed by DNA sequencing (BigDye v3.1, 3130 Genetic Analyzer; Applied Biosystems).

To construct pETDuet-based plasmids encoding both FliH with an /amber mutation at position 7 or 10 and a target protein, FlhA, FlhB, FliO-FLAG, FliH-Cys variant on pTrc99AFF4 and the other encoding FliN (V113C) on pBPA, a photo-cross-linkable unnatural amino acid, into the desired site of the proteins, we used the pEVOL system developed by Young and coworkers (49). The pEVOL plasmid, which encodes evolved *Methano- caldococcus jannaschii* aminocacyl-tRNA synthetase(s) (aaRS)/amber suppressor tRNA pairs, enables the incorporation of pBPA into the *fliQ* coding strand. *Escherichia coli* BL21 (DE3) harboring both pEVOL and the pET-Duet-based plasmid encoding both FliH with an amber mutation and a target protein was grown at 30°C in LB containing 1 mM pBPA until the culture reached mid-log phase. Then, 100 μM IPTG and 0.02% arabinose were added, and the incubation was continued until the culture density reached an OD<sub>600</sub> of ca. 1.4 to 1.5. Equal numbers of cells from each culture were collected by centrifugation. After the cells were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), the cell pellets were resuspended in SDS loading buffer containing 2% SDS normalized by the cell density to give a constant number of cells. The proteins in the culture supernatants were precipitated with 10% trichloroacetic acid (TCA), suspended in a Triton-X-100/SDS loading buffer, and heated at 95°C for 5 min. After SDS-PAGE, Coomassie brilliant blue (CBB) staining or immunoblotting with appropriate antibodies was performed.

**Photo-cross-linking.** To incorporate a p-benzoyl-phenylalanine (PBPA), a photo-cross-linkable unnatural amino acid, into the desired site of the proteins, we used the pEVOL system developed by Young and coworkers (49). The pEVOL plasmid, which encodes evolved *Methano- caldococcus jannaschii* aminocacyl-tRNA synthetase(s) (aaRS)/amber suppressor tRNA pairs, enables the incorporation of pBPA into the *fliQ* coding strand. *Escherichia coli* BL21 (DE3) harboring both pEVOL and the pET-Duet-based plasmid encoding both FliH with an amber mutation and a target protein was grown at 30°C in LB containing 1 mM pBPA until the culture reached mid-log phase. Then, 100 μM IPTG and 0.02% arabinose were added, and the incubation was continued until the culture density reached an OD<sub>600</sub> of ca. 1.4 to 1.5. Equal numbers of cells from each culture were collected by centrifugation. After the cells were washed with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>·H<sub>2</sub>O, 1.8 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4), the cell pellets were resuspended in 400 μl of PBS and then divided into two aliquots. One was transferred to 96-well microtiter plates, followed by UV irradiation at a wavelength of 365 nm using B-100AP (UV Products) for 5 min at room temperature, whereas the other was not subjected to this treatment. The cells were harvested by centrifugation and analyzed by SDS-PAGE and immunoblotting with polyclonal anti-FliH, anti-flaN, anti-FliH<sub>C</sub>, or anti-FlhB<sub>α</sub> antibody or monoclonal anti-FLAG antibody (Wako).

**Disulfide cross-linking.** Disulfide cross-linking experiments were carried out using iodoacetamide as an oxidative reagent. *Salmonella* ΔfliH ΔflaN double null mutant strains harboring two plasmids, one encoding the FliH-Cys variant on pTrc99AFF4 and the other encoding FliN (V113C) on pKg116, were grown at 30°C for 3 to 4 h in LB containing ampicillin and kanamycin. When the cell density had reached an OD<sub>600</sub> of ca. 0.4 to 0.5, 10 μM IPTG and 10 μM sodium iodoacetate were added, and then the incubation was continued until the cell density reached an OD<sub>600</sub> of ca. 1.4 to 1.5. Aliquots of culture proteins containing a constant number of cells were clarified by centrifugation. After being washed with motility buffer, the cells were resuspended in 200 μl of motility buffer and then divided into two aliquots. One microcrystal of 20 mM iodoacetamide in 99% ethanol was added to one sample, and 1 μl of 99% ethanol was added to the other. Samples were gently shaken for 5 min at room temperature, and then oxidation was quenched by adding 2 μl of 1 M N-ethylmaleimide in 99% ethanol. After gentle shaking for 5 min at room temperature, the cells were...
### TABLE 1 Strains and plasmids used in this study

**Strain or plasmid** | **Relevant characteristics** | **Source or reference**
--- | --- | ---
*E. coli* BL21(DE3) | Overexpression of proteins | Novagen
Salmonella strains |
SJW1103 | Wild type for motility and chemotaxis | 48
NH0020 | ΔfliH ΔfliN | This study
NH0022 | ΔfliH ΔfliA | This study
MKM11 | ΔfliH | 11
MKM30 | Δfli | 35
NH0025 | Δfli fliH (T11P) | This study

Plasmids |
pTrc99AFF4 | Modified Ptrc expression vector | 38
pKG116 | Salicylate-inducible expression vector | J. S. Parkinson
pETDuet-1 | P<sub>T7</sub> expression vector designed to coexpress two target proteins | Novagen
pEVOL | Vector for the incorporation of pBPA into the amber TAG codon | 49
pET19b | Expression vector under P<sub>T7</sub> control | Novagen
pUC19 | Cloning vector | Invitrogen
pjSV203 | pET19b/His-FliI-YFP | 37
pMM1702 | pTrc99A/His-FliI | 32
pMMHA004 | pUC19/FliA | 43
pNH001(R85A) | pUC19/FliA(R85A) | 13
pNH001(R94K) | pUC19/FliA(R94K) | 13
pNH001(D208E) | pUC19/FliA(D208E) | 13
pNH002(W7amber) | pUC19/FliI(W7amber) | This study
pNH002(W10amber) | pUC19/FliI(W10amber) | This study
pNH211 | pKG16/FliI(V113C) | This study
pNH284 | pKG16/FliH | This study
pNH284(W7A) | pKG16/FliH(W7A) | This study
pNH284(T11P) | pKG16/FliH(T11P) | This study
pNH333 | pKG16/FliA | This study
pNH333(R85A) | pKG16/FliA(R85A) | This study
pNH333(R94K) | pKG16/FliA(R94K) | This study
pNH333(D208E) | pKG16/FliA(D208E) | This study
pNH332 | pTrc99AFF4/FliI(C206S/C227S) | This study
pNH332(LSC) | pTrc99AFF4/FliI(LSC/C206S/C227S) | This study
pNH332(W7C) | pTrc99AFF4/FliI(W7C/C206S/C227S) | This study
pNH332(W10C) | pTrc99AFF4/FliI(W10C/C206S/C227S) | This study
pNH332(T11C) | pTrc99AFF4/FliI(T11C/C206S/C227S) | This study
pNH332(L15C) | pTrc99AFF4/FliI(L15C/C206S/C227S) | This study
pNH332(P19C) | pTrc99AFF4/FliI(P19C/C206S/C227S) | This study
pNH332(T21C) | pTrc99AFF4/FliI(T21C/C206S/C227S) | This study
pNH332(V23C) | pTrc99AFF4/FliI(V23C/C206S/C227S) | This study
pNH332(V25C) | pTrc99AFF4/FliI(V25C/C206S/C227S) | This study
pNH332(A27C) | pTrc99AFF4/FliI(A27C/C206S/C227S) | This study
pNH332 | pETduet-1/FliH(WT)+FliN | This study
pNH332 | pETduet-1/FliH(W7amber)+FliN | This study
pNH340 | pETduet-1/FliH(W10amber)+FliN | This study
pNH324 | pETduet-1/FliH(WT)+FliA | This study
pNH325 | pETduet-1/FliH(W7amber)+FliA | This study
pNH341 | pETduet-1/FliH(W10amber)+FliA | This study
pNH293 | pETduet-1/FliH(W7amber)+FliB(N269A) | This study
pNH294 | pETduet-1/FliH(W10amber)+FliB(N269A) | This study
pNH342 | pETduet-1/FliH(W10amber)+FliB(N269A) | This study
pNH300 | pETduet-1/FliH(WT)+FlhO-FLAG | This study
pNH301 | pETduet-1/FliH(W7amber)+FlhO-FLAG | This study
pNH343 | pETduet-1/FliH(W10amber)+FlhO-FLAG | This study
pNH302 | pETduet-1/FliH(WT)+FlhP-FLAG | This study
pNH344 | pETduet-1/FliH(W7amber)+FlhP-FLAG | This study
pNH304 | pETduet-1/FliH(WT)+FlhQ-FLAG | This study
pNH305 | pETduet-1/FliH(W7amber)+FlhQ-FLAG | This study

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mixed with nonreducing SDS loading buffer, heated at 95°C for 5 min, and subjected to SDS-PAGE (12.5% SDS-polyacrylamide). After SDS-PAGE, immunoblotting with polyclonal anti-FliH and anti-FlhA antibodies was carried out.

**Fractionation of cell membranes.** Cells were grown exponentially in 50 ml LB at 30°C with shaking. The cells were harvested, resuspended in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM dithiothreitol, and sonicated. After the cell debris was removed by low-speed centrifugation, the cell lysate was ultracentrifuged (100,000 × g, 60 min, 4°C). The soluble and membrane fractions were collected separately. Membranes were resuspended in 300 μl of PBS containing 1% SDS. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad), and equal amounts of membrane proteins (~40 μg) were subjected to SDS-PAGE, followed by immunoblotting with polyclonal anti-FlhA or anti-FliH antibody or monoclonal anti-FLAG antibody.

**RESULTS**

**Isolation of mutations that cause loss of an inhibitory function of FliH in the absence of FliI.** The *Salmonella* fliH null mutant and *fliH fliI* double null mutant are weakly motile, whereas a *fliI* null mutant is nonmotile (11, 35, 40). These observations suggest that FliH causes the arrest of flagellar protein export presumably due to its strong, inappropriate binding to the export gate in an inhibitory mode when FliI is absent (35). Alanine substitution at Trp7 and Trp10 of FliH, which are highly conserved among the FliH orthologues and are of primary importance for export function, abolishes the inhibitory effect of FliH on flagellar protein export (37). The overexpression of the FliH-FliI complex improves the reduced secretion activity of the *fliN* null mutant, whereas that of the FliH(W7A)/FliI or FliH(W10A)/FliI complex does not, suggesting that these two tryptophan residues of FliH are important for the efficient association of FliH with the export gate (37). To test this, weakly motile revertants that lose an inhibitory function of FliH were isolated from the *fliI* null mutant. In total, eight motile revertants were obtained by streaking a colony of the *fliI* null mutant onto a soft agar plate and then incubating at 30°C. The motility of the revertants was significantly better than that of the *fliI* null mutant (Fig. 1A). P22-mediated genetic transformation was measured using a Bio-Rad protein assay kit (Bio-Rad), and equal amounts of membrane proteins (~40 μg) were subjected to SDS-PAGE, followed by immunoblotting with polyclonal anti-FlhA or anti-FliH antibody or monoclonal anti-FLAG antibody.

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C206S/C227S), showed an ~5-kDa shift in mobility (Fig. 2), indicating that these Cys residues are exposed to solvent and accessible to mPEG-maleimide.

To investigate which components of the export gate are directly involved in the interaction with Trp7 and Trp10 of FliH, we carried out targeted photo-cross-linking experiments. We introduced an amber mutation at position 7 or 10 of FliH and found that these FliH amber mutants fully complemented the motility and protein export of a ΔfliH null mutant only in the presence of p-benzoyl-phenylalanine (pBPA), which is a photoreactive phenylalanine (Fig. 3). This result indicates that FliH(W7pBPA) and FliH(W10pBPA) are functional and hence that they retain the ability to bind to the export gate as well as to FliN.

We introduced two plasmids into an E. coli BL21(DE3) strain, one encoding FliH with an amber mutation at either position 7 or position 10 and a target protein, and the other encoding the amber suppressor tyrosyl tRNA and the engineered tyrosyl-tRNA synthetase to incorporate pBPA at the positions of amino acids codons (3, 10, 49). The UV irradiation of the cells grown in the presence of pBPA results in photo-cross-linking of the benzophenone group in FliH(W7pBPA) or FliH(W10pBPA) if they are close to the primary target protein. A band can be assigned as a cross-linked product between FliH and the target protein when it is detected on immunoblots by using both antibodies against FliH and the target protein (Fig. 3). This indicates that these Cys residues are exposed to solvent and accessible to mPEG-maleimide.

FIG 2 Cysteine modification of the FliH-Cys variants by mPEG-maleimide. FliH containing single cysteines at the indicated positions was expressed. After modification by mPEG-maleimide, the reaction was terminated by adding β-mercaptoethanol, and results were analyzed by immunoblotting with polyclonal anti-FliH antibody. The positions of free and modified FliH are indicated.

To confirm that Trp7 and Trp10 are in close proximity at the FliHEN-FliN interface, we next performed site-directed disulfide cross-linking experiments (see Fig. S3 in the supplemental material). It has been shown that mutations of the surface-exposed, hydrophobic residues of Salmonella FliN, Val111, Val112, and Val113, all of which are required for flagellar assembly, substantially weaken the FliH-FliN interaction (23, 41). As the V111C and V112C mutations significantly reduced the binding affinity of FliN for FliH whereas the V113C mutation did not (data not shown), we used FliN(V113C) as a target. Each FliH(C206S/C227S)-Cys variant was coexpressed with FliN(V113C) in a ΔfliH ΔfliN double null mutant strain, and then disulfide cross-linking was induced by adding iodine, followed by SDS-PAGE and finally by immunoblotting with polyclonal anti-FliN and anti-FliH antibodies (see Fig. S3 in the supplemental material). The FliH-FliN cross-linked products were seen when FliH(C206S/C227S) with the L5C, W7C, W10C, T11C, or L15C mutation was coexpressed with FliN(V113C) (see Fig. S3, lanes 2 to 6). Because the molecular masses of FliH and FliN are 25.8 kDa and 14.8 kDa, respectively, the molecular mass of a FliH-FliN heterodimer is 40.6 kDa, which is almost the same as that of the cross-linked products detected on immunoblots. This indicates that these Cys pairs are close to each other in the FliH-FliN interaction. However, no cross-linked products were seen when FliH(C206S/C227S) with the P17C, P19C, T21C, V23C, V25C, or A27C mutation was coexpressed with FliN(V113C), indicating that these pairs are not close enough to form a disulfide bond. These results indicate that Leu5, Trp7, Trp10, Thr11, and Leu15 in FliH are in relatively close proximity to the conserved hydrophobic patch of FliN. Therefore, we can conclude that the photo-cross-linking approach can identify the binding partner of FliH among six export gate component proteins, FliA, FliB, FliO, FliP, and FliR.

We then examined which export gate component binds to FliH(B20). To efficiently detect FliO, FliP, FliQ, and FliR by immunoblotting, we attached a FLAG tag to their C termini. Cell fractionation experiments showed that they were appropriately localized to the membrane fraction (see Fig. S4A in the supplemental material). FliH(W7pBPA) was reproducibly cross-linked with FliA to produce an FliH-FliA cross-linked product with an estimated molecular mass of ca. 100 kDa but not with the others (Fig. 4; see also Fig. S4B in the supplemental material). Because the molecular mass of FlhA is 74.9 kDa, this cross-linked product seems to be the FlhA-FliH heterodimer. We obtained essentially the same result with FliH(W10pBPA) (data not shown). These
results indicate that Trp7 and Trp10 of FliH\(_{EN}\) are in relatively close proximity to FlhA. We observed neither the FliH2-FlhA nor the FliH2-FliN heterotrimer as cross-linked products, although the cross-linked FliH homodimer was observed (see Fig. S4B).

FlhA consists of FlhATM with eight predicted transmembrane helices (residues 1 to 327) and FlhAC (residues 328 to 692) (27). The residues 328 to 351 of FlhA form a flexible linker between FlhATM and FlhAC and are responsible for the interaction with FliJ (1, 34). In an attempt to define the FlhA region involved in the formation of the FliH-FlhA cross-linked product, we used the FlhA(1-351)-YFP fusion protein and FlhAC as the target. Neither FlhA(1-351)-YFP nor FlhAC gave prominent photo-cross-linking with FliH (Fig. 4G and H), indicating that the formation of the FlhA-FliH cross-linked product requires both FlhATM and FlhAC.

**Importance of FliH for the export function of flhA mutants.** It has been shown that mutations at conserved charged residues within FlhATM have more deleterious effect on the protein export function when combined with the \(fliH\ fliI\) double null mutation than by themselves (13). To test the importance of FliH for the function of these FlhA mutants, we analyzed motility of a \(\Delta fliH\ fliI\) strain harboring PUC19-based plasmids encoding wild-type or mutant FlhA and pKG116-based plasmids encoding wild-type FliH (B) or FliH(W7A) (C). Motility of the \(\Delta fliH\ fliI\) strain harboring pKG116-based plasmids encoding wild-type or point mutant FlhA and pTrc99A-based plasmid encoding FliI. Fresh transformants were inoculated onto soft agar plates and incubated at 30°C. V, pUC19; WT, wild-type FlhA; R85A, FlhA(R85A); R94K, FlhA(R94K); D208E, FlhA(D208E).

**DISCUSSION**

and FlhBc provide binding sites for FliH, FliI, FliJ, flagellar chaperones, and export substrates (1, 25, 29, 32, 34, 36, 50). Because the reduced secretion activity of the \(fliH\) null mutant is significantly improved by the overexpression of \(fliH\) or extragenic suppressor mutations in \(flhA\) or \(flhB\), \(fliH\) is thought to be required for the productive association of Fli with the FlhA-FlhB complex (25). FliH binds to a C ring component protein, FliN, for rapid and efficient recruitment of the \(fliH\) complex to the export apparatus (12, 23, 37, 41). Highly conserved Trp7 and Trp10 of FliH are required not only for the interaction with FliN but also for the effective docking of the FliH-C complex to the export gate (37). This is strongly supported by our genetic analysis of a \(fliH\) null mutant showing that FliH(T11P) is unable to be installed into the export apparatus to exert its export-inhibitory action in the absence of FliJ (Fig. 1). Because previous pulldown assays by glutathione S-transferase (GST) affinity chromatography have shown that FliH(W7A) and FliH(W10A) retain the ability to bind to FliH and FliBc at the wild-type levels (37), it remained unknown which membrane protein interacts with these two Trp residues. Here, we performed targeted photo-cross-linking experiments using pBPA and showed that FliH(W7pBPA) and FliH(W10pBPA) formed cross-linked products with FliHb but not with the other export gate proteins (Fig. 4). This result suggests that Trp7 and Trp10 of FliH are in relatively close proximity to FliH. The formation of the FliH-FlhA complex required both FlhATM and FlhAc (Fig. 4G and H). Several point mutations in the extreme N-terminal regions of FlhA (FlhAEN) and FlhB that are close to their respective first transmembrane segment as well as the V404M mutation in FlhAc suppress not only the \(fliH\) null mutation but also the \(fliH\) double null mutation (26, 35). These observations raise the possibility that FlhAc is relatively close to FlhAEN in the tertiary structure. Because the W7A and W10A mutations do not interfere with the interaction of FliH with FlhAc (37), we propose that an interaction between FliH and FlhAc may allow FliH to be in proximity to FlhATM to form the cross-linked product.

It has been shown that most of the mutations of highly conserved residues in putative juxta- and transmembrane helices of FlhA are tolerated but result in loss of function in the absence of FliH and FliJ. This result suggests that these FlhA mutants require FliH and FliJ to exert their export activity (13). In this study, we found that these FlhA mutants are still functional in the absence of FliH (Fig. 5A) and that the overexpression of FliI alone does not restore their export function (Fig. 5D). These results indicate that FliH and FliJ must be in the vicinity of the export gate and that their efficient recruitment to the export gate requires FlhAEN, which is also responsible for the interaction with FliN. The FliI defect is bypassed by the overexpression of FliI or extragenic suppressor mutations in FlhA or FlhB (25). In addition, the requirement of the C ring for the flagellar export process is overcome by the overexpression of FliJ (18, 23) or upregulation of the expression of the FlhD-FlhC complex, which acts as the master regulator in the flagellar gene expression system (8). These observations suggest that the interaction of FlhAEN with FliN increases the local concentration of FliI around the export gate and that the interaction of FlhAEN with FlhA allows the effective docking of FliI to the FlhA-FlhB platform for efficient and rapid protein export under physiological conditions.

The amino acid sequences of the FlhAEN and FlhAc regions are homologous to those of the b and \(\delta\) subunits, respectively, of F\(\beta\)F\(-\)ATP synthase (39). The b and \(\delta\) subunits form the peripheral stalk connecting the \(\alpha_\beta\gamma\) ring complex to the F\(\beta\)F\(\gamma\) unit. Both FliH and the \(\delta\) subunits have an elongated structure and form homodimers (21, 26, 42). An \(\alpha\)-helical coiled-coil structure is responsible for the dimerization of the \(\delta\) subunit (7, 21). Like the b subunit, FliHM, which is responsible for FliH dimerization, contains a sequence predicted to form an \(\alpha\)-helical coiled coil too (11). The \(\delta\) subunit binds to the extreme N-terminal region of the \(\alpha\) subunit of F\(\beta\)F\(\gamma\)ATP synthase (47). FliHC binds to the extreme N-terminal region of FliI (20, 33), whose atomic structure is very similar to those of the \(\alpha\)/\(\beta\) subunits (17). FliI ring formation is stimulated by the binding of FliJ, which shows a striking structural similarity to the \(\gamma\) subunit for its binding to the center of the Fli hexamer ring in a way similar to that of the \(\gamma\) subunit penetrating into the central cavity of the \(\alpha_\beta\) ring (15). A specific interaction of FliI with a linker region of FlhA between FlhAEN and FlhAc is brought about by FliH and FliJ and is required for efficient PMF-driven protein export (34). Taken together, we suggest that FliH firmly anchors the FlhE-FliJ ring complex to the FliA-FlhB platform of the export gate through a specific interaction between FlhAEN and FlhA in a way similar to the peripheral stalk of F\(\beta\)F\(-\)ATP synthase.

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