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Review

Protein export through the bacterial flagellar type III export pathway☆

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ABSTRACT

For construction of the bacterial flagellum, which is responsible for bacterial motility, the flagellar type III export apparatus utilizes both ATP and proton motive force across the cytoplasmic membrane and exports flagellar proteins from the cytoplasm to the distal end of the nascent structure. The export apparatus consists of a membrane-embedded export gate made of FlhA, FlhB, FliO, FliP, FliQ, and FliR and a water-soluble ATPase ring complex consisting of FliH, FliI, and FliJ. FlgN, FliS, and FliT act as substrate-specific chaperones that do not only protect their cognate substrates from degradation and aggregation in the cytoplasm but also efficiently transfer the substrates to the export apparatus. The ATPase ring complex facilitates the initial entry of the substrates into the narrow pore of the export gate. The export gate by itself is a proton-protein antiporter that uses the two components of proton motive force, the electric potential difference and the proton concentration difference, for different steps of the export process. A specific interaction of FlhA with FliJ located in the center of the ATPase ring complex allows the export gate to efficiently use proton motive force to drive protein export. The ATPase ring complex couples ATP binding and hydrolysis to its assembly–disassembly cycle for rapid and efficient protein export cycle. This article is part of a Special Issue entitled: Protein trafficking and secretion in bacteria. Guest Editors: Anastassios Economou and Ross Dalbey.

1. Introduction

Salmonella enterica serovar Typhimurium (thereafter referred to *Salmonella*) can swim in liquid environments by rotating several flagella powered by proton motive force (thereafter referred to PMF) across the cytoplasmic membrane. The flagellum is divided into five parts from the base to the tip: the basal body, hook, hook-filament junction zone, filament, and filament cap (Fig. 1). The basal body consists of the MS ring (FliF), the C ring (FliG, FliM, FliN), the P ring (FlgI), the L ring (FlgH), and the rod (FliE, FlgB, FlgC, FlgF, FlgG), and is embedded in the cell envelope. The basal body is a rotary motor along with the Mot complex that acts as a proton channel to couple the inwardly-directed proton flow through the channel to torque generation. The hook (FlgE) and filament (FliC) extend into the exterior of the cell body. The filament acts as a helical screw to produce thrust. The hook functions as a universal joint to smoothly transmit torque produced by the motor to the filament. The hook-filament junction (FlgK, FlgL) connects the filament with the hook. The filament cap (FliD) is located at the tip of the growing filament to promote filament formation [1–3].

Flagellar assembly begins with the basal body, followed by the hook and finally the filament. For construction of the rod-hook-filament structure beyond the cytoplasmic membrane, flagellar proteins are exported via a specific export apparatus from the cytoplasm to the distal end of the growing structure [1–3]. N-terminal polypeptides of flagellar proteins retain the ability to be secreted via the flagellar export pathway into the culture media [4,5]. Flagellar protein export can occur post translationally [6]. These indicate that N-terminal amino acid sequences of export substrates function as the export signal recognized by the flagellar type III export apparatus. The export apparatus consists of six membrane proteins, FlhA, FlhB, FliO, FliP, FliQ, and FliR, and three soluble proteins, FliH, FliI, and FliJ (Fig. 2A, left panel) [7,8]. These component proteins share substantial sequence and functional similarities with those of the type III secretion system of animal- and plant-pathogenic bacteria, which directly injects effectors into their host cells for their invasion [1–3]. The export apparatus switches substrate specificity from proteins needed for the structure and assembly of the rod and hook (rod- and hook-type class) to those required for filament formation (filament-type class) upon completion of hook-basal body assembly [9,10]. Export of FlgK, FlgL, FliC, and FliD, which belong to the filament-type class, requires assistance of flagellar chaperones: FlgN for FlgK and FlgL, FliS for FliC, and FliT for FliD [11,12]. These substrate-specific chaperones protect their cognate substrates from degradation and aggregation in the cytoplasm [12,13] and transfer them to export component proteins [14–16].

Many protein export apparatuses utilize biological energies such as ATP and PMF for the translocation of proteins across biological membranes [17]. The flagellar type III export apparatus uses both ATP and PMF to drive protein translocation into the central channel of the nascent structure [18,19]. As a diameter of the central channel of the flagellum, which is a physical path, is only 2 nm [20], flagellar proteins must be in an extended conformation. How does the export apparatus convert these two biological energies into the mechanical works

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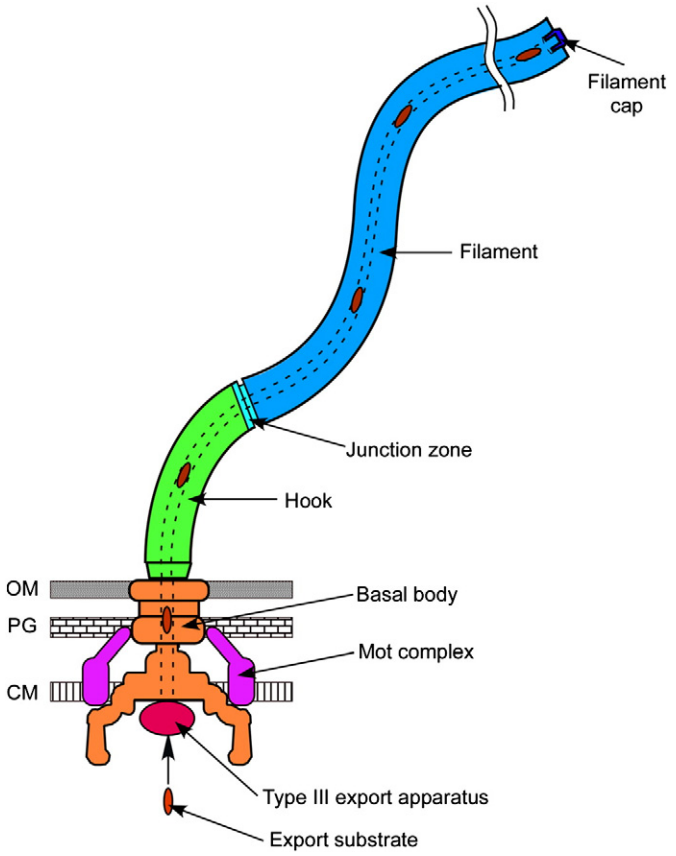

Fig. 1. Schematic diagram of the flagellum. The flagellum consists of a basal body, a hook, a hook-filament junction zone, a filament and a filament cap. Most flagellar component proteins are transported by a specific export apparatus from the cytoplasm to the distal end of the growing flagellar structure where their assembly occurs. OM, outer membrane; PG, peptidoglycan layer; CM, cytoplasmic membrane.

required for unfolding and protein export? In this review, we focus on the structure and function of the *Salmonella* flagellar type III export system.

2. Components of the flagellar export apparatus

2.1. Interactions among FlhA, FlhB, FliO, FliP, FliQ and FliR in the MS ring

FlhA, FlhB, FliO, FliP, FliQ, and FliR are integral membrane proteins with eight, four, one, four, two, and six transmembrane (TM) helices, respectively [21,22]. FlhA, FlhB, and FliO have a large C-terminal cytoplasmic domain [21,23]. FlhA, FliP and FliR associate with the MS ring [24,25]. Immunoelectron microscopy has shown that FliR is in the vicinity of the MS ring [24]. It has been shown that the export apparatus lies at the center of the cytoplasmic face of the MS-C ring complex, and that the C-terminal cytoplasmic domain of FlhA ($FlhA_C$) projects into the cavity within the C ring [26,27]. Consistently, the membrane-embedded part of the export apparatus of the *Salmonella* SPI1-type III secretion system is visualized to be located in the central pore of the basal body inner ring complex [28].

Genetic analyses have shown that the N-terminal TM region of FlhA ($FlhA_{TM}$) associates with FliF [25], FliR [29] and FlhB [30]. It has been also reported that FlhA binds to FliO, FliP, and FliQ [31,32]. The FliR-FlhB fusion protein is partially functional, suggesting that FliR is in close proximity to FlhB [33]. FliO requires FliP stability in the cytoplasmic membrane through TM domain interaction [23], suggesting that FliO and FliP associate with each other. These suggest that FlhA, FlhB, FliO, FliP, FliQ, and FliR interact with each other to form a multi-subunit complex termed the export gate in the central pore of the MS ring (Fig. 2A, left panel).

2.2. FliH, FliI, and FliJ form the ATPase ring complex

FliI is a member of the Walker-type ATPase family [34,35]. The crystal structure of *Salmonella* FliI is remarkably similar to the α and β subunits of F_OF_1-ATPsynthase [36]. Unlike F_OF_1-ATPsynthase, in which the α and β subunits form the $\alpha_3\beta_3$ hetero-hexamer, FliI forms homo-hexamer, thereby fully exerting its ATPase activity [37]. The $FliI_6$ ring is visualized below the MS-C ring complex by electron cryotomography [26]. The first 20 residues of FliI ($FliI_{EN}$) not only regulate FliI ring formation [38] but also are involved in the interaction with FliH [39,40].

The FliJ structure adopts an antiparallel coiled coil structure composed of two long α-helices that show a structural similarity to the two-stranded α-helical coiled coil part of the γ subunit of F_OF_1-ATPsynthase [41]. FliJ binds to the center of the $FliI_6$ ring to form the $FliI_6$–FliJ ring complex in a way similar to the γ subunit binds to the β subunit in F_OF_1-ATPsynthase (Fig. 2B) [41–43].

FliH is divided into three regions: $FliH_N$, $FliH_M$, and $FliH_C$ [44]. The amino acid sequences of $FliH_{NM}$ and $FliH_C$ are homologous to those of the b and δ subunits of F_OF_1-ATPsynthase, respectively [40,45]. The b and δ subunits form the peripheral stalk connecting the $\alpha_3\beta_3\gamma$ ring complex to the membrane-embedded F_O unit (Fig. 2A, right panel). The b subunit forms quite elongated homo-dimer through its α-helical coiled coil motif [45]. FliH too forms homo-dimer through $FliH_M$ with a predicted α-helical coiled coil motif [39,44]. FliH with the W7C, W10C or T11C mutation forms cross-linked homo-dimer [46], suggesting that FliH subunits lie more or less side-by-side in a parallel arrangement. FliH has an elongated structure [47]. $FliH_N$ is quite elongated whereas $FliH_C$ is spherical [48]. The δ subunit binds to the extreme N-terminal region of the α-subunit of F_OF_1-ATPsynthase [40,45]. $FliH_C$ is directly involved in the interaction of the FliH homo-dimer with $FliI_{EN}$ [39,40]. Because two conserved Trp7 and Trp10 residues of FliH, which are essential for FliH function [49], are in relatively close proximity to FlhA [46], the interaction of $FliH_{EN}$ with FlhA seems to be required for stable anchoring the $FliI_6$–FliJ ring complex to the export gate in a way similar to the peripheral stalk of F_OF_1-ATPsynthase (Fig. 2A).

2.3. Assembly of the ATPase complex to the export gate

FliI forms hetero-trimer along with the FliH dimer [39]. Because FliH suppresses ATP hydrolysis by FliI, FliH coordinates ATP hydrolysis by FliI with protein export [39]. The impaired export activity of a *Salmonella fliH* null mutant is significantly enhanced by the over-expression of FliI or extragenic suppressor mutations in FlhA or FlhB [50], suggesting that FliH is required for efficient association between FliI and the FlhA–FlhB platform of the export gate. Consistently, FliH and FliI bind to FlhA and FlhB [8,31,51].

Two C ring component proteins FliM and FliN form a complex consisting of 1 copy of FliM and 4 copies of FliN [52]. The $FliH_2$–FliI complex associates with the FliM–$FliN_4$ complex to form a stable $FliH_2$–FliI–FliM–$FliN_4$ complex through an interaction between FliH and FliN [53], suggesting that the $FliH_2$–FliI complex localizes to the C ring before engaging with the FlhA–FlhB platform of the export gate. Two conserved Trp7 and Trp10 residues of FliH are involved in the FliH–FliN interaction [49]. Because the impaired export activity of *Salmonella fliN* null mutants is considerably improved by the over-expression of FliI [54], the FliH–FliN interaction is presumably required for the efficient assembly of FliI to the FlhA–FlhB platform of the gate. FliJ binds to FliM, and the FliM–FliJ interaction considerably interferes with the interaction of FliH with FliN [53]. Residues 2 to 10 in FliH ($FliH_{EN}$), which are also responsible for the interaction

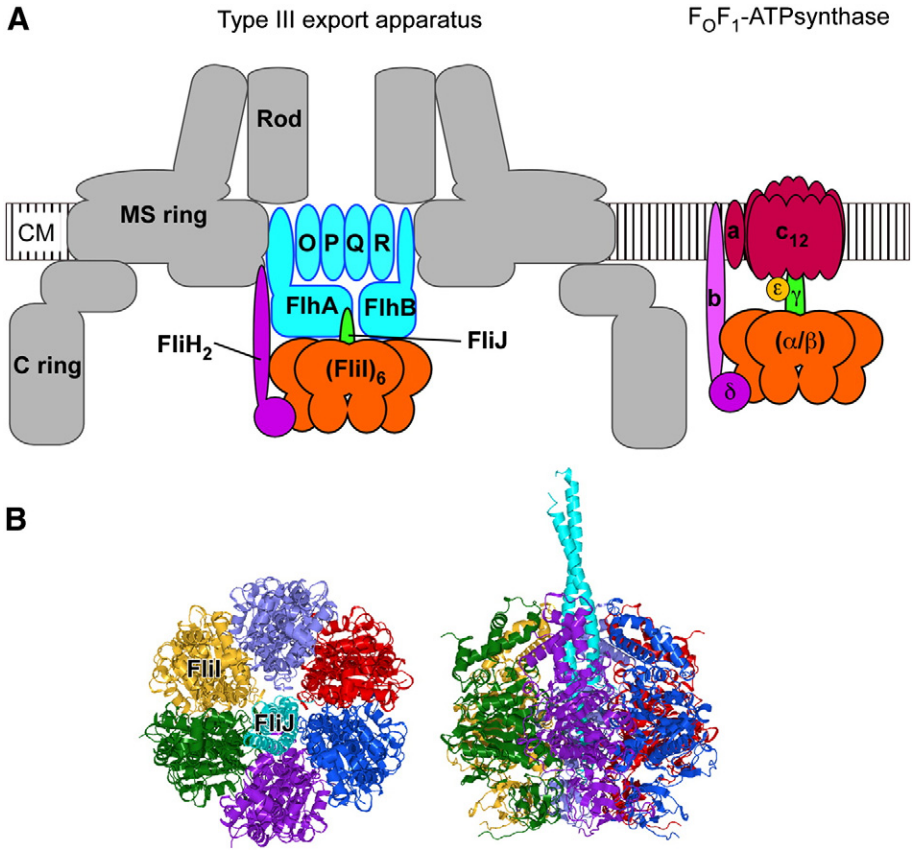

Fig. 2. Hypothetical model for the flagellar type III export apparatus. (A) Cartoons of the flagellar type III export apparatus and F_OF_1-ATPsynthase. FlhA, FlhB, FliO, FliP, FliQ and FliR are located within a central pore of the MS ring. FliI and FliJ form the $FliI_6$-FliJ ring complex, which is remarkably similar to the $\alpha_3\beta_3\gamma$ ring complex of F_OF_1-ATPsynthase. FliH, which is homologous to the peripheral stalk of F_OF_1-ATPsynthase, stably anchors the $FliI_6$-FliJ ring complex to the FlhA-FlhB platform of the export gate. (B) Structural model of the $FliI_6$-FliJ ring complex. Cα backbone trace of the $FliI_6$-FliJ complex. The FliI subunits (PDB ID, 2DPY) are colored blue, violet, green, yellow, orchid and red, and FliJ (PDB ID, 3AJW) is colored cyan.

of FliH with FliN [49], are critical for the FliH–FliJ interaction [44]. FliH binds to the C-terminal region of FliJ [55]. These suggest that the interaction between FliH and FliJ releases the $FliH_2$–FliI–FliJ complex from the C ring.

Trp7 and Trp10 of FliH are in relatively close proximity to FlhA [46]. Pull-down assays by GST affinity chromatography have shown that FliH(W7A) and FliH(W10A) retain the ability to bind to $FlhA_C$ at the wild-type levels [49], suggesting that $FliH_{EN}$ may be in proximity to $FlhA_{TM}$. FliJ binds to a flexible linker ($FlhA_L$) located between $FlhA_{TM}$ and $FlhA_C$ (Fig. 3A) [15,42]. Because FliI binds to $FlhA_C$ and the C-terminal domain of FlhB ($FlhB_C$) [8], the $FliI_6$–FliJ ring complex is formed on the $FlhA_C$–$FlhB_C$ platform and is stably anchored to the platform through the interaction between $FliH_{EN}$ and $FlhA_{TM}$.

3. The C-terminal cytoplasmic domains of FlhB and FlhA mediate an ordered export during flagellar assembly

The flagellar type III export apparatus coordinates protein export with flagellar assembly [3]. Upon completion of hook assembly, a specific interaction between FlhB and the hook length control protein FliK switches substrate specificity of the export apparatus from rod- and hook-type to filament-type substrates [9,10]. $FlhB_C$ plays an important role in substrate recognition [56]. A highly conserved NPTH sequence lies on a flexible loop located between $FlhB_{CN}$ and $FlhB_{CC}$ (Fig. 3B). $FlhB_C$ undergoes autocatalytic cleavage between Asn269 and Pro270 within the NPTH sequence into the $FlhB_{CN}$ and $FlhB_{CC}$ polypeptides by a mechanism involving cyclization of Asp269, but these two polypeptides retain the ability to tightly interact with each other after cleavage [56,57]. This has been demonstrated by X-ray crystallographic analyses of *Salmonella* $FlhB_C$ [58] and its homologues [59,60]. The autocleavage undergoes a major reorientation of the loop away from the conserved Asp residue, resulting in altered electrostatic surface features at the site of the cleavage [58–60]. In addition, this autocleavage induces a significant conformational change in the flexible linker region that connects $FlhB_C$ with $FlhB_{TM}$ [60]. Because the FlhB(N269A) mutation prevents the export apparatus from switching its export specificity [61], these structural rearrangements of $FlhB_C$ are required for the switching process.

FliK is secreted into the culture media during hook assembly [62] and acts as a molecular ruler to measure hook length [63,64]. The N-terminal region of FliK binds to the hook cap with high affinity and the hook with low affinity to measure the hook length [65]. The C-terminal domain of FliK is responsible for the switching of substrate specificity of the export apparatus [66,67]. Extragenic suppressor mutant FlhB proteins that undergo the switching process even in the absence of FliK are much more resistant to the autocleavage than the wild-type [56], indicating that these suppressor mutations affect a conformation of the flexible loop, on which the conserved NPSH motif lies. These suggest that the autocleavage of $FlhB_C$ and the following FliK-driven conformational rearrangement of $FlhB_C$ switches the export specificity of the export apparatus.

$FlhA_C$ consists of four domains, D1, D2, D3, and D4 (Fig. 3A) [68]. Because a truncation of domain D4 of $FlhA_C$ allows filament-type substrates to be exported into the periplasm prior to hook completion [69], domain D4 establishes a strict export order that exactly parallels the assembly order of the rod-hook-filament structure. Domains D1

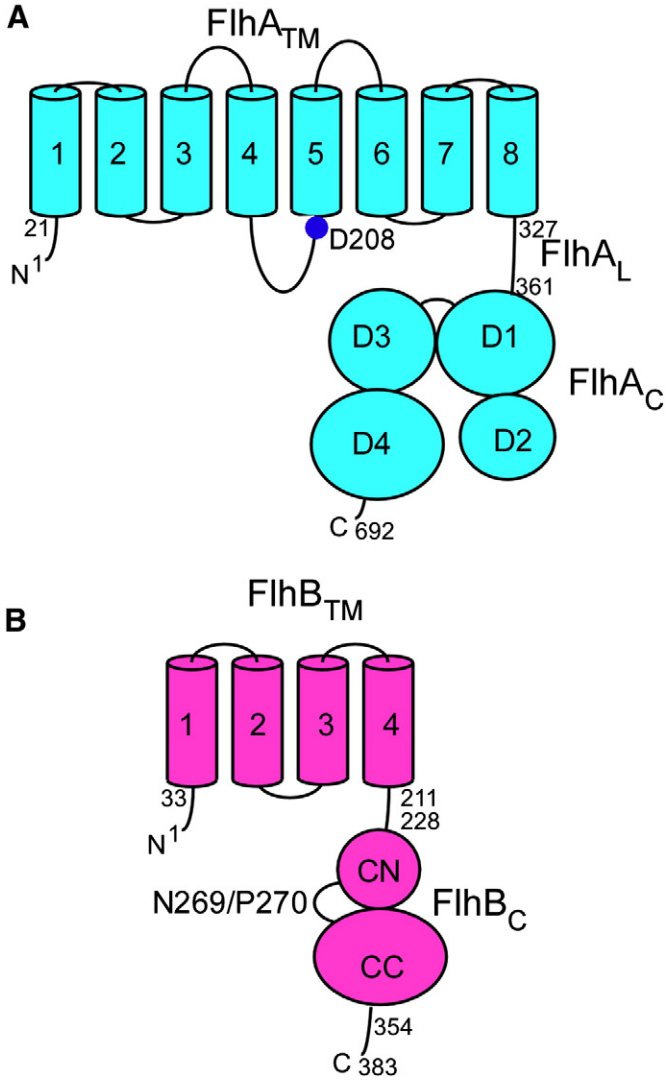

Fig. 3. Topology of (A) FlhA and (B) FlhB. FlhA and FlhB contain an N-terminal transmembrane region ($FlhA_{TM}$ and $FlhB_{TM}$) and a large C-terminal cytoplasmic region ($FlhA_C$ and $FlhB_C$). $FlhA_C$ consists of 4 domains, D1, D2, D3 and D4. A flexible linker ($FlhA_L$) connects $FlhA_C$ with $FlhA_{TM}$. Highly conserved Asp-208 is shown by a closed cycle. $FlhB_C$ is specifically cleaved at the site Asn-269/Pro-270 into two $FlhB_{CN}$ and $FlhB_{CC}$ polypeptides in an autocatalytic manner, but these two polypeptides tightly associates with each other after the autocleavage.

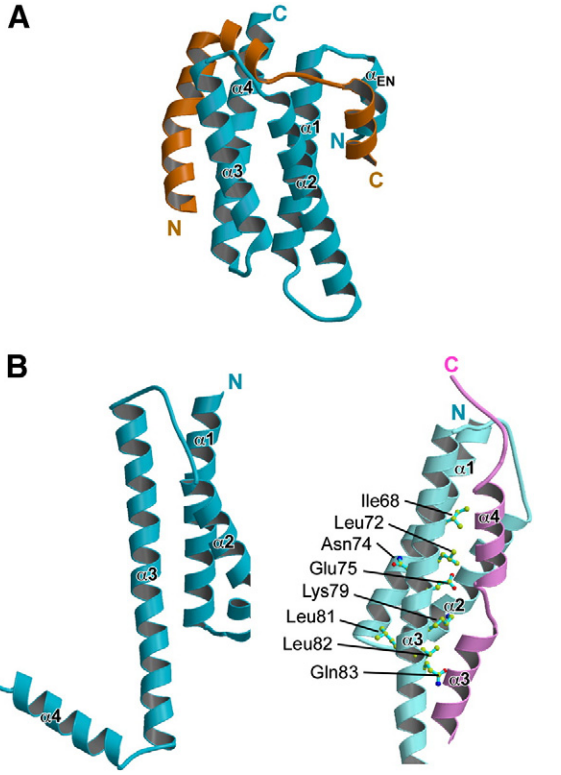

Fig. 4. Crystal structures of FliS and FliT. (A) FliS in complex with the C-terminal fragment of FliC (PDB ID, 10RY) (FliS, light blue; FliC, orange). The FliS structure is formed by anti-parallel four-helix bundle (α1, α2, α3, α4) with a quasi-helical cap (α_{EN}). The interaction of FliS with the C-terminal fragment of FliC induces release of the N-terminal cap from a hydrophobic pocket of anti-parallel four helix bundle. (B) FliT (PDB ID, 3A7M). The FliT structure consists of four α-helices, α1, α2, α3 and α4 (left panel). The core structure of FliT is formed by anti-parallel α-helical bundle composed of α1, α2 and α3. Helices α2 and α3 form a hydrophobic cleft, which interacts with the α4 helix of the neighboring molecule (right panel). The core structure is colored in cyan, and the C-terminal half of α3 and α4 of the neighboring molecule are colored in magenta. Highly conserved, surface-exposed residues are shown by ball and stick representation.

and D2 of $FlhA_C$ provide a binding-site for flagellar chaperones in complexes with their cognate filament-type substrates [15,16]. Certain mutations in a highly conserved hydrophobic dimple located at the interface between domains D1 and D2 of $FlhA_C$ reduce the secretion levels of filament-type substrates but not those of hook-type substrates [M. Kinoshita, T. Minamino, N. Hara, K. Imada, K. Namba, unpublished data], suggesting that domain D4 suppresses the interaction of $FlhA_C$ with these chaperone–substrate complexes during hook assembly and relieves this suppression upon hook completion. However, it remains unknown how it occurs.

4. Interactions of flagellar chaperones with export substrates and export component proteins

4.1. Interactions of flagellar chaperones with their cognate substrates

Crystal structures of *Aquifex aeolicus* FliS and its complex with a C-terminal fragment of FliC ($FliC_C$) have been solved by X-ray crystallography (Fig. 4A) [70]. FliS binds to the C-terminal amphipathic α-helical domain of FliC and forms hetero-dimer. FliS has an antiparallel four-helix bundle with a quasi-helical cap on one end formed by 16 N-terminal residues ($FliS_{EN}$). $FliS_{EN}$ plugs the hydrophobic pocket of the antiparallel α-helical bundle. Upon binding of $FliC_C$ to FliS, $FliC_C$ wraps around the surface of the antiparallel α-helical bundle of FliS in an extended conformation containing three α-helices, and the α-helical segment of $FliC_C$ consisting of residues 499 to 505 moves into the hydrophobic pocket. As a result, $FliS_{EN}$ dissociates from the hydrophobic pocket and is reorganized to form a short helix on one side of the core helix bundle of FliS (Fig. 4A) [70]. Thus, $FliS_{EN}$ seems to act as a molecular plug to cover the hydrophobic binding site of the core structure of FliS when FliS is not bound to $FliC_C$.

4.2. Interactions of flagellar chaperones with FliI

FliT exists in the equilibrium between monomer and dimer under physiological conditions and forms hetero-dimer with FliD in solution [71]. The crystal structure of *Salmonella* FliT consists of four α-helices, α1, α2, α3, and α4 (Fig. 4B, left panel). The core structure termed FliT94 is formed by an anti-parallel α-helical bundle structure composed of α1, α2, and α3. The C-terminal α4 helix binds to the hydrophobic cleft formed by helices α2 and α3, and hence FliT forms homo-dimer in the crystal (Fig. 4B, right panel) [71].

FliT and the FliT–FliD complex bind to FliI [71]. A truncation of the C-terminal α4 helix considerably increases the binding affinity of FliT for FliI but not for FliD [71]. FliT94 binds to $FliI_{EN}$ with high

affinity and to the C-terminal ATPase domain ($FliI_{CAT}$) with low affinity [72]. Highly conserved, surface-exposed Ile68, Lys79, Leu81 Leu82, and Gln83 are involved in the strong interaction of FliT94 with $FliI_{EN}$ [72]. Ile68 is located within a hydrophobic cleft formed by helices α2 and α3, Leu81 and Gln83 are opposite to the hydrophobic cleft, and Lys79 and Leu82 are under the cleft (Fig. 4B, right panel), suggesting that the interaction of helix α4 with the cleft directly controls the binding affinity of FliT for $FliI_{EN}$. Because FliH binds to $FliI_{EN}$ [39,40], FliT94 cannot bind to the $FliH_2$–FliI complex with high affinity [72]. The addition of FliH induces the release of FliI from the FliT94–FliI complex even in the absence of Mg^{2+}-ATP [72]. These suggest that FliI transfers from FliT94 to FliH by direct competition and displacement at a common binding site on $FliI_{EN}$ in an ATP-independent manner.

FliD binds to the hydrophobic cleft of FliT, and the truncation of the C-terminal α4 helix does not affect the interaction of FliT with FliD [71]. This suggests that the FliT–FliD interaction presumably induces the dissociation of the α4 helix from the cleft. Because $FliI_{EN}$ binds to this hydrophobic cleft [72], FliD may block the strong interaction between FliT94 and $FliI_{EN}$. Thus, the interaction of the hydrophobic cleft with its binding partners must be well regulated in a timely manner during protein export.

FliD binds to FliI and significantly enhances or stabilizes the weak interaction between $FliI_{CAT}$ and FliT94 presumably through cooperative interactions among $FliI_{CAT}$, FliD and FliT94 [72]. The FlgN–FlgK complex binds to the $FliH_2$–FliI complex through an interaction between FliI and FlgN [14], suggesting that the FlgN–FlgK complex presumably binds to $FliI_{CAT}$ in a way similar to the FliT–FliD complex. Therefore, it is likely that $FliI_{CAT}$ plays an important role in substrate recognition.

InvC, which is a FliI homologue of the *Salmonella* SPI-1 type III secretion system, releases chaperones from chaperone–substrate complexes and unfolds the substrates in an ATP-dependent manner [73]. In contrast, FliI does not induce FliT release from the FliT–FliD complex although a mixture of FliI and the FliT–FliD complex displays an ATPase activity comparable to that of FliI alone [72]. Because a *Salmonella* Δ*fliH–fliI flhB*(P28T) mutant can form flagella to a considerable degree even in the absence of FliH and FliI [18], the export gate may be involved in chaperone release from the chaperone–substrate complex.

4.3. Interactions of flagellar chaperones with $FlhA_C$

A highly conserved hydrophobic dimple located at the interface between domains D1 and D2 of $FlhA_C$ provides a binding site for the FlgN–FlgK/L, FliS–FliC and FliT–FliD complexes [16]. FliS and FliT require FliC and FliD, respectively, for their interactions with $FlhA_C$ [15] whereas FlgN binds to $FlhA_C$ even in the absence of FlgK [16]. Interestingly, the FlgN–FlgK complex has a much higher affinity for $FlhA_C$ than FlgN alone [16]. These suggest that the newly delivered chaperone–substrate complex induces the dissociation of the empty chaperone from $FlhA_C$.

FliJ binds to $FlhA_L$ [15,42]. FliJ significantly enhances the binding of the FliT–FliD complex to $FlhA_C$ [15]. FliJ interacts with FlgN and FliT but not with FliS [74], suggesting that FliJ may confer an advantage for rapid and efficient export of FlgK, FlgL, and FliD for formation of the junction and cap structures at the tip of the hook prior to filament assembly.

A truncation of the last 20 residues of FlgN, which causes a loss-of-function phenotype, increases the binding affinity of FlgN for FliI, thereby exerting a negative dominance [14]. FlgN120 lacking these 20 residues binds to FlgK and FliJ but not to $FlhA_C$, indicating that this loss-of-function phenotype is a consequence of the impaired ability of FlgN to interact with $FlhA_C$ [16]. This suggests that an interaction between FlgN and $FlhA_C$ is a critical step for efficient export of FlgK and FlgL. Tyr122 of FlgN is a highly conserved residue among FlgN orthologues. The Y122A, Y122C and Y122H mutations significantly decrease the binding affinity of FlgN for $FlhA_C$, reducing the export activity of FlgN [16]. This suggests that this conserved Try122 residue is involved in the interaction of FlgN with the conserved hydrophobic dimple of $FlhA_C$.

5. Energy coupling mechanism of flagellar protein export

A *Salmonella fliH–fliI* double null mutant (Δ*fliH–fliI*) forms flagella albeit at a low probability [18,19]. The FlhB(P28T) bypass mutation significantly enhances motility of and flagellar protein export by the Δ*fliH–fliI* mutant. The amounts of FlgD (hook cap protein) and FliK secreted by the Δ*fliH–fliI flhB*(*P28T*) bypass mutant are even higher than those by wild-type cells [18]. This suggests that the energy derived from ATP hydrolysis by FliI is not absolutely required for the export process. Depletion of PMF across the cytoplasmic membrane by adding carbonyl cyanide *m*-chlorophenylhydrazone abolishes flagellar protein export in both the wild-type and the Δ*fliH–fliI flhB*(*P28T*) bypass mutant [18,19]. This indicates that the export gate utilizes PMF as the energy source for flagellar protein export. Because the FlhB(P28T) mutation considerably increases the probability of flagellar protein entry into the export gate in the absence of FliH and FliI, the ATPase ring complex helps only the initial entry of export substrates into the gate [18]. The binding of Mg^{2+}-ATP to FliI induces FliI ring formation, and ATP hydrolysis and the following release of ADP and Pi destabilizes the ring structure [75]. This suggests that ATP hydrolysis by FliI induces the release and disassembly of the ATPase ring complex from the export gate and the protein about to be exported for successive protein translocation.

PMF consists of two components: the electric potential difference ($\Delta\psi$) and the proton concentration difference (ΔpH). $\Delta\psi$ alone is sufficient to support flagellar protein export in wild-type cells but both $\Delta\psi$ and ΔpH become essential in the Δ*fliH–fliI flhB*(*P28T*) bypass mutant [19,42]. This suggests that these two components of PMF play distinct roles in the export process in the absence of FliH and FliI. The replacement of H_2O by D_2O reduces the levels of FlgD secreted by this bypass mutant, indicating that the rate of proton transfer through the export gate determines the overall rate of flagellar protein export [42], suggesting that the export gate by itself is a proton-protein antiporter that requires both $\Delta\psi$ and ΔpH as the energy sources. Furthermore, genetic analyses combined with pull-down assays by GST affinity chromatography have shown that a specific interaction of FliJ with $FlhA_L$ brought about by the $FliH_x$–$FliI_6$ ring complex turns the export gate into a highly efficient, $\Delta\psi$-driven protein export apparatus [42]. Thus, the interaction of $FlhA_L$ with the ATPase ring complex plays an important role in the energy transduction mechanism.

A temperature-sensitive FlhA(G368C) mutation, which is located in domain D1 of $FlhA_C$, abolishes flagellar protein export at a restrictive temperature of 42 °C but not at a permissive temperature of 30 °C. The G368C mutation blocks the export process after the assembly of the ATPase ring complex to the FlhA–FlhB docking platform of the export gate [51]. This loss-of function phenotype of the *flhA*(*G368C*) mutant results from denaturation of domain D2 of $FlhA_C$ [76]. These suggest that domain D2 is directly involved in the translocation of export substrates into the central channel of the flagellar structure. $FlhA_C$ provides a binding site for flagellar chaperone–substrate complexes [15,16], raising the possibility that domain D2 of $FlhA_C$ coordinates the energy transduction reaction with protein translocation into the central channel of the nascent structure.

Asp208 of FlhA, which is highly conserved among FlhA orthologues, is located in the cytoplasmic juxta-transmembrane region (Fig. 3A) and is essential for FlhA function. Only the conservative D208E replacement permits any function, indicating that the important feature of this residue appears to be either a negative side-chain charge or the ability to bind a proton [29]. Therefore, it is possible that Asp208 of FlhA is involved in the PMF-driven export process.

Many mutations of highly conserved residues located in $FlhA_{TM}$ are tolerated, but result in loss-of-function in the Δ*fliH–fliI* mutant background, even with the FlhB(P28T) bypass mutation that increases the probability of flagellar protein export in the absence of FliH and FliI

[29]. Genetic analysis of the *flhA*(*K203W*) mutant has shown an interaction of a highly conserved loop located between TM-4 and TM-5 of FlhA with the cytoplasmic loop between TM-2 and TM-3 of FliR [29]. These suggest that cooperative interactions of FlhA with FlhB, FliH, FliI and FliR are required for efficient PMF-driven protein export.

6. Mechanism of flagellar protein export

A number of experimental data are gradually accumulating to draw a picture of the flagellar protein export process. Flagellar chaperone binds to its cognate substrate in the cytoplasm [11,12]. The $FliH_2$–FliI complex localizes to the C ring through an interaction between FliH and FliN [53,54]. The chaperone–substrate complex binds to the $FliH_2$–FliI complex through cooperative interactions among $FliI_{CAT}$, the chaperone and the substrate [14,72]. An interaction between FliH and FliJ releases the $FliH_2$–FliI–FliJ-chaperone–substrate complex from the C ring [53]. Upon docking to the FlhA–FlhB platform of the export gate, FliJ facilitates FliI ring formation by binding to the center of the ring [41]. FliH stably anchors the $FliI_6$–FliJ ring complex to the platform through an interaction between $FliH_{EN}$ and $FlhA_{TM}$ [46]. The chaperone–substrate complex binds to a highly conserved hydrophobic dimple of $FlhA_C$ [15,16]. The $FliI_6$ ring supports the initial entry of the N-terminal segment of export substrate into an entry site on the export gate [18]. An interaction of FliJ with $FlhA_L$ fully energizes PMF-driven translocation of the substrate into the central channel of the growing structure [42]. ATP hydrolysis by FliI induces the destabilization of the $FliH_X$–$FliI_6$–FliJ ring structure, followed by the release of the $FliH_2$–FliI complex, FliJ, and the chaperone from the export gate [75].

7. Conclusions

The flagellar type III export apparatus consists of a proton-driven export gate and an ATPase complex. The export process is well regulated by dynamic, specific and cooperative interactions among export components, chaperones, and export substrates in a timely manner. The export gate by itself is a proton-protein antiporter that uses the two components of PMF for distinct steps of the export process. An interaction of FliJ with $FlhA_L$ turns the export gate into a highly more efficient, $\Delta\psi$-driven protein export apparatus [42] but it remains unknown how it occurs. It also remains unknown how the export gate is opened, allowing export substrates to go through a protein conducting channel of the export gate into the central channel of the nascent structure in a PMF-dependent manner. Furthermore, little is known about the function and structure of $FlhA_{TM}$, $FlhB_{TM}$, FliO, FliP, FliQ and FliR. We need to investigate the energy coupling mechanism of the export apparatus in much more detail by genetic, biochemical and biophysical techniques. High-resolution structural analysis of the export gate by X-ray crystallography and electron cryomicroscopy would be essential to advance our mechanistic understanding of the export process.

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